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IL10, TGF Beta1, and IFN Gamma Modulate Intracellular Signaling Pathways and Cytokine Production to Control *Toxoplasma gondii* Infection in BeWo Trophoblast Cells[1]

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ABSTRACT

Considering that interleukin 10 (IL10), transforming growth factor beta1 (TGFB1), and interferon gamma (IFNG) are involved in the susceptibility of BeWo trophoblast cells to *Toxoplasma gondii* infection, the aim of the present study was to investigate the effector mechanisms triggered by these cytokines in the control of *T. gondii* in BeWo cells. For this purpose, infected/uninfected BeWo cells were treated with IL10, TGFB1 (50 ng/ml), and IFNG (20 or 100 ng/ml) in order to verify the phosphorylation of signal transducers and activators of transcription 1 (STAT1), STAT3, and Smad2, parasite intracellular proliferation, as well as the Th1/Th2/IL17A cytokine production. The treatment of BeWo cells with IL10 and TGFB1 favored *T. gondii* proliferation, and these findings were associated with STAT3 and Smad2 phosphorylation, respectively ($P < 0.05$). Also, these cytokine treatments were able to down-modulate TNF alpha (TNFA) and IL6 production ($P < 0.05$). Low concentration of IFNG was unable to control *T. gondii* infection but was able to trigger STAT1 phosphorylation and up-regulate IL6 and IL17A production; whereas a high concentration of IFNG was unable to activate STAT1 but down-modulated IL6 and TNFA and increased *T. gondii* proliferation ($P < 0.05$). IL10, TGFB1, and IFNG regulate a differential *T. gondii* proliferation in BeWo cells because they distinctly trigger intracellular signaling pathways and cytokine production, especially IL6 and TNFA. Our data open new windows to understand the mechanisms triggered by IL10, TGFB1, and IFNG at the maternal-fetal interface in the presence of *T. gondii,* contributing to recognizing the importance of these effector mechanisms involved in the vertical transmission of this parasite.

cytokines, signaling pathways, Toxoplasma gondii, *trophoblast cells*

INTRODUCTION

Toxoplasma gondii is an intracellular protozoan parasite that infects a wide range of warm-blooded vertebrates, including humans [1, 2]. During congenital infection in humans, severe manifestations can be observed, such as retinochoroiditis and miscarriage [3, 4], characterizing an important public health problem in many countries, including Brazil [5, 6].

A Th1-type immune response against *T. gondii* is observed during infection, with the participation of interferon gamma (IFNG) and other proinflammatory cytokines [7–10]. On the other hand, a normal pregnancy is characterized by a preferential Th2-type immune response and T regulatory cell responses [11], with the production of anti-inflammatory cytokines such as interleukin 10 (IL10) and transforming growth factor beta1 (TGFB1) by both maternal and fetal cells, including trophoblast cells [12, 13]. The role of trophoblast cells in the immunology of pregnancy, especially in the presence of intracellular pathogens such as *T. gondii* and *Neospora caninum,* has been studied by our group using well-established cell lines, such as BeWo cells [14–19]. These cells were isolated from a human choriocarcinoma [20] and preserved their trophoblastic characteristics, such as hormone production and cytokine release under in vitro stimulation [21, 22]. Even though BeWo cells are derived from a choriocarcinoma, they are becoming an excellent in vitro model to investigate *T. gondii* infection in trophoblast cells. In fact, our group has investigated several aspects of the immunology of pregnancy using these cells as a trophoblast model, including their cross-talk with monocytes [18] and the role of cytokines and drugs during *T. gondii* infection [14, 15, 17, 19].

Our previous study verified that BeWo cells are highly susceptible to *T. gondii* infection when stimulated with IL10 and TGFB1 [15]. Interestingly, we verified that IFNG was unable to control *T. gondii* infection in BeWo cells [14] or significantly increased the infectivity in these cells [15], although this cytokine is the major molecule involved in the

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immune response against this parasite [8, 10]. Thus, it is possible to assume that BeWo trophoblast cells are resistant to the proinflammatory effects of IFNG, probably in order to maintain the development of the embryo in vivo and to avoid rejection during pregnancy, which could favor *T. gondii* infection in the placental tissues [14, 15].

IL10, TGFB1, and IFNG trigger different intracellular signaling pathways [23]. IL10 and IFNG bind to specific receptors in the plasma membrane, triggering the cytoplasmic Janus kinases (JAK) that are coupled to these receptors, which, in turn, phosphorylate signal transducers and the activation of transcription 3 (STAT3) or STAT1, promoting an anti-inflammatory or proinflammatory response, respectively [24–28]. IL10 and IFNG receptors have been found in human trophoblast [29] and BeWo cells [30], although reduced STAT1 phosphorylation has been observed in human trophoblast cells [31]. On the other hand, TGFB1 binds to its receptors in the plasma membrane and triggers Sma- and Mad-related (Smad) proteins (Smad2 and Smad3) that regulate anti-inflammatory proteins [32–35]. TGFB has been detected in human term placenta [36], and BeWo cells are able to secrete this cytokine [15, 16].

Given that IL10, TGFB1, and IFNG are involved in the increased susceptibility of BeWo trophoblast cells to *T. gondii* infection [15], the aim of the present study was to investigate the effector mechanisms triggered by these cytokines, including intracellular signaling pathways upon release, which could act in the *T. gondii* proliferation process in a human trophoblast model. Studies in human trophoblast cells have demonstrated the intracellular mechanisms potentially interfering in the control of *T. gondii* infection. However, further data are necessary to understand the role of critical cytokines in host-parasite interactions, particularly at the maternal-fetal interface, in order to contribute to the development of new effective drugs to prevent congenital toxoplasmosis.

MATERIALS AND METHODS

Ethics Statement, Cell Culture, and Parasites

The present research protocol was submitted to and approved by the Ethics Committee of the Federal University of Uberlandia, MG, Brazil (protocol number 197/08). The BeWo cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in 75-cm^2 culture flasks in Roswell Park Memorial Institute (RPMI) 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS) (Cultilab) in a humidified incubator at 37°C and 5% CO_2 [19].

Tachyzoites of the *T. gondii* 2F1 strain, which constitutively express cytoplasmic beta-galactosidase and are derived from the highly virulent RH strain, were a gift from Dr. Vern Carruthers (Medical School of Michigan University). The parasites were propagated in BeWo cells maintained in RPMI 1640 medium supplemented with penicillin, streptomycin, and 2% FBS at 37°C and 5% CO_2 [19].

T. gondii *Infection in BeWo Cells Treated with IL10, TGFB1, or IFNG*

In the first set of experiments, BeWo cells were cultured in 6-well plates (1×10^6 cells/well/ml) in RPMI 1640 medium with 10% FBS, at 37°C in 5% CO_2, and after 24 h, the cells were treated as follows: 1) stimulated with recombinant cytokines, rIL10 or rTGFB1 (R&D Systems, Minneapolis, MN), at 50 ng/ml [15] for 1, 3, 6, and 24 h in RPMI 1640 medium with 2% FBS; 2) infected by *T. gondii* at the proportion of five parasites per cell (5:1) for 1, 3, 6, and 24 h in RPMI 1640 medium with 2% FBS; or 3) infected by *T. gondii* (5:1) in RPMI 1640 medium with 2% FBS, washed with medium after 3 h to remove extracellular parasites, and then treated with rIL10 or rTGFB1 (50 ng/ml) for 1, 3, 6, and 24 h in RPMI 1640 medium with 2% FBS, according to previous studies [37, 38]. Uninfected and untreated cells were used as controls and maintained in RPMI 1640 medium with 2% FBS. Next, cells were harvested, centrifuged at $400 \times g$ for 5 min and lysed on ice with lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol, and 200 mM NaCl) supplemented with a protease inhibitor cocktail (Complete; Roche Diagnostic, Mannheim, Germany), 1 mM sodium orthovanadate (Na_3VO_4) and sodium fluoride (NaF) (both from Sigma) and submitted to three freeze-thaw cycles [19]. After centrifugation at $15\,000 \times g$ for 15 min at 4°C, the supernatants were collected, the concentration of total protein was measured using the Bradford assay [39], and the samples were submitted to Western blot analysis.

In a second set of experiments, *T. gondii* proliferation in BeWo cells treated with several concentrations of rIFNG was analyzed because our previous studies demonstrated differential infectivity of these cells in the presence of this cytokine [14, 15]. For this purpose, BeWo cells were cultured in 96-well plates (2×10^4 cells/well/200 μl) in RPMI 1640 medium with 10% FBS at 37°C and 5% CO_2 for 24 h. Next, cells were infected with *T. gondii* tachyzoites (5:1) in RPMI 1640 medium with 2% FBS, and, after 3 h, cells were washed to remove extracellular parasites and treated with increasing concentrations (20, 50, 100, 150, and 200 ng/ml) of rIFNG (Invitrogen Life Technologies, Carlsbad, CA) for an additional 24 h in RPMI 1640 medium with 2% FBS. Cells were analyzed for *T. gondii* intracellular proliferation using a colorimetric beta-galactosidase assay as previously described [19]. *T. gondii* intracellular proliferation data were expressed as the number of tachyzoites calculated in relation to the reference curve of 2F1 strain tachyzoites, ranging from 1×10^6 to 15.625×10^3 parasites.

In a third set of experiments, the intracellular signaling pathway dependent on STAT1 was verified in BeWo cells treated with rIFNG. BeWo cells were cultured in 6-well plates (1×10^6 cells/well/ml) as described above and treated as follows: 1) infected by *T. gondii* (5:1) for 1, 6, and 24 h in RPMI 1640 medium with 2% FBS; 2) treated with rIFNG at 20 or 100 ng/ml for 1, 6, and 24 h in RPMI 1640 medium with 2% FBS; or 3) infected by *T. gondii* (5:1), washed with RPMI medium after 3 h to remove extracellular parasites, and treated with rIFNG at 20 or 100 ng/ml for 1, 6, and 24 h in RPMI 1640 medium with 2% FBS [31]. Uninfected and untreated cells were used as controls and maintained in RPMI 1640 medium with 2% FBS. Next, cells were harvested and analyzed as described above for the first set of experiments.

Western Blot Analysis for Phosphorylated Proteins

Total protein samples (60 μg) were subjected to polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE at 8% or 12%), and the proteins were electrotransferred to polyvinylidene fluoride membranes (Thermo Scientific, Rockford, IL). Blotted membranes were blocked with 4% skimmed milk in Tris-buffered saline solution (TBS, TRIS 25 mM and NaCl 0.15 M, pH 7.4) for 1 h at room temperature and incubated overnight with the following primary antibodies: mouse monoclonal anti-phosphoSTAT3 (Tyr^{705}) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-total STAT3 (1:500; R&D Systems); rabbit polyclonal anti-phosphoSmad2 ($S^{465/467}$) (1:1000; Millipore, São Paulo, SP, Brazil) or mouse monoclonal anti-total Smad2/3 (1:1000; Santa Cruz Biotechnology); rabbit polyclonal anti-phosphoSTAT1 (Tyr^{701}) (1:1000; R&D Systems) or mouse monoclonal anti-total STAT1 (1:500; Invitrogen); or mouse monoclonal anti-beta-actin (1:1000; Santa Cruz Biotechnology) in TBS with 2% skimmed milk. Next, the membranes were incubated with the respective peroxidase-labeled secondary antibodies (1:3000; Jackson ImmunoResearch Laboratories, West Grove, PA) in TBS with 2% skimmed milk for 2 h at room temperature. The reaction was revealed by chemiluminescence (ECL SuperSignal kit; Thermo Scientific) and equal loading of the proteins was confirmed by staining the blots with 1% Ponceau solution. Densitometric analyses were performed using the KODAK software (1D Image Analysis Software 3.5) in order to determine the mean intensity of the bands [19]. The data were expressed as relative density of the ratio between the phosphorylated protein and beta-actin bands.

T. gondii *Infection in BeWo Cells Treated with STAT3, Smad2, or STAT1 Inhibitors*

First, the cytotoxicity of STAT3, Smad2, or STAT1 inhibitors in BeWo cells was verified. Cells were seeded in 96-well plates (1×10^5 cells/well/200 μl) for 24 h and treated or not with increasing concentrations (0.1–10 μM) of STAT3 (WP1066, Santa Cruz Biotechnology), Smad2 (SB431542, Sigma) or STAT1 (Fludarabine, Sigma) inhibitors in RPMI 1640 medium with 10% FBS at 37°C in 5% CO_2 for an additional 24 h. Cell viability was evaluated using the tetrazolium salt colorimetric (MTT) assay as previously described [40]. The data were analyzed as percentage of viable cells in comparison to untreated cells (100% of cell viability).

Next, the BeWo cells were cultured in 96-well plates (2×10^4 cells/well/200 μl) for 24 h, pretreated with WP1066 (0.1–0.5 μM), SB431542 (2.5–10 μM), or Fludarabine (0.1–0.5 μM) for 2 h and incubated with tachyzoites of *T.*

gondii (5:1) for 3 h. Cells were washed to remove extracellular parasites and again treated with WP1066, SB431542, or Fludarabine in the presence or absence of rIL10 (50 ng/ml), rTGFB1 (50 ng/ml), or rIFNG (20 ng/ml) for an additional 24 h, respectively. Infected and untreated cells or infected and cytokine-treated cells were used as controls. In parallel, uninfected BeWo cells were only treated with rIL10 (50 ng/ml), rTGFB1 (50 ng/ml), or rIFNG (20 ng/ml) for 24 h. Untreated and uninfected cells were used as controls. Finally, the cell-free supernatants were collected and stored at −80°C for the subsequent analysis of cytokines by cytometric bead array (CBA). *T. gondii*-infected cells were analyzed for parasite intracellular proliferation by beta-galactosidase assay as previously described [19]. In a set of parallel experiments, BeWo cells (2×10^4 cells/well/200 μl in 96-well plates), infected or not by *T. gondii,* were treated with 100 ng/ml rIFNG for 24 h; the cell-free supernatants were collected for the posterior measurement of cytokines by CBA.

Measurement of Th1, Th2, and IL17A Cytokines by CBA

Human cytokines (IL2, IFNG, tumor necrosis factor alpha [TNFA], IL6, IL4, IL10, and IL17A) were measured in supernatants of BeWo cells using the Th1/Th2/Th17 kit (BD Bioscience, San Jose, CA) by CBA according to the manufacturer's instructions. Samples were analyzed under BD flow cytometry (FACSCalibur; BD Company, San Diego, CA), and data were recorded using specialized BD Cell Quest software and CBA software. The results were expressed as mean fluorescence intensity (MFI) [17, 19, 41].

T. gondii *Intracellular Proliferation in BeWo Cells Treated with IL6 or TNFA*

BeWo cells were cultured in 96-well plates (2×10^4 cells/well/200 μl) for 24 h and primed or not with rTNFA (Millipore) or rIL6 (R&D Systems) at concentrations ranging from 100 to 500 pg/ml for an additional 24 h. Cells were infected by *T. gondii* (5:1) for 3 h, treated again with rTNFA or rIL6 for an additional 24 h, and analyzed for parasite proliferation using the colorimetric beta-galactosidase assay as previously described [19].

Statistical Analysis

All the data were expressed as mean ± SEM of three independent experiments performed in triplicate. Differences among groups were assessed by one-way ANOVA with the Bonferroni multiple comparison post hoc test using GraphPad Prism version 4.0 software (GraphPad Software, Inc., San Diego, CA) being considered significant when $P < 0.05$.

RESULTS

Treatment of BeWo Cells with IL10 and TGFB1 Up-Regulates T. gondii *Infection in a STAT3- and Smad2-Dependent Manner, Respectively*

The intracellular signaling pathways activated by IL10 and TGFB1 in BeWo cells infected by *T. gondii,* which could be involved in the control of parasite proliferation, were investigated (Fig. 1A–F). Higher levels of STAT3 phosphorylation were observed in BeWo cells stimulated with rIL10 after 1, 3, and 6 h, regardless of *T. gondii* infection, when compared to untreated cells ($P < 0.05$; Fig. 1, A and B). In addition, after 1 h of treatment with rIL10, BeWo cells infected by *T. gondii* showed higher STAT3 phosphorylation than treated, but uninfected, cells ($P < 0.05$; Fig. 1, A and B). Furthermore, no STAT3 phosphorylation was observed in BeWo cells treated with rIL10 in the presence or absence of *T. gondii* for 24 h or in untreated and infected cells in all the time points analyzed (Fig. 1, A and B).

Significant Smad2 phosphorylation was detected in uninfected cells treated with rTGFB1 for 3, 6, and 24 h when compared to untreated cells ($P < 0.05$; Fig. 1, D and E). Furthermore, the Smad2 phosphorylation in these cells was time-dependent, with higher phosphorylation after 24 h ($P < 0.05$; Fig. 1, D and E). When BeWo cells were infected by *T. gondii* and treated with rTGFB1, Smad2 phosphorylation was higher in the first hours of treatment (1 and 3 h) in comparison to untreated cells or to the respective controls in the absence of infection ($P < 0.05$; Fig. 1, D and E). Furthermore, no Smad2 phosphorylation was observed in untreated BeWo cells infected by *T. gondii* (Fig. 1, D and E).

In order to confirm these findings, experiments were performed using the inhibitors of these intracellular pathways. First, the concentration of inhibitors that did not alter the cell viability was determined. The MTT analyses demonstrated no change in cell viability when BeWo cells were treated with STAT3 (WP1066) or Smad2 (SB431542) inhibitors, except for 10 μM WP1066 (data not shown). Thus, concentrations ranging from 0.1 to 0.5 μM for WP1066 and 2.5 to 10 μM for SB431542 were selected in further experiments.

BeWo cells treated with rIL10 or rTGFB1 showed increased *T. gondii* proliferation in comparison with untreated cells ($P < 0.05$; Fig. 1, C and F). However, the parasite proliferation decreased significantly when BeWo cells were treated with WP1066 or SB431542 inhibitors in the presence or absence of rIL10 or rTGFB1 when compared with untreated or cytokine-treated cells ($P < 0.05$; Fig. 1, C and F).

Absence of STAT1 Phosphorylation Is Associated with Increased T. gondii *Intracellular Proliferation in BeWo Cells*

Our previous studies demonstrated that IFNG was unable to control *T. gondii* infection [14] or significantly increased the infectivity in BeWo cells [15]. To further understand this phenomenon, experiments were carried out to establish the concentrations of IFNG that cause these effects in infected BeWo cells. The treatment of BeWo cells with 20 ng/ml rIFNG did not control the parasite proliferation when compared to untreated cells (Fig. 2A). Moreover, increased *T. gondii* proliferation was detected in BeWo cells treated with rIFNG (50–200 ng/ml) in comparison to untreated cells ($P < 0.05$; Fig. 2A). Thus, the concentrations of 20 and 100 ng/ml were chosen to verify the STAT1 phosphorylation.

STAT1 phosphorylation was detected in uninfected BeWo cells after treatment with 20 ng/ml rIFNG for 1, 6, and 24 h, although this phosphorylation was significant only after 6 h of treatment ($P < 0.05$; Fig. 2, B and C). Under *T. gondii* infection, however, STAT1 phosphorylation increased after rIFNG treatment when compared to untreated cells or respective controls in the absence of infection ($P < 0.05$; Fig. 2, B and C) at all of the time points analyzed. Furthermore, STAT1 phosphorylation was not observed in untreated and infected cells (Fig. 2, B and C). On the other hand, no STAT1 phosphorylation was detected in uninfected or infected BeWo cells after treatment with 100 ng/ml rIFNG at any time (Fig. 2D).

In order to confirm the effect of STAT1 in the control of parasite proliferation, we performed experiments using the STAT1 inhibitor (Fludarabine) in the presence or absence of 20 ng/ml rIFNG because only this concentration was able to trigger STAT1 phosphorylation in BeWo cells. We first determined the concentration of the inhibitor that did not alter cell viability. The MTT analyses demonstrated decreased cell viability after the treatment of BeWo cells with 5 and 10 μM Fludarabine when compared to untreated cells (data not shown). Fludarabine concentrations ranging from 0.1 to 0.5 μM were selected for use in further experiments. As previously observed, BeWo cells treated with 20 ng/ml rIFNG were not able to control *T. gondii* proliferation in relation to untreated cells (Fig. 2E), but when treated with 0.1–0.5 μM Fludarabine in the absence or presence of rIFNG (20 ng/ml), the parasite proliferation increased in comparison with untreated or only rIFNG-treated cells, respectively ($P < 0.05$; Fig. 2E).

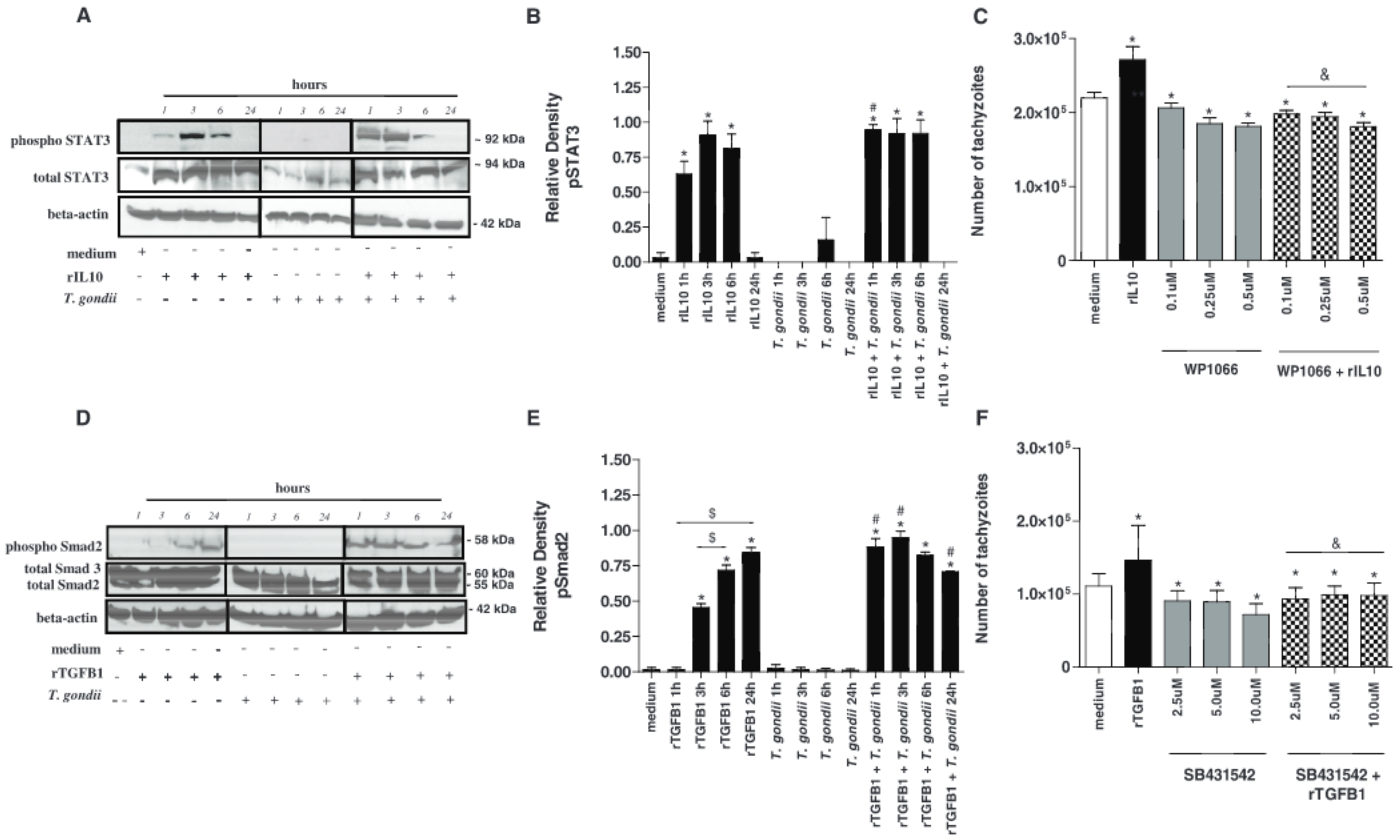

FIG. 1. Detection of STAT3 or Smad2 phosphorylation and *T. gondii* proliferation in BeWo cells (**A**–**F**). Untreated (medium) or cytokine-treated cells were submitted to Western blot analysis to detect STAT3 or Smad2 phosphorylation (phosphoSTAT3 or phosphoSmad2), total proteins, and beta-actin. Representative Western blots for phosphoSTAT3 (**A**) and phosphoSmad2 (**D**) and densitometric analyses (**B**, **E**) of the respective proteins calculated from the ratio between the phosphoproteins and beta-actin bands. In parallel, BeWo cells were pretreated or not (medium) with WP1066 (**C**) or SB431542 (**F**) in the presence or absence of rIL10 or rTGFB1 (both 50 ng/ml), and the number of tachyzoites was determined via a colorimetric microtiter assay. The data were expressed as the mean ± SEM of three independent experiments performed in triplicate. All the data were analyzed by one-way ANOVA with the Bonferroni multiple comparison post hoc test. Significant differences in comparison with medium (*$P < 0.05$), between the different times of rTGFB1 treatment in the absence of infection ($^{\$}P < 0.05$), with the respective cytokine treatments in the absence of infection ($^{\#}P < 0.05$), or with the respective cytokine treatments in the absence of the inhibitors (WP1066 or SB431542) ($^{\&}P < 0.05$).

Treatment of BeWo Cells with TGFB1 and IL10 Down-Modulates IL6 and TNFA Production in a Smad2- and STAT3-Dependent Manner, Respectively

To verify the effect of IL10 and TGFB1 on the production of Th1/Th2/IL17A cytokines in BeWo cells infected or not by *T. gondii*, the levels of cytokines in cell-free supernatants were measured using the CBA assay (Fig. 3A–D). When BeWo cells were infected by *T. gondii* but not treated with rIL10 or rTGFB1, a significant production of IL6 (Fig. 3A) and IL17A (Fig. 3C) ($P < 0.05$) was verified, while no change in TNFA (Fig. 3B) or IL10 (Fig. 3D) release was detected in the supernatant in comparison with untreated and uninfected cells (medium).

Treatment with rIL10 in uninfected cells reduced the levels of IL6 in relation to untreated and uninfected cells (medium) ($P < 0.05$; Fig. 3A). In the presence of infection, rIL10-treated cells showed higher IL6 secretion when compared to untreated and uninfected cells (medium) ($P < 0.05$), but the IL6 release decreased in comparison to untreated and infected cells ($P < 0.05$; Fig. 3A). When the STAT3 inhibitor (WP1066) was added in the presence of rIL10, IL6 levels were reduced in infected BeWo cells in comparison to untreated and uninfected cells (medium) or to the respective controls in the absence of the inhibitor ($P < 0.05$; Fig. 3A). Likewise, rIL10-treated and infected cells demonstrated reduced TNFA production in comparison to untreated and uninfected cells (medium) or only infected cells ($P < 0.05$; Fig. 3B), and the WP1066 treatment promoted an up-regulation of TNFA release when compared to untreated and uninfected cells (medium) or to the respective controls in the absence of the inhibitor, regardless of infection ($P < 0.05$; Fig. 3B). Increased IL17A production in BeWo cells was detected in the presence of rIL10 and *T. gondii* when compared to untreated and uninfected cells (medium) ($P < 0.05$; Fig. 3C). Moreover, IL17A levels were reduced in infected BeWo cells treated with WP1066 when compared to untreated and uninfected cells (medium) or to the respective controls in the absence of the inhibitor ($P < 0.05$; Fig. 3C). IL10 was not determined in the supernatant of BeWo cells treated with rIL10 (Fig. 3D).

At the same time, when BeWo cells were treated with rTGFB1, reduced levels of IL6 were observed regardless of infection in comparison to untreated and uninfected cells (medium) or only infected cells ($P < 0.05$; Fig. 3A), while increased IL17A secretion was detected in infected cells in relation to untreated cells and uninfected cells (medium) ($P < 0.05$; Fig. 3C). In the presence of rTGFB1 and its Smad2 inhibitor (SB431542), up-regulation of IL6 was verified when compared to untreated and uninfected cells (medium) or to the respective controls in the absence of the inhibitor, regardless of infection ($P < 0.05$; Fig. 3A), but rTGFB1 plus SB431542 did not alter the IL17 release in relation to any experimental

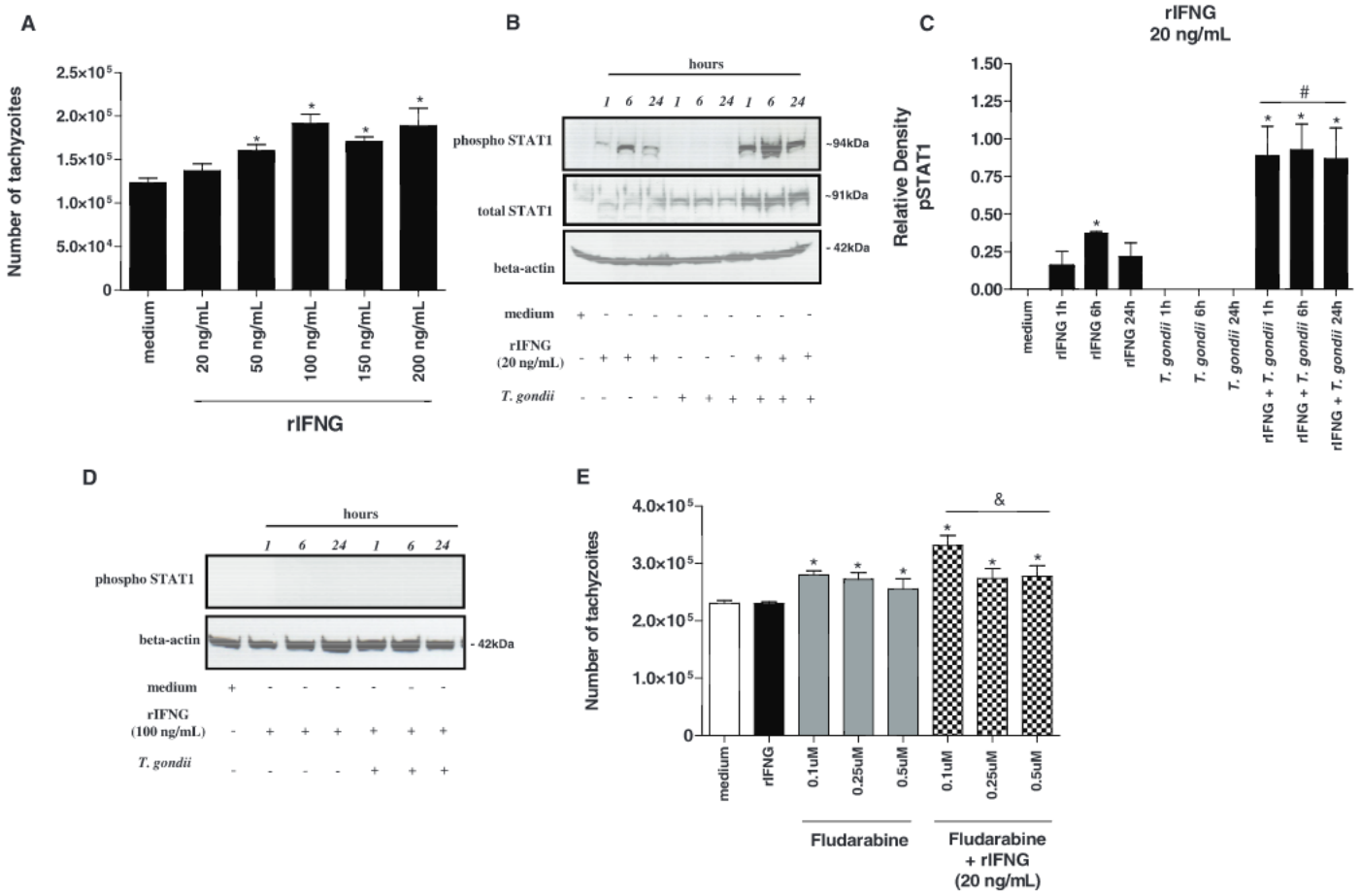

FIG. 2. Detection of STAT1 phosphorylation and *T. gondii* proliferation in BeWo cells (**A**–**E**). Cells were exposed to *T. gondii*, treated or not (medium) with rIFNG (20–200 ng/ml), and the number of tachyzoites (**A**) was determined via a colorimetric microtiter assay. Next, BeWo cells were incubated or not (medium) with rIFNG (20 or 100 ng/ml) for several times in the presence or absence of *T. gondii* infection. All the cells were submitted to Western blot analysis to detect STAT1 phosphorylation (phosphoSTAT1), total protein, and beta-actin. Representative Western blots for phosphoSTAT1 (**B**, **D**) and densitometric analyses (**C**) of the respective protein calculated from the ratio between the phosphoprotein and beta-actin bands. Also, BeWo cells were pretreated or not (medium) with Fludarabine in the presence or absence of rIFNG (20 ng/ml), and the number of tachyzoites (**E**) was determined via a colorimetric microtiter assay. The data were expressed as the mean ± SEM of three independent experiments performed in triplicate. All the data were analyzed by one-way ANOVA with the Bonferroni multiple comparison post hoc test. Significant differences in comparison with medium ($^{*}P < 0.05$), with the respective cytokine treatments in the absence of infection ($^{\#}P < 0.05$), or with the respective cytokine treatments in the absence of the inhibitor (Fludarabine) ($^{\&}P < 0.05$).

condition (Fig. 3C). No change in TNFA (Fig. 3B) or IL10 (Fig. 3D) production was observed in uninfected or infected BeWo cells treated with rTGFB1 and/or Smad2 inhibitor (SB431542) in relation to untreated and uninfected cells (medium) or to the respective controls in the absence of the inhibitor. IL2, IFNG, and IL4 production were not observed in BeWo cells under any experimental conditions.

The IL6 and TNFA Production in BeWo Cells Is Dose-Dependent on IFNG and, Consequently, on STAT1 Phosphorylation

To verify the effect of different concentrations of IFNG on the production of Th1/Th2/IL17A cytokines in BeWo cells infected or not by *T. gondii*, the levels of cytokines in cell-free supernatants were measured using the CBA assay (Fig. 4A–D). As described above, only significant levels of IL6 (Fig. 4A) and IL17A (Fig. 4C) ($P < 0.05$) were detected in the supernatant from untreated and infected cells in comparison with untreated and uninfected cells (medium). Likewise, uninfected or infected BeWo cells treated with 20 ng/ml rIFNG increased the levels of IL6 (Fig. 4A) and IL17A (Fig. 4C) in comparison to untreated and uninfected cells (medium) ($P < 0.05$). No significant change in TNFA (Fig. 4B) and IL10 (Fig. 4D) release was observed in BeWo cells treated with 20 ng/ml rIFNG when compared to untreated and uninfected cells (medium). In the presence of STAT1 inhibitor (Fludarabine), both infected and uninfected BeWo cells treated with 20 ng/ml rIFNG showed down-modulation of IL6 (Fig. 4A) and IL17A (Fig. 4C) and up-regulation of IL10 (Fig. 4D) release in relation to the respective controls in the absence of the inhibitor ($P < 0.05$). Also, treatment with rIFNG and Fludarabine only triggered the up-regulation of TNFA in uninfected cells when compared to untreated and uninfected cells (medium) or to the respective control in the absence of the inhibitor ($P < 0.05$; Fig. 4B).

However, the treatment of BeWo cells with 100 ng/ml rIFNG reduced the levels of IL6 (Fig. 4A) and TNFA (Fig. 4B) in the supernatants, regardless of infection, when compared to untreated and uninfected cells (medium) or to the cells treated with 20 ng/ml rIFNG ($P < 0.05$). No significant change in IL17A (Fig. 4C) and IL10 (Fig. 4D) release was observed in BeWo cells treated with 100 ng/ml rIFNG when compared to

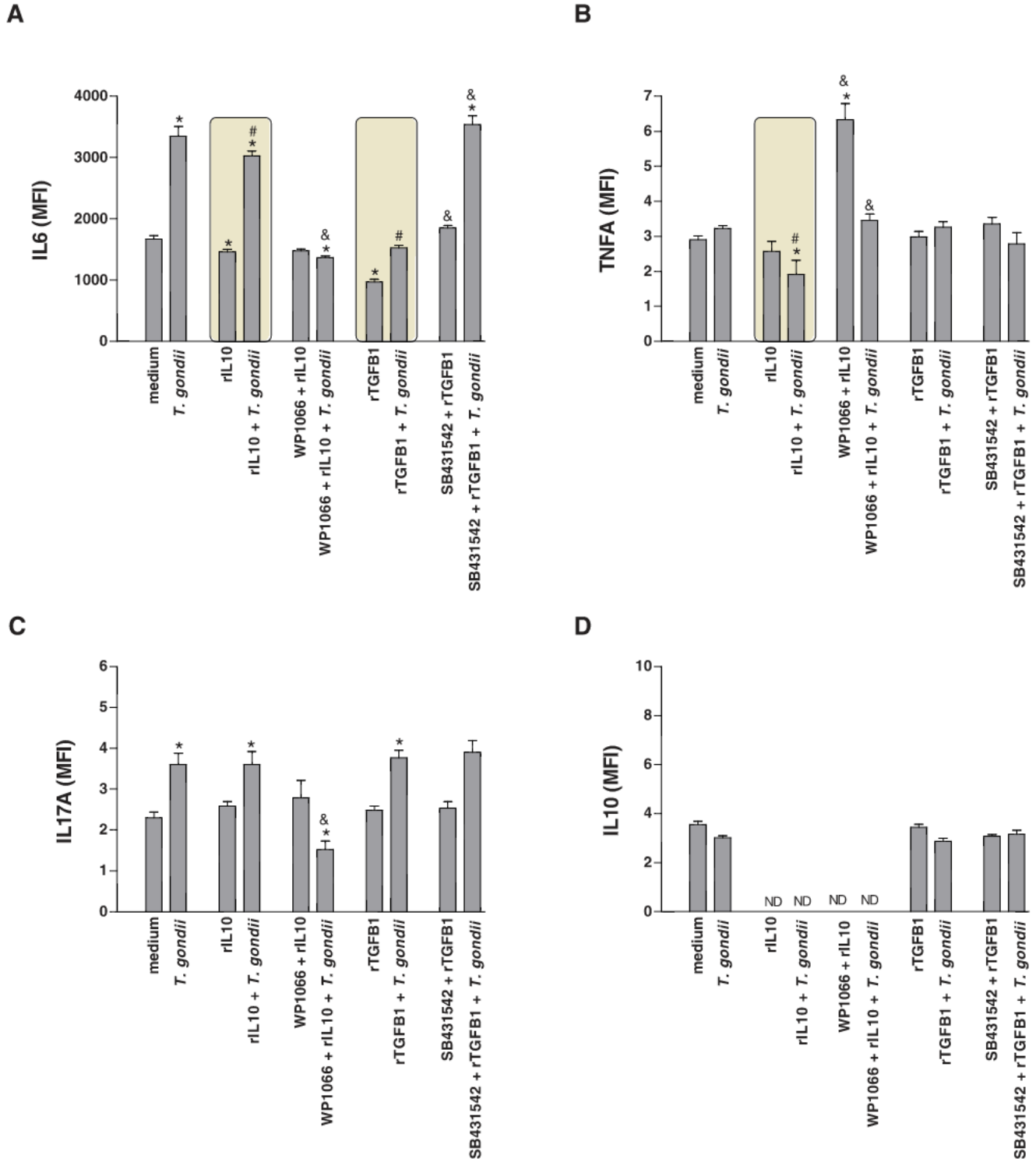

FIG. 3. Cytokine production by uninfected or *T. gondii*-infected BeWo cells under the effect of IL10, TGFB1, or WP1066 and SB431542 inhibitors (**A**–**D**). Levels of IL6 (**A**), TNFA (**B**), IL17A (**C**), and IL10 (**D**) were determined in untreated and uninfected cells (medium), infected cells only, rIL10-treated cells, rTGFB1-treated cells, WP1066-treated cells, and SB431542-treated cells, in the presence or absence of infection. Data were expressed as the mean ± SEM of fluorescence intensity (MFI) of three independent experiments performed in triplicate. All data were analyzed by one-way ANOVA with the Bonferroni multiple comparison post hoc test. Significant differences in comparison with medium ($^{*}P < 0.05$), with untreated and infected cells ($^{\#}P < 0.05$), and with respective cytokine treatments in the absence of the inhibitors (WP1066 and SB431542) ($^{\&}P < 0.05$). ND: not determined. Tan rectangles show the significant effects of IL10 and TGFB1 in the IL6 and TNFA production.

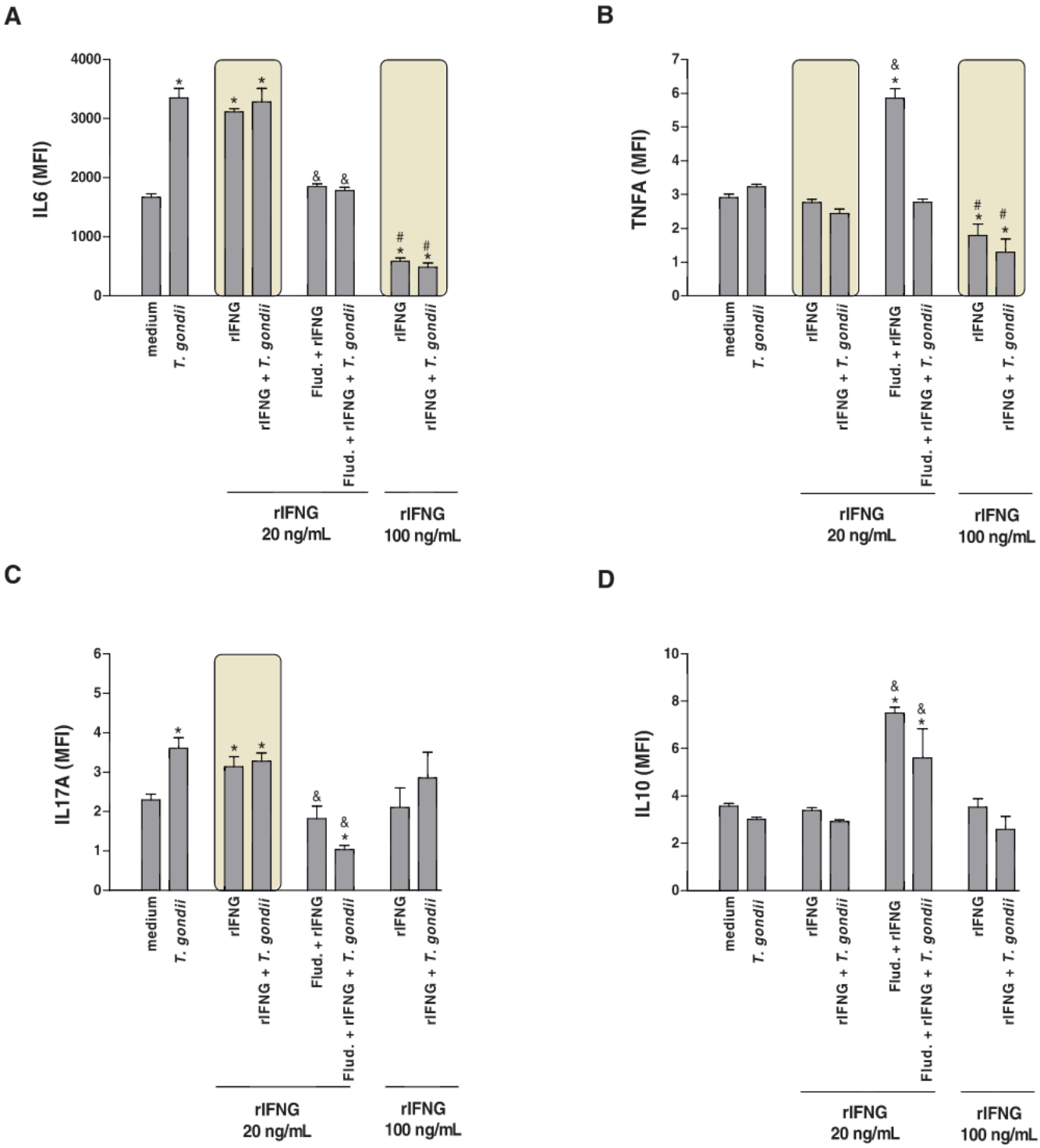

FIG. 4. Cytokine production by uninfected or *T. gondii*-infected BeWo cells under the effect of 20 or 100 ng/ml IFNG and Fludarabine inhibitor (**A**–**D**). Levels of IL6 (**A**), TNFA (**B**), IL17A (**C**), and IL10 (**D**) were determined in untreated and uninfected cells (medium), infected cells only, rIFNG-treated cells, and Fludarabine-treated cells in the presence or absence of infection. Data were expressed as the mean ± SEM of fluorescence intensity (MFI) of three independent experiments performed in triplicate. All the data were analyzed by one-way ANOVA with the Bonferroni multiple comparison post hoc test. Comparisons and significant differences of the following conditions were determined: with medium ($^{*}P < 0.05$), with untreated and infected cells ($^{\#}P < 0.05$), and with respective cytokine treatments in the absence of the inhibitor (Fludarabine) ($^{\&}P < 0.05$). Tan rectangles show the significant effects of different concentrations of IFNG in the IL6, TNFA, and IL17A production.

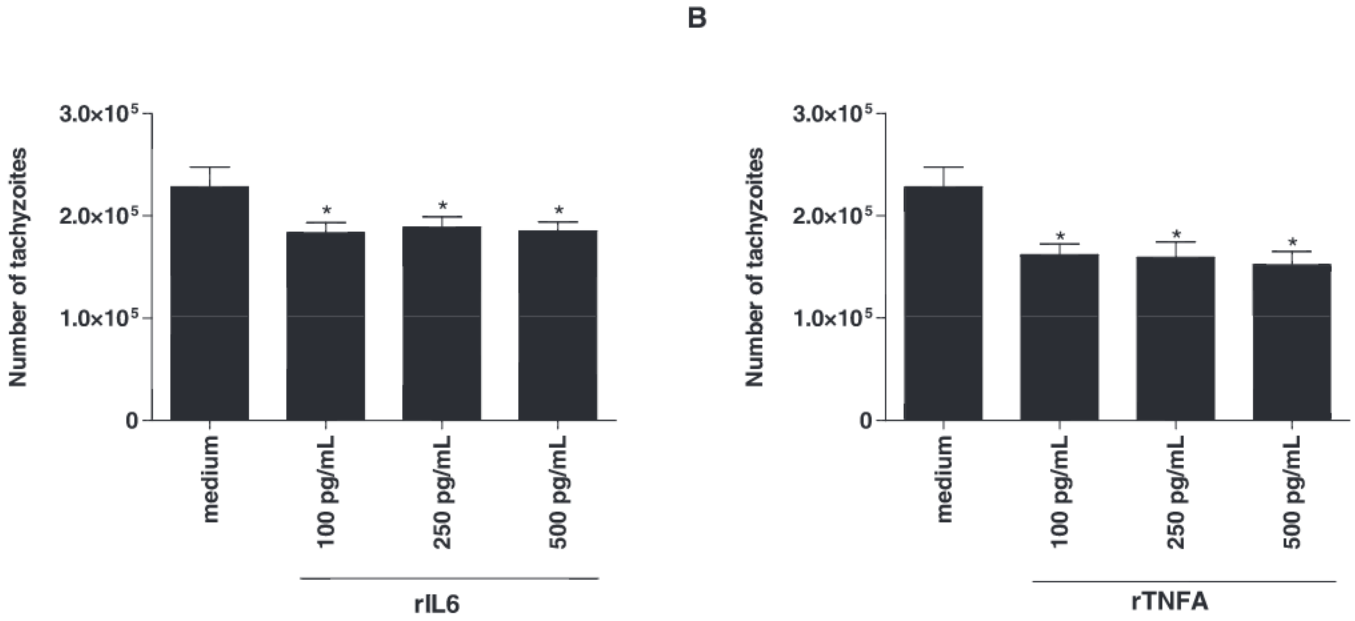

FIG. 5. *T. gondii* proliferation in BeWo cells treated with IL6 (**A**) and TNFA (**B**). BeWo cells were treated or not (medium) with rIL6 or rTNFA, and the number of tachyzoites was determined via a colorimetric microtiter assay. Data were expressed as the mean ± SEM of the number of tachyzoites calculated from a reference curve and are representative of three independent experiments performed in triplicate. All the data were analyzed by one-way ANOVA with the Bonferroni multiple comparison post hoc test. Significant differences in comparison with medium (*$P < 0.05$).

untreated and uninfected cells (medium) or to cells treated with 20 ng/ml rIFNG.

IL6 and TNFA Control T. gondii *Infection in BeWo Cells*

In order to confirm whether TNFA and IL6 are involved in the control of *T. gondii* growth in BeWo cells, cells were infected and treated with these recombinant cytokines. BeWo cells treated with rTNFA or rIL6, at concentrations ranging from 100 to 500 pg/ml, showed significantly reduced parasite proliferation in comparison to untreated cells ($P < 0.05$; Fig. 5, A and B).

Proposed Model of the Intracellular Mechanisms Triggered by IL10, TGFB1, and IFNG in BeWo Cells During T. gondii *Infection*

Based on our results, a proposed model of the effector mechanisms activated by IL10, TGFB1, and IFNG in BeWo cells infected by *T. gondii* is shown in Figure 6. When BeWo cells are treated with rIL10, STAT3 phosphorylation is triggered and promotes the down-modulation of IL6 and TNFA and, consequently, an increase in the number of *T. gondii* tachyzoites (Fig. 6A). In the presence of rTGFB1, Smad2 phosphorylation is triggered with the subsequent reduced IL6 production, and consequently an increased number of *T. gondii* tachyzoites (Fig. 6B). Finally, when BeWo cells are treated with 20 ng/ml rIFNG (low concentration), STAT1 phosphorylation and levels of IL6 and IL17A are up-regulated with no control of parasite proliferation (Fig. 6C, left). However, the treatment of BeWo cells with 100 ng/ml rIFNG (high concentration) does not trigger STAT1 phosphorylation with the subsequent reduction of IL6 and TNFA release, favoring *T. gondii* proliferation (Fig. 6C, right).

DISCUSSION

In the present study, we investigated the effector mechanisms triggered by IL10, TGFB1, and IFNG in BeWo trophoblast cells that could explain the increased *T. gondii* proliferation in these cells under these cytokine treatments. For this purpose, we investigated the intracellular signaling pathways and cytokine production involved in *T. gondii* proliferation in this trophoblast model.

Our data demonstrated early increased STAT3 phosphorylation in BeWo cells treated with rIL10 regardless of *T. gondii* infection. In addition, we also verified the higher parasite proliferation in BeWo cells treated with rIL10, in agreement with our previous study [15]. When we investigated *T. gondii* proliferation in IL10-treated cells in the presence of STAT3 inhibitor, it was reduced to levels smaller than untreated or cells only treated with IL10. It was concluded that the higher *T. gondii* infectivity induced by IL10 in BeWo cells was dependent of STAT3 activation. To the best of our knowledge, the present study for the first time shows increased *T. gondii* infection in BeWo trophoblast cells in a manner dependent on STAT3 phosphorylation induced by IL10. Recent studies have demonstrated STAT3 phosphorylation triggered by IL10 in different experimental models in order to modulate the immune response favoring infection. STAT3 phosphorylation has been shown to be essential for the survival and proliferation of human trophoblast cells from JEG-3 lineage [42]. It has been shown that supernatants of *Lactobacillus rhamnosus* cultures induced STAT3 phosphorylation and IL10 production in human trophoblast cells, contributing to the immunosuppression at the maternal-fetal interface [43]. Mesenchymal stem cells from rat bone marrow down-modulated the maturation of murine dendritic cells when IL10 triggered the JAK1/STAT3 signaling pathway [44]. Furthermore, IL10 and STAT3 are involved in the increased susceptibility to infections caused by *Leishmania amazonensis*, *Leishmania donovani*, the Newcastle disease virus, and *Listeria monocytogenes* [45–48]. According to our present data, IL10 also promoted higher infectivity during *T. gondii* infection in murine dendritic cells [49], and increased IL10 levels were associated with the high susceptibility to reinfection with different recombinant *T. gondii* strains in BALB/c mice [50]. Thus, IL10 and STAT3 are involved in

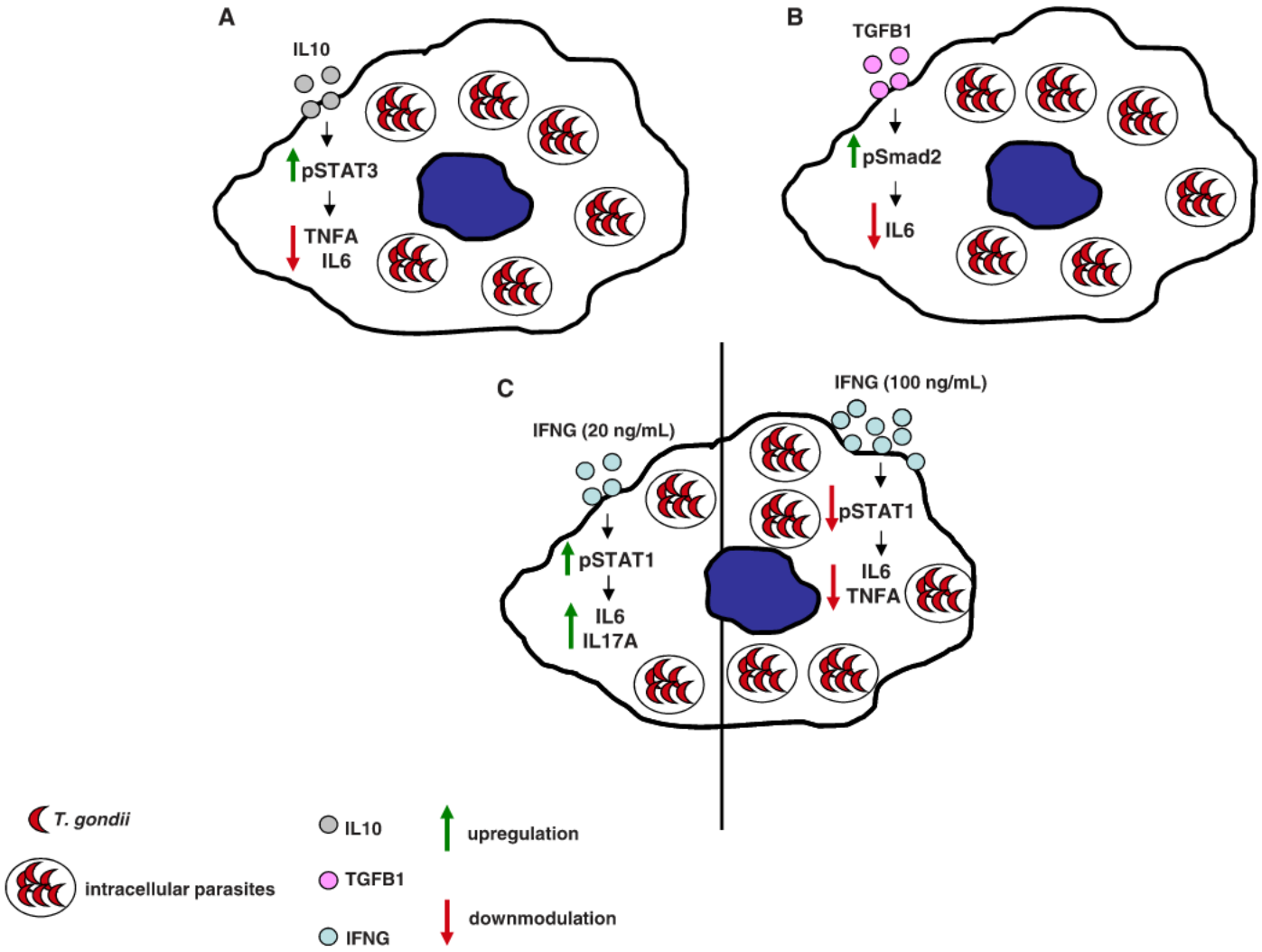

FIG. 6. Proposed model showing the effects of IL10, TGFB1, and IFNG in *T. gondii*-infected BeWo cells. **A**) IL10-treated cells demonstrating STAT3 phosphorylation (up-regulation), down-modulation of TNFA and IL6 release, and an increased number of *T. gondii* tachyzoites. **B**) TGFB1-treated cells showing Smad2 phosphorylation (up-regulation), down-modulation of IL6 release, and a higher number of *T. gondii* tachyzoites. **C**) Left: cells treated with 20 ng/ml IFNG (low concentration) showing STAT1 phosphorylation (up-regulation), up-regulation of IL6 and IL17A release, and, consequently, no control of the *T. gondii* proliferation. Right: cells treated with 100 ng/ml IFNG (high concentration) showing no STAT1 phosphorylation (down-modulation), down-modulation of IL6 and TNFA release, and, consequently, higher *T. gondii* proliferation. Up-regulation: green upward arrow; down-modulation: red downward arrow.

the higher susceptibility to many types of infection, including *T. gondii* in the maternal-fetal interface, and our present findings are the first to show this type of effect in a human trophoblast model.

Similarly to the IL10 data, a previous study by our group also demonstrated that TGFB1 up-regulated *T. gondii* proliferation in BeWo cells [15]. Furthermore, we observed Smad2 phosphorylation induced by TGFB1 in BeWo cells, regardless of *T. gondii*. The number of tachyzoites was significantly reduced when BeWo cells were treated with TGFB1 and Smad2 inhibitor, demonstrating that the higher infectivity by *T. gondii* in BeWo cells in the presence of TGFB1 was dependent on Smad2 activation. Thus, at least in BeWo trophoblast cells, TGFB1 triggers Smad2-dependent intracellular pathways and is significantly associated with increased parasite growth. In agreement with our data, several studies have shown Smad2 phosphorylation triggered by TGFB, but this was during the immune response in T cells [51–53]. The Smad2 phosphorylation induced by TGFB is essential for Th17 cell generation [51, 53]. IFNG production by $CD8^+$ T cells during acute and chronic feline immunodeficiency virus infection was down-modulated by T regulatory cells, and this mechanism of suppression was associated with Smad2 phosphorylation triggered by TGFB [52]. Also, it was previously shown that TGFB signaling can also operate in Smad-independent pathways [54, 55], but future studies are necessary to investigate these alternative pathways in BeWo cells.

Next, we investigated the role of the IL10/STAT3 and TGFB/Smad2 pathways in cytokine production. Down-modulation of TNFA and IL6 levels was detected in BeWo cells treated with IL10. These findings were confirmed in the presence of the STAT3 inhibitor (WP1066) where up-regulation of TNFA was detected in cells treated with IL10 and WP1066, showing that the TNFA release in BeWo trophoblast cells is dependent on STAT3 down-modulation. However, this up-regulatory effect was not observed for IL6 in BeWo cells treated with STAT3 inhibitor, probably because this transcriptional factor is also involved in the induction of IL6 production. Therefore, in our experimental model, the increased *T. gondii* infection in BeWo cells under IL10

treatment was associated with reduced levels of these cytokines, IL6 and TNFA, and this effect was dependent on STAT3 phosphorylation. We also demonstrated that TGFB1 down-modulated IL6 in BeWo cells and was dependent on Smad2 phosphorylation because up-regulation of IL6 was detected in cells treated with TGFB1 and Smad2 inhibitor. Subsequently, we hypothesized that the increased infectivity to *T. gondii* under TGFB1 or IL10 treatments was associated with IL6 or TNFA reduction. This hypothesis was strengthened because a decrease in the parasite proliferation in BeWo cells treated with TNFA and IL6 recombinant cytokines was verified in our experimental model. Higher *T. gondii* proliferation in BeWo trophoblast cells was observed during treatment with IL10 or TGFB1 because these cytokines are able to down-modulate IL6 and TNFA in a manner that is dependent on STAT3 and Smad2 activation, creating an environment of cytokine modulation able to favor or benefit the parasite growth. This study is the first to show it in a human trophoblast model.

Our previous studies demonstrated that BeWo cells produce IL10 and TGFB1 naturally [15, 18], probably to avoid fetal rejection, which, at the same time, favors infection by many pathogens, such as *T. gondii* [15]. It is possible to hypothesize that these cytokines released by trophoblast cells (IL10 and TGFB1) in the maternal-fetal interface can induce some neighboring cells, such as monocytes, to change their cytokine profile and thus promote a milieu of mediators that favor the infection. This was found in a previous study that demonstrated an impaired immune response against bacterial stimuli in human monocytes (THP-1) cultured with trophoblast supernatant, especially reduced IL6 levels, showing the ability of trophoblast cells to modulate the immune response in cells found in the maternal-fetal interface [56]. Also, our previous study showed that the supernatant of BeWo cells induced increased IL10 and IL6 production in THP-1 cells [18]. Therefore, although trophoblast cells are not considered cells with innate or adaptive immunity, they are potentially able to modulate the immune response in the maternal-fetal interface. In this context, it is possible that IL10 and TGFB1 released by trophoblast cells trigger STAT3 and Smad2 in other cells of the maternal-fetal interface, modulating the cytokine profile and favoring the establishment of the infection. However, there are studies that demonstrate immune activation of trophoblast cells, including BeWo cells, as evidenced by the fact that many types of toll-like receptors are expressed and functional in these cells [57]. In this context, it is possible to assume that trophoblast cells are able to modulate the cytokine profile and influence the control of a *T. gondii* infection.

The role of TNFA in the immune response against *T. gondii* is well known [58]. Increased levels of TNFA were observed in infected women, and higher IL10 production was found to prevent an exacerbated immune response, showing the role of IL10 in down-modulating TNFA [59]. The release of TNFA was reduced by endogenous IL10 in mice infected by *T. gondii,* demonstrating a protective mechanism mediated by IL10 that prevents a strong immunopathology [60]. The biological activities of TNFA are mediated by two different receptors, TNFR1 and TNFR2. TNFR1 seems to be the main mediator of TNFA signaling, leading to proinflammatory response [61]. TNFR1 signaling results in the activation of IB kinase (IKK) and, consequently, the activation of nuclear kappa B (NFKB) factor in different physiological and pathological conditions, promoting the secretion of proinflammatory mediators [61, 62]. In addition, TNFR1 also triggers the extracellular signal-regulated kinase (ERK), the c-June N-terminal kinase (JNK) and the p38 mitogen-activated protein kinase (p38 MAPK) in response to TNFA [63]. These pathways can be activated in trophoblast cells because some previous studies demonstrated expression of TNFA and its receptors (both TNFR1 and TNFR2) in human trophoblast isolated from placenta [61] and also in BeWo cells [64]. Thus, when BeWo cells were treated with rTNFA, we speculated that this cytokine triggered NFKB or the MAPKs as described above, promoting the production of proinflammatory mediators that are able to control the parasite infection. However, in the presence of rIL10, down-modulation of TNFA was detected, and it was not possible to verify the paracrine activity of TNFA and its mediators during the infection. Previous studies demonstrated that IL10 down-regulated the activity of TNFA by increasing the release of soluble TNFR1 and TNFR2, reducing the surface expression of both receptors [65]. It may be that IL10 down-modulated TNFA activity in our present study by inducing increased release of soluble TNF receptors, avoiding the signaling of the cytokine. Villous trophoblast and BeWo cells are able to release the soluble forms of these receptors in order to protect against the cytotoxic effects of TNFA [64]. Overall, future investigations are necessary to verify these pathways and the production of proinflammatory mediators in BeWo cells stimulated with TNFA.

Like TNFA, several studies have demonstrated the protective effect of IL6 against *T. gondii* infection [18, 66, 67], although not in trophoblast cells, and our study was the first to show this effect in a human trophoblast model. IL6 has important roles in the maternal-fetal interface that could explain its increased level in trophoblast cells [68–70]. Studies have shown that IL6 participates in the migration and invasion of trophoblasts into the endometrium [71]. Other studies have demonstrated high levels of production of IL6 by BeWo cells and its involvement in the endocrine properties of these cells [22, 72]. However, this cytokine should be at adequate concentrations at the maternal-fetal interface because high levels of IL6 are associated with preeclampsia and inflammatory processes in the amnion [73, 74]. Thus, it is plausible that TGFB1 and IL10 down-modulate IL6 release in order to control the level of this cytokine in BeWo cells, which in turn, promotes the increased proliferation of *T. gondii*, allowing dissemination of the infection in our model. Moreover, previous studies have demonstrated that IL6 triggers intracellular signaling pathways in a manner dependent on STAT1 and STAT3 [75] or ERK1/2 [76]. In our present study, the pathways triggered by IL6 in BeWo cells were not verified, but we detected reduced *T. gondii* proliferation in cells stimulated with rIL6. Therefore, it is possible to hypothesize that IL6 can activate STAT1 or ERK1/2 and promote the parasite control in BeWo trophoblast cells, while this situation becomes dampened when BeWo cells are stimulated with IL10 or TGFB1. Future studies are necessary to verify the signaling pathway triggered by IL6 in BeWo cells.

When we investigated the role of IFNG in the *T. gondii* proliferation, we observed that concentrations lower than 50 ng/ml were unable to control the number of tachyzoites. Interestingly, concentrations higher than 50 ng/ml promoted increased infectivity in BeWo cells. Our previous studies have already shown that IFNG is not able to control *T. gondii* infection [14] or can simply favor the infection in BeWo cells [15], which contrasts with several studies that have demonstrated the effect of this cytokine against the parasite in other cell types [7–10]. To understand the intracellular mechanism triggered by IFNG in BeWo cells, which could clarify the role of this cytokine at the maternal-fetal interface, we investigated the STAT1-dependent intracellular signaling pathway. STAT1 phosphorylation was detected in BeWo cells treated only with

20 ng/ml IFNG. We did not verify any STAT1 activation with 100 ng/ml IFNG. We therefore concluded that higher *T. gondii* proliferation is present when there is no STAT1 phosphorylation. This was confirmed when we used a STAT1 inhibitor in the presence of 20 ng/ml IFNG, demonstrating that STAT1 activation is associated with control of the parasite. To clarify the dose-dependent effect of IFNG on the control of *T. gondii* infection, we measured cytokine release in cells treated with 20 or 100 ng/ml IFNG.

BeWo cells treated with 20 ng/ml IFNG increased IL6 and IL17A production when infected or not with *T. gondii,* and this was confirmed by blocking STAT1 (Fludarabine), showing that the cytokines released (IL6 and IL17A) are dependent on STAT1 activation. IFNG in low concentration was the only cytokine able to trigger IL17A production in BeWo cells, regardless of the presence or absence of infection. This means that, at least in BeWo cells, STAT1 is the major pathway involved in the release of this cytokine, demonstrating the role of IL17A in the control of *T. gondii* infection, as observed with IL6. Additionally, BeWo cells treated with IFNG and Fludarabine showed up-regulation of IL10, even though IL10 levels were not reduced in the absence of the inhibitor. Although IFNG triggered STAT1 phosphorylation when added to cells at a concentration of 20 ng/ml, this cytokine was unable to control *T. gondii* infection, even with increased IL6 and IL17A secretion. On the other hand, BeWo cells treated with100 ng/ml IFNG significantly reduced both IL6 and TNFA. The results suggest that BeWo cells demonstrated increased *T. gondii* infection in the presence of IFNG in high concentrations because IL6 and TNFA were down-modulated, creating a microenvironment favoring parasite proliferation, as observed during the treatments with IL10 and TGFB1.

Our present findings demonstrated that 20 ng/ml IFNG was not able to control *T. gondii* infection even in the presence of STAT1 phosphorylation and high IL6 levels in BeWo cells. Previous studies have shown that trophoblast cell lines such as Jar and JEG have low levels of STAT1 phosphorylation in comparison to some other cell lineages, including HeLa, where IFNG triggered higher STAT1 phosphorylation and interferon regulator factor 1 (IRF1) expression in comparison to trophoblasts [31]. Based in these evidences, we can hypothesize that BeWo cells might present low STAT1 activation in the presence of 20 ng/ml IFNG, which may not be sufficient to control the parasite. Alternatively, it has been demonstrated that 20 ng/ml IFNG promoted increased intercellular adhesion molecule 1 (ICAM1) expression in BeWo cells [77]. Also, *T. gondii* was able to use ICAM1 to infect human intestinal cells as well as BeWo cells by binding the microneme protein 2 of the parasite (MIC2) to ICAM1 of the host cells, demonstrating a key role of ICAM1 in the spreading of *T. gondii* through biological barriers, including the maternal-fetal interface [78]. Based on this evidence, we can speculate that exposure of BeWo cells to low levels of IFNG, even with STAT1 phosphorylation and IL6 production, up-regulates ICAM1, favoring invasion of the parasite and thus failing to control the infection. Interestingly, our previous study demonstrated that IFNG increased the macrophage migration inhibitory factor (MIF) expression in human villous explants from the first trimester, which, in turn, induced higher ICAM1 expression, especially in the syncytiotrophoblast [79]. These data reinforce our hypothesis that 20 ng/ml IFNG does not control *T. gondii* infection in human trophoblast cells because this cytokine is an important regulator of ICAM1.

Based in our findings and all the discussion about the role of IFNG in trophoblast cells infected by *T. gondii*, it is ineluctable to ask which concentration reproduces the physiological condition in the maternal-fetal interface. A previous study showed almost no IFNG secretion in human trophoblast from the first trimester, while the decidual immune cells were the only cells types that produced this cytokine [80]. Then, the local immune cells are the major source of IFNG in the maternal-fetal interface because the decidual natural killer cells, and consequently the trophoblast cells, are stimulated by IFNG in a paracrine manner. Decidual natural killer cells cocultured with trophoblast produce from 20 to 30 ng/ml IFNG, and this production increases significantly when these cells are infected by *T. gondii* (~80 ng/ml) [81]. Therefore, the IFNG concentrations used in our present study are similar to those found during in vivo conditions. It is difficult to ascertain precisely the function of IFNG at the maternal-fetal interface, that is, proinflammatory or anti-inflammatory, because at least in BeWo cells, this cytokine was unable to control the parasite's growth, considering that our data demonstrate that IFNG has different activities, depending on its concentration.

Altogether, our results demonstrate that BeWo cells present high infectivity to *T. gondii* when treated with IL10, TGFB1, and IFNG because these cytokines differentially trigger, in a dependent manner, specific intracellular signaling pathways and, consequently, create a milieu with other cytokines, especially down-modulation of IL6 and TNFA, that dampens an immune response against *T. gondii* and allows its intracellular proliferation. Our findings open up new avenues to understand the effector mechanisms triggered by these key cytokines in a human trophoblast model (BeWo cells) under *T. gondii* infection, helping to elucidate the mechanisms involved in the vertical transmission of this parasite.

REFERENCES

1. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet 2004; 363:1965–1976.
2. Blader IJ, Saeij JP. Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. APMIS 2009; 117:458–476.
3. Jones JL, Lopez A, Wilson M, Schulkin J, Gibbs R. Congenital toxoplasmosis: a review. Obstet Gynecol Surv 2001; 56:296–305.
4. Weiss LM, Dubey JP. Toxoplasmosis: a history of clinical observations. Int J Parasitol 2009; 39:895–901.
5. Carellos EV, de Andrade GM, Vasconcelos-Santos DV, Januario JN, Romanelli RM, Abreu MN, da Silva FM, Loures IR, de Andrade JQ, Caiaffa WT. Adverse socioeconomic conditions and oocyst-related factors are associated with congenital toxoplasmosis in a population-based study in Minas Gerais, Brazil. PLoS One 2014; 9:e88588.
6. Dubey JP, Lago EG, Gennari SM, Su C, Jones JL. Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitology 2012; 139:1375–1424.
7. Gazzinelli RT, Wysocka M, Hayashi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A. Parasite-induced IL12 stimulates early IFN gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. J Immunol 1994; 153:2533–2543.
8. Lang C, Gross U, Luder CG. Subversion of innate and adaptive immune responses by *Toxoplasma gondii*. Parasitol Res 2007; 100:191–203.
9. da Silva RC, Langoni H. *Toxoplasma gondii*: host-parasite interaction and behavior manipulation. Parasitol Res 2009; 105:893–898.
10. Denkers EY. Toll-like receptor initiated host defense against *Toxoplasma gondii*. J Biomed Biotechnol 2010; 2010:737125.
11. Koga K, Aldo PB, Mor G. Toll-like receptors and pregnancy: trophoblast as modulators of the immune response. J Obstet Gynaecol Res 2009; 35: 191–202.
12. Saito S. Cytokine network at the feto-maternal interface. J Reprod Immunol 2000; 47:87–103.
13. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, Petraglia F. Inflammation and pregnancy. Reprod Sci 2009; 16:206–215.
14. Oliveira JG, Silva NM, Santos AA, Souza MA, Ferreira GL, Mineo JR, Ferro EA. BeWo trophoblasts are unable to control replication of *Toxoplasma gondii*, even in the presence of exogenous IFN gamma. Placenta 2006; 27:691–698.
15. Barbosa BF, Silva DA, Costa IN, Mineo JR, Ferro EA. BeWo trophoblast cell susceptibility to *Toxoplasma gondii* is increased by interferon gamma,

interleukin 10 and transforming growth factor beta1. Clin Exp Immunol 2008; 151:536–545.
16. Carvalho JV, Alves CM, Cardoso MR, Mota CM, Barbosa BF, Ferro EA, Silva NM, Mineo TW, Mineo JR, Silva DA. Differential susceptibility of human trophoblastic (BeWo) and uterine cervical (HeLa) cells to *Neospora caninum* infection. Int J Parasitol 2010; 40:1629–1637.
17. Franco PS, Gomes AO, Barbosa BF, Angeloni MB, Silva NM, Teixeira-Carvalho A, Martins-Filho OA, Silva DA, Mineo JR, Ferro EA. Azithromycin and spiramycin induce anti-inflammatory response in human trophoblastic (BeWo) cells infected by *Toxoplasma gondii* but are able to control infection. Placenta 2011; 32:838–844.
18. Castro AS, Alves CM, Angeloni MB, Gomes AO, Barbosa BF, Franco PS, Silva DA, Martins-Filho OA, Mineo JR, Mineo TW, Ferro EA. Trophoblast cells are able to regulate monocyte activity to control *Toxoplasma gondii* infection. Placenta 2013; 34:240–247.
19. Barbosa BF, Paulesu L, Ietta F, Bechi N, Romagnoli R, Gomes AO, Favoreto-Junior S, Silva DA, Mineo JR, Mineo TW, Ferro EA. Susceptibility to *Toxoplasma gondii* proliferation in BeWo human trophoblast cells is dose-dependent of macrophage migration inhibitory factor (MIF), via ERK1/2 phosphorylation and prostaglandin E2 production. Placenta 2014; 35:152–162.
20. Pattillo RA, Gey GO. The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. Cancer Res 1968; 28:1231–1236.
21. Pattillo RA, Hussa RO, Ruckert AC, Kurtz JW, Cade JM, Rinke ML. Human chorionic gonadotropin in BeWo trophoblastic cells after 12 years in continuous culture: retention of intact human chorionic gonadotropin secretion in mechanically versus enzyme-dispersed cells. Endocrinology 1979; 105:967–974.
22. Fujisawa K, Nasu K, Arima K, Sugano T, Narahara H, Miyakawa I. Production of interleukin (IL)-6 and IL8 by a choriocarcinoma cell line, BeWo. Placenta 2000; 21:354–360.
23. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity 2008; 28:477–487.
24. Donnelly RP, Dickensheets H, Finbloom DS. The interleukin 10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. J Interferon Cytokine Res 1999; 19:563–573.
25. Cheon H, Rho YH, Choi SJ, Lee YH, Song GG, Sohn J, Won NH, Ji JD. Prostaglandin E2 augments IL10 signaling and function. J Immunol 2006; 177:1092–1100.
26. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol 2008; 19:414–422.
27. Gough DJ, Levy DE, Johnstone RW, Clarke CJ. IFN gamma signaling-does it mean JAK-STAT? Cytokine Growth Factor Rev 2008; 19: 383–394.
28. Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon gamma. Cytokine 2010; 50: 1–14.
29. Hanna N, Hanna I, Hleb M, Wagner E, Dougherty J, Balkundi D, Padbury J, Sharma S. Gestational age-dependent expression of IL10 and its receptor in human placental tissues and isolated cytotrophoblasts. J Immunol 2000; 164:5721–5728.
30. Entrican G, Wattegedera S, Chui M, Oemar L, Rocchi M, McInnes C. Gamma interferon fails to induce expression of indoleamine 2,3-dioxygenase and does not control the growth of *Chlamydophila abortus* in BeWo trophoblast cells. Infect Immun 2002; 70:2690–2693.
31. Choi JC, Holtz R, Petroff MG, Alfaidy N, Murphy SP. Dampening of IFN gamma-inducible gene expression in human choriocarcinoma cells is due to phosphatase-mediated inhibition of the JAK/STAT1 pathway. J Immunol 2007; 178:1598–1607.
32. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev 2002; 13:413–421.
33. Verrecchia F, Mauviel A, Farge D. Transforming growth factor beta signaling through the Smad proteins: role in systemic sclerosis. Autoimmun Rev 2006; 5:563–569.
34. Kitisin K, Saha T, Blake T, Golestaneh N, Deng M, Kim C, Tang Y, Shetty K, Mishra B, Mishra L. Tgf beta signaling in development. Sci STKE 2007; 2007:cm1.
35. Santibanez JF, Quintanilla M, Bernabeu C. TGF beta/TGF beta receptor system and its role in physiological and pathological conditions. Clin Sci (Lond) 2011; 121:233–251.
36. Frolik CA, Dart LL, Meyers CA, Smith DM, Sporn MB. Purification and initial characterization of a type beta transforming growth factor from human placenta. Proc Natl Acad Sci U S A 1983; 80:3676–3680.
37. Zhou H, Fu G, Yu H, Peng C. Transforming growth factor beta inhibits aromatase gene transcription in human trophoblast cells via the Smad2 signaling pathway. Reprod Biol Endocrinol 2009; 7:146.
38. Finsterbusch M, Khare V, Campregher C, Evstatiev R, Gasche C. An intracytoplasmic IL10 receptor variant permits rapid reduction in STAT3 activation. Genes Immun 2011; 12:575–581.
39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.
40. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65:55–63.
41. Luiza-Silva M, Campi-Azevedo AC, Batista MA, Martins MA, Avelar RS, da Silveira Lemos D, Bastos Camacho LA, de Menezes Martins R, de Lourdes de Sousa Maia M, Guedes Farias RH, da Silva Freire M, Galler R, et al. Cytokine signatures of innate and adaptive immunity in 17DD yellow fever vaccinated children and its association with the level of neutralizing antibody. J Infect Dis 2011; 204:873–883.
42. Oufkir T, Vaillancourt C. Phosphorylation of JAK2 by serotonin 5HT (2A) receptor activates both STAT3 and ERK1/2 pathways and increases growth of JEG-3 human placental choriocarcinoma cell. Placenta 2011; 32:1033–1040.
43. Yeganegi M, Leung CG, Martins A, Kim SO, Reid G, Challis JR, Bocking AD. *Lactobacillus rhamnosus* GR-1-induced IL10 production in human placental trophoblast cells involves activation of JAK/STAT and MAPK pathways. Reprod Sci 2010; 17:1043–1051.
44. Liu WH, Liu JJ, Wu J, Zhang LL, Liu F, Yin L, Zhang MM, Yu B. Novel mechanism of inhibition of dendritic cells maturation by mesenchymal stem cells via interleukin 10 and the JAK1/STAT3 signaling pathway. PLoS One 2013; 8:e55487.
45. Sousa LM, Carneiro MB, Resende ME, Martins LS, Dos Santos LM, Vaz LG, Mello PS, Mosser DM, Oliveira MA, Vieira LQ. Neutrophils have a protective role during early stages of *Leishmania amazonensis* infection in BALB/c mice. Parasite Immunol 2014; 36:13–31.
46. Naiyer MM, Saha S, Hemke V, Roy S, Singh S, Musti KV, Saha B. Identification and characterization of a human IL10 receptor antagonist. Hum Immunol 2013; 74:28–31.
47. Ng SS, Li A, Pavlakis GN, Ozato K, Kino T. Viral infection increases glucocorticoid-induced interleukin 10 production through ERK-mediated phosphorylation of the glucocorticoid receptor in dendritic cells: potential clinical implications. PLoS One 2013; 8:e63587.
48. Pasche B, Kalaydjiev S, Franz TJ, Kremmer E, Gailus-Durner V, Fuchs H, Hrabe de Angelis M, Lengeling A, Busch DH. Sex-dependent susceptibility to *Listeria monocytogenes* infection is mediated by differential interleukin 10 production. Infect Immun 2005; 73:5952–5960.
49. McBerry C, Dias A, Shryock N, Lampe K, Gutierrez FR, Boon L, De'Broski RH, Aliberti J. PD1 modulates steady-state and infection-induced IL10 production in vivo. Eur J Immunol 2014; 44:469–479.
50. Brandao GP, Melo MN, Gazzinelli RT, Caetano BC, Ferreira AM, Silva LA, Vitor RW. Experimental reinfection of BALB/c mice with different recombinant type I/III strains of *Toxoplasma gondii*: involvement of IFN gamma and IL10. Mem Inst Oswaldo Cruz 2009; 104:241–245.
51. Bonnefoy F, Couturier M, Clauzon A, Remy-Martin JP, Gaugler B, Tiberghien P, Chen W, Saas P, Perruche S. TGF beta-exposed plasmacytoid dendritic cells participate in Th17 commitment. J Immunol 2011; 186:6157–6164.
52. Fogle JE, Mexas AM, Tompkins WA, Tompkins MB. CD4(+)CD25(+) T regulatory cells inhibit CD8(+) IFN gamma production during acute and chronic FIV infection utilizing a membrane TGF beta-dependent mechanism. AIDS Res Hum Retroviruses 2010; 26:201–216.
53. Malhotra N, Robertson E, Kang J. SMAD2 is essential for TGF beta-mediated Th17 cell generation. J Biol Chem 2010; 285:29044–29048.
54. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. J Biol Chem 1999; 274:37413–37420.
55. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF beta responses. EMBO J 2002; 21:3749–3759.
56. Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, Chen R, Chavez SL, Romero R, Mor G. Trophoblast–macrophage interactions: a regulatory network for the protection of pregnancy. Am J Reprod Immunol 2007; 57: 55–66.
57. Tangeras LH, Stodle GS, Olsen GD, Leknes AH, Gundersen AS, Skei B, Vikdal AJ, Ryan L, Steinkjer B, Myklebost MF, Langaas M, Austgulen R, et al. Functional toll-like receptors in primary first-trimester trophoblasts. J Reprod Immunol 2014; 106:89–99.
58. Machado FS, Aliberti J. Lipoxins as an immune-escape mechanism. Adv Exp Med Biol 2009; 666:78–87.
59. El-Kady IM. T-cell immunity in human chronic toxoplasmosis. J Egypt Soc Parasitol 2011; 41:17–28.
60. Wilson EH, Wille-Reece U, Dzierszinski F, Hunter CA. A critical role for

IL10 in limiting inflammation during toxoplasmic encephalitis. J Neuroimmunol 2005; 165:63–74.
61. Agius-Calleja J, Muttukrishna S, Jauniaux E. The role of tumor necrosis factor-receptors in pregnancy with normal and adverse outcome. Int J Interferon Cytokine Mediator Res 2012; 4:1–15.
62. Tomar D, Singh R. TRIM13 regulates ubiquitination and turnover of NEMO to suppress TNF induced NFKB activation. Cell Signal 2014; 26: 2606–2613.
63. Olmos G, Lladó J. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators Inflamm 2014; 2014: 861231.
64. Knöfler M, Stenzel M, Husslein P. Shedding of tumor necrosis factor receptors from purified villous term trophoblasts and cytotrophoblastic BeWo cells. Hum Reprod 1998; 13:2308–2316.
65. Joyce DA, Gibbons DP, Green P, Steer JH, Feldmann M, Brennan FM. Two inhibitors of pro-inflammatory cytokine release, interleukin 10 and interleukin 4, have contrasting effects on release of soluble p75 tumor necrosis factor receptor by cultured monocytes. Eur J Immunol 1994; 24: 2699–2705.
66. Silver JS, Stumhofer JS, Passos S, Ernst M, Hunter CA. IL6 mediates the susceptibility of glycoprotein 130 hypermorphs to *Toxoplasma gondii*. J Immunol 2011; 187:350–360.
67. Mirpuri J, Yarovinsky F. IL6 signaling SOCS critical for IL12 host response to *Toxoplasma gondii*. Future Microbiol 2012; 7:13–16.
68. Stephanou A, Myatt L, Eis AL, Sarlis N, Jikihara H, Handwerger S. Ontogeny of the expression and regulation of interleukin 6 (IL-6) and IL1 mRNAs by human trophoblast cells during differentiation in vitro. J Endocrinol 1995; 147:487–496.
69. Tabibzadeh S, Kong QF, Babaknia A, May LT. Progressive rise in the expression of interleukin 6 in human endometrium during menstrual cycle is initiated during the implantation window. Hum Reprod 1995; 10: 2793–2799.
70. Jauniaux E, Gulbis B, Schandene L, Collette J, Hustin J. Distribution of interleukin 6 in maternal and embryonic tissues during the first trimester. Mol Hum Reprod 1996; 2:239–243.
71. Jovanovic M, Vicovac L. Interleukin 6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. Placenta 2009; 30: 320–328.
72. Tsukihara S, Harada T, Deura I, Mitsunari M, Yoshida S, Iwabe T, Terakawa N. Interleukin 1beta-induced expression of IL6 and production of human chorionic gonadotropin in human trophoblast cells via nuclear factor kappaB activation. Am J Reprod Immunol 2004; 52:218–223.
73. Chen LM, Liu B, Zhao HB, Stone P, Chen Q, Chamley L. IL6, TNF alpha and TGF beta promote nonapoptotic trophoblast deportation and subsequently causes endothelial cell activation. Placenta 2010; 31:75–80.
74. Lockwood CJ, Murk WK, Kayisli UA, Buchwalder LF, Huang SJ, Arcuri F, Li M, Gopinath A, Schatz F. Regulation of interleukin 6 expression in human decidual cells and its potential role in chorioamnionitis. Am J Pathol 2010; 177:1755–1764.
75. Briscoe J, Guschin D, Rogers NC, Watling D, Muller M, Horn F, Heinrich P, Stark GR, Kerr IM. JAKs, STATs and signal transduction in response to the interferons and other cytokines. Phil Trans R Soc Lond B 1996; 351: 167–171.
76. Tao Q, Huang H, Chai Y, Luo X, Zhang X, Jia B, Zhang S. Interleukin 6 up-regulates the expression of interleukin 15 is associated with MAPKs and PI3K signaling pathways in the human keratinocyte cell line, HaCaT. Mol Biol Rep 2012; 39:4201–4205.
77. Pfaff AW, Georges S, Abou-Bacar A, Letscher-Bru V, Klein JP, Mousli M, Candolfi E. *Toxoplasma gondii* regulates ICAM1 mediated monocyte adhesion to trophoblasts. Immunol Cell Biol 2005; 83:483–489.
78. Barragan A, Brossier F, Sibley LD. Transepithelial migration of *Toxoplasma gondii* involves an interaction of intercellular adhesion molecule 1 (ICAM1) with the parasite adhesin MIC2. Cell Microbiol 2005; 7:561–568.
79. Ferro EA, Mineo JR, Ietta F, Bechi N, Romagnoli R, Silva DA, Sorda G, Bevilacqua E, Paulesu LR. Macrophage migration inhibitory factor is up-regulated in human first-trimester placenta stimulated by soluble antigen of *Toxoplasma gondii*, resulting in increased monocyte adhesion on villous explants. Am J Pathol 2008; 172:50–58.
80. Piao HL, Wang SC, Tao Y, Zhu R, Sun C, Fu Q, Du MR, Li DJ. Cyclosporine A enhances Th2 bias at the maternal-fetal interface in early human pregnancy with aid of the interaction between maternal and fetal cells. PLoS One 2012; 7:e45275.
81. Zhang L, Zhao M, Jiao F, Xu X, Liu X, Jiang Y, Zhang H, Ou X, Hu X. Interferon gamma is involved in apoptosis of trophoblast cells at the maternal-fetal interface following *Toxoplasma gondii* infection. Int J Infect Dis 2015; 30:10–16.