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# Differential apoptosis in BeWo cells after infection with highly (RH) or moderately (ME49) virulent strains of Toxoplasma gondii is related to the cytokine profile secreted, the death receptor Fas expression and phosphorylated ERK1/2 expression

M.B. Angeloni [a], P.M. Guirelli [a], P.S. Franco [a], B.F. Barbosa [a], A.O. Gomes [a], A.S. Castro [a], N.M. Silva [b], O.A. Martins-Filho [c], T.W.P. Mineo [d], D.A.O. Silva [d], J.R. Mineo [d], E.A.V. Ferro [a, *]

[a] Laboratory of Histology and Embryology, Institute of Biomedical Sciences, Federal University of Uberlândia, Av. Pará, 1720, 38405-320 Uberlândia, MG, Brazil
[b] Laboratory of Immunopathology, Institute of Biomedical Sciences, Federal University of Uberlândia, Av. Pará, 1720, 38405-320 Uberlândia, MG, Brazil
[c] Laboratory of Chagas Disease, René Rachou Research Center, Fundação Oswaldo Cruz, 30190-002 Belo Horizonte, MG, Brazil
[d] Laboratory of Immunoparasitology, Institute of Biomedical Sciences, Federal University of Uberlândia, Av. Pará, 1720, 38400-902 Uberlândia, MG, Brazil

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Introduction: Alterations of apoptosis are commonly associated with pregnancy complications and abortion. Modulation of apoptosis is a relevant feature of Toxoplasma gondii infection and it is related to parasite strain types. The aim of the present study was to evaluate the possible factors that are involved in the differential apoptosis of BeWo cells infected with distinct T. gondii strain types.
Methods: Human trophoblastic cells (BeWo cell line) were infected with RH or ME49 strains, the cytokine production was measured and the phosphorylation of anti-apoptotic ERK1/2 protein was analyzed. Also, cells were treated with different cytokines, infected with RH or ME49 strain, and analyzed for apoptosis index and Fas/CD95 death receptor expression.
Results: ME49-infected BeWo cells exhibited a predominantly pro-inflammatory cytokine profile, whereas cells infected with RH strain had a higher production of anti-inflammatory cytokines. Also, the incidence of apoptosis was higher in ME49-infected cells, which have been treated with pro-inflammatory cytokines compared to cells infected with RH and treated with anti-inflammatory cytokines. Moreover, Fas/CD95 expression was higher in cells infected with either ME49 or RH strain and treated with pro-inflammatory cytokines compared to anti-inflammatory cytokine treatment. The phosphorylation of ERK1/2 protein increased after 24 h of infection only with the RH strain.
Conclusion: These results suggest that opposing mechanisms of interference in apoptosis of BeWo cells after infection with RH or ME49 strains of T. gondii can be associated with the differential cytokine profile secreted, the Fas/CD95 expression and the phosphorylated ERK1/2 expression.


## 1. Introduction

Toxoplasma gondii is an obligate intracellular parasite that can invade and replicate in almost all nucleated cells of warm-blooded animals [1]. Approximately one-third of the human population from all over the world is estimated to be chronically infected by T. gondii and, for that reason, it is considered as one of the most successful parasites that is able to infect humans [2,3]. The population structure of Toxoplasma is dominated by three clonal lineages, designated as strains types I, II and III, according to the virulence to mice and humans, which are mainly found in Europe and North America. While strains of the type I (RH) are virulent and

* Corresponding author. Tel.: þ55 34 3218 2240; fax: þ55 34 3218 2333.
E-mail addresses: ma_bodini@yahoo.com.br (M.B. Angeloni), pamelaguirelli@gmail.com (P.M. Guirelli), pribio85@hotmail.com (P.S. Franco), bellisafb@yahoo.com.br (B.F. Barbosa), angellicagomes@yahoo.com.br (A.O. Gomes), ascastro87@yahoo.com.br (A.S. Castro), neidemsilva@yahoo.com.br (N.M. Silva), oamfilho@cpqrr.fiocruz.br (O.A. Martins-Filho), tiagomineo@gmail.com (T.W.P. Mineo), deiseaosilva@pq.cnpq.br (D.A.O. Silva), jrmineo@ufu.br (J.R. Mineo), eloisa@umuarama.ufu.br (E.A.V. Ferro).

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uniformly lethal to mice, causing severe clinical manifestations of toxoplasmosis, strains of the types II (ME49) and III (VEG) are of moderate and low virulence to murine hosts, respectively, which are able to control acute phase of the disease and are able to establish chronic infections [4]. In South America, however, T. gondii strains show a high diversity of genetic structure and are designated as recombinant strains [5], with virulence patterns yet to be determined. Although the majority of infected healthy individuals have no symptoms, during pregnancy T. gondii can be transmitted to the fetus transplacentally, causing severe disease or even fetal death [5,6].

Apoptosis has been recognized as an important defense mechanism against viral, bacterial and parasitic infections [7,8]. Previous studies demonstrated that T. gondii-infected cells were resistant to apoptosis induced by various proapoptotic stimuli [9]. This phenomenon is considered as a crucial adaptation of the parasite that allows sustained intracellular survival and long-term persistence within the host cells [10]. Also, apoptosis modulation during T. gondii infection is associated with the virulence characteristics of the parasite [11]. Accordingly, in our previous study we demonstrated that the incidence of apoptosis in human trophoblastic cells (BeWo cell line) was differentially modulated by highly (RH) or moderately virulent (ME49) strains of T. gondii since RH-infected BeWo cells had lower incidence of apoptosis compared to ME49 strain or uninfected cells, and that type I strains can inhibit host cell apoptosis [12].

Considering that the apoptosis process is critical for the development and homeostasis of placental tissues and the occurrence of apoptosis in trophoblastic cells is dependent on the T. gondii strain type, in the present study we investigated the profile of cytokines secreted by trophoblastic BeWo cells infected with RH or ME49 strains of T. gondii. Also, we analyzed the incidence of apoptosis in BeWo cells after infection with these T. gondii strains and treatment with different cytokines as well as the Fas death receptor expression and the ERK1/2 phosphorylated protein expression.

## 2. Materials and methods

### 2.1. Cell culture

BeWo cell line (human choriocarcinoma cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (GIBCO, Paisley, UK), supplemented with 25 mM HEPES, 2 mM L-glutamine, 10 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich, St Louis, MO, USA) and 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil) in a humidified incubator at 37 °C and 5%$CO_2$.

### 2.2. Parasites

Tachyzoites of the highly virulent RH strain of T. gondii were originally maintained in Swiss mice by intraperitoneal passages at 48 h intervals [13]. Parasites were obtained from mouse peritoneal exudates, washed twice (720 g, 10 min) in sterile phosphate-buffered saline (PBS) pH 7.2 and cultured in BeWo cells in order to obtain culture-derived parasites. Tachyzoites were harvested by scraping off the cell monolayer after 2e3 days of infection, passed through a 26-gauge needle to lyse any remaining intact host cells, washed (720 g, 5 min) in RPMI medium and the resulting pellet was resuspended in complete medium. Parasites were stained with 0.4% Trypan blue and counted in a hemocytometric chamber for further infection experiments.

Parasites of the moderately virulent ME49 strain of T. gondii were initially obtained by the pepsin digestion of brain cysts from chronically infected Calomys callosus, as previously described [14,12]. Parasites were purified by low centrifugation to remove host cell debris and the supernatant-contained tachyzoites were transferred and maintained in BeWo cell culture in order to obtain culture-derived parasites as described above for further infection experiments.

### 2.3. Cytokine treatment and T. gondii infection

BeWo cells were cultured in 96-well plates ($1 \times 10^5$ cells/well/200 μl) for 24 h at 37 °C and 5%$CO_2$. Cells were then infected with T. gondii, RH or ME49 strain, at 5:1 (parasite:cell) ratio. After 2 h and 24 h of incubation, supernatants were collected and stored at −80 °C for cytokine assays. Cells incubated with medium alone served as controls.

In a second set of experiments, BeWo cells were cultured on 13-mm round glass coverslips into 24-well plates ($1 \times 10^5$ cells/well/200 μl) for 24 h at 37 °C and 5%$CO_2$. Different concentrations of cytokines (15, 20, 25 and 30 ng/ml) were initially tested for cell viability and we selected for further experiments the concentration that showed no change of cell viability (15 ng/ml). Cells were washed with medium and treated with human recombinant cytokines: rMIF (BD Biosciences, San Diego, CA, USA) or rTNF-α (Millipore, São Paulo, SP, Brazil) or rIFN-γ (Invitrogen Life Technologies, Carlsbad, CA, USA) or rIL-6 (Peprotech, Rocky Hill, NJ, USA) or rTGF-β1 (R&D Systems, Minneapolis, MN, USA) or rIL-10 (R&D Systems), all of them at 15 ng/ml for 24 h at 37 °C and 5%$CO_2$. Cells were then infected with T. gondii, RH or ME49 strain, at 5:1 (parasite:cell). After 3 h of infection cells were again treated with cytokines as described above. As controls, cells were uninfected/untreated, treated/uninfected or infected/untreated. After 2 h and 24 h of incubation, medium was gently removed and cells were fixed in 10% buffered formalin for 24 h and were analyzed for apoptosis detection by immunohistochemistry.

In a third set of experiments, BeWo cells were cultured into 6-well plates ($1 \times 10^6$ cells/well/ml) for 24 h at 37 °C and 5%$CO_2$. Cells were washed with medium and treated with human recombinant cytokines as described above. Cells were then infected with T. gondii, RH or ME49 strain, at 5:1 (parasite:cell). After 3 h of infection cells were treated again with cytokines as described above. After 2 h and 24 h of incubation, cells were analyzed for cell surface Fas/CD95 expression by flow cytometric analysis. As controls, cells were uninfected/untreated, treated/uninfected or infected/untreated.

In a fourth set of experiments, BeWo cells were cultured into 6-well plates ($1 \times 10^6$ cells/well/ml) for 24 h at 37 °C and 5%$CO_2$. Cells were washed with medium and infected with T. gondii, RH or ME49 strain, at 5:1 (parasite:cell). After 2 h and 24 h of incubation cells were lysed and subjected to Western blotting assays for phosphorylated ERK1/2 detection. As control, cells were incubated with medium alone. Six independent experiments were performed in triplicate for each condition.

### 2.4. Cytokine assays

#### 2.4.1. Cytokine measurements by enzyme-linked immunosorbent assay (ELISA)

Human cytokines (MIF, TNF-α, IL-12, TGF-β1 and IL-10) were measured using sandwich ELISAs according to the manufacturer's instructions (R&D Systems for MIF, TGF-β1 and IL-10; BD Biosciences for TNF-α and IL-12). Briefly, 96-well plates were coated with capture monoclonal antibody to each cytokine, blocked and incubated with the samples and the respective standards for each cytokine. After washing, plates were incubated with biotinylated detection polyclonal antibody to each cytokine. The assay was developed using streptavidin e horseradish peroxidase and revealed with TMB substrate reagent set (BD Biosciences). Cytokine concentrations were determined via extrapolation from a standard curve obtained from known concentrations of each recombinant cytokine. The sensitivity limits of these assays were 31.3 pg/ml for MIF, IL-10 and TGF-β, and 7.8 pg/ml for TNF-α and IL-12.

#### 2.4.2. Cytokine measurements by cytometric bead array (CBA)

Human IL-6 and IL-17A were measured using cytometric bead array (CBA; BD Biosciences) according to the manufacturer's instructions. Briefly, samples were mixed with cytokine capture beads and incubated with PE-conjugated detection antibody for 3 h at room temperature. After centrifugation, supernatants were carefully aspirated and discarded, and bead pellets were resuspended. Samples were examined under BD flow cytometry (FACSCanto II, BD Company, San Diego, CA, USA) and data were analyzed using BD Cell Quest and CBA software.

### 2.5. Apoptosis detection by immunohistochemistry

Fixed BeWo cells were incubated for 10 min at room temperature with 5% acetic acid and then with 2.5% normal goat serum diluted in 20 mM Tris e HCl buffered saline (TBS, pH 7.2) for 30 min at 37 °C to block non-specific binding sites. Cells were incubated with mouse monoclonal antibody to cytokeratin 18 neo-epitope (clone M30, CytoDEATH, Roche Diagnostics, Mannheim, Germany) diluted 1:250 in TBS for 12 h at 4 °C. Next, cells were incubated with biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Heidelberg, Germany) diluted 1:600 in TBS for 1 h at 37 °C. The reaction signal was amplified using avidin e biotinylated alkaline phosphatase complex at 1:100 (ABC system, Vectastain, Vector Labs Inc., Southfield, MI, USA), developed with fast red-naphthol (Sigma Aldrich) and counterstained with Mayer's hematoxylin. Coverslips were mounted on glass slides and cells were examined under a light microscope to assess immunostained apoptotic cells. Results of apoptosis index were expressed as the mean number of apoptotic cells in 100 counted cells. For the immunostained cells counting, round coverslips containing the adherent cells were divided to form four quadrants, in each quadrant 25 random cells were counted and it was determined how many cells were immunostaining positive for M30 using the 40 of magnification.

### 2.6. Fas (CD95) expression in BeWo cells by flow cytometric analysis

BeWo cells were stained with FITC-conjugated anti-Fas/CD95 antibody (Santa Cruz Biotechnology Inc.) or isotype-matched negative control (Millipore, São Paulo,

SP, Brazil), diluted 1:100 for 30 min at 4 °C. Next, stained cells were washed and fixed with paraformaldehyde and sodium cacodylat. Fluorescence of 20,000 cells in each experimental condition was then analyzed under BD flow cytometry (FACSCanto II, BD Company).

### 2.7. Phosphorylated ERK1/2 detection by Western blotting

BeWo cells were harvested, centrifuged at 400 g for 5 min, homogenized and lysed on ice with lysis buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol, and 200 mM NaCl) supplemented with a protease inhibitor cocktail (Complete®, Roche Diagnostics), 1 mM sodium orthovanadate and 1 mM sodium fluoride (both from Sigma Aldrich). After centrifugation at 13,000 g for 15 min at 4 °C, the supernatants were collected, and the concentration of total protein was measured by Bradford assay [15].

Total protein samples (60 μg) were subjected to polyacrylamide gel electrophoresis under denaturing and non-reducing conditions (SDS-PAGE) at 12% and the proteins were electrotransferred to PVDF membranes (Thermo Scientific, Rockford, IL, USA). Blotted membranes were incubated in blocking buffer [4% fat dry milk in blotting buffer (25 mM Tris, 0.15 M NaCl, and 0.1% Tween 20, pH 7.4)] for 1 h at room temperature and incubated overnight with primary polyclonal goat anti-ERK1/2 phosphorylated (R&D Systems) antibody or monoclonal mouse anti-β-actin (Santa Cruz Biotechnology Inc.), both at 1:1.000 in blotting buffer with 2% fat dry milk. Next, the membranes were exposed to peroxidase-labeled secondary antibodies (rabbit anti-goat-peroxidase or goat anti-mouse-peroxidase; Jackson ImmunoResearch Laboratories, Baltimore, USA) diluted at 1:3.000 in blotting buffer with 2% fat dry milk for 2 h at room temperature. The reaction was revealed by chemiluminescence (ECL kit, GE Healthcare, São Paulo, SP, Brazil) and an equal loading of the proteins was confirmed by staining the blots with 1% Ponceau. Densitometric analyses were performed by using the 1D Image analysis software (KODAK, version 3.5, New Haven, CT, USA) in order to determine the mean intensity of the bands. Data were expressed as a relative density calculated by the ratio between ERK1/2 and β-actin bands.

### 2.8. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences between groups were assessed by one-way ANOVA and Bonferroni or Kruskal Wallis multiple comparison post-tests, when appropriate, by using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Cytokine production is differentially modulated after T. gondii ME49 or RH strain infection in BeWo cells

ME49-infected BeWo cells showed an up-regulation in the secretion of MIF, TNF-α, IL-12, IL-6 and IL-17A in relation to uninfected cells after 2 h of infection (Fig. 1A–E), but the production of IL-10 and TGF-β1 decreased when compared to uninfected controls (Fig. 1F,G). Conversely, RH-infected cells secreted more IL-10 and TGF-β1 than uninfected cells.

After 24 h of infection, ME49-infected cells maintained the higher production of MIF, TNF-α, IL-12, IL-6 and IL-17A (Fig. 1A–E) and lower secretion of IL-10 (Fig. 1F) than uninfected controls. In RH-infected cells the production of MIF, IL-17A, IL-10 and TGF-β1 increased in relation to uninfected cells (Fig. 1A,E,F,G).

These results indicate that cytokine production is differentially modulated after T. gondii ME49 or RH strain infection in BeWo cells, with an opposite cytokine profile for RH or ME49-infected cells. ME49-infected cells exhibited a predominantly pro-inflammatory response whereas RH-infected cells produced more anti-inflammatory cytokines.

### 3.2. Treatment of BeWo cells with MIF, TNF-α, and IFN-γ exacerbates apoptosis in cells infected with ME49 strain, but not with RH strain

In both times analyzed, 2 h and 24 h of infection, RH-infected BeWo cells showed an apoptosis index lower than uninfected cells whereas ME49-infected cells had a higher apoptosis index than controls. In addition the apoptosis index was higher in ME49-infected cells in comparison with RH-infected cells (Fig. 2).

After MIF treatment, ME49-infected cells showed higher apoptosis index than the respective controls after 2 h and 24 h of incubation (Fig. 2A). Also, the apoptosis index of RH-infected cells after MIF treatment increased in relation to infected/untreated cells after 2 h and 24 h of incubation, but decreased after 24 h when compared to uninfected/treated cells only.

The treatment with TNF-α induced a higher apoptosis index for RH-infected cells than in infected/untreated cells after 2 h and 24 h of incubation, but it was lower in comparison to uninfected/treated cells. In contrast, ME49-infected cells showed an apoptosis index higher than uninfected/treated cells after TNF-α treatment (Fig. 2B).

The IFN-γ treatment in RH-infected cells induced a lower apoptosis index compared to uninfected/treated cells after 2 h and 24 h of incubation. Moreover, after 24 h of IFN-γ treatment, the apoptosis index of RH-infected cells was higher than in infected/untreated cells. In the opposite way, ME49-infected cells showed a higher apoptosis index than the respective controls after 2 h and 24 h of IFN-γ treatment (Fig. 2C).

These results indicate that treatment of BeWo cells with pro-inflammatory cytokines like MIF, TNF-α, and IFN-γ exacerbates apoptosis in cells infected with ME49 strain, but not with RH strain of T. gondii.

### 3.3. Treatment of BeWo cells with IL-6, TGF-β1 and IL-10 inhibits apoptosis in BeWo cells infected with ME49 and RH strains of T. gondii

The treatment of BeWo cells with IL-6, TGF-β1 and IL-10 decreased the apoptosis index compared to uninfected/untreated cells, in both times analyzed (Fig. 3A–C).

Cells treated with IL-6 and infected with RH or ME49 strains showed an apoptosis index lower than uninfected/untreated cells after 2 h and 24 h of incubation (Fig. 3A). In addition, after 24 h of IL-6 treatment the apoptosis index increased in ME49-infected cells in comparison to cells uninfected/treated.

The TGF-β1 treatment in RH-infected cells induced lower apoptosis index than cells uninfected/treated after 2 h and 24 h of incubation (Fig. 3B). ME49-infected cells also showed an apoptosis index lower than infected/untreated cells, in both times of TGF-β1 treatment.

Cells treated with IL-10 and infected with RH strain showed lower apoptosis index than infected/untreated cells only after 2 h of incubation (Fig. 3C). Furthermore, ME49-infected cells showed lower apoptosis index in comparison to infected/untreated cells in both times of IL-10 treatment.

These results indicate that the treatment of BeWo cells with IL-6, TGF-β1 and IL-10 inhibits apoptosis in BeWo cells infected with either ME49 or RH strain of T. gondii.

### 3.4. Fas/CD95 expression is higher in BeWo cells infected with ME49 strain of T. gondii and it is related to pro-inflammatory cytokine treatment

Death receptor Fas/CD95 expression was analyzed in BeWo cells after infection with RH or ME49 strains and treatment with cytokines (Fig. 4). After 2 h of incubation, ME49-infected cells exhibited higher Fas/CD95 expression than uninfected and RH-infected cells (Fig. 4A). Moreover, the treatment with MIF, IFN-γ and TNF-α increased Fas/CD95 expression in cells infected with RH or ME49 strain, whereas the treatment with IL-6, IL-10 and TGF-β1 decreased the Fas/CD95 expression.

After 24 h of incubation, cells infected with RH or ME49 strain presented higher Fas/CD95 expression than uninfected cells

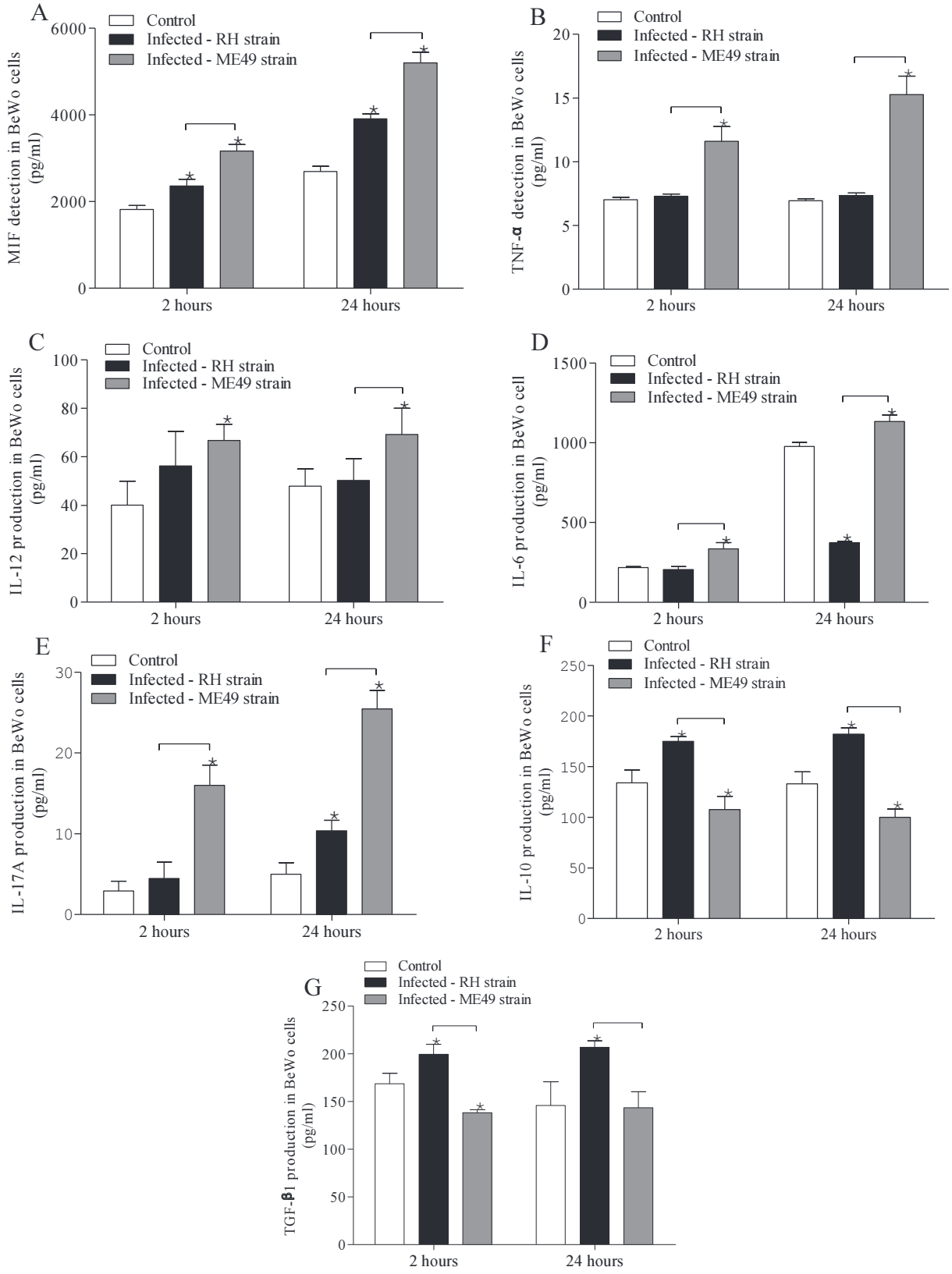


Fig. 1. Cytokine production by BeWo cells infected with T. gondii RH or ME49 strains at 5:1 (parasite:cell) ratio or cultured with medium alone (control). Cell-free supernatants were collected after 2 h and 24 h of infection and the cytokines MIF (A), TNF-a (B), IL-12 (C), IL-6 (D), IL-17A (E), TGF-b1 (F) and IL-10 (G) were measurement by ELISA or cytometric beads array (CBA). The cytokine in medium (pg/ml), represents the cytokine production of a population of 1 × $10^5$ cells. Data are expressed as mean ± SEM of six independent experiments in triplicate. *Comparison between infected and uninfected cells ($P < 0.05$). ○ Comparison between RH and ME49 strains of T. gondii ($P < 0.05$).

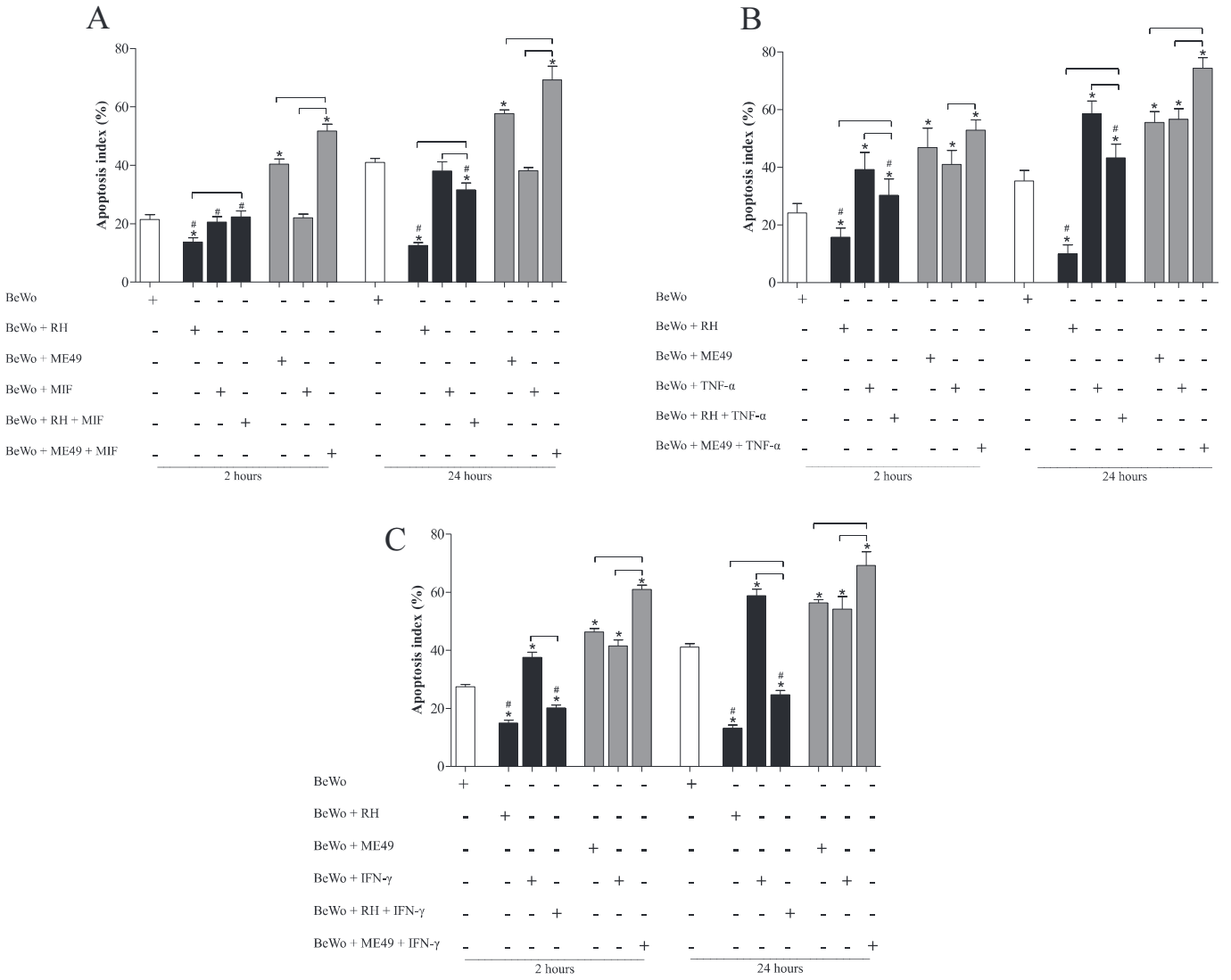


Fig. 2. Influence of T. gondii RH and ME49 strains in the apoptosis index (%) of BeWo cells pre-treated with the recombinant cytokines: MIF (A) or TNF-α (B) or IFN-γ (C) at 15 ng/ml for 24 h, infected for 3 h and treated again with the cytokines described above, for 2 and 24 h. Cells were fixed and the apoptosis index was expressed as the mean number of apoptotic cells in 100 counted cells. Data are expressed as mean ± SEM of six independent experiments in triplicate. (*) Comparison between control (cells uninfected/untreated) and other experimental conditions ($P < 0.05$). (C) Comparison between different conditions for each strain type of T. gondii ($P < 0.05$). (#) Comparison between RH and ME49 strains of T. gondii for each experimental condition ($P < 0.05$).

(Fig. 4B). The IFN-γ treatment induced an increase in the Fas/CD95 expression in uninfected control and cells infected with RH or ME49 strain. The treatment with IL-6, IL-10 and TGF-β1 induced a decrease in the Fas/CD95 expression in cells infected with RH or ME49 strain of T. gondii.

These results indicate that the Fas/CD95 expression is higher in BeWo cells infected with ME49 strain of T. gondii and it is related to a pro-inflammatory cytokine-enriched environment like MIF, IFN-γ and TNF-α.

### 3.5. Increased apoptosis in ME49-infected BeWo cells is directly associated with increased Fas/CD95 expression

The relationship between apoptosis index and Fas/CD95 expression in BeWo cells infected with RH or ME49 strain was investigated in different experimental conditions (Fig. 5). It was observed a direct association between increased apoptosis index and Fas/CD95 expression in ME49-infected BeWo cells, after 2 h and 24 h of infection (Fig. 5). In RH-infected cells, however, the relationship between apoptosis index and Fas/CD95 expression was inversely associated, since RH-infected cells showed higher Fas/CD95 expression and lower apoptosis index (Fig. 5).

The association between apoptosis index and Fas/CD95 expression was also observed after the cytokine treatment (Fig. 5AeF). The MIF treatment in RH- or ME49-infected BeWo cells showed direct association between increased Fas/CD95 expression and apoptosis index after 2 h and 24 h of incubation (Fig. 5A). In addition, in both times analyzed, the treatment with TNF-α and IFN-γ presented direct association between increased apoptosis index and Fas/CD95 expression in uninfected or RH- or ME49-infected cells (Fig. 5B,C).

Similar findings were observed when BeWo cells were treated with IL-6, TGF-β1 and IL-10 (Fig. 5DeF). After IL-6 treatment the lower apoptosis index in uninfected or ME49-infected cells was associated with the reduction in Fas/CD95 expression, in both times analyzed (Fig. 5D).

Also, the TGF-β1 treatment demonstrated direct association between the decreased apoptosis index and Fas/CD95 expression;

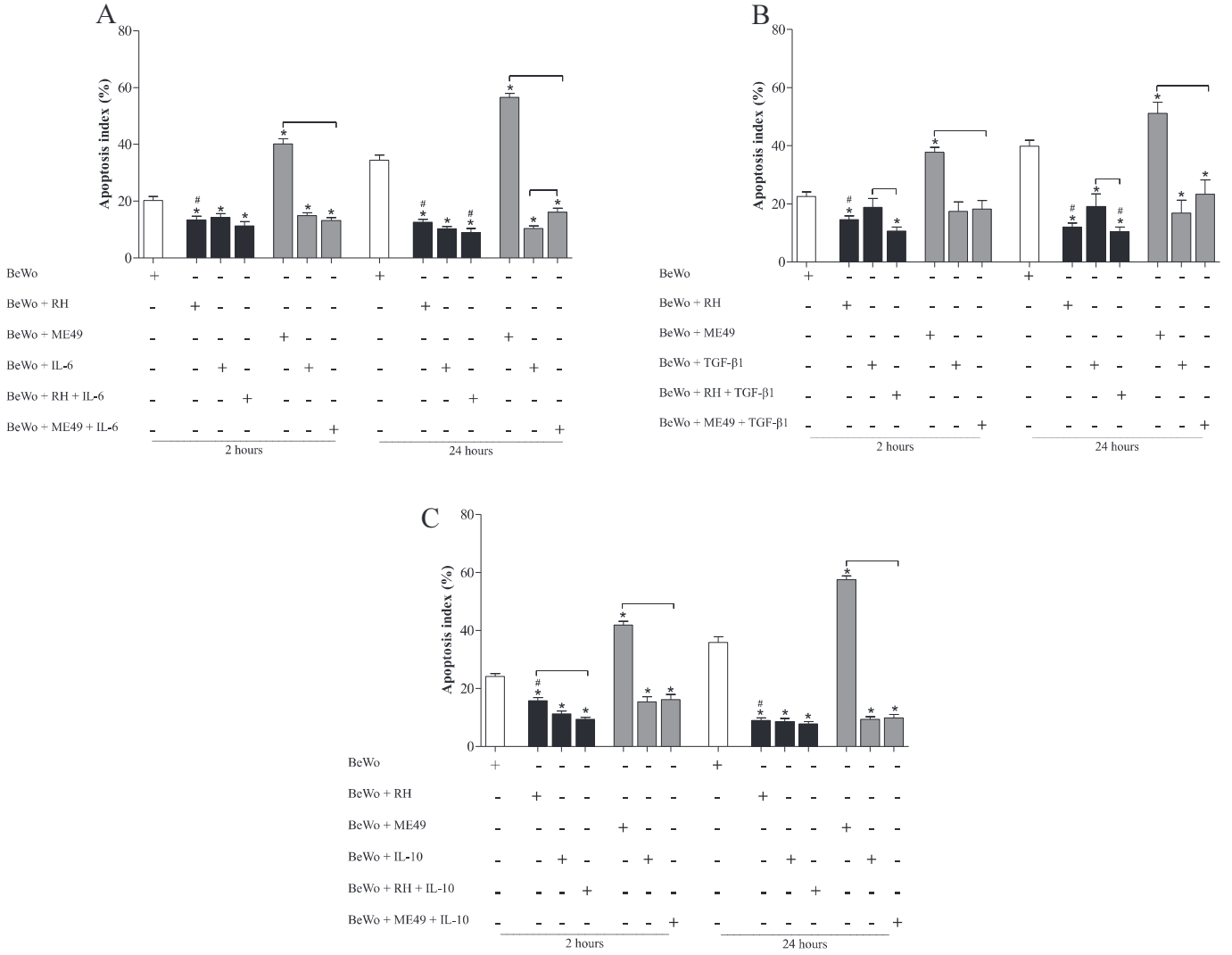


Fig. 3. Influence of T. gondii RH and ME49 strains in the apoptosis index (%) of BeWo cells pre-treated with the recombinant cytokines: IL-6 (A) or TGF-β1 (B) or IL-10 (C) at 15 ng/ml for 24 h, infected for 3 h and treated again with the cytokines described above, for 2 and 24 h. Cells were fixed and the apoptosis index was expressed as the mean number of apoptotic cells in 100 counted cells. Data are expressed as mean ± SEM of six independent experiments in triplicate. (*) Comparison between control (cells uninfected/untreated) and other experimental conditions ($P < 0.05$). (C) Comparison between different conditions for each strain type of T. gondii ($P < 0.05$). (#) Comparison between RH and ME49 strains of T. gondii for each experimental condition ($P < 0.05$).

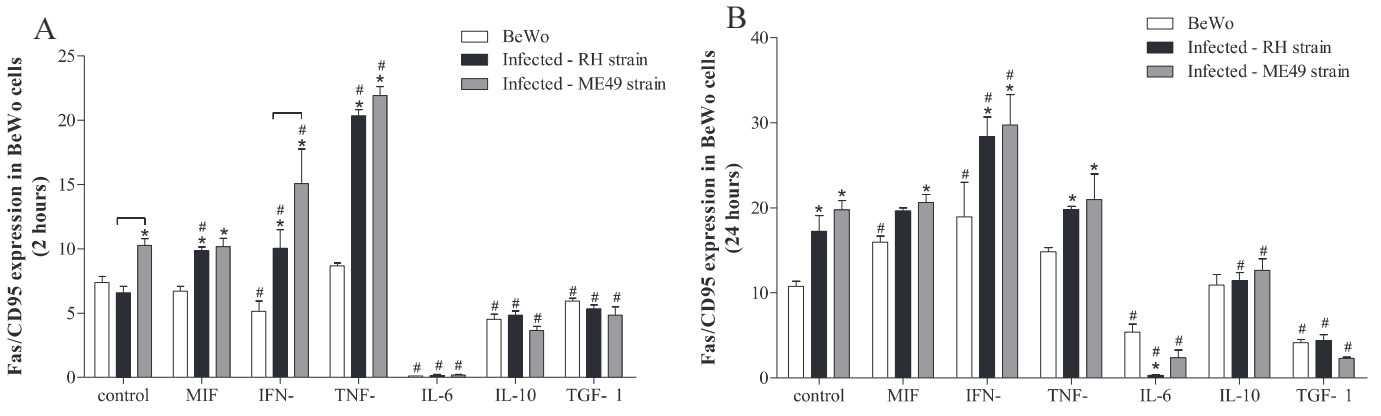


Fig. 4. Influence of T. gondii RH and ME49 strains in the cell surface Fas/CD95 expression (%) in BeWo cells pre-treated with the recombinant cytokines: MIF, TNF-α, IFN-γ, IL-6, TGF-β1 or IL-10 at 15 ng/ml for 24 h, infected for 3 hand treated again with the cytokines described above, for 2 h (A) and 24 h (B). BeWo cells were stained with anti-Fas antibody and analyzed by flow cytometry. Quantitative expression of Fas/CD95 in BeWo cells in different experimental conditions is expressed as mean ± SEM of six independent experiments in triplicate. (*) Comparison between experimental condition and the respective controls ($P < 0.05$). (#) Comparison between treated and untreated conditions ($P < 0.05$). (C) Comparison between RH and ME49 strains for each experimental condition ($P < 0.05$).

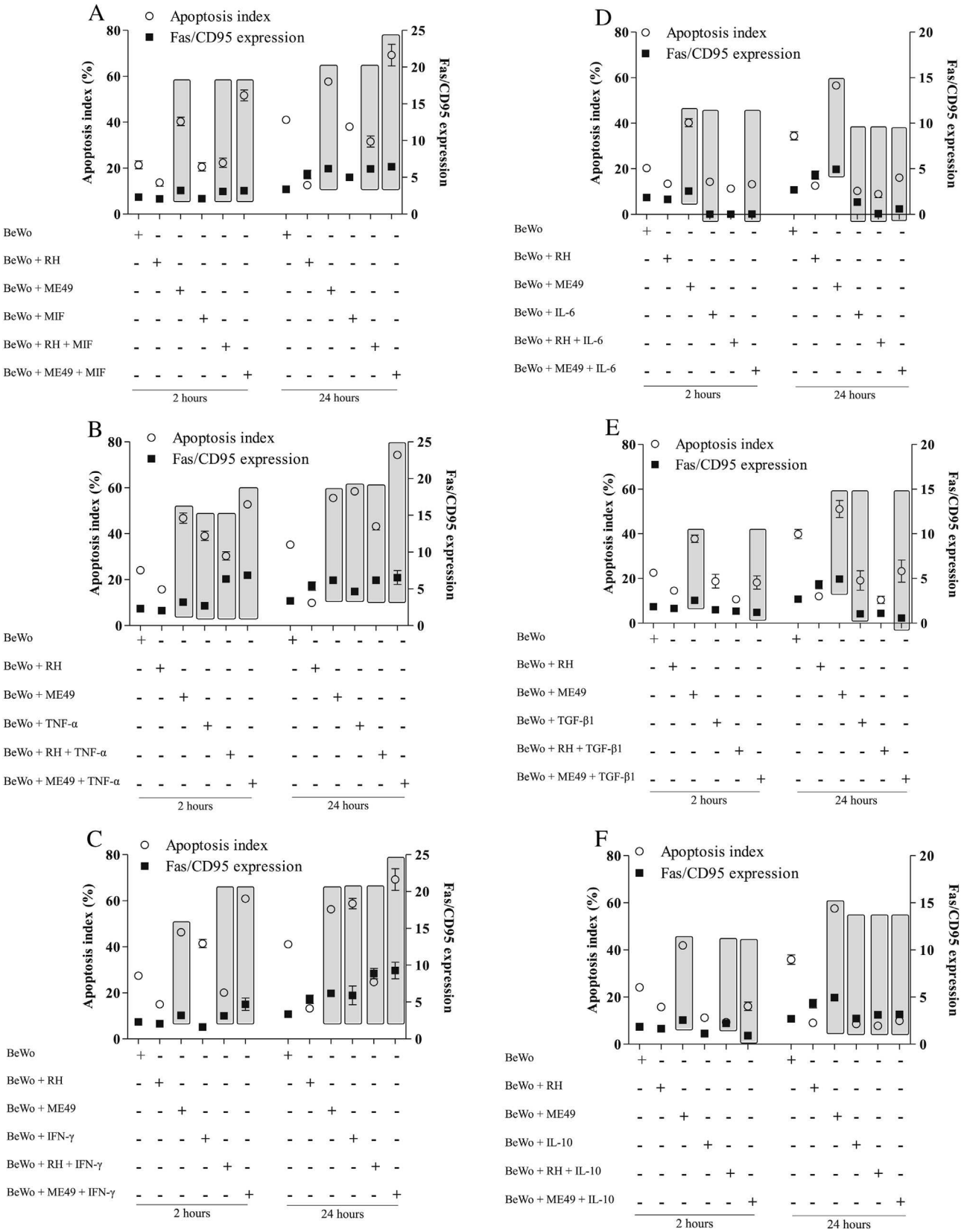


Fig. 5. Relationship between the apoptosis index and the Fas/CD95 expression in BeWo cells. Cells were pre-treated with the recombinant cytokines: MIF (A), TNF-α (B), IFN-γ (C), IL-6 (D), TGF-β1 (E) or IL-10 (F) at 15 ng/ml for 24 h, infected with RH or ME49 strain for 3 h and treated again with the cytokines described above, for 2 h and 24 h. The gray rectangles indicate the conditions in which associations between apoptosis index and the Fas/CD95 expression were observed.

this association was also observed in ME49-infected cells after TGF-b1 treatment for 2 h and 24 h (Fig. 5E).

The IL-10 treatment in RH- or ME49-infected BeWo cells showed direct association between apoptosis index and Fas/CD95 expression after 2 h of incubation (Fig. 5F). After 24 h, it was observed that the lower apoptosis index was associated with the reduction of Fas/CD95 expression in uninfected or RH- or ME49-infected cells.

These results indicate a direct association between increased apoptosis and Fas/CD95 expression in ME49-infected BeWo cells, whereas this relationship is inversely associated in RH-infected BeWo cells.

### 3.6. Phosphorylated ERK1/2 expression was higher in BeWo cells infected with RH strain of T. gondii

To verify whether the differences observed in the apoptosis index of BeWo cells infected with RH or ME49 strain of T. gondii are related to intracellular protein expression, the phosphorylation of the MAPK protein ERK1/2 was analyzed (Fig. 6A). After 2 h of infection, a lower phosphorylation of ERK1/2 was observed in ME49-infected cells compared to uninfected cells (Fig. 6B). In addition, a decreased ERK1/2 phosphorylation was observed in uninfected BeWo cells after 24 h of incubation (Fig. 6B). After 24 h, RH-infected cells showed higher ERK1/2 phosphorylation than uninfected or ME49-infected cells (Fig. 6B).

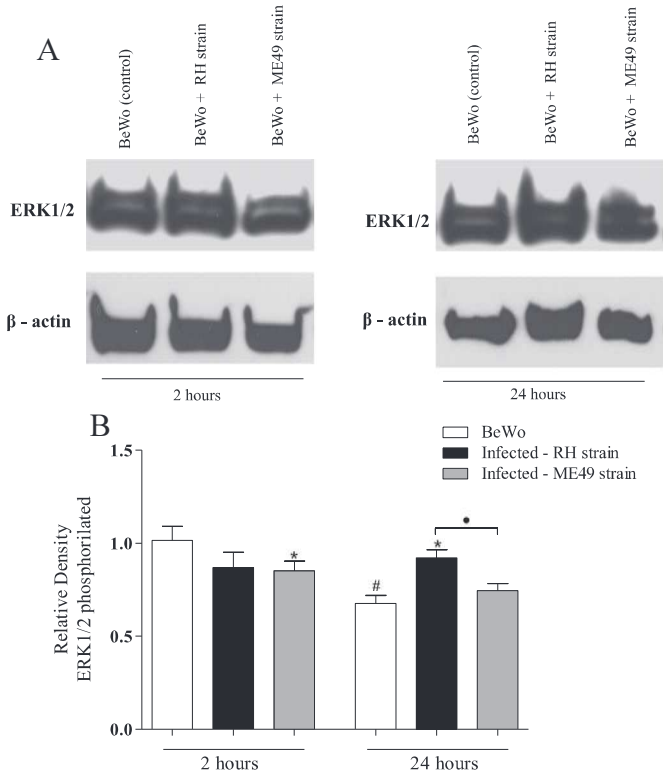


Fig. 6. Detection of phosphorylated ERK1/2 protein in BeWo cells infected with RH or ME49 strain of T. gondii. After 2 and 24 h of infection cells were lysed and samples (60 μg of total protein) were subjected to Western blotting. As control, cells were cultured with medium alone. (A) Representative Western blotting for phosphorylated ERK1/2 and b-actin in BeWo cells, after 2 h and 24 h of infection. (B) Densitometric analyses showing the relative density of phosphorylated ERK1/2 protein obtained by the ratio between the ERK1/2/b-actin bands in BeWo cells. The data were expressed as the mean ± SEM of six independent experiments performed in triplicate. (*) Comparison between infected and uninfected cells ($P < 0.05$). (○) Comparison between RH and ME49 strains of T. gondii ($P < 0.05$). (#) Comparison between 2 h and 24 h of infection ($P < 0.05$).

## 4. Discussion

Alterations of apoptosis in trophoblastic cells can result in pregnancy loss or embryonic damages [16,17]. In our previous study, the apoptosis of BeWo trophoblastic cells was also related to the strain type of T. gondii [12]. In the present study, we evaluated possible factors involved in the differential apoptosis in BeWo cells infected with distinct T. gondii strains. We observed that ME49-infected BeWo cells exhibited a predominantly pro-inflammatory response profile, with higher secretion of MIF, TNF-a, IL-12, IL-17A and IL-6, whereas RH-infected cells showed a higher production of anti-inflammatory cytokines as TGF-b1 and IL-10. These different profiles of cytokine production can be associated to an effective mechanism elicited by the parasite that is observed after infection with moderately virulent strains as ME49 and, conversely, to a strategy of the parasite to evade of the immune response as occurs with highly virulent strains like RH. In agreement with our findings, Robben et al. [18] demonstrated that T. gondii type II strain infection induces high levels of Th-1 polarizing cytokines, mainly IL-12. An in vivo study showed that infection with ME49 strain induces secretion of high levels of the pro-inflammatory cytokine IFN-g [19]. Also, it was demonstrated that T. gondii RH strain can partially antagonize or even subvert inflammatory responses, since parasite acts up-regulating host anti-inflammatory cytokines, such as IL-10 and TGF-b, restricting the production of pro-inflammatory cytokines as TNF-a, IFN-g, IL-12 and IL-6 [20e23]. These data reinforce the strategy used by the parasite to evade anti-parasitic effector mechanisms of the host cell, since the success of T. gondii infection depends on the host immune response modulation.

In the present study, the treatment of BeWo cells with the pro-inflammatory cytokines (MIF, TNF-a and IFN-g) induced an increase in the apoptosis index in ME49-infected cells, in both times analyzed. It is likely that apoptosis has increased after infection with the moderately virulent ME49 strain for the parasite clearance, as an attenuating mechanism of infection. Moreover, cells infected with RH strain and treated with pro-inflammatory cytokines showed a higher apoptosis index than infected/untreated cells, but lower than uninfected/treated cells. These results suggest that even after the treatment with pro-inflammatory cytokines the RH strain of T. gondii is able to inhibit apoptosis of the host cell. Our findings are in agreement with several studies demonstrating that cells infected with RH strain of T. gondii were resistant to apoptosis after treatment of host cells with pro-apoptotic stimuli as irradiation and Fas-mediated apoptosis [9,22,24,25].

The treatment with IL-6 and anti-inflammatory cytokines as IL-10 and TGF-b1 reduced apoptosis in BeWo cells infected with RH or ME49 strain of T. gondii. A previous study showed that apoptosis was inhibited in BeWo cells after treatment with IL-10 [26]. Moreover, treatment of trophoblastic cells with IL-6, IL-10 and TGF-b1 is related to the inhibition of apoptosis by decreasing the death receptor Fas/CD95 [27]. In that sense, we investigated if the expression of the death receptor is related to these differences in the incidence of apoptosis after infection with RH or ME49 strain of T. gondii and under influence of cytokine treatments described above. We observed that ME49-infected cells showed increased Fas/CD95 expression compared to uninfected or RH-infected cells after 2 h of infection. This can be associated with the higher incidence of apoptosis in BeWo cells after infection with ME49 strain of T. gondii. After 24 h of infection, Fas/CD95 expression in RH-infected cells was also higher than uninfected cells, indicating that the inhibition of apoptosis in BeWo trophoblastic cells induced by this strain of T. gondii is not related with the death receptor expression.

These findings can be explained by the ability of type I strains of T. gondii to interact with intracellular pathways of host cells [28,29]. The anti-apoptotic reaction induced by T. gondii infection is associated with up-regulation of a series of anti-apoptotic genes and up-regulating anti-apoptotic proteins [30e32].

Death receptor Fas/CD95 expression was also associated with the cytokine treatments in BeWo cells. Fas/CD95 expression increased after treatment with the pro-inflammatory cytokines (MIF, IFN-g and TNF-a) and these augments were pronounced in ME49-infected BeWo cells. In contrast, Fas/CD95 expression decreased after treatment with IL-6 and the anti-inflammatory cytokines (TGF-b1 and IL-10), even after infection with ME49 strain. These results can be associated to the incidence of apoptosis described in this study, demonstrating that the increased apoptosis indexes in BeWo cells treated with pro-inflammatory cytokines are associated to the increased Fas/CD95 expression. In an opposite way, the decreased apoptosis indexes in BeWo cells treated with IL-6 and the anti-inflammatory cytokines (TGF-b1 and IL-10) are associated with the decreased Fas/CD95 expression. These results are in agreement with another study that showed an up-regulation of the death receptor Fas/CD95 after IFN-g and TNF-a treatment of trophoblast cells, whereas the treatment with IL-10 and IL-6 decreased Fas expression in these cells [27].

Differences in the virulence between strain types of T. gondii can be associated to the host cell intracellular pathways that are manipulated by the parasite [33]. In the present study we also demonstrated that the differential induction of apoptosis observed after infection with RH or ME49 strain can be associated to the increased phosphorylation of the anti-apoptotic MAPK protein ERK1/2. After 24 h of infection, we observed a higher expression of phosphorylated ERK1/2 in RH-infected cells than uninfected or ME49-infected cells. These results suggest that the inhibition of apoptosis observed in BeWo cells after infection with T. gondii RH strain is associated with the increased phosphorylation of the anti-apoptotic ERK1/2 protein. Accordingly, previous studies have demonstrated that RH strain of T. gondii is able to induce ERK1/2 phosphorylation [34e36]. However, there are no studies showing the relationship of ERK1/2 phosphorylation with the decreased incidence of apoptosis observed in cells after infection with RH strain of T. gondii. In addition, Quan et al., [2013] [37] demonstrated that phosphoinisitide 3-kinase is the one of the major routes used by T. gondii to inhibit apoptosis of host cells.

Altogether, our findings demonstrated that opposing mechanisms of interference in apoptosis of human trophoblastic BeWo cells after infection with highly (RH) or moderately virulent (ME49) strains of T. gondii can be associated with the differential cytokine profile secreted, the death receptor Fas/CD95 expression and phosphorylated ERK1/2 protein expression. Therefore, the differences in the incidence of host cell apoptosis are related to virulence factors of T. gondii strains and this can be critical for the outcome of infection in congenital toxoplasmosis.

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