Effect of Macrophage Migration Inhibitory Factor (MIF) in Human Placental Explants Infected with *Toxoplasma gondii* Depends on Gestational Age

Angelica de Oliveira Gomes,*
Deise Aparecida de Oliveira Silva,*
Neide Maria Silva,* Bellisa de Freitas Barbosa,*
Priscila Silva Franco,* Mariana Bodini Angeloni,*
Marise Lopes Fermino,[†]
Maria Cristina Roque-Barreira,[†] Nicoletta Bechi,[‡]
Luana Ricci Paulesu,[‡] Maria Célia dos Santos,[§]
José Roberto Mineo,* and Eloisa Amália Vieira Ferro*

From the Instituto de Ciências Biomédicas,* and the Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina,[§] Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil; the Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos,[†] Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil; and the Dipartimento di Fisiologia,[‡] Università Degli Studi di Siena, Siena, Italy

Because macrophage migration inhibitory factor (MIF) is a key cytokine in pregnancy and has a role in inflammatory response and pathogen defense, the objective of the present study was to investigate the effects of MIF in first- and third-trimester human placental explants infected with *Toxoplasma gondii*. Explants were treated with recombinant MIF, IL-12, interferon- γ , transforming growth factor- β 1, or IL-10, followed by infection with *T. gondii* RH strain tachyzoites. Supernatants of cultured explants were assessed for MIF production. Explants were processed for morphologic analysis, immunohistochemistry, and real-time PCR analysis. Comparison of infected and stimulated explants versus noninfected control explants demonstrated a significant increase in MIF release in first-trimester but not third-trimester explants. Tissue parasitism was higher in third- than in first-trimester explants. Moreover, *T. gondii* DNA content was lower in first-trimester explants treated with MIF compared with untreated explants. However, in third-trimester explants, MIF stimulus decreased *T. gondii* DNA content only at the highest concentration of the cytokine. In addition, high expression of MIF receptor was observed in first-trimester placental explants, whereas

MIF receptor expression was low in third-trimester explants. In conclusion, MIF was up-regulated and demonstrated to be important for control of *T. gondii* infection in first-trimester explants, whereas lack of MIF up-regulation in third-trimester placentas may be involved in higher susceptibility to infection at this gestational age. (Am J Pathol 2011, 178:2792–2801; DOI: 10.1016/j.ajpath.2011.02.005)

Toxoplasma gondii is a protozoan parasite extremely adapted for infection in humans, which accounts for its ubiquitous distribution and high seroprevalence.¹ This parasite has developed mechanisms that make possible a long-lasting parasite-host interaction to ensure its survival without inducing life-threatening disease in the intermediate host.² In contrast, the immune system in immunocompetent hosts controls parasite replication through induction of antibody- and cell-mediated immune responses that preclude disease onset; however, it cannot abolish the infection.² Therefore, *T. gondii* keeps a fine balance between induction and suppression of the immune response to guarantee host survival as a safe harbor for parasite development.² In immunocompromised individuals, however, *T. gondii* can be an important opportunistic pathogen and can cause severe disease such as encephalitis, necrotic lesions in the central nervous system, or retinochoroiditis.^{3,4} In addition, congenital disease can occur in fetuses of mothers primarily infected with *T. gondii* during pregnancy.⁵ Three major strains (types I, II, and III) of *T. gondii* demonstrate widespread distribution. Type II is by far the most prevalent genotype in human congenital toxoplasmosis, although type I is most

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Address reprint requests to Prof. Eloisa A. Ferro, Ph.D. Laboratório de Histologia e Embriologia, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Av Pará 1720, Uberlândia, 38405-320 Minas Gerais, Brazil. E-mail: eloisa@umuarara.ufu.br.

often associated with severe congenital toxoplasmosis.⁶ The type II strain association with severe congenital toxoplasmosis depends on host-parasite interaction. Disease outcome depends not only on the intrinsic virulence of the infecting strain but also on the host immune response and specific susceptibility to infection.⁷

Successful gestation is associated with nonrejection of paternal antigens by the mother, which is achieved through multiple immunologic mechanisms at the interface between the mother and the fetus. Typical examples include inactivation of natural killer cells through HLA-G expression,⁸ tryptophan depletion by indoleamine 2,3-dioxygenase,⁹ expansion of the regulatory T-cell subset during pregnancy,¹⁰ and maternal shift from a Th1 to a Th2 immune response.¹¹ Hormones such as progesterone and glucocorticoids have an important role in Th2 immune responses.^{12,13} However, this type of immune response is not efficient against protozoan parasites, and the mother becomes more susceptible to pathogens such as *T. gondii*.¹⁴ Thus, when the parasitic infection occurs during pregnancy, the maternal body must control the infection and simultaneously create an environment of immune privilege for the fetus.

To understand how the immune system can attempt to resolve this paradox, it was hypothesized that a proinflammatory cytokine that is normally expressed in pregnancy¹⁵ and can be induced rather than inhibited in the presence of glucocorticoid hormones¹⁶ might have an important role in this condition. A cytokine with these characteristics is macrophage migration inhibitory factor (MIF), first described as a factor produced by lymphocytes and associated with inhibition of random macrophage migration during delayed hypersensitivity responses.^{17,18} However, its real importance was neglected for a long time until the interest in MIF was intensified because of its wide range of biological functions. MIF has a multifaceted role in immune responses such as phagocytosis, inflammation, and inhibition of neutrophil apoptosis.^{19,20} In addition, it is an important factor in determining the outcome of infections caused by protozoan parasites such as *Plasmodium chabaudi*,²¹ *P. falciparum*,²² *Leishmania major*,²³ *Trypanosoma cruzi*,²⁴ and *T. gondii*,^{25,26,27} and in helminth (*Taenia crassiceps*)²⁸ and bacterial (*Salmonella typhimurium*)²⁹ infections.

A previous study²⁵ provided evidence that *T. gondii*-soluble antigen induces production and secretion of MIF by human first-trimester villus explants. The objective of the present study was to investigate the effect of MIF on susceptibility to *T. gondii* infection in first- and third-trimester human placental explants.

Materials and Methods

Placenta Samples and Human Chorionic Villus Explant Cultures

Third-trimester placentas (36 to 40 weeks of gestation) were collected after elective cesarean section deliveries, and first-trimester placentas (9 to 12 weeks of gestation) were obtained after authorized termination of pregnancy

in women seronegative for *T. gondii* or other infection. Placental tissues were washed in ice-cold sterile PBS (pH 7.2) and aseptically dissected using a microscope to remove endometrial tissue and fetal membranes up to 1 hour after collection. Terminal chorionic villus containing five to seven free tips per explant was collected as described previously.³⁰ The volume of villus explants was determined as previously described.²⁴ In brief, 800 μ L medium was placed in a pipette and the villus explant was added, which became totally submerged in the medium. The amount of increased volume was assumed as the villus volume. Overall, the volume of villus explants was approximately 10 mm³. Explants were added to a 96-well plate (one per well) and cultured in 150 μ L RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (complete medium) for 24 hours at 37°C and 5% CO₂. The study was approved by the institutional ethics committee.

Parasite

T. gondii RH strain tachyzoites were maintained in Swiss mice via intraperitoneal serial passage at 48-hour intervals.³¹ Mouse peritoneal exudates were harvested in sterile RPMI 1640 medium and washed twice (720 $\times$ g for 10 minutes at 4°C) in medium. Tachyzoites were resuspended in medium, counted in a hemocytometric chamber, and used to infect a BeWo trophoblastic cell line (American Type Culture Collection, Manassas, VA). The parasites were maintained by passages in this cell line for posterior infection of placental explants.

Human Chorionic Villus Explant Treatment and Infection

Villus explants were treated using various concentrations of recombinant MIF (5, 25, and 100 ng/mL), IL-12 (25 ng/mL), interferon- γ (IFN- γ ; 25 ng/mL), transforming growth factor- β 1 (TGF- β 1; 1 and 10 ng/mL), or IL-10 (10 and 25 ng/mL). Alternatively, explants were treated with goat anti-human MIF antibody (10 μ g/mL) or goat IgG (10 μ g/mL) for 30 minutes to verify the effect of MIF blockage. Nontreated explants served as controls. Control and experimental conditions were conducted in parallel.

After 24 hours of incubation with 5% CO₂ at 37°C, explants were infected or not with *T. gondii* tachyzoites (1×10^6 parasites per well) and incubated for 24 hours. Villus explants were then washed with medium and again incubated in the presence or absence of the same stimulus for 24 hours as described. Villus explants were collected for morphologic analysis, immunohistochemistry (IHC) for MIF, MIF receptor (CD74), and *T. gondii* detection. Infected villus explants were processed using real-time PCR to determine parasite burden. Culture supernatants were collected and stored at -80°C for measurement of MIF and NO.

MIF and NO Measurements

MIF was measured in supernatants from villus explant cultures using a double-antibody sandwich enzyme-

linked immunosorbent assay. In brief, plates were coated overnight with capture monoclonal antibody anti-human MIF (R&D Systems Europe Ltd., Abingdon, Oxfordshire, England), blocked, and incubated with samples in duplicate for 2 hours at room temperature. After washing, plates were incubated with biotinylated detection polyclonal antibody anti-human MIF (R&D Systems Europe Ltd.) for 2 hours at room temperature. The assay was developed using streptavidin–horseradish peroxidase (Zymed Laboratories, Inc., South San Francisco, CA), and revealed with 3,3',5,5'-tetramethylbenzidine (Zymed Laboratories, Inc.). MIF concentration was determined via extrapolation from a standard curve obtained from known concentrations of rMIF cytokine standard (R&D Systems Europe Ltd.). Assay sensitivity was 18 pg/mL. Intra-assay and interassay coefficients of variation were 3.86% and 9.14%, respectively.

NO release in supernatants from villus explant cultures was determined using the Griess method.³² In brief, samples were added in triplicate to 96-well plates and mixed 1:1 with 1% sulfanilamide dihydrochloride and 0.1% naphthylethylenediamide dihydrochloride in 2.5% H₃PO₄. Absorbance was read in a plate reader at 570 nm, and concentration was determined with reference to a standard curve of sodium nitrite with concentrations ranging from 5 to 200 μ mol/L.

Villus Explant Protein Content Determination

Frozen villus explants were homogenized in radioimmuno-precipitation assay buffer [50 mmol/L Tris hydrochloride, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS; pH 7.5] plus protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). The homogenate was centrifuged at 15,000 $\times g$ for 15 minutes at 4°C. The supernatants were used for protein content measurement using the Bradford method.³³ Total protein concentration (mg/mL) was used to normalize data of MIF concentration (pg/mL) obtained at enzyme-linked immunosorbent assay, resulting in MIF concentration (pg/mL).

Morphologic Analysis

To verify the integrity of villus explants, fragments of placental explants were fixed in 10% buffered formalin, dehydrated in increasing alcohol concentrations, and embedded in glycol methacrylate (Historesin; LKB Produkter AB, Stockholm, Sweden), and 2- μ m sections were stained using 1% toluidine blue and analyzed at light microscopy. Alternatively, fixed placental explants were embedded in paraffin for IHC. For electron microscopy, explants were fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.2 mmol/L PBS, dehydrated in acetone, and embedded in Epon 812 resin (Fluka Chemie GmbH, Buchs, Switzerland). Sections were stained with 2% uranile³⁴ and 0.5% lead citrate³⁵ contrast medium, and analyzed using an electron microscope (Zeiss EM 109; Carl Zeiss AG, Oberkochen, Germany).

IHC

Paraffin-embedded explant tissues were cut into 4- μ m sections. For antigen retrieval, sections were covered with trypsin solution (0.05% trypsin and 0.1% calcium chloride (Sigma-Aldrich Corp., St. Louis, MO) for 30 minutes at 37°C. Explant sections were incubated with 5% acetic acid at room temperature. For MIF detection, explants were treated with 2.5% normal rabbit serum in Tris-buffered saline solution to block nonspecific sites. Explants were incubated with goat polyclonal antibody anti-human MIF (R&D Systems Europe Ltd.). Negative controls were generated via replacement of the primary antibody with normal goat serum. Samples were washed in Tris-buffered saline solution and incubated with biotinylated rabbit anti-goat IgG (Jackson ImmunoResearch Europe, Ltd., Newmarket, Suffolk, England) for 1 hour at 37°C. Alternatively, for CD74 and *T. gondii* detection, explants were treated with 2.5% normal goat serum in Tris-buffered saline solution to block nonspecific sites. Explants were incubated with mouse anti-human CD74 (eBioscience, Inc., San Diego, CA), or mouse anti-*T. gondii* serum, respectively, overnight at 4°C. Negative controls were generated by replacement of the primary antibody with normal mouse serum. Samples were washed in Tris-buffered saline solution and incubated with biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at 37°C. Amplifications were performed using streptavidin–biotinylated alkaline-phosphatase complex (ABC kit; Vector Laboratories, Inc., Burlington, CA), developed with Fast Red–naphthol (Sigma-Aldrich Corp.), and counterstained using Mayer's hematoxylin.

Quantification of Tissue Parasitism

Tissue parasitism was evaluated at IHC and real-time PCR. Infected and nontreated explants from first- and third-trimester gestations were evaluated at IHC. To score the parasite load in placental explants, quantification was performed in five noncontiguous sections 40 μ m apart. The number of parasites in a whole villus explant was quantified. For each field, parasite immunolocalization was determined as follows: parasites attached to trophoblasts, parasites inside trophoblasts, and parasites inside the mesenchyme. The sum of parasites was considered the total parasite load per tissue. Because the villi were similar in size, it was possible to quantify approximately 10 microscopic fields per villus (original magnification, $\times 400$). Variations in these values were adjusted to obtain 10 microscopic fields for each villus. All were composed of areas of connective tissue surrounded by trophoblasts.

Placental explants previously stimulated and infected with *T. gondii* tachyzoites were collected for parasite quantification at real-time PCR using the 2^{− $\Delta\Delta C_T$} method.³⁶ DNA extraction was performed using lysis buffer (10 mmol/L Tris hydrochloride 5 mmol/L EDTA, 0.2% SDS, and 200 mmol/L NaCl) plus proteinase K (20 mg/mL) and precipitation of DNA with isopropyl alcohol. PCR amplification and analysis were achieved

using a sequence detector (ABI Prism 7500; Applied Biosystems, Inc., Foster City, CA). All reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, Inc.) with a 10- μ L volume in each reaction, which contained 25 ng template cDNA, 2.5 pmol of each primer, and 5 μ L SYBR Green. The cycles were processed according to the manufacturer's instructions. Each sample was tested in duplicate, and all quantifications were normalized on the basis of human β -actin. Primers used for PCR amplification corresponded to the *T. gondii* B1 gene (amplicon 50 pb): forward, 5'-TTCAAGCAGCGTATTGTCGA-3', and reverse, 5'-CATGAACGGATGCAGTTCCT-3', and the β -actin human gene (amplicon 100 pb): forward, 5'-AAGGATTCCTATGTGGGCGA-3', and reverse, 5'-TCCATGTCGTCCCAGTTGGT-3'. *T. gondii* RH strain tachyzoites (10^7 parasites) served as positive control of the reaction, and samples of noninfected placental explants as negative control. Data were analyzed using Data Analysis and Technical Graphics software (Origin version 6.0; Microcal Software, Inc., Northampton, MA).

Quantification of CD74 at Real-Time PCR

First- and third-trimester placental explants previously infected or not with *T. gondii* tachyzoites were collected for CD74 quantification at real-time PCR. Total RNA was isolated from the tissues using Trizol reagent (Life Technologies Corp., Carlsbad, CA) following the manufacturer's instructions. cDNA synthesis was performed in a final volume of 20 μ L using ImProm-II Reverse Transcriptase (Promega Corp., Madison, WI). The reaction mixture contained 4 mg total RNA, 20 pmol oligo dT primer (Life Technologies Corp.), 40 U ribonuclease inhibitor (RNasin; Promega Corp.), 500 mmol/L dNTP mix, and 1 U reverse transcriptase in 1X reverse transcriptase buffer. cDNA was treated with 10 mg RNase (Gibco-BRL, Invitrogen Corp., Carlsbad, CA) and used immediately. PCR amplification and analysis were achieved using a sequence detector (ABI Prism 7500; Applied Biosystems, Inc.). All reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, Inc.), with a 25- μ L volume in each reaction, which contained 2 μ L template cDNA, 5 pmol of each primer, and 12.5 μ L SYBR Green. Primers used for PCR amplification were β -actin (forward, 5'-AGCTGCGTTTTACACCCTTT-3', and reverse, 5'-AAGCCATGCCAATGTTGTCT-3') and CD74 (forward, 5'-CATGGATGACCAACGCGAC-3', and reverse, 5'-TGTA-CAGAGCTCCACGGCTG-3'). The relative expression of each gene was obtained using the comparative CT method, and were normalized using β -actin as an endogenous control. For the infected samples, evaluation of $2^{-\Delta\Delta C_T}$ indicates the fold change in gene expression relative to the uninfected control.

Statistical Analysis

Data are expressed as mean $\pm$ SEM of three independent experiments in triplicate. Differences between the means, from parametric data, were analyzed using one-way analysis of variance and the Bonferroni post hoc test.

Figure 1. A: Data from real-time PCR for MIF receptor (CD74) using RNA from human placental explants infected with *T. gondii*. cDNA contents were normalized on the basis of β -actin levels. Mean values obtained for first- and third-trimester explants were compared using Student's *t*-test (* $P < 0.001$). B and C: CD74 immunolocalization in human placental first-trimester explants (B) and third-trimester explants (C) infected with *T. gondii*. CD74 expression was identified at immunophosphatase staining and counterstaining with Mayer's hematoxylin. Staining for MIF receptor is illustrated by asterisks in trophoblastic cells and by arrows in mesenchymal cells.

Alternatively, Student's *t*-test was used to compare the parasitism index between first- and third-trimester explants. All data were analyzed using commercially available software (PRISM version 4.0; GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

Results

CD74 Expression in Placental Explants

To quantify and verify the expression and localization of MIF receptor in first- and third-trimester placental explants, CD74 was evaluated at real-time PCR and IHC. Compared with third-trimester explants, first-trimester placental explants expressed a larger amount of MIF receptor at PCR (Figure 1A). In addition, IHC revealed that the MIF receptor was localized primarily in the syncytiotrophoblast layer and mesenchymal cells such as Hofbauer cells in first-trimester explants (Figure 1B), whereas expression of MIF receptor was observed primarily in the syncytiotrophoblast layer in third-trimester explants (Figure 1C).

MIF Release by Placental Explants

To verify whether, compared with nontreated and noninfected tissues, placental explants infected with *T. gondii* tachyzoites or treated with several stimuli could increase MIF production, MIF levels were measured in supernatants from tissue cultures after 24 hours of treatment. Compared with controls, in first-trimester placental explants, MIF release was increased in the presence of *T. gondii* ($P < 0.01$) (Figure 2A). Stimulus with IFN- γ also induced elevated levels of MIF release ($P < 0.01$), similar to those observed with *T. gondii* infection in first-trimester placental explants. Furthermore, infection associated

Figure 2. A: MIF production by placental explants after treatment with various stimuli. First- and third-trimester explants were treated with IL-12 (25 ng/mL), IFN-γ (25 ng/mL), IL-10 (10 and 25 ng/mL), TGF-β1 (1 and 10 ng/mL), or medium alone. The explants were infected or not with *T. gondii* and stimulated again. The supernatants were collected after 24 hours, and MIF was measured using an enzyme-linked immunosorbent assay. Data are given as mean ± SEM of three independent experiments in triplicate ($n = 9$). Mean values obtained for different stimuli and infection from first- or third-trimester explants were compared with their respective controls (noninfected and nonstimulated) via one-way analysis of variance with the Bonferroni post hoc test. Results for comparisons are represented by symbols: first trimester, $^{\dagger}P < 0.01$ and $^{\dagger\dagger}P < 0.001$; and third trimester, $^{\ddagger}P < 0.01$. Comparisons between gestational age are indicated by bars. Mean values obtained for first- and third-trimester explants were compared using Student's *t*-test ($^*P < 0.05$ and $^{**}P < 0.01$). B–E: Immunolocalization of MIF in human placental explants. B: First-trimester explant controls (nontreated and noninfected cells). C: First-trimester explants (nontreated and infected with *T. gondii* tachyzoites). D: Third-trimester explant controls (nontreated and noninfected cells). E: Third-trimester explants (nontreated and infected with *T. gondii* tachyzoites). MIF expression was identified at immunoperoxidase staining and counterstaining with Mayer's hematoxylin. Asterisks indicate intracellular MIF staining.

with IFN-γ synergizes MIF release ($P < 0.001$). In contrast, IL-12 tended to increase MIF release in the presence or absence of infection. MIF discharge is not changed by addition of anti-inflammatory cytokines such as IL-10 or TGF-β1; however, when associated with *T. gondii* infection, MIF was up-regulated even in the presence of these regulatory cytokines ($P < 0.001$), in particular when the lowest concentration of IL-10 or TGF-β1 was used. In contrast, similar amounts of MIF were released by third-trimester placental explants from tissue controls and infected tissues (Figure 2A). The increase in MIF release was observed only in the presence of IFN-γ plus *T. gondii* ($P < 0.01$). In addition, there was a tendency

toward increased MIF release in the presence of IL-12 or IFN-γ in the third-trimester placental explants. When comparing gestational age, it was observed that MIF release was higher in first-trimester than in third-trimester explants after *T. gondii* infection ($P < 0.01$), in the presence of IL-10 (10 or 25 ng/mL) ($P < 0.01$ and $P < 0.05$, respectively), and in the presence of IL-10 (10 ng/mL) ($P < 0.01$) or TGF-β1 (1 ng/mL) ($P < 0.01$) plus *T. gondii*.

MIF Expression by Placental Explants

To verify the expression and localization of MIF in placental explants, first- and third-trimester explants were analyzed at IHC. Strong staining was observed for MIF in first-trimester control tissue, whereas MIF was located primarily at the cytotrophoblast layer (Figure 2B). In contrast, infected explants demonstrated intense staining at the syncytiotrophoblast layer and mesenchyme (Figure 2C). In third-trimester placental explants, weak staining was observed at the cytotrophoblast layer of noninfected tissues (Figure 2D); however, the staining was stronger in infected tissues (Figure 2E).

Effect of MIF on Control *T. gondii* Infection

To determine whether MIF has a role in control of *T. gondii* infection in first- and third-trimester placental explants in addition to other stimuli such as IL-12, IFN-γ, TGF-β1, and IL-10, the parasite load was measured using real-time PCR. Compared with unstimulated cells, treatment with MIF (25 and 100 ng/mL) in first-trimester explants resulted in significant reduction in the parasite load ($P < 0.001$) (Figure 3). Other proinflammatory cytokines (IL-12 and IFN-γ) also reduced the infection when compared with unstimulated controls ($P < 0.01$ and $P < 0.05$, respectively). In addition, stimulus with anti-inflammatory IL-10 cytokine (10 and 25 ng/mL) resulted in decreased parasitism ($P < 0.01$ and $P < 0.05$, respectively). Com-

Figure 3. Effect of various stimuli on *T. gondii* load in first- and third-trimester human placenta explants. Explants were treated with MIF (5, 25, or 100 ng/mL), IFN-γ (25 ng/mL), IL-12 (25 ng/mL), IL-10 (10 and 25 ng/mL), TGF-β1 (1 and 10 ng/mL), or medium alone. The explants were infected with *T. gondii* and stimulated again for 24 hours. Control cultures were set up in medium alone. The explants were collected, and parasites were quantified at real-time PCR. Mean values obtained for different stimuli and infection from first- or third-trimester explants were compared with their respective controls (noninfected and nonstimulated) at one-way analysis of variance with the Bonferroni post hoc test. The different comparisons are represented by symbols: first trimester, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$; and third trimester, $^{\ddagger}P < 0.01$ and $^{\ddagger\ddagger}P < 0.001$. Comparisons between gestational age are indicated by bars. Mean values obtained for first- and third-trimester explants were compared using Student's *t*-test ($^*P < 0.05$).

Figure 4. Comparison between MIF release and *T. gondii* load under different cytokine stimuli. MIF release and parasite load in infected and stimulated explants were compared with unstimulated controls at one-way analysis of variance with the Bonferroni post hoc test. Comparisons are represented by symbols: parasitism, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; and MIF release, † $P < 0.05$. **A:** First-trimester explants. **B:** Third-trimester explants. Dotted line represents basal levels of MIF release by control explants (nonstimulated and noninfected).

pared with controls, in third-trimester placental explants, the decrease in parasitism was observed for the highest concentration of MIF (100 ng/mL) ($P < 0.05$) and for IL-12 and TGF-β1 (1 ng/mL) stimuli ($P < 0.01$ and $P < 0.05$, respectively). When gestational ages were compared, the role of MIF in controlling parasitism was more evident in first- than in third-trimester placental explants ($P < 0.05$).

To verify the effect of endogenous MIF blockage on parasite load, experiments were performed using anti-human MIF polyclonal antibody. Decreasing rather than increasing parasitism was observed in both first- and third-trimester placental explants. In addition, after using anti-MIF antibody, MIF release was increased in comparison with noninfected and nontreated explants. Experiments performed to block Fc receptor before using anti-MIF antibody demonstrated that MIF was released at similar levels as in controls (data not shown).

Next was analyzed the relationship between MIF release and parasite load. In first-trimester explants, the increase in MIF release after IFN-γ and small concentrations of IL-10 (10 ng/mL) or TGF-β (1 ng/mL) stimuli was related to a decrease in parasite load (Figure 4A). When higher concentrations of IL-10 (25 ng/mL) or TGF-β (10 ng/mL) were used, MIF release was associated with increasing parasite load. In the presence of IL-12 stimulus, the decrease in MIF release was accompanied by a

decrease in parasitism, when compared with unstimulated explants (Figure 4A). In third-trimester explants, stimulation with IL-12 led to a slight increase in MIF release and a striking decrease in parasite load (Figure 4B). The opposite result was observed with IFN-γ, which led to a high level of MIF release associated with a small decrease in parasite load. Use of increasing concentrations of IL-10 (10 or 25 ng/mL) resulted in a decrease in MIF release associated with an increase in parasite load in third-trimester explants. In contrast, use of increasing concentrations of TGF-β (1 or 10 ng/mL) resulted in an increase in MIF release associated with an increase in parasite load (Figure 4B). Comparison of first- and third-trimester explants demonstrated an opposite relationship in MIF and parasitism values when using increasing concentrations of IL-10 (10 or 25 ng/mL) or TGF-β (1 ng/mL) stimulus.

To further confirm the association between parasitism and gestational age, IHC was performed for *T. gondii* localization and quantification in different tissue layers. A greater number of parasites were observed at the trophoblast layer in third-trimester explants compared with first-trimester explants ($P < 0.01$). In addition, parasitism at the mesenchyme was lower in first-trimester explants compared with third-trimester explants ($P < 0.05$) (Figure 5A). In first-trimester explants, parasites were especially found at

Figure 5. **A:** Quantification of *T. gondii* tachyzoites in human first- and third-trimester placenta explants at IHC. Parasite load was measured in 10 microscopic fields, and three tissue areas were defined for parasite localization as follows: parasites attached to the trophoblast surface, parasites inside the trophoblast, and parasites inside the mesenchyme. The total number of parasites per tissue was also determined. Data are given as mean $\pm$ SEM from two independent experiments performed in triplicate ($n = 6$). Comparisons between gestational age are indicated by bars. Mean values obtained for first- and third-trimester explants were compared using Student's *t*-test (* $P < 0.05$ and ** $P < 0.01$). **B** and **C:** Immunolocalization of *T. gondii* tachyzoites in human placental explants. *T. gondii* was identified at immunoperoxidase staining and counterstaining with Mayer's hematoxylin. **B:** First-trimester explants. **C:** Third-trimester explants. Arrows indicate parasites attached to the trophoblast surface in first-trimester explants and at the mesenchyme in third-trimester explants.

Figure 6. Schema of the proposed model of MIF-dependent *T. gondii* infection control in first-trimester explants (A–C) compared with third-trimester explants (D–F). **A:** Absence of infection or stimulation. MIF is highly expressed by cytotrophoblast cells, and large amounts of MIF receptor are expressed at the syncytiotrophoblast layer. **B:** *T. gondii* infection. MIF is released from the cytotrophoblast and binds to its receptor at the syncytiotrophoblast layer. **C:** Parasite-host cell outcome. Note the small number of parasites in the trophoblast layer and in the mesenchyme, and the large number of parasites attached at the surface of the trophoblast layer. **D:** Absence of infection and stimulation. Small amounts of MIF and its receptor are expressed in the trophoblast. **E:** *T. gondii* infection. MIF production by cytotrophoblast cells is increased; however, there is no increase in MIF release. **F:** Parasite-host cell outcome. Note the large number of parasites in the trophoblast layer and in the mesenchyme, and the small number of parasites attached to the trophoblast layer.

tached to the trophoblast layer (Figure 5B). Conversely, parasites were preferentially located in trophoblasts and mesenchymal tissue in third-trimester explants (Figure 5C).

Morphologic Analysis of Placental Explants

At light microscopy, chorionic villus integrity was verified, as well as the syncytiotrophoblast containing syncytial cells involving the chorionic villus and internally the cytotrophoblast. In addition, fetal blood vessels, mesenchymal cells, endothelial cells, and Hofbauer cells were observed in the connective tissue (data not shown). The structural integrity of the tissues was confirmed at electron microscopy, which demonstrated a viable trophoblast, with no necrosis or tissue degeneration (data not shown).

Production of Nitrite

No production of nitrite was detected in supernatants from first- and third-trimester placental explants in any experimental conditions analyzed (data not shown).

Proposed Model of MIF-Dependent *T. gondii* Infection Control in Placental Explants

A schema of the proposed model of *T. gondii* infection control dependent on MIF in the first-trimester explants in comparison with third-trimester explants is shown in Figure 6. In first-trimester explants and in the absence of infection or stimulation, MIF is highly expressed in cytotrophoblast cells, and there is a great amount of MIF receptor at the syncytiotrophoblast layer (Figure 6A). After *T. gondii* infection, MIF is released from the cytotrophoblasts, and MIF binds to its receptor at the syncytiotrophoblast layer (Figure 6B). This interaction is important in initiation of a signaling cascade essential to parasite control. As a result of this parasite-host cell interaction, it is possible that a small number of parasites reach the fetal circulation (Figure 6C). Third-trimester placental explants, in the absence of infection or stimulation, are characterized by low MIF and CD74 expression (Figure 6D). After *T. gondii* infection, there is an increase in MIF production by cytotrophoblast cells; however, there

is no increase in MIF release (Figure 6E). Consequently, it is possible that a large number of parasites reach the fetal blood vessels (Figure 6F).

Discussion

Trophoblast cells are an important component in prevention of vertical transmission of pathogens because this cell type represents the main barrier in the maternal-fetal interface.³⁷ However, often this barrier can be overcome by certain microorganisms such as *T. gondii*, resulting in potential fetal infection. The mechanisms of transplacental transmission of *T. gondii*, in particular those regarding control of invasion and replication of *T. gondii* in trophoblastic cells, are still largely unknown.

In the present study, first- and third-trimester human explants were used to analyze trophoblast response to *T. gondii*. First, the results demonstrated that expression of MIF receptor in infected explants depends on gestational age. CD74 expression is more intense in first- than in third-trimester explants. In previous studies, trophoblastic cells from the Jar line and term explants expressed CD74 constitutively.³⁸ Second, compared with noninfected and nonstimulated controls, MIF secretion was increased in first-trimester explants infected with *T. gondii*. In addition, IFN- γ was a key cytokine in MIF secretion whether explants were infected or not. A previous study demonstrated that MIF secretion by first-trimester placental explants is stimulated by soluble antigen of *T. gondii* alone or with addition of IFN- γ .²⁵ In the present study, using a model of *T. gondii* infection, it was demonstrated that MIF discharge was up-regulated in the presence of regulatory cytokines such as IL-10 or TGF- β 1 associated with infection. Accordingly, MIF protein is a unique regulatory mediator with ability to sustain the inflammatory response in the presence of endogenous or exogenous anti-inflammatory effects.³⁹ Conversely, results of the present study demonstrated that MIF release was not increased after infection with *T. gondii* in third-trimester explants, although an increase in MIF release was observed in infected explants when IFN- γ was added.

Considering these results of MIF release on the basis of gestational age in the present study, it was observed that the major differences between first- and third-trimester explants occur in the presence of infection. Small concentrations of anti-inflammatory and regulatory stimuli such as TGF- β 1 and IL-10 associated with infection induce MIF secretion in first-trimester explants; however, this effect did not occur in third-trimester explants. In the presence of IFN- γ , however, MIF release was greater in third- than in first-trimester explants. It is probable that these results concerning MIF release after IFN- γ stimulus are related to differences between the amount of IFN- γ receptor at different gestational ages. According to Banerjee et al,⁴⁰ the effect of IFN- γ on T-lymphocyte activation is influenced by the relative membrane density of its two receptors, and in particular IFN γ R2. Transcriptional expression of the *IFNGR2* gene in normal late pregnancy was increased compared with that in normal early

pregnancy. Inversely, IFN- γ production in early placenta is significantly higher than in late pregnancy.⁴⁰

Results of the present study demonstrated that MIF immunostaining is stronger in first- than in third-trimester noninfected placentas, in particular at the cytotrophoblast layer. MIF staining of infected explants from first-trimester pregnancies was located primarily at the syncytiotrophoblast layer, and its localization was similar to that observed for the MIF receptor. It was supposed that infection is an important stimulus for inducing MIF secretion and succeeding MIF association with its receptor, leading to a signaling cascade in response to infection. In contrast, in third-trimester explants, infection with *T. gondii* induced MIF production, as demonstrated at IHC. Nevertheless, MIF immunostaining was observed only in the cytotrophoblast layer, which suggests that MIF was not released because it was confirmed by low levels of this cytokine in the culture supernatants, supporting the lack of concordance between enzyme-linked immunosorbent assay and IHC for MIF of third-trimester infected explants. Decrease in the trophoblast layer number as pregnancy advances³⁷ supports the decrease in MIF secretion and reduction in MIF receptor expression during the gestational course, as observed in the present study.

The results also demonstrated that stimulus with exogenous MIF induces a decrease in tissue parasitism in first-trimester explants. In addition, IFN- γ and IL-10 stimuli decrease tissue parasitism, probably by inducing production of endogenous MIF. In contrast, parasitism control by IL-12 stimulus in first-trimester explants seems to be independent of MIF. In an investigation of the effect of endogenous MIF blockage on parasite load, decreasing rather than increasing parasitism was observed in both first- and third-trimester placental explants. These findings were associated with increased levels of MIF in the culture supernatants, which strengthens the role of this cytokine in control of *T. gondii* infection. Potentiation of the biological activity of cytokines by antibody anti-cytokines has been described for IL-2, IL-3, IL-6, IFN- γ , and TNF.^{41–45} This phenomenon has been associated with formation of stable complexes between cytokines and their respective antibodies that optimize the immune response.⁴¹ It is probable that decreased parasite load after using anti-MIF antibody may be associated with formation of complexes between anti-MIF and endogenous MIF that potentiated the MIF effect. A protector activity of MIF has been proposed in several models of infection owing to its proinflammatory activity in cells infected with bacteria^{29,46} and protozoa.^{22,23,25,26} MIF expression requires interaction with other proinflammatory cytokines such as IFN- γ .²⁵ Subsequently, MIF acts by binding to its receptor complex (CD74–CD44),^{38,47} thereby activating a signal transduction pathway,⁴⁸ gene transcription, and expression of effector molecules such as cytokines²⁴ and adhesion molecules.²⁵ The proinflammatory activity of MIF was recently demonstrated to control *T. gondii* infection because, compared with wild-type mice, MIF^{−/−} mice exhibited greater liver damage, more brain cysts, and less proinflammatory cytokine production, and succumbed to infection faster, which indicates a

critical role of MIF in mediating host resistance against *T. gondii*.^{26,27} To our knowledge, our group was the first to associate MIF with congenital toxoplasmosis, although the entire mechanism responsible for mediating this protection still needs to be clarified.²⁵ It is possible that these mechanisms of control are independent of NO because NO was not detected in the experiments even after MIF stimulation. These data agree with other experimental models that demonstrated that NO is not detected in large amounts in human systems.⁴⁹ The low parasitism observed in the first-trimester explant experiments may be related to a particular role of MIF that mediates a proinflammatory profile after stimulus in the presence of immunosuppressive hormones.³⁹ Alternatively, in third-trimester placental explants, MIF controlled *T. gondii* infection only at the highest concentration of this cytokine. It is probable that in third-trimester explants, MIF activity occurs preferentially via a nonclassical endocytic pathway that depends on high MIF concentrations.^{50,51} In addition, in third-trimester explants, decreased parasite load was observed in the presence of IL-12 and TGF- β 1, although this control seems to be independent of MIF.

In the present study, structural and ultrastructural integrity of cultured trophoblasts was observed in the presence of *T. gondii* in chorionic villus at light and electron microscopy. In accordance with Miller et al,⁵² the tracking of morphologic features is one of the most important tests for assessing viability *in vitro*. Therefore, the possibility that MIF nonregulation in trophoblast cells in third-trimester placental explants relative to nonviable explants can be ruled out.

It is well described in the literature that congenital toxoplasmosis occurs more frequently when the mother acquires the infection during the later stages of pregnancy.⁵³ Results of the present study support this epidemiologic evidence because it was observed that the number of parasites found at the trophoblast layer and mesenchyme was greater in third- than in first-trimester explants. Reproductive biologists have long been interested in differences of susceptibility to infection during gestation. The question is whether the placenta fulfills its barrier function by virtue of its structural characteristics or whether it has a more active role in inducing an immune response against pathogens. It has been proposed that structural differences in placentas during gestation, specifically due to the number of cellular layers interposed between the fetal and maternal blood, could mediate this difference.⁵⁴ The decrease in placenta cellular layers seems to be related to decreased MIF secretion and its receptor expression, as observed in the present study. However, it is not believed that this difference in parasitism is due only to a physical barrier. *T. gondii* is an active parasite able to cross many biological barriers, spreading in several organs including the brain.⁵⁵ It is suggested that variations in MIF regulation during gestation have an important role in differential susceptibility to *T. gondii* infection in the first and third trimesters of pregnancy.

In conclusion, MIF is up-regulated in first-trimester explants, and it is important to control *T. gondii* infection, whereas lack of MIF up-regulation after infection in third-trimester placental explants may be related to higher

susceptibility to infection at this gestational stage. A comprehensive understanding of MIF kinetics in the placental environment will be an important step in clarifying trophoblast responses to infection, in particular in vertical transmission of *T. gondii* during pregnancy.

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