International Journal of Infectious Diseases 30 (2015) 10–16

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Interferon gamma is involved in apoptosis of trophoblast cells at the maternal–fetal interface following *Toxoplasma gondii* infection

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ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form 8 August 2014
Accepted 13 October 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Toxoplasma gondii
Abnormal pregnancy outcomes
Apoptosis
IFN-γ
Trophoblasts

SUMMARY

Objective: To assess whether interferon gamma (IFN-γ) secreted by decidual natural killer (dNK) cells at the maternal–fetal interface is involved in the apoptosis of trophoblast cells (trophoblasts) following *Toxoplasma gondii* infection.
Methods: Human dNK cells were infected with *T. gondii* and then co-cultured with trophoblasts. The infected co-cultured cells were treated with or without IFN-γ neutralizing antibodies. Uninfected co-cultured cells were used as controls. The level of IFN-γ in the supernatant of co-culture was measured by ELISA and the apoptosis of trophoblasts was analyzed by flow cytometry. The expression of caspase 3 and caspase 8 of trophoblasts cells was determined by Western blotting and real-time RT-PCR.
Results: The levels of IFN-γ were increased at $<$24 h following *T. gondii* infection and were maintained thereafter. Caspase 3, caspase 8, and the apoptosis of trophoblasts co-cultured with dNK cells were increased compared with the control. Meanwhile, IFN-γ, caspase 3, caspase 8, and trophoblast apoptosis were up-regulated upon increased ratios of dNK cells to trophoblasts. Compared to the infected group, the levels of IFN-γ, caspase 3, caspase 8, and trophoblast apoptosis were significantly decreased upon treatment with the IFN-γ neutralizing antibody. IFN-γ levels were correlated positively with the apoptosis of trophoblasts ($r = 0.7163$, $p < 0.01$).
Conclusions: The levels of IFN-γ secreted by dNK cells at the maternal–fetal interface were closely correlated with the apoptosis of trophoblasts upon *T. gondii* infection. The apoptosis of trophoblasts induced by the increase in IFN-γ depended on the caspase pathway, which may contribute to the abnormal pregnancy outcomes that occur with *T. gondii* infection.
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1. Introduction

Toxoplasma gondii is an extensive intracellular parasitic protozoon that can infect all warm-blooded vertebrate species and can lead to reproductive failure in humans and animals.[1–3] In particular, the infection of mothers with *T. gondii* during early pregnancy can lead to adverse pregnancy outcomes such as miscarriage, stillbirth, and preterm labor.[4] However, the mechanism by which *T. gondii* infection causes these serious adverse human pregnancy outcomes still needs further study.

Successful pregnancy involves subtle immune regulation mechanisms of the maternal immune system to tolerate the semiallogeneic fetus at the maternal–fetal interface.[5] Uterine decidual natural killer (dNK) cells that are poorly cytolytic and produce low amounts of interferon gamma (IFN-γ) are the major immune cells at the maternal–fetal interface and play an important role in establishing and maintaining a normal pregnancy.[6,7] It has been demonstrated that the number of dNK cells increases significantly after *T. gondii* infection, which may enhance maternofetal transmission of parasites and be related to the adverse pregnancy outcomes.[8]

The balance of Th1/Th2 plays a crucial role in mammalian pregnancy; Th1-type responses are down-regulated during pregnancy in order to induce maternal tolerance.[9] Some studies have reported that *T. gondii* infection is normally controlled by a strong Th1-type response with IFN-γ production,[10,11] and that a predominant Th1 pattern such as up-regulation of IFN-γ leads to rejection of the fetus.[12] We previously showed that IFN-γ was

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http://dx.doi.org/10.1016/j.ijid.2014.10.027
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increased in *T. gondii*-infected mice with abnormal pregnancy outcomes.[13]

IFN-γ is a key Th1-type cytokine that is mainly secreted by dNK cells at the maternal–fetal interface, and plays an important role in controlling excessive trophoblast invasion by inducing apoptosis of extravillous trophoblasts.[14] It has been shown that IFN-γ can inhibit trophoblast invasion, as determined by invasion assays in vitro.[15] On the other hand, an increase in IFN-γ in serum and decidua has also been observed in women with preeclampsia.[16,17] The administration of IFN-γ to pregnant mice resulted in an increased fetal abortion rate.[18] Therefore a moderate level of IFN-γ produced by dNK cells contributes to normal pregnancy, whereas a more generalized IFN-γ response has been considered detrimental to fetal development.

It has been shown that the Fas/FasL system induces apoptosis of trophoblasts,[19,20] providing a mechanism for the maternal immune tolerance to the fetus.[21,22] Balkundi et al. demonstrated that the FasL blocking protein Fas-Fc reduced IFN-γ-induced trophoblast apoptosis.[23] Caspase 3 and caspase 8 have been thought to be essential in Fas-mediated apoptosis.[24–27] While IFN-γ may induce the trophoblast apoptosis mainly through the caspase 3 and caspase 8 pathway,[28,29] the mechanism by which the elevated IFN-γ expression contributes to trophoblast apoptosis in human abnormal pregnancy outcomes following *T. gondii* infection still needs to be investigated.

In this study, *T. gondii*-infected human dNK cells were co-cultured with human trophoblasts to investigate whether IFN-γ is involved in the apoptosis of trophoblasts through the Fas–caspase-mediated pathway.

2. Materials and methods

2.1. Sample collection

Tissues were taken from 36 patients who voluntarily and legally chose to terminate a pregnancy during the first trimester (6–12 weeks of gestation) in the Department of Obstetrics and Gynecology, Chinese Medicine Hospital; written informed consent was obtained (approved by Binzhou Medical University Ethics Committee). Decidua tissue and villi tissue were isolated from fetus and placenta after washing in sterile saline. All procedures were carried out in accordance with the rules of the Chinese Medicine Hospital.

2.2. Isolation and culture of human dNK cells

Decidual tissues were washed with cold phosphate-buffered saline (PBS) solution, cut into 1- to 3-mm pieces with scissors, and then digested with 0.1% collagenase type IV and 25 U/ml DNase I (Sigma-Aldrich, United States) in α-Modified Eagle Medium (α-MEM) at 37 °C for 30 min. The cell suspension was washed once with cold PBS, then passed through a 75-μm nylon mesh filter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and centrifuged at room temperature at 1000 rpm for 5 min. The cells were isolated using density gradient centrifugation on Ficoll–Hypaque Lymph (Sigma-Aldrich, United States) at 2000 rpm for 20 min at 4 °C to isolate the lymphocyte cells. Lymphocytes were collected and washed twice with PBS and then centrifuged for 10 min at 1500 rpm at room temperature. The supernatant was discarded and the dNK cells were purified through positive selection using CD56 antibody-coated magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and magnet-assisted cell separation. Each sample included about 1.5×10^6 ($1.5 \times 10^6 \pm 3.2 \times 10^4$) dNK cells. dNK cells were cultured in α-MEM with 12.5% Fetal Calf Serum (FCS), 12.5% horse serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin, and were incubated for 12 h at 37 °C in a humidified 5% CO_2 incubator.

2.3. Isolation and culture of human trophoblasts

The villi tissues were immediately washed with PBS and cut into 1- to 3-mm pieces. The villi tissues were digested using 0.25% trypsin (Sigma-Aldrich, United States) and 0.02% DNase I (Sigma-Aldrich, United States) three times for 30 min at 37 °C with constant shaking. The dispersed trophoblasts were filtered through a 75-μm nylon mesh filter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and were pushed onto a discontinuous Percoll gradient of 25–65% (GE Healthcare, the UK and Ireland), followed by centrifugation at 2000 rpm for 20 min to separate the different types of cells. These cells, between the density markers of 1.049 g/ml and 1.062 g/ml, were isolated. Cells were washed twice with Hank's solution, equilibrated at 37 °C, and cultured for 1 h in high-glucose, phenol-red-free Dulbecco Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences HyClone Laboratories, Utah, USA) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco, USA), 2.5 mM L-glutamine, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cultured suspension was transferred to culture flasks that had previously been coated with BD Matrigel (1:2, Matrigel:DMEM). The cells were cultured at 37 °C in a humidified 5% CO_2 incubator.

Isolated human trophoblasts were stained with fluorescein isothiocyanate (FITC) -anti-keratin antibody and P-phycoerythrin (PE) -anti-vimentin antibody. The keratin$^+$ vimentin$^-$ cells were considered as trophoblasts. Each sample contained about 1.0×10^6 ($1.0 \times 10^6 \pm 2.9 \times 10^4$) trophoblasts.

2.4. Yellow Fluorescent Protein (YFP) -T. gondii (RH strain) infected trophoblasts co-cultured with dNK cells

YFP-*T. gondii* was a kind gift from Professor Striepen, the Center for Tropical and Emerging Global Diseases of Georgia University, USA. YFP-*T. gondii* was kept in the peritoneal fluid of intraperitoneally infected Kunming mice every 54 h. Tachyzoites were collected under sterile conditions and dNK cells were added to the culture medium of dNK cells at a ratio of 10:1 (*T. gondii*:cells) for 2 h. dNK cells were collected and washed twice with PBS to remove the dissociative *T. gondii*. The total dNK cells were co-cultured with prepared trophoblasts at a ratio of 1:1 for 12, 24, 36, and 48 h, respectively, and at a ratio of 1:5, 1:1, and 5:1 at 24 h. Cells were then collected for analyses. The uninfected group was treated similarly to the infected group, except for the *T. gondii* infection. The culture was maintained in the same conditions as reported previously.

2.5. Blocking experiment

Anti-IFN-γ neutralizing antibody (R&D Systems, USA) was added to infected co-cultured cells at 50 μg/ml every 6 h that were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.6. Caspase 3 and caspase 8 mRNA analysis

The trypsin and DNase I treated villi cells were stained with Human leukocyte antigen-G (HLA-G) –PE antibody (eBioscience, USA), and the HLA-G-positive cells were collected as trophoblasts. The total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was then synthesized using the Reverse-Transcribe kit (Fermentas, USA), in accordance with the manufacturer's instructions. The SYBR Green method was employed, and β-actin was used as an internal control for real-time RT-PCR. The forward and reverse primer sets were designed and synthesized as follows: caspase 3: F-5′-GACTGTGG-CATTGAGACAGAC, R-5′-CTTTCGGTTAACCCGGGTAAG; caspase 8: F-5′-TCCTGCCTGCCTGTACCCCG, R-5′-GCCCAACCTCACGTGCCCAG;

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