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Proteomic profiling of human decidual immune proteins during *Toxoplasma gondii* infection

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ABSTRACT

A *Toxoplasma gondii* infection during pregnancy can result in spontaneous abortion, preterm labor, or congenital fetal defects. The decidual immune system plays a critical role in regulating the immune micro-environment and in the induction of immune tolerance. To better understand the factors that mediate the decidual immune response associated with the *T. gondii* infection, a large-scale study employing TMT proteomics was conducted to characterize the differential decidual immune proteomes from infected and uninfected human decidual immune cells samples. The decidual immune cells from 105 human voluntary abortion tissues were purified, and of the 5510 unique proteins identified, 181 proteins were found to be differentially abundant (> 1.2-fold cutoff, $p < 0.05$) in the *T. gondii*-infected decidual immune cells. 11 proteins of 181 differentially expressed proteins associated with trophoblast invasion, placental development, intrauterine fetal growth, and immune tolerance were verified using a quantitative real-time polymerase chain reaction and western blotting. This systematic analysis for the proteomics of decidual immune cells identified a broad range of immune factors in human decidual immune cells, shedding a new insight into the decidual immune molecular mechanism for abnormal pregnancy outcomes associated with *T. gondii* infection.

1. Introduction

In human pregnancies, a vertical transmission of the intracellular parasite-*Toxoplasma gondii* (*T. gondii*) can lead to devastating consequences [1], such as miscarriages, premature births, or severe congenital birth defects [2, 3]. Accumulating evidence suggests the immune system associated with the maternal-fetal immune system interface is involved in the abnormal pregnancy outcomes caused by *T. gondii* [4, 5]. Nevertheless, the precise mechanisms by which *T. gondii* infections cause abnormal pregnancies remains unclear.

The immune microenvironment on maternal-fetal interface consisted of decidual immune cells, functional molecules, and various major cytokines, all of which play an important role in implantation and embryonic and placental development [6, 7]. Therefore, any disruption to the delicate immunity and tolerance balance in the maternal-fetal interface inevitably leads to abnormal pregnancies [8]. Our previous studies confirmed that a *T. gondii* infection during early pregnancy could regulate the expression of some important functional molecules and the secretion of some major cytokines in the decidual immune cells, further contributing to abnormal pregnancy [9, 10]. *T. gondii* infection was found to cause a down-regulation of some functional molecules, such as the LILRB4 on decidual macrophages [11], NKG2A on dNKs [12], and PD-1 on Tregs [13], and on some cytokines such as TGF-β [14] and IL-10 [13]. However, there has not been any systematic research that is confirmed the quantity and variety of molecules in the human decidual immune cells related to aberrant pregnancies because of the *T. gondii* infection.

Novel tools are now available that allow us to take a “systems biological” approach to determine these differentially expressed proteins when there is a *T. gondii* infection. This was a large-scale study that aimed to characterize the human decidual immune proteome to identify the factors associated with an altered susceptibility to the *T. gondii* infection. 105 human decidual tissue samples from the first trimester were collected from voluntary abortion cases after informed consent was given. After these samples were minced, dissociated, and purified, the human decidual immune cells were extracted ultimately. Using tandem mass tag (TMT) proteomics, the differentially expressed proteins from *T. gondii* infected and uninfected decidual immune cells

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were accurately characterized, and the authenticity and accuracy of the protein expressions detected during the quantitative proteomic examination were further confirmed using real-time PCR and Western blot. The identification of these proteins will facilitate a better understanding of the molecular mechanisms of abnormal pregnancy outcomes underlying *T. gondii* infection.

2. Materials and methods

2.1. Sample collection and preparation

Human decidual tissues from the first trimester [gestational age at sampling, 8–10 weeks] were collected from 105 voluntary abortion cases, after informed consent was given. The sample collection for this study was approved by the Ethics Committee of Binzhou Medical University. All subjects had been visiting the Department of Obstetrics and Gynecology, Yantai Affiliated Hospital of Binzhou Medical University, had not used any abortifacients, and had not suffered from any pregnancy complications. The decidual tissues were immediately washed 5–8 times with a sterile saline solution after induced abortion of indolence, and then were cultured in DMEM/high glucose medium (Hyclone, USA) which contained 10% fetal bovine serum (FBS, Gibco, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin (Sigma-Aldrich, USA).

The decidual tissues were washed thoroughly with a cold phosphate-buffered saline (PBS) solution, minced using sterile scissors into 1 to 3 mm pieces, and dissociated using the Gentle MACS tissue dissociator (Miltenyi Biotec, Germany), after which the suspension was filtered through 100 μm nylon mesh filters. The decidual mononuclear cells were subsequently purified by using Ficoll–Hypaque gradient (SigmaAldrich, United States) at 2000 rpm for 20 min at 20 °C and equally allocated to infected and uninfected groups; after 1 h, the *T.gondii* tachyzoites were added at the ratio of 2:1 (*T. gondii*: cells) to the infected group. The cells from two groups were all cultured in RPMI medium that was supplemented with 10% FBS (FBS; Gibco, USA), 100 IU/ml streptomycin, and 100 IU/ml streptomycin (Sigma, USA) for 24 h at 37 °C in a humidified 5% CO_2 incubator. The cells were centrifuged at 2000 rpm for 10 min to obtain the cell pellet and wash off the unrecruited tachyzoites. The decidual immune cells were snap frozen on liquid nitrogen for 1–2 min, then moved quickly and stored at −80 °C until analysis.

2.2. Maintenance of T. gondii RH tachyzoites

The *T. gondii* RH tachyzoites were maintained in HEp-2 cells in the Minimum Essential Media (MEM) (Hyclone, USA), 5% fetal bovine serum (FBS; Gibco, USA), and 100 IU/ml penicillin/streptomycin (Sigma-Aldrich, USA). After being cultured with 54 h, blowing and sucking the suspension to separated the cells and the tachyzoites, then the tachyzoites suspension was centrifuged at 1500 rpm (433 × *g*) for 10 min, after which the purified tachyzoites were resuspended in MEM and counted using a Neubauer chamber.

2.3. Protein extraction

Three repeated sample pools (n = 35 each) were performed in infected and uninfected group, and there are at least 2×10^7 cells in each sample pool. The sample was sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 12,000 *g* at 4 °C for 10 min. Finally, the supernatant was collected and the protein concentration was determined according to the manufacturer's instructions for the BCA kit.

2.4. Digestion and TMT labeling

The extracted proteins were reduced with 5 mM dithiothreitol for 30 min at 56 °C, after which the samples were alkylated in darkness with 11 mM iodoacetamide for 15 min at room temperature. After being diluted with TEAB, trypsin was added at 1:50 trypsin-to-protein mass ratio at 37 °C overnight. After desalting and vacuum-drying, the peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for the TMT kit. The peptide mixtures were then incubated for 2 h at room temperature, pooled and desalted, and then dried using vacuum centrifugation.

2.5. LC–MS analysis

The labeled peptides were fractionated by high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Following the separation of the peptide with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions, the peptides were combined into 18 fractions and dried using vacuum centrifugation. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo Fisher Scientifc), which had been coupled online to the UPLC. The resulting MS/MS data were searched against a Uniprot human protein database with MaxQuant (v.1.5.2.8), and a functional annotation for the quantified proteins was performed with GO (Gene ontology) and KEGG (Kyoto encyclopedia of genes and genomes) pathway analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [15] partner repository with the dataset identifier PXD008448.

2.6. Quantitative real-time RT-PCR

Total RNA from the decidual immune cells was extracted after homogenization in a TRAzol reagent (Takara Biotechnology, Inc., Japan), according to the manufacturer's protocol. The primers were designed by Takara Biotechnology and the sequences were blasted (Table 1). The cDNA was prepared using PrimeScript™ RT–PCR Kit (Takara) and then amplified using a duplicate SYBR Green real-time PCR reagent (Roche). The β-actin gene was used as an endogenous control for normalization.

Table 1
List primers of identified proteins by real-time PCR.

Cystain B	Forward: CCCTGTGTTTAAGGCCGTGTC
	Reverse: GGAACACTCGCAGGTGTACGAA
AIF-1	Forward: CAGGATGATGCTGGGCAAGA
	Reverse: CCTTCAAATCAGGGCAACTCAGA
HMGN1	Forward: GCCGAAGTGGCTAACCAAGA
	Reverse: GGAGACAGGGACCACTGATAAGAC
NME1	Forward: ACTCCAAGCCTGGGACCATC
	Reverse: AGTTCTGAGCACAGCTCGTGTAATC
GSTO1	Forward: CCTGGTTTGAACGGCTGGA
	Reverse: GCCTCAGGGCTGTTCTGTAAGTAG
IL-1β	Forward: CCAGGGACAGGATATGGAGCA
	Reverse: TTCAACACGCAGGACAGGTACAG
PAI-2	Forward: CCATGGAGCATCTCGTCCAC
	Reverse: GCATTGGCTCCCACTTCATTAAAC
C/EBPβ	Forward: GGCCGGTTTCGAAGTTGATG
	Reverse: AGTTACACGTGGGTTGCGTCAG
Granzyme A	Forward: TCAGGTTGATTGATGTGGGACAG
	Reverse: GACCATGTAGGGTCTTGAATGAGGA
COX2	Forward: CTGGAACATGGAATTACCCAGTTTG
	Reverse: TGGAACATTCCTACCACCAGCA
ICAM-1	Forward: TGTATGAACTGAGCAATGTGCAAGA
	Reverse: CACCTGGCAGCGTAGGGTAA