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Label-free proteomic analysis of placental proteins during *Toxoplasma gondii* infection

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ABSTRACT

Toxoplasma gondii is a ubiquitous, obligate intracellular parasite capable of crossing the placental barrier and causing spontaneous abortion, preterm labor, or significant disease in the surviving neonate. To better understand molecular mechanisms underlying abnormal pregnancy outcomes caused by *T. gondii*, placental proteins extracted from *T. gondii*-infected and -uninfected mice were comparatively analyzed using label-free liquid chromatography–tandem mass spectrometry. Significant difference was observed in the expression of 58 out of 792 proteins in infected placentas ($p < 0.05$) compared with that in uninfected placentas. Quantitative real-time polymerase chain reaction, western blotting, and immunohistochemical staining were used to validate the results of the proteomic analysis. Some placental proteins differentially expressed in infected and uninfected mice were found to be associated with several different biological processes of pregnancy, particularly with trophoblast invasion and placental development. The results provide possible novel insights into the molecular mechanisms for abnormal pregnancy outcomes associated with *T. gondii* infection.

Significance: In order to further explore the mechanisms of abnormal pregnant outcomes caused by *T. gondii* infection, we first applied label-free proteomic technology to analyze the differentially expressed host placental proteins with *T. gondii* infection. The results showed that some differential proteins are associated with trophoblast invasion and placenta development. The findings provide a systemic view of the altered placental proteins and help to declare the molecular mechanisms of abnormal pregnancy outcomes caused by *T. gondii* infection.

1. Introduction

Toxoplasma gondii, an obligate intracellular protozoan parasite, is able to infect a variety of cells in virtually all warm-blooded animals, and approximately one-third of the world population is seropositive for this parasite [1,2]. In particular, primary infection with *T. gondii* during early pregnancy can lead to placental infection and may have profound effects on the mother–fetus relationship and success of the pregnancy, resulting in spontaneous abortion, preterm labor, or serious neurological and ocular sequelae in the surviving offspring [3]. The interplay between immune effectors of successful pregnancy and the anti-infective response during *T. gondii* infection has been extensively discussed elsewhere [4–6]. However, the molecular mechanisms underlying abnormal pregnancy outcomes upon *T. gondii* infection need to be further explored.

The placenta, which is a connection between the mother and fetus, plays a vital role in regulating embryo development. Any major roadblocks occurring in its development may result in pregnancy abnormalities [7]. The maternal–fetal interface consists of (a) the syncytiotrophoblast, which is bathed in the maternal blood and mediates nutrient and gas exchange, and (b) extravillous trophoblasts, which anchor the placenta in the uterine implantation site (decidua) where they are juxtaposed to maternal immune cells [8]. Consequently, previous studies have established that the placenta plays an important role in maintaining immune tolerance at the maternal–fetal interface.

Previous studies have demonstrated that *T. gondii* invades the placenta directly from maternal blood and may disrupt the maternal–fetal immunological balance and lead to abnormal pregnancy [9]. In our previous studies, we found that several decidual immune cells and molecules participate in abnormal pregnancy outcomes caused by *T. gondii* infection [10–12]. However, there was no systematic research to determine molecular mechanisms involved in adverse pregnancy outcomes following *T. gondii* infection. To further accurately explore

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the molecular mechanisms associated with *T. gondii* infection, a quantitative proteomic analysis of placental proteins from *T. gondii*-infected and -uninfected pregnant mice was performed using a label-free liquid chromatography–mass spectrometry (LC–MS) approach. Furthermore, real-time polymerase chain reaction (PCR), western blotting, and immunohistochemistry were used to further confirm differences in protein expression detected during the quantitative proteomic analysis.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), formic acid (FA), acetic acid (HAc), NH_4HCO_3 and NaCl were obtained from Merck (Darmstadt, Germany). Acetone, ethylenediaminetetraacetic acid tetrasodium salt dihydrate, trifluoroacetic acid, phosphate-buffered saline (PBS), and *n*-octyl-β-D-glucopyranoside were purchased from Sigma–Aldrich (St. Louis, MO, USA). For tryptic digestion, iodoacetamide (IAA), urea, and dithiothreitol (DTT) obtained from Sigma–Aldrich. The following antibodies were used: rabbit anti-annexin A2 (Anxa2) and A3 (Anxa3) polyclonal antibodies, rabbit anti-alpha-1-antitrypsin (Serpina1a) polyclonal antibody, rabbit anti-sorting nexin3 (Snx3) polyclonal antibody, rabbit anti-cytokeratin 18 (Krt18) polyclonal antibody (Proteintech, China), rabbit anti-fibrinogen alpha chain (Fga) polyclonal antibody (LifeSpan Biosciences, Seattle, WA, USA), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Zhongshan, China).

2.2. Sample collection

C56BL/6 mice (6- to 8-week-old females and males) were purchased from Jinan Pengyue Experimental Animal Technical Co., Ltd., China. Female mice were housed six per cage, and male mice were housed one per cage at 25 °C with an air humidity of 50%–60% and a 12 h light/dark cycle. All animals were adequately supplied with water sterilized by autoclaving and food for specific pathogen-free mice, purchased from Jiangsu Biological Engineering Co., Ltd. After overnight cohabitation with males at a ratio of 2:1, females with a vaginal plug [gestational day (gd) zero] were kept separately and randomized into two groups of five mice each, the normal pregnancy group and the infected pregnancy group. All experiments were performed according to the ethical standards formulated by the Institutional Animal Experimental Ethics Committee of the Binzhou Medical University, China.

On gd 7, mice in the infected pregnancy group were intraperitoneally injected with 400 *T. gondii* strain RH tachyzoites in 200 μL of sterile PBS, and those in the control group were injected with 200 μL of sterile PBS. All mice were sacrificed at 5 days post-infection (gd 12), and placental tissues were collected from the two groups.

2.3. Protein extraction and precipitation

Placental tissues were stored at −80 °C until analysis. Aliquots of 80 mg of placental tissue powder were homogenized for 60 s in a blender with 400 μL of lysis buffer (Beyotime, China), and 8 μL of phenylmethanesulfonyl fluoride (Beyotime) was added during sample preparation to prevent protein degradation. After homogenization, samples were incubated on ice for 40 min. Tissue lysates were clarified by centrifugation at 12,000 rpm for 10 min, and the supernatant containing extracted proteins was collected for further processing. Aliquots of protein extracts were mixed with 1.4 mL of an ice-cold tri-*n*-butyl phosphate/acetone/MeOH mixture (1:12:1) and incubated at 4 °C for 90 min. Precipitates were pelleted by centrifugation (2800 ×*g*) at 4 °C for 15 min, washed sequentially with 1 mL of acetone and 1 mL of MeOH, and finally air dried.

2.4. On-filter tryptic digestion of proteins

Protein pellets were redissolved in 200 μL of digestion buffer (8 M urea, *n*-octyl-β-D-glucopyranoside in 50% ACN). The equal amounts of protein solution were used for digestion. An on-filter digestion protocol was used for tryptic digestion of the samples using 3 kDa spin filters (Pall Life Sciences, Ann Arbor, MI, USA). Centrifugation was performed at a centrifugal force of 14,000 ×*g* according to the protocol. A volume of 10 μL of 45 mM aqueous DTT was added to all samples, and the mixtures were incubated at 50 °C for 15 min to reduce disulfide bridges. Samples were cooled to room temperature, then 10 μL of 100 mM aqueous IAA was added, and the mixtures were incubated for an additional 15 min at room temperature in the dark to carabamidomethylate cysteines. Samples were transferred to spin filters prewashed with 250 μL of 50% ACN for 15 min and then with 500 μL of water for 20 min. Next, they were centrifuged for 10 min to remove added salts, detergents, and other interfering substances. A volume of 100 μL of 50 mM NH_4HCO_3 in 2% ACN was added, and filters were spun for 10 min, followed by the addition of 100 μL of 50 mM NH_4HCO_3 in 50% ACN and 150 μL of 50 mM NH_4HCO_3 and centrifugation for another 10 min. Finally, 100 μL of 50 mM NH_4HCO_3 (pH 7.8) and 16 μL of trypsin (0.1 μg/μL) were added to the samples. Tryptic digestion was performed at 37 °C overnight in the dark. Digests were spun through the filters for 20 min to collect tryptic peptides. An additional volume of 100 μL of 50% ACN and 1% HAc was added; filters were spun for 10 min, and the resulting filtrate was mixed with the first tryptic peptide filtrate. Collected filtrates were vacuum-centrifuged to dryness using a Speed Vac system ISS110 (Thermo Scientific, Waltham, MA, USA).

2.5. LC–MS analysis

The analyses were performed using a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. Samples were dissolved in water/FA (0.1%), and peptides were separated with reversed-phase LC using an EASY-nLC 1000 system (Thermo Fisher Scientific). A two-column setup consisting of a pre-column and an analytical column was used. The pre-column was a 2-cm EASY-column (ID 100 μm, 5 μm, C18) (Thermo Fisher Scientific), whereas the analytical column was a 10-cm EASY-column (ID 75 μm, 3 μm, C18) (Thermo Fisher Scientific). Peptides were eluted with a 90-min linear gradient from 4% to 100% ACN at 250 nL/min. The mass spectrometer was performed in a positive-ion mode and consecutive high-energy collisional dissociation fragmentation spectra of the 10 most abundant ions. The acquired data (.RAW-files) were processed by Proteome Discoverer software (Version 1.4.0.288, Thermo Fisher Scientific, Bremen, Germany) against the Uniprot-Swissprot database using an extracted FASTA file specified for "mouse" taxonomy. For full proteome data, label-free quantification (LFQ) was performed using MaxQuant (version 1.4.0.1) [13]. Tandem mass spectra were searched with Andromeda against the Swiss-Prot protein database using search settings specified below: maximum 10 ppm and 0.02 Da error tolerance for the survey scan and MS/MS analysis, respectively; enzyme specificity was trypsin; maximum two missed cleavage sites allowed; cysteine carbamidomethylation was set as static modification; Oxidation (M) and Deamidation (N,Q) were set as variable modifications. The protein identifications were based on at least two matching peptides of 95% confidence per protein. Feature-matching between raw files was enabled using a retention time window of 2 min. Averaged LFQ intensity values were used for further data analysis.

2.6. Real-time PCR assay

To confirm changes in protein expression in the array, real-time PCR was performed. Total RNA was extracted from tissue samples using Trizol reagent (Takara Biotechnology, Inc., Japan) and reverse