



Toxoplasma gondii and Downregulation of Gamma Interferon

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ABSTRACT

This study was designed to evaluate the gene expression level of IFN- γ in murine leukocytes treated by *Toxoplasma gondii* products in vivo using quantitative real time-PCR (Q-PCR) method. The mice considered as test groups were treated separately by *Toxoplasma* lysate product (TLP), excretory/secretory products (ESPs) obtained from cell free and cell culture media as well as active tachyzoites. The mice in the control group received phosphate buffered saline (PBS). The peritoneal leukocytes of the mice were harvested separately and their total RNA was extracted, converted to cDNA, and the gene expression level of IFN- γ was measured in all of the test groups relative to the control one. The results showed that the gene expression level of IFN- γ was decreased in the groups of the ESP from cell free medium ($P=0.03$), the TLP ($P=0.02$), and the active tachyzoite ($P=0.16$). Moreover, there was no significant difference statistically for the group of the ESP from cell culture medium ($P=0.33$). The findings revealed for the first time that the TLP and the ESP from cell free medium contain molecules that are capable of downregulating the expression of IFN- γ . Therefore, the question arises as to which molecules in these two products are involved in this process?

Keywords: *Toxoplasma gondii*, Gamma interferon, IFN- γ

INTRODUCTION

Toxoplasmosis, the disease caused by an obligate intracellular parasitic protozoan, *Toxoplasma gondii* (*T. gondii*), with worldwide prevalence, is able to infect a wide variety of vertebrates including humans [1]. Warm-blooded vertebrates as intermediate hosts of the parasite are capable of maintaining the parasite in the form of tissue cysts over their lifetime [2]. This parasite possesses numerous antigens with different properties that play a role in stimulating the host immune system. Surface antigens, cytoplasmic antigens, and excretory/secretory antigens (ESAs) are of most important ones, of which the ESAs are more important than the others. The ESAs are released from the three excretory/secretory organelles of the parasite called microneme, rhoptry and dense granule [3]. Discharge of the contents of these organelles is the most crucial factor in the cell entry and survival of *T. gondii* [4]. The release of these products also triggers cellular and humoral immune responses in the host [5]. Once the parasite enters the tissue cells the stage conversion occurs from tachyzoite to bradyzoite. The most important factor involved in the stage conversion is gamma interferon (IFN- γ) [6]. The parasite within tissue cell releases the ESAs that spread out of the cell into the host body and results in producing a high blood level of IFN- γ in the host [7]. The main sources of IFN- γ production are macrophages and Th-1 lymphocytes. IFN- γ activates various mechanisms against the parasite in the cell body, one of which is an increase in nitric oxide production in the infected cell [8]. Obviously, the parasite survival in the infected cell is depended on the reduction of IFN- γ effects on it. Many studies have shown that *T. gondii* can reduce the effects of IFN- γ on the infected cell through different mechanisms, but

whether the parasite products possess one such function is still unknown. Therefore, this study was designed to evaluate the gene expression level of IFN- γ in murine leukocytes treated by the parasite products in vivo using quantitative real time- PCR (Q-PCR).

MATERIALS AND METHODS

Parasite

T. gondii tachyzoite strain RH was used in this study. This parasite was maintained in laboratory by passaging in laboratory mice as well as keeping at -196 in a cryoprotectant solution consisted of dimethyl sulfoxide (DMSO) (10%), RPMI-1640 medium (60%), fetal bovine serum (FBS) (30%). In addition, after taking out the parasite from freezing environment, the parasite was started up on the murine fibroblasts cell culture (4).

Mouse

Outbred Swiss Webster mice were used to keep the parasite in vivo. Moreover, inbred female, 20-25 grams, 8-10 weeks old, BALB/c mice were used for experiment. The university research ethics committee (UREC) of the Isfahan University of Medical Science approved the use of the mice in this study.

T. gondii lysate product (TLP)

The murine fibroblast cell culture was used to obtain a large number of tachyzoites. The harvested parasites were two times washed by centrifugation at 1500 \times g, 4 $^{\circ}$ C, for 10 min in RPMI-1640 medium (Gibco, Inc.) and then the precipitate containing the parasite was suspended in 2-3 ml of the same medium. Using sonication method in ultrasonic bath the parasites were lysed under the condition of 25 kHz, 30 s on and 10 s off for 5 min. In order to remove the parasite debris, the solution was centrifuged at 15000 \times g for 15 minutes and the supernatant was collected in a sterile tube. The sample was sterile filtered using 0.22- μ m pore size filters (Denville Inc.), and the amount of its protein was measured by the Bradford method [9], lyophilized in the presence of trehalose (5% w/v) (Sigma, Inc.) and maintained at -80 $^{\circ}$ C until use. No protease inhibitor was added to this product.

The ESP from cell culture medium

The culture of murine peritoneal leukocytes was used for this purpose. At first, 2.4×10^8 of peritoneal leukocytes harvested from the peritoneal cavity of Swiss Webster mice were two times washed by centrifugation at 1500 \times g, 4 $^{\circ}$ C, for 10 min in RPMI-1640 medium and then about 4×10^6 cells was poured into each well of 24-well cell culture plates to which 4×10^6 active tachyzoites/well was added. The plates were incubated at 37 $^{\circ}$ C, 5 % CO₂, and 95 % humidity in a cell culture incubator for 48 h. The supernatants of the wells was then harvested, pooled, centrifuged at 15000 \times g, 4 $^{\circ}$ C, for 15 min, and the supernatant was collected, sterile filtered using 0.22- μ m pore size filters, and its protein concentration was measured by the Bradford method (9), lyophilized in the presence of trehalose (5% w/v) and kept at -80 $^{\circ}$ C until use. Neither FBS was added to the cell culture medium nor was protease inhibitor added to this product.

The ESP from cell free medium

A large number of active tachyzoites were harvested from the murine fibroblast cell culture and were three times washed by centrifugation at 1500 \times g, 4 $^{\circ}$ C, for 10 min in RPMI-1640 medium and the number of tachyzoites was adjusted to 6×10^6 tachyzoites in 2 ml RPMI-1640 medium in each tube. The tubes were incubated under mild shaking at 37 $^{\circ}$ C for 3 h and the supernatants were collected in sterile tubes, centrifuged at 15000 \times g, 4 $^{\circ}$ C, for 15 min, the supernatants were collected again, pooled, sterile filtered using 0.22- μ m pore size filters, and its protein concentration was measured by the Bradford method [9], lyophilized in the presence of trehalose (5% w/v) and kept at -80 $^{\circ}$ C until use. No protease inhibitor was added to this product.

Injection to mice

Each of the lyophilized powders was dissolved in 2 ml of phosphate buffered saline (PBS; pH=7.4). Fifty BALB/c mice were divided into 5 groups. Four groups each received one of the products including the TLP, the ESP from cell culture, and the ESP from cell free medium at doses of 100-1000 μ g for 1-10 mice, respectively, once a week for 3 weeks, and the fourth one received 1000-10000 active tachyzoites for 1-10 mice, respectively, only once and three days before samples collection. The aforementioned groups were considered as test groups. The fifth group was also considered as control one and received PBS at doses of 100-1000 μ l for 1-10 mice, respectively, once a week for 3 weeks. All of the injections were performed intraperitoneally. No adjuvant was used for the injections.

Sample collection

Three days after the last injection the mice were euthanized and their peritoneal cavity was washed by PBS and the harvested fluid was poured into RNAlater (Qiagen, Inc.), a RNA stabilizer solution, and kept at -20 °C until use.

Total RNA extraction

The total RNA was extracted by Total RNA Purification Kit (Jena Bioscience, Inc.) according to manufacturer instruction. On-column DNA digestion was used to eliminate all DNA in the samples using RNase-Free DNase Set kit (Qiagen, Inc.). With NanoDrop® ND-1000 spectrophotometer the purity and concentration of total RNA was evaluated. At last, the total RNA was stored at -20 °C until use.

Complementary DNA synthesis

The total RNA was converted to complementary DNA (cDNA) using AccuPower® CycleScript RT PreMix (dN6) kit (Bioneer, Inc.) according to manufacturer instruction. Random hexamer primers were annealed at 15 °C for 1 min, cDNA was synthesized at 45 °C for 4 min.

Primer design

For this objective, the mRNA sequences of gene of interest including murine IFN- γ mRNA as target gene and the mRNA sequence of murine hydroxymethylbilane synthase (HMBS) as housekeeping gene were extracted from the GeneBank® database. The primers were designed by Beacon Designer™ software according to SYBR® Green method, one of which spanned an exon-exon junction. The primer sequences have been shown in the Table 1.

Table1. The sequences of primers designed in this study

Gene	Accession number	Primer	Sequence	Slope: Efficiency
HMBS	NM_013551.2 ^a	Forward	CCGAGCCAAGGACCAGGATA	-3.368: 0.976
		Reverse	TCAGGTACAGTTGCCCATCTTTC	
IFN- γ	NM_008337.3	Forward	GTATTGCCAAGTTTGAGGTCAACAAC	-3.382: 0.972
		Reverse	CCGAATCAGCAGCGACTCC	

^{a,b} The primers were designed for the identical region of HMBS transcript variants. This region was found using MEGA6 software.

Quantitative real time-PCR (Q-PCR)

The Q-PCR was performed with Applied Biosystems StepOne™ Real-Time PCR System and using qPCR GreenMaster with UNG kit (Jena Bioscience, Inc.) according to manufacturer instruction. Initial denaturation was carried out at 95 °C for 2 min, and afterwards 40 cycles of denaturation were utilized at 95 °C for 15 s, annealing-extension at 60.2 °C for 45s.

Data analysis

The normal distribution of data was confirmed by Kolmogorov–Smirnov (K–S) statistical test. Subsequently, the melting curve of reactions was evaluated for unwanted amplicons. Afterwards, the gene expression of IFN- γ in the test groups was examined relative to the control one using the REST-2009 software (Qiagen Inc.). This software also utilizes t-test to show significant difference statistically between the test groups and the control one at a level of difference lower than 0.05. The standard error of mean (SEM) for IFN- γ Δ Ct was calculated for the groups as well.

RESULTS

The findings showed that the gene expression level of IFN- γ was decreased in the groups of the ESP from cell free medium (P=0.03), the TLP (P=0.02), and the active tachyzoite (P=0.16) relative to the control one. Moreover, there was no significant difference statistically regarding the expression level of the target gene in the group of the ESP from cell culture medium (P=0.33). The relative expression values of IFN- γ in the aforementioned groups have been shown in Figure 1. The values of SEM for all of the groups have been presented in Table 2.

Table2. The standard error of mean (SEM) for IFN- γ Δ Ct in the groups under study

Average \pm SEM				
TLP	ESP-CF	ESP-CC	AT	Control
3.89 \pm 0.36	4.6 \pm 0.42	4.78 \pm 0.75	3.44 \pm 0.69	3.2 \pm 0.12

TLP: *Toxoplasma gondii* lysate product, ESP-CF: excretory/secretory product from cell free medium, ESP-CC: excretory/secretory product from cell culture medium, AT: active tachyzoite

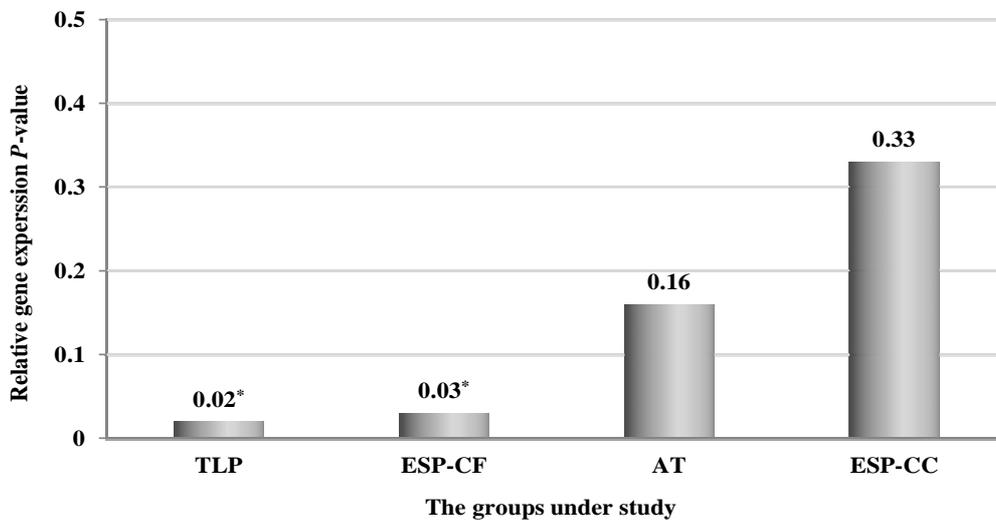


Fig.1. The relative gene expression of IFN- γ in the groups under study. The columns marked with P-value relative to the control group ($P < 0.05$ is significant, indicated with asterisk [*]). TLP: *Toxoplasma gondii* lysate product, ESP-CF: excretory/secretory product from cell free medium, AT: active tachyzoite, ESP-CC: excretory/secretory product from cell culture medium

DISCUSSION

A study showed that the alteration in the host cell gene expression differs among *T. gondii* strains and this is the reason why genotype II strains develop more tissue cysts than the other genotype strains [10, 11]. Cell-mediated immune responses, especially IFN- γ production, are the most important strategy by which the host inhibits the growth of *T. gondii* in the tissue cells [6]. A study revealed that a decrease in the expression level of IFN- γ can activate the tissue cysts developed by the parasite [12], therefore, this cytokine is crucial for stage conversion of this parasite and also keeping the parasite in the cystic form [13]. The amino acid tryptophan is critical for rapid replication of the parasite and IFN- γ keeps this amino acid out of reach of the parasite by the induction of an enzyme which catalyzes this amino acid called indoleamine 2,3-dioxygenase [14], resulting in the slow parasite replication that this form is called bradyzoite. Moreover, one study demonstrated that the mice lack of IFN- γ gene can bear a high burden of free tachyzoites which give rise to the infected mice death [15]. In addition, researchers elsewhere showed that mice infected with *T. gondii* may die because of the significant amount of IFN- γ production [16]. Given that the anti-toxoplasmic effect of this cytokine is related to the induction of nitric oxide (NO) production [17], therefore, the parasite should inhibit the IFN- γ effects on the infected cell. One of these mechanisms is the upregulation of suppressor of cytokine signaling 1 (SOCS1) molecule that inhibits the signaling pathway of IFN- γ into the infected cell [18]. Another mechanism by which this parasite limits the effect of IFN- γ is the use of some ESPs which traffic from parasitophorous vacuole to the host cell nucleus such as ROP16 and ROP18 that alter the host cell gene expression patterns, respectively, through the upregulation of signal transducer and activator of transcription factor 3 (STAT3) and the downregulation of nuclear factor- κ B (NF- κ B) [19, 20]. The STAT3 has multifunctional effects, of which apoptosis inhibition and reduction of IL-12 production are some examples, helping the parasite to survive in the infected cell [21, 22]. This parasite also dysregulates IFN- γ -inducible genes in the infected cells and inhibits the effect of this cytokine on these cells [23]. Surprisingly, the findings of the present study showed that the two *T. gondii* products, the TLP and the ESP from cell free medium, reduced the gene expression level of IFN- γ to the lowest level possible (Fig. 1). However, in the group of the ESP from cell culture medium it showed no significant difference statistically relative to the control one and it may be due to the impurity of this product with the secretions of the cultured cells. Nevertheless, the expression level of the target gene in the group of the ESP from cell culture medium was evidently about one-third of the control group (Fig. 1).

CONCLUSION

The results of this study showed a new aspect of host-*T. gondii* interaction about the down regulation of IFN- γ expression. The results of the current study revealed for the first time that the *Toxoplasma* lysate product (TLP) and the excretory/secretory product (ESP) from cell free medium contain molecules that are capable of down regulating IFN- γ expression. Therefore, the question arises as to which molecules in these two products are involved in this process?

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