

## Manipulation of IL-10 gene expression by *Toxoplasma gondii* and its products

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### Abstract

**Background:** This study was designed to evaluate whether or not *T. gondii* and its derivatives can change the gene expression level of IL-10 in murine leukocytes in vivo.

**Methods:** Fifty BALB/c mice were divided into 5 groups, four of which received the excretory/secretory product (ESP) from cell culture medium, the ESP from cell free medium, the Toxoplasma lysate product (TLP) and the active tachyzoites, respectively. The fifth group was considered as control and received PBS. The peritoneal leukocytes from the mice were collected. Their total RNA were extracted and converted to cDNA and the gene expression levels of IL-10 in the samples were evaluated by quantitative real time-PCR using the REST-2009 software.

**Results:** The findings showed a decrease in the expression level of IL-10 in the TLP group ( $p=0.004$ ). Moreover, the IL-10 gene expression level was upregulated in the group of the ESP from cell culture medium ( $p=0.04$ ) and the active tachyzoite group ( $p=0.04$ ). The expression of IL-10 gene in the group of ESP from cell-free medium was not significant compared to the control one ( $p=0.45$ ).

**Conclusion:** *T. gondii* and its derivatives are able to increase (the active *T. gondii* tachyzoite and the ESP from cell culture medium) and decrease (the TLP) the gene expression level of IL-10 in a murine model. The question remains to be examined in further study about which molecules are involved in this process.

**Keywords:** IL-10, *Toxoplasma gondii*, Gene expression.

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### Introduction

*Toxoplasma (T.) gondii* is a parasitic protozoan which infects wide-ranging vertebrates, of which felines host the sexual stage of the parasite and other warm-blooded vertebrates do the asexual stage. Toxoplasmosis, the disease caused by this parasite, is spread all over the world (1). The signs of the disease are mostly limited to a mild fever and lymphadenopathy in healthy individuals while it can be highly important in those who suffer from AIDS/HIV<sup>+</sup> disease (2). The interaction between the host immune system and the

parasite is the most important factor involved in the development of the disease manifestations. Most importantly, the studies showed that the manipulation of the infected host cell transcription factors by the parasite help it to evade from the host immune responses and to survive in the cell safely as well (3). One of the mechanisms by which the parasite escapes from the host immune responses is antigen shedding, a mechanism during which the parasite excretory/secretory products (ESP) release into the host body and evoke the immune system (4). The antibodies produced by

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host immune responses to ESP push the parasite to be encysted at the early stage of infection (5). Another strategy is to mimic apoptosis induced by the parasite in the infected cell in order not to be recognizable by host immune cells (6). On the other hand, this parasite has so many strains; the stimulation of host immune responses by them are different (7).

This study was designed to evaluate whether or not *T. gondii* and its derivatives are able to change the gene expression level of IL-10 in murine leukocytes in vivo.

## Methods

### Parasite

The RH strain of *T. gondii* tachyzoite was maintained in our laboratory. Murine fibroblast cells were cultured and cryopreserved according to the methods described by Daryani et al. (4).

### Mouse

Swiss Webster female, 8 weeks old, 20-25 gram, and inbred BALB/c mice were used in this study. The former was used for parasite maintenance and the latter for the experiment. The use of the mice was approved by the university research ethics committee (UREC) of the Isfahan University of Medical Science.

### *T. gondii* lysate product (TLP)

A number of tachyzoites obtained from the peritoneal fluid of the infected mice were washed for three times with RPMI-1640 medium (Sigma, Inc.) by centrifugation at 1500×g, 4°C, for 10 min. The parasites were divided into smaller parts in separate tubes and lysed by sonication at 25 kHz, 30s on and 10s off for 5min in an ultrasonic bath filled with cold water (2-4°C). The tubes were centrifuged at 15000×g, 4°C, for 15 min and their supernatant was collected in a tube, sterile filtered with 0.22µm pore size filters (Denville Inc.) and stored at -20°C until use. No protease inhibitor was added to this product to be with no alteration.

### The ESP from cell culture medium

The peritoneal cavities of some healthy mice were washed with RPMI-1640 medium to harvest murine peritoneal leukocytes. The leukocytes were washed for three times with RPMI-1640 medium. One active tachyzoite for one leukocyte was considered in cell culture plates and the plates were incubated at 37°C, 5% CO<sub>2</sub> and 95% humidity for 48h (4). After this time, the culture media in all of the wells were collected, pooled, centrifuged at 15000 ×g, 4°C, for 15min and the supernatant was harvested, sterile filtered using 0.22µm pore size filters and stored at -20°C until use. No protease inhibitor was added to this product to be with no alteration. Also, no serum was used in the cell culture medium to avoid impurity of this product with serum proteins.

### The ESP from cell-free medium

A large number of fresh tachyzoites harvested from murine fibroblast cell culture medium were washed for three times with RPMI-1640 medium, divided into smaller parts each containing  $6 \times 10^6$  tachyzoites in separate tubes. The tubes were incubated under mild shaking in a shaking incubator at 37 °C for 3 hours (4). The tubes were then centrifuged at 15000×g, 4°C, for 15min. The supernatants were harvested, pooled and sterile filtered using 0.22µm pore size filters and kept at -20°C until use. No protease inhibitor was added to this product to be with no alteration.

### Protein concentration measurement

The concentration of protein in the products was measured according to the method described by Bradford (8).

### Injection to mice

Fifty BALB/c mice were divided into 5 groups, four of which received the ESP from cell culture medium (100-1000µg for 1-10 mice), the ESP from cell free medium (100-1000µg for 1-10 mice), the TLP (100-1000µg for 1-10 mice) and the active tachyzoites (1000-10000 active *T. gondii*

tachyzoites for 1-10 mice), respectively. The remaining group was considered as control and received PBS (100-1000 $\mu$ l for 1-10 mice). The injections were performed intraperitoneally. Moreover, except for the group receiving active tachyzoite, the injections were done once a week in the other groups for three times. For the group of active tachyzoite, the injection was carried out only once. No adjuvant was injected into mice.

#### Sample collection

Three days after the last injection, the mice were euthanized and their peritoneal leukocytes were harvested in separate tubes and immediately 2 ml of RNeasy lysis solution (Qiagen Inc.) was added to them and were kept at -20°C until use.

#### Total RNA extraction

The extraction of total RNA from the samples was performed using Total RNA Purification Kit (Jena Bioscience Inc.) according to the manufacturer instruction. The impurity of the samples with genomic DNA was removed by RNase-Free DNase Set kit (Qiagen Inc.). The purity and concentration of the extracted RNAs were evaluated by NanoDrop® ND-1000 spectrophotometer. The extracted RNAs were then kept at -20°C until use.

#### cDNA synthesis

It was performed by AccuPower® CycleScript RT PreMix (dN6) kit (Bioneer Inc.) according to the manufacturer instruction. Random hexamer primers were utilized. The reaction condition was as follows: primers were annealed at 15 °C for 1 min and followed by cDNA was synthesized at 45 °C for 4 min. Then, the enzyme reverse transcriptase (RT) was heat-

inactivated at 95 °C for 5 min.

#### Primer design

The sequence related to mRNA of murine IL-10 as target gene on chromosome 1 and the mRNA sequence of murine hydroxymethylbilane synthase (HMBS) as housekeeping gene on chromosome 9 were found from the website of the National Center for Biotechnology Information (NCBI). The specific forward and reverse primers were designed by the software AlleleID® based on SYBR Green method in view of one of the primers spanned an exon-exon junction. The primer sequences have been shown in Table 1.

#### Quantitative real time-PCR (Q-PCR)

It was carried out with Applied Biosystems StepOne™ apparatus as well as using qPCR GreenMaster with UNG kit (Jena Bioscience Inc.) according to the manufacturer instruction. Time and temperature for Q-PCR were 95°C for 2 min, followed by 40 amplification cycles with denaturation at 95°C for 15s, annealing-extension at 60.2°C for 45s.

#### Data analysis

Data were tested using Kolmogorov-Smirnov (KS) statistical test to evaluate the normal distribution of the data. The melting curve of each reaction was examined to confirm the the accuracy of the reactions. The gene expression level of IL-10 in the test groups was compared to that in the control one using the REST-2009 software (Qiagen Inc.). In addition, the *P*-values were calculated by the same software. In all of the groups under study, the standard error of mean (SEM) was calculated for  $\Delta$ Ct of IL-10.

Table 1. The sequences of the designed primers in this study

Gene	NCBI Reference Sequence	Primer	Sequence
HMBS	NM_013551.2 <sup>a</sup>	Forward	CCGAGCCAAGGACCAGGATA
	NM_001110251.1 <sup>b</sup>	Reverse	TCAGGTACAGTTGCCCATCTTTC
IL-10	NM_010548.2	Forward	TGCTATGCTGCCTGCTCTTA
		Reverse	GCAACCCAAGTAACCCTTAAAGTC

<sup>a,b</sup>MEGA4 software was used to design the primers based on their homologous regions

## Results

The results of the current study revealed that there was no significant difference statistically regarding the gene expression level of IL-10 in the group of the ESP from cell-free medium ( $p=0.45$ ). However, in the group of the TLP, a decrease in the expression level of IL-10 was observed ( $p=0.004$ ). Moreover, the IL-10 gene expression level was upregulated in the group of the ESP from cell culture medium ( $p=0.04$ ) and the active tachyzoite group ( $p=0.04$ ) (Fig. 1). The SEM calculated values have been shown in Table 2.

## Discussion

A study showed that the presence of IL-10 in the *T. gondii*-infected host body is far important to warrant the survival of the host because overexpression of IFN- $\gamma$  can be detrimental for the host (9). It has been demonstrated that IL-10 inhibits the production of nitrite oxide (NO) by IFN- $\gamma$ -activated macrophage to kill *T. gondii* in the infected cell (10). One of the serious

problems that can be observed in the infection with this parasite is a miscarriage. Studies showed that the injection of IL-10 to pregnant mice can avoid the dangerous effects of the parasite on the fetus during pregnancy (11,12). In addition, the evidence suggested that the aforementioned molecule is increased in the infection with this parasite and its high amount in the sample collected by bronchoalveolar lavage from infected animals has been reported (13). Moreover, when mice without IL-10 gene is infected with a limited number of the parasite, were reinfected, the observed immune responses were similar to that observed in the wild type of the infected mice (14). This indicates that this molecule does not play an important role in starting secondary immune responses against the parasite. In a study it was observed that the parasite improves the symptoms of *Dermatophagoides farinae*-induced allergy in susceptible mice by inducing the IL-10 gene expression (15). Furthermore, in the last-mentioned study both IFN- $\gamma$  and IL-10

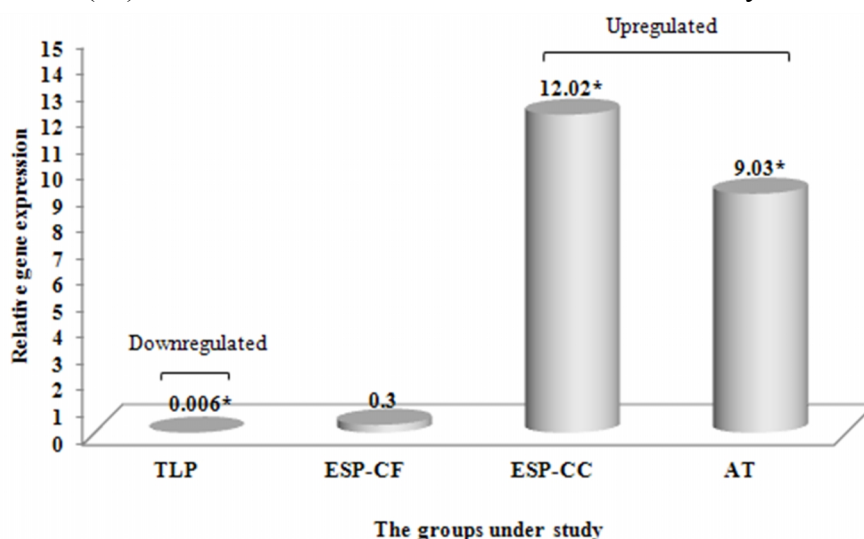


Fig. 1. The relative expression of IL-10 gene in the groups under study

Asterisk (\*) shows a significant difference statistically ( $p < 0.05$ ). 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

Table 2. The standard error of mean (SEM) for IL-10  $\Delta$ Cts in groups under study

Average $\pm$ SEM				
TLP	ESP-CF	ESP-CC	AT	PBS
4.01 $\pm$ 0.68	2.9 $\pm$ 0.84	3.88 $\pm$ 0.95	3.22 $\pm$ 1.01	4.15 $\pm$ 0.93

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, PBS: phosphate buffered saline

were upregulated simultaneously. Consequently, it can be concluded that the parasite can play an effective role in the modulation of immune responses (16). Unlike most studies that confirmed the increased expression of IL-10 in *T. gondii* infection, a study showed that the intestinal stage of this parasite can decrease the expression of this molecule (17). Moreover, the researchers showed that the vaccine made from a type of rhoptry molecule, ROP38, can greatly reduce the production of IL-10 by the immunized murine spleen cells (18). Contrary to the latter study, a study showed that murine spleen cells stimulated with *T. gondii* produce a high amount of IL-10 (19). Likewise, the results of another study showed that serum level of IL-10 in mice receiving the *T. gondii* ESAs rises (20). In a study conducted by Matowicka-Karna et al. (21) it was observed that serum level of IL-10 in patients infected with this parasite is fivefold higher than that in healthy individuals. It has been demonstrated that a considerable increase in the serum level of IL-10 is detectable during *T. gondii* infection at the chronic stage of the disease (19). The results of one study indicated that irradiated *T. gondii* tachyzoites induce production of IL-10 (22). An increase in the production of IL-10 in *T. gondii* infection is important from several aspects: IL-10 induces signal transducer and activator of transcription factor 3 (STAT3) which results in inhibiting the apoptosis of the infected cells and ensuring the survival of the parasite within the host cell (23). Induced STAT3 has an inhibitory effect on macrophage activity through preventing the production of proinflammatory cytokines (24,25). Most importantly, it seems that activation of STAT3 is crucial for parasite because the parasite through a rhoptry molecule called ROP16 activates independently IL-10 (26). As discussed above, the majority of the studies showed that *T. gondii* is capable of enhancing the production of IL-10 but a few ones also showed a decrease in the production of IL-10 in the infected hosts. The findings of the present study revealed

that active *T. gondii* tachyzoites and the ESP from cell culture medium upregulate the IL-10 expression. Strangely, we observed that the gene expression level of IL-10 was reduced in the group treated with the TLP so that it was close to zero (Fig. 1). This is likely due to the difference of this product with the other ones. Only the body of the parasite was destroyed in this product. Studies showed that the parasite has a large number of miRNAs that the role of very few of them has been known (27). These molecules are involved in the regulation of the gene expression. So in the *T. gondii* lysate product (TLP) these molecules are present and probably the reason for an excessive decrease in the gene expression of IL-10 is the same.

### Conclusion

In conclusion, we showed that *T. gondii* and its derivatives are able to increase (active *T. gondii* tachyzoite and the ESP from cell culture medium) and decrease (the TLP) the gene expression level of IL-10 in a murine model. The question remains to be examined in further study about which molecules are involved in this process.

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