# Interleukin-10 and immune response in mice infected with the cyst strain of *Toxoplasma gondii*

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**Abstract**

Early *in vitro* studies have shown that specific anti-*Toxoplasma gondii* antibodies are lytic for the parasite. However, it can persist in chronic latent infections, and it is suggested that parasite survival is dependent on induction of IL-10 in order to down regulate the host protective immune response. In the present study, Balb/c mice were infected orally with the cyst strain of *T. gondii* and the changes in the serum levels of interleukin-10 (IL-10) and anti-*Toxoplasma* antibodies were determined at weekly intervals throughout the 35 days post-infection, using a double sandwich ELISA and the indirect ELISA technique respectively. IgM peaked during early infection, while IgG peaked at the fourth week post-infection. IL-10 peaked at 28 days post-infection. There was a strong positive correlation between IgG anti-*Toxoplasma* antibodies and IL-10 ($r = 0.704$, $P < 0.05$). Tissue cysts were detected in spleens that were enlarged at two weeks post-infection. Mortality was found to be dependant on the infective dose, this being highest in mice infected with 10,000 cysts.

**Key words**: *Toxoplasma gondii*, cyst strain, interleukin-10, immune response

## Introduction

Toxoplasmosis is due to infection with the intracellular protozoan *Toxoplasma gondii*. The cat is the most important definitive host for *T. gondii* and about 200 species of mammals and birds infected with the meronts or extra intestinal tissue stages of the parasite, serve as intermediate hosts. It is present more frequently in warm, moist climates than in cold, dry climates. Epidemiological studies generally based on serological tests show that human infection rises with age, with the peak prevalence in individuals ≥ 35 years of age. Chronic latent infection in multiple organs exists even though a strong and lasting immune response is induced (Wong & Remington, 1993). In immunocompetent individuals infection is often asymptomatic. However, it is an important cause of ocular and neurological lesions in congenitally infected infants and children (Remington & Desmonts, 1990). These infections can range from quite mild to fatal. Prenatal infection of the central nervous system can result in hydrocephalus.

Tachyzoites, bradyzoites, and sporozoites are the three infectious stages of *T. gondii*. The ingestion of food or water contaminated by cat faeces with mature oocysts containing sporozoites and consuming undercooked meat with tissue cysts are the two major pathways of post-natal transmission of toxoplasmosis (Dubey & Beattie, 1988). After ingestion of oocysts and cysts, both sporozoites and bradyzoites respectively convert to tachyzoites inside host cells. Once within its host, the tachyzoite is capable of infecting all cell types except the mature red blood cell. The conversion of tachyzoites to bradyzoites and vice versa is of biological and clinical significance because bradyzoites are less susceptible to chemotherapy and reactivation of bradyzoites to tachyzoites is considered the cause of fatal toxoplasmosis in AIDS patients (Dubey, 1998).

In mice, tissue cysts were formed between 5 to 6 days and 6 to 7 days after ingestion of bradyzoites and oocysts respectively (Dubey, 1997). It has been proposed that bradyzoites escape from cysts but fail to result in toxoplasmic encephalitis in immunocompetent hosts whereas with impaired host immunity, active infection can result. Thus, it seems likely that there is immune regulation of the infection throughout the body including the central nervous system.

Infection with large doses of parasites or some virulent strain of *T. gondii* is rapidly fatal to the mouse. For example, mice infected with the virulent RH strain of *T. gondii* normally die within a week if left untreated. However, a cyst

strain of *T. gondii* originally isolated from a slow loris (*Nycticebus coucang*) in Singapore produces chronic infection with cysts in the brain and other tissues (Zaman & Goh, 1968).

The infection has increased in recent years with the advent of acquired immunodeficiency syndrome (AIDS). As the immune status of a human immunodeficiency virus (HIV) infected individual deteriorates, toxoplasmosis reactivates resulting in toxoplasmic encephalitis and disseminated toxoplasmosis (Israelski & Remington, 1992). In AIDS patients seropositive for *T. gondii*, 30% may develop toxoplasmic encephalitis. *T. gondii* is one of the most important opportunistic pathogens in patients with AIDS (McCabe & Remington, 1988), those undergoing chemotherapy for cancer, or those on corticosteroids for an inflammatory or auto-immune disease. Although chemotherapy has some effect in reducing the clinical signs and symptoms of toxoplasmosis, it does not seem to eliminate the infection.

*T. gondii* being a facultative intracellular parasite can survive and even replicate within phagocytes. Since it is mainly intracellular or within cysts it is inaccessible to circulating antibodies and elimination is probably mediated by cellular immune mechanisms. In murine models, and likely in humans, CD4+ and CD8+ T cells are both important in the immune response to *T. gondii*, at least part of which is through cytokine production (Suzuki & Remington, 1991). Elevated levels of cytokines in human and mice infected with toxoplasmosis suggest that they play a role in the immune response and immunopathology (Hunter, 1994). Previous studies have shown that the initial immune response preventing parasite multiplication is T cell-dependent and requires the production of IFN-γ (Suzuki, 1988). The tachyzoites are unaffected by normal human serum but sera containing anti-*T. gondii* antibodies induce parasite lysis through activation of the classical complement pathway (Schreiber & Feldman, 1980; Suzuki & Kobayashi, 1985). IFN-γ is produced in both resistant and susceptible strains of mice during the course of infection (Suzuki, 1993) and has been detected in acutely infected humans and congenitally infected newborns (Raymonds, 1990). Administration of IFN-γ protected mice against acute *T. gondii* infection (McCabe, 1984) and decreased the pathology associated with toxoplasmic encephalitis (Suzuki, 1990). However, Interleukin-10 (IL-10) inhibits IFN-γ activation of macrophages that inhibit multiplication of intracellular *Toxoplasma* (Gazzinelli, 1992). In toxoplasmosis, it has been shown to be a potent inhibitor of IFN-γ by spleen cells from uninfected SCID mice or SCID mice infected with *T. gondii* (Sher, 1993; Hunter, 1994). There is suggestion that parasite induced production of IL-10 may be an important strategy to avoid cell-mediated immune killing (Gazzinelli, 1992).

A study of the relationship between serum levels of IL-10 and anti-*Toxoplasma* antibody levels in mice experimentally infected with a parasite strain known to induce chronic infection will provide information on immune response and immunopathology in chronic toxoplasmosis. The findings may also provide leads for the design of more effective anti-*Toxoplasma* drugs.

## Materials and Methods

*Parasites*

The cyst strain *T. gondii* obtained from the Microbiology Department, National University of Singapore, was maintained in mice fed orally with cysts recovered from the brain. This cyst strain was originally isolated from a slow loris (*Nycticebus* coucang) in Singapore (Zaman & Goh, 1968). The highly virulent RH strain of *T. gondii*, originally isolated from the brain of a child with toxoplasmic encephalitis by Sabin, was used as a source of antigen for the enzyme-linked immunosorbent assay (ELISA).

*Preparation of T. gondii tissue cysts*

Mice infected with the cyst strain of *T. gondii* two months earlier were killed and the brains were obtained. Normal saline was added as diluent and the brains were homogenized with a glass rod. Quantification of the cyst density in the homogenate was carried out. Two μl of homogenate was applied onto a microscope glass slide and allowed to dry. After fixing with methanol, the specimen was stained with Giemsa. The number of cysts present on the slide was determined. The homogenate was then mixed with penicillin (100 units/mL) and streptomycin (100 μg/mL) for half an hour before being fed to mice.

*Preparation of T. gondii antigen*

Tachyzoites of the RH strain of *Toxoplasma* were harvested in phosphate-buffed saline (PBS), pH 7.2, from the peritoneal cavity of Balb/c mice infected 4 days earlier. Peritoneal fluid containing *Toxoplasma* was passed though a 3

µm pore size polycarbonate membrane (Nucleopore Corp., Pleasanton, A) to remove host cell debris. The cell free tachyzoites were washed 3 times with PBS at 2000x G for 20 minutes and resuspended in sterile distilled water. The resulting preparation was placed in an ice bath and sonicated with three 30-sec pulses at low speed in a biosonic sonicator (Microson, Misonic). The solution was stored at 4°C overnight. Cellular debris and unlysed cells were removed by centrifugation at 2000x G for 20 min at 4°C. The supernatant was used as the source of antigen and is referred to as sonicate.

*Protein determination for Toxoplasma antigen*

*Toxoplasma* antigen protein concentration in the sonicate was determined using the Bradford method. Seven micro centrifuge tubes were aliquoted with colorimetric standard protein solution (2 g/ml BSA), with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 µl. 100 µl of 0.15M NaCl was added to dilute the solution. Five µl of *Toxoplasma* soluble antigen (sonicate) was also diluted with 100 µl 0.15M NaCl and labelled as unknown sample. One more micro centrifuge tube with 100 µl of 0.15M NaCl served as blank control. 1 ml Coomassie brilliant blue solution was then added to each tube and vortexed. After 2 minutes of incubation, the absorbance at $A_{450}$ of all the solutions was determined using 1-cm-path-length microcuvette.

A standard curve was plotted with absorbance at 450 nm versus protein concentration. The concentration of *Toxoplasma* antigen in the stock solution (sonicate) was determined using the standard curve and found to be 200 µg/ml.

*Experimental animals*

Balb/c mice of similar age and size obtained from Institute for Medical Research, Kuala Lumpur were used. The animals were randomly assigned to three groups of 10 mice per group. Animals in Group 1 were each fed with 1,000 *T. gondii* cysts, while animals in Group 2 were each fed with 10,000 cysts of *T. gondii* prepared as described previously, via stomach tube. Animals in Group 3 (control) were each given 0.5ml normal saline.

*Collection of serum*

Each week, two mice were randomly selected from each group until 5 weeks post-infection and blood specimens were obtained through cardiac puncture. The sera were collected and stored at -20°C until used.

*Production of positive and negative serum*

The sediment obtained from the production of RH strain *T. gondii* soluble antigen was used for immunization. The pellet was mixed with Freund's complete adjuvant (Gibco, BRL) and administered by weekly subcutaneous injection to the mice. Seven days post-infection serum was obtained via tail bleeding and ELISA was used to measure anti-*T. gondii* antibody titres. In addition, isotyping was carried out to monitor changes in antibodies in immunized and control mice. Immunized mice were killed when there was at least a 3-fold increase in antibody titre.

Negative serum was obtained from uninfected mice. Blood was collected from 4 uninfected mice through cardiac puncture, and the sera obtained were stored at -20°C until used.

*Antibody isotyping*

Mouse monoclonal sub-isotyping kit-K5150 (American Qualex Antibodies) was used for the detection of mouse monoclonal antibody subclasses. This double antibody detection system is a highly sensitive enzyme immuno-assay method incorporating specific primary antisera. The antibodies subclasses were IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA.

*Indirect ELISA*

Optimal concentrations of antigen, positive and negative sera, conjugated second antibodies and substrates were determined by prior chequer-board titration. 100 µl antigen solution was dispensed into each well of an Immulon microtitre plate which was then sealed with plastic wrap and incubated overnight at room temperature or for 2 hr at 37°C. The plate was then washed three times with PBS Tween-20, sealed and stored at -20°C until used. Prior to use, each well was filled with blocking buffer and incubated for 1 hour at 37°C. After washing with PBS Tween-20 three times residual liquid was removed by wrapping the plate in paper tissue and gently flicking it faced down onto several paper towels. 50 µl of diluted serum sample were then added into each well and then incubated for 1 hour at 37°C. The serum was pre-incubated with peritoneal antigen obtained from uninfected mice overnight at 4°C before use. The antigen plates were pre-incubated with 0.5% skim milk before being used. After washing three

times with PBS-Tween-20, 100 μl antimouse-antibody peroxidase conjugate were added into each well. The plate was then incubated at $37^0$C for 1 hour again.

After the final washing, 50 μl of peroxidase solution (substrate) were added into each well. The reaction was stopped with 50 μl of 2M $H_2SO_4$ after 1 hour incubation in the dark at room temperature. The absorbance at $A_{450}$ was read using a microtitre reader, Dynex MRX. An absorbance reading three times higher than the mean negative reading was taken as positive.

*Assay for IL-10*

Interleukin-10 present in the sera samples was determined using a commercial solid phase sandwich ELISA kit based on a monoclonal antibody specific for mIL-10 (BioSource International, Inc.).

*Detection of T. gondii tissue cysts*

Two mice from each group were sacrificed at weekly intervals. Tissue imprints including blood, peritoneal exudates, liver, spleen, and brain were applied onto microscope glass slides. The tissue imprints were dried, fixed with methanol and stained with Giemsa. The slides were then washed with tap water and left to dry. The presence of *Toxoplasma* cysts or tachyzoites was examined for under light microscopy.

**Results**

*Preparation of positive control sera*

Mice immunized with antigen prepared from *T. gondii* tachyzoites had IgM antibody levels twice as high as that in control animals at 2 weeks post-immunization and this was sufficient for use in the ELISA as positive control. IgA antibody titres were not significantly different from those of control animals. IgG positive control was obtained from mice that were immunized four weeks previously.

*Antibody response in mice chronically infected with 1,000 cysts of T. gondii*

Balb/c mice infected with 1,000 cysts of the *T. gondii* cyst strain showed an increasing IgM titre early after infection. The IgM titre peaked at the first week post-infection and declined during the following weeks. Sera from animals of the control group gave consistently low readings in the IgM-ELISA. IgG titre increased more gradually than the IgM. Serum IgG levels increased slowly to peak at the fourth week before declining at the fifth week (Table 1).

*IL-10 levels in infected mice*

After oral infection with 1,000 cysts of *T. gondii* IL-10 levels increased dramatically at the first two weeks and reached a peak at the fourth week, before declining subsequently (Table 1). The highest IL-10 concentration in the serum was 304.49 pg/ml. In the control group consistently low levels of between 5.05 to 7.10 pg/ml were observed.

In mice infected with 10,000 cysts, the IL-10 level was 397.25 pg/ml at the first week but the concentration declined slowly subsequently (Table 2). IL-10 production did not follow the trend for IgG or IgM.

**Discussion**

*Toxoplasma* tachyzoite antigens were used in this study to generate positive control antibodies for the ELISA assay as these could induce greater immune response much more rapidly compared to cyst antigens as shown in previous studies.

IgM response seen in mice infected either with 1,000 or 10,000 cysts may play an important role in parasite elimination at the early stage of infection. Since IgM activates the complement system leading to opsonization and phagocytosis of the parasite, it may play an important role in the elimination of extracellular parasites that are released into the blood stream during the haematogenous spread of the parasite.

The switching of antibody class from IgM to IgG during infection is considered to be important since the parasite invades host cells, and IgG promotes phagocytosis and block parasite entry into host cells. However, the humoral immune response may not be sufficient to eliminate intracellular *T. gondii*.

The significant increase in IL-10 levels in response to the infection indicates that there is immunological regulation by cytokines in mice infected with the cyst strain of *T. gondii*. IL-10 was initially characterized based on its production by the $T_h2$ subset of CD4+ T cell. It may also be produced by other T cell subsets as well as by B cells and macrophages and exert a number of immunosuppressive effects. IL-10 inhibits the ability of IFN-γ to activate macrophages which prevent multiplication of intracellular *T. gondii*. In this study IL-10 and IgG levels showed a similar pattern of increase from week 2 to reach the peak at week 4 post-infection in mice infected with 1,000 cysts. This was then followed by a similar pattern of decline after week 4 post-infection. In mice infected with

**Table 1. Mean ELISA IgM and IgG anti-*Toxoplasma gondii* antibody levels* and IL-10 levels in control and experimental mice infected orally with 1,000 cysts of *T. gondii***

| Weeks post-immunisation | Mean IgM OD reading | | Mean IgG OD reading | | Mean IL-10 (pg/ml) | |
|---|---|---|---|---|---|---|
| | Control | Immunised | Control | Immunised | Control | Immunised |
| 0 | 0.170 | 0.171 | 0.212 | 0.209 | 5.05 | 7.09 |
| 1 | 0.163 | 0.330 | 0.212 | 0.263 | 6.08 | 121.07 |
| 2 | 0.171 | 0.297 | 0.215 | 0.258 | 7.04 | 210.71 |
| 3 | 0.168 | 0.249 | 0.208 | 0.847 | 5.08 | 225.99 |
| 4 | 0.169 | 0.187 | 0.212 | 1.042 | 7.10 | 304.49 |
| 5 | 0.168 | 0.154 | 0.208 | 0.372 | 7.09 | 95.29 |

*Enzyme-linked immunosorbent assay optical density (OD) absorbance readings at 450 nm

**Table 2. Mean ELISA IgM and IgG anti-*Toxoplasma gondii* antibody levels* and IL-10 levels in control and experimental mice infected orally with 10,000 cysts of *T. gondii***

| Weeks post-immunisation | Mean IgM OD reading | | Mean IgG OD reading | | Mean IL-10 (pg/ml) | |
|---|---|---|---|---|---|---|
| | Control | Infected | Control | Infected | Control | Infected |
| 0 | 0.170 | 0.171 | 0.212 | 0.210 | 7.08 | 7.11 |
| 1 | 0.163 | 0.195 | 0.212 | 0.246 | 7.10 | 397.25 |
| 2 | 0.171 | 0.291 | 0.215 | 0.408 | 7.15 | 248.85 |
| 3 | 0.168 | 0.434 | 0.208 | 0.452 | 7.01 | 141.64 |

*Enzyme-linked immunosorbent assay optical density (OD) absorbance readings at 450 nm

10,000 cysts there was a more intense and greater production of both IL-10 and IgG antibodies. Peak levels of IL-10 for mice infected with 1,000 cyst was 304.49 pg/ml achieved at week 4 post-infection compared to 397.25 pg/ml achieved at week 1 post-infection in mice infected with 10,000 cysts. However, in the mice infected with the higher infective dose the IgG and IgM levels continued to increase even when the IL-10 levels started to decline after the peak level at week 1 post-infection. At the lower infective dose, Pearson correlation analysis indicates there was a strong positive relationship between IgG and IL-10 ($r = 0.704$; $P = 0.011$) but poor relation between IgM and IL-10 ($r = 0.203$; $P = 0.528$). It has been shown that IL-10 inhibits, but tumour necrosis alpha (TNF-α) and IL-12 enhanced IFN-γ production (Hunter *et al*., 1994). IFN-γ is known to protect against *T. gondii* infection in mice. IL-10 will therefore indirectly promote the survival of *T. gondii* through its inhibition of IFN-γ production. As IgG also declined in tandem with IL-10, even in the presence of continued infection with the parasite, it can be assumed that the infection has reached equilibrium with the host-defence mechanisms.

In the case of high dose parasite infection (mice infected with 10,000 cysts), the mortality rate was high; 50% of the mice died at the second week post-infection and 80% in the weeks thereafter. The humoral response differed from that in mice infected with the lower dose (1,000 cysts). There was a greater humoral immune response with a higher production of both IgM and IgG antibodies. IgM and IgG increased throughout the post-infection period until the mice died by week 3 post-infection. This may be due to persistently high parasite load present in the infected animals. At autopsy on the first week post-infection the spleen was enlarged. Large amounts of tissue cysts were detected in spleen based on impression smears made at the first week.

In heavy infections with 10,000 cysts the IL-10 induced did not follow the trend of IgG and IgM production. While the IL-10 levels declined from the peak at week 1 post-infection, both IgG and IgM levels continued to increase until death of the animals. It is possible that the initial induction of a high level of IL-10 permitted the uptake of the infection and even though high levels of IgG and IgM were induced, these could

not prevent the fatal outcome in response to a heavy parasite load.

Increased IL-10 was detected in mice acutely or chronically infected with *T. gondii*. This suggests that the survival of *T. gondii* may provoke the production of IL-10 within the infected host. Although the antibodies helped to eliminate some of the parasite during early infection, a depressed cell-mediated immunity is caused by increased production of IL-10 contributing to prolonged infection. Therefore, the aim of any form of immunological modulation or immunotherapy of this disease must be to boost both the humoral and the cell-mediated immune response in an attempt to achieve stronger anti-*Toxoplasma* activity.

The study on antibody levels against the cyst stage of *T. gondii* should be carried out so that broader understanding of such responses can be obtained. Anti IL-10 antibody treatment can be explored experimentally in mice.

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