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Infection of Murine Macrophages with *Toxoplasma gondii* Is Associated with Release of Transforming Growth Factor β and Downregulation of Expression of Tumor Necrosis Factor Receptors

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***Toxoplasma gondii* is capable of invading and multiplying within murine peritoneal macrophages. Previous studies have shown that treatment of macrophage monolayers with recombinant gamma interferon but not tumor necrosis factor (TNF) is associated with intracellular killing of *T. gondii* by macrophages. Furthermore, infection of macrophages with *T. gondii* prevents their stimulation for mycobactericidal activity by TNF. Since transforming growth factor β (TGF-β) is known to suppress a number of functions in macrophages, we investigated the influence of infection with *T. gondii* on macrophage TNF receptors and on production of TGF-β. Infection with *T. gondii* was associated with increased production of TGF-β and downregulation of TNF receptors. This effect was observed early after infection and was partially inhibited by anti-TGF-β_1 antibody.**

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of invading all cells in the human body. It multiplies within a vacuole in the cytoplasm of its host cell (1, 20). Although toxoplasmosis is in general clinically asymptomatic in healthy individuals, it may cause severe complications in the offspring of acutely infected pregnant women and in immunocompromised patients. *T. gondii* is recognized as a major opportunistic pathogen and the most common cause of focal intracerebral lesions in patients with AIDS (12). In this group of individuals, disruption of cysts that persist in multiple tissues after primary infection, with release of proliferative forms of the parasite, causes severe disseminated toxoplasmosis and encephalitis (10, 21).

The importance of cytokines in inducing anti-toxoplasma activity in macrophages is well established (7, 15, 18). A number of studies have demonstrated the role of gamma interferon (IFN-γ) in inducing intracellular killing of toxoplasmas in macrophages (15, 18, 23). Alternatively, previous studies showed that treatment of toxoplasma-infected macrophage monolayers with other cytokines, such as recombinant tumor necrosis factor alpha (TNF), had no effect on the intracellular survival of the parasite (9). Coinfection of macrophages with *T. gondii* and *Mycobacterium avium* prevented the TNF-mediated mycobacteriostatic and mycobactericidal activity (6) observed in macrophages infected only with *M. avium*, suggesting that the presence of intracellular *T. gondii* may influence TNF mRNA by interfering with the signal transduction. To examine this question, we sought to determine the effect of *T. gondii* infection of macrophages on TNF receptors.

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MATERIALS AND METHODS

Organism. *T. gondii* RH was maintained by serial passage in the peritoneal cavity of Swiss Webster mice. Tachyzoites were collected from the peritoneal cavity of infected mice as previously described (7).

Murine macrophages. Murine macrophages were obtained as previously described (7, 15). Briefly, peritoneal macrophages were harvested from 6-week-old female Swiss Webster mice (Simonson, Gilroy, Calif.) by injecting 5 ml of saline into the abdominal cavity and removing the cell suspension after 5 min. Adherent cells were cultivated in plastic tissue culture plates (Flow Laboratories, Inc., McLean, Va.) or on 12-mm glass coverslips in RPMI 1640 (GIBCO) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), penicillin (100 U/ml), and streptomycin (100 μg/ml) (standard medium). The preparation was more than 97% pure as assessed by the ability to absorb neutral red.

Infection with *T. gondii*. Macrophage monolayers were washed after 2 h of adherence and then incubated for 24 h in test media. Monolayers containing approximately 5×10^5 macrophages were challenged for 60 min with 5×10^6 *T. gondii* RH trophozoites. Uningested organisms were removed by washing, and monolayers were incubated in standard medium. Infected macrophage monolayers were monitored for differential detachment of cells. Depending on the experiment and the course of infection, monolayers treated with anti-transforming growth factor β_1 (TGF-β_1) antibody were assessed at 2, 6, and 20 h of infection by counting the number of tachyzoites per 100 macrophages in Giemsa-stained preparations.

Expression of TNF receptors on macrophages. Expression of TNF receptors on macrophages was determined as previously described (4). Briefly, murine TNF was iodinated

with Na^{125}I by the chloramine-T method (ICN, Costa Mesa, Calif.) to yield a specific activity of approximately 40 Ci/ml. The protein concentration of the preparation, estimated by trichloroacetic acid precipitation, exhibited full biological activity as evidenced by the L929 (TNF-sensitive cells) assay as previously described (5).

The binding assay was performed as follows. Adherent macrophages were infected with *T. gondii* for 1 h, and the extracellular organisms were removed by washing. Expression of TNF receptors on macrophages was determined by adding ^{125}I-TNF (10 pmol) at 1, 2, 4, 6 and 20 h after infection, in monolayers containing approximately 10^5 cells.

Control experiments for nonspecific binding of the ligand were performed in wells in the presence of a 200-fold excess of unlabeled TNF. Equilibrium was achieved at 4°C, and the specific binding peaked in 3 h and declined thereafter. Measurements were performed in duplicate, and the background was always <20% of the total.

Concentration of TNF and TGF-β_1. The murine TNF concentration in the culture supernatant was measured by an enzyme-linked immunosorbent assay (Genzyme, Cambridge, Mass.). The MVILu mink lung cell line (American Type Culture Collection, Rockville, Md.) was used to measure TGF-β activity as previously described (22). Supernatants were assayed either untreated or after treatment with 0.12 N HCl for 15 min at room temperature followed by neutralization with 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 0.144 M NaOH. Samples were added to approximately 5 × 10^3 MVILu cells per well in 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass.). The plates were incubated at 37°C with 5% CO_2 for 24 h and pulsed during the last 6 h with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; ICN). Cells were harvested with a cell harvester, and [^{3}H]thymidine incorporation was compared with incorporation in cells treated with known concentrations of recombinant TGF-β_1. The lower detection limit of the bioassay was 10 pg of TGF-β_1 per ml. Experiments were run in parallel with polymyxin B to neutralize the effect of endotoxin. No difference was seen in TGF-β_1 production between polymyxin B-treated and experimental groups (data not shown).

The specificity of the assay was demonstrated by inhibiting activity with chicken anti-TGF-β_1 antibody (R and D System, Minneapolis, Minn.).

Effect of anti-TGF-β_1 antibodies on the expression of TNF receptors. To determine the influence of secreted TGF-β_1 on the expression of TNF receptors by infected macrophages, a chicken anti-TGF-β_1 antibody (0.25 μg of the antibody per μl neutralizes 2 ng of TGF-β_1) was added to monolayers before they were infected with *T. gondii*, at a concentration sufficient to neutralize 10^4 U/ml of TGF-β_1. The concentration of antibody was maintained constant during all the experiments.

Statistics. Each experiment was repeated at least twice. The values were obtained on duplicate wells, and means ± standard deviations were calculated. The significance of the experimental results versus controls at identical time points was tested by Student's *t* test.

RESULTS

Effect of *T. gondii* infection on the binding of TNF to murine macrophages. To examine whether the unresponsiveness observed might be related to the regulation of expression of TNF receptors on infected macrophages, we infected murine macrophages with *T. gondii* and determined ^{125}I-TNF binding at various times thereafter.

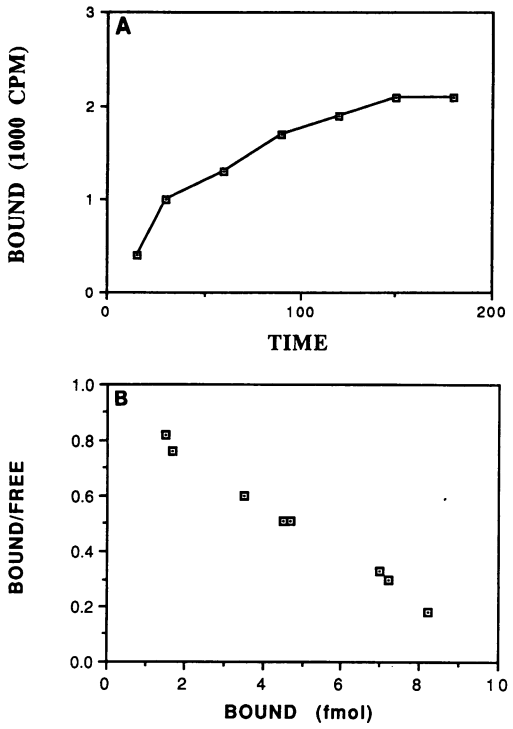

FIG. 1. (A) Kinetics of binding of TNF to murine peritoneal macrophages. (B) Scatchard plot analysis of the binding of TNF to receptors on mouse peritoneal macrophages. Cells were incubated with increasing concentrations of ^{125}I-TNF at 4°C for 1 h.

The specific binding of ^{125}I-TNF and Scatchard analysis are shown in Fig. 1. The results indicate that macrophages had a K_d of 1.3 × 10^{-10} M and an average of 22,300 ± 1,600 receptors per cell. As shown in Fig. 2, infection of macrophages with *T. gondii* was associated initially with upregulation in the expression of TNF receptors but macrophages at 2 h following infection have a significant downregulation in the expression of TNF receptors. At 6 h after infection,

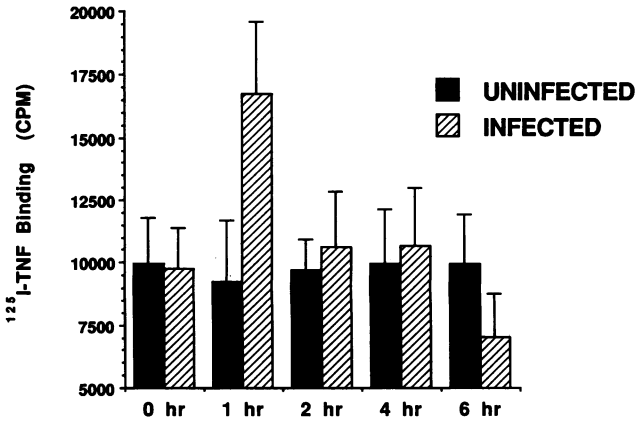

FIG. 2. Binding of ^{125}I-TNF to murine peritoneal macrophages infected with *T. gondii*. Binding was determined at different time points to compare the effects of infection with *T. gondii* on the expression of TNF receptors on macrophages.

TABLE 1. Effect of supernatant from monolayers containing *T. gondii*-infected macrophages on the binding of ^{125}I-TNF to uninfected macrophages

Incubation period (h) of macrophages and supernatant	Length of infection (h) of monolayers	^{125}I-TNF binding[a] (cpm)	% Change
1	0[b]	12,580 ± 3,642	
	1	13,808 ± 2,716	0
	2	14,388 ± 3,431	0
	6	13,441 ± 3,549	0
2	0	12,552 ± 3,217	
	1	6,280 ± 2,976	−49.9[c]
	2	6,615 ± 3,109	−47.3[c]
	6	7,405 ± 2,810	−41.0[c]
6	0	13,470 ± 3,543	
	1	6,268 ± 1,247	−53.5[c]
	2	6,565 ± 1,537	−51.3[c]
	6	6,128 ± 1,534	−54.5[c]

[a] Binding at control time zero, 11,951 ± 2,629.
[b] Control uninfected.
[c] $P < 0.05$.

macrophages expressed significantly fewer receptors for TNF than did control macrophages and infected macrophages at 1 h after infection ($P < 0.05$).

Effect of supernatant on TNF binding. Since not all macrophages in the monolayers were infected with *T. gondii* (we determined that in these experiments approximately 50 to 60% of the cells were infected), it seemed plausible to hypothesize that the observed downregulation of expression of TNF receptors would be more significant if only infected cells were considered, unless the downregulation in the number of receptors was secondary to the secretion by infected cells of suppressor molecules that would then affect all macrophages in the monolayers. To examine this question, we carried out experiments with uninfected macrophages exposed to supernatants obtained from infected macrophage monolayers at 1, 2, and 6 h after infection. Culture supernatants were obtained, filtered through a filter (pore size, 0.22 μm; Millipore, Bedford, Mass.), and then incubated with uninfected macrophages for a time. Then supernatant was removed, and the expression of TNF receptors was determined. As shown in Table 1, incubation of uninfected macrophages with such supernatants was associated with significant downregulation of the expression of TNF receptors. However, a significant decrease in the number of receptors was observed only following incubation for between 2 and 6 h. Of note, the decrease in ^{125}I-TNF binding to monolayers exposed to supernatants was comparable to the decrease in binding to monolayers infected with *T. gondii*.

Therefore, factors released by infected macrophages were apparently responsible for the downregulation of TNF receptors on infected as well as uninfected cells in the monolayer.

Control supernatants, obtained from uninfected macrophage monolayers at the same time points, had no effect on the binding of ^{125}I-TNF to mouse peritoneal macrophages (data not shown).

Production of TNF and TGF-β_1 following infection with *T. gondii*. Previous studies in other laboratories have revealed that murine macrophages infected with *Trypanozama cruzi* or *Leishmania braziliensis* and *L. amazonensis* produce high concentrations of TGF-β (2, 22). Since TGF-β has been shown to suppress the macrophage response to cytokines such as TNF and IFN-γ (8, 24), we examined whether production of TGF-β_1 increased in macrophages infected with *T. gondii* and also measured TNF production by the same macrophages.

As shown in Table 2, macrophages infected with *T. gondii* produce high concentrations of TGF-β_1. Interestingly, most of the released TGF-β_1 was found in a biologically active form (Table 2). In addition, TGF-β_1 was detected in the supernatants of the monolayers as early as 2 h following infection.

Infected macrophages were also shown to produce TNF. However, release of TNF was greater after 2 h of infection and decreased subsequently.

Effect of anti-TGF-β_1 antibody on *T. gondii*-mediated downregulation of TNF receptors. To determine whether release of TGF-β_1 *T. gondii*-infected macrophages was related to the downregulation of TNF receptors, we carried out experiments with anti-TGF-β_1 antibody. Anti-TGF-β_1 antibody was added to macrophage monolayers at time zero (immediately before addition of toxoplasmas to monolayers), and expression of TNF receptors was determined 2, 6, and 20 h after infection with *T. gondii*.

The results, shown in Fig. 3, demonstrate that the presence of anti-TGF-β_1 antibody had a significant effect on the expression of TNF receptors by infected macrophages. Anti-TGF-β_1 antibody partially inhibited the *T. gondii*-mediated suppression of TNF binding, with a more significant effect observed at 20 h after infection.

Anti-TGF-β_1 had no significant effect on tachyzoite proliferation during the 6 h of the assay.

DISCUSSION

T. gondii is an intracellular parasite, capable of invading and surviving in a number of mammalian cells (19, 20). Previous studies have demonstrated that human peritoneal

TABLE 2. Production of TNF and TGF-β by mouse peritoneal macrophages infected with *T. gondii*

Source of supernatant	Concn[a] of:		
	TNF	TGF-β	TGF-β plus acid treatment[b]
Control (uninfected macrophages)	35 pg/ml	ND	61 pg/ml
2 h postinfection	247 ± 149 pg/ml	7.1 ± 0.4 pg/ml	8.9 ± 1.7 ng/ml
6 h postinfection	163 ± 99 pg/ml	9.3 ± 0.7 ng/ml	11.2 ± 3.0 ng/ml
20 h postinfection	ND	7.4 ± 1.2 ng/ml	10.2 ± 1.7 ng/ml

[a] Limits of the assays: TNF, 30 pg; TGF-β, 10 pg.
[b] Acid treatment activates all TGF-β present in the culture supernatant. ND, not detected.

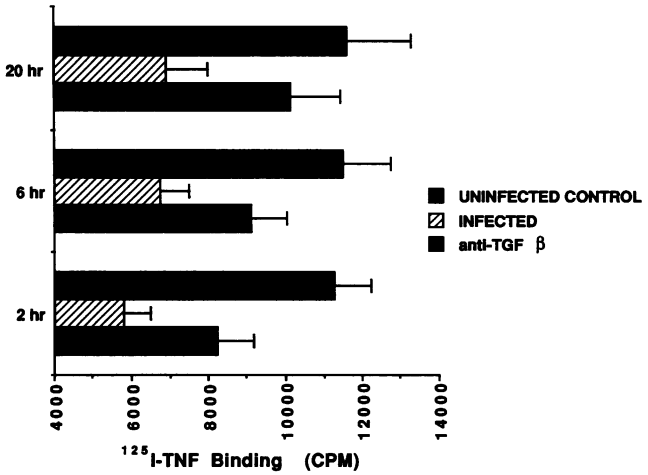

FIG. 3. Effect of anti-TGF-β_1 antibody on ^{125}I-TNF binding to macrophages infected with *T. gondii*. Anti-TGF-β_1 was added immediately before infection, and the concentration was maintained constant during the entire experiment. The effect was compared with that of uninfected controls and infected monolayers not treated with anti-TGF-β_1 antibody.

macrophages, while allowing infection by *T. gondii*, consistently demonstrated toxoplasmacidal activity (11). In contrast, mouse macrophages are unable to kill *T. gondii* and require activation with cytokines to do so (6, 8). However, a study by Murray et al. (16) showed that macrophages obtained from patients with AIDS fail to inhibit or kill intracellular *T. gondii* but can be stimulated to do so in vitro when treated with recombinant IFN-γ.

Results of previous studies have indicated that IFN-γ is a critical cytokine for induction of toxoplasmacidal activity in macrophages, although it has been suggested that costimulation with TNF is required to obtain maximal IFN-γ effect (14). Despite this, TNF by itself does not have the ability to activate murine macrophages in vitro to kill *T. gondii* (9), and, at least in one system, *M. avium* infection together with *T. gondii* causes macrophages to become unresponsive to subsequent stimulation with TNF (6). The reason for the *T. gondii*-mediated effect on infection on macrophage function is unknown, but recent evidence accumulated in studies with other intracellular pathogens suggests that release of suppressive molecules such as TGF-β_1 by infected macrophages can interfere with the host immune response and facilitate the intracellular survival of the pathogenic organism (2, 3, 22).

Our results reveal that infection of murine peritoneal macrophages is associated with downregulation of the expression of TNF receptors. This downregulation was observed early following infection and was dependent on the secretion of inhibitory factors by infected macrophages. Infection of peritoneal macrophages with *T. gondii* induced the production of high concentrations of TGF-β, and the use of anti-TGF-β_1 antibody was associated with a significant decrease of *T. gondii*-mediated downregulation of the expression of TNF receptors on macrophages. Although uninfected macrophages also produced TGF-β_1, this cytokine was released in an inactive form, in contrast with the situation in *T. gondii*-infected cells.

The basis for the survival of *T. gondii* within macrophages is poorly understood. Studies of the interaction of *T. gondii* with cultured murine and human macrophages indicate that the organism is capable of interfering with a number of microbial mechanisms in the cell. For example, virulent strains of *T. gondii* suppress the release of the oxidative burst by macrophages (25), and, once inside the vacuole, they inhibit phagosome-lysosome fusion (13). Our present findings that infection of macrophages with *T. gondii* induces production and release of TGF-β_1 add to the previously known mechanisms associated with intracellular survival of the parasite. Production of TGF-β_1, mostly in an active form, can explain at least in part the inability of recombinant TNF to activate macrophages to kill intracellular *T. gondii*. In addition, the observed suppression of TNF receptors following infection prevents a possible autocrine effect of TNF on infected cells. In fact, similar observations obtained with in vitro and in vivo models of *Leishmania* (2) and *Trypanozoma cruzi* (22) infections are consistent with the hypothesis that intracellular pathogens are capable of inducing the production of suppressive molecules such as TGF-β and interleukin-10, which would impair the microbicidal activity of macrophages. Our findings are also consistent with previously published results by us and others (15, 17) that the ability of recombinant IFN-γ to activate macrophages to kill *T. gondii* is significantly greater if monolayers are treated with IFN-γ before rather than after infection, indicating that infection with *T. gondii* is associated with macrophage unresponsiveness to subsequent stimulation. Although previous studies have demonstrated that TGF-β inhibits the macrophage response to IFN-γ and TNF, results of our experiments involving anti-TGF-β_1 antibody suggest the presence of other inhibitory mechanisms.

It is interesting to speculate that the ability to trigger the production of suppressive molecules (such as TGF-β) in macrophages may vary with the strain of *T. gondii* and with the macrophage population and perhaps can explain differences in susceptibility to toxoplasma infection between mouse and human macrophages (11).

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