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# Production of Gamma Interferon by Natural Killer Cells from *Toxoplasma gondii*-Infected SCID Mice: Regulation by Interleukin-10, Interleukin-12, and Tumor Necrosis Factor Alpha

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**Previous studies of mice have implicated natural killer (NK) cells as mediators of protective activity against *Toxoplasma gondii* through their production of gamma interferon (IFN-γ). In the present study, we have compared NK-cell activity in infected and uninfected SCID mice. Our data reveal that infection results in increased levels of IFN-γ in serum and elevated NK-cell activity but that these NK cells were not cytotoxic for *T. gondii*-infected P815 cells. Treatment with anti-IFN-γ antibody abrogated the increase in NK-cell activity and resulted in earlier mortality of infected mice. In vivo treatment with anti-asialo GM1 antiserum reduced NK cell activity and levels of IFN-γ in serum but did not alter time to death. Spleen cells from infected mice produced higher levels of IFN-γ than those from uninfected mice when stimulated in vitro with live *T. gondii* or parasite antigen preparations. Further analysis revealed that interleukin 10 (IL-10) inhibited, whereas tumor necrosis factor alpha (TNF-α) and IL-12 enhanced, IFN-γ production by spleen cells from infected or uninfected mice. The combination of IL-12 and TNF-α induced higher levels of IFN-γ from whole spleen cells of infected mice than from those of uninfected mice. Depletion of the adherent cell population from the spleen cells of infected mice led to a significant reduction in the levels of IFN-γ produced after stimulation with IL-12 plus TNF-α. Similar results did not occur with cells from uninfected mice. These data indicate that other cytokines produced by the adherent cell population from infected mice may be involved in maximal production of IFN-γ by NK cells stimulated with IL-12 and TNF-α. To assess the importance of endogenous IL-12, a polyclonal anti-IL-12 was administered to infected SCID mice. This treatment led to earlier mortality, indicating that endogenous IL-12 mediates resistance to *T. gondii*.**

*Toxoplasma gondii* is an intracellular parasite that normally stimulates a strong and lasting protective immune response but results in a latent infection in multiple tissues and organs (41). 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 (9, 25, 38). The importance of this infection has grown in recent years with the advent of AIDS. It appears that as the immune status of a human immunodeficiency virus-infected individual deteriorates, the infection is able to reactivate and results in toxoplasmic encephalitis and disseminated toxoplasmosis (21).

Mice with severe combined immune deficiency (SCID), when infected with *T. gondii*, develop a progressive disease with necrotic lesions in their central nervous system and other organs and die approximately 20 days postinfection (19, 22, 29). We have recently demonstrated the presence of cytokine transcripts for gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 10 (IL-10) in the central nervous system of SCID mice infected with *T. gondii* (19). Schluter et al. (29) demonstrated elevated levels of IFN-γ and TNF-α protein in the serum and cerebrospinal fluid of SCID mice infected with *T. gondii*. On the basis of these data, we employed cytokine-neutralizing antibodies to investigate the role of these cytokines in SCID mice (19). These studies revealed that endogenous TNF-α and IFN-γ are involved in a T-cell-independent mechanism(s) of resistance to *T. gondii* whereas endogenous IL-10 inhibited the resistance of SCID mice against *T. gondii*. Since these mice lack functional $CD4^+$ and $CD8^+$ T cells, the local production of IFN-γ transcripts and the finding of an IFN-γ-dependent mechanism of resistance suggest a protective role for natural killer (NK) cells in these mice.

An IFN-γ-dependent mechanism of resistance in SCID mice infected with *Listeria monocytogenes* has previously been described (2). Treatment of these mice with an IFN-γ-neutralizing monoclonal antibody or depletion of NK cells with anti-asialo GM1 antisera resulted in earlier mortality and heavier bacterial burdens (1). NK cells from uninfected SCID mice were capable of producing IFN-γ upon stimulation with either bacterial lysate or live bacteria (40), and this activity was inhibited by IL-10 and enhanced by TNF-α (2). Recent work by Sher et al. (32) has demonstrated similar findings for NK cells derived from uninfected SCID mice and stimulated with *T. gondii*. This group also identified the importance of macrophage production of IL-12 for induction of IFN-γ synthesis in vitro by NK cells derived from uninfected mice and stimulated with *T. gondii* and found that the protective effect of exogenous IL-12 in SCID mice infected with *T. gondii* was dependent on IFN-γ and NK cells (10).

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Hauser et al. (12) have reported increased NK cell activity in normal mice infected with *T. gondii* and found that these cells were cytotoxic for extracellular tachyzoites (14). On the basis of these results, these investigators hypothesized a role for NK cells in resistance to the acute phase of infection. More recently, Denkers et al. (6) found that, in the absence of $CD8^+$ T cells, increased NK cell numbers mediate protection against *T. gondii* in β2-microglobulin-deficient mice via production of IFN-γ. Cytotoxicity has been proposed as an effector mechanism of protective immunity, and $CD8^+$ T cells as well as lymphokine-activated killer cells have been shown to have the capacity to lyse a variety of *T. gondii*-infected cells (11, 35, 36). Whether NK cells are able to act as cytotoxic effector cells against *T. gondii*-infected cells is unknown, although they have been demonstrated to acquire cytotoxicity for *T. gondii*-infected cells after stimulation with IL-2 (35).

The aim of our study was to gain a better understanding of the T-cell-independent mechanisms of resistance against *T. gondii* in SCID mice by comparing NK cell activities of infected and uninfected mice. In addition, we have analyzed the roles of IL-10, TNF-α, and IL-12 in production of IFN-γ by NK cells from infected and uninfected mice and assessed the importance of endogenous IL-12 in mediating resistance against *T. gondii* in vivo.

## MATERIALS AND METHODS

**Mice.** Female CB-17S *scid/scid* (SCID) mice obtained from the Department of Comparative Medicine, Stanford University Medical Center (Stanford, Calif.) were 6 to 10 weeks of age when used for experiments and were maintained in filter-top cages within laminar-flow hoods.

***T. gondii.*** Mice were infected orally with 20 cysts of the ME49 strain as previously described (20). For in vitro studies, RH strain tachyzoites were purified as previously described (30) and used for the preparation of toxoplasma lysate antigen (TLA) (3). Live parasites, or parasites incubated at 56°C for 30 min (heat killed), or TLA was used to provide direct stimulation of spleen cells in vitro.

**Histology.** Tissues were removed from animals that had died as a consequence of *T. gondii* infection, fixed overnight in 4% formaldehyde–70% ethanol–0.8 N acetic acid, and embedded in paraffin for histopathological analysis as previously described (19). Hematoxylin and eosin stain of sections and immunohistochemistry of *T. gondii* were used to assess pathological changes in the spleen, lungs, heart, brain, liver, and kidneys as previously described (19).

**Antibodies.** Rat anti-mouse IFN-γ-neutralizing monoclonal antibody (XMG 1.2) (5) was used to deplete IFN-γ in SCID mice. Doses of 2 mg per mouse in a volume of 1 ml were administered intraperitoneally 24 h preinfection and on days 4 and 9 postinfection. For depletion of NK cells in vivo, 50 μl of a rabbit anti-asialo GM1 antiserum (WAKO, Richmond, Va.) was administered intraperitoneally 4 days and 1 day prior to infection and thereafter every 3 days in a volume of 0.2 ml for the duration of the experiment. The dose recommended by the manufacturer was a minimum of 20 μl every 5 days. A rat isotope control (anti-β-galactosidase) or a rabbit serum which had been previously demonstrated to be negative for toxoplasma antibodies by protein blot was used for controls. Neutralizing rat monoclonal antibodies to murine IL-10 (JES5-2A5) (28) and TNF-α (MP6-XT22) (34) were used for the in vitro neutralization of these cytokines in the stimulation assays at a concentration of approximately 120 μg/ml. Antibodies were diluted in sterile, endotoxin-free saline (Abbott Laboratories, Chicago, Ill.). All monoclonal antibodies employed in this study were purified by fast protein liquid chromatography (FPLC)–anion-exchange chromotography under low endotoxin conditions and were provided by John Abrams of DNAX (Palo Alto, Calif.). Polyclonal sheep anti-murine IL-12 was supplied by the Immunology Department of the Genetics Institute, Inc. (Cambridge, Mass.) and was administered intraperitoneally at a dose of 200 μg per mouse 24 h prior to infection and every 2 days thereafter. Neutralizing antibody was produced by immunizing sheep (S7) with 100 μg of native mouse IL-12 on alternating weeks. The initial injection was administered subcutaneously at multiple sites in complete Freund's adjuvant; subsequent injections were administered subcutaneously with incomplete Freund's adjuvant. Test bleeds were taken prior to injection starting at week 4 and then titers were determined against mouse IL-12-coated plates. Bound antibody was detected with a rabbit anti-goat immunoglobulin (Ig)-horseradish peroxidase conjugate (Zymed, San Francisco, Calif.), and color was developed with *o*-phenylenediamine substrate at a wavelength of 490 nm. Immunoglobulin was then purified from pooled antisera by binding to protein G (Pierce, Rockford, Ill.) with an FPLC system (Pharmacia Fine Chemicals, Piscataway, N.J.). The polyclonal antibody against IL-12 used in these studies did not react with IFN-γ by protein blot, alter reactivity of IFN-γ or IL-1β in an enzyme-linked immunosorbent assay (ELISA), or inhibit the ability of the combination of IFN-γ and TNF-α to activate macrophages to inhibit replication of *T. gondii* (data not shown). Sheep IgG (Sigma) was used as a control for in vivo and in vitro experiments.

**Cytokines.** Recombinant mouse IL-10 ($10^7$ U/mg) was provided by Satish Menon and Kevin Moore of the Department of Immunology, DNAX. Recombinant murine IFN-γ ($5.2 \times 10^6$ U/mg) and TNF-α ($1.2 \times 10^7$ U/mg) were supplied by R. Shephard, Genentech (South San Francisco, Calif.). Recombinant murine IL-12 was supplied by S. Wolf of the Genetics Institute.

**Target cells.** YAC-1 and P815 cells were obtained from the American Type Culture Collection (Rockville, Md.). To investigate whether NK cells could lyse *T. gondii*-infected cells, we chose the P815 cell line as the target. We routinely use infected P815 cells as targets for *T. gondii*-specific $CD8^+$ T cells (36) and uninfected P815 cells are frequently used to assay the lytic activity of NK cells activated with IL-2. P815 cells were infected with tachyzoites of *T. gondii* as previously described (36). Purified tachyzoites of the RH strain were irradiated with 1,300 ergs of UV light and used to infect target cells at a ratio of 12 parasites to 1 target cell overnight at 37°C. Extracellular parasites were removed by centrifugation with Lympholyte-M (Cedarlane, Hornby, Ontario, Canada), and the percentage of cells infected was estimated by use of cytocentrifuge preparations.

**Cytotoxicity assays.** The cytotoxicity assay was carried out as previously described (35). Briefly, $2 \times 10^6$ to $3 \times 10^6$ target cells were labeled with 100 μCi of $Na^{51}CrO_4$ by incubation for 1 h at 37°C. They were then washed three times in complete RPMI (10% heat-inactivated fetal calf serum [Sigma], 1,000 U of penicillin per ml, 10 μg of streptomycin per ml, 0.25 μg of amphotericin B [Fungizone; BioWhittaker, Walkersville, Mass.] per ml) and resuspended at a cell density of $5 \times 10^4$ $ml^{-1}$ in complete medium, and 100 μl was plated into individual wells of 96-well, round-bottomed plates (Costar, Cambridge, Mass.). Effector cell populations were obtained from the spleens of groups of three infected or uninfected SCID mice. Effector cells were added at appropriate concentrations, and the plates were centrifuged at $50 \times g$ for 5 min and incubated for 6 h at 37°C. Thereafter, plates were centrifuged

at 200 × *g* for 5 min, 100 μl of supernatant was harvested from each well, and γ emissions were counted with a Gamma 5500 B counter (Beckman Instruments, Palo Alto, Calif.). The percentage of specific $^{51}Cr$ release was calculated with the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Experimental release was obtained from wells that contained target and effector cells, spontaneous release was obtained from wells which lacked effector cells, and maximum release was obtained by lysis of target cells with 100 μl of 5% Triton X-100. Results are expressed as the means of triplicate wells.

**In vitro stimulation of spleen cells.** Single-cell suspensions of spleen cells were prepared as previously described (3). Spleens were dissociated in complete RPMI to give a single-cell suspension. The cells were washed twice by centrifugation and resuspended in complete RPMI. Erythrocytes were lysed with ammonium chloride. After two further washes, cells were counted and plated in 96-well plates at a cell density of $3 \times 10^5$ to $4 \times 10^5$ cells per well in a final volume of 200 μl. Depletion of NK cells was carried out by incubating spleen cells with anti-asialo GM1 antiserum at a dilution of 1:10 in cytotoxicity medium (Cedarlane) for 1 h at 4°C followed by a 1:8 dilution of rabbit complement (Cedarlane) at 37°C for 1 h. This treatment resulted in a 90 to 95% reduction in asialo GM1-positive cells as revealed by fluorescence-activated cell sorter (FACS) analysis. Adherent cells were removed to enrich for NK cells by incubating the whole spleen cell populations in tissue culture flasks (Nunc, Naperville, Ill.) at 37°C for 3 h. Cells were incubated with cytokine-neutralizing antibodies for 1 to 2 h prior to the addition of live parasites or TLA.

**ELISA for IFN-γ.** A two-site ELISA was employed to assay levels of IFN-γ as previously described (28, 37). Reagents were supplied by John Abrams (DNAX). Typically, the sensitivity of this assay was 19 to 38 pg/ml.

**Statistics.** Statistical analysis (unpaired *t* test, paired non-parametric Mann-Whitney *t* test, and Wilcoxon signed rank test) was performed with INSTAT software. A *P* value of $<0.05$ was considered significant.

## RESULTS

**NK cell activity.** Spleen cells from mice infected with *T. gondii* for 5 days had higher NK-cell activity than uninfected mice as measured by lysis of YAC-1 cells (Fig. 1A). To determine whether this increased NK-cell cytotoxic activity was accompanied by induction of cytotoxic activity against *T. gondii*-infected target cells, either uninfected or *T. gondii*-infected P815 cells were used in $^{51}Cr$ release assays. Spleen cells from SCID mice infected with *T. gondii* had no significant cytotoxic activity against either infected or uninfected target cells. In addition, NK cells enriched by removal of the adherent spleen cell population (98% asialo GM1 positive as assessed by FACS) were not cytotoxic against either infected or uninfected P815 cells.

**Effect of in vivo neutralization of IFN-γ.** The NK-cell activity in infected mice treated with the IFN-γ-neutralizing antibody was comparable to that in uninfected controls (Fig. 1A). Administration of anti-IFN-γ abrogated measurable levels of IFN-γ in serum compared with those in infected control mice treated with an isotype control antibody and led to earlier mortality (Fig. 2). IFN-γ concentrations in sera (in picograms per milliliter, means ± standard deviations) from SCID mice of the indicated categories were as follows: uninfected control, 0; infected control, 844 ± 185; infected plus anti-IFN-γ, 0; infected plus anti-asialo GM1, 214 ± 91. Uninfected mice or

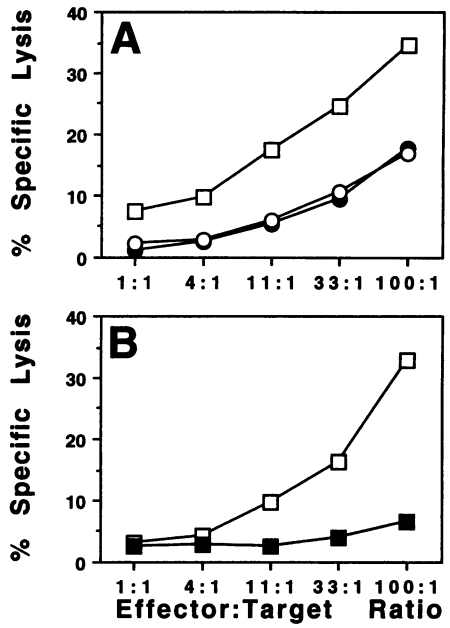


FIG. 1. YAC-1-specific NK-cell activity of SCID mice infected with *T. gondii*. Spleen cells from groups of three uninfected mice (●), infected mice (□), or mice infected and treated with anti-IFN-γ (○) (A) or infected (control) mice (□) or mice infected and treated with anti-asialo GM1 (■) (B) were pooled and assayed for NK-cell activity by $^{51}Cr$ release from YAC-1 target cells, and the percent specific lysis was calculated as described in Materials and Methods. Similar results were observed in two further experiments.

mice infected for 5 days were sacrificed, and serum samples from four mice per group were assayed in duplicate for IFN-γ with an ELISA. Uninfected and infected control mice received an isotype control monoclonal antibody plus rabbit serum, while mice treated with anti-IFN-γ also received rabbit serum. Mice treated with anti-asialo GM1 received the isotype control monoclonal antibody. Similar results were observed in two separate experiments in which pooled sera were used.

**Effect of in vivo depletion of NK cells.** In vivo administration of anti-asialo GM1 considerably decreased the NK-cell activity

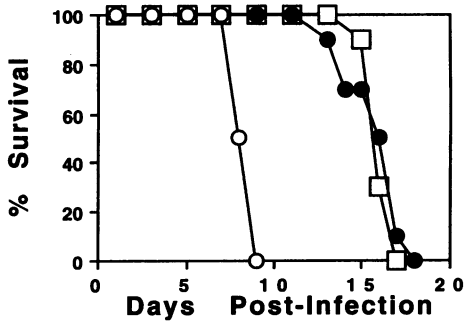


FIG. 2. Effects of administration of monoclonal antibody to IFN-γ or of antiserum for asialo GM1 on time to death of SCID mice infected with *T. gondii*. SCID mice were infected with *T. gondii* as described in Materials and Methods and administered either rabbit serum (□), a monoclonal antibody to IFN-γ (○), or antiserum to asialo GM-1 (●) as described in Materials and Methods. Experimental groups consisted of 10 mice, and time to death was monitored on a daily basis.

TABLE 1. Effects of in vitro depletion of NK cells on IFN-γ production

| Exptl group[a] | IFN-γ concn (pg/ml)[b] | |
|---|---|---|
| | No in vitro depletion of NK cells | In vitro depletion of NK cells |
| Uninfected | 1,140 (±127) | 64 (±22) |
| Infected | 2,000 (±312) | 453 (±13) |
| Infected and treated with anti-asialo GM1 | 302 (±56) | 0 |

[a] Groups of five uninfected mice, infected mice, or mice infected and treated with anti-asialo GM1 were sacrificed 5 days postinfection, and spleen cells were prepared as described in Materials and Methods. NK cells were depleted in vitro by incubation with anti-asialo GM1 followed by addition of rabbit complement as described in Materials and Methods. Cells were plated at a density of $3 \times 10^5$ cells in a final volume of 200 μl. Cultures were stimulated with 1 μg of TLA per ml, and supernatants were collected after 24 h and assayed for IFN-γ with an ELISA.

[b] All experiments were performed in duplicate; values are means for two experiments, and the ranges are given in parentheses. Similar results were observed in two other experiments.

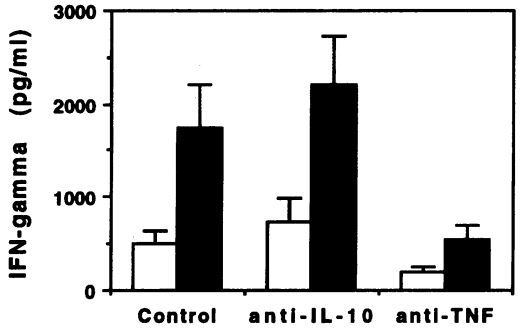


FIG. 3. Effect of inclusion of cytokine-neutralizing antibodies on IFN-γ production by spleen cells stimulated with live parasites. Data presented are the means ± standard errors of nine separate experiments. Spleen cells from groups of three to five uninfected mice (open bars) or mice infected for 5 days (solid bars) were pooled, prepared as described in Materials and Methods, and plated at a cell density of $3 \times 10^5$ cells per well before stimulation with $2 \times 10^5$ live RH tachyzoites. Levels of IFN-γ in unstimulated cultures were typically <39 pg/ml. In duplicate cultures, anti-IL-10, anti-TNF-α, or an isotype control (control) were added, prior to stimulation with live tachyzoites, to culture supernatants to give a final concentration of 120 μg/ml. Supernatants were collected after 24 to 48 h and assayed for IFN-γ by ELISA.

(Fig. 1B) and reduced levels of IFN-γ in serum in comparison with levels in infected controls (unpaired $t$ test; $P < 0.0001$) (see "Effect of in vivo neutralization of IFN-γ" above). However, this treatment did not alter time to death of infected mice (Fig. 2). The clinical status of the anti-asialo GM1-treated animals, as assessed by weight loss, piloerection, and paresis was observed to deteriorate 4 to 5 days prior to the deterioration observed in controls. Depletion of NK cells in mice infected with a more severe challenge, 50 or 100 cysts orally or 20 cysts intraperitoneally, also did not affect time to death in comparisons with controls. Histological analysis of the spleens, lungs, kidneys, brains, and livers of animals that died revealed the presence of severe inflammation in the lungs and necrotic foci associated with the presence of *T. gondii* tachyzoites in the brains. No obvious increase in severity of pathology was associated with *T. gondii* infection in anti-asialo GM1-treated mice compared with controls.

**IFN-γ production in vitro.** Stimulation of spleen cells from uninfected mice or mice infected with TLA or live *T. gondii* resulted in significant production of IFN-γ. The highest levels of IFN-γ production were consistently observed in cultures derived from infected mice (Table 1) and were independent of the stimulus used. While in vivo treatment with anti-asialo GM1 decreased NK-cell activity (Fig. 1B) and reduced levels of IFN-γ in serum (see "Effect of in vivo neutralization of IFN-γ" above), stimulation in vitro of spleen cells from these mice, with either TLA or live parasites, resulted in production of significant quantities of IFN-γ (Table 1). Incubation of whole spleen cells with anti-asialo GM1 in vitro followed by complement significantly decreased (paired nonparametric $t$ test; $P = 0.03$) the amount of IFN-γ produced in all experimental groups (Table 1).

**Effect of IL-10, IL-12, and TNF-α on IFN-γ production.** Addition of IL-10 to spleen cells derived from infected or uninfected SCID mice and stimulated with live parasites or TLA inhibited IFN-γ production. Maximal inhibition was observed at a concentration of 200 U of IL-10 per ml, and 50% inhibition occurred between 0.5 and 5.0 U/ml. The addition of anti-IL-10 to *T. gondii*-stimulated spleen cell cultures resulted in increased levels of IFN-γ. This increase was observed whether TLA or live parasites were used as a stimulus but was most apparent when spleen cells from infected SCID mice were employed (Fig. 3). The effects of anti-IL-10 on IFN-γ production varied. In some experiments there was a remarkable increase, while in others there was little or no increase. The reason for this variability is unclear. Data from nine separate experiments were submitted to statistical analysis. When spleen cells from infected mice were used, inclusion of anti-IL-10 significantly increased the production of IFN-γ (paired nonparametric Mann-Whitney $t$ test; $P < 0.004$, mean [± standard deviation] percent increase = 46% ± 38%). Furthermore, statistical analysis of nine separate experiments with spleen cells from uninfected mice revealed that addition of anti-IL-10 resulted in a trend towards increased IFN-γ production by cells from uninfected mice (paired nonparametric Mann-Whitney $t$ test; $P = 0.0504$, mean percent increase = 31% ± 56%).

The addition of TNF-α to spleen cells from infected or uninfected mice resulted in increased IFN-γ production by spleen cells stimulated with TLA or live parasites. Maximal activity was observed at 500 U of TNF-α, and half-maximal activity was observed between 5 and 50 U/ml. Interestingly, the addition of exogenous TNF-α consistently resulted in two- to threefold higher levels of IFN-γ when added to spleen cells from infected mice than when added to spleen cells from uninfected mice. The addition of anti-TNF-α decreased production of IFN-γ by spleen cells from both infected and uninfected mice (Fig. 3). Statistical analysis of nine separate experiments revealed that for spleen cells from infected or uninfected mice stimulated with *T. gondii*, the mean percentage decrease in IFN-γ production was approximately 65% (paired nonparametric Mann-Whitney $t$ test; $P < 0.001$) for both data sets.

In a subsequent series of experiments, the addition of anti-IL-12 in vitro abrogated production of IFN-γ induced by the stimulation of spleen cells from infected or uninfected mice with either TLA, live parasites, or heat-killed parasites (Table 2). Furthermore, stimulation with TLA or live parasites following depletion of the adherent cell population to enrich for NK cells induced minimal levels of IFN-γ (19 to 40 pg/ml). Addition of macrophages isolated from the peritoneum of SCID mice restored the ability of the enriched NK cell populations to produce IFN-γ. These data confirm those of

TABLE 2. Effects of anti-IL-12 on production of IFN-γ by spleen cells from SCID mice[a]

| Treatment | IFN-γ concn (pg/ml) | |
|---|---|---|
| | Uninfected mice | Infected mice |
| IgG | 0 | 0 |
| HKT + IgG | 2,525 (±375) | 4,609 (±882) |
| HKT + anti-IL-12 | 0 | 910 (±260) |

[a] Spleen cells from groups of four uninfected SCID mice or SCID mice infected for 5 days were pooled, prepared as described in Materials and Methods, and plated at a cell density of $4 \times 10^5$ cells per well. Sheep IgG or sheep anti-IL-12 was added to wells at a concentration of 100 μg/ml 30 min before stimulation with $2 \times 10^5$ heat-killed *T. gondii* (HKT). Experiments were performed in two (sheep IgG) or six separate wells, and supernatants were collected after 48 h and assayed for IFN-γ by ELISA. Data presented are means, with standard errors given in parentheses. Similar results were observed in three repeat experiments.

Sher and Gazzinelli and their coworkers (10, 32), who used spleen cells from uninfected mice.

Direct stimulation of SCID spleen cells with the combination of IL-12 and TNF-α resulted in a remarkable increase in IFN-γ production over that observed with either cytokine alone (Fig. 4). Maximal IFN-γ production was observed with concentrations of 0.1 to 1.0 ng of IL-12 per ml in combination with 10 to 50 U of TNF-α per ml. The addition of higher levels of TNF-α resulted in sharp declines in IFN-γ production. When spleen cells from infected mice were stimulated with IL-12 plus TNF-α, they produced significantly higher levels of IFN-γ than did spleen cells from uninfected mice (unpaired *t* test; $P < 0.004$) (Fig. 4). Depletion of the adherent cell population from spleen cells of uninfected mice, to enrich for NK cells, did not significantly alter IFN-γ production in response to the combination of IL-12 and TNF-α. However, removal of the adherent cell population from spleen cell populations of infected mice and subsequent stimulation with

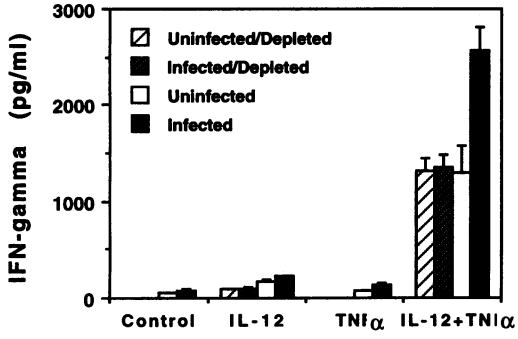


FIG. 4. Stimulation of spleen cells from uninfected and infected SCID mice with IL-12 and TNF-α. Whole spleen cells from groups of five infected or uninfected mice were prepared as described in Materials and Methods, plated at a cell density of $3 \times 10^5$ per well, and stimulated with either IL-12 (1 ng/ml) or TNF-α (50 U/ml) alone or with both in combination in a final volume of 200 μl. Adherent cells were depleted, by adherence to plastic, from spleen cells of infected (Infected/Depleted) or uninfected (Uninfected/Depleted) mice, and nonadherent cells were plated at a cell density of $3 \times 10^5$ per well and stimulated with either IL-12 (1 ng/ml) or TNF-α (50 U/ml) alone or with both in combination in a final volume of 200 μl. Control supernatants are those from unstimulated cells. Culture supernatants were collected after 48 h and assayed for IFN-γ production by ELISA. All experiments were performed in duplicate. The data presented are the means ± standard errors of the pooled data from two experiments.

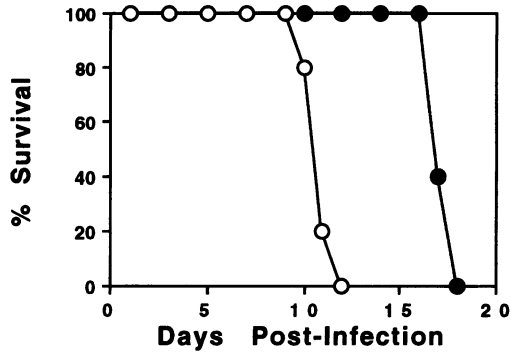


FIG. 5. Effect of administration of polyclonal antibody to IL-12 on time to death of SCID mice infected with *T. gondii*. SCID mice were infected with *T. gondii* as described in Materials and Methods and administered either sheep immunoglobulins (●) or a polyclonal antibody to IL-12 (○) as described in Materials and Methods. Experimental groups consisted of five mice, and time to death was monitored on a daily basis. Similar results were observed in a repeat experiment.

IL-12 and TNF-α resulted in a remarkable reduction in the levels of IFN-γ produced (unpaired *t* test; $P < 0.0006$). The reduced levels were not significantly different from the levels observed with whole spleen cells or NK-cell-enriched populations from uninfected mice (Fig. 4).

**Effect of anti-IL-12 on survival.** The results described above demonstrate an important role for IL-12 in vitro to induce NK-cell production of IFN-γ. Other workers have reported on the protective activity of exogenous IL-12 in *T. gondii*-infected SCID mice (10). As a consequence, we decided to determine whether endogenous IL-12 mediates resistance to *T. gondii* in SCID mice. Administration of a polyclonal anti-mouse IL-12 to infected mice resulted in earlier mortality than did administration to control mice (Fig. 5) (Wilcoxon signed rank test; $P = 0.0039$). Similar results were observed in a repeat experiment (data not shown).

## DISCUSSION

In the present study, we have demonstrated that *T. gondii* infection in SCID mice results in increased NK-cell activity against YAC-1 target cells and that IFN-γ was required for this increased NK activity. IFN-γ has previously been reported to have a regulatory role on the activity of human NK cells that enhances their cytolytic activity against tumor target cells in vitro (7). Since treatment of infected SCID mice with anti-IFN-γ abrogated the increase in NK-spleen-cell activity, it is likely that IFN-γ production by NK cells in response to *T. gondii* infection in vivo regulates the increased NK-cell activity. Comparison of results in infected and uninfected SCID mice revealed that spleen cells from infected mice produced higher levels of IFN-γ when exposed in vitro either to a lysate antigen preparation of *T. gondii* or to live tachyzoites, reflecting the increased NK-cell activity induced by infection. Since we were unable to demonstrate a cytolytic activity of NK cells for infected P815 cells, a result also reported recently by Denkers et al. (6), it seems likely that the main protective activity of NK cells in this model is through production of IFN-γ. However, NK cells may be capable of lysing other infected cells, and NK cells have been reported to lyse extracellular tachyzoites of *T. gondii* (14).

That treatment with anti-asialo GM1 antiserum, in six separate experiments, did not result in earlier mortality in

SCID mice infected with *T. gondii* administered per os was surprising, since this treatment markedly decreased NK-cell activity (as assessed by cytotoxicity for YAC-1 cells) and levels of IFN-γ in serum. Our results appear to be due to the failure of the treatment to completely eliminate the NK-cell population, since there were low levels of IFN-γ in the sera of the anti-asialo GM1-treated mice. Moreover, the source of the IFN-γ produced by spleen cells from infected mice to which anti-asialo GM-1 had been administered was removed by in vitro depletion of asialo GM-1-positive cells. Although these results illustrate that the use of anti-asialo GM1 in vivo did not completely ablate NK-cell activity, they indicate that the low levels of NK-cell activity and the low levels of IFN-γ present in the sera of these mice are sufficient to mediate resistance during the early stage of the infection.

When the role of IL-10 was examined, we found that addition of exogenous IL-10 significantly decreased the capacity of spleen cells from uninfected and infected mice to produce IFN-γ in response to exposure to TLA or live parasites. These results confirm previous studies in which spleen cells from uninfected SCID mice were used (32). However, that addition of anti-IL-10 in vitro to SCID spleen cells stimulated with *T. gondii* resulted in increased production of IFN-γ, particularly with spleen cells from infected mice, demonstrates for the first time the important contribution of endogenous IL-10 in regulation of IFN-γ production by NK cells. Since SCID mice lack several of the recognized sources of IL-10, such as B cells and $CD4^+$ T cells (16), it appears that macrophages are the primary source of the endogenous IL-10 present. The inhibitory activity of IL-10 in our system is similar to that found in previous studies in which IL-10 inhibited synthesis of IFN-γ by human and mouse NK cells (18, 32, 39). This action of IL-10 on NK cells appears to be through its effect on accessory cells (8, 18, 32, 39). Because of the importance of TNF-α and IL-12 in production of IFN-γ by NK cells, it appears that IL-10 inhibits production of IFN-γ by NK cells through its ability to decrease production of both TNF-α and IL-12 by macrophages (10, 17, 39). This activity of IL-10 may explain our previous results, which revealed that administration of anti-IL-10 delayed time to death of *T. gondii*-infected SCID mice (19).

Endogenous TNF-α is also important for production of IFN-γ by NK cells from both infected and uninfected mice, since neutralization of TNF-α in vitro considerably reduced IFN-γ production. We have recently reported that administration of anti-TNF-α led to earlier mortality in SCID mice acutely infected with *T. gondii* (19). In attempting to understand the role of TNF-α in resistance to *T. gondii* in SCID mice, our results and those of others (32, 39) reveal that TNF-α is required as a stimulatory factor with IL-12 for production of IFN-γ by NK cells but alone is unable to stimulate purified NK cells to produce IFN-γ. Thus, the protective activity associated with endogenous TNF-α in vivo (19) may work through augmentation of production of IFN-γ by NK cells. However, TNF-α is also critical for the activation of macrophages by IFN-γ to inhibit replication of *T. gondii* (24, 33).

Of interest is that whereas whole spleen cell populations from infected SCID mice produced higher levels of IFN-γ in response to the combination of IL-12 and TNF-α than did those from uninfected mice, these differences were not apparent after removal of the adherent cell population. These data indicate that the adherent cell populations from infected mice are producing other factors that markedly increase the responsiveness of NK cells to IL-12 and TNF-α. This may be related to the results reported by Chan et al. (4), who demonstrated that an accessory cell was required for IL-12 to induce IFN-γ production by resting human peripheral blood lymphocytes. These data highlight the importance of the accessory cell population, and our finding that in vivo infection increases the ability of this population to induce IFN-γ production by NK cells in response to IL-12 and TNF-α requires further study.

Recent studies have demonstrated the protective effect of administration of exogenous IL-12 on *T. gondii* infection in SCID mice (10) and on *Leishmania major* infection in BALB/c mice (15). Our data on decreased survival time of infected SCID mice to which anti-IL-12 was administered demonstrate the importance of endogenous IL-12 in mediating resistance to *T. gondii*. These results indicate that the in vitro data demonstrating the importance of IL-12 in driving IFN-γ production by NK cells presented here and by other groups (10, 39) are relevant to the protective immune response in vivo.

Of interest in regard to our findings reported here are previous results in which sonicate and subcellar fractions of tachyzoites induced production of IFN-γ by human NK cells (31). Administration of different *T. gondii* antigen preparations to immunocompetent mice also resulted in increased NK-cell activity (13). Those results indicate that NK cells may play a protective role in humans and immunocompetent mice similar to that observed in SCID mice. A prompt NK-cell response, before other arms of the immune system are primed to produce IFN-γ, may result in the early appearance of high levels of IFN-γ, which activate nonspecific defense mechanisms, such as the macrophage. Since macrophages are likely a first line of defense against *T. gondii*, their initial response to live parasites, parasite antigen, or IFN-γ produced by NK cells may influence the subsequent development of different T-helper subtypes (27). Production of TNF-α and IL-12 would lead to activation of NK cells to produce IFN-γ, while IL-12 alone could act to stimulate $CD4^+$ and $CD8^+$ T lymphocytes to produce IFN-γ (17, 23, 26). However, the production of IL-10 by macrophages could severely impair the accessory cell function of macrophages and inhibit the development of protective NK- and T-cell responses.

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