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Interleukin-12 Enhances Murine Survival against Acute Toxoplasmosis

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Protective immunity against *Toxoplasma gondii* is mediated by the host cellular immune response. Interleukin-12 (IL-12), a recently described cytokine that stimulates NK cells to produce gamma interferon (IFN-γ), is able to enhance host protection against this parasite in SCID mice. Administration of IL-12 to A/J mice significantly increased survival over that of control mice when IL-12 was delivered early in the course of acute infection. If it was administered at day 3 or thereafter, there was no observed difference in mortality between treated and control mice. Antibody depletion of IL-12 increased susceptibility to infection, as measured by mortality, only when the IL-12 was administered before day 3 postinfection. Mice treated with IL-12 at day 0 postinfection exhibited a significant rise above the control in both IL-2 and IFN-γ production. Once infection has been established in the host (3 days), administration of exogenous IL-12 is unable to alter parasite-induced downregulation of IFN-γ production. Thus, IL-12 appears to play an important, but transitory, role in protection against acute infection with *T. gondii* in the normal murine host.

Cell-mediated immunity is critical in host resistance to infection with the obligate intracellular protozoan *Toxoplasma gondii* (11). Both the $CD4^+$ and, in particular, $CD8^+$ T-cell responses are involved in resolution of infection (1, 7, 14, 17). However, NK cells appear to play a critical role in the response to acute infection (16). These NK cells produce interferon gamma (IFN-γ), which in turn activates macrophages into a microbicidal state. Recent studies in SCID mice show that interleukin-12 (IL-12), a heterodimeric cytokine, is essential for stimulation of NK cell IFN-γ production and that macrophages appear to be an important source of this cytokine (8). Furthermore, exogenous IL-12 is able to extend the life of SCID mice infected with *T. gondii* cysts. In this report, we show that exogenous IL-12 is able to enhance protection in normal mice against acute *T. gondii* infection only when administered at the time of parasite challenge. Similarly, antibody directed at IL-12 increases host susceptibility only when administered shortly after infection.

MATERIALS AND METHODS

Animals and infection. Female A/J mice (Jackson Laboratory, Bar Harbor, Maine) were infected intraperitoneally with either 10^4 (50% lethal dose [LD_{50}]) or 5×10^4 (LD_{90}) P-strain tachyzoites. Parasites used in these studies were obtained from tissue culture in human fibroblast cells and were <50th passage.

In vivo IL-12 treatment. Infected mice treated with the LD_{90} of P-strain parasites were given 0.33 μg of recombinant murine (rm) IL-12 (kindly supplied by Genetics Institute, Cambridge, Mass.) in 0.2 ml of saline via daily intraperitoneal injection beginning on day 0, 3, or 6 postinfection. Mice were observed daily for evidence of infection (ruffled fur, weight loss, and lethargy), and mortality was used as the endpoint. Control mice were treated with an equal volume of saline.

In vivo depletion studies. For IL-12 depletion, mice were treated intraperitoneally with 200 μg of rabbit anti-IL-12 antibody (kindly supplied by Genetics Institute) beginning on day 0 or 3 postinfection with the LD_{50} of parasites. Antibody treatment was continued on an alternate-day basis for 14 days. Control mice were treated with either a similar quantity of normal rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) or an equal volume of saline for the same period.

For IFN-γ depletion, mice were treated with 3 mg of rat anti-mouse IFN-γ antibody (XMG6; American Type Culture Collection) weekly. Control mice were given an equal quantity of rat immunoglobulin G (Sigma). Depletion of NK cells was accomplished by administering 50 μl of anti-asialo GM1 antibody (Wako Chemicals) on alternate days throughout the experiment. The antibody treatment was started 2 days prior to parasite challenge.

Phenotypic analysis. Splenocytes were analyzed for T-cell phenotype by a fluorescence-activated cell sorter (FACS; Ortho Diagnostics), using direct immunofluorescence. Cells (10^6) were incubated with 1 μg of fluorescein isothiocyanate-labelled anti-CD4 or anti-CD8 monoclonal antibody (MAb) (PharMingen, San Diego, Calif.) or a 1:80 dilution of anti-asialo GM1 antibody (Wako Chemicals). After 45 min of incubation at 37°C, the cells were washed with cold 3% bovine serum albumin in phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde in PBS, and analyzed by FACS scan (Beckton Dickinson).

Assay for IL-2 and IFN-γ production. Infected mice treated with IL-12 or saline were splenectomized, and after homogenization of the tissue, the erythrocytes were lysed by hypotonic shock with 0.2% ammonium chloride. The cells were washed and cultured at a concentration of 10^6 in Iscove medium (LTI, Gaithersburg, Md.) with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah) in 24-well plates. Wells were stimulated with 0.3 μg of whole parasite extract per ml prepared by repeat freeze-thaw (four times). The lysed parasites were centrifuged at $600 \times g$, and the soluble fraction was filtered. The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.). For IL-2 assay, cell culture supernatant was collected by centrifugation after 24 h and stored at −70°C until used. IL-2 analysis was performed in a CTTL assay (5). To determine IFN-γ production, supernatants from cultured splenocytes were collected at the completion of 48 h of incubation and stored as described

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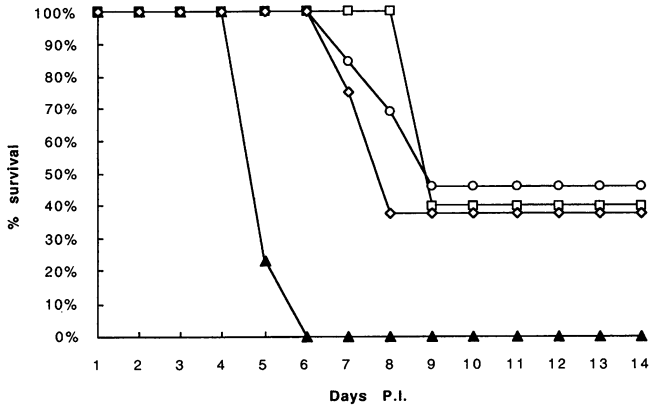

FIG. 1. Depletion of IL-12 by antibody in mice following acute infection with *T. gondii*. Inbred A/J mice (n = 6 to 10) were infected via the intraperitoneal route with 10^4 P-strain tachyzoites (LD_{50}). Mice were treated with rabbit anti-IL-12 antibody (200 μg) on alternate days beginning on either day 0 (▲) or 3 (□) postinfection (P.I.). An equal quantity of either normal rabbit antibody (○) or saline (◇) was similarly administered. Antibody therapy was continued through day 14, when the experiment was terminated. Results represent combined data from two experiments.

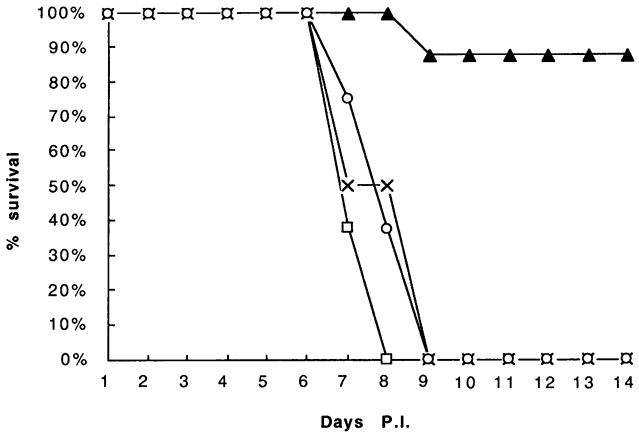

FIG. 2. Effect of exogenous IL-12 on survival of mice following acute infection with *T. gondii*. Inbred A/J mice (n = 6 to 10 per group) were acutely infected via the intraperitoneal route with 5×10^4 P-strain tachyzoites (LD_{90}). Following infection, mice were treated on alternate days with exogenous rmIL-12 (0.33 μg) beginning on either day 0 (▲), 3 (□), or 6 (×). Control mice were treated with saline beginning on day 0 (○). Treatment was continued through day 14 and terminated. Results represent combined data from two experiments. P.I., postinfection.

above. IFN-γ titers were assayed by enzyme-linked immunosorbent assay (Endogen Inc., Boston, Mass.).

Detection of cytokine mRNA. Total cellular RNA was extracted from the splenocytes of mice by the guanidinium thiocyanate method, as described previously (4). Total RNA (10 μg) was fractionated in a 1.2% agarose–formaldehyde gel and transferred to a nylon membrane (Hybond-N^+; Amersham, Arlington Heights, Ill.) with 0.05 M NaOH by capillary transfer for 2 h. The membrane was prehybridized in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.4])–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt solution–20 μg of salmon sperm DNA per ml at 65°C for 1 h. The filter was hybridized separately with cDNA probes for murine IFN-γ (kindly supplied by Kevin Moore, Genentech, San Francisco, Calif.) and human β-actin (Clontech, Palo Alto, Calif.). Probe was labelled with [α-^{32}P]dATP to a specific activity of $>10^9$ cpm/μg by a random-primer labelling method (Boehringer, Mannheim, Germany). The filter was washed twice with 2× SSPE–0.1% SDS for 10 min at room temperature, twice with 2× SSPE–0.1% SDS, and finally with 0.1× SSPE–0.1% SDS at 52°C for 10 min. Autoradiography was performed at −70°C for 24 h.

Statistical analysis. Student's t test was used for the analysis of data in these experiments.

RESULTS

Antibody directed at IL-12 was used to determine whether this cytokine was important in eliciting a protective response in normal mice against acute *T. gondii* infection. Mice were depleted of IL-12 with antibody on alternate days following intraperitoneal infection. As shown in Fig. 1, all mice treated with anti-IL-12 antisera on day 0 postchallenge died from *T. gondii* infection by day 6 compared with control mice receiving either nonspecific antibody or saline. The time postchallenge at which administration of anti-IL-12 altered the host response was crucial. If delivered shortly after infection and continued on an alternate-day schedule, the antibody had a significant effect in blocking host protective immunity ($P < 0.01$). If, however, the antibody was delivered beginning on day 3 postinfection and on alternate days thereafter, there was no significant difference in survival between the IL-12-depleted mice and control mice that were treated with saline beginning on day 0.

The ability of exogenous IL-12 to enhance protection in normal mice was then evaluated by administration of this cytokine to acutely infected mice. To perform this experiment, mice were infected and administered rmIL-12 beginning either immediately postinfection (day 0) or on day 3 or 6 postinfection, and administration was continued on a daily basis thereafter. As shown in Fig. 2, those mice receiving IL-12 on day 0 postinfection survived (87.5% survival), whereas all mice receiving IL-12 starting on day 3 or 6 postinfection died by day 8 or 9 ($P < 0.001$).

Splenocytes were phenotyped by FACS analysis for the expression of either $CD4^+$, $CD8^+$, or NK cells. As shown in Table 1, mice receiving IL-12 on day 0 exhibited a substantial rise in their NK cell population (9.8%) compared with mice receiving their first dose of IL-12 on day 3 (4.5%) or no IL-12 (2.3%). During this period, there was a small drop in the number of cells expressing $CD4^+$ but no change in the $CD8^+$ count.

TABLE 1. Phenotypic analysis of T-cell subsets in mice acutely infected with *T. gondii* and treated with rmIL-12[a]

Treatment	Change (%) in given cell population		
	$CD4^+$	$CD8^+$	NK
IL-12			
Day 0	20.8	13.6	9.8
Day 3	18.6	15.3	4.5
Saline control	15.7	12.6	2.3

[a] Inbred A/J mice were infected with an LD_{90} of P-strain tachyzoites via intrapertioneal inoculation. Alternate-day treatment with rmIL-12 began on day 0 or 3 postinfection. Mice were sacrificed on day 5 postinfection, and splenocytes were evaluated for T-cell phenotype expression by FACS analysis after fluorescein isothiocyanate incubation. Results represent the averages of three mice per group.

TABLE 2. Analysis of cytokine production in mice acutely infected with *T. gondii* and treated with rmIL-12[a]

Treatment	IL-2 (pM)		IFN-γ (U/ml)	
	Control	+Tg	Control	+Tg
IL-12				
Day 0	50	200	200	400
Day 3	<5	<5	50	100
Saline control	<5	<5	<5	50

[a] Inbred A/J mice were infected with an LD_{90} of P-strain tachyzoites via intraperitoneal inoculation. Alternate-day treatment with rmIL-12 began on day 0 or 3 postinfection. Mice were sacrificed on day 5 postinfection, and splenocytes were isolated and cultured in vitro in the presence of *T. gondii* parasite extract (+Tg). After 24 h for IL-2 and 48 h for IFN-γ, levels of these cytokines were determined by biologic analysis.

Splenocytes from mice treated with exogenous IL-12 were assayed for the production of both IL-2 and IFN-γ. As shown in Table 2, mice receiving IL-12 beginning on day 0 and on alternate days thereafter had detectable levels of IL-2 on day 5 when splenocytes were incubated in both the presence and the absence of parasite antigen. Significantly greater quantities of IL-2 were produced by splenocytes incubated in the presence of parasite antigen. Splenocytes obtained from mice that had IL-12 therapy initiated on day 3 postinfection failed to produce detectable quantities of IL-2 in culture as determined by our assay. Similarly, control mice not treated with IL-12 failed to produce IL-2 in both the presence and the absence of parasite antigen. Both parasite antigen-stimulated and unstimulated splenocytes from mice receiving IL-12 on day 0 postinfection showed significant rises in levels of IFN-γ. In comparison, mice receiving IL-12 on day 3 postinfection had an observed, but lesser, rise in IFN-γ levels.

To further delineate the requirement for NK cells and IFN-γ in this model, IL-12-treated mice were MAb depleted of either NK cells or IFN-γ. As shown in Fig. 3, depletion of IFN-γ resulted in a significant increase in mortality, with all mice dying by day 5 postinfection. Similarly, treatment with antibody to NK cells resulted in all mice succumbing to infection by day 8. Control mice treated with IL-12 and a nonspecific immunoglobulin G exhibited significantly greater survival postinfection.

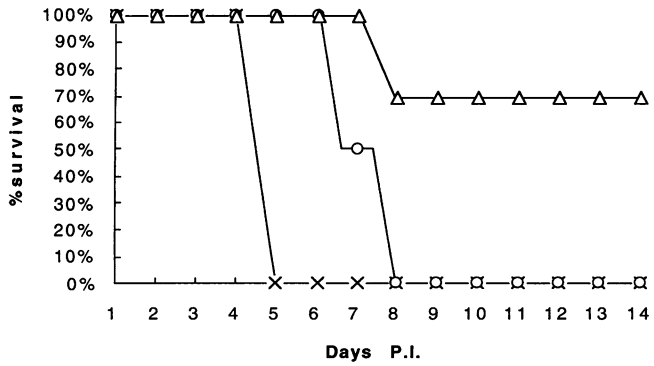

FIG. 3. Antibody depletion of IFN-γ and NK cells with MAb. Three groups of mice (n = 6) were treated with either 3 mg of anti-IFN-γ (XMG6) (×), 50 μl of anti-asialo GM1 antibody (○), or an equal amount (3 mg) of irrelevant immunoglobulin G (△). The antibody treatment was started 2 days prior to administration of daily IL-12 therapy (0.33 μg per mouse) and challenge with an LD_{90} of parasites. P.I., postinfection.

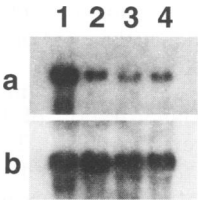

FIG. 4. Quantitative cytokine (IFN-γ) mRNA analysis following acute infection with *T. gondii* and treatment with rmIL-12. Inbred A/J mice were challenged with an LD_{90} of P-strain tachyzoites via intraperitoneal inoculation and were treated with rmIL-12 daily. Mice that were treated (n = 3 per group) with IL-12 beginning on day 0 (lane 1) or 3 (lane 2) or with saline (lane 4) were sacrificed on day 5 postinfection. Another group of mice (n = 3) that were infected with *T. gondii* and treated with IL-12 beginning on day 3 were sacrificed on day 8 (lane 3). This allowed mice in both day 0 and 3 groups to receive equal quantities of rmIL-12 prior to sacrifice. The cellular RNA from the spleen cells was extracted, and message for IFN-γ (a) and a β-actin control (b) was determined by Northern blot analysis.

Quantitative expression of cytokine message was evaluated by Northern (RNA) blot. For this experiment, mice were infected on day 0 and treatment with IL-12 was initiated on either day 0 or 3. Exogenous IL-12 was administered on a daily basis once therapy began. Mice infected on day 0 and treated with IL-12 on day 0 were sacrificed on day 5. Mice infected on day 0 and not treated with IL-12 until day 3 were sacrificed on either day 5 or 8. This allowed mice to receive equal quantities of exogenous IL-12 prior to evaluation for cytokine message expression. As shown in Fig. 4, splenocytes obtained from mice receiving IL-12 at the time of infection (day 0) expressed a substantial increase in the message for IFN-γ at the time of assay. For the mice that were infected but were not administered IL-12 until day 3, there was no difference in the quantity of expressed message for IFN-γ.

DISCUSSION

Our observations indicate that IL-12 is an important mediator of host defense during the acute phase of *T. gondii* infection in normal inbred mice. Administration of recombinant IL-12 to normal mice on day 0 postinfection significantly decreases morbidity and prolongs life against acute infection. These findings confirm the recent observations of Gazzinelli et al. (8), who observed marked prolongation of life in SCID mice following administration of IL-12. However, in normal mice, it appears that the effect of exogenous IL-12 occurs only at the onset of infection. Thereafter (by day 3 postinfection), the beneficial effect of this cytokine is no longer apparent, as determined by mortality. Similarly, antibody to IL-12 blocks the protective effect of this cytokine when administered on the day of infection but not thereafter.

The mechanism by which IL-12 increases host protection against *T. gondii* infection appears to be the induction of NK cells. Depletion of these cells in mice treated with IL-12 reverses the protective effect of this cytokine (Fig. 3). It would appear that the NK cells produce greater quantities of IFN-γ, enhancing the microbicidal activity of macrophage (3). Additionally, IL-12 can augment the helper T-cell response to produce more IL-2 (18). In our studies, we have observed a rise in both IL-2 and IFN-γ in the supernatants of cultured splenocytes following administration of IL-12 to the host on day 0 postinfection. We did not, however, observe a rise in the

number of splenocytes expressing the $CD4^+$ T-cell phenotype. This is perhaps due to the involvement of immune organs (i.e., lymph nodes and intestinal epithelial cells) other than the spleen. Our results do show a significant rise in the NK cell population following administration of IL-12, in particular, when the IL-12 is delivered on day 0. The NK cell response observed on day 3 (4.5%) may not reflect the peak NK cell response since we harvested splenocytes and measured for cell phenotype at day 5 in both conditions (IL-12 administered on days 0 and 3).

In these studies, administration of exogenous IL-12 at the time of infection resulted in an increase of both IL-2 and IFN-γ levels. Depletion of IFN-γ with MAb reversed the protective effect of the IL-12. However, delayed administration (day 3) of exogenous IL-12 failed to augment the levels of IL-2 and IFN-γ in the infected mice. There is abundant evidence for defects in cellular immunity during *T. gondii* infection. Depressed lymphocyte proliferation to parasite antigens has been observed in infants with congenital toxoplasmosis and in adults with acute toxoplasmosis (2, 15). Generalized immunosuppression also occurs in mice infected with *T. gondii* (18, 21). Preliminary results from our laboratory demonstrate that decreased IL-2 production can be detected in the culture supernatant of splenocytes derived from mice acutely infected with *T. gondii* and stimulated in vitro with mitogen (9). Both macrophages and T cells from acutely infected mice produce a soluble factor that is able to inhibit the proliferative response of naive T cells to mitogen. The same culture supernatants contain increased levels of IL-10, and partial neutralization of the immunosuppressed condition can be observed following administration of anti-IL-10 antibody (13). Since IL-10 is a potent antagonist of the effects of IFN-γ, it may be important in mediating the immunosuppression observed. Recent studies by D'Andrea et al. (6) suggest that IL-10 is able to inhibit the production of IFN-γ by suppressing IL-12 synthesis in accessory cells. Perhaps the host primary response to *T. gondii* involves the production of macrophage-derived IL-12, which in turn stimulates the proliferation of NK cells and IFN-γ. Once acute infection has been established, the parasite stimulates the production of IL-10 in the host, which in turn inhibits IL-12 production and reduces IFN-γ and IL-2 levels.

Our results indicate that the time of IL-12 administration is critical for its ability to alter host infection. If IL-12 is administered shortly after infection, it is able to significantly enhance protection against acute toxoplasma infection. If, however, this cytokine is administered on day 3 or later postinfection, the ability to enhance the protective response is lost. This reduced protective response is perhaps due to the parasite having established itself within the host, making the role of NK cell-mediated protection less important. This confirms earlier studies of Hughes et al. (10), who demonstrated that although NK cells are present beyond day 3 of infection, they do not appear to be important in modulating host protection against infection. Previous studies from our laboratory (12) and by others (17) indicate that, beyond day 3, the host $CD8^+$ T-cell response in association with the production of high-titer IFN-γ is necessary for host survival. Thus, the role of IL-12 may be transitory and important only in modulating the host immune response to the initial phase of acute primary infection.

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