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Immunology

Interleukin 12 is required for the T-lymphocyte-independent induction of interferon γ by an intracellular parasite and induces resistance in T-cell-deficient hosts

(*Toxoplasma gondii*/natural killer cells)

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ABSTRACT **Immunity against the intracellular protozoan *Toxoplasma gondii* is highly dependent on interferon γ (IFN-γ). We have previously shown that, in addition to T lymphocytes, natural killer (NK) cells can be stimulated by the parasite to produce this cytokine by a reaction requiring adherent accessory cells and tumor necrosis factor α. We now demonstrate that a recently characterized cytokine, interleukin 12 (IL-12), is also necessary for parasite-induced IFN-γ synthesis by NK cells. Anti-IL-12 antibodies completely inhibited *T. gondii* or bacterial endotoxin-stimulated IFN-γ production by NK-enriched spleen cells from severe combined immunodeficient mice. Moreover, potent NK cytokine responses were induced by the combination of IL-12 and tumor necrosis factor α. In addition, adherent spleen cells from *scid/scid* mice or thyoglycollate-elicited macrophages from BALB/c animals produced high levels of both IL-12 (p40) and tumor necrosis factor α mRNAs when exposed to either live tachyzoites, parasite extracts, or endotoxin, confirming that these cytokines are produced by accessory cells. Finally, *in vivo* studies showed that treatment with recombinant IL-12 results in prolonged survival of scid mice after infection with *T. gondii* by means of a response dependent on both IFN-γ and NK cells. Together the data argue that IL-12 is required for the T-cell-independent triggering of NK cells by intracellular parasites and that the cytokine may be useful for inducing this protective pathway in immunodeficient hosts.**

Intracellular pathogens typically induce strong T-lymphocyte responses leading to the production of interferon-γ (IFN-γ) (1). Recent studies indicate that natural killer (NK) cells can be triggered by many of the same intracellular parasites to synthesize this cytokine in the absence of T cells (2). This T-cell-independent pathway of IFN-γ production has been most extensively studied with *Listeria monocytogenes*, an intracellular bacterium that stimulates production of the cytokine from splenocytes of mice with severe combined immunodeficiency (SCID) (3). The *Listeria*-induced IFN-γ response was found to be mediated by NK cells and to depend on soluble factors released from macrophage accessory cells (4).

In addition to *Listeria*, a wide variety of different intracellular bacteria, including *Corynebacterium parvum*, *Salmonella*, and *Mycobacteria*, have been shown to stimulate T-cell-independent IFN-γ synthesis by NK cells (2). Recently, we have shown that the same pathway is triggered by an intracellular protozoan, *Toxoplasma gondii* (5). In experimental hosts, this normally harmless parasite induces strong T-cell-dependent immunity against itself, as well as nonspecific resistance against other pathogens and tumors (6–10). Previous studies have shown that IFN-γ produced by both $CD4^+$ and $CD8^+$ cells plays an essential role in the protective response against *T. gondii* (11–14), and the loss of this function is thought to explain the reactivated toxoplasmosis seen in AIDS and other immunodeficient states (15–17). The ability of the parasite to stimulate T-cell-independent IFN-γ production from NK cells *in vitro* suggests that induction of the same pathway *in vivo* could be used as a strategy for controlling infection in T-lymphocyte-deficient hosts.

Our previous work had indicated that, in common with *Listeria*, *T. gondii* induces NK-cell responses by stimulating accessory cell tumor necrosis factor α (TNF-α) synthesis, which, although required, is not sufficient for triggering IFN-γ production (5). We now show that interleukin (IL)-12, a recently identified heterodimeric cytokine, is essential for stimulation of NK-cell IFN-γ synthesis and that macrophages are its likely source. Moreover, we show that this cytokine, by inducing NK-dependent IFN-γ production *in vivo*, can confer nonspecific immunity against *T. gondii* in T-cell-deficient *scid/scid* mice. Together these findings suggest that IL-12 has major potential for the induction of microbial resistance in immunocompromised hosts.

MATERIALS AND METHODS

Experimental Animals. CB-17 SCID, BALB/c, and Swiss–Webster mice were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD), or from Harlan–Sprague–Dawley. The animals were maintained in a modified barrier facility in microisolator cages and used between 5 and 8 weeks of age.

Parasites and Extract Preparation. Tachyzoites of the RH strain of *T. gondii* were obtained from infected monolayers of human foreskin fibroblasts and washed five times in complete medium (RPMI 1640/10% fetal calf serum/2 mM glutamine/penicillin/streptomycin at 100 μg/ml/10 mM Hepes/50 mM β-mercaptoethanol/indomethacin at 10 μg/ml) before use. As previously shown control preparations from noninfected fibroblasts had no biological activity (5). For *in vitro* experiments, parasites were irradiated at 15 kR (1 R = 0.258 mC/kg) from a ^{57}Cs source to prevent multiplication. A soluble extract was prepared by sonication of RH strain tachyzoites harvested from Swiss–Webster mice, as described (13). Cysts of *T. gondii* strain ME-49 (used to study the protective role of IL-12 *in vivo*) were partially purified (18) from brains of BALB/c mice.

Reagents, Cytokines, and Antibodies. *Escherichia coli* lipopolysaccharide was obtained from Sigma. Recombinant

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Abbreviations: NK, natural killer; mAb, monoclonal antibody; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; IL-2 and IL-12, interleukins 2 and 12, respectively; SCID, severe combined immunodeficiency; rIL-12, recombinant IL-12.

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human IL-2 and murine IFN-γ were donated by Cetus and Genentech, respectively. Recombinant TNF-α was purchased from Genzyme. Murine recombinant IL-12 (rIL-12) was prepared as described (19). Rat IgG1 neutralizing monoclonal antibodies (mAbs) produced against either TNF-α (mAb XT-22/11) or IFN-γ (mAb XMG1.6) and an isotype-matched control mAb (GL113) were provided by DNAX. Rabbit anti-asialoganglioside GM1 antibodies were purchased from Wako Bioproducts (Wako, TX). Anti-IL-12 polyclonal antibodies were obtained from rabbits immunized with rIL-12 in complete Freund's adjuvant. Preimmunization sera from the same rabbits (pre-bleed) or an irrelevant rabbit antiserum (control serum) were used as controls in the *in vitro* assays.

Cell Preparation. Spleens were removed asceptically, and single-cell suspensions were prepared in complete medium after lysis of red cells (3). Purified bone-marrow-derived *scid* NK cells were produced by using a modification of a published procedure (4, 5). Peritoneal exudate cells were obtained from BALB/c mice injected 5 days previously with 1.5 ml of 2.3% thioglycollate broth, and the cells were then washed in complete medium containing indomethacin at 5 μg/ml.

Cytokine Assays. Cells (0.1 ml at 6×10^6 cells per ml) were distributed into 96-well flat-bottom microtiter plates (Costar), treated with the different culture additions, and brought to a final 0.2 ml vol with complete medium. Each assay was run in duplicate, triplicate, or quadruplicate. After incubation for 48 hr at 37°C in a 5% CO_2 atmosphere, 50 μl of supernatant was removed and assayed for IFN-γ in a two-site ELISA assay (20) using a rat anti-IFN-γ mAb (HB170) and a polyclonal rabbit serum specific for the cytokine. IFN-γ levels were calculated by reference to a standard curve constructed with recombinant cytokine.

Cytokine mRNA Measurements. Unfractionated spleen cells from *scid* mice or adherent inflammatory macrophages (5×10^6 cells per sample) were incubated in the presence of different stimuli, as indicated in Fig. 2. The cells were then pelleted by centrifugation and resuspended in 0.5 ml of RNAzol B (Tel-Test, Friendswood, TX) for RNA isolation, as described (21). One microgram of total RNA was reverse-transcribed by using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). The reaction mixture was then diluted 1:8, and 10 μl was used for specific semi-quantitative amplification of cytokine mRNA with *Taq* DNA polymerase (Promega). Southern transfers of PCR products were subsequently probed with internal cytokine-specific oligonucleotides and visualized by using the ECL chemiluminescent detection system (Amersham). Nucleotide sequences for sense primers, antisense primers, and probes for hypoxanthine phosphoribosyltransferase (23 cycles), TNF-α (32 cycles), IFN-γ (27 cycles), and IL-2 (32 cycles) were obtained from Svetic *et al.* (21) and cycle numbers, indicated within the parentheses were determined experimentally. Nucleotide sequences for sense primers, antisense primers, and probes and the cycle numbers for IL-12 were as follows: p35, GGCTACTAGAGAGACTTCTTCC, GTGAAGCAGGATGCAGAGCTTC, GCAGATCATTCTAGACAAGGGC (35 cycles); p40, CGTGCTCATGGCTGGTGCAAAG, CTTCATCTGCAAGTTCTTGGGC, TCTGTCTGCAGAGAAGGTCACA (35 cycles).

***In Vivo* Measurement of IL-12 Protective Function.** *Scid* mice were infected when 6 to 8 weeks old by i.p. inoculation with 10 ME-49 strain cysts and treated with either rIL-12 or phosphate-buffered saline. In a second experiment, therapy with recombinant cytokine was combined with either rat (IgG1), control (GL113), or anti-IFN-γ (XMG6) mAbs or rabbit anti-asialoganglioside GM1 antibodies to deplete NK cells. Similar treatments of uninfected mice did not affect survival.

RESULTS

Because of its previously described function as a stimulator of NK-cell proliferation, cytokine synthesis, and cytotoxic activity, we examined the role of IL-12 as a cofactor in the induction by *T. gondii* of NK-cell IFN-γ production. Live tachyzoites or soluble parasite extracts stimulated splenocytes from *scid* mice to produce high levels of IFN-γ previously shown by us to be NK-cell derived. Fig. 1 shows that

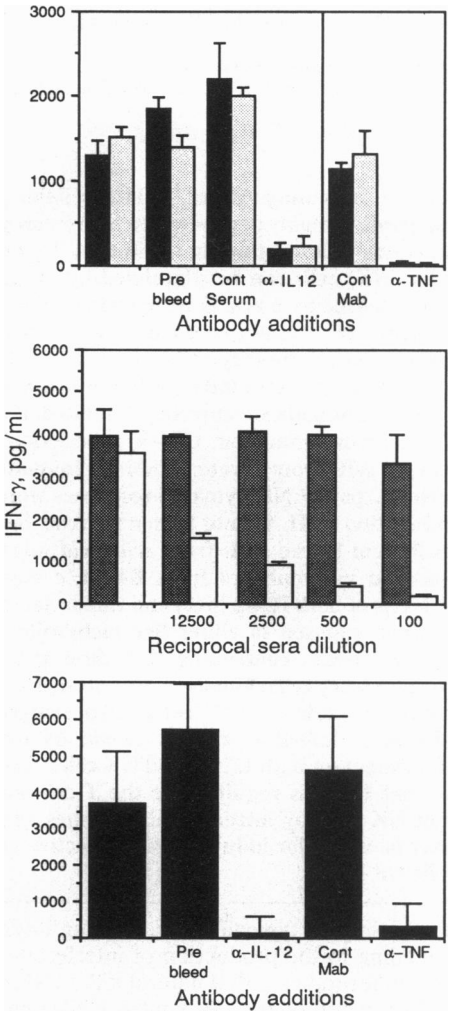

FIG. 1. *T. gondii* induces a T-cell-independent IFN-γ response in NK cells that depends on both IL-12 and TNF-α produced by macrophages. (*Top*) Inhibition of *scid* mouse splenic IFN-γ responses by antibodies to IL-12 (α-IL12) or TNF-α (α-TNF). Spleen cells from CB-17 *scid/scid* mice were stimulated with either irradiated live RH strain tachyzoites (▨) or tachyzoite extract (■) in the presence of rabbit anti-IL-12 antibodies (1:100 dilution; α-IL-12), preimmunization sera from the same animals (Pre-bleed), a rabbit antiserum (Cont Serum) prepared against an irrelevant antigen (schistosome paramyosin), or rat anti-TNF-α (α-TNF) or β-galactosidase (Control; Cont Mab) mAbs (100 μg/ml). After incubation for 48 hr supernatants were removed and assayed for IFN-γ. (*Middle*) Titration of the effects of the anti-IL-12 (□) versus pre-bleed serum (■) on IFN-γ synthesis induced by the *T. gondii* extract. Results are the means and SDs of assays done on triplicate culture wells. (*Bottom*) Inhibitory effect of anti-IL-12 (α-IL-12) or anti-TNF-α (α-TNF) antibodies on the response of bone-marrow-derived NK cells to supernatants of macrophages exposed to tachyzoite extract. Control supernatants from macrophages not exposed to *T. gondii* failed to stimulate an IFN-γ response. Similarly, tachyzoite extracts or irradiated live parasites were without effect when added directly to the purified NK cells in the absence of macrophages (data not shown). Results are representative of data obtained in three experiments.

rabbit antiserum against murine rIL-12 inhibited this response in a dose-dependent manner, reaching almost complete neutralization at the highest concentration tested. In contrast, preimmune sera from the same animals, as well as rabbit antisera against an irrelevant protein, were without effect. As described (5), the *T. gondii*-induced IFN-γ response was also completely inhibited by a neutralizing mAb prepared against TNF-α.

Supernatants from macrophage populations stimulated with *T. gondii* have previously been shown by us (5) to induce IFN-γ production from purified bone-marrow-derived NK cells in a TNF-α-dependent manner. As shown in Fig. 1 *Bottom* this response was totally inhibited by either anti-IL-12 or anti-TNF-α antibodies, indicating a dual requirement for these cytokines in NK triggering.

Cytokine mRNA levels were assayed to confirm the induction of IL-12 synthesis in *scid* spleen cells exposed to *T. gondii* (Fig. 2 *Left*). Up-regulation of mRNAs encoding the p40 subunit of IL-12 was observed in splenocytes exposed to either live tachyzoites, soluble tachyzoite extract, or bacterial lipopolysaccharide. This enhanced expression of p40 was also seen in adherent cells purified from the spleen cultures (data not shown). Constitutive expression of the gene encoding the IL-12 (p35) subunit was seen, as predicted (19, 22). As expected, TNF-α and IFN-γ mRNA synthesis was also induced by either the parasite or endotoxin stimuli, whereas no increase in IL-2 transcription was detected.

The results of Fig. 2 *Right* implicate the macrophage as the likely source of both the IL-12 and TNF-α measured in these assays. Thus, when stimulated with either *T. gondii* or endotoxin, purified peritoneal macrophages expressed increased levels of IL-12 (p40) and TNF-α mRNAs. This expression was markedly augmented (6- to 8-fold for p40, 3- to 5-fold for TNF-α) when the macrophages were simultaneously exposed to exogenous IFN-γ, the endproduct of the NK-cell response.

The above findings suggested that the T-cell-independent induction of IFN-γ by *T. gondii* depends on the dual production of IL-12 and TNF-α from macrophages. Consistent with this hypothesis is the observation (Fig. 3) that IL-12 synergizes with TNF-α in inducing IFN-γ synthesis in both unfractionated scid spleen cells as well as in purified bone-marrow-derived NK cells. As expected from previous studies (19, 22, 23), at higher levels, IL-12 alone induced some cytokine production in both cell populations. In contrast, TNF-α alone was without effect at all concentrations tested.

To further investigate the requirement for TNF-α in the induction of IFN-γ synthesis by NK cells, bone-marrow or spleen-derived NK cells were exposed to different concentrations of IL-12 with or without anti-TNF-α mAb. As shown in Fig. 4 *Top*, anti-TNF-α blocked the low level of IFN-γ synthesis induced by IL-12 alone on scid spleen cells, as well as the higher synergistic effect stimulated by IL-12 plus TNF-α. Nevertheless, when bone-marrow-derived NK cells

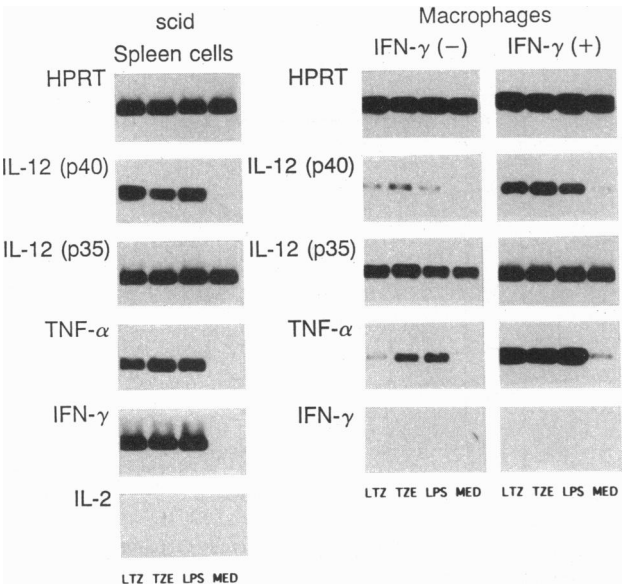

FIG. 2. *T. gondii* triggers *scid* spleen cells or peritoneal macrophages to express IL-12 (p40) and TNF-α mRNAs. (*Left*) Unfractionated spleen cells from scid mice were incubated with live tachyzoites (LTZ), tachyzoite extracts (TZE), bacterial endotoxin (LPS), or medium (MED) for 6 hr at 37°C. Semiquantitative reverse transcriptase-PCR was then done on RNA from each sample, and the products were visualized after Southern transfer. (*Right*) Thioglycollate-elicitated macrophages from BALB/c mice were purified by adherence to plastic and incubated with either tachyzoites (LTZ), tachyzoite extracts (TZE), bacterial endotoxin (LPS), or medium (MED) for 6 hr in the presence or absence of IFN-γ (200 units/ml). Cytokine mRNA levels were then determined, as described above. Data are representative of three experiments. HPRT, hypoxanthine phosphoribosyltransferase.

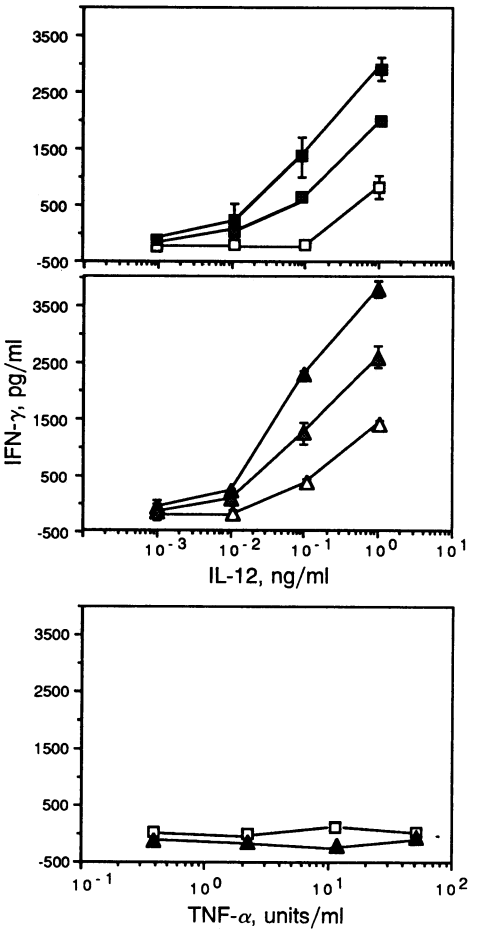

FIG. 3. Synergistic effect of IL-12 and TNF-α on induction of IFN-γ synthesis by NK cells. Spleen cells (*Top*) or bone-marrow cells (*Bottom*) from *scid* mice were used as sources of either unfractionated or purified NK cells, respectively. Cell preparations were cultured in the presence or absence of different concentrations of IL-12 and/or TNF-α, and IFN-γ synthesis was measured in the supernatant at 48 hr. *Top* and *Middle* show the effect of different concentrations of IL-12 in the absence (open symbols) or presence of 5 (shaded symbols) or 25 (closed symbols) units of recombinant murine TNF-α per ml. TNF-α in the absence of IL-12 failed to stimulate NK cells to produce measurable amounts of IFN-γ (*Bottom*). Data are the means and SDs of triplicate culture assays; these experiments were repeated three times with similar results.

were used (*Bottom*) TNF-α neutralization failed to inhibit IL-12-induced IFN-γ synthesis, while clearly blocking the synergistic response induced by IL-12 plus TNF-α. This discrepancy is discussed below.

To test whether IL-12 could stimulate the T-cell-independent pathway of NK IFN-γ synthesis *in vivo*, *scid* mice were injected with rIL-12 during infection with a avirulent strain of *T. gondii*. Control infected mice receiving PBS alone all died between 9 and 16 days of infection (Fig. 5). In contrast, most rIL-12-injected mice survived for 30 days or longer. This prolongation of survival was blocked when the animals were simultaneously treated with either anti-IFN-γ or asialoganglioside GM1 antibodies (*Bottom*), suggesting that the effect of rIL-12 depends on IFN-γ and NK cells. Cyst formation is an indicator of chronic *Toxoplasma* infection in immunocompetent hosts. Early-stage cysts were observed in brain sections of rIL-12-infected *scid* mice, whereas only tachyzoites, the rapidly replicating form of the parasite, were evident in equivalent sections from infected control mice (data not shown).

DISCUSSION

IL-12 (originally designated NKSF for NK stimulatory factor) has previously been shown to exert a wide range of effects on NK cells, including enhancement of cytolytic activity and proliferation and the induction of IFN-γ synthesis (19, 22–24). The IL-12 heterodimer has recently been shown to be selectively up-regulated in stimulated adherent peripheral blood mononuclear cells (25). The results presented here indicate that IL-12 is also synthesized by highly enriched tissue macrophage populations when specifically triggered by exposure to an intracellular parasite or bacterial lipopolysaccharide and that its production is enhanced by IFN-γ activation. More importantly, our findings establish that this IL-12 response is essential for the T-cell-independent induction of NK-cell IFN-γ synthesis, a reaction previously shown in both the *Listeria* and *Toxoplasma* models to depend on another monokine, TNF-α (3, 4, 17). The selective triggering of this pathway can now be explained as resulting solely from a synergy between the two cytokines. Once initiated, the NK-cell response could be augmented by its own endproducts, such as IFN-γ (Fig. 2 *Right*), TNF-α, or by T-cell-derived IL-2 (3, 4, 17).

Although TNF-α clearly synergizes with IL-12 in the induction of NK-cell IFN-γ responses, IL-12 on its own (Figs. 3 and 4) or in combination with IL-2 (22) can also trigger IFN-γ production. Nevertheless, it is possible that in the latter situations TNF-α endogenously synthesized by NK cells (26) upon stimulation with IL-12 promotes the response by the same synergistic mechanism. In support of this hypothesis, we showed that activation of *scid* spleen cells by IL-12 alone is completely blocked by anti-TNF-α mAb. In contrast, neutralization of TNF-α had no significant effect on IL-12-induced IFN-γ synthesis by bone-marrow-derived NK cells. The latter NK-cell populations were derived in the presence of exogenous IL-2 and, in contrast to spleen cells (Fig. 2), express high levels of TNF-α mRNA before exposure to rIL-12 (data not shown). Therefore, the bone-marrow-derived NK cells were probably already primed by TNF-α before exposure to IL-12 and anti-TNF-α mAb.

In addition to *T. gondii*, a number of different microorganisms that infect macrophages can trigger T-cell-independent IFN-γ synthesis by NK cells. We speculate that the induction of IL-12 by macrophages is a common property of these parasites. Although bacterial endotoxin can also stimulate IL-12 production (Fig. 2), it is clear from previous studies that

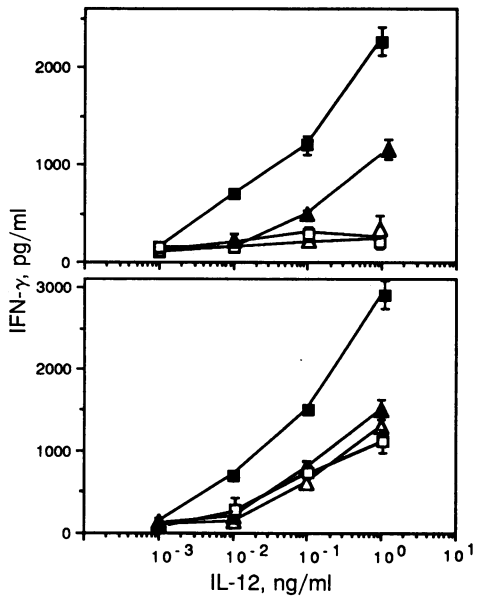

FIG. 4. Inhibitory effect of anti-TNF-α mAb on IL-12-induced IFN-γ synthesis by NK cells. Spleen cells (*Upper*) or bone marrow (*Lower*) from *scid* mice were cultured with different concentrations of IL-12 with (□) or without (△) TNF-α (25 units/ml) and anti-TNF-α (50 μg/ml) (□, △) or control (50 μg/ml) mAb (■, ▲). IFN-γ synthesis was measured in the supernatants obtained at 48 hr. The data were replicated as in Fig. 3.

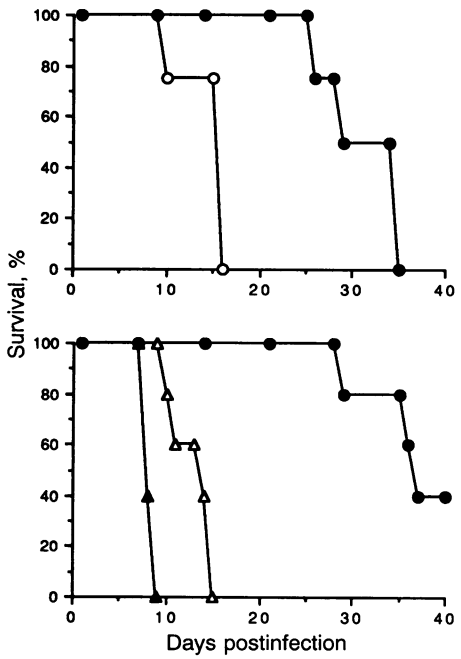

FIG. 5. rIL-12 protects *scid* mice against *T. gondii* infection by inducing NK-cell IFN-γ production. (*Upper*) *In vivo* measurement of IL-12 protective function. CB-17 *scid* mice were infected by i.p. inoculation with 10 cysts of *T. gondii* (ME-49 strain). The animals (four per group) were also i.p. injected with rIL-12 (1 μg/ml) (●) or phosphate-buffered saline/0.5% of bovine serum albumin (○) every day throughout the experiment beginning 2 days before infection. (*Lower*) Protective effect of rIL-12 is abolished by simultaneous neutralization of IFN-γ or depletion of NK cells. Therapy with recombinant cytokine was combined with weekly injections (2 mg per mouse) of either control (GL113) (●) or anti-IFN-γ (XMG-6) mAbs (▲). Alternatively, treatment with IL-12 was combined with thrice weekly injections of rabbit anti-asialoganglioside GM1 antibodies (Wako Bioproducts) (50 μl per mouse) to deplete NK cells (△). Survival was monitored daily, and cumulative mortality was calculated at the end of 40 days. Similar results were obtained in a second experiment.

lipopolysaccharide is not responsible for the IFN-γ response induced by *T. gondii* as well as other pathogens (2, 5, 17). Regardless of the nature of the stimulus, the induction of this T-cell-independent pathway may be crucial in explaining the strong cell-mediated immunity triggered by many of these organisms. Thus, the early production of IFN-γ by NK cells, by suppressing the proliferation of Th2 cells (27), could determine the subsequent selection of Th1 $CD4^+$ T lymphocytes (T. M. Scharton and P. A. Scott, personal communication) responsible for the synthesis of protective cytokines such as IL-2 and IFN-γ. Additionally, the induction of IFN-γ synthesis by NK cells could contribute to innate resistance and, in the case of organisms such as *T. gondii* may provide a means for limiting virulent-pathogen replication in the interval before the appearance of T-cell-dependent control of infection. Indeed, the ability of anti-IFN-γ to reduce the resistance of *scid* mice to strain ME-49 challenge (Fig. 5) argues that this pathway may provide a partial barrier to *T. gondii* infection *in vivo*.

The finding that IL-12 is an important mediator of IFN-γ synthesis by NK cells suggested that rIL-12 could be used to augment natural resistance to the parasite in T-cell-deficient hosts. Indeed, injection of the cytokine into *scid* mice caused a marked prolongation in the survival of mice infected with *T. gondii* (Fig. 5 *Top*) and resulted in the appearance of immature cysts in the brains of the same animals. This prolongation of survival was found to depend on both IFN-γ- and asialoganglioside GM1-positive cells (Fig. 5 *Bottom*), arguing for the involvement of the T-cell-independent NK-cell pathway. Treatment with higher doses of IL-12 or combination with different cytokines or chemotherapy may lead to a greater resistance against the parasite. Regardless, the initial data presented here establish the feasibility of using IL-12 for inducing resistance against opportunistic infections in immunocompromised hosts and support previous studies suggesting that this cytokine could be used to augment IFN-γ-dependent immunity in AIDS patients (28).

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