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In the Absence of Endogenous IFN-γ, Mice Develop Unimpaired IL-12 Responses to *Toxoplasma gondii* While Failing to Control Acute Infection

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The relationship between IFN-γ and IL-12 in generating innate immune responses and resistance to acute *Toxoplasma gondii* infection was assessed in *T. gondii*-exposed IFN-γ knockout (gko) mice. Gko mice, in contrast to wild-type (wt) animals, rapidly succumbed to infection with either the avirulent ME49 strain or, surprisingly, an attenuated temperature-sensitive mutant strain, ts4. Microscopic examination of peritoneal exudates from infected gko mice demonstrated that mortality is associated with unchecked tachyzoite replication. Nevertheless, both wt and gko animals developed a peritoneal inflammatory response that in gko animals was greater due to a 5- to 10-fold increase in the number of granulocytes recruited to the site of infection. In addition, IL-12 production in gko mice was both unimpaired and functional since a significant, albeit lower than wt, IL-12-dependent NK cell response developed in these animals. Regardless, no evidence for an IFN-γ-independent protective function for IL-12 or NK cells was apparent since in vivo treatment of gko mice with an IL-12-neutralizing mAb ablated the NK cell response, but did not decrease survival. Together, these data identify distinct functions for IL-12 and IFN-γ in host resistance to *T. gondii*: IL-12 precedes and initiates synthesis of IFN-γ, while the latter lymphokine directly controls parasite growth and diminishes the contribution of IL-4- and IL-5-producing T cell subsets. *The Journal of Immunology,* **1996, 157: 4045–4054.**

Infection with the intracellular protozoan *Toxoplasma gondii* is characterized by an acute phase during which the rapidly dividing tachyzoite form disseminates throughout the body, a result of the parasite's ability to invade and replicate within virtually all nucleated cells. If left unchecked by the immune system, extensive tissue damage occurs at this stage. In contrast, chronic infection is associated with the dormant bradyzoite form of the parasite and is largely asymptomatic. The latter parasite stage resides in tissue cysts that are maintained indefinitely in the central nervous system and other host tissues (1, 2).

The absence of pathology in chronic toxoplasmosis is attributed to the early induction of a strong cell-mediated immune response that limits parasite replication in acute infection and later acts to prevent reactivation of encysted bradyzoites. Innate immune mechanisms play a critical role in the initial control of tachyzoite growth (3, 4). Thus, despite the absence of T cells, SCID[3] or athymic nude mice are resistant to infection for several weeks (5). This transient control of parasite expansion is attributed to NK cell production of IFN-γ since the animals succumb more rapidly following depletion of either response element (6–8). IFN-γ is known to be essential for adaptive as well as innate immunity to *T. gondii* and is thought to function by activating macrophages and possibly nonphagocytic host cells to limit intracellular growth of tachyzoites (9, 10). IFN-γ may also contribute to the selective development of Th1-type adaptive immunity to *T. gondii*, as has been described in several other intracellular infection models (11, 12). Given its central role in resistance to *Toxoplasma*, we and others have sought to define the exact mechanism(s) by which the parasite stimulates IFN-γ production during early infection.

In vitro studies with splenocytes from SCID mice have been used to identify the T cell-independent pathway by which *T. gondii* stimulates IFN-γ synthesis (7, 8, 13). These cells produce high levels of the cytokine when exposed to live tachyzoites or tachyzoite extracts. The observed response is dependent upon the synthesis of IL-12, TNF-α, and IL-1β produced by macrophages in the cultures (7, 8, 13). Of these monokines, IL-12 appears to play the dominant role, promoting resistance to infection, in both immunologically compromised and intact animals. Thus, treatment of infected animals with IL-12-specific neutralizing mAbs has been shown to both reduce IFN-γ synthesis and ablate resistance in wt and SCID mice (7, 14, 15). Conversely, administration of rIL-12 augments IFN-γ production as well as control of acute infection (7, 15–17).

Based on the above evidence, we have speculated that the induction of IFN-γ synthesis by NK cells following *T. gondii* infection is indirect, involving a secondary effect of IL-12 rather than direct stimulation by the organism (3, 4). Nevertheless, the actual sequence of IL-12 and IFN-γ production and the distinct functions of these cytokines have not yet been defined during in vivo infection. According to the model proposed above, enhanced IL-12 synthesis precedes IFN-γ production, and the antiparasitic effects of IL-12 are attributed, in large part, to its potent IFN-γ-inducing

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[3] Abbreviations used in this paper: SCID, severe combined immunodeficient; gko, interferon-γ knockout mice; NO, nitric oxide; PEC, peritoneal exudate cell; STAg, soluble tachyzoite antigen; wt, wild-type mice.

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activity. In contrast, Carrera et al. recently published that bone marrow macrophages are incapable of synthesizing IL-12 p40 mRNA in response to *Toxoplasma* unless the cells have been primed with IFN-γ (18). We have obtained comparable results with both resident peritoneal as well as bone marrow macrophages (our unpublished results) and previously reported that the production of IL-12 by *T. gondii*-stimulated inflammatory macrophages is enhanced greatly in the presence of IFN-γ (14). Together, the latter observations suggest that IFN-γ derived from a parasite-stimulated cell population such as NK cells might be required to initiate IL-12 production by macrophages.

The suggestion that IFN-γ synthesis may precede IL-12 production, combined with the known role of IL-12 in host resistance to *T. gondii*, has underscored the question of whether IL-12 itself is capable of directly controlling *T. gondii* replication. In fact, a direct contribution of IL-12 to immunity to this parasite has been suggested by Khan et al. (17) based on the observation that host survival is enhanced by IL-12 treatment of mice with a defective IFN regulatory factor-1 gene. The latter study is supported by other data demonstrating that resistance to another intracellular pathogen, *Listeria monocytogenes*, can be induced in the absence of endogenous IFN-γ (19).

In the present work, we have directly examined the relationship between IFN-γ and IL-12 in innate resistance to *T. gondii*. Our approach was to analyze host resistance, cytokine synthesis, and NK cell function in *T. gondii*-infected mice with a targeted disruption of the IFN-γ gene. These knockout mice (gko) fail to synthesize detectable levels of IFN-γ (20) and therefore allow an analysis of the host immune response to *T. gondii* in the absence of this biologically important cytokine. Our results formally establish that the early induction of IL-12 and other innate immune responses in acute *T. gondii* infection does not require IFN-γ. At the same time, the findings of this study underscore the critical importance of the latter cytokine in host resistance to this parasite.

Materials and Methods

Experimental animals

Breeding pairs of mice with a targeted disruption of the IFN-γ gene (gko mice) (20) were generously provided by Dyana Dalton and T. Stewart (Genentech, San Bruno, CA). Breeding stock backcrossed on either the C57BL/6 or BALB/c backgrounds were obtained and further backcrossed to the seventh generation for each strain. The genotype of the mice was determined by PCR of DNA isolated from tail snips or blood using the following primers synthesized by Operon Technologies (Alameda, CA): Neo.sense, 5′-TTG AAC AAG ATG GAT TGC ACG CAG G; Neo.anti-sense, 5′-GGC TGG CGC GAG CCC CTG ATG CTC T; IFN-γ.sense, 5′-AGA AGT AAG TGG AAG GGC CCA GAA G; and IFN-γ.anti-sense, 5′-AGG GAA ACT GGG AGA GGA GAA ATA T. Products of 250 bp were indicative of a wt (+/+ phenotype); 550 bp, a gko phenotype (−/−); and both 250 and 550 bp, a heterozygous (+/−) phenotype. Knockout (−/−) and wt (+/+) animals were obtained routinely from homozygous or heterozygous inbreeding. Mice were bred and housed in specific pathogen-free conditions and utilized for experiments at 5 to 10 wk of age.

Parasites and parasite Ags

Tachyzoites of the virulent RH and temperature-sensitive mutant ts4 strains were maintained in vitro by infection of human foreskin fibroblasts and biweekly passage in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 1% FCS (HyClone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cysts of the avirulent ME49 strain (initially provided by Dr. J. Remington, Palo Alto Research Foundation, Palo Alto, CA) were harvested from the brains of C57BL/6 mice that had been inoculated with approximately 20 cysts i.p. 1 mo prior. For experimental infections, gko or wt mice received 20 ME49 cysts or PBS (BioWhittaker, Walkersville, MD). Control inoculations with normal brain suspensions failed to elicit detectable inflammatory responses, NK cell cytotoxicity, or significant increases in cytokine levels (data not shown). Mice exposed to the ts4 strain received 2×10^4 tachyzoites i.p. in 0.5 ml of PBS. Mice challenged with RH were inoculated with 2×10^6 irradiated (15 krad) tachyzoites i.p. in 0.5 ml of PBS at day 0 and day 14. Soluble tachyzoite Ag (STAg) was prepared from sonicated tachyzoites of the RH strain after centrifugation at 10,000 × *g* in the presence of protease inhibitors and dialysis against PBS. The preparations were then filtered through a 0.2-μm membrane and stored at −80°C (21).

Differential cell counts

Cytocentrifuge smears were prepared from 1.5×10^5 PEC in a Cytospin (Shandon Lipshaw, Pittsburgh, PA) set for 5 min at 1000 rpm. Slide preparations were fixed in absolute methanol for 5 min and then stained with Diff-Quik (Baxter Healthcare Corporation, McGaw Park, IL), a modified Wright-Giemsa stain, as specified by the manufacturer. Differential analyses were performed on 200 to 400 cells using an oil immersion (×100 objective). In some experiments, the eosinophil and neutrophil composition was verified by staining preparations with Fast Green FDF/Neutral Red (Sigma Chemical Co., St. Louis, MO).

In vivo rIL-12 and anti-IL-12 treatments

Mice were treated i.p. with rIL-12 (generously provided by Genetics Institute, Cambridge, MA; 5.6×10^6 U/mg) at doses of 1, 0.5, or 0.1 μg in 500 μl of PBS for 5 consecutive days beginning on the day of parasite challenge. Control animals received an equal volume of saline. For IL-12 depletion, mice were treated 1 day before infection with 1 mg of anti-IL-12 mAb from a cell line (C17.8-rat IgG2a) generously provided by M. Wysocka and G. Trinchieri (Wistar Institute, Philadelphia, PA). The ascites used was obtained from Harlan Bioproducts for Science (Indianapolis, IN) from nude mice inoculated with the hybridoma. Control mice were treated with normal rat Ig (Sigma Chemical Co.).

Serum preparation

Blood was collected from mice at the time of sacrifice and allowed to clot at room temperature for 2 h. Serum was then separated from the individual samples following a 5-min centrifugation at 5000 rpm and analyzed for cytokines within 24 h following preparation.

Macrophage preparation and in vitro culture

Inflammatory macrophages were obtained from uninfected gko or wt mice that had been injected 4 days previously with 1.5 ml of 3% thioglycolate (Sigma Chemical Co.). The resulting PEC were harvested and plated at 2×10^5 per well in 96-well plates for 2 h, and the nonadherent cell population was removed. The adherent cells were then incubated in the presence of medium alone, LPS (100 ng/ml; Sigma Chemical Co.), RH tachyzoites (0.1 or 1 per cell), or STAg (5 μg/ml), as previously described (22). Supernatants were harvested at 24 h, and IL-12 p40 was measured by ELISA, as described below.

Cell cultures from infected animals

Single cell suspensions were prepared from spleen and peritoneal cells harvested at various time points postinfection. Spleen cells from animals exposed to irradiated RH were collected at d35 (21 days following the second exposure to the parasite). Peritoneal cells were cultured at 4×10^5 cells, and spleen cells at 8×10^5 per well in a total volume of 200 μl in a medium consisting of RPMI 1640 (BioWhittaker) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (mM), and 2-ME (5×10^{-5} M) in the presence or absence of STAg (5 μg/ml). Supernatants were harvested 48 h later for IL-12 and nitrite determinations or at 72 h for IFN-γ, IL-4, and IL-5 measurements.

Measurement of nitric oxide

Nitrite (NO_2^-) levels were used as an indicator of reactive nitrogen intermediates in samples, and were measured by the Griess assay (23). Briefly, 100-μl aliquots of supernatant were added to 96-well plates followed by 100 μl of a 1:1 mixture of 1% sulfanilamide dihydrochloride (Sigma Chemical Co.) in 2.5% H_3PO_4 and 0.1% naphthylethylenediamide dihydrochloride (Sigma Chemical Co.) in 2.5% H_3PO_4. After a 10-min incubation at room temperature, the absorbance of the samples (A_{550}) was read spectrophotometrically and units of nitrite (range of sensitivity: 4 to 250 μM) were determined by comparison with a standard curve generated with sodium nitrite ($NaNO_2$) (Sigma Chemical Co.).

Cytokine assays

The following procedures were used to quantitate levels of cytokines in sera, cell supernatants, or spleen and peritoneal cell populations.

IFN-γ, IL-4, IL-5 protein. IFN-γ and IL-5 were assayed by two-site ELISA, as previously described (24, 25). Cytokine levels were quantitated

by reference to standard curves produced with rIFN-γ (generously provided by Genentech) or rIL-5 (donated by DNAX Research Institute, Palo Alto, CA). IL-4 was measured by the CT4S bioassay (26) and quantitated by reference to a standard curve generated with rIL-4 (1 U = 45 pg) (DNAX Research Institute). Neutralizing mAb (11B11) (27) was added to test supernatants as a control for the specificity of the bioassay.

IL-12 p40/p75 protein. Levels of IL-12 p40 were assayed by two-site ELISA, as previously described (28), using mAb from cell lines originally generated by M. Wysocka and G. Trinchieri. The assay was sensitive to a range of 20 to 2500 pg/ml. Reagents for ELISA measurement of IL-12 p75 protein were generously provided by David H. Presky at Hoffmann-La Roche (Nutley, NJ). In this assay, rat anti-mouse p75 (9A5) mAb was used as the coating Ab, and peroxidase-labeled rat anti-mouse IL-12 p40 (5C3) was used as the developing Ab (29). The assay was sensitive in a range of 20 to 1200 pg/ml. rIL-12 (provided by Genetics Institute) was used as a standard in both assays.

Measurement of NK cell functional activity

Assays of NK cell-mediated target lysis were conducted as previously described (30). Briefly, YAC-1 lymphoma target cells were incubated for 1 h with 500 μCi of $Na^{51}CrO_4$ (Amersham, Arlington Heights, IL), followed by extensive washing in RPMI media. The target cells were resuspended at 1×10^5/ml in RPMI media (as described above) and 50 μl plated into single wells of a 96-well round-bottom plate. Effector cells were obtained from spleens and peritoneal exudates of individual animals. E:T ratios of 100:1, 50:1, 25:1, 12:1, and 6:1 were used in a total volume of 150 μl. Plates were incubated for 5 h at 37°C in a CO_2 incubator. Chromium release into the supernatants was determined with a gamma counter (Beckman Instruments, Palo Alto, CA). The specific ^{51}Cr release was determined as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%. Maximum chromium release and spontaneous release were calculated from wells in which 100 μl of 10% SDS (Sigma Chemical Co.) or media alone, respectively, was added in place of effector cells.

Statistical analyses

Statistical determinations of the difference between means of experimental groups were performed using an unpaired, two-tailed Student's *t* test.

Results

Enhanced susceptibility of gko mice to acute T. gondii *infection*

Mice treated with neutralizing anti-IFN-γ mAb show increased susceptibility to infection with an avirulent strain of *T. gondii* (9). To determine whether targeted disruption of the IFN-γ gene also leads to a loss in resistance to the parasite, gko mice on the BALB/c or C57BL/6 backgrounds were infected with cysts of the ME49 strain of *T. gondii* and their survival compared with that of littermate controls. As shown in Figure 1*A*, BALB/c gko mice all succumbed within 9 days of parasite inoculation, while 100% of the infected control littermates survived during the 30 days of the experiment. Essentially identical results were obtained when gko mice bred onto the C57BL/6 background were challenged with ME49 (mean survival time 9 ± 0, $n = 4$). Because of their greater availability, BALB/c gko mice were used in all subsequent experiments. To determine whether the acute susceptibility of gko mice to ME49 extends to other strains of *T. gondii*, gko mice were infected with the normally avirulent, temperature-sensitive isolate, ts4 (31). Unexpectedly, we found that gko mice were highly susceptible to infection with this normally attenuated parasite strain and succumbed with a time course (Fig. 1*B*) comparable with that observed with ME49-infected gko animals. The above observations confirm the importance of IFN-γ in resistance to *T. gondii* as well as the absence of redundant mechanisms of parasite elimination in mice with the gko defect. Unless otherwise indicated, the ME49 strain was used for the remainder of the experiments presented.

To determine whether the death of infected gko animals is associated with increased parasite growth, peritoneal cells were recovered from the mice at days 0, 3, 5, and 7 after inoculation with ME49, and the presence of intracellular tachyzoites was measured. As shown in Figure 1*C*, parasites were found in peritoneal exudates of both wt and gko animals, although significantly more infected cells were detected in the gko mice. In these animals, a dramatic increase in the number of infected cells occurred between 3 and 5 days postinfection (Fig. 1C), and by day 7 more than 50% of the recovered cells were infected (data not shown). Moreover, histopathologic examination at the time of death revealed the presence of tachyzoites in lung and liver sections from gko animals, indicating that metastasis of the infection had occurred (data not shown). gko mice inoculated with ts4 showed similar evidence of parasite expansion. At 5 days, 25% of peritoneal macrophages were infected in these animals, while no organisms were detected in exudates from wt mice that had been exposed to ts4.

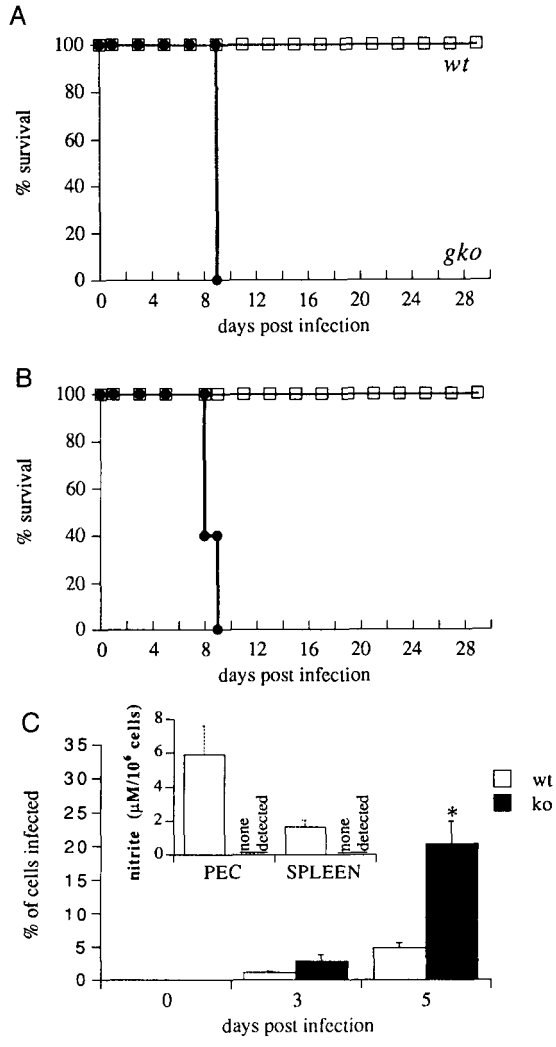

FIGURE 1. Lack of resistance of gko mice to *T. gondii* infection. Gko mice were infected with cysts of the ME49 strain (*A* and *C*) or tachyzoites of the ts4 strain (*B*). Cumulative mortality was followed in four mice per group (*A* and *B*). The number of infected peritoneal cells was assessed in wt (open bars) and gko (solid bars) mice by evaluating Diff-Quik-stained Cytospin smears of exudates harvested at days 0, 3, and 5 following infection (*C*). A significant difference (Student's *t* test, $p \leq 0.05$) in the number of infected cells is denoted by an asterisk. Comparable results were obtained with exudates from the ts4 strain (data not shown). Spontaneous production of NO (measured as nitrite) was assessed in 48-h supernatants of peritoneal cells harvested from 5-day-infected wt (open bars) and gko (solid bars) animals (*C, inset*).

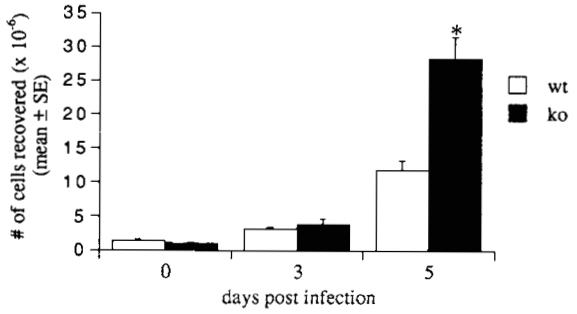

FIGURE 2. Cellular infiltration at the site of infection in wt (open bars) and gko (solid bars) mice at 3 and 5 days following i.p. inoculation with ME49. PEC were collected and enumerated in a hemocytometer. Data shown reflect the mean ± SD for five mice per group. An asterisk denotes a statistically significant difference ($p \leq 0.05$) between the recoveries from wt and gko animals. Similar results were observed in two additional experiments.

Previous studies have suggested that nitric oxide (NO) is an important mediator of IFN-γ-dependent resistance in *T. gondii* infection (17, 32–34). Consistent with this hypothesis, spontaneously produced NO was detectable in cultures of spleen and peritoneal cells harvested from infected wild-type animals, but was virtually absent from equivalent cultures derived from gko mice (Fig. 1*C*, *inset*).

Altered inflammatory response to T. gondii *in gko mice at the site of infection*

To characterize differences in the response to *T. gondii* in gko mice that might correlate with their enhanced susceptibility to infection, we examined the composition of the inflammatory cell populations elicited by *T. gondii* at the site of infection, the peritoneal cavity. Inoculation of both wt and gko mice with cysts of ME49 resulted in the influx of a mixed leukocyte population that was first evident at 3 days and increased dramatically by 5 days postinfection (Fig. 2). At this later time point, exudates from gko mice contained over twice the number of cells as the corresponding exudates from wt animals. A comparable increase in peritoneal cell numbers was observed when gko mice were inoculated with ts4 tachyzoites (data not shown). Thus, *T. gondii*-elicited inflammation is in part IFN-γ independent.

Before inoculation with parasites, exudates from wt and gko mice were indistinguishable, consisting primarily of macrophages with smaller numbers of granulocytes, lymphocytes, and mast cells (Figs. 3 and 4). By 5 days after infection with ME49, the composition of peritoneal populations from both wt and gko mice was clearly altered (Figs. 3 and 4). In exudates from wt animals, this change consisted of an influx of macrophages and, to a lesser extent, lymphocytes and granulocytes. Granulocyte infiltration was particularly pronounced by 5 days postinfection, as indicated by a 50-fold increase in this cell type. At this time point, exudates from wt mice consisted of approximately 70% large mononuclear cells, 17% neutrophils, 10% small lymphocytes, and ≤2% eosinophils and mast cells. In exudates from gko mice, however, the changes between cell populations were even more striking, with granulocyte levels approaching 40% of the total exudate population (Fig. 3). Moreover, the increase in peritoneal eosinophils was more pronounced in gko mice, suggesting that Th2-type responses might be enhanced in these animals. In addition, the number of mast cells recovered from gko mice at 5 days postinfection ($12.0 \pm 0.8 \times 10^4$) was significantly higher ($p < 0.05$) than that recovered from

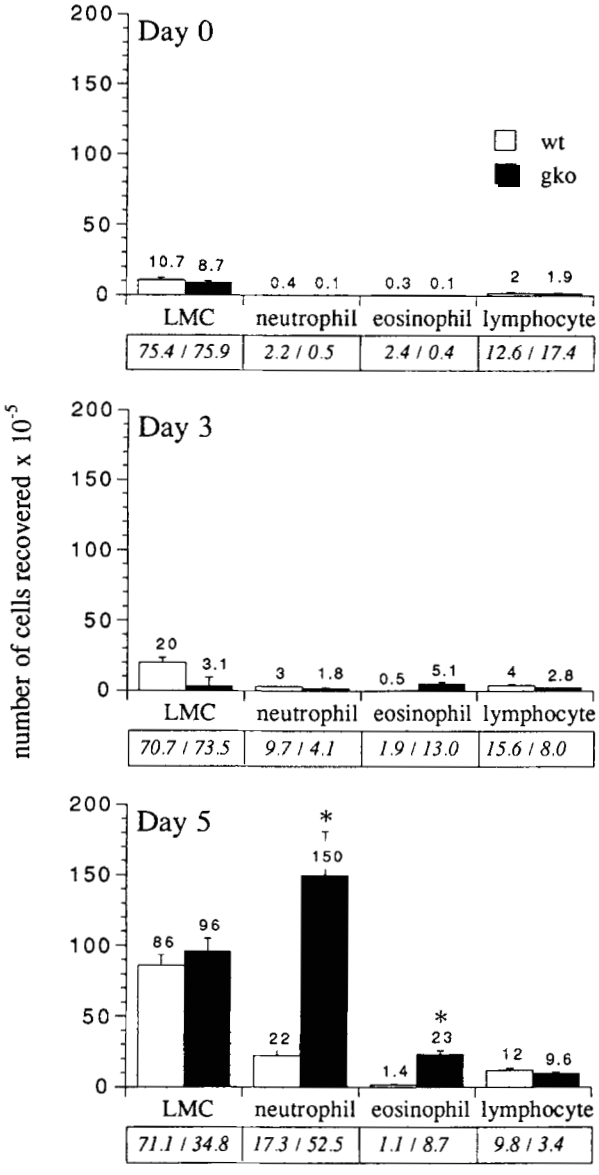

FIGURE 3. Qualitative analysis of the PEC present in wt (open bars) and gko (solid bars) before and 3 and 5 days following infection with ME49. Cytospin smears of peritoneal cells were prepared and stained with Diff-Quik reagent, as described in *Materials and Methods*. The values printed above the data bars are the actual number of cells recovered × 10^{-5}, as calculated by multiplying the percentage of each cell type (large mononuclear cells (LMC), neutrophil, eosinophil, or small lymphocyte) by the total number of cells recovered for each animal. Below each graph is the mean percentage, expressed as a function of the total cell composition, for each cell type. Data shown reflect the mean ± SE for five mice per group. An asterisk indicates a statistically significant difference between the values observed in wt and gko samples ($p \leq 0.05$).

naive gko mice ($6.4 \pm 1.4 \times 10^4$) or from 5-day-infected wt mice ($8.5 \pm 1 \times 10^4$).

As noted above (Fig. 1*C*), tachyzoite-infected cells were evident in both wt and gko mice, with significantly higher numbers in the gko animals. Intracellular parasites were most prevalent in macrophages and monocytes, although a significant number of infected neutrophils was also observed (wt, 9.6 ± 1.2% (range 7.4–13.6%); gko, 14.5 ± 2.7% (range 8.9–21.9%)). Interestingly, infected neutrophils from either gko or wt mice rarely contained more than two

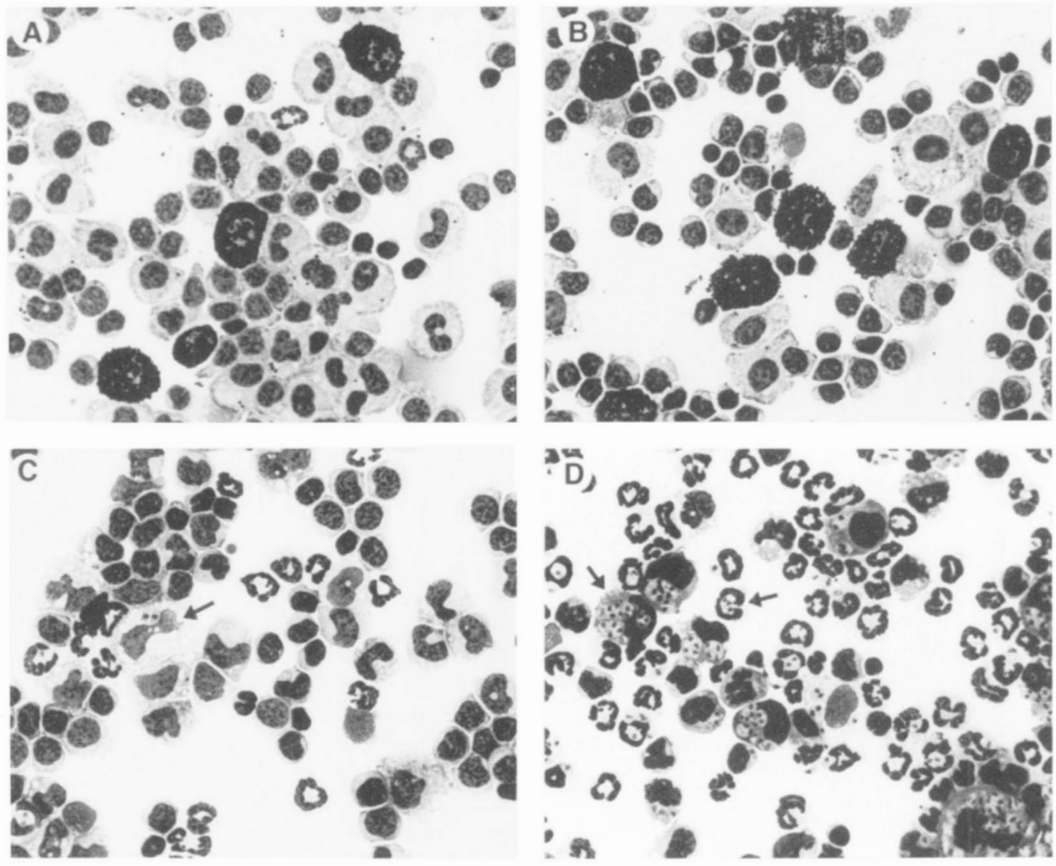

FIGURE 4. Photomicrographs of stained Diff-Quik smears comparing the composition of peritoneal exudates from wt (*A* and *C*) and gko (*B* and *D*) mice (original magnification is ×630). Cells were collected before (*A* and *B*) and 5 days after (*C* and *D*) i.p. inoculation of ME49.

parasites, whereas eight or more intracellular tachyzoites were typically observed in macrophages/monocytes (Fig. 4).

Unaltered IL-12 response of gko-derived inflammatory macrophages to T. gondii

Previous work from this laboratory indicated that the synthesis of IL-12 by inflammatory macrophages stimulated with *T. gondii* is markedly up-regulated in the presence of IFN-γ (7, 14). This finding raised the question of whether or not the production of IL-12 observed in the absence of in vitro exposure to IFN-γ may have resulted from in vivo priming of macrophages by the cytokine. We therefore tested the ability of inflammatory macrophages derived from gko mice to produce IL-12 in response to either *T. gondii* (RH strain) infection or to a soluble tachyzoite extract (STAg). As shown in Table I, gko- and wt-derived macrophages produced indistinguishable levels of IL-12 p40 when triggered by RH infection, STAg, or LPS. Thus, endogenous IFN-γ is not required for the induction of IL-12 by *T. gondii*-stimulated macrophages.

Table I. *In vitro production of IL-12 p40 by thioglycollate-elicited inflammatory cells from wt and gko mice*

Stimulus		IL-12 p40 (pg/ml)[a]	
		Wild Type	gko
Medium		638 ± 1105	1152 ± 501
LPS	100 ng/ml	9084 ± 1112	9860 ± 293
T. gondii	RH 1:10[b]	3078 ± 302	4299 ± 611
	RH 1:1	3689 ± 365	4508 ± 579
	STAg 5 μg/ml	13851 ± 1757	13509 ± 961

[a] IL-12 p40 production was measured in 24-h culture supernatants of thioglycollate-elicited peritoneal cells from gko or wt animals. Cells from five mice were pooled and cultured in triplicate in the presence of the indicated stimuli. Data are the mean ± SD of measurements on individual cultures. Similar results were observed in two additional experiments.

[b] Ratio of tachyzoites to host cells.

T. gondii*-infected mice display normal IL-12 production*

To determine whether the in vivo initiation of IL-12 responses by *T. gondii* requires IFN-γ, gko and wt mice were inoculated with ME49, spleen and peritoneal cells were recovered from the mice at time 0 and at 3 and 5 days postinfection, and the spontaneous production of IL-12 p40 was measured. As shown in Figure 5, significant IL-12 responses were detected in spleen and PEC cultures from infected wt as well as gko mice. Notably, the levels of IL-12 p40 detected in the PEC cultures were indistinguishable at days 3 and 5 postinfection and in spleen cell cultures at day 3 postinfection. These in vivo data confirm that the initial induction of IL-12 by *T. gondii* is largely independent of IFN-γ. Nevertheless, significantly less ($p < 0.05$) IL-12 p40 was detected in spleen cell cultures harvested from gko mice as compared with wt mice at 5 days postinfection. The latter data are consistent with a role for IFN-γ in augmenting IL-12 responses later in infection. Indeed, splenic lymphocytes from 5-day-infected animals are known to produce substantial levels of the former cytokine (14).

The IL-12 protein is a 75-kDa heterodimer that depends on both the p40 and p35 subunits for its bioactivity. To confirm that, in addition to p40, the intact p75 IL-12 molecule is synthesized normally in gko mice, the latter protein was measured by means of a specific ELISA in sera from infected animals. IFN-γ and IL-12 p40 were assayed simultaneously in the same samples (Table II).

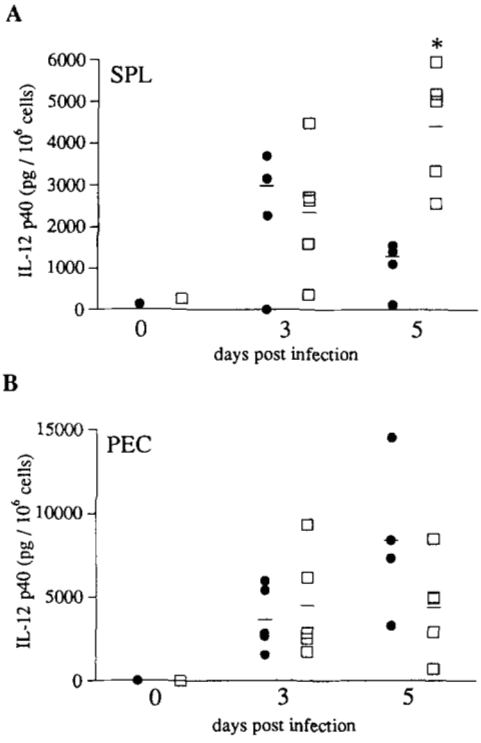

FIGURE 5. IL-12 p40 production is increased in cultures of splenic (*A*) and peritoneal (*B*) cells harvested from wt (squares) and gko (circles) mice following infection with ME49. IL-12 p40 levels were measured by ELISA in 48-h supernatants, as described in *Materials and Methods*. Data shown are the individual measurements in four to five mice per group. An asterisk indicates a statistically significant difference between the values observed in wt and gko samples. Similar results were obtained in two additional experiments.

Table II. *Serum levels of IL-12 p40, IL-12 p75, and IFN-γ in wt and gko mice following infection with* T. gondii

			IL-12 p40[a]	IL-12 p75	IFN-γ
		n	pg/ml		
wt	D0	3	1737 ± 27	52 ± 3	<65
	D3	5	6033 ± 657[b]	102 ± 7[b]	1095 ± 377
	D5	5	12275 ± 1544[b]	159 ± 26	2411 ± 525
gko	D0	3	1417 ± 43	45 ± 15	<65
	D3	5	1499 ± 229	182 ± 20	<65
	D5	3	7523 ± 2505	227 ± 14	<65

[a] IL-12 p40, IL-12 p75, and IFN-γ were measured in individual serum samples (*n*) from gko and wt mice at the indicated times following infection as described in *Materials and Methods*. Data are the mean ± SE for the individual mice. Similar results were observed in a second experiment.

[b] Indicates a statistically significant difference ($p \leq 0.05$) between the gko and wt value.

Serum levels of p75 were found to be elevated in both wt and gko mice at 3 and 5 days post-ME49 infection, with significantly higher levels detected in the gko animals at both time points. As expected, serum p40 levels were also increased in both strains of mice and far exceeded the levels of p75 in the same samples (14, 35). In addition, as predicted, sera from gko mice lacked detectable IFN-γ protein, while levels of this cytokine were dramatically elevated in wt mice at days 3 and 5 following infection.

T. gondii-*infected gko mice exhibit IL-12-dependent NK cell activation in vivo*

Cells from mice infected with *T. gondii* exhibit markedly enhanced NK cell responses, as measured by increased cytotoxicity against sensitive cell lines (8, 36, 37). It has been demonstrated that the NK cell response to the intracellular protozoan *Leishmania major* is IL-12 dependent (38). Based on these results, we predicted that the splenic NK cell response to *T. gondii* would also be IL-12 dependent and used NK cell-mediated cytotoxicity as in vivo indicator of IL-12 function in *T. gondii*-infected mice. As predicted, at 5 days following infection with ME49, wt mice developed a strong NK cell response that was IL-12 dependent, since neutralization of this cytokine blocked the development of the cytotoxic response in these animals (Fig. 6*A*, *left*). More importantly, as shown in Figure 6*A*, *right*, an IL-12-dependent splenic NK cell response was also apparent, albeit at diminished levels, in gko mice. Similar results were observed with cells from ts4-infected gko mice (6*B*, *right* and *left*). Together, these findings demonstrate that *T. gondii* is capable of inducing IFN-γ-independent production of IL-12 and that the cytokine produced is immunologically active.

Inability of IL-12 to influence survival of gko animals

To analyze the relationship between IL-12 and IFN-γ in resistance to *T. gondii*, we performed experiments in which IL-12 levels were modulated artificially and the control of infection assessed. Rather than using ME49 in these studies, we utilized the avirulent, temperature-sensitive isolate, ts4, a parasite that we anticipated might be influenced more readily by IL-12 since it is rapidly controlled by the innate immune system in conventional animals (39). IL-12 levels were altered in gko mice by administering exogenous rIL-12 at 0.1, 0.5, or 1 μg per day on days 1 to 5 postinfection, doses that we and others have shown to augment survival in immunocompromised mice (7, 16, 17). Alternatively, mice were treated with a single injection of an IL-12-neutralizing mAb at the time of infection, a protocol that consistently renders wt mice susceptible to infection (14). As shown in Figure 7, neither of these treatments altered the survival of the animals, suggesting that, in the absence of IFN-γ, IL-12 is not capable of promoting resistance to *T. gondii*. Furthermore, this hypothesis was supported by the observation that anti-IL-12 treatment substantially augmented parasite replication in the PEC of wt but not gko mice (wt ≤ 2% vs wt anti-IL-12 16 ± 8% ($p \leq 0.05$), gko 33 ± 6% vs gko anti-IL-12 32 ± 3% ($p \leq 0.05$)).

Role of endogenous IFN-γ in regulation of Th2 cytokine production

While infection with *T. gondii* induces high levels of Th1-associated cytokines, Th2 lymphokines such as IL-4 and IL-5 are usually difficult to detect. It has been unclear whether this lack of Th2-associated cytokine production is due to an inability of *T. gondii* to stimulate the production of these cytokines or is a result of the persistent production of IFN-γ inhibiting their synthesis. To distinguish between these two possibilities, we evaluated IL-4, IL-5, and IFN-γ production in cells from *T. gondii*-exposed gko mice. Since ME49 is lethal to these animals, radiation-attenuated RH strain tachyzoites were used instead to allow prolonged study of the mice. As seen in Figure 8, spleen cells harvested from gko animals at 35 days after primary exposure to irradiated RH secreted augmented levels of IL-4, as compared with splenocytes from wt animals. The observed activity was neutralized completely by the addition of an IL-4-specific mAb, confirming that the enhanced proliferation of the cell line is due to bioactive IL-4 (data not shown). Moreover, cell cultures from infected gko mice also produced substantially more IL-5 than equivalent cultures from wt animals. The IFN-γ deficiency resulted in enhanced Th2 cytokine

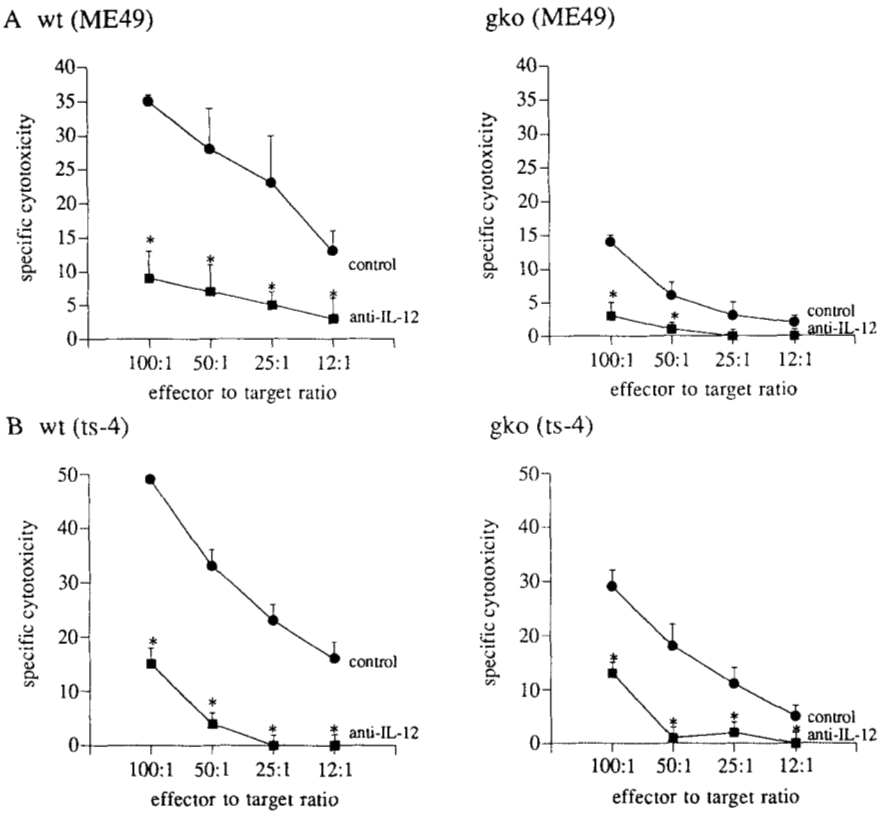

FIGURE 6. Comparison of NK cell cytotoxic responses in wt (*left panels*) and gko (*right panels*) mice with or without in vivo treatment with anti-IL-12 (C17.8.2 mAb). Mice were infected with the ME49 (*panel A*) or ts4 (*panel B*) strains of *T. gondii*. Five days later, the NK cell cytotoxic response against the target cell line YAC-1 was assessed, as described in *Materials and Methods*. Data shown are the means ± SD for measurements determined in three mice per group. An asterisk indicates statistically significant differences between the values observed in mice treated with rat Ig (control) or anti-IL-12 mAb for each panel (Student's *t* test, $p < 0.05$). Statistically significant differences were also apparent in corresponding wt and gko samples at E:T ratios of 100:1, 50:1, and 25:1 (e.g., *right* (solid circle) vs *left* (solid circle)). Background cytotoxic activity, as measured in spleen cells from naive or brain-treated animals, was less than 5% at all E:T ratios.

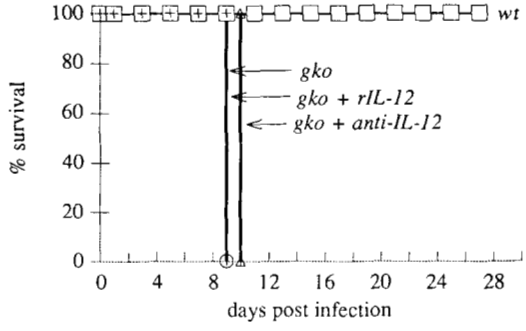

FIGURE 7. Failure of IL-12 or anti-IL-12 mAb treatment to alter acute survival in gko mice following infection with the ts4 strain of *T. gondii*. Survival was compared in gko mice (three to four animals per group) that were treated with PBS (open circle), rIL-12 (cross), or anti-IL-12 (open triangle) daily, over the first 5 days of infection, as described in *Materials and Methods*. The rIL-12-treated groups received 0.1, 0.5, or 1 μg/ml of IL-12 by the i.p. route, each of which gave identical survival curves. The experiments shown used gko mice on the BALB/c background. Essentially identical results were observed in a second experiment using gko mice on the C57BL/6 background.

production in both BALB/c (Fig. 8*A*) and C57BL/6 (Fig. 8*B*) gko mouse strains. Together with our previous observations of increased eosinophils and mast cells in the peritoneal cavity of ME49-infected gko mice, these observations support the hypothesis that the absence of Th2 responses during *T. gondii* infection is, at least in part, due to cross-regulation by IFN-γ.

Discussion

Previous studies using neutralizing mAb indicate that early production of IFN-γ is required for the control of acute *T. gondii* infection in vivo (9, 14). Support for a direct antiparasitic effect of this cytokine is provided by the observations that IFN-γ-treated macrophages display increased killing activity against intracellular tachyzoites and that this cytokine induces tryptophan starvation and parasite killing in human fibroblasts (40, 41). However, the existence of the latter control pathway has not been confirmed in experimental models (42). Since *T. gondii* infects virtually all nucleated cells, it is unlikely that either of these mechanisms completely describes the means by which infected hosts control the parasite. In the present study, we utilized gko mice to determine whether IFN-γ-independent mechanisms of resistance exist during acute infection, as well as to evaluate the IFN-γ dependence of previously described innate immune responses. In particular, we have addressed the possibility that IL-12, a cytokine also implicated in resistance to infection, might also contribute directly to control of the parasite (4, 17). Overall our data demonstrate that early IL-12 production is induced in the absence of IFN-γ, but that the former cytokine is unable, by itself, to stimulate effective immunity against *T. gondii*. Thus, our findings support the hypothesis that IFN-γ, rather than IL-12, is the final effector cytokine of host resistance to this infection.

Mortality studies with the ME49 strain of *T. gondii* indicated that gko mice are exquisitely sensitive to acute infection with the parasite (Fig. 1*A*). One might argue that the acute susceptibility of gko mice to cyst challenge with ME49 was predictable based on previous IFN-γ neutralization studies. This was not obvious, however, since a number of redundant host effector mechanisms have been detected by the study of *T. gondii* infection in knockout animals (17, 43). Indeed, an IFN-γ-independent mechanism of host resistance has been revealed in IL-12-treated mice with a defective IFN regulatory factor-1 gene (17). Our results clearly indicate that this undefined effector function is not triggered in gko mice. A possible explanation is that host protection in IFN regulatory

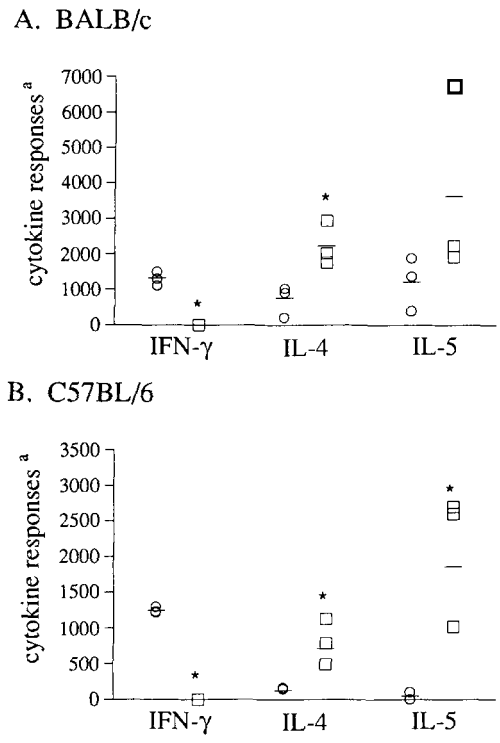

FIGURE 8. Th2 cytokine production by gko (squares) and wt (circles) spleen cells harvested 35 days following in vivo challenge with irradiated RH tachyzoites. IFN-γ-deficient and wt mice on the BALB/c (*A*) and C57BL/6 (*B*) backgrounds were inoculated i.p. at days 0 and 14 with 2×10^6 irradiated (15 krad) tachyzoites. At 35 days following the first inoculation, the animals were killed, and single cell suspensions of spleen cells were prepared from three individual mice. Cells were cultured with STAg (5 μg/ml) at 8×10^5/well in 96-well plates and cytokine levels in supernatants at 72 h. [a], Cytokine units were as follows: IL-4 = mU/ml, IL-5 = pg/ml, and IFN-γ = pg/ml $\times 10^{-1}$. An asterisk indicates a statistically significant difference between the values observed in wt and gko samples ($p \leq 0.05$).

factor-1 knockout mice involves unconventional compensatory mechanisms not present in gko animals, since the immunologic phenotypes of these mice are not identical. For example, the former mice harbor a defective $CD8^+$ T cell compartment (44) and may develop unique resistance mechanisms related to the loss of this important effector cell population, as described previously in *T. gondii*-infected class I MHC-deficient animals (43).

The exquisite susceptibility of gko mice to ME49 infection confirms the pivotal role of IFN-γ in control of *T. gondii* growth. An unexpected finding, however, was the rapid mortality of gko animals challenged with the ts4 strain. Ts4 is a temperature-sensitive mutant of the highly virulent RH strain, which is considered to be both an avirulent and nonpersistent strain since normal mice survive high doses of the parasite ($\geq 10^5$) and by 2 mo following infection appear to be free of the organism (31, 39, 45). Based on the latter properties, one might predict that the avirulent phenotype of this strain is due to its inability to replicate extensively at ambient body temperature or in the presence of a febrile host response. However, Waldeland et al. (39) previously demonstrated that ts4 infection is fatal for athymic, nude mice and suggested that the limited persistence of the attenuated strain is dependent on host immunity. Nevertheless, the interpretation of this study was complicated by the limited success of thymic reconstitution studies as well as the authors' observation of decreased body temperature in athymic nude mice. Our experiments in gko mice formally establish that the avirulent phenotype of the ts4 strain is dependent upon IFN-γ-dependent mechanisms of immunity in the host (Fig. 1*B*).

Synthesis of IL-12 and IFN-γ is often coordinately regulated, a finding that may relate to the ability of IFN-γ to enhance IL-12 production, and vice versa (14). Indeed, recent studies on IL-12 induction in macrophages suggest that priming by IFN-γ may be necessary for activation of the IL-12 p40 promoter by LPS and other stimuli (46). In the case of *T. gondii*, we (unpublished observations) and others (18) have noted that quiescent macrophage populations (e.g., bone marrow-derived or resident peritoneal macrophages) fail to synthesize IL-12 after in vitro stimulation with STAg unless the cells are also exposed to IFN-γ. On the other hand, inflammatory macrophage populations elicited by thioglycolate injection produce high levels of IL-12 after exposure to *T. gondii* in the absence of in vitro priming with IFN-γ (14, 22). Nevertheless, the latter results are difficult to interpret since thioglycolate may stimulate IFN-γ production in vivo. Thus, on the basis of the in vitro evidence described above, it was unclear whether gko mice would be able to produce IL-12 following *T. gondii* infection.

As shown in Figure 5 and Table II, infection of gko animals with the ME49 strain of *Toxoplasma* clearly results in the stimulation of IL-12 p40 and p75 production in vivo. Similar experiments performed in IFN-γ receptor knockout mice or in mice infected with ts4, instead of the ME49 strain, confirm these findings (data not shown). Moreover, thioglycolate-elicited cells from gko animals were unimpaired in their ability to produce IL-12 upon in vitro stimulation with STAg or tachyzoites (Table I). Taken together, these data formally prove that endogenous IFN-γ is not required for *T. gondii*-induced IL-12 synthesis. Nevertheless, at 5 days postinfection, spleen cells from gko mice infected with ME49 synthesized less IL-12 p40 than cells from wt animals. The latter observation is consistent with an important role for IFN-γ in enhancing the induction of IL-12 during *T. gondii* infection in vivo.

Studies with intracellular bacteria have suggested that pathogens differ in the degree to which the IL-12 response they induce depends upon IFN-γ. Thus, it has been reported that the IL-12 stimulated by bacillus Calmette-Guérin (BCG) requires endogenous IFN-γ (12). In contrast, data in a recent review article from the same laboratory indicate that the IL-12 stimulated by *L. monocytogenes* and *Mycobacterium tuberculosis* does not require prior exposure to IFN-γ (47). Our data argue that *T. gondii* belongs in the second category of pathogens, a conclusion consistent with the potency of the parasite as a stimulus of cell-mediated immunity. Nevertheless, it is possible that to induce IL-12, *T. gondii*, *M. tuberculosis*, and *L. monocytogenes* use unknown macrophage-priming stimuli distinct from IFN-γ and perhaps analogous to that provided by thioglycolate elicitation.

While our experiments clearly indicate that IL-12 is induced normally in gko mice, it remained possible that the cytokine fails to function in these animals. This interpretation was evaluated by assessing whether gko mice raise an NK cell cytotoxic response following *T. gondii* infection. NK cell killing function was largely IL-12 dependent in both wt and gko mice. Nevertheless, this response was diminished in gko mice, a finding consistent with the observation of Hunter et al. (48) that anti-IFN-γ mAb treatment reduces NK cell cytotoxicity in SCID mice. Together, these data suggest that the NK cell response to this parasite is primarily IL-12 dependent but is induced optimally in the presence of IFN-γ. Our findings regarding the IL-12-dependent nature of the *T. gondii*-induced NK cell response are similar to those reported for the protozoan *L. major* (38) but contrast with the IL-12-independent NK cell activation mechanism described for murine viruses such

as lymphocytic choriomeningitis virus and murine cytomegalovirus (49). The basis for this difference is unclear but may relate to the dominant induction and function of IFN-$\alpha\beta$ during viral infection. In the case of *T. gondii* infection, the exact role of IFN-γ in augmenting NK cell activity remains to be defined. It is possible that IFN-γ indirectly regulates NK cell function via its ability to augment IL-12 synthesis and/or it functions through affecting levels of receptor for the latter cytokine (50). Preliminary studies measuring IL-12Rβ mRNA expression in wt and gko mice suggest that *T. gondii* induces up-regulation of transcripts for the receptor and that this increase is largely IFN-γ dependent (T. A. Wynn, T. Scharton-Kersten, and L. Showe, unpublished observation).

Importantly, our data failed to reveal any evidence for an IFN-γ-independent protective role of NK cells in these animals. Thus, while in vivo administration of a neutralizing mAb against IL-12 ablated the cytotoxic response in gko mice, such treatment failed to affect the progression of disease, as measured by mortality (Fig. 7) or the number of infected peritoneal cells (data not shown). Overall, these data argue that IFN-γ production, rather than cytotoxicity, is the major function of NK cells in the effector mechanism of resistance to *T. gondii*. Consistent with this hypothesis, we recently observed that perforin knockout mice (51), which lack detectable NK cell activity following *T. gondii* infection, are not defective in their ability to control the infection (E. Y. Denkers, G. Yap, H. Charest, T. Scharton-Kersten, B. A. Butcher, and A. Sher, manuscript in preparation).

Recent studies in our laboratory have been aimed at identifying the cells induced to synthesize IL-12 following exposure to *T. gondii*. In vitro studies using STAg-stimulated macrophages indicate that parasite invasion is not required for IL-12 synthesis. This finding is also supported by the in vivo observation that most of the IL-12-producing cells in the peritoneum are uninfected. Further characterization of the IL-12-producing cells by intracellular staining, using morphology and light scatter properties to distinguish cell populations, indicates that both macrophages and granulocytes are capable of producing IL-12 during acute toxoplasmosis (Scharton-Kersten et al., manuscript in preparation). As documented in the present study, neutrophils are a major component of the early inflammatory response to the parasite in both wt and gko mice (Fig. 3) and thus may contribute to the initiation of cell-mediated immunity through the production of IL-12.

While increased granulocyte infiltration occurred in both gko and wt mice following infection, this response was exaggerated in gko animals (Fig. 3). By 5 days postinfection, the number of peritoneal neutrophils increased by 50-fold in wt mice, as compared with 1500-fold in gko mice. Similarly, the number of eosinophils increased by only 5-fold in wt mice, but over 200-fold in gko animals. The time frame, 5 days, in which the increase in granulocyte infiltration took place in both wt and gko mice was slower than that observed for nonreplicating inflammatory agents such as thioglycolate or peptone-protease (52). The reason for this difference is unclear but may reflect a dose effect in which a critical number of parasites must amass to induce a response. In this regard, tachyzoites have been shown to secrete metabolites that are directly chemotactic for human neutrophils in vitro (53). Therefore, it is probable that the increased granulocytic inflammatory response seen in gko animals is the result of increased parasite replication leading to increased chemotaxis. Alternatively, the absence of IFN-γ in gko mice may lead to increased expression of granulocyte-specific chemokines (e.g., kc or mip-2) or adhesion molecules involved in cellular infiltration (54, 55). Although IL-12 has been shown to facilitate hemopoiesis, including granulocyte differentiation, this activity has been demonstrated to be IFN-γ dependent (56) and thus does not explain the increased granulocyte infiltration observed in infected gko mice. The latter conclusion is supported by experiments in which treatment of infected gko animals with anti-IL-12 mAb failed to alter the composition of peritoneal exudates (data not shown).

As noted above, the inflammatory exudates from infected gko mice contain large numbers of eosinophils as well as mast cells (Fig. 3), suggesting an augmentation of Th2 responses as a consequence of the absence of IFN-γ. This is consistent with the previous observation of decreased IFN-γ and increased IL-4 production by spleen cells from acutely infected mice treated with an anti-IL-12 mAb (14). Direct analysis of the production of IL-4 and IL-5 in gko mice revealed small increases in IL-4 and no detectable IL-5 at 5 days of infection. Nevertheless, the short life span of infected, gko mice limited our ability to study cytokine production at time points in which conventional T cell responses develop in vivo. To address this issue we analyzed cytokine production by spleen cells from gko mice that were exposed to radiation-attenuated parasites in vivo (Fig. 8). The results of these studies indicate that, in the absence of IFN-γ, *T. gondii* stimulates enhanced synthesis of IL-4 and IL-5 and support the concept that the parasite is intrinsically capable of triggering Th2 cytokine production, but that this response is normally attenuated by its potent induction of IFN-γ.

The findings presented in this work underscore the central role played by IFN-γ in resistance to *T. gondii*. Indeed, our data argue that IFN-γ is also a crucial host factor in the determination of parasite virulence. Thus, the ts4 strain, a mutant previously considered to be fully attenuated, was found to be highly virulent when inoculated into gko mice, a finding that suggests that the susceptibility of the organism to IFN-γ-mediated effector mechanisms, rather than its temperature sensitivity, is responsible for its limited survival in vivo. Since our data also reveal that IL-12 is the key initiator of cell-mediated immunity in this model, one might speculate that the ability of different *T. gondii* isolates to induce IL-12 synthesis may itself be a major virulence determinant.

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