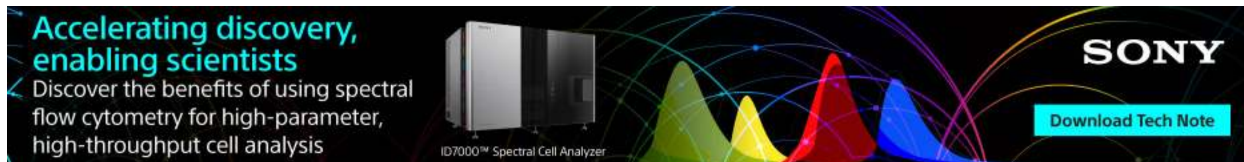

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J Immunol 2000; 165:628-631; ;
doi: 10.4049/jimmunol.165.2.628
http://www.jimmunol.org/content/165/2/628

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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CUTTING EDGE

Cutting Edge: IL-12 Is Required for the Maintenance of IFN-γ Production in T Cells Mediating Chronic Resistance to the Intracellular Pathogen, *Toxoplasma gondii*

George Yap, Michael Pesin, and Alan Sher[1]

IL-12 is required for the development of IFN-γ-dependent resistance to intracellular pathogens but is not thought to play a major role in its maintenance. To directly assess the requirement for continuous IL-12 signaling in long-term cell-mediated immunity, recombinant cytokine was transiently administered to IL-12 p40-deficient mice during the first 2 wk of infection with the intracellular pathogen *Toxoplasma gondii*. As expected, these animals survived the acute phase and established chronic infections. However, 4–6 wk after IL-12 withdrawal, the mice exhibited increased brain cyst burdens and succumbed to toxoplasmic encephalitis. Reactivation was associated with a loss of T-dependent IFN-γ production without a concomitant increase in Th2 cytokine expression. Importantly, parasite Ag-induced IFN-γ synthesis by purified T cells from these animals could be restored by in vitro exposure to IL-12. These results argue that endogenous IL-12 is required for the long-term maintenance of IFN-γ-dependent resistance against intracellular pathogens. *The Journal of Immunology,* 2000, 165: 628–631.

Interferon-γ production by T lymphocytes and NK cells is critical for host resistance to intracellular pathogens. In vitro as well as in vivo studies have identified the heterodimeric monokine IL-12 as the most potent and important inducer of IFN-γ synthesis (1). Developing T cell precursors are thought to be exposed to this cytokine during their interaction with APC in the lymphoid microenvironment early in infection (2). IL-12 binding to its high affinity receptor (composed of β1 and β2 subunits) expressed by Ag-primed cells transduces signals through STAT-4, resulting in IFN-γ gene transcription (3). Although the latter pathway has been shown to be important for the induction of IFN-γ synthesis and Th1 development, it is unclear whether continued exposure to IL-12 is required to maintain the type 1 cytokine production phenotype of Ag-primed Th1 lymphocytes. The observation that Th1 lymphocytes, in contrast to Th2 cells, selectively retain responsiveness to IL-12 as a consequence of persistent IL-12 β2-chain receptor expression suggests an active and ongoing regulation of the Th1 differentiation program (4). The latter hypothesis is also consistent with the IL-12 dependence for optimal IFN-γ responses previously observed in murine Th1 clones (5). On the other hand, more recent studies (6) that document stable inheritance of epigenetic modifications (demethylation) of the IFN-γ promoter region in type 1 CD8 T cells argue that this state of differentiation, or at least the commitment to it, may be passively achieved. These considerations raise the issue of whether continued IL-12 exposure is required to maintain type 1 T cell responses.

A number of studies have utilized neutralizing mAbs to IL-12 to address the requirement for this monokine in the expression of chronic resistance to *Leishmania* (7) and *Toxoplasma* (8) or immunity to reinfection with *Listeria* (9) and *Histoplasma* (10). The general conclusion has been that, while neutralization of endogenous IL-12 early during infection severely impairs the development of acute IFN-γ production and host resistance, the same treatment regimens fail to significantly reverse established resistance or vaccination-induced immunity. These findings argue that following initial priming by microbial Ags and exposure to IL-12, type 1 differentiated lymphocytes exert their effector function (IFN-γ secretion) in an IL-12 autonomous fashion. In contrast, numerous reports (11, 12) indicate that chronic type 1 cytokine-associated autoimmune/inflammatory diseases can be readily reversed by IL-12 deprivation, thus presenting a scenario wherein IFN-γ producing T cells reactive to Ag of foreign vs self origin apparently follow different rules (13).

Since the apparent IL-12 independence of IFN-γ responses during chronic infection could stem from a technical failure resulting in incomplete neutralization of the cytokine, we decided to revisit this question using mice genetically deficient in endogenous IL-12. The experimental design was to infect these animals with *T. gondii* while supplementing the mice with recombinant IL-12 to allow the development of acute Th1-dependent resistance. We then asked whether cessation of IL-12 administration would result in a loss in Th1 effector function and reactivation of chronic infection. Our results demonstrate that the maintenance of pre-established resistance and T-dependent IFN-γ responses requires the continued presence of IL-12.

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Received for publication March 30, 2000. Accepted for publication May 18, 2000.

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Materials and Methods

Mice and experimental infection

IL-12 p40-deficient animals (14), originally donated by Dr. Jean Magram (Hoffman La Roche, Nutley, NJ) and backcrossed onto the C57BL/6 background for five generations as well as control C57BL/6 mice, were obtained through the National Institute of Allergy and Infectious Diseases Taconic Contract. For experimental infection, each mouse received 20 cysts of the ME49 strain of *T. gondii* given i.p. (15).

In vivo treatment with recombinant cytokine

Recombinant murine IL-12 (lot no. 6D23F3.4.6) was a gift from the Genetics Institute (Cambridge, MA). Mice received daily i.p. injections of 0.25 μg of IL-12 in 0.25 ml of PBS or vehicle alone starting day 1 to day 14 of acute infection.

Spleen cell cultures and measurement of IFN-γ production

At indicated time points after infection, spleen cells (3×10^6/ml) were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT), antibiotics, L-glutamine, and 2-ME (5 micromolar). Splenocytes were stimulated with 10 μg/ml of soluble *T. gondii* tachyzoite Ag (STAg).[2] Neutralizing mAb to IL-12 (C17.8.2) (16) or control rat Ig (Sigma, St. Louis, MO) was used at 20 μg/ml. In some experiments, splenocytes from IL-12 reconstituted, IL-12-deficient mice (day 45) were stimulated with IL-12 (10 ng/ml), STAg (10 μg/ml), or both. In other experiments, T cells were purified and sort fractionated on the basis of CD44 expression levels by FACS (FACSVantage, Becton Dickinson Biosciences, San Diego, CA) after staining with FITC anti-CD4 (RM4-5) and FITC anti-CD8 (53-6.7) and PE anti-CD44 (IM7) (PharMingen). Sort-purified T cells (1×10^6/ml) were cultured with irradiated IL-12-deficient splenocytes (1×10^7/ml) and stimulated with IL-12, STAg, or a combination as described above. Culture supernatants were collected at 48 h poststimulation, and the levels of IFN-γ were measured using a previously described two-site ELISA assay (15).

Analysis of lymphokine mRNA expression in brain tissue

Total RNA from brain tissue was extracted using RNAStat reagent (Tel-Test, Friendswood, TX). Fifty micrograms of total RNA was used per reaction for RNase protection assay. The mCK-1 probe set (PharMingen, Dan Diego, CA) used included probes for IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN-γ, L32, and GADPH.

Results and Discussion

Exogenous IL-12 provides only transient protection in T. gondii-*infected IL-12-deficient mice*

To critically evaluate the requirement for IL-12 in the maintenance of chronic resistance to *T. gondii*, recombinant cytokine was administered daily to IL-12 p40 deficient mice for 2 wk following i.p. infection with ME49 strain cysts. As shown in Fig. 1*A*, whereas PBS control-treated cytokine-deficient animals succumbed to acute infection during the first 10–12 days, IL-12-treated mice survived the acute phase and showed no evidence of morbidity for a subsequent 3-wk period despite cessation of IL-12 administration. Interestingly, a significant fraction (40%) of wild-type (WT) mice treated in parallel with exogenous IL-12 succumbed after the first week of infection (Fig. 1*A*). This acute death is likely to be the result of the excessive cytokine levels achieved as observed previously in *T. gondii*-infected IL-10-deficient mice (16).

Despite the early efficacy of IL-12 treatment, IL-12 p40-deficient mice began to show symptoms of reactivation (ruffled fur, tilted gait, seizures) 25 days after the cessation of IL-12 treatment, and all succumbed within the next 15 days (Fig. 1*B*). The delayed mortality of all IL-12 reconstituted IL-12-deficient mice was consistently observed in four independent experiments. To investigate the basis of the loss of chronic resistance observed in mice that received transient IL-12 supplementation, WT and IL-12-treated mice were sacrificed at days 15, 30, and 45, and the numbers of

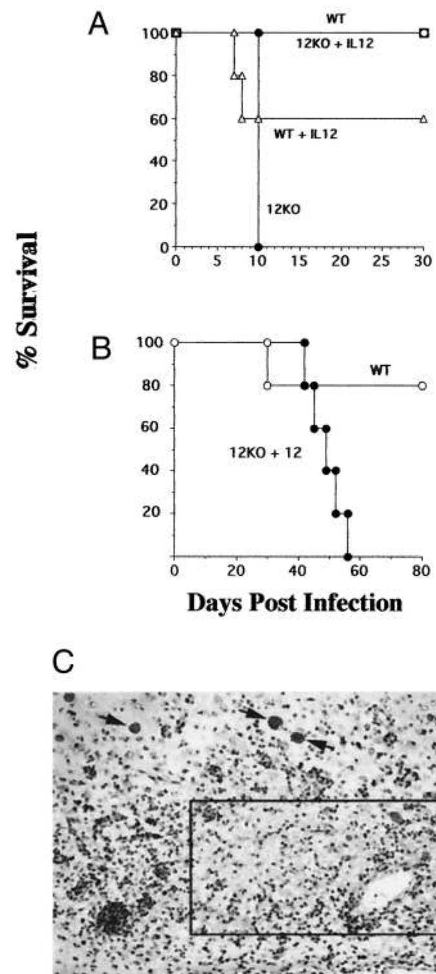

FIGURE 1. IL-12 p40-deficient mice treated with IL-12 exhibit transient resistance to *T. gondii* infection. Mice were inoculated with 20 cysts of the ME49 strain of *T. gondii* i.p. Some mice were treated with IL-12 daily from day 1 to 14. Each treatment group was comprised of five mice. Data shown are representative of four independent experiments performed. *A,* Short-term survival of *T. gondii*-infected C57BL/6 and IL-12-deficient mice treated with IL-12 or PBS. *B,* Long-term survival of *T. gondii*-infected WT or IL-12-reconstituted IL-12-deficient C57BL/6 mice. *C,* Histopathology of brain sample from a representative moribund IL-12-deficient mouse at day 50. Micrograph shows large necrotic lesion (shown in box) with surrounding inflammatory infiltrates and numerous small immature cysts (indicated by arrows).

cysts in the brain were enumerated as a measure of the infection level. As shown in Fig. 2*A*, when assayed 1 day after cessation of cytokine repletion, cyst counts in IL-12-reconstituted IL-12-deficient mice were significantly lower than the numbers determined for untreated WT mice. Nevertheless, the brain cyst counts in the IL-12-deficient mice steadily increased and by day 45 were 5- to 7-fold higher than the cyst numbers in WT brains. At the time of death and necropsy, cyst counts exceeding 50,000 per brain were observed in representative IL-12-deficient mice succumbing from reactivation (data not shown). As expected, histologic examination of brain tissue from late stage moribund mice revealed the presence of massive necrotizing lesions with numerous immature cysts (Fig. 1*C*). Thus, in the absence of continued IL-12 reconstitution, parasite-infected IL-12-deficient mice exhibit a gradual loss of resistance resulting in death associated with a fulminating toxoplasmic encephalitis.

[2] Abbreviations used in this paper: STAg, soluble *T. gondii* tachyzoite Ag; WT, wild type.

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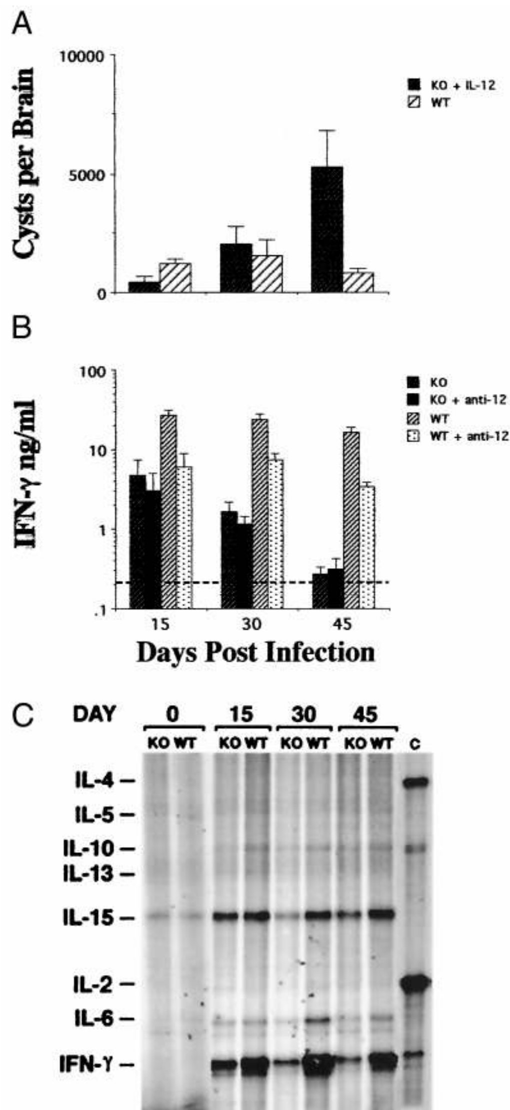

FIGURE 2. Following cessation of IL-12 reconstitution, parasite reactivation in the CNS and loss of IFN-γ responses ensues in *T. gondii*-infected IL-12-deficient mice. *A,* Cysts were enumerated in brain suspensions from IL-12 reconstituted (days 1–14), IL-12 deficient mice (knockout (KO) + IL-12) and WT animals ($n = 5$) sacrificed on days 15, 30, and 45 postinfection. *B,* IFN-γ responses of splenocytes from the same IL-12 deficient (filled bars) and WT animals (open bars) stimulated with STAg (10 μg/ml) in the presence of neutralizing mAb to IL-12 (solid filling) or control rat Ig (hatched filling). Data shown in *A* and *B* are representative of three separate experiments performed. Broken line indicates level of IFN-γ produced by naive WT splenocytes. *C,* Ribonuclease protection assay of cytokine transcript expression in brain extracts. Each lane is representative of three to four mice analyzed individually per time point. *Lane C* contains positive control mouse RNA (2 μg) for IL-4, IL-10, IL-2, IFN-γ, and housekeeping genes provided by the manufacturer and was processed in parallel with the brain RNA samples.

IL-12 withdrawal results in a loss of parasite-induced IFN-γ production without concomitant switch to a Th2 cytokine profile

The gradual loss of chronic resistance in the IL-12-deficient mice was accompanied by a steady diminution in the capacity of splenocytes to mount an IFN-γ response when restimulated with a *Toxoplasma* Ag extract, STAg. As shown in Fig. 2*B*, splenocytes from 15-day infected IL-12-reconstituted IL-12-deficient mice displayed significant IFN-γ responses to STAg in vitro and, as expected, this cytokine production

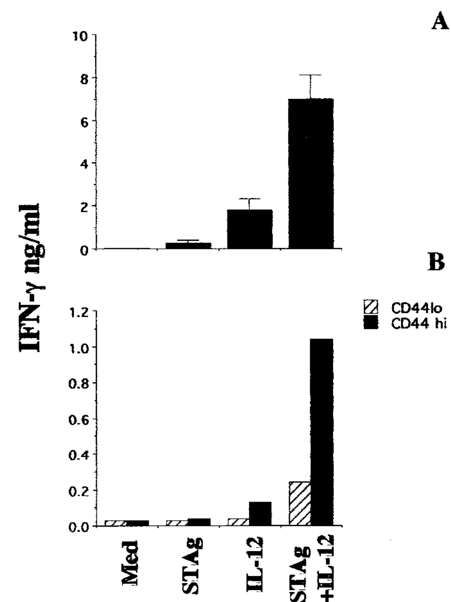

FIGURE 3. Splenocytes (*A*) or purified $CD4^+$ and $CD8^+$ $CD44^{high}$ T lymphocytes (*B*) from day 45 infected IL-12-reconstituted IL-12-deficient animals produce high levels of IFN-γ upon combined in vitro exposure to IL-12 and STAg. Data shown in *A* are mean ± SD of cytokine levels measured by ELISA in spleen cultures from four individual mice/group and are representative of three experiments performed. Results shown in *B* are representative of two independent sorting experiments.

was not significantly altered by in vitro neutralization of IL-12. However, the IFN-γ levels observed were significantly lower than those measured in STAg-stimulated WT splenocyte cultures, where neutralizaton of IL-12 reduced IFN-γ production to the levels observed in the IL-12-deficient spleen cell cultures. Nonetheless, as the infection proceeded, splenocytes from IL-12-deficient mice produced less IFN-γ, and by day 45 they failed to synthesize levels above the background measured in cultures of STAg stimulated splenocytes from naive IL-12-deficient mice. As expected, splenocytes from infected WT mice maintained their STAg-induced IFN-γ responses during this period. At all time points tested, Ab depletion experiments performed on pooled splenic lymphocytes showed that $CD4^+$ and $CD8^+$ T cells account for all of the IFN-γ produced (data not shown).

To investigate whether the loss of Ag induced IFN-γ production by T cells in the IL-12-deficient mice is accompanied by a switch to a Th2-like cytokine phenotype, a ribonuclease protection assay of the lymphokine expression profiles was conducted using mRNA extracted from brain, the major anatomical site for latent infection. As shown in Fig. 2*C*, no IL-4 or IL-5 transcripts were detected as late as 30 days after IL-12 withdrawal and no increase in IL-10 mRNA levels relative to day 15 was evident. Furthermore, no IL-4 protein was detected in culture supernatants of STAg restimulated splenocytes from IL-12 deficient mice at day 45 (data not shown). Thus, the loss of IFN-γ response, also evident in the brain (Fig. 2*C*), is not due to the replacement of a Th1 with a Th2 type T cell population.

IL-12 responsive T. gondii*-reactive T cells persist in IL-12-deficient mice after IL-12 withdrawal*

To assess whether *T. gondii*-reactive T lymphocytes that have the capacity to make IFN-γ are present in IL-12 reconstituted IL-12-deficient mice, splenocytes from these mice were stimulated on day 45 with STAg, IL-12, or STAg, and IL-12 and IFN-γ production was measured. As shown in Fig. 3*A*, IL-12 plus STAg, but not IL-12 or STAg alone, stimulated high levels of IFN-γ, comparable

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to that observed in STAg-stimulated splenocytes cultures from mice sacrificed shortly after IL-12 withdrawal (see Fig. 2*B*). To document that the IFN-γ response arises from Ag-primed T cells, total splenic $CD4^+$ and $CD8^+$ lymphocytes were FACS fractionated on the basis of CD44 expression and restimulated with STAg and IL-12 in vitro. IFN-γ production in response to the combined stimulus was enriched in the $CD44^{bright}$, effector/memory T cell population (Fig. 3*B*). The above observations reveal the existence, within previously IL-12-reconstituted IL-12-deficient infected mice, of a cryptic population of parasite Ag-reactive and IL-12-responsive T lymphocytes with potential effector function (IFN-γ synthesis). Although the precise lineage of this population remains to be determined, clonal descendants of T lymphocytes activated during the acute phase of infection probably comprise a significant fraction of these cells (17, 18). A further implication is that the loss of IFN-γ responses observed in the IL-12-deficient mice cannot be due to a wholesale death of IL-12-responsive type 1-committed T lymphocytes, a mechanism previously proposed to explain the therapeutic effects of anti-IL-12 treatment on experimental autoimmune disease (19).

The major conclusion of the present study is that endogenous IL-12 is required for the long-term maintenance of a protective type 1 T cell response against the intracellular pathogen, *T. gondii*. Nevertheless, for a 2-wk period after IL-12 withdrawal, pathogen-specific T cells retain the capacity to produce IFN-γ and mediate control of infection. The eventual loss of IFN-γ synthesis and host resistance is likely to result from the exit of these terminally differentiated effectors from lymphoid organs into brain tissue, where they recognize their targets and die in situ (20). Because *T. gondii* is never cleared by the immune response, recruitment of T cells into the effector pool continues. We propose that during the subsequent generation of effectors from Ag-primed precursors, de novo IL-12 signaling is required to confer competence to the IFN-γ transcriptional machinery within these cells. Consistent with this hypothesis, resumption of in vivo IL-12 treatment of cytokine-deficient mice (days 31–42) significantly decreased brain cyst counts to 5,700 $\pm$ 642 per brain from the level (40,700 $\pm$ 3,681 per brain) observed at the same time of assay (day 45) in untreated mice ($p < 0.0001$, four mice per group) and increased ex vivo STAg-induced IFN-γ production from 0.17 $\pm$ 0.09 ng/ml (untreated) to 4.78 $\pm$ 0.88 ng/ml (IL-12 treated, days 31–42). A candidate transcription factor that may mediate the downstream effects of IL-12 during this differentiation process is the recently identified IL-12-inducible transactivator of IFN-γ synthesis, T-bet (21). In the absence of such signaling, the parasite Ag-reactive effector cells that emerge are IFN-γ negative and, therefore, nonprotective.

It has been previously proposed that the type 1 T cells that mediate resistance to infection and autoimmune inflammation differ in their requirement for IL-12 for sustained IFN-γ production (13). The greater difficulty in reversing chronic resistance to infection or vaccine-induced immunity with anti-IL-12 mAb may reflect a larger steady-state pool of IL-12-independent effectors. An additional confounding factor could be that higher levels of endogenous IL-12 are induced during microbial infections, precluding complete neutralization. Nonetheless, the picture that emerges from this study as well as a previous report (22) documenting a role for IL-12 in sustaining DNA vaccine-induced protection against *L. major*, is that long-term maintenance of type 1 responses requires endogenous IL-12, regardless of whether the cognate Ag(s) is of microbial or self origin. The preponderance of experimental evidence (11, 12, 22) for such IL-12 dependence in vivo raises a basic question as to how the fidelity of cytokine phenotypes is achieved during the generation of effector T cell pools from long-lived memory lymphocytes (23).

Acknowledgments

We thank Dr. Joseph Sypek (Genetics Institute) for facilitating the donation of recombinant IL-12, Ruth Swofford and Pat Caspar for their expert assistance, and Drs. Dragana Jankovic, Marika Kullberg, and Thomas Wynn for helpful discussions.

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