Endogenous Interleukin 12 (IL-12) Regulates Granuloma Formation Induced by Eggs of *Schistosoma mansoni* and Exogenous IL-12 both Inhibits and Prophylactically Immunizes against Egg Pathology

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Summary

Morbidity in humans infected with *Schistosoma mansoni* results primarily from the deposition of parasite eggs in portal areas where they induce a granulomatous response. In mice infected with this helminth granuloma formation is a $CD4^+$ T helper (Th) cell–dependent process that is associated with a strong Th2 cytokine response which appears to evolve through a Th0 phase. In this report, we asked whether endogenously synthesized or exogenously induced interferon (IFN)γ through its suppression of Th2 cell expansion exerts a regulatory role on egg pathology. Depletion of IFN-γ or natural killer cells resulted in a marked enhancement of granuloma formation around intravenously injected eggs and was associated with increased Th2 and decreased Th1 and interleukin (IL)12 mRNA expression. Similar changes occurred when egg-injected mice were treated with neutralizing monoclonal antibodies specific for IL-12 indicating a role for this cytokine in the regulation of the granulomatous response. In contrast, treatment with exogenous rIL-12 profoundly inhibited primary granuloma formation while increasing IFN-γ, IL-2, IL-10, and IL-12 pulmonary mRNA levels and suppressing IL-4, IL-5, IL-6, and IL-13 mRNA expression. Cytokine depletion studies indicated that the effects of IL-12 could be attributed primarily to increased IFN-γ. Importantly, IL-12 also inhibited secondary granuloma formation in mice presensitized with eggs demonstrating a role for the cytokine in reversing established Th2-type responses. Moreover, mice sensitized with eggs in combination with IL-12 to precommit them toward a Th1 response developed only minimal granulomas upon subsequent egg challenge. The latter findings suggest that simultaneous vaccination with antigen plus IL-12 may provide a strategy for the prevention of schistosome egg pathology as well as other diseases stemming from Th2 cytokine production.

Schistosomiasis is a chronic helminthic disease that affects more than 200 million people worldwide (1). The morbidity in schistosome infections is primarily due to fibrosis resulting in large part from the granulomatous response to parasite eggs in tissues (2). In *Schistosoma mansoni*–infected mice, egg granuloma formation has been characterized as a $CD4^+$ T cell–dependent delayed-type hypersensitivity reaction (3, 4) and based on previous analyses of this process, should result from Th1-like (IFN-γ and IL-2) cytokine responses (5). Unexpectedly, egg deposition was found to associate primarily with increases in Th2 cytokine (IL-4, IL-5, and IL-10) expression (6, 7). Two recent studies examined the kinetics of cytokine mRNA (8) and protein expression (9) after the injection of parasite eggs and concluded that the egg-specific Th2 response is preceded by a Th0 phase of cytokine expression. Moreover, cytokine-depletion studies indicated that both IL-2 and IL-4 are required for granuloma formation in naive animals and for the development of T cells expressing IL-4 and IL-5 mRNA (8). Neutralization of IL-2 or IL-4, while suppressing granuloma formation, did not affect or slightly enhanced expression of IFN-γ and IL-2 mRNA, thereby confirming a predominant role for Th2 cells in granuloma formation (8).

If Th2 responses play a dominant role in granuloma formation, then it may be possible to regulate schistosome pathology by the use of immunomodulators that suppress Th2 cytokine expression. In this regard, IFN-γ has been shown to have potent downregulatory effects on Th2 cell expansion

(10, 11) and has been shown in several systems to have a major role in selectively promoting Th1 cell differentiation (12, 13). It was recently demonstrated that a non-T, non-B cell, the NK cell, can provide a major source of this immunoregulatory cytokine (14). In addition, IL-12, a newly characterized cytokine, produced primarily by macrophages and B cells (15–17), has been shown to play an important role in the induction of IFN-γ synthesis by NK cells (18, 19) as well as activated T cells (18) and appears to selectively stimulate Th1 cell differentiation (20, 21).

Several observations are consistent with a possible regulatory role for IFN-γ in schistosome egg pathology. Thus, this cytokine is expressed during the early stages of liver granuloma formation in infected mice (22, 23) and in the first few days of pulmonary granuloma formation after intravenous egg injection (8). Moreover, NK cells have been demonstrated within granulomas (24) and thus could provide a source of the initial IFN-γ response. Lastly, IFN-γ inhibits egg granuloma formation in an in vitro model (25).

The aim of this study was to directly assess the regulatory functions of NK cells, IFN-γ, and IL-12 in granuloma formation in vivo. As described below, depletion of NK cells, IFN-γ, or IL-12 increased primary granuloma size and Th2 cytokine mRNA expression while decreasing Th1 cytokine mRNA levels, indicating a major role for this pathway in endogenous regulation of the granulomatous response. More importantly, exogenous IL-12 was shown to cause a near complete inhibition of both primary and secondary granuloma formation while suppressing the egg-induced Th2 response. Finally, we demonstrate that IL-12 administered during primary stimulation with eggs precommits the response in the Th1 direction and thus protects mice from subsequent pathology due to secondary exposure to eggs. Together, these results suggest that IL-12–induced immunomodulation may have major potential as a strategy for preventing egg-induced pathology in schistosomiasis.

Materials and Methods

Laboratory Hosts. 6-wk-old female C3H/HeN and C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Antibody and Cytokine Reagents. A neutralizing mAb specific for murine IFN-γ (XMG 1.6) and a rat IgG control (GL113) directed against *Escherichia coli* β-galactosidase (kindly provided by Dr. John Abrams, DNAX, Palo Alto, CA) were purified by precipitation with ammonium sulfate from ascites. In some experiments, mice were injected intraperitoneally with 1 or 2 mg XMG1.6 antibody/dose in 0.5 ml of saline at −1, +3, and +6 d after the initial intravenous injection of *S. mansoni* eggs. For elimination of NK cells (14, 26), either a rabbit anti-asialo GM_1 polyclonal serum (Wako Chemicals, Richmond, VA) or mAb PK136 directed against NK1.1 was utilized. Animals were treated with 1.2 mg anti-asialo GM_1 on days −4 and −1. Mice treated with mAb PK136 were given 1 mg on the same days. Control animals were injected with GL113 or normal rabbit serum (Sigma Chemical Co., St. Louis, MO) on a similar regimen. The gamma globulin fraction of serum was obtained by 50% ammonium sulfate precipitation followed by dialysis with PBS, pH 7.2. Animals depleted of IL-12 were treated on days −1 and +3 with 0.5 mg each of mAbs C15-6.7.5 and C15-1.2.1 (kindly provided by M. Wysocka and G. Trinchieri, Wistar Institute, Philadelphia, PA). Mice depleted of IL-10 were injected intraperitoneally with 2.5 mg each of mAbs SXC-1 and SXC-2 on days −1, +3, +6, and +10. Animals treated with rIL-12 (a gift from S. P. Wolf, Genetics Institute Inc., Cambridge, MA) were injected intraperitoneally with 0.25 μg/day on days 0–3 and 6–7 after intravenous injection of parasite eggs.

Induction of Pulmonary Granulomas. The induction of synchronous egg-induced granulomas was performed as described previously (27). Briefly, *S. mansoni* eggs were extracted from the livers of infected mice (Biomedical Research Institute, Rockville, MD) and enriched for mature eggs (8). To induce primary granulomas, mice were injected intravenously with 5,000 eggs. In some experiments, mice were sensitized by intraperitoneal injection of 5,000 parasite eggs with a 1-wk regimen of rIL-12 or saline. Animals were killed 1–18 d after egg injection and the right lung placed in 1 ml of RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and frozen on dry ice in preparation for RNA extraction.

Histopathology and Serology. For measurement of granulomas, the left lung was inflated with Bouin-Hollande fixative and processed routinely. The size of the pulmonary granulomas was determined in histological sections stained by Litt's (28) modification of the Dominici stain. The diameters of each reaction containing a single egg were measured with an ocular micrometer. Numbers of eosinophils and other cell types were evaluated in the same sections.

Isolation and Purification of mRNA. RNase-free plastic and water were used throughout. The right lung was homogenized in 1 ml RNA STAT-60 using a tissue polytron (Omni International Inc., Waterbury, CT) and total RNA isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water containing 1 mM EDTA and quantitated spectrophotometrically. RNA gel electrophoresis was performed when necessary to confirm that RNA was intact and that the concentration had been determined correctly.

RT-PCR Detection of Cytokine mRNAs. A reverse transcriptase-PCR procedure was performed to determine relative quantities of mRNA for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, IL-12 (p35 and p40 subunits), IL-13, and Hypoxanthine-guanine phosphoribosyl transferase (HPRT)[1]. A brief description of the procedure employed follows. Reverse transcription of 1 μg of RNA was performed as previously described (8). The final cDNA preparation was diluted 1:8 and used in the PCR. The primers and probes for all genes except IL-13 (sequence kindly provided by Robert Coffman, DNAX), p40, and NK1.1 have been previously published (8, 29, 30). Primers and probes for p40, IL-13, and NK1.1 were as follows: p40 sense, CGTGCTCATGGCTGGTGCAAAG, antisense, GAACACATGCCCACTTGCTG, probe, GCTCAGGATCGCTATTAC; IL-13 sense, CTCCCTCTGACCCTTAAGGAG, antisense, GAAGGGGCCGTGGCGAAACAG, probe, TCCAATTGCAATGCCATCTAC; NK1.1 sense, CTACCTCGGTTTAAAGCCACC, antisense, GAAGCACAGCTCTCAGGAGTCAC, probe, GTTTCTCAAGTTTCCAACACTTG. The PCR reaction was as previously described (8). PCR reaction conditions were strictly defined for each cytokine primer pair such that a linear relationship between input RNA and final PCR product was obtained. Both positive and negative controls were included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated with cDNA or previous PCR products. The number of PCR cycles selected for each cytokine was

[1] *Abbreviation used in this paper:* HPRT, Hypoxanthine-guanine phosphoribosyl transferase.

as follows: IL-2 (33), IL-4 (33), IL-5 (32), IL-6 (32), IL-10 (32), IFN-γ (28), p35 (30), p40 (33), IL-13 (33), NK1.1 (30), and HPRT (21).

Analysis and Quantitation of PCR Products. After the appropriate number of PCR cycles, the amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with nonradioactive cytokine-specific probes as previously described (8). The chemiluminescent signals were quantified using a scanner (model 600 ZS; Microtek, Torrance, CA). The amount of PCR product was determined by comparison of signal density to that of standard curves generated from simultaneously amplified step-wise dilutions of cDNA obtained from samples with high amounts (as determined from initial experiments) of specific cytokine mRNA. Fold increase was calculated as the reciprocal of the equivalent dilution of control cDNA and results were normalized for the relative quantity of total mRNA by comparison to HPRT when necessary. Mean fold changes and standard errors were calculated from the signal densities obtained from four to eight animals analyzed at each time point.

Statistics. Statistical significance was determined by Student's *t* test and significance was determined with p values <0.05. All experiments were repeated one or more times with similar results.

Results

Depletion of IFN-γ Increases Th2-like Cytokine Expression and Granuloma Formation. To assess the role of IFN-γ in pulmonary granuloma formation, C3H/HeN mice were injected intravenously with parasite eggs and IFN-γ neutralized with mAb XMG1.6. Mice were killed 1, 3, 6, and 14 d after injection of eggs. Neutralization of IFN-γ resulted in increased granuloma size ($p < 0.05$) by day 14 which is routinely the peak time of granulomatous inflammation during primary responses (Fig. 1 *A*). Analysis of lung cytokine mRNA re-

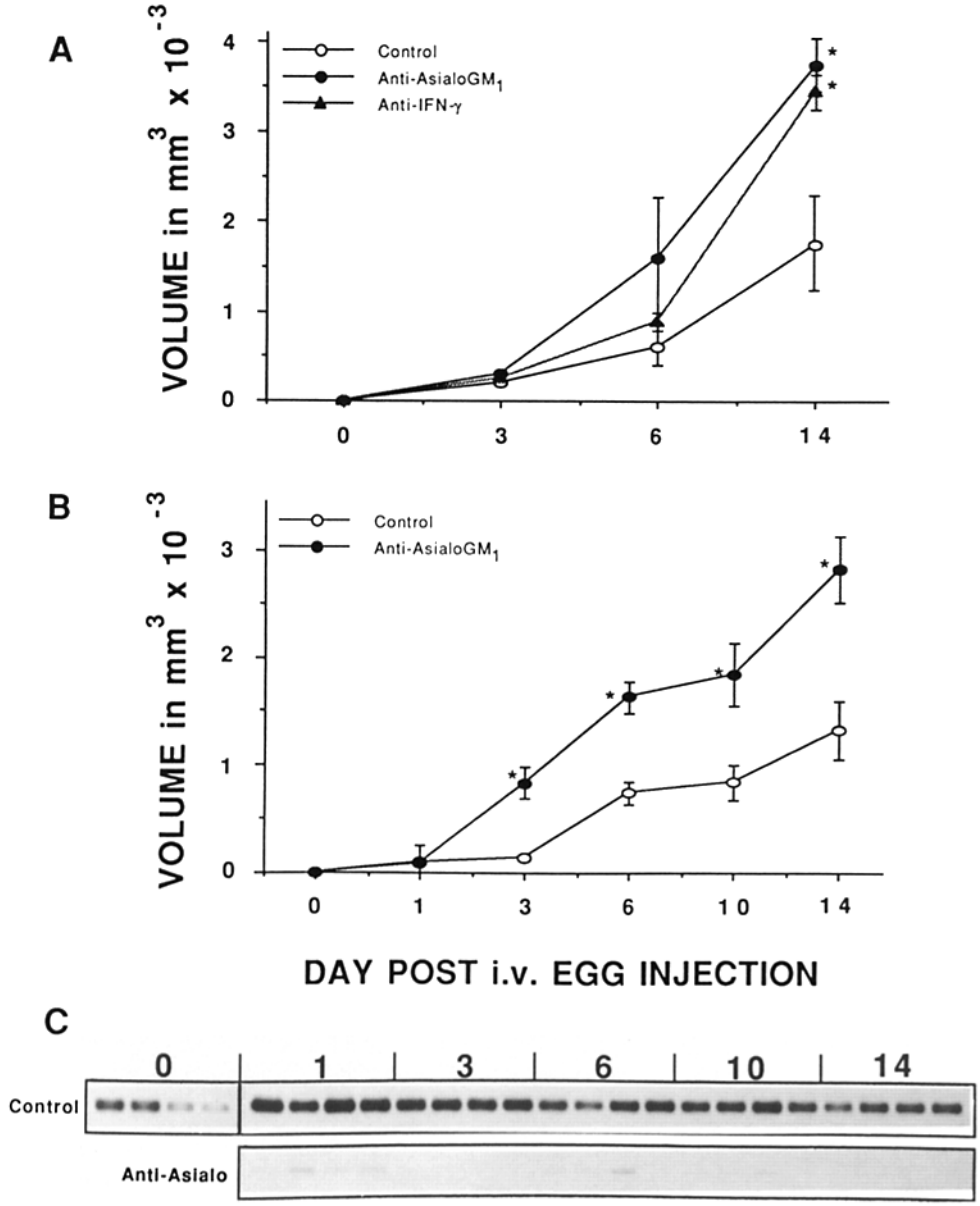

Figure 1. Depletion of IFN-γ or NK cells increases pulmonary schistosome egg granuloma formation. C3H/HeN (*A*) or C57BL/6 (*B* and *C*) mice were injected intravenously with 5,000 eggs and treated with either control Abs, or neutralizing mAbs to IFN-γ (1 mg/dose XMG1.6) or depleted of NK cells by injection with anti-asialo GM_1 as described in Materials and Methods. Four animals/group were killed at the times indicated and mean granuloma volume ± SE (*A* and *B*) and NK1.1 mRNA levels (*C*) measured in the lungs. (*) The depleted group is significantly different ($p < .05$) from the control by Student's *t* test.

sponses demonstrated a significant reduction in IFN-γ, IL-2, and IL-10 mRNA at late time points, whereas levels of the Th2 cytokines IL-4, IL-5, IL-6, and IL-13 were elevated at nearly all times (Fig. 2).

NK Cells Suppress Granulomatous Inflammation and Th2 Cytokine mRNA Expression. To investigate the contribution of NK cells in the primary granulomatous response and in Th1/Th2 subset selection, C3H/HeN animals were depleted of NK cells by treatment with anti-asialo GM_1 Abs. This procedure, similar to IFN-γ neutralization, significantly enhanced granuloma size by day 14 (Fig. 1 *A*). Anti-asialo GM_1–treated mice also demonstrated reduced (10-fold) IFN-γ mRNA expression, an effect that was most pronounced at early time points (Fig. 2). As was observed in IFN-γ–depleted mice, a significant increase in Th2 cytokine mRNA expression occurred at most time points. To confirm the effect of anti-asialo GM_1 on NK cells, egg-injected C57BL/6 mice were treated with the Ab in a second experiment and alterations in granuloma size (Fig. 1 *B*) correlated with decreased expression of NK1.1 mRNA (Fig. 1 *C*). Treatment with the polyclonal serum again increased granuloma size (Fig. 1 *B*). As predicted (24), message for NK1.1 rapidly increased after injection of parasite eggs into control animals. This elevation in NK1.1 message was completely abrogated in animals treated with anti-asialo GM_1 (Fig. 1 *C*). In additional experiments, treatment with PK136, a mAb specific for $NK1.1^+$ cells also significantly enhanced granuloma formation, although the changes were less marked than in the asialo GM_1–treated animals (data not shown) as has been observed previously in other comparisons of these two reagents (31).

Mice Injected with Parasite Eggs Show Increased Expression of IL-12 p40 mRNA. Since IL-12 plays a major role in the regulation of IFN-γ expression by NK and T cells, we examined the induction of this cytokine during the early stages of granuloma formation. As shown in Fig. 2, a modest but highly significant increase in IL-12 p40 mRNA was detected after injection of eggs, reaching a maximum by day 3 and returning to baseline levels by day 14. The p35 subunit of IL-12 was expressed constitutively in the lungs of untreated animals and was slightly downregulated after injection of eggs (data not shown). It is interesting to note that depletion of NK cells or IFN-γ completely blocked the increased p40 mRNA expression, suggesting a primary role for NK cell–derived IFN-γ in inducing IL-12 expression in this model.

Neutralization of Endogenous IL-12 during Pulmonary Granuloma Formation Significantly Increases Granuloma Size While Suppressing Th1 and Increasing Th2 Cytokine mRNA Levels. To assess the role of endogenous IL-12 in primary granuloma formation, neutralizing antibodies to IL-12 were administered to mice injected with eggs as described in Materials and Methods. A dramatic increase ($p < 0.05$) in granuloma size

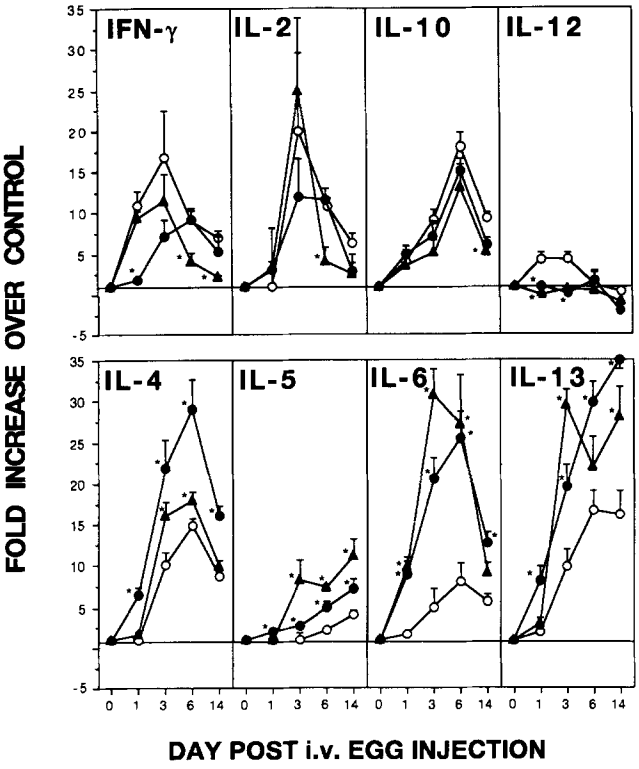

Figure 2. Depletion of NK cells or IFN-γ increases Th2 and decreases Th1 cytokine mRNA expression in the lungs of mice injected with parasite eggs. C3H/HeN mice were injected with 5,000 eggs and treated with neutralizing mAbs to IFN-γ (1 mg/dose, *filled triangles*) or depleted of NK cells with anti-asialo GM_1 Abs (1.2 mg/dose, *filled circles*) as above. mRNA levels are expressed relative to levels in uninjected controls at day 0 (given an arbitrary value of 1) and are expressed as the mean ± SE of determinations on lungs from four individual mice. (*) The treated group is significantly different from the control group at that time point.

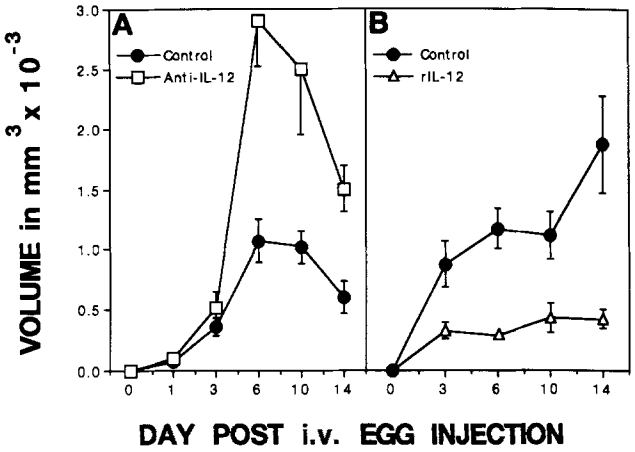

Figure 3. Neutralization of endogenous IL-12 increases whereas administration of exogenous rIL-12 decreases primary egg granuloma formation. In two separate experiments, C57BL/6 mice were injected with 5,000 eggs and either depleted of IL-12 (*A*) or given recombinant IL-12 (*B*) during the first week of granuloma formation. In *A*, mice were injected intraperitoneally with 1 mg control mAb GL113 (*filled circles*) or with 0.5 mg of each anti-IL-12 mAb (C15 6.7.5 and C15 1.2.1, *open squares*) on days −1, and +3. In *B*, mice were treated with saline (*filled circles*) or 0.25 μg/day of rIL-12 (*open triangles*) during the first week of primary granuloma formation as described in Materials and Methods. Four to five mice/group were killed at each time point and granuloma volumes determined.

was seen on days 6, 10, and 14 after injection of eggs (Fig. 3 *A*). The pattern of cytokine mRNA expression was similar to that seen in mice depleted of IFN-γ (Figs. 2 and 4 *A*). There was no effect on the early Th1 response (days 1–6). However, both IFN-γ (days 10–14) and IL-2 (day 10) mRNA expression fell near or below baseline levels suggesting an important role for IL-12 in stimulating late Th1-like cytokine responses. By contrast, expression of the Th2 cytokines IL-4, IL-5, IL-6, and IL-13 was significantly elevated at several time points. It is interesting to note that as described above, expression of IL-10 did not follow the Th2 pattern but rather closely resembled the pattern of expression exhibited by the Th1 cytokines, with depressed expression by days 10 and 14 in animals in which IL-12 was neutralized. IL-12 p35 or p40 mRNA expression was not significantly affected by the depletion of endogenously expressed IL-12.

Recombinant IL-12 Markedly Inhibits Primary Granuloma Formation While Increasing Th1 and Decreasing Th2 Cytokine mRNA Levels. The results from depleting IL-12 suggested that IL-12 primarily acts during the growth stages of granuloma formation, presumably by influencing the expansion of NK cells or the development of Th1 and/or $CD8^+$ cells expressing IFN-γ. We therefore studied the effects of administering rIL-12 during the first week of granuloma formation to determine whether it would suppress egg-induced Th2 responses and thus inhibit the granulomatous response. The results revealed a dramatic reduction in granuloma size at all time points (Fig. 3 *B*). Examination of the cytokine mRNA responses in the lungs of these mice revealed pronounced increases in the Th1 cytokines at nearly all time points (Fig. 4 *B*). IFN-γ mRNA levels were elevated by day 3 whereas IL-2 mRNA increased by day 6. Both mRNAs remained elevated through day 14. Exogenous rIL-12 also dramatically enhanced IL-12 p40 mRNA expression. Consistent with the results from the anti-IL-12 experiment (Fig. 4 *A*), IL-12 appeared to stimulate expression of IL-10. In contrast, mRNA levels for the other Th2 cytokines were almost completely suppressed in rIL-12–treated animals. A more than 10-fold reduction in IL-4 mRNA levels was observed by day 6, whereas IL-5, IL-6, and IL-13 were suppressed at nearly all time points, falling below constitutive levels.

Neutralization of IFN-γ but not IL-10 Dramatically Reverses the Suppressed Granuloma Formation and Th2 Cytokine mRNA Expression Induced by IL-12. Together, the data described above suggest a major role for IL-12 and IFN-γ in limiting granulomatous inflammation, presumably by blocking Th2 cell expansion and cytokine expression. However, it was possible that the increased IL-10 levels in IL-12–treated mice might be responsible for the depressed granuloma size, given the known inhibitory effects of the former cytokine on inflammatory gene expression (32). To distinguish between these mechanisms, mice were treated with or without rIL-12 in combination with neutralizing mAbs to IFN-γ or IL-10. As expected, mice treated with rIL-12 displayed suppressed, whereas animals given anti-IFN-γ displayed increased granulomatous reactions (Fig. 5 *A*). Neutralization of IL-10 had only a small effect on granuloma size, increasing the mean

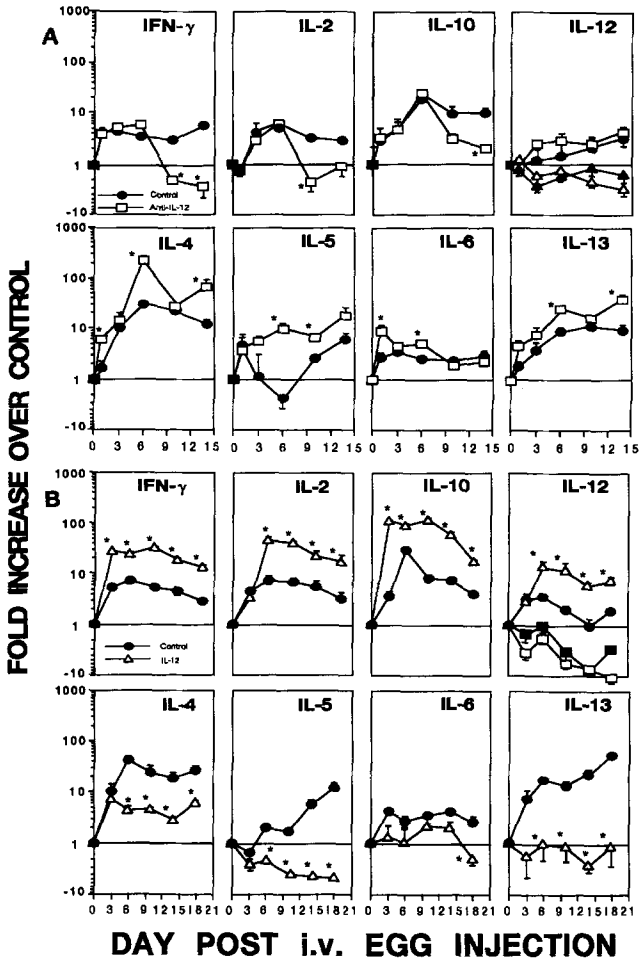

Figure 4. Effect of neutralization of endogenous IL-12 or addition of rIL-12 on cytokine mRNA expression in the lungs of egg-injected mice. Mice were treated as described in Fig. 3 except that the right lung was analyzed by RT-PCR for cytokine gene expression. (*A*) Egg-injected mice were treated with a control mAb (GL113, *filled circles*) or with neutralizing mAbs to IL-12 (*open squares*). For IL-12 mRNA expression, the p40 mRNA subunit is represented as filled circles (control) and open squares (anti-IL-12) whereas the p35 subunit is represented by the filled (control) and open (anti-IL-12) triangles. (*B*) Egg-injected mice were treated with saline (*filled circles*) or with rIL-12 (*open triangles*). For IL-12 mRNA expression, the p40 mRNA is represented by filled circles (*CONTROL*) or open triangles (*rIL-12*) and the p35 subunit as filled (*CONTROL*) and open (*rIL-12*) squares. (*) Denotes a significant difference ($p < .05$) between the two groups. There was a high constitutive level of p35 mRNA in all control animals that was gradually decreased (3–10-fold) after injection of eggs.

volume by ~10–15%. Mice treated with rIL-12 plus neutralizing mAbs to IFN-γ developed granulomas similar to those seen in mice treated with anti-IFN-γ alone. In contrast, mice treated with rIL-12 plus neutralizing abs to IL-10 developed granulomas similar to those found in animals treated with IL-12 alone. These data suggest that the suppressive effects of IL-12 are primarily mediated through IFN-γ secretion.

Analysis of cytokine expression in rIL-12 plus anti-IFN-γ–treated mice revealed several interesting observations. As

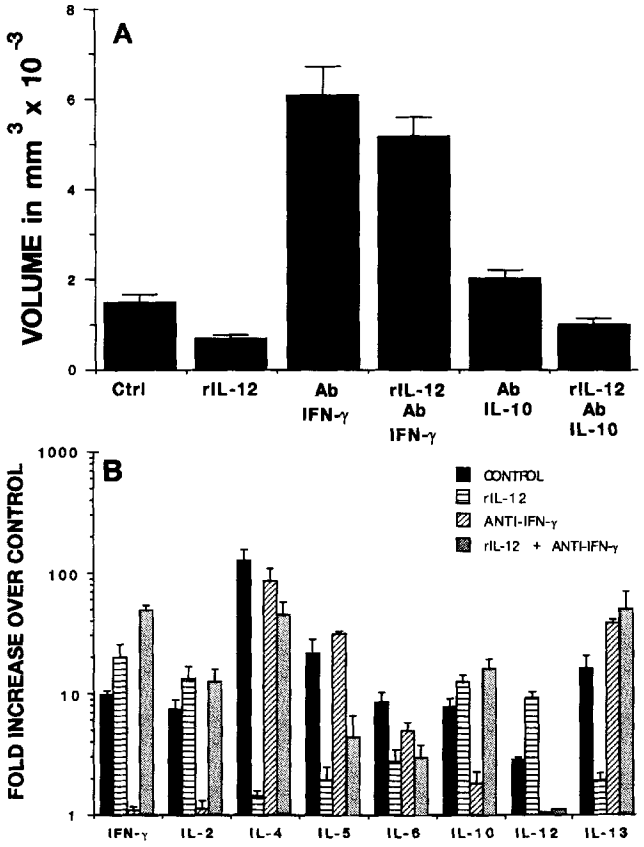

Figure 5. Neutralization of IFN-γ but not IL-10 reverses the suppression of granuloma formation and Th2 cytokine expression by rIL-12 during primary granuloma formation. C57BL/6 mice were injected intravenously with eggs and treated with a control mAb (GL113) and saline, rIL-12 (0.25 μg/dose), anti-IFN-γ mAb (2 mg/dose), anti-IL-10 mAbs (2.5 mg each/dose), or with a combination of IL-12 plus anti-IFN-γ or IL-12 plus anti-IL-10. A total of eight mice per group was killed on day 14, the time of peak granuloma formation and granuloma volumes (*A*) and cytokine mRNA expression (*B*) assessed in the lungs of each animal. The values shown are the means ± SE.

expected, mice treated with rIL-12 displayed suppressed IL-4, IL-5, IL-6, and IL-13 mRNA levels whereas IFN-γ, IL-2, IL-10, and IL-12 were slightly enhanced (Fig. 5 *B*). In addition, as seen in Fig. 2, neutralization of IFN-γ resulted in depressed IFN-γ, IL-2, IL-10, and IL-12 mRNA, whereas levels of IL-4, IL-5, IL-6, and IL-13 were either unaffected or slightly enhanced. Animals depleted of IFN-γ and simultaneously treated with rIL-12 showed enhanced rather than suppressed IFN-γ, IL-2, or IL-10 mRNA expression, suggesting that IL-12 may have a direct effect on the induction of these cytokines. Moreover, the same mice displayed restored IL-4 and IL-13 mRNA expression, approaching the levels seen in control and IFN-γ-depleted animals. Nevertheless, only a modest reversal of the IL-5 mRNA suppression was observed. It is interesting to note that IL-12 p40 mRNA appeared to be largely dependent upon IFN-γ for its induction as evidenced by its reduced expression in rIL-12 injected animals given anti-IFN-γ.

rIL-12 Blocks the Accumulation of Eosinophils in Granulomas. Histological examination of granulomas indicated that eosinophils were almost completely absent from the small lesions found in rIL-12–treated animals (Fig. 6). Not surprisingly, mice treated with neutralizing mAbs to IFN-γ displayed dramatically increased numbers of eosinophils, reaching nearly 50% of the total granuloma cell population, which correlated with increased mRNA levels of IL-5, a cytokine important in the differentiation of eosinophils (33). Although mice treated with rIL-12 plus anti-IFN-γ mAb displayed slightly increased numbers (12%) of eosinophils, their numbers did not approach the levels seen in IFN-γ–depleted animals (50%) or controls (22%). Moreover, these mice failed to show increased IL-5 mRNA levels (Fig. 5 *B*).

rIL-12 Suppresses Granuloma Formation and Egg-induced Th2 Responses in Sensitized Mice. To determine whether IL-12 would reverse established Th2 cell responses and suppress granuloma formation in a secondary response, mice were sensitized intraperitoneally with eggs and challenged 1 mo later with eggs together with rIL-12. In sensitized controls, there was a vigorous granulomatous response that was on average six to eight times greater than that seen in unsensitized animals (Fig. 7 *A*). Sensitized mice treated for 1 wk with rIL-12 after intravenous challenge of eggs showed a greater than fourfold reduction in granulomatous inflammation. Analysis of cytokine mRNA expression again demonstrated a significant increase in IFN-γ, IL-2, and IL-10 mRNA expression at nearly all time points whereas mRNA levels for the Th2 cytokines IL-4, IL-5, and IL-13, although not altered at early time points (days 3–6), were slightly but significantly suppressed (two- to fourfold) from days 10 through 14 (Fig. 7 *B*). It is interesting to note that sensitized controls, but not IL-12–treated mice, displayed depressed IL-12 p40 and p35 mRNA expression at all time points.

IL-12 Administered during Egg Sensitization Precommits the Cytokine Response in the Th1 Direction and Effectively Blocks Secondary Granulomatous Inflammation. Since our initial studies indicated that we could ablate Th2-like cytokine expression and suppress granuloma formation by early treatment with rIL-12 (Figs. 3 *B* and 4 *B*), we asked whether this approach could be used to develop a vaccine that would immunomodulate subsequent egg pathology. For these studies, mice were

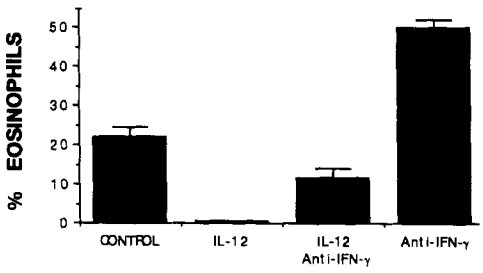

Figure 6. IL-12 suppresses granuloma eosinophil numbers. Granulomas in the mice described in Fig. 5 were analyzed for eosinophil numbers that were recorded as the mean (± SE) percent of cells present.

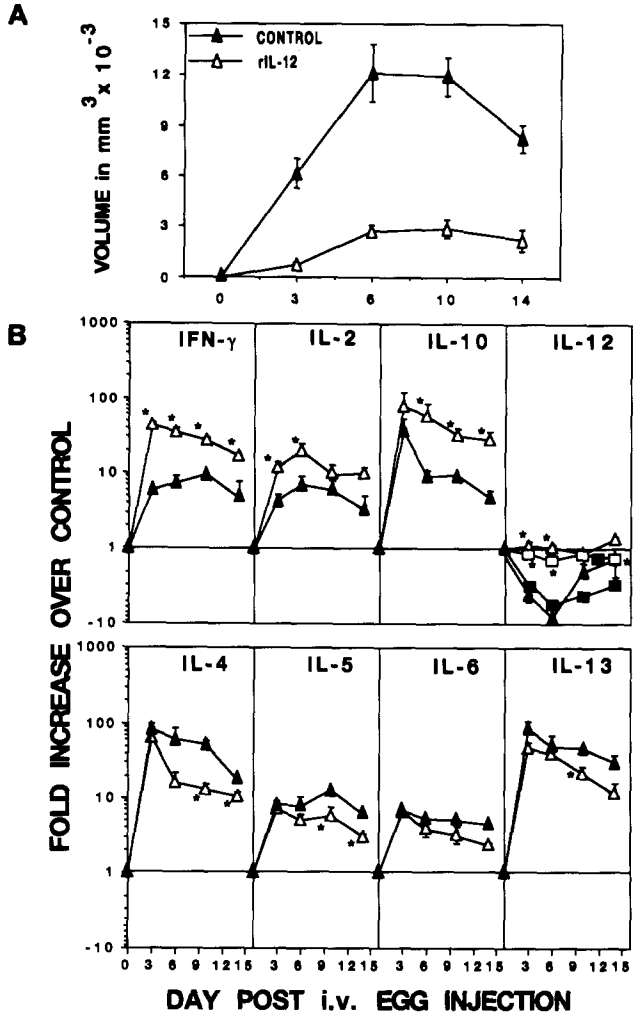

Figure 7. Treatment of mice with rIL-12 suppresses secondary egg granuloma formation while significantly increasing Th1 and decreasing Th2 cytokine mRNA expression. C57BL/6 mice were sensitized by intraperitoneal injection of 5,000 eggs and 4 wk later challenged intravenously with 5,000 eggs. Different animal groups were treated with saline (*filled triangles*) or rIL-12 (0.25 μg/dose, *open triangles*) after challenge. Four mice per group were killed and granuloma volumes (*A*) and cytokine mRNA expression analyzed by RT-PCR (*B*). mRNA for p35 in the control and rIL-12–treated animals is indicated by filled and open squares, respectively, and p40 by triangles.

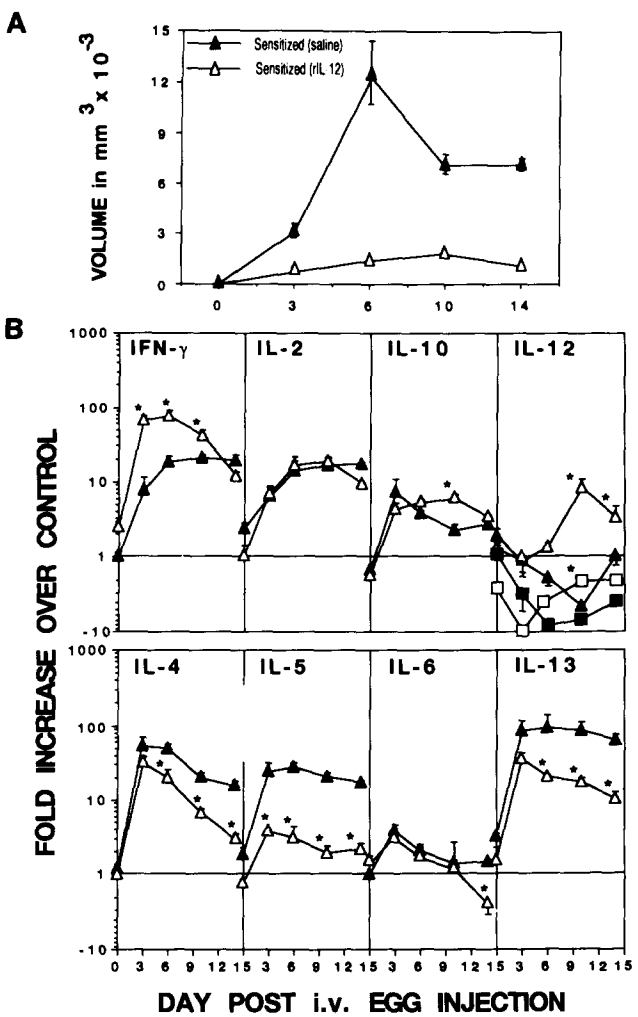

Figure 8. Sensitization of mice with parasite eggs plus IL-12 prevents secondary granuloma formation, is associated with increased IL-12 and Th1 cytokine expression, and dramatically decreased Th2 cytokine mRNA levels. C57BL/6 mice were sensitized by intraperitoneal injection of 5,000 eggs with (*open triangles*) or without (*filled triangles*) IL-12 (0.25 μg/dose). 4 wk after the last injection of IL-12, mice were challenged intravenously with 5,000 parasite eggs in the absence of additional exogenous cytokine and four animals per group killed for histological examination (*A*) and RT-PCR analysis (*B*) of cytokines at the times indicated. The different cytokines are indicated with the same symbols used in Fig. 7.

sensitized as described above except that one group received rIL-12 during the first week of sensitization. 1 mo after the last rIL-12 injection, the mice were challenged intravenously with eggs. As shown in Fig. 8 *A*, mice sensitized in the presence of rIL-12, were almost completely protected from the secondary granulomatous response. Only a small accumulation of cells around eggs was observed which was almost completely eliminated by day 14. The mRNA response indicated a strong anamnestic IFN-γ response and suppressed Th2 cytokine expression in egg plus IL-12–sensitized mice (Fig. 8 *B*). It is interesting to note that IL-12 p40 mRNA expression was dramatically increased only in mice previously sensitized with IL-12, indicating a potential role for IL-12 in controlling Th1/Th2 cytokine patterns in these mice. The p35 chain was again constitutively expressed at high levels in untreated mice and was downregulated from 2- to 10-fold in both groups after the injection of eggs, although a significant level of p35 mRNA was still detected even at these later times. Mice injected with IL-12 alone failed to display upon subsequent egg challenge the alterations in granuloma formation and cytokine expression observed in animals presensitized with eggs plus IL-12 (data not shown).

Discussion

By demonstrating that granuloma formation can be enhanced or inhibited as a consequence of IFN-γ regulation, the data presented in this study support the concept that Th2 responses play a central role in egg-induced pathology. The latter hypothesis was based on the observation of enhanced Th2 cytokine synthesis induced during the formation of granulomas in infected mice (6, 7, 22) or in animals injected with schistosome eggs (8, 9, 34) and on the inhibition of egg pathology caused by in vivo depletion of either IL-4 or IL-2 (8), two cytokines required for the generation of Th2 responses in vitro (10, 35–37). As demonstrated here, manipulations involving IFN-γ or IL-12 in altering the granulomatous response also modified the expression of Th2 cytokines and in particular IL-4, a mediator previously implicated in the pathogenesis of egg lesions (6–8, 23, 34, 38–40). Effects on three other Th2 cytokines, IL-5, IL-6, and IL-13 were also noted. IL-5, while important in the recruitment of eosinophils, does not play a significant role in controlling the size of the lesions (33). Similarly, we have been unable to inhibit granuloma formation by in vivo depletion of IL-6 or by blocking the IL-6 receptor (our unpublished observations). In this study, IL-13, which has both structural and functional homology to IL-4 (41–44) was markedly induced by egg injection. The contribution of IL-13 to egg pathology has not been assessed and it is conceivable that it, as well other uncharacterized Th2 products, may participate directly in the formation of granulomatous lesions.

Previous reports suggested that IFN-γ has a regulatory role in granuloma formation in vitro (25) and in vivo (34, 45). In this study, we demonstrated that parasite eggs induce a rapid IFN-γ response that appears to interfere with the development of IL-4–producing Th2 cells, thus resulting in reduced granuloma formation. Our data argue that NK cells are a likely source of the early IFN-γ since animals depleted of these cells demonstrate a marked reduction in IFN-γ mRNA as well as an increase in granuloma size. As was observed in IFN-γ–depleted animals, the increase in granuloma size was associated with an increase in Th2 cytokine mRNA expression. Together these observations suggest that the early endogenous production of IFN-γ by NK cells through its regulation of T cell responses is a critical factor influencing granulomatous inflammation. Moreover, they support previous studies in mice infected with *Leishmania major* (14) indicating that the NK cell–IFN-γ pathway is a major determinant of Th cell subset selection.

IL-12 has potent IFN-γ stimulating activities on NK cells as well as on $CD4^+$ and $CD8^+$ T lymphocytes (18, 46, 47). In addition, it has been demonstrated in vitro to play a major role in directing the differentiation of Th1 effector $CD4^+$ cells (20, 21, 48, 49). Biologically active IL-12 (50, 51) is a heterodimer, consisting of a constitutively expressed L chain (p35) and a H chain (p40) that is induced in macrophages and several other cell types by a number of stimuli (15, 17). After primary injection of eggs, IL-12 p40 mRNA was induced rapidly in the lungs (Fig. 2). It is interesting to note that this induction was completely blocked in animals depleted of IFN-γ or NK cells, suggesting an essential role for NK cell–derived IFN-γ in regulating IL-12 mRNA expression. The latter hypothesis is consistent with the known upregulatory effects of exogenous IFN-γ on IL-12 gene expression in vitro (17). Moreover, depletion of endogenous IL-12 resulted in an enhanced granulomatous response that was associated with an increase in Th2 cytokine mRNA levels similar to that observed in IFN-γ or NK cell–depleted mice.

The above observations suggest the following mechanism for endogenous regulation of Th activity in primary granuloma formation. Immediately after egg injection there is a rapid IL-12–independent NK cell–mediated IFN-γ response. The induced IFN-γ triggers IL-12 expression (presumably from macrophages) and together, IFN-γ and IL-12 direct Th1 cell expansion and cytokine expression while dampening the evolving Th2 response. IL-12 p40 mRNA expression itself is not significantly affected by depletion of IL-12, suggesting that it is primarily dependent upon the secretion of IFN-γ by NK cells. Therefore, in this model, endogenous IL-12 does not appear to significantly affect the early NK cell response, but rather plays a more important role at later times by directing the development of T cells expressing IFN-γ. The later conclusion is supported by the failure of anti-IL-12 mAb treatment to affect the early IFN-γ mRNA response mediated by NK cells (Fig. 4 *A*).

Our results indicate that IFN-γ, IL-12, and NK cells are important immunomodulators of primary granulomatous inflammation in the lung. Therefore, we hypothesized that it should be possible to stimulate IFN-γ expression and thus simultaneously inhibit granuloma formation by treatment with exogenous rIL-12. Animals injected with rIL-12 had significantly smaller granulomas, and this change correlated with an almost complete suppression of Th2-like mRNA responses (Fig. 4 *B*). It is interesting to note that expression of IL-10 was elevated in IL-12–treated animals, suggesting that IL-10, a potent downregulatory cytokine (32), could also contribute to the suppressed granulomatous response. However, depletion of IFN-γ, but not IL-10 restored Th2 mRNA responses in IL-12–treated mice, arguing that the major suppressive effects of IL-12 on granuloma formation and Th2 cytokine expression were mediated through IFN-γ. Nevertheless, mice treated with IL-12 and depleted with anti-IFN-γ mAb continued to show elevated IFN-γ, IL-2, and IL-10 mRNA levels, suggesting that in contrast to its IFN-γ–dependent effect on Th2 responses, IL-12 directly stimulates Th1 cytokine and IL-10 expression. A direct role for IL-12 in Th1 cell differentiation has been hypothesized from in vitro studies using TCR transgenic $CD4^+$ T cells (20, 21). Our present findings strongly support the existence of these mechanisms in vivo.

As indicated above, treatment with IL-12 caused a marked increase in IL-10 mRNA expression in the lungs of egg-injected mice. Since IL-10 is usually categorized as a Th2 cytokine (52), this finding was somewhat unexpected. Indeed, in an early series of experiments, IL-12 was found to suppress all soluble egg antigen induced recall responses, in-

cluding IL-10, in draining lymph node cells of mice intradermally injected with schistosome eggs (Oswald, I. P., P. Caspar, D. Jankovic, T. A. Wynn, E. J. Pearce, and A. Sher, manuscript in preparation). This discrepancy probably reflects a difference in the source of IL-10 in the two experimental situations, T cells providing most of the cytokine in the recall experiments and macrophages supplying most of the IL-10 expressed in tissues. Regardless, it is unlikely that IL-12–induced IL-10 plays a role in the suppression of granuloma formation since anti-IL-10 treatment failed to reverse the reduction in egg granuloma size induced by exogenous IL-12 (Fig. 5).

Eosinophils were almost completely absent from granulomas of animals treated with rIL-12. This observation correlated directly with the decreased expression of IL-5, a cytokine that controls the differentiation of eosinophils (33). By contrast, animals depleted of IFN-γ (Fig. 6) or IL-12 (data not shown) displayed increased eosinophils in the lesions as well as elevated IL-5 mRNA levels (Figs. 2, 4 *A*, and 5 *B*). It is interesting to note, however, that the large granulomas in animals treated with IL-12 and anti-IFN-γ mAb contained few eosinophils (Fig. 6) and that IL-5 mRNA expression (Fig. 5 *B*) remained low, suggesting that IL-12 may effect IL-5 production and/or the differentiation and recruitment of eosinophils directly. Alternatively, neutralization of IFN-γ, while sufficient to affect granuloma size, may have been insufficient to effect IL-5 and eosinophil development. Studies on the effects of IL-12 on eosinophils in nematode-infected mice yielded similar findings (53).

In addition to effecting primary granuloma formation, IL-12 profoundly inhibited the vigorous anamnestic granulomatous response in animals presensitized with eggs, and this suppression again correlated with a shift in Th1/Th2 cytokine mRNA expression (Fig. 7). The latter observations are of basic interest because they indicate that IL-12 can alter established Th2 responses. In the granuloma model the mechanism of this reversal is unclear. One possibility is that IL-12 alters cytokine expression of Th2 cells in vivo causing them to shift to a Th1- or Th0-like pattern. This explanation is consistent with recent data showing that IL-12 can induce IFN-γ production in human Th2 cell lines (48) as well as clones (54). A second and more likely alternative is that in vivo IL-12 treatment drives the differentiation of the remaining egg-specific Th0 lymphocytes in sensitized animals into Th1 cells. Since in our experiments the egg challenge was performed in a different site than the sensitization, it is also possible that IL-12 injection results in the preferential recruitment of Th1 or Th0 cells from the peritoneum into the lungs. The above considerations are important since they may impact on the feasibility of using IL-12 therapeutically to suppress granulomatous responses or allergic disorders (55, 56) in which Th2-like responses dominate.

The ability of IL-12 to suppress both primary and secondary egg-induced Th2 responses suggested the possibility of prophylactically immunizing mice against granulomatous inflammation by sensitizing them to egg antigens in the presence of exogenous IL-12. This resulted in nearly complete inhibition of granuloma formation after challenge with eggs in the absence of additional IL-12. As predicted, pulmonary cytokine responses showed a reversal in Th expression pattern similar to that observed in animals treated with IL-12 during primary egg injection. These results suggest that when given during priming, IL-12 effectively switches the Th phenotype of the subsequent memory population, thereby altering the cytokine response during secondary antigenic stimulation. Similar findings have recently been obtained in the *L. major* mouse model (26). Together, the data from both parasite experimental models suggest that IL-12 may have important use as an immunomodulatory agent during vaccination, altering both the quality and quantity of protective cell-mediated responses. Indeed, it is tempting to speculate that the action of certain bacterial adjuvants such as Bacillus Calmette-Guérin may relate to their ability to induce IL-12 during immunization.

In the case of schistosomiasis, administration of egg antigens plus IL-12 could potentially be used as an "anti-pathology" vaccine for preventing granulomatous disease (57). Studies are now in progress to determine whether IL-12 can reverse granuloma formation during natural infection and whether combined egg antigen–IL-12 vaccination can lead to a lasting protection against egg-induced tissues responses, fibrosis, and morbidity. If successful, this approach might lead to a method for controlling schistosomal disease that does not depend on interruption of infection.

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