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Interleukin-12 Promotes Activation of Effector Cells That Induce a Severe Destructive Granulomatous Form of Murine Experimental Autoimmune Thyroiditis

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Granulomatous inflammatory lesions are a major histopathological feature of a wide spectrum of human infectious and autoimmune diseases. Experimental autoimmune thyroiditis (EAT) with granulomatous histopathological features can be induced by mouse thyroglobulin (MTg)-sensitized spleen cells activated *in vitro* with MTg and anti-interleukin-2 receptor (anti-IL-2R), anti-IL-2, or anti-interferon-γ (anti-IFN-γ) monoclonal antibody (MAb). These studies suggested that IFN-γ-producing T cells requiring IL-2 for growth may negatively regulate activation of granulomatous EAT effector cells. As IL-12 promotes activation of IFN-γ-producing Th1 cells, the present study was undertaken to determine the role of IL-12 in activation of effector cells for granulomatous EAT. MTg-sensitized cells activated *in vitro* with MTg, anti-IL2R MAb, and IL-12 induced severe, destructive granulomatous thyroiditis with neutrophil inflammation, fibrin deposition, and necrosis. Many glands ultimately underwent atrophy and became fibrotic; some also showed fibrinoid necrosis and a mixed inflammatory cell infiltration of blood vessel walls indicative of a necrotizing vasculitis. Induction of severe granulomatous EAT by IL-12 required MTg *in vitro* and was unrelated to the IL-12-induced increase in IFN-γ production. IL-12 markedly increased IFN-γ production but did not induce a shift to a Th1-dominant phenotype, as other Th1 and Th2 cytokines were generally unaffected and both Th1 and Th2 cytokines were expressed in recipient thyroids. Addition of IL-12 or neutralization by anti-IL-12 at various times indicated that IL-12 exerted its primary effects in the final 24 hours of the 72-hour culture and was not required in recipient mice. Cells cultured with anti-IL-12, MTg, and anti-IL2R MAb transferred mild lymphocytic EAT but little or no granulomatous EAT. Thus, IL-12 profoundly regulates the *in vitro* activation of effector cells that induce histologically distinct autoimmune inflammatory lesions in the thyroid. *(Am J Pathol 1998, 152:1347–1358)*

Experimental autoimmune thyroiditis (EAT)[1] is a chronic inflammatory autoimmune disease that can be induced in genetically susceptible mice by injection of mouse thyroglobulin (MTg) and adjuvant.[1] EAT can also be induced by MTg-sensitized spleen cells activated *in vitro* with MTg.[2,3] MTg-sensitized cells cultured with MTg alone induce a mild lymphocytic form of EAT in which the thyroid is infiltrated primarily by T lymphocytes and other mononuclear cells.[1–4] Lymphocytic EAT lesions are chronic as the extent of thyroid damage remains essentially unchanged from 21 to 60 days after cell transfer.[5] When donor cells are activated with MTg in the presence of anti-interleukin-2 receptor (anti-IL-2R), anti-IL-2, or anti-interferon-γ (anti-IFN-γ) monoclonal antibodies (MAbs), recipient mice develop a histologically distinct, more severe form of EAT characterized by granulomatous histopathology, with follicular cell proliferation, large numbers of histiocytes, multinucleated giant cells, and variable numbers of neutrophils in addition to T lymphocytes.[3–6] Granulomatous thyroid lesions undergo spontaneous resolution so that by 50 to 60 days after cell transfer, most thyroids have only mild lymphocytic infiltration.[5] $CD8^+$ T cells are necessary for the histological resolution of granulomatous EAT.[5] $CD4^+$ T cells have been shown to be the primary effector cells for both lymphocytic and granulomatous EAT.[1,4,7]

We previously hypothesized that effector T cells for granulomatous EAT might predominate in cultures when activation or effector function of a T cell subset, which normally acts to negatively regulate the activity of these effector cells, is blocked.[4] For example, anti-IL-2 or anti-IL2R MAb would block activation of such a regulatory cell if that cell required IL-2 for growth. As neutralization of IFN-γ, a cytokine produced by the Th1 subset of $CD4^+$ T

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cells as well as by NK cells and $CD8^+$ T cells,[8] also promotes selection of cells that induce granulomatous EAT,[6] we hypothesized that effector cells for granulomatous EAT may be primarily Th2-like CD4+ T cells.[4,6]

IL-12, a cytokine produced primarily by APCs, is known to play a key role in promoting the development and activation of Th1 CD4+ T cells.[9–11] One major effect of IL-12 is to increase IFN-γ production by Th1 and NK cells, which may inhibit the development and/or activation of IL-4-producing Th2 cells and promote activation of IFN-γ-producing Th1 cells.[9–12] IL-12, by promoting activation of Th1 cells, can exacerbate Th1-mediated autoimmune diseases such as experimental allergic encephalomyelitis (EAE)[13] and spontaneous diabetes.[14] IL-12 has also been shown to decrease immunopathology mediated by Th2 cells.[12,15,16] If the primary effector cells for granulomatous EAT are Th2-like cells the function of which is suppressed by Th1 cytokines, donor cells activated with MTg and IL-12 would be expected to induce less severe granulomatous EAT in recipient mice, whereas addition of anti-IL-12 might result in preferential activation of Th2-like cells that would transfer granulomatous EAT. In contrast to these expectations, the results presented here indicate that sensitized donor cells cultured with MTg and IL-12 induce an extremely severe and destructive granulomatous EAT in recipient mice, whereas addition of anti-IL-12 suppressed activation of the effector cells for granulomatous, but not lymphocytic, EAT.

Materials and Methods

Mice

CBA/J mice were obtained through Mr. Clarence Reeder at the National Institutes of Health, Bethesda, MD. Female mice, 6 to 10 weeks old, were used for all experiments.

Reagents and Antibodies

Recombinant murine IL-12 and polyclonal sheep anti-murine IL-12 antibody were provided by Dr. Stanley Wolf of the Immunology Department of Genetics Institute, Cambridge, MA. Other antibodies used in these studies included anti-IL2R MAb M7/20, produced from cells supplied by Dr. G. Gaulton, University of Pennsylvania (Philadelphia, PA),[4,17] anti-IFN-γ MAb, XMG 1.2,[18] anti-IFN-γ MAb R4–6A2 (American Type Culture Collection (Rockville, MD) HB 170), and anti-IL-12 MAbs C17.8 and C17.15, provided by Dr. G. Trinchieri, Wistar Institute (Philadelphia, PA). Sheep and rat Ig were obtained from Sigma Chemical Co. (St. Louis, MO).

Immunization

MTg was prepared as previously described.[2] Donor mice were immunized twice with 150 μg of MTg and 15 μg of lipopolysaccharide (LPS) intravenously at 10-day intervals.[2,4]

Cell Transfer System

Seven days after the second injection of MTg and LPS, spleen cells from donor mice were cultured at 10^7 cells/ml in RPMI 1640 containing 25 mmol/L HEPES buffer (Cell and Immunobiology Core Facility, University of Missouri), 5% fetal calf serum (Sigma), 2 mmol/L glutamine, vitamin solution, nonessential amino acids, 1 mmol/L sodium pyruvate, and 5×10^{-5} mol/L 2-mercaptoethanol. Cells were cultured with 25 μg/ml MTg alone or with MTg together with cell culture supernatant containing anti-IL2R MAb M7/20 (5% by volume) as previously described.[4] Other antibodies or IL-12 were added as indicated for individual experiments. After 72 hours, cells were harvested and washed, and 3×10^7 to 3.5×10^7 cells were injected intravenously into irradiated (600 rad) recipient mice.[4] Recipient thyroids were generally evaluated for EAT 18 to 20 days later, the time of maximal severity of both lymphocytic and granulomatous EAT in this adoptive transfer model.[2,4] In the kinetics experiments in Table 2, EAT was assessed from 3 to 50 days after cell transfer as indicated in the table. In the experiments shown in Table 6, recipient mice were given anti-IL-12, anti-IFN-γ, or rat or sheep Ig (150 to 200 μg/injection) every 2 to 4 days beginning on the day of cell transfer as indicated in the table.

Evaluation of EAT

Thyroid lesions were scored quantitatively for EAT severity (the extent of inflammation and destruction of thyroid follicles) using a scale of 1+ to 5+ modified from that previously described.[4] A severity score of 1+ EAT indicates an infiltrate of at least 125 cells in one or several foci; 2+ indicates 10 to 20 foci of cellular infiltration involving destruction of up to one-fourth of the gland; 3+ indicates that one-fourth to one-half of the gland's normal follicular structure is destroyed; 4+ indicates that greater than one-half of the gland is destroyed; and 5+ indicates virtually complete destruction of the thyroid with few or no remaining follicles.

Thyroid lesions were also evaluated qualitatively. The inflammatory infiltrate in conventional (lymphocytic) EAT consists primarily of lymphocytes and some plasma cells with relatively few neutrophils. Thyroids designated G in the tables had granulomatous lesions characterized by proliferation and enlargement of thyroid follicular cells, with small to moderate numbers of neutrophils in the perifollicular stroma, lymphocytes, epithelioid histiocytes, and multinucleated giant cells. The more severe (4+ to 5+) granulomatous lesions had intense inflammation dominated by neutrophils (designated N in the tables), which also included microabscess formation, necrosis, fibrin deposition, multinucleated giant cells, numerous histiocytes, and focal fibrosis that predominated over the follicular cell proliferative changes and mononuclear infiltrates characteristic of the milder granulomatous lesions. The granulomatous inflammation in thyroids graded 4+ to 5+ characteristically extended beyond the thyroid to involve the adjacent connective tissue and

Table 1. Effect of IL-12 and Anti-IL-12 on Activation of Effector Cells for Induction of Lymphocytic and Granulomatous EAT

Culture	Number of mice with EAT severity:*						Number of mice/total		IFN-γ (U/ml)†	Anti-MTg IgG‡
	0	1+	2+	3+	4+	5+	G	N		
MTg	3	2	0	0	0	0	0/5	0/5	2	0.438 ± 0.041
MTg and IL-12	0	0	0	1	2	2	5/5	4/5	131	0.816 ± 0.051
MTg and anti-IL-12	1	4	0	0	0	0	0/5	0/5	0	0.479 ± 0.053
MTg and M7/20	0	0	0	1	3	1	5/5	1/5	61	0.691 ± 0.082
MTg, M7/20, and IL-12	0	0	0	0	0	5	5/5	5/5	825	0.819 ± 0.066
MTg, M7/20, and anti-IL-12	1	2	2	0	0	0	2/5	0/5	0	0.638 ± 0.080
MTg, M7/20, IL-12, and anti-IL-12	0	0	1	2	2	0	5/5	0/5	37	0.504 ± 0.058
NoAg, M7/20, and IL-12	3	0	0	0	0	0	0/3	0/3	800	0.012 ± 0.004

Spleen cells from donors immunized with MTg and LPS were cultured 72 hours with 25 μg/ml MTg (or, in line 8, no antigen) with or without anti-IL-2R MAb M7/20 (5% final concentration of culture supernatant). IL-12 was added to cultures where indicated at 20 ng/ml; anti-IL-12 was used at a final concentration of 10 μg/ml. Cells (3.5×10^7) were transferred intravenously to 600-rad-irradiated CBA/J recipients. G, number of mice/total with granulomatous changes in the thyroid. N, number of mice/total with neutrophil inflammatory lesions (as defined in Materials and Methods). *P* values are <0.01 (line 1 *versus* line 2), <0.05 (line 4 *versus* line 5), and <0.01 (line 4 *versus* line 6 and line 5 *versus* line 7).

*Number of recipient mice with various degrees of severity of EAT 19 days after cell transfer.

†IFN-γ in 72-hour culture supernatants as determined by ELISA.

‡MTg-specific IgG in sera of individual recipient mice as determined by ELISA. Results are expressed as OD_{410} ± SEM of 1:1600 dilutions of serum.

muscle. Mice with very severe 5+ granulomatous thyroid lesions had marked reductions in serum T3 and T4, whereas thyroid function in mice with less severe lesions was not compromised (data not shown).

ELISAs

Serum levels of MTg-specific IgG autoantibodies were assessed by ELISA using serum from individual mice as previously described.[4,6] Alkaline-phosphatase-conjugated antibodies specific for total mouse IgG or for mouse IgG1, IgG2A, and IgG2B were used at previously determined optimal dilutions (1:6000 or 1:8000), which gave an optical density < 0.05 with normal mouse serum (diluted 1:50) on an MTg-coated plate or with a 1:100 dilution of anti-MTg sera on plates coated with an irrelevant antigen (ovalbumin). Levels of IFN-γ produced by cells during the 72-hour *in vitro* culture were evaluated by double-sandwich ELISA using MAb R46A2 as the capture antibody and biotinylated XMG 1.2 as the detection antibody.

Reverse Transcription Polymerase Chain Reaction Amplification

RNA isolation, reverse transcription polymerase chain reaction (RT-PCR) amplification, and primer sequences were described in detail previously.[19,20] To determine the relative initial amounts of target cDNA, each cDNA sample was serially diluted, and each dilution was amplified with cytokine-specific primers. HPRT was used as a housekeeping gene to verify that equivalent amounts of RNA were amplified. PCR products were collected before the amplifications reached the plateau phase, separated by electrophoresis in 3% agarose gels, and visualized by ultraviolet light after ethidium bromide staining. Densitometry analysis was performed using an IS-1000 Digital Imaging System (San Leandro, CA). Dilutions of samples within the linear relationship (usually 1/25 dilution) were used for analysis, and the densitometric units for each cytokine band were normalized to those obtained for the corresponding hypoxanthine-guanine phosphoribosyltransferase band. For comparison of relative levels of mRNA transcripts between different samples, samples were reverse transcribed and amplified at the same time using aliquots of reagent from the same master mix.[19,20]

Statistical Analysis

Statistical significance of differences in EAT severity between groups was determined by the Wilcoxon rank sum test. Student's *t*-test was used to compare the groups in Figure 4. $P < 0.05$ was considered statistically significant.

Results

Cells Cultured with IL-12 Induce Severe Granulomatous EAT with Intense Neutrophil Inflammation, and Culture with Anti-IL-12 Suppresses Activation of Cells to Induce Granulomatous EAT

In initial experiments, spleen cells from MTg/LPS- immunized CBA/J mice were cultured with MTg or with MTg and the anti-IL2R MAb M7/20 in the presence or absence of 20 ng/ml recombinant IL-12, 10 μg/ml anti-IL-12, or 10 μg/ml sheep Ig (Table 1). As described previously,[4] cells cultured with MTg alone (line 1) induced no or mild (1+) lymphocytic EAT in recipient mice. Addition of anti-IL-12 (line 3) or sheep Ig (data not shown) had no effect on disease transfer, whereas the same donor cells cultured with MTg together with IL-12 (line 2) induced severe (4 to 5+) granulomatous EAT with marked neutrophil inflammatory changes after transfer to recipient mice. Donor cells cultured with MTg in the presence of anti-IL2R MAb M7/20 induced moderately severe granulomatous EAT (3 to 5+; Table 1, line 4; Figure 1, A and B); addition of 20 ng/ml IL-12 to the cultures (Table 1, line 5) resulted in

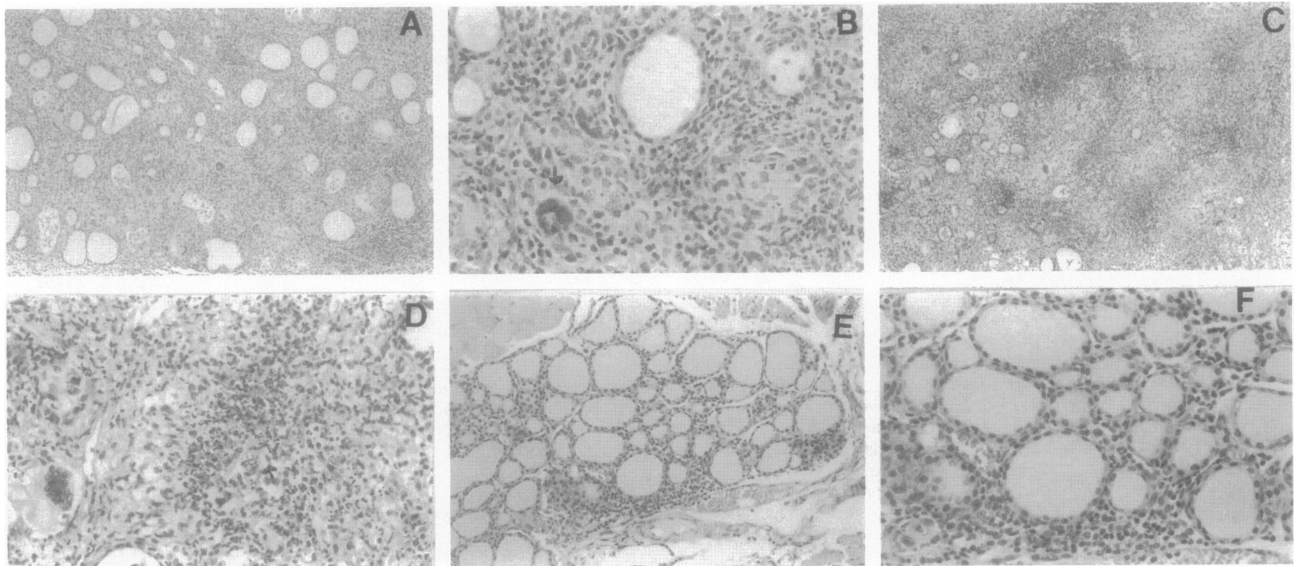


Figure 1. Effect of IL-12 and anti-IL-12 on granulomatous EAT. Hematoxylin and eosin (H&E)-stained thyroid sections are shown from recipients of cells cultured with MTg and M7/20 (**A** and **B**); MTg, M7/20, and 20 ng/ml IL-12 (**C** and **D**); or MTg, M7/20, and anti-IL-12 (10 μg/ml; **E** and **F**). **A** and **B**: Granulomatous thyroiditis (4+) with marked destruction of thyroid follicles, follicular cell proliferation with cells filling the follicle lumen, neutrophils, lymphocytes, and multinucleated giant cells (**arrow**). H&E; magnification, ×100 (**A**) and ×400 (**B**). **C**: More severe (5+) granulomatous thyroiditis with few remaining follicles. H&E; magnification, ×100. **D**: Higher-power micrograph of **C** demonstrating marked neutrophil accumulation, histiocytes including giant cells, and microabscesses (**arrow**). H&E; magnification, ×400. **E** and **F**: Mild (1+) thyroiditis with primarily lymphocytic interstitial infiltrate between thyroid follicles. H&E; magnification, ×100 (**E**) and ×400 (**F**)..

induction of much more severe (5+) granulomatous EAT lesions, with widespread neutrophil inflammation, fibrin deposition, necrosis, and extension of the inflammation beyond the thyroid to involve the adjacent connective tissue and muscle. These massively enlarged thyroids were virtually completely destroyed with almost no remaining follicles (Figure 1, C and D). The effect of IL-12 was antigen dependent, as cells cultured with M7/20 and IL-12 and no MTg induced no EAT (Table 1, line 8). Although cells cultured with IL-12 produced high amounts of IFN-γ, the IL-12-induced increase in IFN-γ did not require MTg in culture; ie, increased production of IFN-γ is apparently not sufficient to induce increased effector function by the donor cells.

In contrast to the potentiating effects of IL-12, cells cultured with MTg and M7/20 in the presence of 10 μg/ml anti-IL-12 induced less severe, primarily lymphocytic, EAT compared with the granulomatous EAT induced by cells cultured with MTg and M7/20 (Table 1, line 4 *versus* line 6; Figure 1, E and F). Anti-IL-12 (10 μg/ml) effectively neutralized the 20 ng/ml IL-12 added to cells cultured with MTg and M7/20 as the EAT induced by these cells was similar in severity and histopathology to that induced by cells cultured with MTg and M7/20 alone (Table 1, line 4 *versus* line 7). Anti-IL-12 also inhibited the IL-12-induced increase in IFN-γ production (Table 1, line 5 *versus* line 7) and inhibited IFN-γ production by cells cultured with MTg and M7/20 alone (line 4 *versus* line 6). EAT induced by cells cultured with sheep Ig together with MTg and M7/20 was indistinguishable from that induced by cells cultured with MTg and M7/20 (data not shown).

IL-12 and anti-IL-12 had no significant effects on MTg-specific or concanavalin-A-induced T cell proliferation (data not shown). Recipients of cells activated in the presence of IL-12 often, but not always, had increased anti-MTg autoantibody responses compared with controls, whereas anti-MTg autoantibody responses were unaffected or, in some experiments, slightly decreased by addition of anti-IL-12 to culture (Table 1). The increase in anti-MTg autoantibody responses in recipients of cells cultured with IL-12 included all IgG subclasses, IgG1, IgG2a, and IgG2b, although in most experiments the increase in the IgG2a subclass was more pronounced (data not shown). These results indicate that IL-12 strongly potentiates the activation of MTg-primed donor cells *in vitro* to induce a very severe inflammatory and destructive granulomatous form of EAT. In contrast, neutralization of endogenous IL-12 *in vitro* inhibits the activation of cells able to induce granulomatous EAT but apparently has little, if any, effect on their ability to induce mild lymphocytic EAT.

Although as little as 0.2 ng/ml IL-12 was sufficient to activate cells to transfer severe granulomatous EAT and to increase IFN-γ production by cultured cells, maximal effects were consistently obtained using 2 to 5 ng/ml IL-12 in culture (data not shown). To ensure maximal activation of EAT effector cells, 5 ng/ml IL-12 was used for all subsequent experiments.

Histopathology and Kinetics of Granulomatous EAT Development in Recipients of Cells Cultured with MTg, M7/20, and IL-12

Recipients of cells activated with MTg, M7/20, and IL-12 had few or no inflammatory cells in their thyroids 3 or 5 days after cell transfer, but by 7 to 8 days, most mice had EAT (1+ to 4+) with widespread follicular cell prolifera-

Table 2. Kinetics of Granulomatous EAT Induced by Cells Activated with IL-12 *in Vitro*

	Day	Number of mice with EAT severity: 0	1+	2+	3+	4+	5+	Number of mice/total G	N	Anti-MTg IgG
Experiment 1	3	3	0	0	0	0	0	0/3	0/3	0.004 ± 0.001
	5	6	1	0	0	0	0	1/7	0/7	0.043 ± 0.090
	7–8	2	2	6	1	1	0	10/12	0/12	0.219 ± 0.025
	10–11	0	0	0	3	4	0	7/7	1/7	0.462 ± 0.067
	14	0	0	0	1	3	0	4/4	3/4	0.533 ± 0.100
	19–21	0	0	0	0	0	13	13/13	13/13	0.868 ± 0.034
	35	0	1	0	0	0	3	3/4	3/4	0.829 ± 0.097
	50	0	0	0	0	2	5	7/7	5/7	0.832 ± 0.025
Experiment 2	20–21	0	0	0	3	5	0	8/8	2/8	0.867 ± 0.092
	35	0	7	1	1	0	0	2/8	0/8	0.950 ± 0.043
	50	7	3	0	0	0	0	0/8	0/8	0.798 ± 0.083

See Table 1 footnotes. Cells were cultured with MTg, M7/20, and 5 ng/ml IL-12. Recipient thyroids were removed at the indicated times after cell transfer. Granulomatous changes on days 7 and 8 were mild.

tion with neutrophils in the perifollicular stroma and within the lumens of some residual follicles (Table 2; Figure 2, A and B). The follicular cell proliferation consisted of enlargement of the cell cytoplasm and an increase in the number of cells within the follicle to form multiple layers that eventually filled the entire lumen. By 10 to 14 days, the inflammatory process had progressed to a severity of 4+ in most mice. Thyroids given the N designation (usually first evident 14 days after cell transfer) had overt necrosis of thyroid follicles associated with broad areas containing numerous neutrophils, with nuclear debris and fibrin deposition. Many follicles now contained intraluminal microabscesses. Numerous large epithelioid histiocytes, as well as multinucleated giant cells, were present within the interstitial stroma (Figure 2, C and D). By 19 to 21 days, there was an even greater degree of inflammation (5+), consisting mainly of widespread neutrophil infiltration that extended beyond the thyroid into the adjacent skeletal muscle and connective tissue. Thyroid follicles were almost completely destroyed (Figure 2, E and F). In contrast to the resolution typical of less severe granulomatous thyroid lesions that contain more apparently uninvolved follicles,[5] thyroids from animals with essentially no remaining follicles at day 21 had extensive fibrosis, with inflammation and follicle destruction, 35 to 50 days after cell transfer (Figure 2, G and H). In contrast, as reported previously,[5] when thyroid lesions at day 21 were less severely destroyed, lesions resolved so that by days 35 to 50, most mice had only mild lymphocytic EAT or relatively uninvolved glands (Table 2, experiment 2). Of particular interest, several thyroids examined 50 days after cell transfer showed areas of fibrinoid necrosis and neutrophil infiltration of blood vessel walls indicative of a necrotizing vasculitis (Figure 2, I and J).

$CD4^+$ T Cells Are Required for Development of EAT Induced by Cells Cultured with IL-12, and the Effect of IL-12 Is Unrelated to IFN-γ Production by Cells in Culture

Previous studies have shown that development of both lymphocytic and granulomatous forms of EAT is dependent on the activity of $CD4^+$ EAT effector cells.[4,7] As shown in Table 3, lines 1 and 2, depletion of $CD4^+$ T cells in recipient mice also completely prevented development of the very severe 5+ granulomatous EAT lesions induced when cells were activated with MTg in the presence of IL-12.

A major function of IL-12 is to increase IFN-γ production by $CD4^+$ and $CD8^+$ T cells and NK cells,[9] and addition of IL-12 to cultures of MTg-primed spleen cells markedly increased IFN-γ production by cultured cells (Table 1). However, the fact that antigen was required for IL-12 to activate cells to induce EAT but not to induce IFN-γ production (Table 1) suggests that the ability of IL-12 to induce increased EAT effector function may be unrelated to its ability to increase IFN-γ production *in vitro*. To more directly address this question, cells were activated with MTg and IL-12 in the presence of anti-IFN-γ MAb. As shown in Table 3, lines 3 to 6, neutralization of IFN-γ produced during the 72-hour culture did not diminish the ability of cells to induce severe granulomatous EAT in recipient mice. In contrast, neutralization of IL-12 with anti-IL-12 also markedly reduced IFN-γ production and also abrogated the effects of IL-12 on EAT induction. The results in Table 3 suggest that the ability of IL-12 to potentiate the activation of effector cells for granulomatous EAT requires $CD4^+$ T cells and is not dependent on IFN-γ production by the cells during the 72-hour culture.

Effect of Delayed Addition of IL-12 or Delayed Neutralization of IL-12 on Activation of EAT Effector Cells

To begin to address the mechanism by which IL-12 potentiates the activation of EAT effector cells *in vitro*, it was of interest to determine whether IL-12 was required throughout the 72-hour culture period. IL-12 was therefore added at culture initiation as in the previous experiments or at various times thereafter. As shown in Table 4, experiments 1 and 2, addition of IL-12 at culture initiation or 24 or 48 hours later resulted in a similar potentiation of the ability of cells to induce severe granulomatous EAT

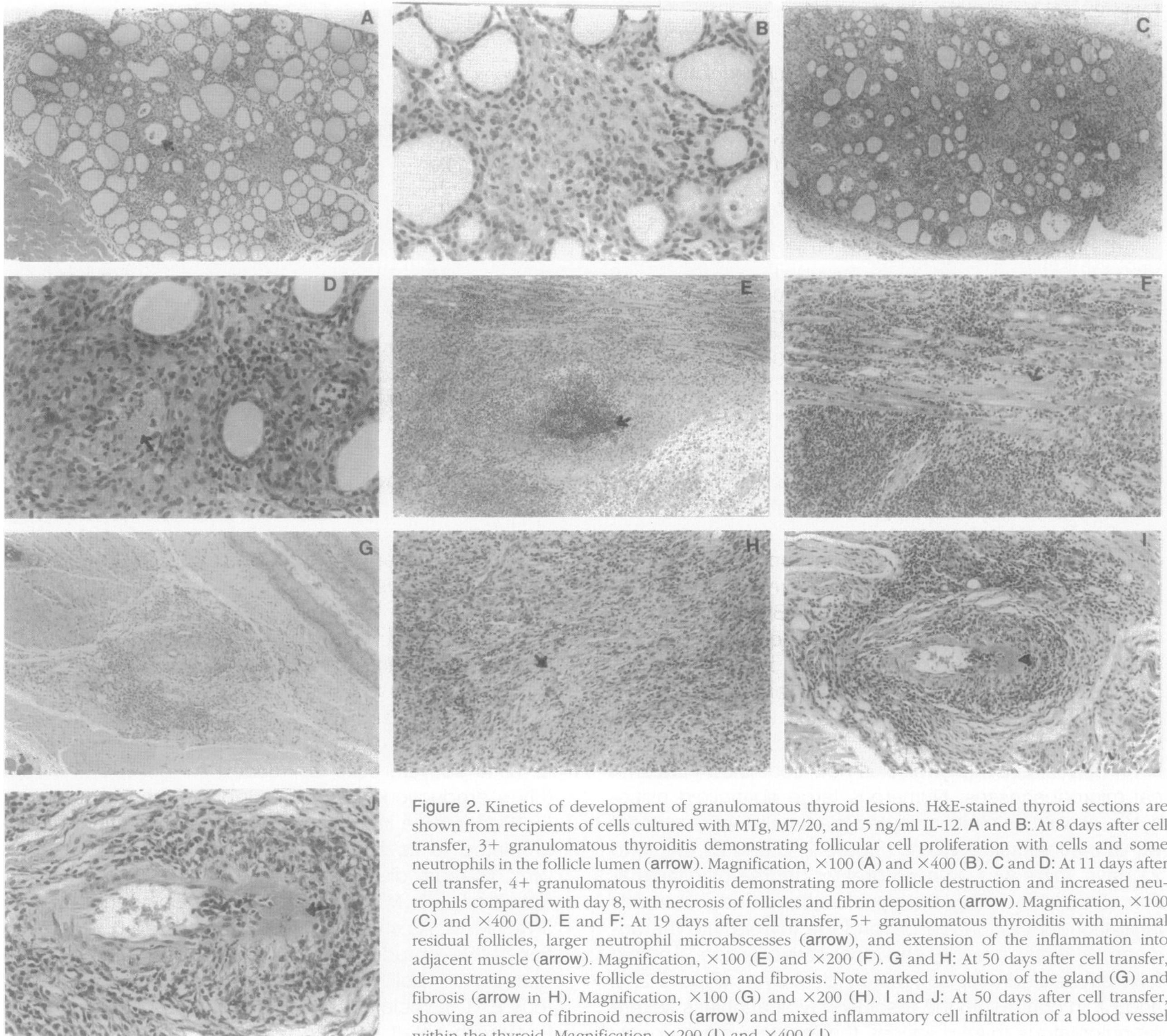


Figure 2. Kinetics of development of granulomatous thyroid lesions. H&E-stained thyroid sections are shown from recipients of cells cultured with MTg, M7/20, and 5 ng/ml IL-12. A and B: At 8 days after cell transfer, 3+ granulomatous thyroiditis demonstrating follicular cell proliferation with cells and some neutrophils in the follicle lumen (arrow). Magnification, ×100 (A) and ×400 (B). C and D: At 11 days after cell transfer, 4+ granulomatous thyroiditis demonstrating more follicle destruction and increased neutrophils compared with day 8, with necrosis of follicles and fibrin deposition (arrow). Magnification, ×100 (C) and ×400 (D). E and F: At 19 days after cell transfer, 5+ granulomatous thyroiditis with minimal residual follicles, larger neutrophil microabscesses (arrow), and extension of the inflammation into adjacent muscle (arrow). Magnification, ×100 (E) and ×200 (F). G and H: At 50 days after cell transfer, demonstrating extensive follicle destruction and fibrosis. Note marked involution of the gland (G) and fibrosis (arrow in H). Magnification, ×100 (G) and ×200 (H). I and J: At 50 days after cell transfer, showing an area of fibrinoid necrosis (arrow) and mixed inflammatory cell infiltration of a blood vessel within the thyroid. Magnification, ×200 (I) and ×400 (J).

with neutrophil inflammatory lesions in recipient mice. In other experiments, addition of IL-12 as late as 8 hours before transfer (64 hours after culture initiation) was as effective as IL-12 added at culture initiation (data not shown). Addition of IL-12 either 2 (data not shown) or 4 (Table 4, experiment 3) hours before transfer had no effect on the ability of cells to induce EAT, suggesting that IL-12 mediates its effects on cells during culture and not after their transfer to recipient mice. The results of several experiments of this type suggest that the critical time during which IL-12 mediates its effects in this system is approximately 48 hours after culture initiation. Although addition of IL-12 48 hours after culture initiation was as effective as adding IL-12 at day 0 in some experiments (eg, Table 4, experiments 1 and 2), in other experiments (eg, Table 4, experiment 3), a greater effect was observed when IL-12 was added at day 0. Again, the effects of IL-12 on EAT were unrelated to IFN-γ production, as the amount of IFN-γ detected in 72-hour culture supernatants when IL-12 addition was delayed was similar to that for cells cultured in the absence of IL-12 (possibly because 72 hours was needed to allow optimal accumulation of IFN-γ). These results suggest that IL-12 acts primarily during the final 24 hours of the 72-hour culture period to potentiate activation of cells that induce very severe inflammatory granulomatous EAT .

An alternative approach to define the time period during which IL-12 functions to potentiate EAT effector cell activation was to add IL-12 to culture at time 0 and then neutralize its activity by addition of anti-IL-12 at various times thereafter. As shown in Table 5, experiments 1 and 2, neutralization of exogenous IL-12 (5 ng/ml) by addition of 5 μg/ml anti-IL-12 at 0 or 24 hours significantly diminished the ability of cells to induce severe granulomatous EAT in recipient mice compared with cells cultured with IL-12 and sheep Ig. In each of the experiments in Table 5, neutralization of IL-12 at 48 hours resulted in the transfer of granulomatous EAT that was either comparable (ex-

Table 3. Enhancement of EAT by IL-12 Requires $CD4^+$ T Cells and Is Not Influenced by Neutralization of IFN-γ in Culture

Culture	Number of mice with EAT severity: 0	1+	2+	3+	4+	5+	Number of mice/total G	N	IFN-γ (U/ml)	Anti-MTg
IL-12	0	0	0	0	0	5	5/5	5/5	ND	0.729 ± 0.110
IL-12	5	0	0	0	0	0	0/5	0/5	ND	0.005 ± 0.003
—	0	2	3	0	0	0	2/5	0/5	80	0.727 ± 0.050
IL-12 + sheep Ig	0	0	0	1	1	3	5/5	5/5	740	0.856 ± 0.031
IL-12 + anti-IL-12	0	2	2	0	0	0	1/4	0/4	43	0.540 ± 0.056
IL-12 + anti-IFN-γ	0	0	0	0	0	5	5/5	5/5	0	0.927 ± 0.029

See Table 1 footnotes. All cells were cultured with MTg plus M7/20 with or without IL-12 at 5 ng/ml; antibodies were added as indicated at a concentration of 10 μg/ml (sheep Ig, anti-IL12, and anti-IFN-γ). Recipient mice in line 2 were given anti-CD4 MAb 1 day after cell transfer.

Table 4. Effect of Delayed Addition of IL-12 on Induction of EAT and IFN-γ Production

Experiment	IL-12	Number of mice with EAT severity: 0	1+	2+	3+	4+	5+	Number of mice/total G	N	IFN-γ	Anti-MTg
1	–	0	0	2	2	1	0	5/5	0/5	185	0.275 ± 0.045
	0	0	0	0	1	2	2	5/5	3/5	500	0.585 ± 0.074
	24	0	0	0	2	1	2	5/5	3/5	250	0.662 ± 0.094
	48	0	0	0	1	2	2	5/5	3/5	185	0.607 ± 0.019
2	–	0	1	3	1	0	0	4/5	0/5	350	0.388 ± 0.072
	0	0	0	0	0	3	2	5/5	4/5	1460	0.811 ± 0.042
	24	0	0	0	0	3	2	5/5	3/5	420	0.761 ± 0.032
	48	0	0	0	0	4	1	5/5	3/5	260	0.809 ± 0.037
3	–	0	0	1	3	1	0	5/5	0/5	75	0.456 ± 0.081
	0	0	0	0	0	1	4	5/5	4/5	750	0.509 ± 0.077
	48	0	0	0	1	3	1	5/5	1/5	65	0.586 ± 0.099
	68	0	0	2	2	1	0	5/5	1/5	85	0.328 ± 0.048

See Table 1 footnotes. MTg plus M7/20 was added to all cultures; IL-12 (5 ng/ml) was added at the indicated times after culture initiation and cells were harvested at 72 hours. *P* values: experiment 1, line 1 *versus* lines 2, 3, and 4, $P = 0.08$; experiment 2, line 1 *versus* lines 2, 3, and 4, $P < 0.01$; experiment 3, line 1 *versus* line 2, $P < 0.02$, line 1 *versus* lines 3 and 4 and line 2 *versus* line 3, $P = 0.9$, and line 2 *versus* line 4, $P < 0.02$.

periments 1 and 3) or reduced in severity (experiment 2) relative to lesions induced by cells cultured with MTg and M7/20 and no IL-12. The amount of anti-IL-12 was apparently sufficient to neutralize both exogenous and endogenous IL-12, as cultures in which anti-IL-12 was added, together with 5 ng/ml IL-12 or 24 hours later, transferred less severe granulomatous EAT than cells cultured in the absence of IL-12. In two separate experiments (Table 5, experiments 2 and 3), neutralization of exogenous IL-12 only during the final 8 hours of culture still partially inhibited the ability of cells to induce granulomatous EAT. These results, like those in Table 4, suggest that IL-12 exerts its primary effects during the last 24 hours of the 72-hour culture. Again, the effects of IL-12 on EAT are unrelated to the increased IFN-γ production, as neutralization of IL-12 at 48 hours prevented the IL-12-induced effects on EAT but had little effect on the amount of IFN-γ detected in 72-hour culture supernatants, presumably because the signal for induction of IFN-γ by IL-12 had occurred in the first 24 hours.

Effect of IL-12 on Induction of Other Cytokines

As the induction of increased IFN-γ by IL-12 appears to be unrelated to its ability to increase EAT effector activity, it was important to determine whether production or expression of other cytokines by the MTg-activated cells was influenced by addition of IL-12 or anti-IL-12 to culture. To examine cytokine gene expression by cells cultured with MTg and anti-IL-2R in the presence or absence of IL-12 or anti-IL-12, RNA was isolated from cells after 72 hours of culture. Addition of IL-12 at culture initiation (d0) or after 24 hours (d1) increased IFN-γ mRNA expression in all experiments but had no consistent effects on expression of other cytokines (Figure 3, A–C). In one experiment (Figure 3A), IL-4 mRNA expression was decreased in cells cultured with MTg, anti-IL2R, and IL-12, but in other experiments (Figure 3, B and C, and data not shown), IL-12 had no effect on IL-4 mRNA expression. Tumor necrosis factor (TNF)-α and IL-2 mRNA expression was increased in some but not all experiments, whereas IL-10 mRNA expression was slightly increased in most experiments by addition of IL-12. Addition of anti-IL-12 at culture initiation or at 24 hours decreased the expression of IFN-γ and, in most experiments, TNF-α mRNA. IL-4 mRNA expression was consistently increased, whereas there were no consistent effects of anti-IL-12 on IL-2 and IL-10 mRNA expression (Figure 3). Addition of IL-12 at 48 hours (not shown) had little or no effect on expression of mRNA for any cytokines at 72 hours although, as shown above, addition of IL-12 at 48 hours did potentiate the ability of cells to transfer severe granulomatous EAT (Table 4).

To determine whether activation of cells in the presence of IL-12 increased expression of Th1 cytokines in the target organ, mRNA was isolated from recipient thy-

Table 5. Effect of Neutralization of IL-12 at Various Times after Culture Initiation on Induction of EAT and IFN-γ Production

Experiment	IL-12	Anti-IL12	Number of mice with EAT severity: 0	1+	2+	3+	4+	5+	Number of mice/total G	N	IFN-γ (U/ml)	Anti-MTg
1	−	−	0	0	0	1	3	1	5/5	1/5	150	0.339 ± 0.075
	+	−	0	0	0	0	0	5	5/5	5/5	725	0.552 ± 0.067
	+	(24)	0	1	1	2	1	0	3/5	0/5	300	0.397 ± 0.068
	+	(48)	0	0	0	1	2	2	5/5	2/5	575	0.573 ± 0.037
2	−	−	0	0	0	2	3	0	5/5	2/5	120	0.684 ± 0.036
	+	−	0	0	0	0	1	4	5/5	5/5	650	0.794 ± 0.033
	+	+(0)	0	3	2	0	0	0	2/5	0/5	50	0.404 ± 0.064
	+	+(24)	0	4	1	0	0	0	2/5	0/5	625	0.111 ± 0.042
	+	+(48)	0	1	2	1	0	0	3/4	0/4	450	0.311 ± 0.073
	+	+(64)	0	0	0	2	3	0	5/5	2/5	600	0.487 ± 0.064
3	−	−	0	1	2	2	0	0	5/5	0/5	60	0.418 ± 0.067
	+	−	0	0	0	0	1	3	4/4	4/4	350	0.529 ± 0.018
	+	+(48)	0	0	1	2	1	0	4/4	0/4	375	0.443 ± 0.080
	+	+(64)	0	0	0	1	3	1	5/5	2/5	475	0.469 ± 0.057

See Table 1 footnotes. MTg plus M7/20 was added to all cultures; IL-12 (5 ng/ml) was added where indicated (+) at initiation of culture. Anti-IL-12 (5 μg/ml) was added at 0, 24, 48, or 64 hours as indicated, and cells were harvested at 72 hours. *P* values: experiment 1, line 1 *versus* line 2, $P < 0.05$; line 1 *versus* line 3, $P < 0.1$; line 2 *versus* line 3, $P < 0.01$, line 2 *versus* line 4, $P > 0.1$; experiment 2, line 1 *versus* lines 2, 3, 4, or 5, $P < 0.05$, line 2 *versus* lines 3, 4, or 5, $P < 0.01$, line 2 *versus* line 6, $P < 0.05$; experiment 3, line 1 *versus* lines 2 and 4, $P < 0.02$, line 2 *versus* line 3, $P = 0.05$, and line 2 *versus* line 4, $P > 0.1$.

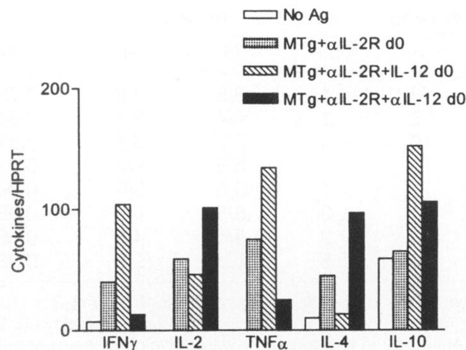
roids 19 days after cell transfer and cytokine gene expression was determined by RT-PCR (Figure 4). As thyroid size is influenced by disease severity, the total amount of RNA obtained from each thyroid is not constant. Thus, cytokine gene mRNA expression was expressed as a relative value to a housekeeping gene HPRT.[19,20] Thyroids obtained from recipients of cells activated in the presence of IL-12 expressed significantly ($P \leq 0.005$) higher levels of Th1 cytokines IFN-γ, IL-2, and TNF-α compared with thyroids of recipients of cells activated in the absence of IL-12. The Th2 cytokines IL-10 and IL-13 were increased to a lesser extent ($P \leq 0.2$) in recipients of cells activated in the presence of IL-12. Both groups expressed similar levels of IL-4 mRNA. These results suggest that recipients of effector cells activated in the presence of IL-12 express more Th1 cytokines in their thyroids than cells activated in the absence of IL-12 (consistent with the increased inflammation in the former thyroids), but there is not a pronounced skewing to a Th1-dominant cytokine expression as Th2 cytokine expression is not decreased.

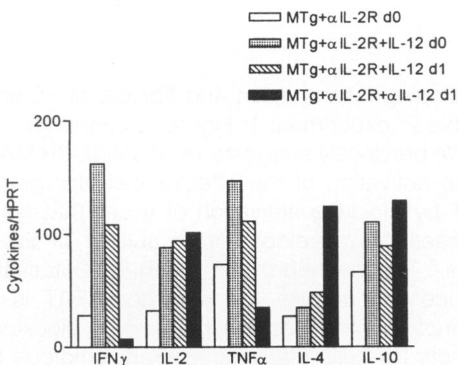
Effect of Neutralization of IL-12 or IFN-γ in Recipient on Granulomatous EAT Induced by Cells Activated in the Presence of IL-12 in Vitro

As shown above, addition of IL-12 during *in vitro* activation of MTg-sensitized spleen cells results in activation of effector cells that induce a very severe destructive granulomatous form of EAT. Although IL-12 can promote the development and activation of Th1 CD4+ T cells[9–11] and may inhibit development of IL-4-producing Th2 cells,[9–12] IL-12 did not appear to induce obvious differentiation of MTg-activated splenic T cells to a Th1 phenotype. IFN-γ was the only Th1 or Th2 cytokine that was consistently increased by addition of IL-12 to culture (Figure 3), and this was apparently unrelated to the ability of IL-12 to potentiate activation of EAT effector cells (Tables 1, 3, 4, and 5). However, cells activated in the presence of IL-12 do produce IFN-γ in recipient mice (Figure 4), which may be important for development of severe granulomatous EAT. To address this possibility, donor spleen cells were activated with MTg, anti-IL-2R, and IL-12, and recipient mice were given anti-IFN-γ, anti-IL-12, or control Ig every 4 days beginning on the day of cell transfer (Table 6). Recipient mice given anti-IFN-γ developed slightly more severe granulomatous EAT than did mice given control Ig, whereas anti-IL-12 had no effect on EAT severity. Thyroids of most anti-IFN-γ-treated recipients had more extensive necrosis and fibrin deposition and increased infiltration of their thyroids by eosinophils (not shown). Eosinophils were rarely observed in thyroid infiltrates of control or anti-IL-12-treated mice. These results suggest that production of IFN-γ in recipient mice is not essential for development of severe granulomatous EAT induced by cells activated in the presence of IL-12. Moreover, although production of endogenous IL-12 *in vitro* is apparently essential for activation of effector cells that induce severe granulomatous EAT (Tables 1, 3, and 5), neutralization of IL-12 after cells are transferred to recipient mice has no effect on their ability to induce severe granulomatous EAT.

Discussion

The results presented here demonstrate that addition of IL-12 to MTg-sensitized spleen cells during *in vitro* activation with MTg or with MTg and anti-IL-2R MAb results in activation of EAT effector cells that induce a very destructive inflammatory EAT with granulomatous histopathology. The ability of IL-12 to potentiate EAT required antigen (MTg; Table 1) and was apparently unrelated to the ability of IL-12 to increase IFN-γ production in culture (Tables 1, 3, 4, and 5). Although this suggests that the potentiating effect of IL-12 on EAT effector cells may be IFN-γ independent, these results do not exclude a role for





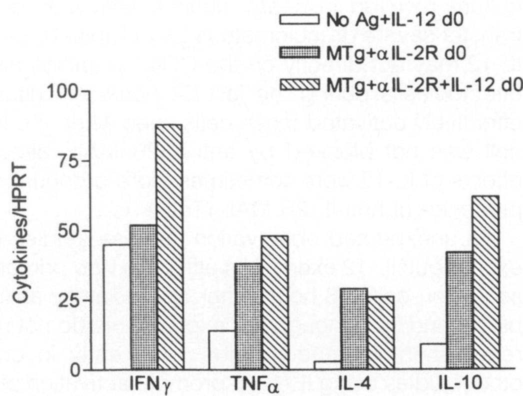


Figure 3. Cytokine gene mRNA expression by spleen cells. Seven days after the second immunization with MTg and LPS, donor spleen cells were cultured 72 hours with MTg, anti-IL2R MAb, IL-12, or anti-IL-12 as indicated. Where indicated, cells were cultured in the absence of MTg. IL-12 or anti-IL-12 was added at culture initiation (d0) or 24 hours after culture initiation (d1). Results are representative of three separate experiments and are expressed as the ratio of cytokines to HPRT mRNA densitometric units (1/25 cDNA dilutions).

IFN-γ in granulomatous EAT as IFN-γ-expressing cells are present in recipient thyroids (Figure 4). However, development of severe granulomatous EAT is not absolutely dependent on IFN-γ as cells from IFN-γ −/− mice activated with MTg and IL-12 induce severe EAT with granulomatous histopathology in IFN-γ −/− recipients,[21] and severe granulomatous EAT developed in recipient mice given anti-IFN-γ (Table 6). IFN-γ-independent effects of IL-12 have been described by others. IL-12, in the presence of IL-4, promoted IL-4 production by murine Th2 cells,[22,23] exacerbated Th2-dependent granulomatous inflammation in IFN-γ-negative mice,[24] and had similar effects on IL-4 and IL-10 production in both IFN-γ-positive and -negative mice.[25] Neutralization of IFN-γ also did not interfere with the IL-12-dependent development of Th1 cells[11,26] or with the ability of IL-12 added *in vitro* to potentiate adoptive transfer of EAE.[13,27]

Neutralization of endogenous IL-12 during activation of effector cells *in vitro* markedly suppressed the ability of cells to transfer granulomatous EAT (Tables 1 and 5), suggesting that IL-12 may be essential to activate the effector cells for severe granulomatous EAT. However, neutralization of IL-12 after transfer to recipient mice (Table 6) had no effect, suggesting that IL-12 does not directly influence development of granulomatous thyroid lesions. Neutralization of endogenous IL-12 had no effect on the ability of cells activated with MTg alone or with MTg and anti-IL-2R to induce mild lymphocytic EAT (Table 1), suggesting that endogenous IL-12 may not be required for induction of lymphocytic EAT. Recent studies have demonstrated an important role for IL-12 in inflammation[28–30] and Th1-mediated autoimmune diseases.[13,14,30–33] Our results suggest that IL-12-deficient mice might develop the mild lymphocytic form of EAT but that their cells would be unable to be activated to induce severe granulomatous EAT. Studies to address this issue are in progress.

Although we previously suggested that the effector cells for granulomatous EAT may be primarily Th2-like $CD4^+$ T cells,[3,6] the present results suggest this is unlikely. As IL-12 induces differentiation or activation of Th1 cells,[9,26] the ability of IL-12 to promote, and of anti-IL-12 to inhibit, activation of cells that induce granulomatous EAT suggests that Th1-like cells are more likely to be granulomatous EAT effector cells. However, in contrast to some other studies,[12,14,28–30] IL-12 did not promote obvious differentiation of MTg-sensitized cells to a Th1 phenotype as cells cultured with MTg and IL-12 produced increased amounts of IFN-γ, but expression or produc-

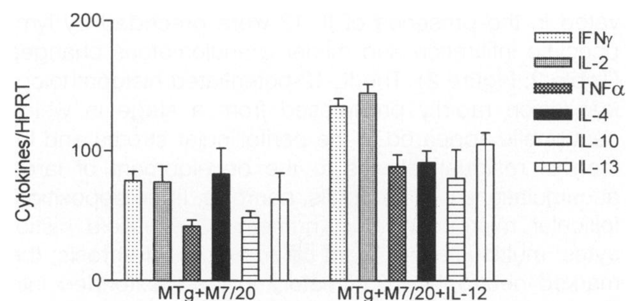


Figure 4. Cytokine gene mRNA expression in thyroids of recipient mice with granulomatous EAT. Individual thyroid lobes were obtained 19 days after cell transfer from four recipients of cells activated with MTg and anti-IL2R MAb (4+ EAT) and from five recipients of cells activated with MTg, anti-IL2R, and IL-12 (5+ EAT). Results are representative of several separate experiments and are expressed as the ratio of cytokines to HPRT mRNA densitometric units (1/25 cDNA dilutions) + SEM of individual thyroids. All samples compared for a particular cytokine:HPRT ratio were amplified at the same time. $P = \leq 0.005$ for IFN-γ, IL-2, and TNF-α; $P > 0.05$ for IL-4, IL-10, and IL-13.

Table 6. Development of Severe Granulomatous EAT in Recipient Mice Given Anti-IL-12 or Anti-IFN-γ

Experiment	Treatment of recipients	Number of mice with EAT severity:						Number of mice/total		Anti-MTg
		0	1+	2+	3+	4+	5+	G	N	
1	Rat/Sheep Ig	0	0	1	3	3	1	8/8	3/8	0.791 ± 0.023
	Anti-IL-12	0	0	0	1	2	1	4/4	2/4	0.648 ± 0.047
	Anti-IFN-γ	0	0	0	0	0	3	3/3	3/3	0.976 ± 0.029
2	Rat Ig	0	0	1	2	1	1	5/5	2/5	0.917 ± 0.045
	Anti-IL-12	0	0	1	1	2	1	5/5	3/5	0.942 ± 0.033
	Anti-IFN-γ	0	0	1	0	1	3	5/5	4/5	0.984 ± 0.011
3	Rat Ig	0	0	0	0	3	2	5/5	4/5	0.669 ± 0.049
	Anti-IFN-γ	0	0	0	0	0	5	5/5	5/5	0.984 ± 0.023

See Table 1 footnotes. Donor spleen cells were cultured 72 hours with 25 μg/ml MTg, M7/20, and 5 ng/ml IL-12. A total of 3×10^7 to 3.5×10^7 cells were transferred to recipient mice. Recipients were given 200 μg of sheep or rat Ig, anti-IL-12, or anti-IFN-γ (XMG 1.2) on days 0, 2, 4, 7, 10, and 12 (experiment 1) or 150 μg on days 0, 4, 8, 12, and 16 (experiments 2 and 3). Anti-IL-12 in experiment 1 was polyclonal sheep anti-IL-12 (Genetics Institute) and in experiment 2 was rat anti-IL-12 MAb (C17.8 plus C17.15). Thyroids were removed 15 days after cell transfer in experiments 1 and 3 and 19 days after cell transfer in experiment 2. Most of the anti-IFN-γ-treated mice had eosinophils in thyroid infiltrates. *P* values: experiment 1, line 1 *versus* line 3, $P = 0.05$; all others, $P > 0.1$.

tion of other Th1 and Th2 cytokines was not consistently affected (Figure 3). Moreover, even though thyroid-infiltrating cells of recipients of cells cultured in the presence of IL-12 had increased expression of Th1 cytokines compared with recipients of cells activated in the absence of IL-12, Th2 cytokine gene expression remained high (Figure 4). These and other results[4,6] are more consistent with the idea that granulomatous EAT effector cells may express both Th1- and Th2-like characteristics. IL-12 may increase chemokine expression, which could promote recruitment of neutrophils and macrophages to the thyroid,[28] resulting in increased damage to thyroid follicles. Moreover, studies with IL-4 and IFN-γ −/− and +/+ mice indicate that effector cells activated in the presence of IL-12 can induce severe EAT with granulomatous histopathology involving expression of distinct cytokines and mediators of inflammation in the thyroid.[20,21]

Recently, donor cells cultured with MTg and anti-IL-2R MAb have tended to induce less severe (3 to 4+) granulomatous EAT than in our earlier studies.[4] This has facilitated the recognition of the accelerating and potentiating effects of IL-12 on granulomatous EAT. The extensive 5+ granulomatous inflammatory lesions observed 19 to 21 days after transfer of effector cells activated in the presence of IL-12 were preceded by lymphocytic infiltration and milder granulomatous changes (Table 2; Figure 2). The IL-12-potentiated histopathological lesion rapidly progressed from a stage in which neutrophils appeared in the perifollicular stroma and lumen of residual follicles to the development of large accumulations of neutrophils, necrosis, fibrin deposition, follicular microabscesses, numerous epithelioid histiocytes, multinucleated giant cells, and focal fibrosis; the marked neutrophil inflammatory infiltrate extended into the adjacent muscle and connective tissue. By this stage, most thyroid follicles had disappeared. Whereas granulomatous EAT induced by cells activated with MTg and anti-IL-2R MAb in the presence or absence of IL-12, in which some recognizable thyroid follicles remain, resolves 35 to 60 days after cell transfer[5] (Table 2, experiment 2), thyroids with very severe neutrophil inflammatory lesions induced by IL-12-activated cells exhibit continuing inflammation and fibrosis at 35 and 50 days (Table 2, experiment 1; Figure 2, G and H).

We previously suggested that anti-IL-2R MAb may promote activation of the effector cells for granulomatous EAT by blocking activation of a cell that normally suppresses the development or activity of these effector cells.[4] The mechanism by which IL-12 activates cells to induce very severe granulomatous EAT is apparently distinct from the mechanism by which blocking the IL-2R selects for cells that induce granulomatous EAT. Thus, anti-IL-2R MAb was required at culture initiation,[4] whereas addition of IL-12 only during the final 24 hours of culture resulted in nearly optimal activation of cells to transfer severe granulomatous EAT (Table 4). Exogenous IL-12 may act directly on the $CD4^+$ granulomatous EAT effector cells during the last 24 hours of culture. IL-12 effectively activated these cells even when the inhibitory cell was not blocked by anti-IL-2R MAb, although the effects of IL-12 were sometimes more pronounced in the presence of anti-IL-2R MAb (Table 1).

An unexpected observation in these studies was that exogenous IL-12 exerted its effects *in vitro* primarily, if not solely, on cells 48 hours after activation by antigen (Tables 4 and 5), although our experiments do not rule out a requirement for endogenous IL-12 early in culture. In other studies using IL-12 to promote activation of $CD4^+$ T cells *in vitro*,[13,22,26,34,35] IL-12 was present continuously in culture. IL-12 has also been shown to act relatively late to potentiate the generation and differentiation of cytotoxic T lymphocytes from thymocytes in the presence of IL-4 *in vitro*.[36] IL-12 can induce differentiation of $CD4^+$ T cells[9–11,22,26] as well as growth of activated, but not resting, T cells by an IL-2-independent mechanism.[34,35,37,38] Maximal IL-12R expression by activated T cells occurs 2 to 4 days after antigen stimulation,[34,39] consistent with our finding that IL-12 mediates its effects on EAT effector cells 48 to 64 hours after antigen activation *in vitro* (Tables 4 and 5). This delayed action suggests that IL-12 may promote differentiation rather than growth of recently activated MTg-specific effector T cells, consistent with our finding that IL-12 did not induce detectable increases in MTg-specific T cell proliferation in

the presence or absence of anti-IL-2R MAb (data not shown). However, the MTg-induced proliferation of these cells is very low,[4] so IL-12 may have induced biologically significant increases in proliferation that were not detected in our assays.

IL-12 appears to mediate its effects on cells during culture, as addition of IL-12 to cultures 2 to 4 hours before cell transfer (Table 4) or administration of anti-IL-12 to recipient mice (Table 6) had no effect on EAT severity. This suggests that IL-12 is not simply binding to cells in culture and exerting its effects in the recipient mice. As IL-12 has been reported to induce homotypic aggregation of Th1 cells by an LFA-1/ICAM-dependent interaction,[40,41] expression of cell surface and adhesion molecules on cells cultured in the presence *versus* the absence of IL-12 was examined by flow cytometry. These studies provided no evidence for an IL-12-induced increase in adhesion (LFA-1, ICAM, VLA-4, or CD44) or co-stimulatory molecules (B7–1 or B7–2) after 72 hours of culture (data not shown). The increase in EAT effector function by culturing cells with IL-12 is most likely due to a direct effect on the $CD4^+$ EAT effector cells. Recipients of cells activated in the presence of IL-12 do not develop thyroid lesions when recipient mice are given anti-CD4 MAb (Table 3), and depletion of $CD8^+$ T cells from either donor or recipient mice did not diminish the effects of IL-12 on development of granulomatous EAT (data not shown). Additional studies, currently in progress, are needed to determine the mechanism by which IL-12 induces increased effector function by these cells and whether some of the effects of IL-12 may be mediated through other cells, such as NK cells or B cells present in these cultures.

The ability of IL-12 and anti-IL-12 to regulate the activation of EAT effector cells to induce histologically distinct thyroid lesions may have important implications for understanding how distinct types of autoimmune inflammatory responses can be regulated. Granulomatous inflammatory lesions are a major histopathological feature of a spectrum of human diseases ranging from noncaseating granulomas in sarcoidosis and granulomas of parasitic infections to destructive lesions associated with mycobacterial infections, giant cell arteritis, and the necrotizing vasculitis of Wegener's granulomatosis. The mechanisms underlying development of these lesions are poorly understood, and current treatments of granulomatous vasculitis with corticosteroids and cytotoxic drugs often result in serious toxicity and are not always effective. In the present studies, sensitized lymphocytes activated with MTg in the presence of IL-12 induced granulomatous histopathological changes in the thyroid that resulted in a progressive destructive neutrophil inflammation that in some cases proceeded to fibrinoid necrosis and a necrotizing vasculitis resembling the histopathological lesions of Wegener's granulomatosis. These severe granulomatous autoimmune lesions could be abrogated by neutralization of IL-12. IL-12 can exacerbate several other autoimmune diseases[13,14,30] and can augment the recruitment of inflammatory cells and chemokines[28] or alter the ratios of Th1 and Th2 cells in inflammatory responses.[12,15,16,24,30] Studies with the EAT model described here may ultimately lead to new concepts and more specific treatment for some human granulomatous autoimmune diseases.

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