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Induction of Experimental Autoimmune Thyroiditis in IL-12−/− Mice

Kemin Chen,* Yongzhong Wei,* Gordon C. Sharp,*‡ and Helen Braley-Mullen2*†§

Granulomatous experimental autoimmune thyroiditis (G-EAT) is induced by transfer of mouse thyroglobulin (MTg)-sensitized spleen cells activated in vitro with MTg and anti-IL-2R or MTg and IL-12. Previous work suggested that IL-12 was required in vitro for development of G-EAT. To determine whether IL-12 was also required during the induction and/or effector phases, DBA/1 mice with a disrupted IL-12-P40 gene (IL-12−/−) were used for EAT induction. Cells from MTg-sensitized IL-12−/− donors activated in vitro by MTg or MTg and anti-IL2R induced severe EAT in recipient mice. Compared with effector cells from IL-12−/− donors, effector cells from IL-12+/− donors induced thyroid lesions dominated by lymphocytes with minimal granulomatous changes. Thyroids of recipients of IL-12−/− cells expressed less IFN-γ mRNA and more TGF-β, IL-4, and IL-10 compared with recipients of IL-12+/+ cells. When IL-12 was added during in vitro activation, cells from both IL-12−/− and IL-12+/+ donors induced severe G-EAT, and expression of all cytokines except IL-12 was comparable in thyroids of both IL-12−/− and IL-12+/+ recipients. Transfer of cells from IL-12+/+ or IL-12−/− donors into IL-12+/+ or IL-12−/− recipients indicated that IL-12 expressed in thyroids was derived from recipients. Thus, endogenous IL-12 is not absolutely essential for the sensitization and activation of EAT effector cells to induce severe EAT, although it is required in vitro to promote activation of cells to induce severe granulomatous histopathology. The Journal of Immunology, 2001, 167: 1720–1727.

Experimental autoimmune thyroiditis (EAT) is an organ-specific autoimmune disease that can be induced in genetically susceptible strains of mice by injection of mouse thyroglobulin (MTg) and adjuvant (1), or by the transfer of MTg-primed donor spleen cells activated with MTg in vitro (2, 3). In our cell transfer model, cells activated with MTg alone generally induce a chronic lymphocytic form of EAT in which the thyroid is infiltrated primarily by T lymphocytes and other mononuclear cells (2–4). A histologically distinct and generally more severe granulomatous form of EAT (G-EAT) is induced when MTg-sensitized donor spleen cells are activated with MTg together with IL-12 and/or anti-IL-2R mAb (4, 5). Thyroid lesions in G-EAT are characterized by follicular cell proliferation, large numbers of histocytes, multi-nucleated giant cells, and variable numbers of neutrophils in addition to T lymphocytes and other mononuclear cells (2–4). A histologically distinct and generally more severe granulomatous form of EAT (G-EAT) is induced when MTg-sensitized donor spleen cells are activated with MTg together with IL-12 and/or anti-IL-2R mAb (4, 5). Thyroid lesions in G-EAT are characterized by follicular cell proliferation, large numbers of histocytes, multi-nucleated giant cells, and variable numbers of neutrophils in addition to T lymphocytes and other mononuclear cells (2–4). G-EAT lesions are more acute, reaching peak severity 19–21 days after cell transfer, with nearly complete resolution of inflammation or development of fibrosis 35–60 days after transfer (6–8). CD4+ T cells are the primary effector cells for both forms of EAT (4, 7, 9).

We recently demonstrated that MTg-sensitized donor cells activated in vitro with MTg and IL-12 induce a very severe destructive form of G-EAT (5). IL-12 plays a key role in promoting the development and activation of the Th1 subset of CD4+ T cells (10–12), and one major effect of IL-12 is to induce IFN-γ production from Th1 and NK cells (12). Although G-EAT effector cells activated in the presence of exogenous IL-12 produce high amounts of IFN-γ, they also produce Th2 cytokines (5, 13). IFN-γ is not required for induction of severe G-EAT (14), and both Th1 and Th2 cytokines are expressed in recipient thyroids when cells are activated with MTg and IL-12 (5, 13, 14). These results suggest that IL-12 does not promote activation of G-EAT effector cells solely by inducing their polarization to a Th1 phenotype, but a Th0-like CD4+ T cell population may be important for induction of G-EAT (5, 7, 13). Our earlier results suggested that IL-12 plays a critical role for the in vitro activation of MTg-sensitized cells to transfer G-EAT, because cells activated with MTg and anti-IL-2R mAb or IL-12 in the presence of anti-IL-12 transferred only mild lymphocytic EAT to recipient mice (5). Furthermore, Zaccone et al. (15) showed that IL-12−/− mice were relatively resistant to EAT induction.

To more clearly define the role of IL-12 in activation of G-EAT effector cells, IL-12-deficient DBA/1 mice with a disruption in the IL-12p40 locus (16) were used as donors and recipients. The results indicate that MTg-sensitized donor cells from IL-12−/− mice can be activated to transfer severe EAT, although thyroid lesions induced in the absence of IL-12 have very mild granulomatous features compared with those induced in the presence of IL-12. Thyroids of IL-12−/− recipients of cells from IL-12−/− mice activated in the absence of IL-12 in vitro expressed predominantly a Th2-like pattern of cytokines, whereas recipients of cells from IL-12+/+ or IL-12−/− mice activated with IL-12 in vitro expressed both Th1 and Th2 cytokines in their thyroids.
Materials and Methods

**Mice**

Mice with a disrupted IL-12 p40 gene (IL-12−/−) and backcrossed onto the DBA/1 (H-2b) genetic background were developed and screened by Dr. Jeanne Magram of Hoffman-LaRoche (Nutley, NJ) (16). Breeding pairs of IL-12−/− mice and homozygous IL-12−/− DBA/1 mice were provided by Dr. Magram and subsequently bred under specific pathogen-free conditions in the animal facilities at the University of Missouri (Columbia, MO). Mice were maintained under specific pathogen-free conditions until used for experiments at which time they were transferred to conventional housing. Male IL-12−/− or IL-12+/+ mice were used as donors, and either male or female mice were used as recipients. Mice were generally 7- to 10-wk old at the time of transfer.

**EAT induction**

EAT was induced as previously described (2, 4, 5). Briefly, donor mice were injected i.v. with 150 μg MTg and 15 μg LPS (Escherichia coli 0111: B4, Sigma, St. Louis, MO) twice at 10- to 14-day intervals. Seven days after the second immunization, donor spleen cells were cultured in 60-mm petri dishes at 107 cells/ml at 37°C as described previously in detail (4, 5). Medium was RPMI 1640 containing 25 mM HEPES buffer (Cell and Immunobiology Core Facility, University of Missouri, 5% FCS (Sigma, St. Louis, MO), sodium pyruvate, glutamine, nonessential amino acids, vitamins (all obtained from Fisher Scientific, St. Louis, MO), and 5 × 10−5 M 2-ME. Cells were cultured with MTg (25 μg/ml) alone or with MTg together with culture supernatant containing the anti-IL-2R mAb M7/20 (4, 5) or 5 μg/ml IL-12 (Intergen, Gaithersburg, MD) (5). Cells were harvested, washed twice with balanced salt solution, and 3–3.5 × 106 cells were transferred i.v. into (500 rad) irradiated syngeneic recipients. EAT-susceptible DBA/1 strain used here can induce severe G-EAT after adoptive transfer, and one lobe was stored at −80°C before processing. Frozen thyroid were homogenized in TRIzol, and RNA was extracted and reverse transcribed as previously described (13, 14). Diluted cDNA (1/5, 1/25) was amplified using 94°C for 30 s for denaturing, 60°C for 30 s for annealing, and 72°C for 1 min for extension. To determine the relative initial amounts of target cDNA, each cDNA sample was serially diluted 1/100 dilution of normal mouse serum on MTg-coated plates. Levels of IFN-γ, IL-2, and IL-10 produced by cells during the 72-h in vitro culture were evaluated by double sandwich ELISA as previously described (13). For some experiments, spleen cells were activated using plate-bound anti-CD3.

### RT-PCR of cytokine mRNA

Thyroid lobes were removed from individual mice at different times after adoptive transfer, and one lobe was stored at −80°C before processing. Frozen thyroids were homogenized in TRIzol, and RNA was extracted and reverse transcribed as previously described (13, 14). Diluted cDNA (1/5, 1/25) was amplified using 94°C for 30 s for denaturing, 60°C for 30 s for annealing, and 72°C for 1 min for extension. To determine the relative initial amounts of target cDNA, each cDNA sample was serially diluted 1/100 dilution of normal mouse serum on MTg-coated plates. Levels of IFN-γ, IL-2, and IL-10 produced by cells during the 72-h in vitro culture were evaluated by double sandwich ELISA as previously described (13). For some experiments, spleen cells were activated using plate-bound anti-CD3.

### Statistical analysis

All experiments were repeated at least three times. Statistical analysis of data was performed using an unpaired two-tailed Student’s t test. Values involving up to 25% of the gland, 3+ indicates that 25–50% of the gland is infiltrated, 4+ indicates destruction of >50% of the gland by infiltrating inflammatory cells, and 5+ indicates almost complete destruction of the gland with few or no remaining follicles. Thyroid lesions were also evaluated qualitatively. Thyroids given the lymphocytic designation had infiltrates consisting primarily of mononuclear cells, with few polymorphonuclear cells (PMNs), minimal enlargement of thyroid follicular cells and no extension of the inflammation into the surrounding muscle or connective tissue. Thyroids designated as granulomatous had enlargement and proliferation of thyroid follicular cells, with numerous histiocytes, multinucleated giant cells, and increased numbers of PMNs in addition to the mononuclear cell infiltration. The more severely inflamed granulomatous thyroids (4+–5+ severity scores) also had microabscess formation, necrosis, and focal fibrosis, and the inflammation generally extended beyond the thyroid to involve adjacent muscle and connective tissue (5, 7, 8). Infiltration of the thyroid by eosinophils was also evident in many recipients of cells from IL-12−/− donors.

**ELISAs**

Serum levels of MTg-specific IgG autoantibodies in individual donor or recipient mice was determined by ELISA as previously described (4). The contribution of various IgG subclasses to the total IgG autoantibody response was assessed using alkaline-phosphatase-conjugated Abs specific for mouse IgG1, IgG2a, and IgG2b. The secondary Abs were used at previously determined optimal dilutions (1/6000–1/8000) that detected optimal Ab activity on MTg-coated plates while giving minimal activity (OD < 0.05) on plates coated with an irrelevant protein (OVA) or of a 1/100 dilution of normal mouse serum on MTg-coated plates.

### Table I. Cells from IL-12-deficient mice induce primarily lymphocytic EAT when cells are activated with MTg or MTg + anti-IL2R mAb

<table>
<thead>
<tr>
<th>Donors &amp; Activation</th>
<th>In Vitro Activationb</th>
<th>EAT Severityc</th>
<th>Anti-MTGd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
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<td>MTg</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>−/−</td>
<td>MTg</td>
<td>0 0</td>
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<tr>
<td>+/+</td>
<td>MTg + M7/20</td>
<td>0 0</td>
<td>0</td>
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<tr>
<td>−/−</td>
<td>MTg + M7/20</td>
<td>0 0</td>
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<tr>
<td>+/+</td>
<td>MTg + M7/20</td>
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<td>0</td>
</tr>
<tr>
<td>−/−</td>
<td>MTg + M7/20</td>
<td>0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

a IL-12−/− or IL-12+/+ donors were immunized twice at 10-day intervals with 150 μg MTg + 15 μg LPS.

b Spleen cells from donor mice were activated 72 h with MTg (25 μg/ml) or with MTg and anti-IL2R mAb M7/20. Cells (3.5 × 107) from IL-12−/− donors were transferred to (500-rad) irradiated IL-12−/− recipients, and cells from IL-12−/− donors were transferred to irradiated IL-12−/− recipients. Lines 1–4 represent one experiment, and lines 5 and 6 are a separate experiment.

c Number of mice with various degrees of severity of EAT 19–21 days after cell transfer. G, Number of mice granulomatous changes in thyroids. p values are: 0.78 (line 1 vs line 2), 0.06 (line 3 vs line 4), and <0.001 (line 5 vs line 6).

d Anti-MTG IgG1 and IgG2a in sera of recipient mice (1/1600) serum dilutions. Values are expressed as mean OD410 ± SEM.
Results

Cells from MTg-sensitized IL-12−/− donors can induce severe EAT in recipient mice

As noted in the introduction, our previous studies suggested that endogenous IL-12 was required for the in vitro activation of MTg-sensitized donor cells to transfer G-EAT, because CBA/J donor cells activated in the presence of MTg and anti-IL-12 induced only mild lymphocytic EAT (5). However, in those experiments, endogenous IL-12 was present during donor sensitization and in recipient mice. To determine whether G-EAT could be induced when both donor and recipient mice lacked IL-12, IL-12+/+ and IL-12−/− donor mice were immunized with MTg and LPS, and their cells were activated in vitro with MTg alone or with MTg and anti-IL2R mAb and transferred to irradiated syngeneic recipients (Table I). As reported previously (7, 8), DBA/1 mice are very susceptible to G-EAT induction compared with other strains of mice such as AKR and CBA/J. In most experiments, spleen cells from MTg-sensitized IL-12−/− donor mice induced very severe EAT after activation in vitro with MTg alone or with MTg and anti-IL2R, and most thyroid had typical granulomatous histopathology and extensive infiltration of the thyroids by PMN (Fig. 1, A and B). In 4 of 6 experiments (e.g., Table I, lines 2 and 4), cells from IL-12−/− donors activated with MTg or MTg and anti-IL2R transferred EAT that tended to be less severe, but was not statistically different in severity from that induced by cells from IL-12+/+ donors. However, thyroid lesions in recipients of IL-12−/− donor cells were qualitatively different, in that they were primarily lymphocytic (Fig. 1, C and D), with only mild granulomatous histopathologic features, consisting of proliferation of thyroid follicular cells and increased PMN accumulation in some thyroids. These thyroids often had many infiltrating plasma cells, and had fewer PMNs and more eosinophils than thyroids of IL-12+/+ mice. In 2 of 6 experiments (Table I, line 6), cells from IL-12−/− donors were markedly deficient compared with IL-12+/+ donor cells in their ability to induce EAT, and induced only mild lymphocytic EAT in recipient mice. The reason for this difference compared with other experiments is unknown, but it was apparently not due solely to poor donor sensitization, because cells from the same donors transferred severe G-EAT when exogenous IL-12 was added to culture (Table II, lines 7 and 8). These results indicate that cells from IL-12−/− donor mice can be sensitized and activated by MTg or MTg and anti-IL2R in vitro to transfer severe EAT in the absence of endogenous IL-12. However, endogenous IL-12 increased EAT severity in some experiments, and was required in vitro to activate effector cells that induced severe granulomatous histopathologic lesions in recipient mice.

Cells activated in the presence of exogenous IL-12 in vitro induce very severe G-EAT (5). To determine whether exogenous IL-12 added in vitro would be sufficient for the induction of G-EAT in IL-12−/− mice, MTg-primed spleen cells from IL-12+/+ or IL-12−/− donors were cultured with MTg and IL-12 before transfer. When IL-12 was added during in vitro activation, cells from both IL-12+/+ and IL-12−/− donors induced very severe G-EAT in IL-12+/+ or IL-12−/− recipients (Table II), and there was little difference either qualitatively or in the extent of thyroid follicle destruction in recipients of IL-12+/+ or IL-12−/− cells. Thyroid lesions had more severe granulomatous changes than those induced by cells from IL-12+/+ donors activated in the absence of exogenous IL-12, with widespread follicular cell proliferation, numerous PMNs and epithelioid histiocytes, lymphocytes, multinucleated giant cells, necrosis, microabscess formation, and fibrosis (Fig. 1, E–H). Taken together, these results suggest that endogenous IL-12 is not essential for the sensitization and activation of effector cells to induce severe lymphocytic EAT (Table I). However, the presence of IL-12 during in vitro activation is needed for cells from IL-12−/− donors to induce severe granulomatous thyroid histopathology.

Because IL-12 has been reported to regulate Ab production (18–21), the anti-MTg autoantibody responses of donor and recipient mice were also measured. Both the autoantibody levels and the IgG subclass distributions were similar for both IL-12+/+ and IL-12−/− donors (data not shown) and recipients of IL-12+/+ or IL-12−/− donor cells (Tables I and II). The lack of effect of IL-12 deficiency on IgG2a autoantibody responses may be due to

FIGURE 1. Histology of thyroids from IL-12−/− and IL-12+/+ mice. MTg-sensitized donor cells from IL-12+/+ and IL-12−/− mice were activated with MTg and anti-IL2R (no exogenous IL-12) (A–D) or with MTg and 5 ng/ml IL-12 (E–H). In the absence of exogenous IL-12, granulomatous thyroid lesions (4–5+ severity) were induced in IL-12+/+ recipients of IL-12+/+ cells (A and B), whereas lesions induced in IL-12+/− recipients of IL-12−/− cells were mostly lymphocytic (4+ severity) (C and D). Severe granulomatous thyroid lesions developed in recipients of both IL-12+/+ (5+, E and F) and IL-12−/− (5+, G and H) cells when MTg-sensitized effector cells from IL-12+/+ or IL-12−/− donors were activated in vitro by MTg and IL-12. Hematoxylin and eosin staining. Magnification: A, C, E, and G, ×100; B, D, F, and H, ×400.
the fact that IL-12<sup>−/−</sup> mice produce sufficient IFN-γ to promote switching to IgG2a.

Cytokine mRNA expression and protein production by spleen cells from donor mice

IL-12 is a pivotal molecule in immune responses based in part on its ability to influence the differentiation of CD4<sup>+</sup> T cells to a Th1 phenotype (10–12). To determine whether cytokine gene expression was altered in the absence of endogenous IL-12, mRNA expression of Th1 and Th2 cytokines was examined in MTg-sensitized spleen cells of IL-12<sup>+/+</sup> and IL-12<sup>−/−</sup> donors activated by MTg with or without addition of exogenous IL-12. As expected, IL-12 mRNA was undetectable in IL-12<sup>−/−</sup> spleen cells, and IFN-γ expression was decreased in IL-12<sup>−/−</sup> cells compared with IL-12<sup>+/+</sup> cells. However, activation of splenocytes with MTg or MTg and anti-IL-2R in the presence or absence of IL-12 resulted in similar expression of IL-2, IL-4, and IL-10 by both IL-12<sup>−/−</sup> and IL-12<sup>−/+</sup> cells (data not shown). These results are consistent with our earlier studies (5) indicating that IL-12 does not induce a shift of donor effector cells to a Th1-dominant phenotype.

IL-2, IFN-γ, and IL-10 production by donor spleen cells stimulated with anti-CD3, MTg, MTg and anti-IL-2R, or MTg and IL-12 was also determined by ELISA. As shown in Table III, cells from MTg-sensitized IL-12<sup>−/−</sup> mice activated by MTg or anti-CD3 in the absence of exogenous IL-12 secreted less IFN-γ than IL-12<sup>+/+</sup> cells. However, similar amounts of IFN-γ were produced by IL-12<sup>+/+</sup> and IL-12<sup>−/−</sup> cells when cells were activated in the presence of IL-12. IFN-γ production by IL-12<sup>+/+</sup> spleen cells was also high when cells were activated with MTg or anti-CD3 together with IL-18, but IL-18 was much less effective than IL-12 for inducing IFN-γ production by IL-12<sup>−/−</sup> spleen cells in vitro. There were no consistent differences in the amounts of IL-10 or IL-2 produced by IL-12<sup>−/−</sup> cells compared with IL-12<sup>−/+</sup> cells under any of the activation conditions. IL-2 was not detected in 72-h supernatants of splenocytes cultured in the presence of IL-12 (Table III) presumably due to their increased consumption of IL-2 (5). When supernatants were tested at 24 h rather than at 72 h, IL-12<sup>−/+</sup> and IL-12<sup>−/−</sup> cells produced similar amounts of IL-2 (Table III, experiment 4; and data not shown). Spleen cells from IL-12<sup>−/+</sup> and IL-12<sup>−/−</sup> mice produced no detectable IL-4 or IL-5 under any of these activation conditions (data not shown).

The origin of IL-12 mRNA expression in recipient thyroids

This model in which G-EAT is induced in recipient mice by adoptive transfer of in vitro activated donor spleen cells (4, 5, 7) provides an opportunity to address the origin of particular molecules or cells in G-EAT development. For example, we previously showed that IL-4 and IFN-γ mRNA expressed in recipient thyroids was derived entirely from the transferred donor cells (14, 17). To

<table>
<thead>
<tr>
<th>Donors&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Recipients&lt;sup&gt;6&lt;/sup&gt;</th>
<th>EAT Severity&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Anti-MTg&lt;sup&gt;4&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>1+</td>
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</table>

<sup>4</sup> Donor IL-12<sup>+/+</sup> or IL-12<sup>−/−</sup> mice were immunized as described in Table I footnote a. Spleen cells from donor mice were activated 72 h with 25 μg/ml MTg + 5 ng/ml IL-12.

<sup>5</sup> Spleen cells from the indicated donors were transferred (3.5 × 10<sup>7</sup> cells) to (500-rad) irradiated IL-12<sup>+/+</sup> or IL-12<sup>−/−</sup> recipients as indicated. Lines 1–4 represent a single experiment, lines 5 and 6 a second experiment, and lines 7 and 8 a third experiment. Donor cells in Expt. 3 (lines 5 and 6) were from the same donors used in Expt. 2 (lines 5 and 6) in Table I.

<sup>6</sup> See Table I, footnote c. All p values are >0.05.

<sup>7</sup> See Table I, footnote d.

Table III. Cytokine production by spleen cells of IL-12<sup>+/+</sup> and IL-12<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>In Vitro Activation</th>
<th>IFN-γ (U/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>+/-</td>
<td>-/-</td>
<td>+/-</td>
</tr>
<tr>
<td>1</td>
<td>MTg + anti-IL-2R&lt;sup&gt;4&lt;/sup&gt;</td>
<td>61</td>
<td>8</td>
<td>686</td>
</tr>
<tr>
<td></td>
<td>MTg + IL12</td>
<td>500</td>
<td>505</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>MTg</td>
<td>79</td>
<td>8</td>
<td>1053</td>
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<td>23</td>
<td>2977</td>
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<tr>
<td></td>
<td>MTg + IL12</td>
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<td>505</td>
<td>1285</td>
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<td>3</td>
<td>MTg</td>
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<td>240</td>
<td>282</td>
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<td>MTg + IL-18</td>
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<td>Anti-CD3 + IL-18</td>
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<td>3569</td>
</tr>
</tbody>
</table>

<sup>4</sup> Spleen cells from MTg-sensitized IL-12<sup>+/+</sup> or IL-12<sup>−/−</sup> donors were activated 72 h as indicated. IL-12 was added at 5 ng/ml and IL-18 at 25 ng/ml. Supernatants were collected and assayed for IFN-γ, IL-10, or IL-2 by ELISA. IL-4 was not detected in any of these culture supernatants, and IL-2 was not significantly above background in supernatants from cells cultured with IL-12 except in Expt. 4, where IL-2 was determined after 24 h.

<sup>6</sup> The indicated cytokines were not detected in supernatants designated –.
determine whether IL-12 expressed in recipient thyroids was derived from donors or recipients, cells from IL-12−/− donors were transferred to IL-12−/− or IL-12+/+ recipients, and cells from IL-12+/+ donors were transferred to both IL-12+/+ and IL-12−/− recipients (Fig. 2). As expected, when both donors and recipients were IL-12−/−, IL-12 mRNA was not detected in recipient thyroids (Fig. 2, A and B). Interestingly, IL-12 mRNA was also not detected in recipient thyroids when cells from IL-12+/+ donors were transferred to IL-12−/− recipients, whereas IL-12 mRNA was detected in thyroids when IL-12+ cells were transferred to IL-12+/+ recipients. Therefore, whether the spleen cells were from IL-12+/+ or IL-12−/− donors, IL-12 mRNA was detected in recipient thyroids only when the recipients were IL-12−/− (Fig. 2C). IL-12 mRNA was not detected in thyroids of irradiated recipients without EAT (Fig. 2C, normal). These results demonstrate that IL-12 mRNA expressed in recipient thyroids is derived from the recipients, and indicate that up-regulation of IL-12 mRNA in the thyroid is not required for development of severe G-EAT because IL-12+/+ donor cells induced severe G-EAT in IL-12−/− recipients lacking IL-12 expression in the thyroid.

Expression of cytokines in recipient thyroids from IL-12+/+ and IL-12−/− mice

To assess cytokine gene expression in the target organ, expression of cytokine gene mRNA in individual thyroids was determined by RT-PCR. HPRT was used as a housekeeping gene to normalize cytokine gene expression in thyroids with different degrees of G-EAT severity. When cells were activated with MTg and anti-IL2R (no exogenous IL-12), IFN-γ mRNA was lower in thyroids of recipients of IL-12−/− cells compared with recipients of IL-12+/+ cells at both days 11 and 21 (Fig. 3, A and B). TNF-α and inducible NO synthase (iNOS) mRNA transcripts mirrored those of IFN-γ, with lower levels in thyroids of recipients of IL-12−/− cells (Fig. 3, A and B). Expression of IL-2 mRNA was lower in thyroids of IL-12−/− compared with IL-12+/+ recipients at day 11, but was comparable for both strains at day 21. Interestingly, intrathyroidal expression of IL-18 was comparable for both IL-12+/+ and IL-12−/− mice (Fig. 3, A and B). Expression of all these cytokines in thyroids of both IL-12−/− and IL-12+/+ mice was above their naive cohorts, because thyroids of normal mice or irradiated mice not given effector cells did not express detectable mRNA for any cytokines (Fig. 4 and data not shown). These results suggest that cells producing IFN-γ and proinflammatory cytokines could differentiate and migrate to the thyroid in the absence of exogenous IL-12, but their expression was higher in thyroids of IL-12−/− mice. In contrast, IL-4, IL-10, and TGF-β1 mRNA was increased in thyroids of IL-12−/− mice compared with IL-12+/+ mice (Fig. 3, C and D). However, IL-5 (Fig. 3, C and D) and IL-13 (data not shown) transcripts were comparable between IL-12−/− and IL-12+ mice. The increased expression of IL-4, IL-10, and TGF-β1, and the decreased expression of IFN-γ and proinflammatory cytokine mRNA, in thyroids of IL-12−/− mice suggests that TGF-β and some Th2 cytokines were preferentially expanded in thyroids of IL-12−/− mice when donor cells were activated in the absence of exogenous IL-12.

As described above (Table II), when effector cells were activated by MTg in the presence of exogenous IL-12, severe G-EAT (4−5+ ) developed in both IL-12−/− and IL-12+/+ recipient mice. To determine whether activation of cells in the presence of exogenous IL-12 would affect cytokine gene expression in thyroids of IL-12−/− and IL-12+/− mice, cytokine gene mRNA expression was analyzed in thyroids of recipients of effector cells activated in vitro by MTg and IL-12. Expression of mRNA for all cytokines was comparable in thyroids of recipients of both IL-12−/− and IL-12+/− cells (Fig. 4, A and B). Therefore, the expression of proinflammatory cytokines and the extent of granulomatous histopathology in thyroids of IL-12−/− mice was comparable to that of IL-12+/− mice when exogenous IL-12 was added during in vitro activation of donor cells.

Discussion

The adoptive transfer model of EAT has facilitated the analysis of pathophysiological mechanisms involved in induction of autoimmunity. A role for IL-12 in G-EAT was suggested from our earlier studies, because neutralization of endogenous or exogenous IL-12 in vitro resulted in the transfer of only mild lymphocytic EAT in CBA/J mice (5). However, those studies did not enable us to determine whether endogenous IL-12 was required in vivo either for sensitization of donor cells or for development of thyroiditis in recipient mice. In the present study, the role of IL-12 during the induction and effector stages of EAT was examined using IL-12-deficient DBA/1 mice as donors and recipients of MTg-sensitized cells activated in vitro in the presence or absence of exogenous IL-12. The results indicate that endogenous IL-12 is not required for donor sensitization or for development of thyroid lesions in recipient DBA/1 mice. However, exogenous or endogenous IL-12 is needed during in vitro activation to generate effector cells that

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**FIGURE 2.** Analysis of IL-12 mRNA expression in thyroids from IL-12+/+ and IL-12−/− recipient mice with EAT induced by transfer of donor spleen cells activated in vitro by MTg, MTg and anti-IL2R (M7/20), or MTg and IL-12. Activation conditions are given in B, A and C. Cells activated with MTg and IL-12. Individual thyroid lobes were obtained from five IL-12+/+ or IL-12−/− recipient mice 11 or 19 days after cell transfer (A) or 19−21 days after cell transfer (B and C). A and B, IL-12+/+ donor cells transferred to IL-12+/+ recipients and IL-12−/− donor cells to IL-12−/− recipients. C, Cells from IL-12+/+ donors transferred to IL-12+/+ (+/+), IL-12−/− mice (±/−); and cells from IL-12−/− donors transferred to IL-12−/− mice (−/−) or IL-12+/+ (±/+,−/−) recipients. RT-PCR was performed as described in Materials and Methods. Results are expressed as the mean ratio of IL-12 densitometric U/HPRT ± SD (×100) using a 1/25 dilution of cDNA, and are representative of three independent experiments.
can induce thyroid lesions with severe granulomatous histopathologic features.

In the adoptive transfer EAT model, the donor spleen cells are the source of all detectable IL-4 and IFN-γ expressed in recipient thyroids (14, 17). The donor cells also contribute the autoantibody-producing cells that play a necessary, but as yet undefined, role in G-EAT (4, 7, 17). However, the irradiated recipients do make important contributions to the inflammatory cell infiltrate in G-EAT thyroids. For example, CD8+ T cells outnumber CD4+ T cells in recipient thyroids (7, 22). CD8+ T cells play an important regulatory role in G-EAT (6, 7, 13, 23) but contribute minimally, if at all, to cytokine gene expression (13) or to thyroid damage (6, 13, 23). The intrathyroidal CD8+ T cells can be derived entirely from the irradiated recipients (23). The recipients probably also contribute macrophages, PMNs, and eosinophils to the thyroid infiltrate in G-EAT, and as shown here, they are the source of the cells that express IL-12 mRNA in the thyroid (Fig. 2).

IL-12 is produced by activated macrophages and dendritic cells, and one of its major functions is to induce IFN-γ production by T and NK cells (12, 24). Because IL-12 p40 mRNA is not expressed in thyroids before infiltration of inflammatory cells, recipient IL-12-producing macrophages and/or dendritic cells may become activated when encountering MTg-primed effector CD4+ T cells. Thyroids of mice with G-EAT have many macrophages, but few dendritic cells (4, 5, 8). However, the thyroid-infiltrating macrophages apparently do not need to produce IL-12, because both IL-12−/− and IL-12+/+ recipients can develop severe G-EAT with

![FIGURE 3](http://www.jimmunol.org/) 
Levels of IFN-γ, IL-2, TNF-α, iNOS, and IL-18 (A and B), or IL-10, TGFβ, IL-4, and IL-5 (C and D) mRNA transcripts in thyroids of IL-12−/− and IL-12+/+ recipients 11 or 19 days after transfer of MTg-primed donor spleen cells activated in vitro by MTg and M7/20. IL-12−/− donor cells were transferred to IL-12+/+ recipients and IL-12−/− donor cells to IL-12−/− recipients. cDNA from IL-12+/+ or IL-12−/− cells was prepared and amplified as described in Materials and Methods. Bars are means of data for thyroids of five individual mice ± SD. Results are expressed as the mean ratio of cytokine densitometric U/HPRT ± SD (×100), and are representative of three independent experiments. A significant difference between IL-12−/− and IL-12+/+ thyroids is indicated by an asterisk (p < 0.05).

![FIGURE 4](http://www.jimmunol.org/) 
Expression of cytokine mRNA in thyroids of IL-12+/+ vs IL-12−/− recipient mice with 5+ G-EAT induced by MTg-primed splenocytes activated in vitro by MTg and IL-12 or in thyroids of irradiated recipient IL-12+/+ and IL-12−/− mice not given effector cells. Bars are means of data for thyroids of five individual mice ± SD. Results are expressed as the mean ratio of cytokine densitometric U/HPRT ± SD (×100), and are representative results from three independent experiments. A significant difference between IL-12+/+ and IL-12−/− thyroids is indicated by an asterisk (p < 0.05).
macrophages infiltrating the thyroids (Table II). In IL-12−/− thyroids, macrophages presumably produce other inflammatory mediators that contribute to thyroid damage.

The role of IL-12 in autoimmune diseases is complex. IL-12 can have different effects on an autoimmune disease depending on whether it is administered (or neutralized) during the inductive or effector stage, given systemically or locally, or used in vitro for activation of effector cells. For example, exogenous IL-12 can either promote or inhibit collagen-induced arthritis depending on the adjuvant and the time of administration (16, 25–27), it can promote or inhibit diabetes in nonobese diabetic (NOD) mice (28, 29), inhibit experimental autoimmune uveitis (EAU) (30), and can inhibit G-EAT (our unpublished results). Neutralization or antagonism of IL-12 can inhibit diabetes in NOD mice (31) and development of experimental autoimmune encephalomyelitis (EAE) (32, 33) and EAU (34). Development of collagen-induced arthritis, EAE, EAU, and experimental autoimmune myasthenia gravis is reduced in IL-12−/− mice (16, 35–37). However, IL-12−/− NOD mice develop diabetes similar to wild-type mice (38), indicating that IL-12 is not essential for development of all Th1-mediated autoimmune diseases. Using an adoptive transfer model of EAE similar to that used here, IL-12 was critical for sensitization of donor cells, whereas neutralization of IL-12 in culture had minimal effects on disease transfer (35). In the EAU adoptive transfer model (36), IL-12−/− donor cells produced Th2 cytokines and did not transfer EAU when activated with Ag alone, but IL-12−/− cells did induce EAU when IL-12 was added during in vitro activation (36). The studies in EAU are consistent with those reported here, and indicate that the role of endogenous IL-12 in some autoimmune disease models can be replaced by IL-12 added exogenously during in vitro activation of effector cells. In another model in which EAT was induced by immunization with MTg and CFA, IL-12−/− mice were resistant to the induction of EAT (15). The difference between those results and those reported here could be due to the use of different adjuvants and/or different mouse strains (15, 25, 26, 39). In fact, the use of the highly EAT-susceptible DBA/1 strain of IL-12−/− mice in the current study was probably critical for demonstrating that effector cell sensitization and the final effector phase of G-EAT can be IL-12-independent.

IL-12 is a key cytokine in determining whether a Th1 or Th2 response will evolve following Ag challenge (10–12). To begin to address the mechanisms involved in the development of severe EAT in IL-12−/− mice, and the role of IL-12 in vitro in promoting the activation of effector cells, the cytokine profile in thyroids of recipient mice was determined. In vitro activated splenocytes from IL-12−/− donor mice did not have an obvious polarization to a Th2 response, because they produced no detectable IL-4, and IL-10 production was comparable to that of IL-12+/+ splenocytes (Table III). However, when IL-12−/− splenocytes were activated by MTg in the absence of exogenous IL-12, IL-12−/− recipient thyroids had increased expression of TGF-β, IL-10, and IL-4 mRNA, and decreased expression of IFN-γ and proinflammatory cytokine mRNA (Fig. 3). This suggests a role for IL-12 in the generation of optimal Th1 inflammatory responses in the target organ. Spleen cells of IL-12−/− mice produced low amounts of IFN-γ (Table III), and IFN-γ mRNA was expressed at low levels in IL-12−/− thyroids (Fig. 3). However, the low expression of IFN-γ in thyroids of IL-12−/− compared with IL-12+/+ mice is unlikely to explain the absence of severe granulomatous histopathology, because IFN-γ−/− mice develop very severe G-EAT even when donor cells are activated in the absence of exogenous IL-12 (14). The IFN-γ expressed in thyroids of IL-12−/− mice with EAT but not in normal thyroids is induced by cytokines other than IL-12. IL-18 can induce IFN-γ expression in the absence of endogenous IL-12 (40–42). Although IL-18 was relatively ineffective in inducing IFN-γ production by IL-12−/− cells in vitro (Refs. 31, 36 and Table III), IL-18 might contribute to IFN-γ production in vivo, because IL-12−/− and IL-12+/+ thyroids expressed comparable amounts of IL-18 mRNA.

Thyroids of IL-12−/− mice (when IL-12 is not added exogenously) have a cytokine profile similar to IFN-γ−/− mice, i.e., low or absent IFN-γ mRNA expression, low expression of iNOS and TNF-α, increased expression of some Th2 cytokines, and increased infiltration by eosinophils (14). This profile is also similar to that reported for IFN-γ−/− and IL-12−/− mice in EAU (36). However, despite the similarities in inflammatory mediator expression in thyroids of IFN-γ−/− and IL-12−/− mice, thyroids of IFN-γ−/− recipient mice activated in the absence of exogenous IL-12 in vitro have severe granulomatous histopathology, whereas thyroids of IL-12−/− recipients activated in the same way do not. This may be because cells from IFN-γ−/− mice produce sufficient endogenous IL-12 in vitro to activate effector cells to induce severe G-EAT by an IFN-γ-independent mechanism.

Polarized Th1 or Th2 reactions can be found in granulomatous lesions of known or unknown etiology (43–45). Granuloma formation can be IL-12-dependent or -independent, and the role of IL-12 in granuloma formation may vary depending on the inducing agent (45–48). In our model, in the absence of IL-12 in vitro, granulomatous changes in the thyroid were minimal (Table I), and thyroids expressed increased Th2 cytokines (Fig. 3). IFN-γ−/− mice also develop severe G-EAT with a Th2 predominant profile (14). This may suggest that a predominant intrathyroidal Th2 cytokine profile accompanied by eosinophil infiltration and decreased iNOS expression is observed when IFN-γ is low, but this is not necessarily associated with the extent of the granulomatous changes or extent of damage to the thyroids.

Locally produced cytokines and other inflammatory mediators induce much of the damage to the target tissue or organ in autoimmune disease. Our study provides additional insight into understanding the multiple pathways that can be used to achieve damage to a tissue or organ during an autoimmune inflammatory response. IL-12−/− mice generally do not have reduced severity of EAT despite expressing different cytokines in the target organ. These and other studies with cytokine gene knockout mice have demonstrated that many redundancies and alternative pathways can be used to achieve damage to a particular tissue or organ in autoimmune disease (49). Because many protocols currently being tested for potential therapy of autoimmune disease involve alterations in cytokines and other inflammatory mediators such as iNOS and chemokines, a better understanding of how different cytokines and mediators can lead to an apparently similar degree of organ damage is important.

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References