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Transgenic Expression of TGF-β on Thyrocytes Inhibits Development of Spontaneous Autoimmune Thyroiditis and Increases Regulatory T Cells in Thyroids of NOD.H-2h4 Mice

Shiguang Yu,*†‡ Yujiang Fang,*† Gordon C. Sharp,†§ and Helen Braley-Mullen*†§

Transgenic NOD.H-2h4 mice expressing TGF-β under control of the thyroglobulin promoter were generated to assess the role of TGF-β in the development of thyrocyte hyperplasia. In contrast to nontransgenic littermates, which develop lymphocytic spontaneous autoimmune thyroiditis (L-SAT), all TGF-β transgenic (Tg) mice given NaI water for 2–7 mo developed thyroid lesions characterized by severe thyroid epithelial cell hyperplasia and proliferation, with fibrosis and less lymphocyte infiltration than in nontransgenic mice. Most Tg mice produced less anti-mouse thyroglobulin autoantibody than did wild type (WT) mice. T cells from Tg and WT mice were equivalent in their ability to induce L-SAT after transfer to SCID or TCRα−/− mice. WT lymphocytes could transfer experimental autoimmune thyroiditis or L-SAT to Tg mice, indicating that the transgenic environment did not prevent migration of lymphocytes to the thyroid. Thyroids of Tg mice had higher frequencies of Foxp3+ regulatory T cells (Tregs) compared with nontransgenic WT mice. Transient depletion of Tregs by anti-CD25 resulted in increased infiltration of inflammatory cells into thyroids of transgenic mice. Treg depletion also resulted in increased anti-mouse thyroglobulin autoantibody responses and increased expression of IFN-γ and IFN-γ-inducible chemokines in thyroids of Tg mice. The results suggest that spontaneous autoimmune thyroiditis is inhibited in mice expressing transgenic TGF-β on thyrocytes, at least in part, because there is an increased frequency of Tregs in their thyroids. *The Journal of Immunology, 2010, 184: 5352–5359.

Several disease processes, including some autoimmune diseases, as well as fibrosis and chronic inflammation, are characterized by dysregulated expression of or response to TGF-β (1). Effects of TGF-β on lymphocytes and other cells, such as epithelial cells, can be stimulatory or inhibitory, depending on the cytokine milieu (2). For example, TGF-β inhibits T cell proliferation and promotes activation and function of regulatory T cells (Tregs) (3, 4). TGF-β also influences the recruitment, adhesion, and activation of circulating leukocytes (5), and it can suppress the production of proinflammatory cytokines by T cells and macrophages (2, 6). TGF-β can also be proinflammatory (e.g., in combination with IL-6 and Ag), TGF-β induces autoimmune inflammation mediated by Th17 cells (7–9).

NOD.H-2h4 mice develop lymphocytic spontaneous autoimmune thyroiditis (L-SAT) characterized by the infiltration of the thyroid by B and T lymphocytes (10–15). IFN-γ is important for the development of L-SAT, because IFN-γ−/− NOD.H-2h4 mice do not develop typical L-SAT; instead, they develop thyroid epithelial cell hyperplasia and proliferation (TEC H/P) and fibrosis (16, 17). TEC H/P has an autoimmune basis, because lymphocytes are required for its development, and mice with TEC H/P produce anti-mouse thyroglobulin autoantibodies (17). Splenocytes from IFN-γ−/− mice with severe TEC H/P transfer severe TEC H/P and fibrosis to NOD.H-2h4.SCID mice (17). Proliferating thyrocytes produce active TGF-β, and anti–TGF-β inhibits TEC H/P and fibrosis in SCID recipients of IFN-γ−/− donor splenocytes with severe TEC H/P, indicating that TGF-β promotes the development of TEC H/P and fibrosis in IFN-γ−/− NOD.H-2h4 mice (18).

To directly determine whether TGF-β plays a role in the development of TEC H/P and fibrosis, TGF-β transgenic (Tg) NOD.H-2h4 mice expressing active TGF-β on thyrocytes were generated. All Tg IFN-γ−/− NOD.H-2h4 mice developed moderate to severe TEC H/P and fibrosis, consistent with the role of TGF-β in promoting thyrocyte hyperplasia (18). Unexpectedly, the expression of transgenic TGF-β on thyrocytes of wild type (WT) NOD.H-2h4 mice inhibited the development of L-SAT, and the transient depletion of Tregs promoted the development of typical L-SAT in Tg mice.

Materials and Methods

Generation of Tg mice

The TGF-β rat thyroglobulin promoter construct was provided by Dr. Leonard Kohn (Ohio University, Athens, OH). The SalI sites of the TGF-β rat thyroglobulin promoter construct were used to excise the cassette for microinjection. This construct contains two G to C point mutations in the TGF-β coding sequence that result in Cys to Ser amino acid substitutions at residues 223 and 225 of TGF-β1, leading to bioactive TGF-β1 (19–21). The construct was directly injected into superovulated NOD.H-2h4 mice (University of Missouri Transgenic Core), resulting in two founder Tg NOD.H-2h4 females. The founders were mated with NOD.H-2h4 males, and Tg+ offspring were selected by PCR analysis of tail DNA, as previously described (16, 18). The Tg founders and progeny were genotyped by PCR.
analysis of mouse tail DNA using primers specific for the rat thyroglobulin promoter 5′-AGA GCA CTG CTT GCC ACT GTG C-3′ (forward) and 5′-GCT GTT GTA CAA AGG CAC CAC-3′ (reverse) located in the mouse TGF-β genomic sequence and the transgenic vector. These primers amplify a 340-bp band in mice expressing the TGF-β transgene.

Evaluation of TEC H/IP and L-SAT severity scores

Male mice were used for all experiments. At various intervals after receiving NaI water, thyroids were removed, and one thyroid lobe from each mouse was fixed in formalin, sectioned, and stained with H&E. All slides were independently scored by at least two individuals, one of whom had no knowledge of the experimental groups. The other thyroid lobe was snap-frozen in liquid nitrogen and stored at -70°C for use in immunohistochemical staining or for isolation of RNA for RT-PCR. The extent of infiltration of thyroids by lymphocytes was scored on a scale of 0 to 4+, as previously described (11, 12). Briefly, a score of 0 indicates a normal thyroid or mild follicular changes with a few inflammatory cells (lymphocytes) infiltrating the thyroids. A 1+ severity score is defined as an infiltrate of ≤125 inflammatory cells in one or several foci; 2+ represents 10–20 foci of inflammatory cell infiltration, each the size of several follicles causing replacement or destruction of up to one quarter of the gland. A 3+ score indicates that one quarter to one half of the follicles are destroyed or replaced by infiltrating inflammatory cells, and a 4+ score indicates that more than one half of the gland is destroyed. Because thyroids in all Tg mice had follicular hyperplasia and fibrosis which were never observed in thyroids of WT NOD.H-2h4 mice that did not express the transgene, the severity scores given in the Tables and figures indicate the extent of infiltration of thyroids by inflammatory cells, primarily lymphocytes [i.e., L-SAT severity scores (11, 12)].

Anti-mouse thyroglobulin autoantibody determination

Mouse thyroglobulin (MTg)-specific autoantibodies were determined by ELISA using serum from individual mice, as previously described (11, 16, 17). Serum were diluted 1/50 or 1/100. Normal mouse serum used at the same dilutions always gave an OD value <0.05. OD values > 0.1 for experimental sera were considered positive.

Immunohistochemical staining

TGF-β staining was done as described previously (18) using anti–TGF-β (AB-101-NA, R&D Systems, Minneapolis, MN) to stain active TGF-β (22) and chicken IgY as isotype control. Frozen thyroid sections were used for immunohistochemical staining as previously described (12, 16). The following primary antibodies were used: anti-C-CD4 (GR 1.5; ATCC, Manassas, VA), anti-CDB (53.6; ATCC), and anti-B220 (Invitrogen, Eugene, OR). Biotinylated goat anti-rat IgG (Invitrogen) was used as secondary Ab, and 0.3% hydrogen peroxide was used to block endogenous peroxidase. Sections were dehydrated using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and peroxidase activity was visualized using the Nova-Red substrate (Vector Laboratories). Slides were counterstained with hematoxylin. Negative controls used IgG isotype controls as primary Ab, with the remaining steps performed as described above. These controls were always negative.

Confocal laser scanning double-immunofluorescence microscopy

Foxp3 and CD4 double immunofluorescence staining was done using frozen sections of thyroids, as previously described (18, 23). After blocking with 2% BSA, sections were incubated with anti-Foxp3 (rabbit anti-Foxp3, provided by Dr. Alexander Y. Rudensky, Memorial Sloan-Kettering Cancer Center, New York, NY (23)) for 40 min at room temperature and visualized with 1:500 diluted Alexa 568-conjugated anti-rabbit Ab ( Molecular Probes, Eugene, OR, Invitrogen). CD4 staining was visualized with 1:500 diluted Alexa 488-conjugated anti-CD4 (Invitrogen). Slides were observed using a Bio-Rad Radiance 2000 (Hercules, CA) confocal system coupled to an Olympus IX70 inverted microscope (Center Valley, PA). The frequency of Foxp3+ cells among thyroid-infiltrating CD4+ T cells was quantified by manually counting all cells in two or three randomly selected high-power fields (~300 magnification), and Foxp3+ cells were expressed as a percentage of the total CD4+ T cells. Results represent data from three individual thyroids in each group and are expressed as the mean ± SE.

Semi-quantitative and quantitative RT-PCR

Total RNA was isolated from thyroids or splenocytes using TRIzol (Invitrogen), and cDNA was synthesized as previously described (12, 16). Semi-quantitative RT-PCR was done as previously described (12, 16), using the housekeeping gene β-actin to correct for differences in the amount of RNA in different samples. Samples were electrophoresed, stained with ethidium bromide, and densitometry analysis was performed using a digital imaging system. Samples within the linear relationship between input cDNA and final PCR products (usually 1/25 dilution of cDNA) were collected, and densitometric units for each cytokine band were normalized to that for the corresponding β-actin band (16). Results are expressed as ratios of cytokine/β-actin. A ratio of 100 indicates a 1:1 ratio between a particular cytokine and β-actin. Quantitative RT-PCR was done using AB-solute QPCR SYBR Green ROX mix (ABgene, Lafayette, CO) and ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). A series of five standards with defined values were included in every reaction, and a standard curve was obtained to calculate the amount of gene amplified. A dissociation curve was generated at the end of each PCR to verify the amplification of a single product. The level of β-actin expression for each sample was used for data normalization (23).

TGF-β ELISA

Active TGF-β in serum or in supernatants of thyrocyte cultures of Tg or nontransgenic mice was analyzed using a TGF-β ELISA kit (eBioscience, San Diego, CA). Samples were activated by treating with 1 N HCl and neutralized with 1 N NaOH, according to the manufacturer’s instructions. Results from four or five samples in each group are expressed as the mean ± SE.

Flow cytometry

Spleen or cervical lymph node (CLN) cells from experimental mice were analyzed by flow cytometry (FACScan), as described previously (15), CD4-FITC, CD25-PE-Cy5, and Foxp3 Treg kits were obtained from eBioscience (23).

Adoptive transfer experiments

For some experiments, splenocytes (3 × 10^7 cells) from naive WT NOD.H-2h4 mice or from WT NOD.H-2h4 mice with spontaneous autoimmune thyroiditis (SAT) were transferred i.v. to Tg mice. Recipients were irradiated (300 rad) prior to cell transfer, given 0.05% NaI water, and thyroids were removed 8 wk later (11, 12). In other experiments, T cells from WT or Tg NOD.H-2h4 mice were obtained by passage of splenocytes through nylon wool (24). Enriched T cells were transferred i.v. to irradiated (300 rad) TCR α/β NOD.H-2h4 mice lacking detectable CD4+ and CD8+ T cells. Recipients were given NaI water, and thyroids were removed 8 wk later.

Transfer of experimental autoimmune thyroiditis to Tg mice

For some experiments, WT NOD.H-2h4 mice were immunized with MTg and LPS, as previously described (25). Splenocytes from immunized donors were activated in vitro with MTg and 5 ng/ml IL-12. Cells were transferred i.v. to irradiated (500 rad) Tg or nontransgenic littermates, and thyroids were removed 4 wk later (25).

Depletion of Tregs

As previously described (23), some mice were given three weekly injections of 0.5 mg rat anti-mouse CD25 mAb PC61 (ATCC) or isotype control, beginning 9–15 d after birth. At 8 wk of age, treated and control mice were given 0.05% NaI water, and thyroids were removed 8 wk later.

Statistical analysis

The Wilcoxon rank-sum test was used for analyzing differences in disease severity scores between groups of mice, and the Student t test was used for all other analyses. A value of p < 0.05 was considered significant.

Results

Generation of transgenic NOD.H-2h4 mice expressing TGF-β on thyrocytes

The rat thyroglobulin promoter/active TGF-β construct was directly injected into superovulated NOD.H-2h4 mice to produce transgenic mice expressing TGF-β on thyrocytes. Transgenic founders were crossed with WT NOD.H-2h4 mice, and the progeny were examined for expression of TGF-β by RT-PCR and immunohistochemical staining (Fig. 1). Quantitative analysis of TGF-β by real-time PCR indicated that thyroids of Tg NOD.H-2h4 mice expressed 3–5-fold more TGF-β than did the thyroids of nontransgenic mice (Fig. 1A). Immunohistochemical staining showed that thyrocytes of transgenic mice strongly expressed active TGF-β, whereas thyroids of nontransgenic littermates expressed little, if any, active TGF-β (Fig. 1C versus 1G). Salivary
glands (Fig. 1D), spleen (Fig. 1E), and CLN (not shown) of transgenic mice expressed little or no TGF-β, indicating that the TGF-β transgene was expressed specifically on thyrocytes. The latent form of TGF-β was comparably expressed in thyroids of transgenic and nontransgenic mice (data not shown).

Transgenic TGF-β expressed by thyrocytes is not detected in serum or supernatants of thyrocyte cultures

Other investigators reported that serum levels of TGF-β were increased in Tg mice (19), suggesting that transgenic TGF-β might exert some of its functions peripherally. Serum levels of active TGF-β in individual serum samples from six Tg and six WT NOD.H-2h4 mice were determined by ELISA. Transgenic and nontransgenic mice had similar low levels of serum TGF-β (698 ± 170 pg/ml versus 571 ± 104 pg/ml; p > 0.5). In addition, supernatants of cultured thyrocytes from Tg and nontransgenic littersmates produced similar amounts of active TGF-β (466 ± 168 pg/ml versus 381 ± 47 pg/ml; p > 0.5), as determined by ELISA. These results suggest that transgenic TGF-β expressed by thyrocytes is not secreted and are consistent with reports that serum levels of TGF-β1 in Tg NOD mice expressing TGF-β1 in pancreatic islets did not differ from those in nontransgenic littersmates, although the development of diabetes was inhibited by the transgenic TGF-β (26).

Transgenic expression of TGF-β on thyrocytes inhibits development of L-SAT in WT NOD.H-2h4 mice

SAT in WT NOD.H-2h4 mice is characterized by infiltration of thyroids by T and B lymphocytes, and almost all WT NOD.H-2h4 mice develop L-SAT 2 mo after receiving 0.05% NaI in their drinking water (11, 12). In contrast, all Tg NOD.H-2h4 mice developed moderate to severe TEC H/P (Fig. 2A, 2B, asterisks) with extensive collagen deposition (fibrosis) (Fig. 2C) 2 mo after receiving NaI water; however, they developed only minimal L-SAT (Fig. 2B, arrows), even after receiving NaI in their water for as long as 6–7 mo (Table I). Nontransgenic littersmates, like WT NOD.H-2h4 mice, developed typical L-SAT, with clusters of infiltrating lymphocytes (Fig. 2D, 2E, arrows), and they did not have TEC H/P or fibrosis (Fig. 2D–F, Table I). Thyroid lesions in Tg mice are similar to TEC H/P lesions that develop in IFN-γ−/− NOD.H-2h4 mice (17), except that thyroids in the transgenic mice are smaller, TEC H/P develops much earlier, and fibrosis is more extensive. Notably, most lymphocytes in thyroids of Tg mice accumulated near the periphery of the thyroid (data not shown), whereas most lymphocytes infiltrated the gland in nontransgenic mice (Fig. 2D, 2E, arrows). Thus, L-SAT severity is markedly reduced and TEC H/P and fibrosis are increased in transgenic mice overexpressing TGF-β on thyrocytes.

Anti-MTg autoantibody responses are reduced in transgenic mice expressing TGF-β on thyrocytes

All IFN-γ−/− mice with severe TEC H/P and fibrosis and WT NOD.H-2h4 mice with L-SAT produce anti-MTg autoantibodies, but autoantibody levels are generally higher in WT mice with L-SAT than in IFN-γ−/− mice with TEC H/P (17). Because Tg WT NOD.H-2h4 mice given NaI water for 2–7 mo have TEC H/P, fibrosis, and minimal L-SAT (Table I), it was important to determine whether...
anti-Mtg autoantibody production was influenced in mice expressing TGF-β in the thyroid. Although there was considerable variation in anti-Mtg autoantibodies in individual mice, Tg mice generally had lower levels of anti-Mtg autoantibodies compared with nontransgenic mice (Table I). Because transgenic expression of TGF-β was confined to the thyroid, these results were unexpected and suggest that the immune response to Mtg might be influenced by expression of the transgene in the thyroid.

To determine whether Tg mice could develop anti-Mtg autoantibody responses comparable to those of WT mice after immunization with Mtg and adjutant, groups of age-matched transgenic and nontransgenic mice were immunized with Mtg and LPS, and serum was collected 14 d later. Both groups produced comparable levels of anti-Mtg autoantibody (data not shown), suggesting that expression of transgenic TGF-β in the thyroid did not influence the ability of B cells to respond to immunization with Mtg and adjutant.

T cells from transgenic and nontransgenic mice induce comparable L-SAT after transfer to TCRα−/− or SCID mice

To determine whether autoreactive T cell activation was reduced in Tg mice, T cells were purified from spleens of transgenic and nontransgenic mice and transferred to TCRα−/− NOD.H-2h4 mice. Recipients were given NaI water, and thyroids were removed 2–7 mo later, as indicated.

**Table I. Development of L-SAT in Tg− and Tg+ NOD.H-2h4 mice given NaI water for 2–7 mo**

<table>
<thead>
<tr>
<th>Micea</th>
<th>L-SAT Severitya</th>
<th>Anti-Mtg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>Tg−</td>
<td>2–3 mo NaI</td>
<td>1</td>
</tr>
<tr>
<td>Tg−</td>
<td>2–3 mo NaI</td>
<td>9</td>
</tr>
<tr>
<td>Tg+</td>
<td>4–5 mo NaI</td>
<td>1</td>
</tr>
<tr>
<td>Tg−</td>
<td>4–5 mo NaI</td>
<td>7</td>
</tr>
<tr>
<td>Tg−</td>
<td>6–7 mo NaI</td>
<td>0</td>
</tr>
<tr>
<td>Tg+</td>
<td>6–7 mo NaI</td>
<td>9</td>
</tr>
</tbody>
</table>

*aSeven- to 8-wk-old male Tg NOD.H-2h4 mice or nontransgenic littermates were given 0.05% NaI in their water at 8 wk of age, and thyroids were removed 2–7 mo later.

*bNumbers of mice with different degrees of severity of L-SAT; p < 0.01 (row 1 versus row 2, row 3 versus row 4, and row 5 versus row 6).

**Table II. Development of L-SAT in TCRα−/− recipients of splenocytes from Tg− or Tg+ Donors**

<table>
<thead>
<tr>
<th>Cell Transfera</th>
<th>L-SAT Severitya</th>
<th>Anti-Mtg IgGa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive Tg−</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Naive Tg+</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Naive Tg−</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Naive Tg+</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*aSplenocytes (105) from naive Tg− and Tg+ mice (8–10 wk old) were transferred to irradiated (300 rad) TCRα−/− mice. Mice were given NaI in their drinking water, and thyroids were removed 2 mo later for evaluation of thyroid histopathology.

**Table III. Thyroiditis Severity of Tg− and Tg+ NOD.H-2h4 mice that are recipients of WT spleen cells**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Experimental Autoimmune Thyroiditis Severitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg−</td>
<td>0</td>
</tr>
<tr>
<td>Tg+</td>
<td>0</td>
</tr>
<tr>
<td>Tg−</td>
<td>0</td>
</tr>
<tr>
<td>Tg+</td>
<td>0</td>
</tr>
<tr>
<td>Tg−</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table IV. Anti-Mtg IgG production in splenocytes from Tg− and Tg+ NOD.H-2h4 mice**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Anti-Mtg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg−</td>
<td>0.574 ± 0.125</td>
</tr>
<tr>
<td>Tg+</td>
<td>0.469 ± 0.104</td>
</tr>
</tbody>
</table>

**Table V. Anti-Mtg IgG production in splenocytes from Tg− and Tg+ NOD.H-2h4 mice**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Anti-Mtg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg−</td>
<td>0.239 ± 0.047</td>
</tr>
<tr>
<td>Tg+</td>
<td>0.244 ± 0.055</td>
</tr>
</tbody>
</table>

**Table VI. Anti-Mtg IgG production in splenocytes from Tg− and Tg+ NOD.H-2h4 mice**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Anti-Mtg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg−</td>
<td>0.803 ± 0.206</td>
</tr>
</tbody>
</table>

**Table VII. Anti-Mtg IgG production in splenocytes from Tg− and Tg+ NOD.H-2h4 mice**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Anti-Mtg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg−</td>
<td>1.258 ± 0.197</td>
</tr>
<tr>
<td>Tg+</td>
<td>0.778 ± 0.220</td>
</tr>
</tbody>
</table>
and CLNs of transgenic and nontransgenic mice were evaluated by immunohistochemical staining. The frequency of Foxp3+ T cells among total intrathyroidal CD4+ T cells was significantly increased in thyroids of Tg mice compared with nontransgenic litters (Fig. 3A–D, p < 0.05). However, the total number of Foxp3+ cells in the thyroid was not increased because there were fewer infiltrating CD4+ T cells in thyroids of Tg mice compared with their transgene-negative litters. The frequency of CD4+ CD25+Foxp3+ cells was marginally increased in CLNs of transgenic mice compared with nontransgenic mice, but there was no difference in the frequency of CD4+Foxp3+ cells in spleens of the two groups of mice (Fig. 3E). Thyroids of young (4 wk) transgenic or nontransgenic mice with no thyroid inflammation did not have detectable Foxp3+ cells, but CLNs of young transgenic mice, similar to the older mice in Fig. 3E, had an increased frequency of Foxp3+ T cells compared with their nontransgenic litters (Fig. 3F).

**Transient depletion of Tregs increases L-SAT and anti-MTg autoantibody responses in Tg mice**

To determine whether the increased frequency of Foxp3+ cells in thyroids of Tg mice might play a role in the suppression of L-SAT, groups of transgenic and nontransgenic mice were given rat IgG or anti-CD25 to transiently deplete Tregs, as previously described (23). Mice were given NaI water at 8 wk, and thyroids were removed 8 wk later. Compared with Tg mice that received rat IgG (Fig. 4A–E, Table V), thyroids of Tg mice given anti-CD25 had greatly increased lymphocyte infiltration, although they also had proliferating thyrocytes and fibrosis (Fig. 4F–J, Table V). MTg autoantibodies in Treg-depleted transgenic and nontransgenic mice were always higher than those in mice given rat IgG (p < 0.01). Consistent with our previous studies (23), transient depletion of CD25+ T cells had little effect on the severity of L-SAT in nontransgenic WT NOD.H-2h4 mice (Table V). Thyroids of Treg-depleted Tg mice had clusters of CD4+ T cells and B cells (Fig. 4I, 4J), similar to that seen in nontransgenic NOD.H-2h4 mice (Fig. 2D, 2E). These results suggest that the development of L-SAT is inhibited in mice overexpressing TGF-β on thyrocytes, at least in part, because they have an increased frequency of Tregs in their thyroids and CLNs.

**Treg depletion increases expression of IFN-γ and IFN-γ-inducible chemokines in thyroids of Tg mice**

Most Tg mice develop TEC H/P with minimal lymphocyte infiltration of thyroids. As shown above, transient depletion of Tregs results in increased migration of lymphocytes to thyroids of Tg mice. To determine whether Treg depletion resulted in increased expression of cytokines or chemokines in thyroids of Tg mice, RNA was isolated from thyroids of WT and Tg mice given rat Ig or anti-CD25. As expected, the expression of TGF-β in thyroids of transgenic mice was not influenced by Treg depletion. Thyroids of both groups of transgenic mice expressed comparable amounts of TGF-β mRNA, and this was always greater than in thyroids of nontransgenic litters (Fig. 5). Consistent with the decreased infiltration of T cells, thyroids of Tg mice treated with rat Ig expressed less IFN-γ than thyroids of nontransgenic litters with L-SAT (Fig. 5). Consistent with the increased T cell infiltration, IFN-γ expression in thyroids of Treg-depleted Tg mice was increased, but Treg depletion had no effect on SAT severity or expression of IFN-γ in thyroids of nontransgenic litters. Thyroids of Tg mice with minimal lymphocyte infiltration always had lower expression of IFN-γ-inducible chemokines CXCL9,
results are consistent with our earlier studies indicating that overexpression of TGF-β by thyrocytes of IFN-γ−/− mice correlates with the severity of TEC H/P, and neutralization of TGF-β inhibits the development of TEC H/P and fibrosis (18). Of particular interest, transient depletion of Tregs in mice expressing TGF-β in the thyroid resulted in greater SAT severity scores, with increased infiltration of CD4+ T cells and B cells into thyroids (Fig. 4, Table V). However, transient depletion of Tregs does not reduce fibrosis in thyroids of Tg mice.

Because TGF-β has multiple functions, its overexpression by thyrocytes could inhibit development of L-SAT by multiple mechanisms. IFN-γ-inducible chemokines are important for recruiting inflammatory cells to the site of inflammation (28–30), and TGF-β interferes with the functions of IFN-γ (31, 32). TGF-β mRNA expression was inversely correlated with the expression of IFN-γ and IFN-γ-inducible chemokines, such as CXCL9, CXCL10, and CCL5, in thyroids of transgenic mice (Fig. 5). Therefore, one mechanism by which TGF-β inhibits the development of L-SAT is to inhibit upregulation of IFN-γ and IFN-γ-inducible chemokines in the thyroid, resulting in the migration of fewer lymphocytes to thyroids.

Because expression of the TGF-β transgene was confined to the thyroid (Fig. 1), and there was no evidence that the active TGF-β was secreted by the thyrocytes and was present in the circulation, it was surprising that anti-MTg autoantibody responses were consistently reduced in Tg mice compared with their nontransgenic littermates (Table I). Although the reason for these results is not understood, it is possible that the increased frequency of Tregs in the Tg mice inhibited the expansion of the autoreactive B cells in response to the thyroid-expressed autoantigen, as shown recently in another model (33). This hypothesis is consistent with the observation that anti-MTg autoantibody responses were always higher after Treg depletion (Table V).

Mice with transgenic expression of TGF-β in pancreatic islets were described by other investigators (26, 34, 35). NOD mice expressing transgenic TGF-β in pancreatic β cells were relatively resistant to passive transfer of diabetes with diabetogenic T cells compared with their nontransgenic littermates (26, 35). In our studies, activated autoreactive T cells from Tg and nontransgenic mice were equivalent in their ability to induce L-SAT after transfer to SCID (data not shown) or TCRα−/− mice (Table II). In addition, adoptively transferred lymphocytes from nontransgenic WT mice could migrate to and infiltrate thyroids of Tg mice (Table IV), and splenocytes from WT mice could be activated in vitro to transfer severe G-EAT to Tg mice (Table III). Tg recipients of activated G-EAT effector cells also had less thyrocyte hyperplasia (Fig. 2G, 2H) and fibrosis (Fig. 2I).

These results indicate that transferred T cells can migrate to thyroids of transgenic mice expressing TGF-β on thyrocytes.

**Table V. SAT severity is increased in Tg+ NOD.H-2b4 mice given anti-CD25**

<table>
<thead>
<tr>
<th>Mice</th>
<th>SAT Severity</th>
<th>Anti-MTg IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg+/rat-Ig</td>
<td>6 2 0 0</td>
<td>0.144 ± 0.059</td>
</tr>
<tr>
<td>Tg+/anti-CD25</td>
<td>0 0 4 10</td>
<td>0.536 ± 0.079</td>
</tr>
<tr>
<td>Tg / rat-Ig</td>
<td>4 4 4 2</td>
<td>0.246 ± 0.069</td>
</tr>
<tr>
<td>Tg / anti-CD25</td>
<td>0 0 5 4</td>
<td>0.617 ± 0.111</td>
</tr>
</tbody>
</table>

*See Table I; p < 0.01 (row 1 versus row 2); p = 0.11 (row 2 versus row 4); p = 0.02 (row 3 versus row 4).

*See Table I; p < 0.01 (row 1 versus row 2 and row 3 versus row 4); p = 0.551 (row 2 versus row 4).

**Discussion**

The results of this study demonstrated that transgenic expression of TGF-β on thyrocytes inhibits the development of L-SAT in WT NOD.H-2b4 mice, and all Tg mice develop TEC H/P. These results are consistent with our earlier studies indicating that overexpression of TGF-β by thyrocytes of IFN-γ−/− mice correlates with the severity of TEC H/P, and neutralization of TGF-β inhibits the development of TEC H/P and fibrosis (18). Of particular interest, transient depletion of Tregs in mice expressing TGF-β in the thyroid resulted in greater SAT severity scores, with increased infiltration of CD4+ T cells and B cells into thyroids (Fig. 4, Table V). However, transient depletion of Tregs does not reduce fibrosis in thyroids of Tg mice.

Because TGF-β has multiple functions, its overexpression by thyrocytes could inhibit development of L-SAT by multiple mechanisms. IFN-γ-inducible chemokines are important for recruiting inflammatory cells to the site of inflammation (28–30), and TGF-β interferes with the functions of IFN-γ (31, 32). TGF-β mRNA expression was inversely correlated with the expression of IFN-γ and IFN-γ-inducible chemokines, such as CXCL9, CXCL10, and CCL5, in thyroids of transgenic mice (Fig. 5). Therefore, one mechanism by which TGF-β inhibits the development of L-SAT is to inhibit upregulation of IFN-γ and IFN-γ-inducible chemokines in the thyroid, resulting in the migration of fewer lymphocytes to thyroids.

Because expression of the TGF-β transgene was confined to the thyroid (Fig. 1), and there was no evidence that the active TGF-β was secreted by the thyrocytes and was present in the circulation, it was surprising that anti-MTg autoantibody responses were consistently reduced in Tg mice compared with their nontransgenic littermates (Table I). Although the reason for these results is not understood, it is possible that the increased frequency of Tregs in the Tg mice inhibited the expansion of the autoreactive B cells in response to the thyroid-expressed autoantigen, as shown recently in another model (33). This hypothesis is consistent with the observation that anti-MTg autoantibody responses were always higher after Treg depletion (Table V).

Mice with transgenic expression of TGF-β in pancreatic islets were described by other investigators (26, 34, 35). NOD mice expressing transgenic TGF-β in pancreatic β cells were relatively resistant to passive transfer of diabetes with diabetogenic T cells compared with their nontransgenic littermates (26, 35). In our studies, activated autoreactive T cells from Tg and nontransgenic mice were equivalent in their ability to induce L-SAT after transfer to SCID (data not shown) or TCRα−/− mice (Table II). In addition, adoptively transferred lymphocytes from nontransgenic WT mice could migrate to and infiltrate thyroids of Tg mice (Table IV), and splenocytes from WT mice could be activated in vitro to transfer severe G-EAT to Tg mice (Table III). Tg recipients of activated G-EAT effector cells also had less thyrocyte hyperplasia (Fig. 2G, 2H) and fibrosis (Fig. 2I).

These results indicate that transferred T cells can migrate to thyroids of transgenic mice expressing TGF-β on thyrocytes.
TGF-β can promote apoptosis of T cells (35, 36). Transgenic expression of TGF-β in pancreatic islets was shown to increase apoptosis of islet-infiltrating T cells, suggesting that this could be one mechanism by which TGF-β prevents diabetes (35). In the current study, the numbers of TUNEL+ apoptotic cells in thyroids of WT nontransgenic mice with SAT severity scores of 2–3 were very low and did not differ from those detected in thyroids of Tg mice with severe TEC H/P and fibrosis (data not shown). Therefore, there is no evidence to suggest that transgenic TGF-β expressed in the thyroid prevents the development of L-SAT by inducing apoptosis of thyroid-infiltrating inflammatory cells.

The production of TGF-β by Tregs is important for the inhibition of immune responses and the expansion of Tregs in vivo (4, 37), and TGF-β can promote the conversion of peripheral CD4+CD25−Foxp3− T cells into Foxp3+expressing CD4+CD25+ Tregs (27). Although the frequency of CD4+CD25+Foxp3+ T cells in spleens of Tg mice did not differ from that in WT nontransgenic mice, the frequency of Foxp3+ T cells among total thyroid-infiltrating CD4+ T cells was increased in thyroids of Tg mice (Fig. 3A, 3D). The increased frequency or activity of Tregs apparently plays an important role in the suppression of SAT in Tg mice, because transient depletion of Tregs by the administration of anti-CD25 resulted in markedly increased infiltration of lymphocytes into thyroids (Fig. 4F, 4G, 4I, 4J). Anti-MTg autoantibodies and expression of IFN-γ and IFN-γ-inducible chemokines were also increased after Treg depletion (Fig. 5, Table V). Treg depletion did not reverse all of the thyroid abnormalities in Tg mice because their thyroids still had TEC H/P and fibrosis, which were absent in thyroids of nontransgenic mice. It is not entirely clear why transient Treg depletion had such dramatic effects in promoting lymphocyte migration to thyroids of Tg mice, because activation of autoreactive T cells in the spleen was not reduced in Tg mice compared with their nontransgenic littermates (Table II), and activation of T cells able to induce L-SAT in TCRα−/− mice was not greater after Treg depletion (data not shown). The same transient Treg depletion had essentially no effect on L-SAT severity or expression of IFN-γ or IFN-γ-inducible chemokines in nontransgenic WT mice (Fig. 5, Table V). It is possible that Treg depletion in Tg mice lowered the threshold of activation of autoreactive T cells, as suggested in other studies (38). Anti-MTg autoantibody responses were consistently increased in transgenic and nontransgenic mice after Treg depletion (Table V). Although increased autoantibody responses in Tg mice after Treg depletion are consistent with an increased severity of L-SAT; autoantibody responses were also increased after Treg depletion in nontransgenic litters in which L-SAT severity and the production of IFN-γ or IFN-γ–inducible chemokines in the thyroid were not affected. It is unknown why transient Treg depletion consistently resulted in increased autoantibody responses in transgenic and nontransgenic mice. Taken together, the results presented in this article suggest that transgenic overexpression of TGF-β on thyrocytes inhibits the development of L-SAT in NOD.H-2h4 mice.
and this is due, at least in part, to an increased frequency and/or activity of Tregs in thyroids of Tg mice.

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Disclosures

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References