The Link between Graves’ Disease and Hashimoto’s Thyroiditis: A Role for Regulatory T Cells

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Hyperthyroidism in Graves’ disease is caused by thyroid-stimulating autoantibodies to the TSH receptor (TSHR), whereas hypothyroidism in Hashimoto’s thyroiditis is associated with thyroid peroxidase and thyroglobulin autoantibodies. In some Graves’ patients, thyroiditis becomes sufficiently extensive to cure the hyperthyroidism with resultant hypothyroidism. Factors determining the balance between these two diseases, the commonest organ-specific autoimmune diseases affecting humans, are unknown. Serendipitous findings in transgenic BALB/c mice, with the human TSHR A-subunit targeted to the thyroid, shed light on this relationship. Of three transgenic lines, two expressed high levels and one expressed low intrathyroidal A-subunit levels (Hi- and Lo-transgenics, respectively). Transgenics and wild-type littermates were depleted of T regulatory cells (Treg) using antibodies to CD25 (CD4+ T cells) or CD122 (CD8+ T cells) before TSHR-adenovirus immunization. Regardless of Treg depletion, high-expressor transgenics remained tolerant to A-subunit-adenovirus immunization (no TSHR antibodies and no hyperthyroidism). Tolerance was broken in low-transgenics, although TSHR antibody levels were lower than in wild-type littermates and no mice became hyperthyroid. Treg depletion before immunization did not significantly alter the TSHR antibody response. However, Treg depletion (particularly CD25) induced thyroid lymphocytic infiltrates in Lo-transgenics with transient or permanent hypothyroidism (low T4, elevated TSH). Neither thyroid lymphocytic infiltration nor hypothyroidism developed in similarly treated wild-type littermates. Remarkably, lymphocytic infiltration was associated with intermolecular spreading of the TSHR antibody response to other self thyroid antigens, murine thyroid peroxidase and thyroglobulin.

These data suggest a role for Treg in the natural progression of hyperthyroid Graves’ disease to Hashimoto’s thyroiditis and hypothyroidism in humans. (*Endocrinology* 148: 5724–5733, 2007)

GRAVES’ DISEASE, ONE of the most common autoimmune diseases affecting humans, is caused by autoantibodies that induce thyrotoxicosis by mimicking the action of TSH and activating the TSH receptor (TSHR). Although such thyroid-stimulating autoantibodies are pathognomonic of Graves’ disease, autoantibodies to thyroid peroxidase (TPO) and thyroglobulin (Tg) are also present. The latter two autoantibodies are the classical markers of Hashimoto’s thyroiditis, a condition in which thyroid lymphocytic infiltration and thyrocyte damage may progress to hypothyroidism. Moreover, many Graves’ patients have mild lymphocytic thyroiditis. In some instances, thyroiditis in Graves’ disease becomes sufficiently extensive as to cure the hyperthyroidism with resultant hypothyroidism.

The relationship between Graves’ disease and Hashimoto’s thyroiditis has been debated for decades. Although initially considered to be two separate diseases, the present view is that they represent the opposite sides of the same coin, or the two ends of a spectrum. On the other hand, whole-genome scanning studies in humans have revealed distinct differences between loci linked to, or associated with, these two autoimmune thyroid diseases (for example, Ref. 1). Moreover, animal models of Graves’ disease and Hashimoto’s thyroiditis are studied as two distinct entities. Not surprisingly, the pathophysiological relationship between TSHR, TPO, and Tg autoantibodies remains an enigma. For example, why do TPO and Tg autoantibodies arise in Graves’ disease? Do TSHR, TPO, and Tg autoantibodies arise independently or through intermolecular spreading, and if the latter, what is the primary antigen? Is the TSHR the autoantigen associated with lymphocytic infiltration in Graves’ disease?

Thyroid-stimulating antibodies, the proximal cause of Graves’ hyperthyroidism, arise from the breakdown in self-tolerance to the TSHR, a G protein-coupled receptor with seven transmembrane-spanning domains (reviewed in Ref. 2). However, the autoantigen that drives the immune response in Graves’ disease is not the full-length receptor but the A-subunit (3, 4), an ectodomain component that is shed after intramolecular cleavage of the receptor (reviewed in Ref. 2). Recently, we generated transgenic mice with the human A-subunit targeted to the thyroid gland (5). The founder transgenics were crossed with BALB/c mice, a strain that is susceptible to immunization with adenovirus express-
ing either the TSH holoreceptor or its A-subunit (4, 6). Unlike wild-type littersmates, the transgensics failed to develop T cell responses or TSHR antibodies after low-dose A-subunit adenovirus (A-subunit-Ad) immunization. However, tolerance to the human A-subunit was partially overcome by immunization with high doses of adenovirus expressing the A-subunit or the holoreceptor (5).

Development of tolerance is a complex process that includes central and peripheral mechanisms acting in concert to eliminate self-reactive lymphocytes (7). T cell deletion by central tolerance may not eliminate all self-reactive cells. Another potent mechanism involves regulatory T cells (Treg), such as naturally occurring CD25+/CD122+ T cells or CD8+ CD122+ cells, that control autoreactive effector T cells in the periphery (8, 9). In the present study, we used A-subunit transgenic animals to probe the influence of A-subunit transgene expression levels and Treg depletion on the immune response to TSHR-Ad immunization. We report that Treg are a major factor in the influence of A-subunit transgene expression levels and study, we used A-subunit transgenic animals to probe the active effector T cells in the periphery (8, 9). In the present study, we used A-subunit transgenic animals to probe the influence of A-subunit transgene expression levels and Treg depletion on the immune response to TSHR-Ad immunization. We report that Treg are a major factor in the influence of A-subunit transgene expression levels and study, we used A-subunit transgenic animals to probe the active effector T cells in the periphery (8, 9).

Materials and Methods

Human TSHR A-subunit transgenic mice

Mice with the human TSHR A-subunit targeted to the thyroid using the bovine Tg promoter were described previously (5). Founders were bred to BALB/cj (Jackson Laboratories, Bar Harbor, ME) to generate five separate lines. Transgene-positive offspring were repeatedly crossed to BALB/cj, and three lines (60.6, 50.6, and 51.9) maintained as heterozygotes were studied (F6–F9 generation). Wild-type littermates were used as controls. Because Tg is a recognized abbreviation for the thyroid autoantigen thyroglobulin, we refer to our transgenic mice as A-subunit transgenic (or Tg-ic). The copy number of the A-subunit transgene was determined by the Murine Genetic Analysis Laboratory Center for Comparative Medicine (University of California, Davis, CA). The low-expression transgenic line was accepted for archiving and cryopreservation by the Mutant Mouse Regional Resource Center (University of California, Davis) and is available under the following designation: C57-bg-Tg(TG-TSHR)51.9smcl, no. 014125.

Human A-subunit protein expression in transgenic thyroids

Intrathyroidal expression of human TSHR A-subunit was previously demonstrated by RT-PCR (5). To examine A-subunit protein expression, we used a murine anti-pentahistidine (anti-5H) antibody (Qiagen, Valencia, CA) to detect the C-terminal 6-histidine (6H) tag encoded by the transgene. Immunohistochemistry was performed by the Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO) as follows: Paraffin-embedded sections of murine thyroid tissue were dewaxed and rehydrated followed by heat-induced epitope retrieval (steam at 97 °C for 30 min in 10 mM citrate buffer, pH 6.0). Sections were cooled and treated with 3% H2O2 (to quench endogenous peroxidase) followed by anti-5H (1:100), biotinylated rabbit antimouse IgG, and horseradish peroxidase-streptavidin (Dako North America, Inc., Carpinteria, CA). Color was developed with 3,3′ diaminobenzidine (Dako), and H2O2 and the sections were counterstained with Mayer’s hematoxylin.

The concentrations of human A-subunit protein were measured in thyroid extracts prepared by homogenization (three glands per transgenic line or wild-type littersmates) in buffer containing protease inhibitors (Roche Applied Science, Indianapolis, IN) and the 14,000 × g supernatant retained. We modified our ELISA for detecting TSHR antibodies in mouse sera (10) to estimate human A-subunit concentrations in these extracts. To distinguish the mouse and human A-subunits, anti-5H was used to detect the 6H-tagged transgenic protein. Duplicate supernatant aliquots (1:5 to 1:30) were preincubated with an equal volume of biotinylated anti-5H (Qiagen; 1:1000). In the absence of thyroid extract, this anti-5H dilution yielded an OD of about 1.00 on ELISA wells coated with A-subunit (1 mg/ml) expressed in eukaryotic cells and purified by affinity chromatography (11). Preincubated test samples were transferred to A-subunit-coated wells and, after further incubation and washing, subsequently exposed to horseradish peroxidase-streptavidin (BD Biosciences, San Jose, CA). Color was developed with o-phenylene diamine and H2O2 and stopped with H2SO4 and OD values were read at 490 nm. A-subunit concentrations were expressed as milligrams per thyroid gland estimated from a standard curve generated in the same assay using non-plate-bound A-subunit (0–20 mg/ml). Tg concentrations were measured by a similar inhibition assay using ELISA wells coated with murine (Tg) 1 mg/ml and a polyclonal mouse antihuman Tg that cross-reacts with mTg (generously provided by Dr. Terry F. Davies, Mount Sinai Medical Center, New York, NY; and Dr. Yaron Tomer, University of Cincinnati, Cincinnati, OH). The standard curve, generated using 0–20 mg/ml mTg and the polyclonal anti-Tg at 1:1500, was used to calculate mTg concentrations. Total protein concentrations were determined by Bradford assay (12).

Adenovirus immunization

RGD-Ad encoding either the TSH holoreceptor or the A-subunit (5) were used in this study and are referred to as TSHR-Ad. The same adenovirus stock was always used in an individual experiment. Control immunizations were performed with adenovirus lacking an insert [control adenovirus (Con-Ad)] (13). Viruses were propagated in HEK293 cells and purified by CsCl density gradient centrifugation, and viral particle concentration was determined by absorbance at 260 nm (14). A-subunit transgenic mice and wild-type littersmates (7–10 wk old) were immunized three times at three-weekly intervals with high doses of TSHR-Ad or Con-Ad (~108 particles per injection). Blood was drawn 1 wk after two injections, and animals were euthanized 1 month after the third injection to harvest blood and thyroid glands. Animal studies were approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center and performed with the highest standards of care in a pathogen-free facility.

Depletion of Treg

Rat hybridomas PC61 (anti-CD25) and TM β1 (15) (anti-CD122; generously provided by Dr. K. Yui, Nagasaki University, Nagasaki, Japan; and Dr. T. Tanaka, Osaka University, Osaka, Japan, respectively) were injected into nude mice to induce ascites. The antibodies were purified on HiTrap protein G HP columns (Amersham, Piscataway, NJ) and their efficacy tested in BALB/c mice (Charles River Japan Laboratory Inc., Tokyo, Japan). Splenocytes from untreated and injected mice were compared by FACScan flow cytometry (CellQuest software; BD Biosciences, Mountain View, CA) using the following antibodies: fluorescein isothiocyanate (FITC)-anti-CD4 (H129.19) and phycoerythrin-anti-CD25 (7D4) (BD Biosciences, San Jose, CA) or FITC-conjugated-anti-CD122 (SH; eBioscience, San Diego, CA) and phycoerythrin-conjugated anti-CD8 (53–6.7, BD Biosciences). Four days after ip injection of anti-CD25 (500 μg PC61, CD25+ CD24– T cells were reduced from 8 to 2.1% (supplemental Fig. S1, a and b, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo. endojournals.org) in accordance with previous observations (16). After injecting anti-CD122 (TM β1, 250 μg), CD122–, CD8+ T cells were reduced from 17.5 to 2.9% (supplemental Fig. S1, c and d). From these data, Treg depletion was performed by ip injections of 500 μg/mouse anti-CD25 or 250 μg/mouse anti-CD122 4 d before adenovirus immunization. These studies were conducted according to the principles and procedures in the Guideline for the Care and Use of Laboratory Animals, Nagasaki University.
**Serum T₄, thyroid histology, and TSH**

Total T₄ was measured in undiluted mouse serum (25 ml) by RIA using a kit (Diagnostic Products Corp., Los Angeles, CA). Thyroids were fixed in buffered formaldehyde (pH 7.4) and paraffin-embedded, and serial sections were stained with hematoxylin and eosin (Research Animal Diagnostic Laboratory, University of Missouri, Columbia, MO). Serial thyroid sections were examined without knowing the immunization employed or the origin (transgenic or wild type) of the tissue. Lymphocytic infiltration was assessed as a percentage of the tissue involved. TSH levels in some mice were determined (with a fee for service) by Dr. Roy Weiss (Thyroid Unit, University of Chicago, Chicago, IL) in undiluted serum (50 µl) by RIA (17).

**TSHR antibodies**

TSHR antibody levels were measured by inhibition of TSH binding to the TSHR (abbreviated TBI) using a commercial kit according to the manufacturer’s protocol (Kronus, Boise, ID). In brief, aliquots (25 µl) of mouse serum were incubated with detergent-solubilized TSH; 125I-TSH was added and the TSHR-antibody complexes were precipitated with polyethylene glycol. TBI values were calculated from the formula:

\[
\frac{(TSH \text{ binding in test serum} - \text{nonspecific binding})}{(TSH \text{ binding in control serum} - \text{nonspecific binding})} \times 100
\]

**Autoantibodies to mTg**

Autoantibody binding to mTg was measured using ELISA wells coated with mTg (1 µg/ml); the positive control was a cross-reacting polyclonal mouse antihuman Tg (mTg and antibody from Drs. Davies and Tomer; see above). Test sera were diluted 1:100, antibody binding was detected with horseradish peroxidase-conjugated mouse anti-IgG (Sigma-Aldrich, St. Louis, MO), and the signal was developed with o-phenylenediamine and H₂O₂. The data are expressed as OD 490 nm.

**Autoantibodies to mTPO**

The cDNA for mTPO (18) (provided by Dr. S. Ohtaki, Japan) was transferred to the vector pHMCMV6 (19). Expression of the mTPO-pHMCMV6 plasmid was tested by transiently transfecting COS-7 cells using FuGene HD (Roche) and antihuman TPO antibody in mice using adenovirus (20) that cross-reacts with mouse TPO. Antibody binding was detected using FITC-conjugated affinity-purified goat antiamoouse IgG (Caltag Laboratories, Burlingame, CA) and analysis by flow cytometry. Cells stained with propidium iodide (1 µg/ml) were excluded from analysis. Sera (diluted 1:50) from immunized transgenics and wild-type littermates were tested for mTPO binding in the same way. Data are expressed as percent positive cells in the gated fraction (M2).

**Statistical analyses**

The statistical significance of differences between the magnitude of responses in multiple groups was determined by ANOVA and testing between two groups by Mann Whitney rank sum test or, when normally distributed, by Student’s t test.

**Results**

**Thyroids from transgenic mice express different levels of human TSHR A-subunit**

At the time of previous studies on human TSHR A-subunit transgenic mice bred from five founders (5), we had no information on the level of A-subunit expression. Retaining three of these transgenic lines, we have now determined by immunohistochemistry that thyrocytes from transgenic lines 50.6 and 60.6 express high levels of human TSHR A-subunits, with extensive leakage (or secretion) into the follicular lumens (Fig. 1A, top panels). Although the TSHR holoreceptor is expressed on the basal surface of the thyrocyte, the soluble A-subunit is a secreted protein (21). Thyroids from 51.9 transgenics have a much lower A-subunit expression, similar to that in wild-type mice (Fig. 1A, bottom panels). In thyroid extracts, the highest A-subunit protein level (measured by ELISA inhibition) was in transgenic line 50.6, followed by line 60.6, with minimal or undetectable levels in 51.9 and wild-type mice (Fig. 1B). In contrast, as controls, thyroid concentrations of mTg and total protein were similar in all three transgenic lines and wild-type littermates. The differences in human A-subunit expression among transgenic lines were unre-
lated to transgene copy number, with two copies present in 50.6 transgenics and five copies in the 51.9 and 60.6 transgenics. In subsequent studies, we focused on human TSHR A-subunit transgenic lines 50.6 and 51.9, termed high (Hi) and low (Lo) expressors, respectively.

Breaking tolerance in A-subunit transgenic mice

In our previous studies on A-subunit transgenic mice before categorization into Hi- and Lo-expressors, tolerance was partially broken by immunizing with high doses of TSHR-Ad (A-subunit or TSH holoreceptor) (5). We have now addressed the question of breaking tolerance in Hi vs. Lo A-subunit expressor transgenics. In addition, we tested the outcome of Treg depletion using anti-CD25 or anti-CD122 before each immunization with high-dose TSHR-Ad (A-subunit or holoreceptor) (Fig. 2A). As expected, wild-type littermates had a robust TBI response 1 wk after the second injection and 1 month after the third injection (Fig. 2B). In contrast, Hi-expressor transgenics remained tolerant at both time intervals, even after Treg depletion with anti-CD25 (Fig. 2C). Unlike the Hi-expressor transgenics, TSHR-Ad immunization of Lo-expressors did induce TBI activity; Treg depletion using anti-CD25 or anti-CD122 did not alter the TSHR antibody response (Fig. 2D). Although some responses tended to be higher in some CD25-treated compared with untreated transgenics, the differences were not significantly different.

Incidentally, categorization into A-subunit Hi- and Lo-expressor lines permitted reanalysis of previously reported data (5). Consistent with the present findings, TBI activity was induced in Lo- but not Hi-expressor mice with high-dose TSHR-Ad immunization (supplemental Fig. S2).

Thyroid function and pathology in Lo and Hi A-subunit expressor transgenics

TSHR antibodies detected in the TBI assay may also have thyroid stimulatory activity with resultant thyrotoxicosis. We, therefore, assessed thyroid function by determining se-

Fig. 2. A, Overview of antibody administration to deplete Treg before immunization with TSHR-Ad, obtaining blood 1 wk after the second immunization and euthanasia 1 month after the third immunization. B–D, Induction of TSHR antibodies in A-subunit Hi- or Lo-expressor transgenic mice depleted of Treg before immunization with high-dose TSHR-Ad. Mice were untreated (H11002) or injected with anti-CD25 (H9251 CD25) or H9251 CD122 to deplete Treg, and 4 d later immunized with TSHR-Ad (10^10 particles per injection). Some mice received Con-Ad (Con). Sera were tested 1 wk after two adenovirus injections (Inj 2x) and 1 month after the third immunization (3x). TSHR antibodies were measured as percentage inhibition of TSH binding to the TSHR (TBI). Values for individual transgenics are shown: ○, wild-type mice (B); ●, Hi-expressor transgenics (C); speckled circles, Lo-expressor transgenics (D). The shaded area represents the mean ± 2 SD TBI levels for Con-Ad-immunized wild-type mice.
rum T₄ levels in the mice immunized with TSHR-Ad. As expected in wild-type littermates, high-dose TSHR-Ad induced elevated serum T₄ in about one third of mice (Fig. 3A). Consistent with the absence of TSHR antibodies (Fig. 2), all Hi-expressor transgenics remained euthyroid, even after Treg depletion with anti-CD25 (Fig. 3B). Despite detectable TBI activity, all Lo-expressor transgenics remained euthyroid, even after Treg depletion with anti-CD25 (Fig. 3B). Some recovery in T₄ levels was evident at euthanasia, 4 wk after the third immunization. However, four of seven mice pretreated with anti-CD25 had extremely low or undetectable serum T₄ levels (Fig. 3C). Because of these unusual findings, we measured serum TSH levels in the Lo-expressors and wild-type littermates for which sufficient serum was available at euthanasia. TSH was markedly elevated in both mice with very low T₄ levels (Fig. 3D, arrows), confirming their hypothyroid status.

**Thyroid lymphocytic infiltration in A-subunit transgenics with Treg depletion**

Thyroid pathology provided the explanation for the hypothyroidism described above. In scores of wild-type mice of different strains (including BALB/c) immunized with TSH holoreceptor or A-subunit-Ad, neither we nor others ever observed significant thyroid lymphocytic infiltration (4, 6, 22, 23). Consistent with these observations, wild-type littermates and euthyroid Lo-expressor transgenics immunized with TSHR-Ad had normal thyroid histology (Fig. 4, A and B). Hyperthyroid wild-type mice developed thyroid hyperplasia without infiltrating lymphocytes (Fig. 4C), as observed by ourselves and others (4, 6, 22, 23). Some Lo-expressor transgenics pretreated with anti-CD122 before TSHR-Ad immunization had modest lymphocytic infiltrates (Fig. 4D). Most striking, however, thyroids of hypothyroid Lo-expressor transgenics Treg depleted with anti-CD25 had massive lymphocyte infiltrates, encompassing much of the thyroid (Fig. 4, E and F). These findings are consistent with a shift from Graves’ disease to Hashimoto’s thyroiditis.

The extent of thyroid lymphocytic infiltration in both experiments was quantified as the percentage of the thyroid area invaded by lymphocytes. All Lo-expressor transgenics depleted of Treg with anti-CD25 before TSHR-Ad immunization had thyroid lymphocytic infiltration. In this group, extensive infiltrates, encompassing more than 50% of the

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**Fig. 3.** Serum T₄ and TSH in A-subunit transgenics depleted of Treg before immunization with high doses of TSHR-Ad. As shown in Fig. 2A, mice were untreated (−) or injected with anti-CD25 (αCD25) or anti-CD122 (αCD122) 4 d before each TSHR-Ad immunization. Sera were tested 1 wk after two adenovirus injections (Inj 2x) and 1 month after the third immunization (3x). Some mice were injected with Con-Ad (Con). Data are shown for individual animals. A–C, T₄ levels (µg/dl) in wild-type littermates (A), Hi-expressor transgenics (Tgic-Hi) (B), and Lo-expressor transgenics (Tgic-Lo) (C); D, TSH values (mU/liter) in wild-type and Lo-expressor transgenics. Serum TSH levels were determined when sufficient serum was available (at euthanasia). Arrows indicate elevated TSH values in hypothyroid mice. For each panel, the shaded area represents the mean ± 2 SD T₄ levels for Con-Ad-immunized wild-type littermates.
thyroid, were observed in four mice (Fig. 5, right), all of whom were hypothyroid at the time of euthanasia (Fig. 3C). Smaller infiltrates (less than 20% of the gland) were present in euthyroid animals, including some transgenics pretreated with anti-CD122, as well as in one wild-type mouse depleted of Treg using anti-CD25.

**Intermolecular spreading of autoantibodies to other thyroid autoantigens**

The appearance of extensive lymphocytic infiltration and hypothyroidism suggested a shift in this animal model of Graves’ disease toward Hashimoto’s thyroiditis. Immunological hallmarks of Hashimoto’s thyroiditis are autoantibodies to TPO and Tg. Indeed, in association with thyroid lymphocytic infiltration, autoantibodies to murine Tg were present in all anti-CD25-treated Lo-expressor transgenics but in no other groups of transgenics or wild-type mice (Fig. 6A). Similarly, autoantibodies to mTPO were positive in four of six anti-CD25-treated transgenics (Fig. 6B).

**Discussion**

Because of human diversity as well as for obvious ethical limitations, syngeneic animal models of autoimmune diseases are invaluable investigative tools. Previously, we derived transgenic mice with the human TSHR A-subunit targeted to the thyroid gland. We studied animals derived from five transgenic founders, all bred to BALB/c, a genetic background susceptible to Graves’ hyperthyroidism induced by TSH holoreceptor (6) or A-subunit-Ad (4, 22, 23). Unlike their wild-type littermates, A-subunit transgenic mice were resistant to immunization with low-dose A-subunit-Ad, although high-dose A-subunit or holoreceptor adenovirus immunization elicited low-level immune responses (5). In the present report, using immunohistochemistry and analysis of thyroid extracts, we categorized the extent of intrathyroidal A-subunit expression in progeny from the five founders and have studied one Lo-expressor and two Hi-expressor transgenic lines. The data obtained provide novel insight into tolerance and the role of Treg in the pathogenesis of thyroid autoimmune disease.

Immunoization of the transgenic lines with TSHR-Ad indicated that Hi-expressor mice had a depressed or absent immune response compared with Lo-expressors. These data are consistent with the Hi-expressers having a greater degree of central tolerance, a process in which self-reactive T cells, which bind with high affinity to peptides from self-antigens expressed in the thymus, are deleted. Intrathyroid expression of the TSHR has been reported for humans (24, 25) and rats (26), but A-subunit mRNA was undetectable in thymic tissue from Hi-expressor mice (not shown). Although some self-antigens, for example Tg (27), can be studied in thymic tissue (predominantly nonexpressing thymocytes), others are studied in thymic medullary epithelial cells (28). We cannot, therefore, exclude a role for peripheral tolerance in the A-subunit transgenics. However, as in mice transgenic for hen
Therefore, breaking tolerance is inversely related to the extent of A-subunit transgene expressed in the thyroid, and Treg do not appear to play an important role in this process.

The above studies on tolerance led to serendipitous findings providing novel insight into the role of Treg in the progression of Graves’ hyperthyroidism to severe autoimmune thyroiditis and hypothyroidism. A clinical relationship between these two diseases has long been recognized. For example, without surgical or radioiodine thyroid ablation, the long-term natural course of hyperthyroid Graves’ disease is not uncommonly hypothyroidism (30). As in Hashimoto’s thyroiditis, diffuse lymphocytic infiltration is present in Graves’ thyroid, although typically much less extensive and without thyroid follicle destruction. Although considerable progress has been made in understanding the pathogenesis of Graves’ disease and Hashimoto’s thyroiditis, the mechanism underlying a shift in the balance between these two phenotypes remains enigmatic.

In experimental animals, Graves’ disease and Hashimoto’s thyroiditis are generally studied as separate diseases, the former induced by immunization with the TSHR, the latter with Tg or, less commonly, TPO. Unlike in human disease, Graves’ hyperthyroidism induced in mice has previously not been associated with significant thyroid lymphocytic infiltration (4, 6, 22, 23) or the appearance of TPO and Tg autoantibodies. With minimal or no lymphocytic infiltration, it is self-evident that there are no reports of progression of hyperthyroidism to hypothyroidism in these mice. Lymphocytic thyroiditis is readily induced using adjuvant combined with self protein, murine Tg (31) or mTPO (32). Despite extensive lymphocytic infiltration, in most studies, the animals remain euthyroid. Indeed, transgenic mice with thyroid-restricted expression of the chemokine CCL21 develop massive lymphocytic infiltration (B and T cell) thyroid infiltration without thyroid autoantibodies, and thyroid function remains unaffected (33). Targeting TPO by immunizing with a particular mTPO peptide (34) or by expressing a pathogenic TPO-specific T cell receptor in transgenic mice lacking normal T cells or B cells (Rag knockout) (35) can cause thyroiditis and hypothyroidism. However, until the present report, no animal model has included all the pathological features of human autoimmune thyroid disease, namely hyperthyroidism, massive lymphocytic infiltration leading to hypothyroidism, and autoantibody spreading from the TSHR to TPO and Tg, the other major thyroid-specific autoantigens.

The present study suggests that although Treg do not appear to be responsible for tolerance in our A-subunit transgenic mice, they (particularly CD25+ Treg) are the pathogenetic key to the shift in the balance from Graves’ hyperthyroidism to Hashimoto’s hypothyroidism. Previous studies have demonstrated a role for CD4+ CD25+ Treg depletion in experimentally induced thyroiditis. Tg immunization of BALB/c mice, normally resistant to induction of thyroiditis, develop thyroiditis in conjunction with CD25 Treg depletion (36, 37). Mild thyroiditis was induced by CD25+ T cell depletion before immunization with TSHR-expressing adenovirus in thyroiditis-susceptible C57BL/6 (but not BALB/c) mice (16). Part of this strain difference may involve the genetically controlled

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**Fig. 6.** Intermolecular autoantibody spreading from the TSHR to other thyroid autoantigens. Wild-type littermates and Lo-expressor transgenics (Tgic-Lo) were pretreated with anti-CD25 (αCD25) or anti-CD122 (αCD122) before immunization with TSHR-Ad or Con-Ad (Con) (Fig. 2A). Autoantibody spreading to mTg (A) and mTPO (B) occurs in Tgic-Lo animals Treg depleted with αCD25 before immunizations with A-subunit-Ad (A-sub) or TSHR-Ad. These groups of animals also had the greatest degree of thyroid lymphocytic infiltration (Fig. 5). Autoantibodies to mTPO were assayed by flow cytometry using mTPO-expressing COS-7 cells. Data are expressed as the percent positive gated cells. Murine Tg autoantibodies were detected by ELISA. The dashed horizontal line represents the mean ± 2 SD for control-Ad-immunized mice. * Values significantly greater than for other transgenic groups (ANOVA, P < 0.05).
thymic development of Treg (CD4⁺CD25⁺FoxP3⁺), with higher percentages in BALB/c than C57BL/6 mice (38). In thyroiditis-susceptible NOD mice expressing a human HLA-DR3 transgene, anti-CD25 treatment enhances iodide-induced thyroiditis (39). However, it should be emphasized that none of these studies involving Treg depletion led to severe thyroiditis or to hypothyroidism. In contrast, after CD25 Treg depletion, all Lo-expressor mice studied developed hypothyroidism after the second immunization (Fig. 3C). Moreover, all four mice whose hypothyroidism persisted 1 month after the final immunization had extensive thyroiditis encompassing 60–80% of the gland.

Besides inducing extensive thyroid lymphocytic infiltration and hypothyroidism in Lo-expressor A-subunit transgenics, immunization had another unexpected result that mimics human thyroid autoimmune disease, namely spreading of the humoral autoantibody response from the TSHR to other thyroid-specific autoantigens. We provide a hypothesis to explain these intriguing findings (Fig. 7). Unimmunized wild-type and transgenic mice are tolerant to the TSHR, TPO, and Tg (all self murine proteins) and transgenics are tolerant to the human A-subunit. After immunization with human TSHR-Ad, wild-type and transgenic mice develop T cells that recognize human TSHR peptides. Presumably, cross-reactivity to mouse TSHR peptides is limited or absent. The immune response is also restrained by Treg. Therefore, lymphocytes rarely infiltrate the thyroid in mice of this genetic background (BALB/c). However, after Treg depletion (particularly CD25 T cells), restraint on the immune response is diminished. In transgenics (but not wild-type mice), lymphocytes home to the thyroid, the source of their target peptides, the human A-subunit. The infiltrating lymphocytes (likely including cytotoxic cells and generating cytokines) cause thyrocyte damage that can lead to overt hypothyroidism. Release of thyroid antigens in this inflammatory milieu breaks tolerance to other thyroid antigens. Thus, in the Lo-expressor A-subunit transgenics, CD25 (but not CD122) Treg depletion was accompanied by strong autoantibody responses to mTg and mTPO. Previously, very low-level mTg autoantibodies were observed in a single human TSHR-DNA-vaccinated DR3⁺ NOD mouse (40), a background susceptible to developing spontaneous thyroiditis and Tg autoantibodies. Consequently, our findings provide the first unequivocal evidence for intermolecular autoantibody spreading in thyroid autoimmunity.

Incidentally, it should be noted that hyperthyroidism developed only in wild-type littermates immunized with TSHR-Ad and not in Lo-expressor transgenics. The relatively low proportion of hyperthyroid wild-type mice is anticipated; to break tolerance in A-subunit transgenic animals, immunizations required high-dose adenovirus immunizations, which generate fewer hyperthyroid animals than low-dose immunizations (41). Extensive thyroiditis explains why Lo-expressor transgenics depleted of CD25 Treg became hypothyroid. However, it is less clear why Lo-expressor transgenics that were not Treg depleted remained euthyroid. It is possible that sufficient A-subunit protein is generated in the thyroid to neutralize thyroid-stimulating antibodies.

What evidence is available of a role for Treg in human thyroid autoimmunity? In humans, the number and function of Treg are still unclear, depending on the Treg markers and assays employed as well as the disease. Abundant Treg were found infiltrating the thyroid gland of Graves’ patients in one study, but the suppressor function of peripheral Treg was decreased (42). In another study, intra-thyroidal Treg were reduced compared with those in peripheral blood, possibly because of increased apoptosis (43). Despite the limited number of studies (and in some cases the limited number of patients investigated), these data are consistent with our findings for the association between thyroiditis and Treg in mice and (incidentally) with the early studies of Volpe and Iitaka (44) concerning a suppressor T cell defect. Future studies on the evolution of Graves’ disease into Hashimoto’s thyroiditis with hypothyroidism will be of interest to determine whether this shift is accompanied by an alteration in Treg number or function.

In summary, the present study provides the first description of a complete animal model of autoimmune thyroid disease mimicking all the clinical and pathological features of human disease. Moreover, it describes an immunological mechanism whereby induction of thyroid-stimulating antibodies and Graves’ hyperthyroidism can be diverted to spreading of the immune response to endogenous thyroid autoantigens (mTPO and mTg) with extensive lymphocytic infiltration and hypothyroidism. Our data, taken together with other studies on experimental thyroiditis, support the clinical observation that Hashimoto’s thyroiditis in humans is only rarely associated with TSHR autoantibodies, the antithesis of Graves’ disease in which autoantibodies to Tg and TPO are commonly present. This finding has two important clinical implications. First, in Graves’ disease, TPO and Tg autoantibodies are secondary to the immune response to the
TSHR. Second, lymphocytic infiltration in Graves’ disease is likely to reflect spreading of the immune response to TPO and to Tg.

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