A Unique Mouse Strain That Develops Spontaneous, Iodine-Accelerated, Pathogenic Antibodies to the Human Thyrotrophin Receptor

Basil Rapoport, Holly A. Aliesky, Bianca Banuelos, Chun-Rong Chen and Sandra M. McLachlan

*J Immunol* 2015; 194:4154-4161; Prepublished online 30 March 2015; doi: 10.4049/jimmunol.1500126

http://www.jimmunol.org/content/194/9/4154

Supplementary Material http://www.jimmunol.org/content/suppl/2015/03/28/jimmunol.1500126.DCSupplemental

References This article cites 47 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/194/9/4154.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

Why *The JI*?

• *Rapid Reviews!* 30 days* from submission to initial decision

• *No Triage!* Every submission reviewed by practicing scientists

• *Speedy Publication!* 4 weeks from acceptance to publication

*average
A Unique Mouse Strain That Develops Spontaneous, Iodine-Accelerated, Pathogenic Antibodies to the Human Thyrotrophin Receptor

Basil Rapoport, Holly A. Aliesky, Bianca Banuelos, Chun-Rong Chen, and Sandra M. McLachlan

Abs that stimulate the thyrotropin receptor (TSHR), the cause of Graves’ hyperthyroidism, only develop in humans. TSHR Abs can be induced in mice by immunization, but studying pathogenesis and therapeutic intervention requires a model without immunization. Spontaneous, iodine-accelerated, thyroid autoimmunity develops in NOD.H2b/d mice associated with thyroglobulin and thyroid-peroxidase, but not TSHR, Abs. We hypothesized that transferring the human TSHR A-subunit to NOD.H2b/d mice would result in loss of tolerance to this protein. BALB/c human TSHR A-subunit mice were bred to NOD.H2b/d mice, and transgenic offspring were repeatedly backcrossed to NOD.H2b/d mice. All offspring developed Abs to thyroglobulin and thyroid-peroxidase. However, only TSHR-transgenic NOD.H2b/d mice (TSHR/NOD.H2b/d) developed pathogenic TSHR Abs as detected using clinical Graves’ disease assays. In as humans, TSHR/NOD.H2b/d female mice were more prone than male mice to developing pathogenic TSHR Abs. Fortunately, in view of the confounding effect of excess thyroid hormone on immune responses, spontaneously arising pathogenic human TSHR Abs cross-react poorly with the mouse TSHR and do not cause thyrotoxicosis. In summary, the TSHR/TSHR Abs. Fortunately, in view of the confounding effect of excess thyroid hormone on immune responses, spontaneously arising pathogenic human TSHR Abs cross-react poorly with the mouse TSHR and do not cause thyrotoxicosis. In summary, the TSHR/NOD.H2b/d mouse strain develops spontaneous, iodine-accelerated, pathogenic TSHR Abs in female mice, providing a unique model to investigate disease pathogenesis and test novel TSHR Ag-specific immunotherapies aimed at curing Graves’ disease in humans.

The Journal of Immunology, 2015, 194: 4154–4161.

G
raves’ disease is the prototypic autoimmune disease in which the humoral arm of the immune system directly causes organ overactivity (reviewed in Ref. 1). The phenotypic expression of hyperthyroidism results from the stimulatory effect of a single type of autoantibody on a single autoantigen, the thyrotropin (TSH) receptor (TSHR). Graves’ disease is one of the most common autoimmune diseases, affecting ∼1% of the population in their lifetimes, with a very strong predilection toward females (3–7:1 female/male ratio in different countries) (2). There is no cure for the disease. Hyperthyroidism can be treated, either by inhibiting thyroid hormone synthesis with thiouamide drugs or by radioiodine or surgical thyroid ablation, all with the attendant risks of negative side effects or, even more commonly, permanent hypothyroidism requiring lifelong thyroid hormone ingestion.

Immune intervention to cure Graves’ disease by inducing immune tolerance to the TSHR has been a long-standing goal, but it is very difficult to approach experimentally. An important barrier to studying the pathogenesis of Graves’ disease, as well as investigating novel therapies, is that this disease only occurs in humans. Not even the closely related great apes (chimpanzees, gorillas, and orangutans) experience Graves’ disease (3). For 40 y, immunization of different animal species with thyroid extracts, and later with recombinant TSHR protein together with adjuvant, did generate Abs, but none had the conformational specificity capable of activating the TSHR. In 1996, a breakthrough occurred with the demonstration that in vivo expression of the TSHR was necessary to induce thyroid-stimulating Abs (TSAb) in mice, with resultant hyperthyroidism (4). Subsequently, different vectors and immunization approaches have been used to express TSHR in vivo leading to TSAb induction and hyperthyroidism, for example, in some mouse strains (5–9), hamsters (10) and rhesus monkeys (11).

All the foregoing approaches involving in vivo TSHR expression in animals are of limited use in studying approaches to induce tolerance to the TSHR, a necessary and essential requirement for eliminating TSAb and consequent hyperthyroidism without suppressing or ablating normal thyroid function. A suitable animal model to study potential immunotherapeutic strategies requires TSAb to arise spontaneously and stably to self (syngeneic)-Ag. In contrast, the majority of previous induced animal models have used xenogeneic (human) TSHR with a transient TSAb response. Another consideration for an ideal animal model to study modulation of spontaneously arising TSAb to self-TSHR would be to avoid the effects of consequent hyperthyroidism. Thyroid hormone excess, or thyrotoxicosis, has widespread effects on virtually all aspects of the immune system (see Discussion).

We now report the development of a novel mouse model in which functional TSAb arise spontaneously to the TSHR in the absence of the confounding influence of thyrotoxicosis. These animals represent a major advance that will facilitate study of approaches toward the goal of using immunotherapy to induce tolerance to the
TSHR, and thereby reverse the development of TSAb to cure, not treat, Graves’ disease in humans.

Materials and Methods

Generating NOD.H2h4 mice expressing the human TSHR A-subunit

NOD.H2h4 mice (The Jackson Laboratory, Bar Harbor, ME) and transgenic BALB/c mice expressing low intrathyroidal levels of the human TSHR A-subunit (line 51.9; subsequently referred to as TSHR-Tgic) (12) were bred at Cedars-Sinai Medical Center. Male TSHR-Tgics were crossed to female NOD.H2h4 mice to generate N1 progeny. Expression of the transgene was determined by PCR (13). Transgenic male N1 pups were bred to wild-type NOD.H2h4 females to generate N2 mice, and the same procedure was repeated to produce the N3 and N4 generations. At this stage, to introduce the NOD.H2h4 Y chromosome, we crossed wild-type NOD.H2h4 males to female N4 Tgic-NOD.H2h4 mice. Thereafter, we reverted to crossing Tgic-NOD.H2h4 male offspring with wild-type NOD.H2h4 females. Genome scanning (The Jackson Laboratory) was performed on tail DNA from the N2, N3, N5, and N6 generations to select males with the highest proportion of NOD.H2h4 genes to breed the next generation. N7 mice were bred from two N6 males with 99.3 or 99.5% NOD.H2h4 genes (Supplemental Fig. 1).

Data are reported for parental strains and offspring from N1 to N8 backcrosses. Unless indicated otherwise (and excluding all breeding mice), from 8 wk of age water was supplemented with 0.05% sodium iodide (NaI) for 16 wk at which time (age 24 wk) TSHR-Tgic and non-Tgic offspring (N1-N8) as well as parental strains were euthanized to harvest blood and thyroid tissue. Where indicated, additional NOD.H2h4 mice were maintained on regular or NaI water for up to 32 wk. All mouse studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center. TSHR/NOD.H2h4 mice have been archived by the Mutant Mouse Regional Resource Center under the designation NOD.Cg-Tg(TG-TSHR)51.9Smcl, MMRRC:037586.

TSHR Ab assays

TSHR Abs were measured using three assays: ELISA, TSH binding inhibition (TBI) assay, and TSAb bioassay.

ELISA. The assay for TSHR Abs (IgG class) has been reported previously (7). Recombinant TSHR A-subunit protein secreted by CHO cells with an amplified transgene (14) was purified from culture supernatants by affinity chromatography (15). ELISA wells were coated with A-subunit protein (5μg/ml) and incubated with test sera (1:100 dilution; duplicate aliquots). The positive control used in this assay was serum from BALB/c mice immunized with mouse Tg and CFA (19). Thyroglobulin Ab (TgAb) data are presented as the OD at 490 nm.

Thyroid peroxidase (TPO) Abs (TPOAbs) were measured using CHO cells stably expressing mouse TPO (18). Sera (1:50 dilutions) were incubated with mouse TPO-CHO cells, and binding was detected with FITC-conjugated affinity-purified goat anti-mouse IgG (A3673; Sigma Chemical), the signal developed with o-phenylenediamine, and the reaction stopped using 20% H2SO4. The negative control was serum from 8-wk-old NOD.H2h4 mice on regular water; the positive control was serum from BALB/c mice immunized with mouse Tg and CFA (19). Thyroglobulin Ab (TgAb) data are presented as the OD at 490 nm.

Flow cytometry was performed (10,000 events) using a FACSscan with CELLQUEST Software (Becton Dickinson, San Jose, CA). Data are reported as the geometric mean.

Serum thyroxine, TSH, and thyroid histology

Serum total thyroxine (T4) was measured in 25 μl mouse serum by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). T4 values were computed from kit standards and expressed as micrograms per deciliter (μg/dl). TSH was measured by radioimmunoassay (21) (Dr. S. Refetoff, University of Chicago; fee for service). Thyroid glands were preserved in zinc fixative (BD Pharmingen, San Diego, CA), paraffin-embedded, and serial sections stained with H&E (IDEXX BioResearch Lab Animal and Biological Materials Diagnostic Testing, Columbia, MO).

Autoantibodies to thyroglobulin and thyroid peroxidase

Thyroglobulin (Tg) was isolated from murine thyroid glands as previously described (18). ELISA wells (Immulon 4HBX; Thermo Scientific, Rochester NY) were coated with mouse Tg (1.5μg/ml) and incubated with test sera (duplicate aliquots, 1:100 dilution). Ab binding was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (A3673; Sigma Chemical), the signal developed with o-phenylenediamine, and the reaction stopped using 20% H2SO4. The negative control was serum from 8-wk-old NOD.H2h4 mice on regular water; the positive control was serum from BALB/c mice immunized with mouse Tg and CFA (19). Thyroglobulin Ab (TgAb) and thyroid peroxidase (TPO) Abs (TPOAbs) were measured using CHO cells stably expressing mouse TPO (18). Sera (1:50 dilutions) were incubated with mouse TPO-CHO cells, and binding was detected with FITC-conjugated affinity-purified goat anti-mouse IgG (M30101; Invitrogen, Carlsbad, CA). Cells staining with propidium iodide (1 μg/ml) were excluded from analysis. The negative control for IgG class Ab binding to mouse TPO-CHO cells was serum from 8-wk-old NOD.H2h4 mice. Positive controls were mouse mAbs 15 and 64 to human TPO (20), provided to us by Dr. J. Ruf (Marseille, France), that recognize mouse TPO (18, 20). Flow cytometry was performed (10,000 events) using a FACSscan with CELLQUEST Software (Becton Dickinson, San Jose, CA). Data are reported as the geometric mean.

FIGURE 1. Concepts underlying the hypothesis that expressing the human TSHR A-subunit transgene in NOD.H2h4 mice would generate a strain that produces spontaneous, iodine-accelerated, TSHR Abs. Recipient NOD.H2h4 mice experience thyroditis associated with TgAb and TPOAb (18, 22–24), but not to the TSHR. The donor BALB/c mice do not develop thyroid autoimmune, and the human TSHR A-subunit targeted to the thyroid gland is a self-Ag (13). However, regulatory T cell depletion before human TSHR A-subunit adenovirus immunization breaks tolerance to endogenous mouse thyroid Ags Tg and TPO (12).
Statistics

Significant differences between responses in different groups were determined by Mann–Whitney rank sum test or, when normally distributed, by Student t test. Multiple comparisons were made using ANOVA. Tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

Results

The concept of developing a mouse that fulfills the criteria described earlier arose from a number of factors (Fig. 1). First, unlike the conventional NOD mouse that develops diabetes, the NOD.H2A4 strain develops spontaneous, iodine-accelerated, autoimmune thyroiditis in association with autoantibodies to Tg (22–24) and, at a later stage, to TPO (18), but not to the TSHR. Second, we previously generated transgenic BALB/c mice with the human TSHR A-subunit selectively expressed in the thyroid gland, a self-Ag as evident by tolerance to human TSHR A-subunit immunization (13). This component of the TSH holoreceptor is the antigenic target of TSAb that causes Graves’ disease (7, 14, 25). Regulatory T cell depletion with anti-CD25 before TSHR A-subunit adenovirus immunization led to massive thyroiditis associated with Ab spreading from the TSHR to the other two thyroid autoantigens, Tg and TPO (12). We therefore hypothesized that transferring the TSHR A-subunit transgene locus from the BALB/c transgenics to the NOD strain would lead to the spontaneous generation of pathogenic TSHR autoantibodies.

**TSHR Abs develop in TSHR transgenic (not wild-type) NOD.H2A4 offspring**

TSHR Abs detected by ELISA were present in some transgenic offspring derived by crossing human TSHR A-subunit–expressing BALB/c mice to the NOD.H2A4 strain and repeated backcrossing to NOD.H2A4 mice (N1–N8 generations; Fig. 2A). Neither wild-type offspring lacking the TSHR transgene nor the parental NOD.H2A4 mice developed TSHR Abs. In contrast, TgAbs were detectable in some parental NOD.H2A4 mice and N1-N8 backcross offspring regardless of whether they contained the TSHR A-subunit transgene (Fig. 2B). TPOAbs also develop in some N1-N8 backcross offspring independent of the A-subunit transgene (Supplemental Fig. 2).

Consistent with the standard protocol for NOD.H2A4 mice, from 8 wk of age mice were provided with NaI-supplemented water for 16 wk. However, we maintained a smaller number of animals on regular water. As for the Nal group, only transgenic backcross offspring, not the parent NOD.H2A4 strain or the nontransgenic offspring, developed TSHR Abs detectable by ELISA (Supplemental Fig. 3). TSHR Abs were undetectable in the N1+N2 generation on regular water but were clearly present in the same generation of mice on NaI water (Supplemental Fig. 3 versus Fig. 2A). These data confirm previous observations for Tg autoantibodies that dietary iodide enhances (or accelerates) but is not responsible for the development of thyroid autoimmunity in NOD.H2A4 mice (18, 23). In particular, TgAbs are detectable much earlier than TPOAbs in NOD.H2A4 mice maintained on NaI (18). Because NaI accelerates thyroid autoimmunity, backcrossed mice after the N6 generation were maintained on NaI-supplemented water.

**High TBI in male NOD.H2A4 mice associated with elevated TSH**

Although measuring TSHR Abs by ELISA is a convenient initial screening method to determine whether tolerance to the receptor is maintained or broken, this assay only detects nonfunctional TSHR Abs. Pathogenic, functional TSHR Abs do not recognize adherent, purified TSHR Ag on the ELISA plate (26). The latter autoantibodies can only be detected by competition for TSH binding to the native holoreceptor or by activating the receptor expressed on the surface of intact cells, thereby generating cAMP.

In preliminary studies, sera from male and female parental NOD.H2A4 mice without the A-subunit transgene were tested for TBI Abs. Unexpectedly, high TBI values were detected in male, but not in female, mice (Fig. 3A). Greater TSH levels in males than in females is a recognized phenomenon in a number of mouse strains with normal thyroid function and without thyroid autoimmunity (21, 22). Because the high TBI levels observed in...
Among the female mice, none of the parental NOD.H2h4 TSHR autoantibodies detected by the TBI assay limited was not a handicap in view of the far greater female background values in male mice severely limited interpretation of the data, after the N6 generation we focused primarily on females. Although reducing the number of mice available for study, this limitation was not a handicap in view of the far greater female incidence of Graves’ disease in humans.

**TSHR autoantibodies detected by the TBI assay**

Among the female mice, none of the parental NOD.H2h4 or nontransgenic backcrossed offspring in the N1+2 to N7+8 generations had elevated TBI autoantibodies (Fig. 4A). In contrast, TBI autoantibodies were detected in some female mice expressing the TSHR A-subunit transgene from the N1+2 to the N7+8 backcrosses (Fig. 4A). In these female mice, the proportion of TBI positivity increased from 2 of 13 in the N1+N2 generation to 5 of 10 in the N7+N8 generation.

Turning to the male mice, because the high background levels in the TBI assay limited interpretation of the data, we studied fewer males in later generations. With this proviso, none of the male TSHR/NOD.H2h4 transgenics attained TBI values greater than those observed in their nontransgenic littermates (Fig. 4B). Moreover, none of the male TSHR/NOD.H2h4 transgenics attained TBI values increasing above the high background to approach values observed in some of the female transgenics (Fig. 4A). These data suggest that the human TSHR A-subunit locus is more conducive to the spontaneous development of pathogenic TSHR autoantibodies in females than in male TSHR/NOD.H2h4 transgenic mice.

**TSHR autoantibodies detected in the TSAb bioassay**

Additional evidence for pathogenic TSHR Ab levels arising spontaneously in NOD.H2h4 mice transgenic for the human TSHR A-subunit came from functional bioassays for TSAb. As mentioned earlier, because of the confounding influence of high TSH levels even in nontransgenic male NOD.H2h4 mice, we tested sera from female TSHR/NOD.H2h4 transgenic mice for their ability to stimulate cAMP generation in monolayers of CHO cells expressing the TSH holoreceptor. TSAb was clearly increased in some (4/9) female transgenic offspring in the N7+N8 generation versus 0/13 in the nontransgenic offspring (Fig. 5). TSAb values correlated with TBI levels in the transgenic NOD.H2h4 N7+N8 offspring ($r = 0.865$, $p = 0.003$).

**Serum T4 levels in NOD.H2h4 mice with the human TSHR A-subunit transgene**

Spontaneously arising autoantibodies to the human TSHR transgene had little, if any, effect on the murine TSHR. Three female NOD.H2h4 mice transgenic for the TSHR A-subunit in the N5-N8 generations had serum T4 levels greater than the normal range established in nontransgenic NOD.H2h4 littermates (Fig. 6A). However, for a number of reasons, a diagnosis of hyperthyroidism cannot be clearly established. First, an elevated serum T4 of similar magnitude was also observed in one female non-

---

**FIGURE 3.** Greater TSH levels in male than in female parental NOD.H2h4 mice are a confounding influence on measuring TSHR Abs in the TSHR binding inhibition (TBI) assay. Sera from male and female mice were assayed at 32 wk of age for TBI (A) and TSH (B). Values for TBI (% inhibition of TSH binding to the TSH holoreceptor) and TSH (mU/l) are shown as the mean + SEM ($n = 5$ sera/group). Significance of differences: *$p = 0.032$ (rank sum test) (B), **$p < 0.002$ (t test) (A).

**FIGURE 4.** Detection of TSHR Abs by the TBI assay in female, not in male, backcrossed mice with the A-subunit transgene. Sera from 24-wk-old NOD.H2h4 littermates with and without the human TSHR A-subunit transgene were tested in the TBI assay. Values in female (A) and male (B) mice represent percentage binding inhibition of radiolabeled TSH to the TSH holoreceptor. Shaded area represents the mean ± 2 SD for all nontransgenic female (A) and male (B) mice. Number of female mice studied: NOD.H2h4, 13; nontransgenic: N1+N2, 23; N3+N4, 19; N5+N6, 18; N7+N8, 20; transgenics: N1+N2, 13; N3+N4, 12; N5+N6, 11; N7+N8, 11. Number of male mice studied: NOD.H2h4, 7; nontransgenic: N1+N2, 6; N3+N4, 18; N5+N6, 12; N7+N8, 7; transgenics: N1+N2, 14; N3+N4, 10; N5+N6, 9; N7+N8, 3. NOD, parental NOD.H2h4 mice; N1-N8, generations of backcrossing to NOD.H2h4.
transgenic littermate. Second, although the two females with the highest T4 levels were positive for TSAb, the former levels are disproportionately low relative to those (12–20 μg/dl) attained in BALB/c females with similar TSAb levels after TSHR A-subunit adenovirus immunization (e.g., 6, 7, 27, 28). Finally, on histological examination, the mouse thyroid follicular cells were not hypertrophic (cuboidal or columnar) as typically observed in hyperthyroid mice in the induced model of Graves’ disease. The extent of thyroid lymphocytic infiltration did not differ between the NOD.H2b4 males with and without the A-subunit transgene (up to 35% of total thyroid volume; representative histology shown in Fig. 7) and was insufficient to severely decrease thyroid reserve.

Consistent with serum T4 sex differences observed in other mouse strains (21, 29), the normal range for this parameter in nontransgenic NOD.H2b4 male mice was higher than in female mice. On this basis, none of the NOD.H2b4 males with the TSHR A-subunit transgene had increased serum T4 levels (Fig. 6B). The goal of immunotherapy using this novel strain (that does not develop hyperthyroidism) is shown schematically in Fig. 8.

**Discussion**

We report the generation of a strain of mice that develop spontaneous, iodine-accelerated, pathogenic TSHR autoantibodies. This TSHR/NOD.H2b4 strain was generated by transferring the human TSHR A-subunit transgene locus from BALB/c mice (12, 13) to NOD.H2b4 mice. In the former BALB/c strain, the human TSHR A-subunit is self, whereas NOD.H2b4 mice are genetically predisposed to spontaneous development of autoimmune thyroiditis in association with autoantibodies to the thyroid-specific Ags Tg and TPO (18, 22–24). However, autoantibodies to the mouse TSHR do not arise spontaneously in NOD.H2b4 mice, which remain euthyroid despite moderate lymphocytic infiltration. The reason for immunological tolerance (at least at the humoral level) to the TSHR in NOD.H2b4 mice is unknown but may relate to the very low level of TSHR expression on thyrocytes. In contrast, Tg, the primary autoantigen in the development of thyroiditis in these animals, is by far the dominant protein generated and secreted by the thyroid. Our hypothesis, validated in this report, was that a higher level of TSHR expression in the thyroid, particularly in a secreted form available for presentation to the immune system, would break tolerance to this self-Ag in a strain, namely, NOD.H2b4 mice, that spontaneously develop thyroid autoimmunity.

The TSHR differs from the closely related luteinizing hormone and follicle-stimulating hormone receptors in undergoing intramolecular proteolytic cleavage leading to disulfide bonded A- and B-subunits. Some of the TSHR autoantibody binding A-subunits are subsequently shed from the cell surface (30, 31), and there is strong evidence that shed A-subunits contribute to the induction and affinity maturation of functional TSHR Abs (7, 32, 33). In studies on the recombinant TSHR, the isolated A-subunit lacking the transmembrane component of the receptor is not retained at the cell surface but is secreted into the extracellular milieu (14). Because the secreted, recombinant TSHR A-subunit is essentially the same as the A-subunit shed from the holoreceptor, we previously generated two lines of transgenic BALB/c mice with the human TSHR A-subunit targeted to the thyroid, one a being a high expression and the other a low expressor (12, 13). In these mice, the human TSHR A-subunit is a self-Ag. Consequently, tolerance to the human TSHR is difficult to break in the high-expressor transgenics but can be broken by adenovirus immunization in the low expressors (12, 13). Therefore, to optimize the possibility of a spontaneous loss of tolerance to the A-subunit transgene, we
used the low-expressor transgenic as the parent strain to backcross the A-subunit locus onto an NOD.H2<sup>bkd</sup> background.

In NOD.H2<sup>bkd</sup> mice expressing the human TSHR A-subunit transgene, autoantibodies to this Ag measured by ELISA arose spontaneously in both males and females. Such Abs lack bioactivity (26). Only TSHR Abs measured in the TBI and TSAb assays are of pathogenic significance and clinically relevant. However, unlike the ELISA, the TBI and TSAb assays also detect TSH. As reported in some other mouse strains (21, 29), TSH levels in male NOD.H2<sup>bkd</sup> mice were much higher than in females, thereby introducing spuriously high background levels that limit interpretation of the TBI and TSAb data in male mice. For this reason, in later backcrosses, we focused on female TSHR/NOD.H2<sup>bkd</sup> mice. It is, therefore, fortunate for the future study of these mice that Graves’ disease, like many autoimmune conditions, primarily affects women (2). Indeed, although the confounding influence of endogenous TSH precludes making firm conclusions, our data suggest a bias toward pathogenic TSHR autoantibody generation in female TSHR/NOD.H2<sup>bkd</sup> mice (Fig. 4). By the N7/N8 backcross, 40–50% of female TSHR/NOD.H2<sup>bkd</sup> mice developed TBI and TSAb, a sufficient proportion for future investigations described later.

The variability in developing pathogenic TSHR Abs by N7/N8 backcross mice is not likely to be due to extensive heterogeneity in their genetic background for two reasons: 1) genome-wide screens of the N6 males selected to generate N7 mice revealed 99.3 and 99.5% NOD.H2<sup>bkd</sup> genes, implying <1% BALB/c genes; and 2) virtually all N7+N8 mice produced TgAb in comparable amounts.
with the parent NOD.H2b/d strain. More likely, the variability is an inherited (albeit unexplained) characteristic of the NOD strain used to generate NOD.H2b/d mice; NOD mice are well-known to exhibit variability in diabetes and breeding (e.g., 34), and variable degrees of spontaneous thyroiditis develop in another NOD-derived strain, NOD.H2b (35).

An important, and initially puzzling, feature of the TSHR/NOD.H2b/d mice was the minimal degree of hyperthyroidism despite the presence of TSAb activity in their sera. Serum T4 levels greater than the normal range were observed in three female TSHR/NOD.H2b/d mice, but increased values also occurred in some nontransgenic NOD.H2b/d female littermates. Lymphocytic thyroiditis was not extensive and was similar in NOD.H2b/d mice with and without the A-subunit transgene. Coexisting thyroiditis could, therefore, not explain this lack of hyperthyroidism. Rather, there is strong evidence for two other factors that limit the thyroid response to pathogenic TSHR autoantibodies. First, the transgenic human A-subunit, lacking the transmembrane component of the receptor, cannot respond functionally to the TSHR autoantibodies that it induces. Only activation of the endogenous mouse TSH holoreceptor can cause hyperthyroidism. Second, different mouse strains do not respond equally well to autoantibodies induced by human TSHR adenovirus immunization, used in most models. For example, despite similar TSAb activities in serum, severe hyperthyroidism occurs readily in BALB/c mice, whereas C57BL/6 mice largely remain euthyroid (6, 28, 36). The parental NOD.H2b/d mouse strain used in this study is similar to C57BL/6 mice. In a previous study, only a small proportion of mice of the former strain became hyperthyroid, with modest serum T4 elevations that were disproportionately low relative to the high TSH autoantibody values (37). Therefore, TSAb to the human TSHR in NOD.H2b/d mice, whether induced (37) or arising spontaneously with iodine-acceleration (as in this model), appear to cross-react minimally with the mouse TSHR.

Genome-wide array studies in recombinant inbred mice derived from BALB/c and C57BL/6 mice (strongly and weakly responsive to induced TSHR Abs, respectively) have revealed linkage of this phenotype to the IgH V region gene locus (16). These genes are, in turn, linked to H chain C region polymorphisms (or allotypes) (e.g., 38–40). It may be relevant that C57BL/6 and NOD.H2b/d mice (both low responders to TSAb generated to the human TSHR) have the IgG2a IgH-C allotype [b], whereas BALB/c mice (strong responders to human TSHR-specific TSAb) bear the [a] allotype (41).

In addition to TSHR Abs and hyperthyroidism induced by immunization (see Introduction), a transgenic mouse model of Graves’ disease has been reported with the H and L chain variable regions of a human IgM mAb with weak TSAb activity (B6B7) (42, 43). These TSAb are generated by the inserted IgM transgenes and do not arise spontaneously in response to a self-Ag. Moreover, the IgM TSAb transgenic mouse model requires LPS administration to expand the transgenic B cell population. Other animal models have involved the i.p. injection of hybridoma cells secreting a potent hamster monoclonal TSAb (44) or injection of purified, extremely potent mouse monoclonal TSAb IgG (45). Valuable insights were obtained from all three studies into (for example) immune parameters and/or the pathogenic changes accompanying TSAb-induced hyperthyroidism. However, none of these approaches permits study of the spontaneous loss of tolerance to self-Ag leading to TSHR autoantibody production by normal B cells.

It may not be appreciated that the absence of overt hyperthyroidism in TSHR/NOD.H2b/d mice is not detrimental but, instead, is an advantage for studies for several reasons. TSAbs are the proximate cause of Graves’ disease. Therefore, the critical goal in studying the pathogenesis of, or potential therapy for, Graves’ disease using TSHR/NOD.H2b/d mice is not to focus on hyperthyroidism that develops secondary to TSAb, but on the modulation of the primary development of TSHR autoantibodies, preferably by restoring TSHR self-tolerance (Fig. 8). There are many animal models of hyperthyroidism and/or thyrotoxicosis, and numerous therapies can be used to reverse hyperthyroidism. However, it should be noted that thyrotoxicosis (excess thyroid hormone) alters the phenotype and function of Ag-presenting dendritic cells by increasing the expression of costimulatory molecules required to initiate Ab production (46). Thyrotoxicosis also polarizes dendritic cells leading to impaired function of regulatory T cells, a major change that may influence the emergence of pathogenic autoantibodies (47). Consequently, the critical goal in studying the pathogenesis or therapy of our new model is to focus on preventing the development of TSHR autoantibodies without the confounding influence of thyroid hormone fluctuations.

In conclusion, we have developed a unique model, the TSHR/NOD.H2b/d strain, that develops spontaneous, iodine-accelerated, TSAb detectable by ELISA. High TS levels interfere with assays for pathogenic Abs in males. However, in female TSHR/NOD.H2b/d mice, pathogenic Abs can be detected in assays used clinically for human Graves’ disease that involve inhibition of TSH binding to its receptor, as well as activation of the TSHR. This mouse model, which has nontransgenic T and B cells, represents a significant advance for studies of Graves’ pathogenesis and will facilitate investigating potential approaches for TSHR Ag-specific immunotherapy to cure, rather than treat, Graves’ disease in humans.

Acknowledgments
We thank Dr. Jean Ruf (INSERM Unit U555, Faculté de Médecine Timone, Université de la Méditerranée, Marseille, France) for generously providing mouse mAbs to human TPO.

Disclosures
The authors have no financial conflicts of interest.

References