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A unique mouse strain that develops spontaneous, iodine-accelerated, pathogenic antibodies to the human thyrotrophin receptor [1]

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Abstract

Antibodies that stimulate the thyrotropin receptor (TSHR), the cause of Graves' hyperthyroidism, only develop in humans. TSHR antibodies 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.$H2^{h4}$ mice associated with thyroglobulin and thyroid-peroxidase, but *not* TSHR, antibodies. We hypothesized that transferring the human (h)TSHR A-subunit to NOD.$H2^{h4}$ mice would result in loss of tolerance to this protein. BALB/c hTSHR A-subunit mice were bred to NOD.$H2^{h4}$ mice and transgenic offspring were repeatedly backcrossed to NOD.$H2^{h4}$ mice. All offspring developed antibodies to thyroglobulin and thyroid-peroxidase. However, only TSHR-transgenic NOD.$H2^{h4}$ mice (TSHR/NOD.$H2^{h4}$) developed pathogenic TSHR antibodies as detected using clinical Graves' disease assays. As in humans, TSHR/NOD.$H2^{h4}$ females were more prone than males to developing pathogenic TSHR antibodies. Fortunately, in view of the confounding effect of excess thyroid hormone on immune responses, spontaneously arising pathogenic (h)TSHR antibodies cross-react poorly with the mouse TSHR and do not cause thyrotoxicosis. In summary, the TSHR/NOD.$H2^{h4}$ mouse strain develops spontaneous, iodine-accelerated, pathogenic TSHR antibodies in females, providing a unique model to investigate disease pathogenesis and test novel TSHR-antigen specific immunotherapies aimed at curing Graves' disease in humans.

INTRODUCTION

Graves' disease is the prototypic autoimmune disease in which the humoral arm of the immune system directly causes organ overactivity (reviewed in 1). The phenotypic expression of hyperthyroidism results from the stimulatory effect of a *single* type of autoantibody on a *single* autoantigen, the TSH receptor (TSHR). Graves' disease is one of the most common autoimmune diseases, affecting approximately 1% of the population in their lifetimes, with a very strong predilection towards females (female to male ratio of 3–7 to 1 in different countries)(2). There is no cure for the disease. Hyperthyroidism can be

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treated, either by inhibiting thyroid hormone synthesis with thionamide drugs or by radio-iodine or surgical thyroid ablation, all with the attendant risks of side-effects or, even more commonly, permanent hypothyroidism requiring life-long thyroid hormone ingestion.

Immune intervention to cure Graves' disease by inducing immune tolerance to the TSHR has been a long-standing goal, but 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) develop Graves' disease (3). For 40 years, immunization of different animal species with thyroid extracts, and later with recombinant TSHR protein together with adjuvant, did generate antibodies, 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 antibodies (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. In order to study potential immuno-therapeutic strategies, a suitable animal model requires TSAb to arise spontaneously and stably to self (syngeneic) antigen. In contrast, the majority of previous *induced* animal models have employed 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 (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 towards the goal of using immunotherapy to induce tolerance to the TSHR and, thereby, reverse the development of TSAb so as to cure, not treat, Graves' disease in humans.

METHODS AND MATERIALS

Generating NOD.*H-2*^{h4} mice expressing the human TSH receptor A-subunit

NOD.*H2*^{h4} 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.*H2*^{h4} mice to generate N1 Tgic-NOD.*H2*^{h4} x non-Tgic-NOD.*H2*^{h4} progeny. Expression of the transgene was determined by polymerase chain-reaction (13). Transgenic male N1 pups were bred to wild-type NOD.*H2*^{h4} 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.$H2^{h4}$ Y chromosome, wild-type NOD.$H2^{h4}$ males were crossed to female N4 Tgic-NOD.$H2^{h4}$ mice. Thereafter, we reverted to crossing Tgic-NOD.$H2^{h4}$ male offspring with wild-type NOD.$H2^{h4}$ 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.$H2^{h4}$ genes to breed the next generation. N7 mice were bred from two N6 males with 99.3 or 99.5 % NOD.$H2^{h4}$ genes (Supplementary Fig. S1).

Data are reported for parental strains and offspring from N1 to N8 backcrosses. Unless indicated otherwise (and excluding all breeding mice), from 8 weeks of age water was supplemented with 0.05% sodium iodide (NaI) for 16 weeks at which time (age 24 weeks) TSHR-Tgic and non-Tgic offspring (N1 to N8) as well as parental strains were euthanized to harvest blood and thyroid tissue. Where indicated, additional NOD.$H2^{h4}$ mice were maintained on regular or NaI water for up to 32 weeks. All mouse studies were performed in accordance with the guide-lines of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center. TSHR/NOD.$H2^{h4}$ mice are being cryopreserved by the Mutant Mouse Regional Resource Center under the designation NOD.Cg-Tg(TG-TSHR)51.9Smcl, MMRRC:037586).

TSHR antibody assays

TSHR antibodies were measured using three assays:-

a. *ELISA*: The assay for TSHR antibodies (IgG class) has been reported previously (7). Recombinant TSHR A-subunit protein secreted by Chinese Hamster Ovary cells (CHO) with an amplified transgenome (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 TSHR A-subunit adenovirus. Antibody binding was detected with horseradish peroxidase-conjugated mouse anti-IgG (A 3673, Sigma Chemical Co., St. Louis, MO) and the signal was developed with o-phenylenediamine and H_2O_2. Data are reported as the optical density (OD) at 490 nm.

b. *TSH binding inhibition (TBI) assay*: TBI levels were measured in 25μl mouse serum using a clinical assay kit (Kronus Inc, Star ID). The data are reported as the % inhibition of ^{125}I-TSH binding to the TSH holoreceptor.

c. *TSAb bioassay*: An in-house bioassay was used to measure cAMP generation by Chinese hamster ovary (CHO) cells expressing the human TSHR (7, 16). To permit testing double the volume of serum normally used in immunized mice (10μl versus 5μl), the assay was modified as follows: 25μl test mouse serum + 75μl normal human serum was precipitated with 300μl 20% polyethylene glycol 4000 (PEG; Sigma-Aldrich) in water and resuspended in 240μl Ham's F12 medium supplemented with 10 mM Hepes, pH 7.4, 1 mM isobutylmethylxanthine and 0.3% bovine serum albumin. Duplicate aliquots (110μl) were applied to human TSHR-expressing CHO cell monolayers in 96 well plates. After incubation (90 min, 37°C), the medium was aspirated, intracellular cAMP was extracted with ethanol,

evaporated to dryness and resuspended in 200μl Dulbecco's PBS. Aliquots (12μl) were assayed using the LANCE cAMP kit (PerkinElmer, Boston MA). TSAb activity was expressed as a percentage of cAMP values attained with PEG precipitated IgG from wild-type BALB/c mice. As a positive control, IgG from normal BALB/c mice was supplemented with human monoclonal TSHR M22 (17).

Autoantibodies to thyroglobulin and thyroid peroxidase (TgAb and TPOAb)

Thyroglobulin was isolated from murine thyroid glands as previously described (18). ELISA wells (Immulon 4HBX, Thermo Scientific, Rochester NY) were coated with mouse thyroglobulin (1.5μg/ml) and incubated with test sera (duplicate aliquots, 1:100 dilution). Antibody binding was detected with horse radish peroxidase-conjugated goat anti-mouse IgG (A3673, Sigma Chemical Co., St. Louis MO), the signal developed with o-phenylenediamine and the reaction stopped using 20% H_2SO_4. The negative control was serum from 8 week old NOD.$H2^{h4}$ mice on regular water; the positive control was serum from BALB/c mice immunized with mouse thyroglobulin and complete Freund's adjuvant (19). TgAb data are presented as the optical density (OD) at 490 nm.

TPOAb were measured using CHO cells stably expressing mouse-TPO (18). Sera (1:50 dilution) were incubated with mouse TPO-CHO cells and binding was detected with fluorescein isothyocyanate-conjugated affinity purified goat anti-mouse IgG (M30101, Invitrogen, Carlsbad, CA). Cells staining with propidium iodide (1μg/ml) were xcluded from analysis. The negative control for IgG class antibody binding to mouse TPO-CHO cells was serum from 8 week old NOD.$H2^{h4}$ mice. Positive controls were mouse monoclonal antibodies #15 and #64 to human TPO (20), provided to us by Dr. P. Carayon and Dr. J Ruf (Marseille, France), that recognize mouse TPO (18,20). Flow cytometry was performed (10,000 events) using a FACScan with CELLQUEST Software (Becton Dickinson, San Jose, CA). Data are reported as the geometric mean (Geo Mean).

Serum thyroxine (T4), TSH and thyroid histology

Serum total thyroxine (T4) was measured in 25μl mouse serum by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). T4 values were computed from kit standards and expressed as 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 hematoxylin and eosin (IDEXX BioResearch Lab Animal and Biological Materials Diagnostic Testing, Columbia, MO).

Statistics

Significant differences between responses in different groups were determined by Mann Whitney rank sum test or, when normally distributed, by Student's t test. Multiple comparisons were made using analysis of variance (ANOVA). Tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

RESULTS

The concept of developing a mouse that fulfills the criteria described above arose from a number of factors (Fig. 1). First, unlike the conventional NOD mouse that develops diabetes, the NOD.*H2*^{h4} strain develops spontaneous, iodine-accelerated, autoimmune thyroiditis in association with autoantibodies to thyroglobulin (Tg)(22–24) and, at a later stage, to thyroid peroxidase (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' antigen as evident by tolerance to human TSHR A-subunit immunization (13). This component of the TSH holoreceptor is the antigenic target of TSAb that cause Graves' disease (7,14,25). Regulatory T-cell depletion with anti-CD25 prior to TSHR A-subunit adenovirus immunization led to massive thyroiditis associated with antibody 'spreading' from the TSHR to the other two thyroid autoantigens, thyroglobulin (Tg) and thyroid peroxidase (TPO) (12). We, therefore, hypothesized that transferring the TSHR A-subunit transgene locus from the BALB/c transgenics to the NOD.*H2*^{h4} strain that spontaneously develops thyroid autoimmunity would lead to the spontaneous generation of pathogenic TSHR autoantibodies.

TSHR antibodies develop in TSHR transgenic (not wild-type) NOD.H2^{h4} offspring

TSHR antibodies detected by ELISA were present in some transgenic offspring derived by crossing hTSHR A-subunit expressing BALB/c mice to the NOD.*H2*^{h4} strain and repeated backcrossing to NOD.*H2*^{h4} mice (N1 to N8 generations)(Fig. 2A). Neither wild-type offspring lacking the TSHR transgene nor the parental NOD.*H2*^{h4} mice developed TSHR antibodies. In contrast, TgAb were detectable in some parental NOD.*H2*^{h4} mice and N1-N8 back-cross offspring regardless of whether or not they contained the TSHR A-subunit transgene (Fig. 2B). TPOAb also develop in some N1-N8 back-cross offspring independent of the A-subunit transgene (Supplemental Fig. S2).

Consistent with the standard protocol for NOD.*H2*^{h4} mice, from 8 weeks of age mice were provided with NaI supplemented water for 16 weeks. However, we maintained a smaller number of animals on regular water. As for the NaI group, only transgenic back-cross offspring, not the parent NOD.*H2*^{h4} strain or the non-transgenic offspring, developed TSHR antibodies detectable by ELISA (Supplemental Fig. S3). TSHR antibodies 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. S3 versus Fig. 2A). These data confirm previous observations for thyroglobulin autoantibodies that dietary iodide enhances (or accelerates) but is not responsible for the development of thyroid autoimmunity in NOD.H2^{h4} mice (18,23). In particular, TgAb are detectable much earlier than TPOAb in NOD.*H2*^{h4} mice maintained on NaI (18). Because NaI accelerates thyroid autoimmunity, back-cross mice after the N6 generation were maintained on NaI supplemented water.

High TSH binding inhibition (TBI) in male NOD.H2^{h4} mice associated with elevated TSH

Although measuring TSHR antibodies 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 antibodies. Pathogenic, functional TSHR receptor antibodies do not

recognize adherent, purified TSHR antigen 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.H2^{h4} mice *without* the A-subunit transgene were tested for TSH binding inhibitory (TBI) antibodies. Unexpectedly, high TBI values were detected in male, but not in female, mice (Fig. 3A). Higher 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 non-transgenic NOD.H2^{h4} males could be caused by endogenous TSH and not by TSHR autoantibodies, we measured the former in the sera of male and female wild-type NOD.H2^{h4} mice. Indeed, significantly higher TSH levels in male than in female mice (Fig. 3B) revealed the spurious nature of the TBI measurements in the males. A similar TSH-dependent high background was also observed in the bioassay for thyroid stimulatory antibodies (TSAb)(data not the shown).

The foregoing observations indicated the need to study male and female mice separately when testing sera for pathogenic TSHR autoantibodies. In addition, because the high TBI and TSAb 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 TSH binding inhibition (TBI) assay

Among the female mice, none of the parental NOD.*H2*^{h4} or non-transgenic back-cross offspring in the N1+2 to N7+8 generations had elevated TSH binding inhibitory (TBI) autoantibodies (Fig. 4A). On the other hand, TBI autoantibodies were detected in some female mice expressing the TSHR A-subunit transgene from the N1+2 to the N7+8 back-crosses (Fig. 4A). In these female mice, the proportion of TBI positivity rose from 2/13 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.*H2*^{h4} transgenics had TBI values above those observed in their non-transgenic littermates (Fig. 4B). Moreover, none of the male TSHR/NOD.*H2*^{h4} transgenics attained TBI values rising 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.*H2*^{h4} transgenic mice.

TSHR autoantibodies detected in the TSAb bioassay

Additional evidence for pathogenic TSHR Ab levels arising spontaneously in NOD.*H2*^{h4} mice transgenic for the human TSHR A-subunit came from functional bioassays for thyroid stimulating antibodies (TSAb). As mentioned above, because of the confounding influence

of high TSH levels even in non-transgenic male NOD.*H2*^{h4} mice, we tested sera from female TSHR/NOD.*H2*^{h4} transgenic mice for their ability to stimulate cAMP generation in monolayers of CHO cells expressing the TSH holoreceptor. TSAb was clearly elevated in some (4 of 9) female transgenic offspring in the N7+N8 generation, versus 0/13 in the non-transgenic offspring (Fig. 5). TSAb values correlated with TBI levels in the transgenic NOD.*H2*^{h4} N7+N8 offspring (r=0.865, p=0.003).

Serum T4 levels in NOD.H2^{h4} 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.*H2*^{h4} mice transgenic for the TSHR A-subunit in the N5-N8 generations had serum T4 levels above the normal range established in non-transgenic NOD.*H2*^{h4} 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-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- 20μg/dL) attained in BALB/c females with similar TSAb levels following TSHR A-subunit adenovirus immunization (for example 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.*H2*^{h4} mice 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 non-transgenic NOD.*H2*^{h4} males was higher than in females. On this basis, none of the NOD.*H2*^{h4} males with the TSHR A-subunit transgene had elevated serum T4 levels (Fig. 6B).

DISCUSSION

We report the development of a strain of mice which develop spontaneous, iodine-accelerated, pathogenic TSHR autoantibodies. This TSHR/NOD.*H2*^{h4} strain was generated by transferring the human TSHR A-subunit transgene locus from BALB/c mice (12,13) to NOD.*H2*^{h4} mice. In the former BALB/c strain, the human TSHR A-subunit is ‘self’ whereas NOD.*H2*^{h4} mice are genetically predisposed to spontaneous development of autoimmune thyroiditis in association with autoantibodies to the thyroid-specific antigens thyroglobulin and thyroid peroxidase (18,22–24). However, autoantibodies to the mouse TSHR do not arise spontaneously in NOD.*H2*^{h4} mice, which remain euthyroid despite moderate lymphocytic infiltration. The reason for immunological tolerance (at least at the humoral level) to the TSHR in NOD.*H2*^{h4} mice is unknown but may relate to the very low level of TSHR expression on thyrocytes. In contrast, thyroglobulin, 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 the present 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-antigen in a strain, namely NOD.$H2^{h4}$ 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 antibodies (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-expressor and the other a low-expressor (12,13). In these mice the human TSHR A-subunit is a ‘self’ antigen. 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 back-cross the A-subunit locus on to a NOD.$H2^{h4}$ background.

In NOD.$H2^{h4}$ mice expressing the human TSHR A-subunit transgene, autoantibodies to this antigen measured by ELISA arose spontaneously in both males and females. Such antibodies lack bioactivity (26). Only TSHR antibodies 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.$H2^{h4}$ 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 back-crosses, we focused on female TSHR/NOD.$H2^{h4}$ 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 towards pathogenic TSHR autoantibody generation in female TSHR/NOD.$H2^{h4}$ mice (Fig. 4). By the N7/N8 backcross, 40–50% of female TSHR/NOD.$H2^{h4}$ mice developed TBI and TSAb, a sufficient proportion for future investigations described below.

It should be noted that the variability in developing pathogenic TSHR antibodies 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.$H2^{h4}$ genes, implying less than 1% BALB/c genes; 2) Virtually all N7+N8 mice produced TgAb in comparable amounts to the parent NOD.$H2^{h4}$ strain. More likely, the variability is an inherited (albeit unexplained) characteristic of the NOD strain used to generate NOD.$H2^{h4}$ mice; NOD mice are well known to exhibit variability in diabetes and breeding (for example 34) and variable degrees of spontaneous thyroiditis develop in another NOD-derived strain, NOD.$H2^{k}$ (35).

An important, and initially puzzling, feature of the TSHR/NOD.$H2^{h4}$ mice was the minimal degree of hyperthyroidism despite the presence of TSAb activity in their sera. Serum T4 levels above the normal range were observed in three female TSHR/NOD.$H2^{h4}$ mice but elevated values were also occurred in some non-transgenic NOD.$H2^{h4}$ female littermates. Lymphocytic thyroiditis was not extensive and was similar in NOD.$H2^{h4}$ mice with and without the A-subunit transgene. Co-existing 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.$H2^{h4}$ mouse strain used in the present 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 TSHR autoantibody values (37). Therefore, TSAb to the human TSHR in NOD.$H2^{h4}$ mice, whether induced (37) or arising spontaneously with iodine-acceleration (as in the present 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 antibodies, respectively) have revealed linkage of this phenotype to the immunoglobulin heavy chain variable region gene locus (16). These genes are, in turn, linked to heavy chain constant region polymorphisms (or ‘allotypes’)(for example 38–40). It may be relevant that C57BL/6 and NOD.$H2^{h4}$ 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 antibodies and hyperthyroidism induced by immunization (Introduction), a transgenic mouse model of Graves’ disease has been reported with the H and L chain variable regions of a human IgM monoclonal antibody 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 antigen. Moreover, the IgM TSAb transgenic mouse model requires LPS administration to expand the transgenic B cell population. Other animal models have involved the intraperitoneal 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 antigen leading to TSHR autoantibody production by normal B-cells.

It may not be appreciated that the absence of overt hyperthyroidism in TSHR/NOD.$H2^{h4}$ mice is not detrimental but, instead, is an advantage for studies for several reasons. TSAb 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.$H2^{h4}$ 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 antigen-presenting dendritic cells by increasing the expression of co-stimulatory molecules required to initiate antibody production (46). Thyrotoxicosis also polarizes dendritic cells leading to impaired function of regulatory T-cells (Treg), 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.$H2^{h4}$ strain, that develops spontaneous, iodine-accelerated, TSHR Ab detectable by ELISA. High TSH levels interfere with assays for pathogenic antibodies in males. However, in female TSHR/NOD.$H2^{h4}$ mice, pathogenic antibodies 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 non-transgenic T- and B-cells, represents a significant advance for studies of Graves' pathogenesis and will facilitate investigating potential approaches for TSHR-antigen specific immunotherapy to cure, rather than treat, Graves' disease in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TBI	Inhibition of TSH binding to the TSHR
Tg	thyroglobulin
TPO	thyroid peroxidase
TSAb	thyroid stimulating antibody
TSH	thyrotropin
TSHR	thyrotropin receptor
hTSHR	human thyrotropin receptor

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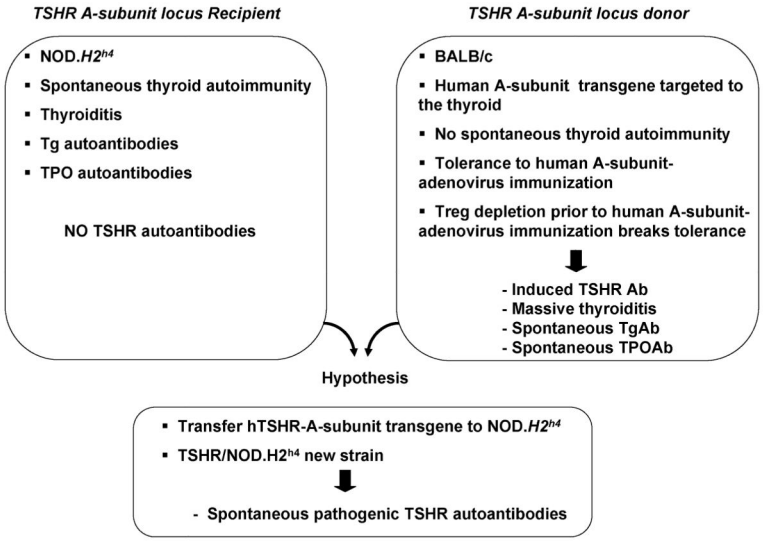

Figure 1.

Concepts underlying the hypothesis that expressing the human TSHR A-subunit transgene in NOD.*H2*^{h4} mice would generate a strain that produces spontaneous, iodine-accelerated, TSHR antibodies. Recipient NOD.*H2*^{h4} mice develop thyroiditis associated with antibodies to thyroglobulin (TgAb) and thyroid peroxidase (TPOAb) (18,22–24), but not to the TSHR. The donor BALB/c mice do not develop thyroid autoimmunity and the human TSHR A-subunit targeted to the thyroid gland is a ‘self’ antigen (13). However, regulatory T-cell depletion prior to human TSHR A-subunit adenovirus immunization breaks tolerance to endogenous mouse thyroid antigens Tg and TPO (12).

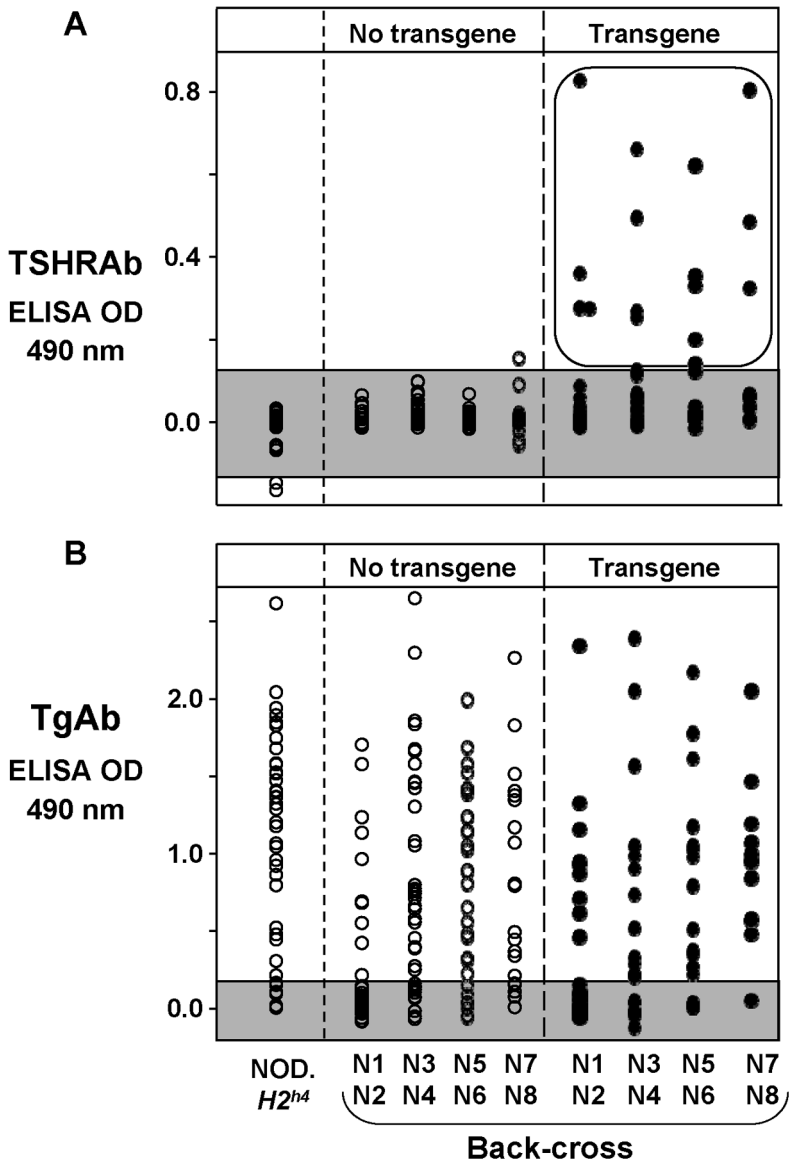

Figure 2.

TSHR Ab develop spontaneously (iodine enhanced) on backcrossing human TSHR A-subunit-BALB/c transgenic mice to NOD.*H2*^{h4} mice. The A-subunit transgene in the BALB/c mice is targeted to the thyroid and the present study utilized mice expressing the transgene at a low level in which tolerance to the 'self' human A-subunit can be broken using a moderate adenovirus dose (12,13). Backcross generations are labeled N1 to N8 with data grouped for N1+N2, N3+N4, N5+N6 and N7+N8. Values for TSHRAb and TgAb, measured by ELISA (OD 490 nm), are shown for individual mice (males and females) at 24 weeks of age (NaI-supplemented drinking water begun at 8 weeks).

Panel A: Only the offspring carrying the A-subunit transgene develop spontaneous TSHR antibodies. The shaded area represents the mean + 2 SD for antibody levels in NOD.*H2*^{h4} littermates not carrying the A-subunit transgene. Number of mice studied: NOD.*H2*^{h4}, 42;

Non-transgenic: N1+N2, 26; N3+N4, 33; N5+N6, 30; N7+N8, 21; Transgenics: N1+N2, 28; N3+N4, 22; N5+N6, 20; N7+N8, 12.

Panel B: Thyroglobulin antibodies (TgAb) develop in offspring regardless of whether or not they carried the A-subunit transgene. The shaded area represents the mean ± 2 SD for antibody levels in parental TSHR A-subunit BALB/c transgenic mice. Number of mice studied as for TSHRAb by ELISA except for: NOD.$H2^{h4}$, 47; Non-transgenic: N1+N2, 28; N3+N4, 37; Transgenics: N1+N2, 31.

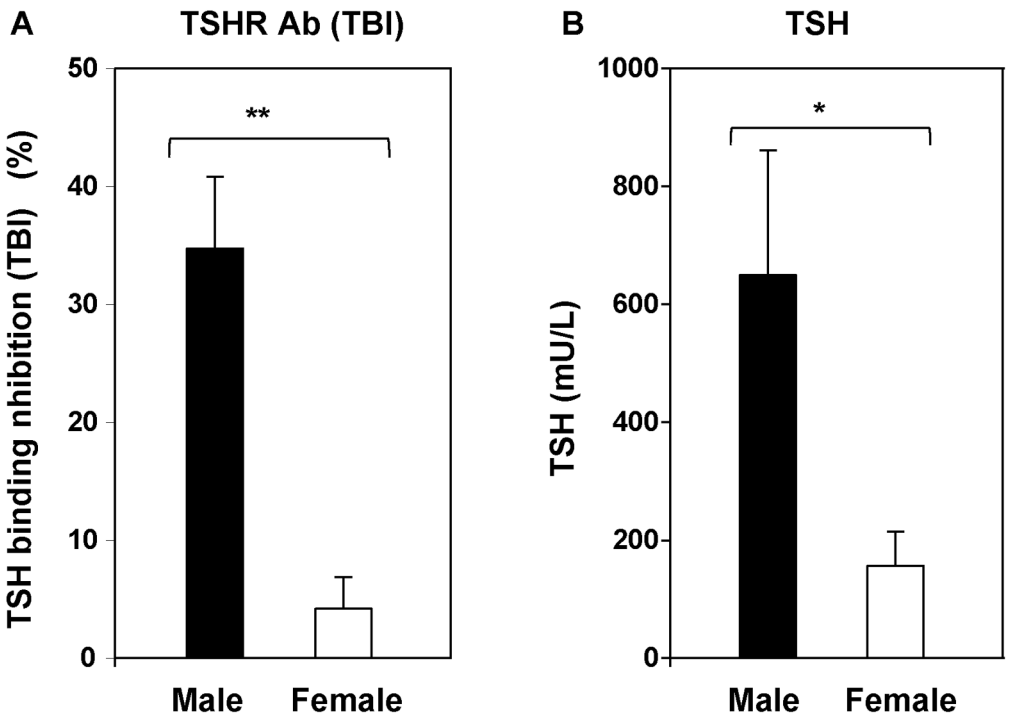

Figure 3.

Higher TSH levels in male than in female parental NOD.$H2^{h4}$ mice are a confounding influence on measuring TSHR antibodies in the TSHR binding inhibition (TBI) assay. Sera from male and female mice were assayed at 32 weeks of age for TBI (panel A) and TSH (panel 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 per group). Significance of differences: Panel A, ** $p<0.002$ (t test); Panel B, * $p=0.032$ (Rank Sum test).

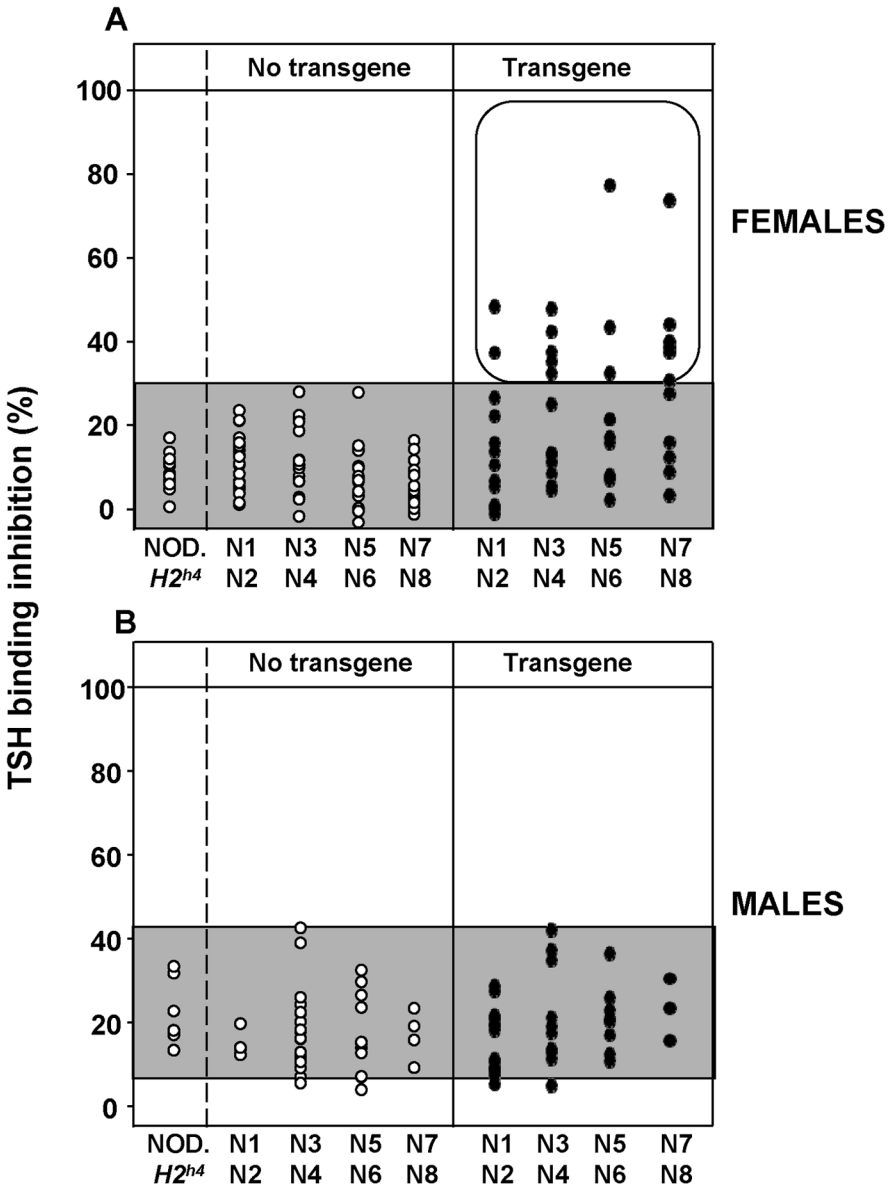

Figure 4.

Detection of TSHR antibodies by the TBI assay in female, not in male, backcross mice with the A-subunit transgene. Sera from 24 week old NOD.$H2^{h4}$ littermates with and without the human TSHR A-subunit transgene were tested in the TSH binding inhibition (TBI) assay. Values in female (panel A) and male (panel B) mice represent % binding inhibition of radiolabeled TSH to the TSH holoreceptor. NOD, parental NOD.$H2^{h4}$ mice; N1 to N8, generations of back-crossing to NOD.$H2^{h4}$. The shaded area represents the mean ± 2SD for all non-transgenic females (panel A) and males (panel B). Number of female mice studied: NOD.$H2^{h4}$, 13; Non-transgenic: 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.$H2^{h4}$, 7; Non-transgenic: 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.

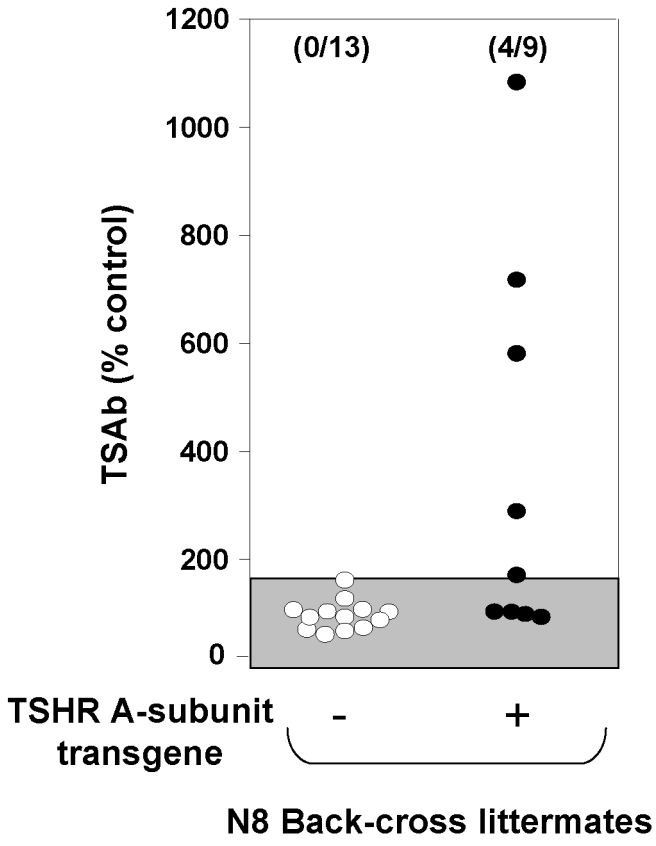

Figure 5.

Detection of TSHR antibodies by thyroid stimulating bioassay in female backcross mice expressing the A-subunit transgene. Sera from 24 week old NOD.$H2^{h4}$ littermates (N7 and N8 generations) with (+) and without (−) the human TSHR A-subunit transgene were tested for thyroid stimulating antibody (TSAb) activity (Methods). TSAb activity was expressed as a percentage of intracellular cAMP values attained with IgG from wild-type BALB/c mice. Data are shown for individual mice. The numbers in parentheses indicate the number of mice in each group. The shaded area represents the mean ± 2SD for values from littermates without the transgene.

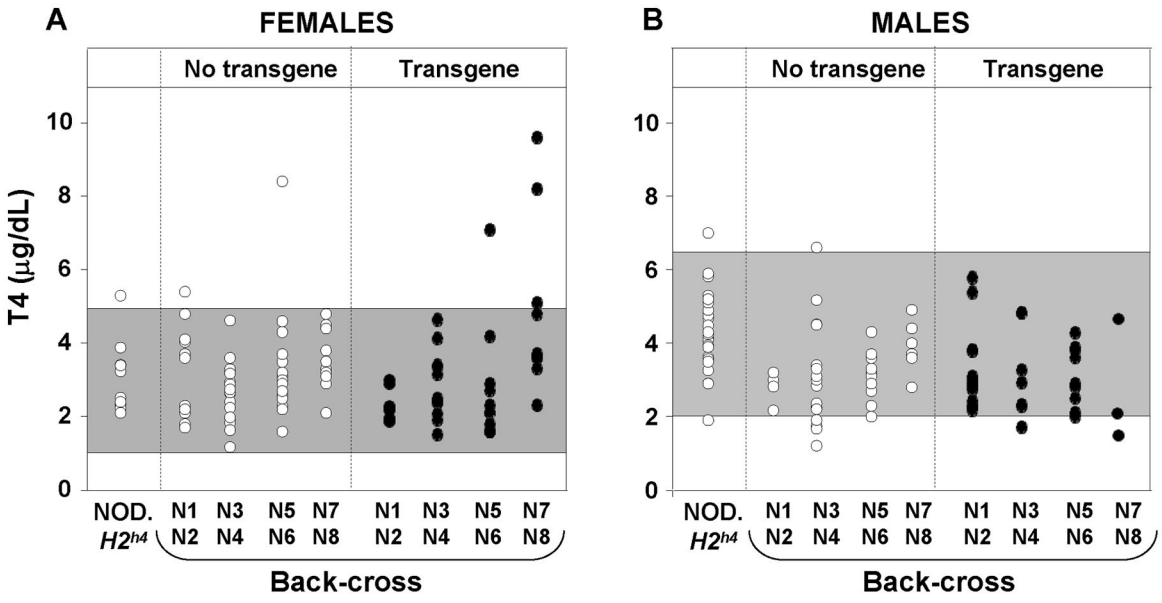

Figure 6.

Serum T4 in transgenic and non-transgenic NOD.$H2^{h4}$ female (panel A) and male (panel B) littermates. Mice at the indicated backcross generation (N1-N8) were tested at 24 weeks of age. T4 values (µg/dL) are shown for individual mice. The shaded panel represents the mean ± 2SD for all non-transgenic females (panel A) and all non-transgenic males (panel B). Number of female mice studied: NOD.$H2^{h4}$, 11; Non-transgenic: N1+N2, 15; N3+N4, 19; N5+N6, 17; N7+N8, 14; Transgenics: N1+N2, 6; N3+N4, 12; N5+N6, 11; N7+N8, 9. Number of male mice studied: NOD.$H2^{h4}$, 25; Non-transgenic: N1+N2, 5; N3+N4, 17; N5+N6, 12; N7+N8, 7; Transgenics: N1+N2, 14; N3+N4, 7; N5+N6, 9; N7+N8, 3.

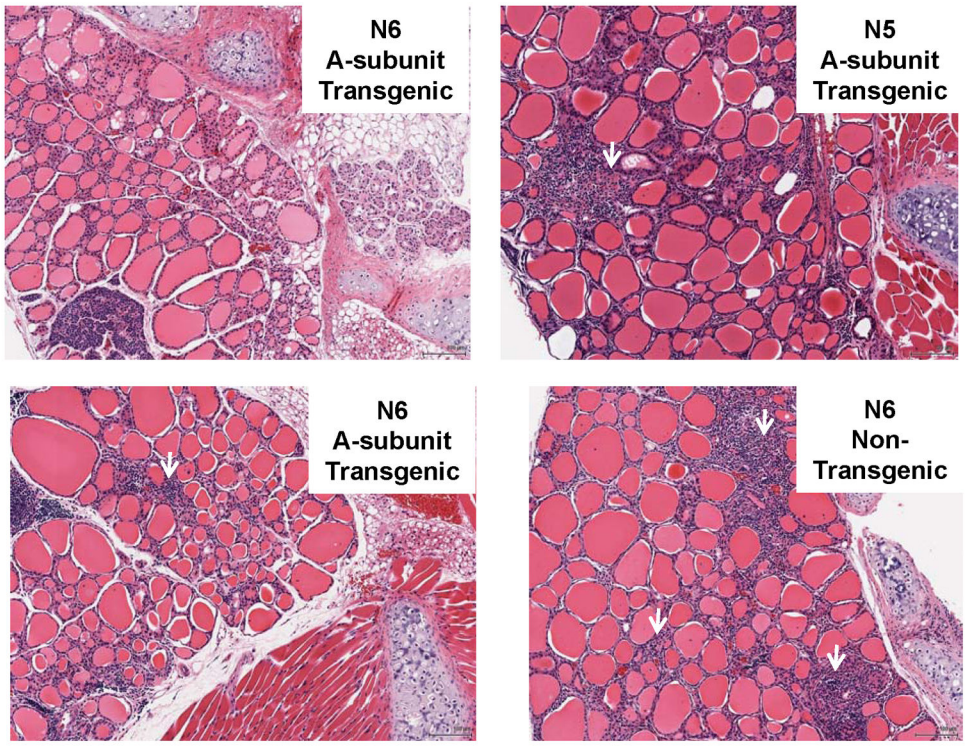

Figure 7.

Thyroiditis in transgenic and non-transgenic NOD.$H2^{h4}$ mice. Typical examples of lymphocytic infiltration, varying from minimal to moderate, are shown on thyroid histology (hematoxylin and eosin, 10x magnification) from N5 and N6 mice (3 transgenic and one non-transgenic). Arrows (white) indicate lymphocytic infiltrates.

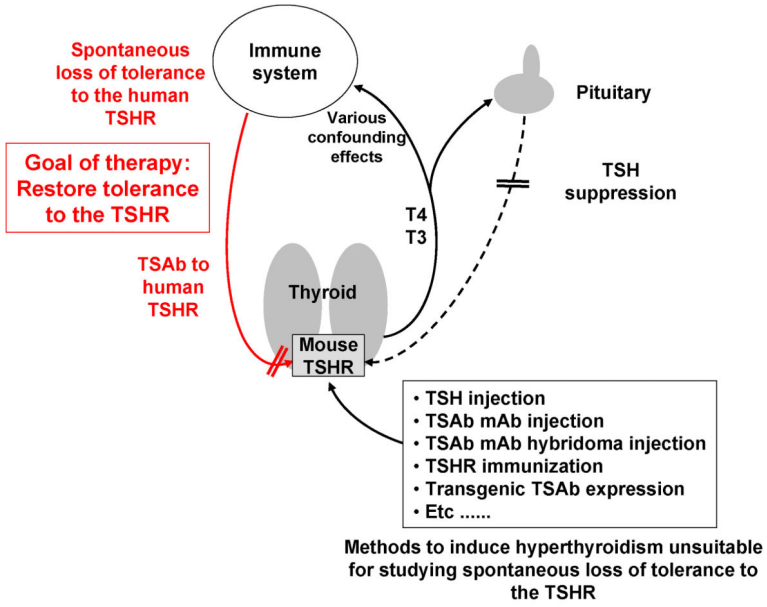

Figure 8.

The goal of immunotherapy using TSHR/NOD.$H2^{h4}$ mice is the restoration of tolerance to the TSHR. Methods used to induce hyperthyroidism in mice, unsuitable for inducing self-tolerance to the TSHR, include: TSH injection (for example 48), TSAb mAb injection (45), hamster TSAb mAb hybridoma injection (44), TSHR immunization approaches to express TSHR in mice (5–9), hamsters (10) and rhesus monkeys (11), and expressing TSAb (B6B7) in a transgenic mouse (42,43). Confounding effects of hyperthyroidism on the immune system include altering the phenotype and function of antigen-presenting dendritic cells (46) and polarizing dendritic cells leading to impaired function of regulatory T-cells (Treg), a major change that may influence the emergence of pathogenic autoantibodies (47).