Title

Nanoparticles Bearing TSH Receptor Protein and a Tolerogenic Molecule Do Not Induce Immune Tolerance but Exacerbate Thyroid Autoimmunity in hTSHR/NOD.H2h4 Mice.

Journal

The Journal of Immunology, 202(9)

ISSN

0022-1767

Authors

McLachlan, Sandra M
Aliesky, Holly A
Rapoport, Basil

Publication Date

2019-05-01

DOI

10.4049/jimmunol.1900038

Peer reviewed

HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2020 May 01.

Published in final edited form as:

J Immunol. 2019 May 01; 202(9): 2570–2577. doi:10.4049/jimmunol.1900038.

Nanoparticles bearing TSH receptor protein and a tolerogenic molecule do not induce immune tolerance but exacerbate thyroid autoimmunity in hTSHR/NOD.*H2*^{h4} mice^

Sandra M McLachlan[*,+], **Holly A Aliesky**[*], and **Basil Rapoport**[*,+]

[*]Cedars-Sinai Medical Center, University of California, Los Angeles, CA

[+]UCLA School of Medicine, University of California, Los Angeles, CA

Abstract

Transgenic NOD.*H2*^{h4} mice that express the human (h)TSHR A-subunit in the thyroid gland spontaneously develop pathogenic TSHR autoantibodies resembling those in patients with Graves' disease. Nanoparticles coupled to recombinant human TSHR A-subunit protein and a tolerogenic molecule (ITE; ligand for the endogenous aryl-hydrocarbon receptor) were injected intra-peritoneally four times at weekly intervals into hTSHR/NOD.*H2*^{h4} mice with the goal of blocking TSHR antibody development. Unexpectedly, in transgenic mice, injecting TSHR A-subunit-ITE nanoparticles (not ITE-nanoparticles or buffer) accelerated and enhanced the development of pathogenic TSHR antibodies measured by inhibition of TSH binding to the TSHR. Non-pathogenic TSHR antibodies (ELISA) were enhanced in transgenics and induced in wild-type littermates. Serendipitously, these findings have important implications for disease pathogenesis: development of Graves' TSHR antibodies is limited by the availability of A-subunit protein which is shed from membrane bound TSHR, expressed at low levels in the thyroid. The enhanced TSHR antibody response following injected TSHR A-subunit protein-nanoparticles is reminiscent of the transient increase in pathogenic TSHR antibodies following the release of thyroid autoantigens after radio-iodine therapy in Graves' patients. However, in the hTSHR/ NOD.*H2*^{h4} model, enhancement is specific for TSHR antibodies, with antibodies to thyroglobulin and thyroid peroxidase remaining unchanged. In conclusion, despite the inclusion of a tolerogenic molecule, injected nanoparticles coated with TSHR A-subunit protein enhanced and accelerated development of pathogenic TSHR antibodies in hTSHR/NOD. NOD.*H2*^{h4}. These findings emphasize the need for sufficient TSHR A-subunit protein to activate the immune system and the generation of stimulatory TSHR antibodies in genetically predisposed individuals.

INTRODUCTION

The goal of autoantigen specific immunotherapy has been approached in several ways in diseases for which one or more autoantigens have been identified. A variety of approaches are being employed to specifically target autoimmunity including appropriate variety of

^The study was supported by the following grants: NIH DK 54684 (SMM) and DK19289 (BR).

Correspondence: Sandra M McLachlan, 468 21st Place, Santa Monica, CA 90402; Phone number: 310-899-0408; sandra.mclachlan@ucla.edu.

adjuvants, altered peptide ligands, and multi-peptides [reviewed in (1)]. Moreover, an epitope-specific vaccine has been developed that modulates T cell responses and suppresses progression of arthritis in two murine models of rheumatoid arthritis (2). Many years ago, Kong and colleagues demonstrated that raising the circulating level of thyroglobulin (Tg), either by injecting thyroid stimulating hormone (TSH) or Tg, maintained peripheral tolerance and suppressed the induction of experimental thyroiditis [reviewed in (3)]. Similarly, Braley-Mullen and colleagues suppressed the development of thyroiditis (but not antibodies) in guinea pigs by injecting splenocytes coupled to Tg before immunization with thyroglobulin (4).

Recently, a novel approach was used to treat type I diabetes that arises spontaneously in NOD mice: injecting nanoparticles coupled to the insulin beta chain (a major autoantigen) together with a tolerogenic small molecule (5). The tolerogenic small molecule (ITE), the ligand for the endogenous aryl-hydrocarbon receptor, has also been used together with myelin antigen to suppress induced experimental encephalomyelitis (6).

Unlike autoimmune conditions for which multiple autoantigens have been identified, the unequivocal autoimmune target in Graves' disease is the thyrotropin receptor (TSHR) with hyperthyroidism being directly caused by stimulating autoantibodies to the TSHR (TSAb) [reviewed in (7)]. Moreover, the TSHR A-subunit, cleaved and shed from the membrane bound receptor, is the autoantigen responsible for the initiation and/or enhancement of immune responses leading to production of stimulating TSHR antibodies (8, 9). To attempt immunotherapy in Graves' disease, unlike for insulin and myelin basic protein, the extremely low level of TSHR expression in thyroid tissue precludes purification of sufficient antigen for this purpose. However, recombinant TSHR A-subunit protein generated in eukaryotic (not prokaryotic) cells is recognized by human TSAb (10) and such protein can be (and has been) used to test the efficacy of immune-specific therapy.

We have developed a unique strain of NOD.$H2^{h4}$ mice that express the human (h) TSHR A-subunit in the thyroid and the thymus and consequently, unlike their wild-type littermates, spontaneously develop pathogenic stimulating TSHR antibodies, as in Graves' patients (11). Like their non-transgenic counterparts, hTSHR/NOD.$H2^{h4}$ mice also spontaneously develop autoantibodies to the other two major thyroid autoantigens, thyroglobulin (Tg) (12–14) and thyroid peroxidase(TPO)(15). These transgenic mice provide the tools to test the outcome of injecting nanoparticles coated with hTSHR A-subunit protein plus ITE to specifically block the development of pathogenic TSHR antibodies. Unlike with insulin and myelin basic protein, this approach exacerbated, rather than ameliorated the autoimmune response. This finding provides insight into a limiting factor critical for the spontaneous development of TSAb.

METHODS

Reagents:

Recombinant human (*h*)TSHR A-subunit protein (amino acid residues 22-289; residues 1-21 being the signal peptide) secreted by Chinese Hamster Ovary cells (CHO) with an amplified transgenome (16) was purified from culture supernatants by affinity chromatography (17)

and dialyzed against 10 mM Tris, pH 7.4, 50 mM NaCl. Two different conformational forms of this recombinant hTSHR protein can be purified separately from the culture medium based on their reciprocal recognition by Graves' patients' autoantibodies and mouse mAb 3BD10, namely "active" hTSHR A-subunits by the former and "inactive" hTSHR A-subunits by the latter (10). To preclude the possibility of passive TSHR antibody neutralization, inactive hTSHR-289 (hereafter referred to as 'A-subunit protein') was coupled to nanoparticles. For ELISA, mouse Tg (mTg) was isolated from murine thyroid glands as previously described (15).

The following reagents were purchased:- a) Citrate stabilized uncoupled gold particles, 60 nm in diameter (Ted Pella Inc, Redding CA; catalog #15709-20); b) ITE, endogenous aryl hydrocarbon receptor agonist ($C_{14}H_{10}N_2O_3S$; Tocris Bioscience, Bristol, UK); c) Methoxy-Poly (Ethylene-glycol)-Thiol, Average MW 5,000 (mPEG-SH-5000; Laysan Bio Inc, Arab, Alabama).

Nanoparticles coupled to ITE alone or ITE + hTSHR A subunit protein.

Nanoparticles (np) coupled to thyroid autoantigens were prepared using citrate stabilized uncoupled gold particles (see above) as described by Yeste et al. (5, 6), slightly modified as described below. Two types of np were prepared: np-ITE-TSHR (human TSHR A-subunit) and, as a control, np-ITE alone. TSHR protein (1 mg/ml in phosphate buffered saline, pH 7.4) was pre-mixed with ITE (1 mg/ml in water). Dissolving 10 mg of ITE required initial dissolving in ~0.2 ml DMSO followed by the addition of water and prolonged warming and vortexing. The TSHR-ITE mixture was added drop by drop while stirring to the gold np (1:6 vol/vol; typically 0.4 ml of TSHR-ITE or ITE alone with 2.4 ml of np) and incubated for 30 min at room temperature with stirring continued. After the addition of freshly dissolved mPEG-SH-5000 (45 μg/ml; typically 10 μl for a final concentration of 0.16 μg/ml), the incubation was continued for another 30 min at room temperature while stirring. The nanoparticles were recovered by centrifugation, the pellets washed with water and resuspended in 1.1 ml water and stored at 4 C for the first injection (50 μl of np + 150 μl of phosphate buffered saline) the following day. After the second injection one week later the sample was discarded and a fresh preparation made for the third and fourth injections.

It should be noted that we attempted unsuccessfully to couple purified mouse Tg to np-ITE using the protocols reported by Yeste et al (5, 6) together with a number of in-house variations that we thought could be effective. However, unlike for purified TSHR A-subunit protein, perhaps because of the very large size of Tg (~330 KDa dimer) and physical characteristics, addition of this protein to the nanoparticles formed an oily smear adherent to the polypropylene tube with very poor nanoparticle recovery.

Mice studied:

NOD.$H2^{h4}$ mice (originally from The Jackson Laboratory, Bar Harbor, ME) and transgenic TSHR/NOD.$H2^{h4}$ mice (11), that express low levels of the human TSHR A-subunit in the thyroid and thymus, were bred at Cedars-Sinai Medical Center. [Sperm from hTSHR/NOD.$H2^{h4}$ have been frozen by MMRRC; Strain Name**:** NOD.Cg-Tg(TG-TSHR)51.9Smcl/Mmmh**;** Stock Number: 037586]. The two types of nanoparticles, 200 μl of np-ITE-TSHR

or np-ITE, and phosphate buffered saline as an additional control, were injected intraperitoneally (ip) four times at weekly intervals starting in 7-week old mice, following the approach used by Quintana and colleagues for NOD mice. (5). (Fig. 1A). From the age of 8 weeks, mice were provided with drinking water containing sodium iodide (NaI, 0.05%). Blood was drawn from the tail vein after 8 weeks on iodide (mice aged 16 weeks). Mice were euthanized to obtain blood and thyroid tissue after 16 weeks on iodide (mice aged 24 weeks).

A total of 65 mice was studied, 29 TSHR/NOD.$H2^{h4}$ (10 males, 19 females) and 36 NOD.$H2^{h4}$ mice (15 males and 21 females). More females than males were investigated because high levels of TSH that develop in males preclude measuring pathogenic TSHR antibodies in males (11). All mouse studies were approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center and conducted in accordance with mandated standards of humane animal care.

Autoantibodies to Tg and TPO (TgAb and TPOAb):

ELISA wells (Immulon 4HBX, Thermo Scientific, Rochester NY) were coated with mouse Tg (1.5 μg/ml) and incubated with test sera (1:100 dilution) in duplicate. 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 (Sigma Chemical Co.) and the reaction stopped with 20% (v/v) 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 Tg and complete Freund's adjuvant (18). TgAb values are presented as the optical density (OD) at 490 nm.

TPOAb were measured using Chinese hamster ovary (CHO) cells stably expressing murine TPO (15). Sera (1:50 dilution) were incubated with mouse TPO-CHO cells and binding was detected with fluorescein isothiocyanate-conjugated affinity purified goat anti-mouse IgG (M30101, Invitrogen, Carlsbad, CA. The negative control was serum from 8-week old NOD.$H2^{h4}$ mice on regular water; the positive control was a previously characterized TPOAb positive serum from NOD.$H2^{h4}$ mice on iodide for 16 weeks. Flow cytometry was performed (10,000 events) using a BD FACScanto II with FACSDiva Software (Becton Dickinson, San Jose, CA) for collection and FloJo v.X.07 software (FloJo, Ashland, OR) for analysis. Data are reported as the geometric mean (Geo Mean).

TSHR antibody assays:

TSHR antibodies were measured in two ways:-

a) Non-pathogenic TSHR antibodies (ELISA): As reported previously, ELISA wells were coated with A-subunit protein ('inactive' form, described above; 5 μg/ml) and incubated with test sera (1:100 dilution). The positive control was serum from BALB/c mice immunized with TSHR A-subunit adenovirus (for example (9) and the monoclonal antibody (mAb) 3BD10 (17) . Antibody binding was detected with horseradish peroxidase-conjugated mouse anti-IgG (A3673, 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) Pathogenic TSHR antibodies(TSH binding inhibition assay): The levels of antibodies that inhibit the binding of TSH to the TSHR (TSH binding inhibition, TBI) were measured as previously described [for example (11)] in 25μl mouse serum using a clinical assay (Kronus Inc, Star ID). The data are reported as the % inhibition of ^{125}I-TSH binding to the TSH holoreceptor.

Serum T4 and thyroid histology:

T4 levels were measured (10 μl aliquots) by ELISA (Mouse/Rat Thyroxine (T4) ELISA, Calbiotech, El Cajon, CA); data are reported as μg T4/dL. 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 were determined by the 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

Enhancement of non-pathogenic TSHR antibodies by np-ITE-TSHR

The schedule for injecting mice with np-ITE-TSHR, np-ITE or buffer alone is shown in Fig. 1A. The outcome of injecting transgenic hTSHR/NOD.$H2^{h4}$ and wild-type (wt) NOD.$H2^{h4}$ mice with np-ITE-TSHR was unexpected considering the previous tolerogenic property of ITE when used together with insulin (5) or myelin basic protein (6). Instead, we observed enhancement rather than a reduction in TSHRAb levels. In particular, non-pathogenic TSHRAb levels (measured by ELISA) were markedly enhanced in transgenic hTSHR/NOD.$H2^{h4}$ and wild-type NOD.$H2^{h4}$ injected with np-ITE-TSHR compared with the levels in mice that received buffer or np-ITE (Fig. 1B). Incidentally, the mean levels of non-pathogenic TSHRAb in mice that received buffer or np-ITE were lower than those in untreated mice in our previous study (11), reflecting variability in this spontaneous model of TSHRAb generation. In the same mice, the levels of TgAb (Fig 1C) and TPOAb (Supplemental Fig. S1) that develop spontaneously in wild type NOD.$H2^{h4}$ mice were unchanged following np-ITE-TSHR injection. These thyroid autoantibody measurements were made at euthanasia in 24-week old mice (Fig. 1A), the time at which TSHRAb are usually measured (11). Because of relatively small numbers, data for male and female mice are not shown separately for TPOAb despite our recent findings that such an approach is preferred (19). TgAb were detectable at lower levels at the earlier time point (16-week old mice)(Supplemental Fig. S2), consistent with other studies (12, 13, 15).

Because we observed such high levels of non-pathogenic TSHRAb by ELISA in 24-week old mice injected with np-ITE-TSHR, we also assayed sera obtained from the same mice at 16 weeks of age. Surprisingly, the levels were higher at the younger age (Fig. 2A). Previously, following subcutaneous injection of the same TSHR A-subunit protein in soluble form, rather than coupled to nanoparticles, we observed enhanced non-pathogenic TSHRAb measured by ELISA in 25-week old transgenic and wild-type NOD.$H2^{h4}$ (20). In view of our present findings, we tested stored sera drawn from these mice. In contrast to the np-ITE-TSHR injected mice, levels of these antibodies were lower at 17 than at 25 weeks, particularly in the wild-type NOD.$H2^{h4}$ mice (Fig. 2B).

Accelerated development of pathogenic TSHRAb by np-ITE-TSHR

Pathogenic TSHRAb, measured by TSH binding inhibition (TBI), can only be studied in female mice because high serum TSH levels in males give spurious TBI values (11). Injecting np-ITE-TSHR antigen enhanced TBI levels in hTSHR/NOD.$H2^{h4}$ mice but (as previously reported (11, 20) TBI activity did not exceed the cut-off point for positivity in wild-type NOD.$H2^{h4}$ littermates (Fig. 3A). TBI activity was detected, as expected, in transgenic mice aged 24 weeks and also in some np-ITE-TSHR injected mice aged 16 weeks. The importance of this finding is emphasized by our data for transgenic hTSHR/NOD.$H2^{h4}$ and wild-type NOD.$H2^{h4}$ mice injected subcutaneously with TSHR protein in which TBI activity was below the assay cut-off point at 17 weeks and was only positive in transgenic mice aged 25 weeks (Fig 3B). Similar observations were made for stored sera drawn from uninjected TSHR/NOD.$H2^{h4}$ mice on variable selenium diets, namely TBI activity was only positive in 24-week but not in 16-week old mice. (Supplemental Fig. S3).

Comparative summary of differing protocols for delivering TSHR protein

The foregoing two protocols for injecting TSHR A-subunit protein differ in the route of delivery as well as the number and timing of injections. Four weekly intra-peritoneal injections of np-ITE-TSHR into transgenic hTSHR/NOD.$H2^{h4}$ and wild-type NOD.$H2^{h4}$ mice induced an early response followed by a decline in non-pathogenic TSHRAb measured by ELISA (Fig. 2A). In contrast, following subcutaneous injection of soluble TSHR A-subunit protein 3 times every 4 weeks, TSHRAb levels rose progressively and more slowly (Fig 2B). Pathogenic TSHR antibodies measured in the TBI assay were detectable earlier in the transgenic hTSHR/NOD.$H2^{h4}$ mice injected with np-ITE-TSHR (16 weeks) (Fig. 3A) than with soluble TSHR (25 weeks) (Fig. 3B).

Serum T4 in mice injected with np-ITE-TSHR

T4 levels were similar in males and females and in transgenic and wild-type NOD.$H2^{h4}$ mice (Fig. 4A versus B). In particular, T4 levels were not elevated in female transgenics with positive TBI values, even in the group with increased TBI levels following injection of np-ITE-TSHR (Fig. 4A, compare left and right panels). As previously suggested, TSHRAb specific for the human TSHR A-subunit (expressed by the transgene), fail to stimulate the endogenous mouse TSHR (11). In our experience, NOD.$H2^{h4}$, whether wild-type or transgenic for the hTSHR A-subunit, always develop thyroiditis, with a variable range of thyroid lymphocytic infiltration [typically 10-35% of thyroid volume (11)]. In the present

study, thyroid histology was similar in female transgenic mice injected with np-ITE-TSHR or np-ITE (Supplementary Fig. S4).

DISCUSSION

Our goal in using nanoparticles bearing TSHR A-subunit protein together with a tolerogenic molecule (ITE) was to block or ameliorate the spontaneous development of pathogenic TSHR antibodies in hTSHR/NOD.$H2^{h4}$ mice, a potential approach for treating Graves' disease in humans. Despite the presence of pathogenic TSHRAb, these mice do not develop hyperthyroidism. However, the absence of thyrotoxicosis is an advantage, not a disadvantage of the model. The 'holy grail' of immunotherapy in Graves' disease is to blunt or abolish the spontaneous development of pathogenic, stimulating TSHR antibodies, the direct cause of the disease. The potential effect of excess thyroid hormones on the immune system would be an unwanted, confounding factor, as discussed previously (11).

The thyroiditis-susceptible transgenic hTSHR/NOD.$H2^{h4}$ strain expresses low levels of *human* TSHR A-subunit mRNA in the thymus and the thyroid gland (21) and the mice spontaneously develop TSHRAb, both pathogenic and non-pathogenic (11). As anticipated, the wild type NOD.$H2^{h4}$ mice do not express the human TSHR and TSHRAb do not develop spontaneously in these animals (summarized in Table 1). Higher human TSHR A-subunit mRNA levels were detected in a different line of NOD.$H2^{h4}$ mice to expressing a higher level of the transgene (21); however, these high-expressor NOD. $H2^{h4}$ mice developed only low levels of non-pathogenic TSHRAb and no pathogenic TSHRAb. The amount of human A-subunit protein was very small, ~3 ug/thyroid even in mice with the high expressor transgene, three hundred times less than that of Tg, the most abundant component of the thyroid, and was undetectable in serum (22). Overall, these data indicate that spontaneous development of TSHRAb requires TSHR A-subunit protein expressed by the transgene, but too high a level of TSHR mRNA expression in the thymus protects against development of pathogenic TSHRAb [as discussed previously (21)].

Valuable information has been obtained from mouse models of TSHRAb induced by TSHR DNA vaccination or TSHR-adenovirus [for example (23–25)]. Two approaches have been recently been used in the TSHR A-subunit adenovirus model to modulate the clinical effects of TSHRAb: first, injecting cyclic peptides that mimic one of the cylindrical loops of the TSHR reduced the effects of hyperthyroidism without altering the levels of TSHRAb (26, 27); second, injecting HLA-DR3 mice with TSHR-specific "apitopes" (Antigen Processing Independent epitopes reduced non-pathogenic TSHRAb levels (28).

Despite these potentially promising approaches, we used the spontaneous model of TSHRAb generation rather than an induced model for the following reason: our comparison of antigen-specific TSHRAb modulation using TSHR A-subunit adenovirus versus spontaneous TSHRAb development highlighted the potential problems of induced models (29). For this reason, we followed a protocol previously reported to inhibit the spontaneous development of IDDM type 1 in NOD mice, namely injecting nanoparticles coupled to proinsulin together with ITE (5). The NOD.$H2^{h4}$ strain is closely related to NOD, differing predominantly in a segment on Chromosome 17 including MHC region genes from the

B10.A4R strain (30, 31). Surprisingly, unlike the antigen-specific suppression of type 1 diabetes in NOD mice, the outcome of injecting nanoparticles coupled to TSHR A-subunit protein and ITE in hTSHR/NOD.$H2^{h4}$ mice was completely different. This antigen-specific approach induced non-pathogenic TSHR antibodies in wild-type, non-transgenic NOD.$H2^{h4}$ mice and accelerated spontaneous development of non-pathogenic and pathogenic TSHR antibodies in transgenic hTSHR/NOD.$H2^{h4}$ mice.

Two questions arise from these observations: First, why did this treatment induce or enhance, rather than suppress, development of TSHR antibodies? Second, what are the implications of our observations of accelerated and enhanced pathogenic TSHRAb development?

Considering first the inability to suppress TSHR antibody development using np-ITE-TSHR, one issue is the route used for injection. Following the approach described by Quintana et al (5) to suppress IDDM type 1 in NOD mice, we injected nanoparticles coated with TSHR-A-subunit + ITE **intraperitoneally**. The intra-peritoneal approach was used in order to follow the identical protocol reported by Quintana and colleagues (5). However, **intravenous** injection of antigen-coated autologous lymphocytes specifically inhibited a variety of immune responses (for example (32, 33). Consequently, intraperitoneal rather than intravenous injection may have been a major factor preventing antigen specific nanoparticles from blocking development of TSHR antibodies.

In addition to the injection route, a second (possibly more important) issue is the use of “naked” antigen-coated nanoparticles. As emphasized by Miller and colleagues (34), injecting un-encapsulated antigen-coated nanoparticles can have adverse outcomes such as anaphylaxis in pre-sensitized mice. Indeed, the successful use of encapsulated antigen-specific nanoparticles emphasizes the importance of initially concealing the specific autoantigen prior to uptake of the particles by antigen presenting cells (35).

As mentioned above, rather than inducing tolerance, injecting hTSHR A-subunit protein coupled nanoparticles in transgenic hTSHR/NOD.$H2^{h4}$ mice **accelerated** the development of non-pathogenic and pathogenic TSHRAb (summarized in Table 1). Without this manipulation, TSHR antibodies are only detectable in these mice at 24 weeks of age, the same time point as TPOAb (36), later than TgAb which are usually detected at age 16 weeks after iodide exposure for 8 weeks [for example (12, 13, 15, 19)]. In the present study, following np-TSH-ITE injection, both pathogenic and non-pathogenic TSHRAb are detectable in transgenic hTSHR/NOD.$H2^{h4}$ mice earlier, at the same time as TgAb. Development of antibodies to other thyroid autoantigens, Tg and TPO, was not accelerated in these mice whether injected with TSHR antigen in soluble form or on nanoparticles. The two approaches, A-subunit bound to nanoparticles versus soluble A-subunit protein, differ in the number of injections and the intervals between injections. However, nanoparticle bound TSHR A-subunit protein appears to be extremely effective at enhancing the development of TSHR antibodies (pathogenic and no-pathogenic).

Turning to the second major question, serendipitously, our findings have important implications for the pathogenesis of Graves’ disease. Of the three thyroid autoantigens, Tg,

TPO and the TSHR, very limited amounts of TSHR are expressed in the thyroid (for example (37) and correspondingly very small amounts of shed A-subunit are available to stimulate the immune system (reviewed in (38). The inability of hTSHR/NOD.$H2^{h4}$ mice to develop TSHR antibodies to the *endogenous* mouse TSHR is likely due, at least in part, to insufficient shed *mouse* TSHR A subunit. The requirement for additional TSHR antigen is shown by transgenic intrathyroidal expression of small amounts of human TSHR A-subunit that permitted transgenic mice to spontaneously develop pathogenic and non-pathogenic TSHR antibodies (11). On the other hand, the presence of a high expressor TSHR A-subunit transgene in NOD.$H2^{h4}$ mice prevented development of pathogenic TSHR antibodies (21). We have suggested that the "goldilocks" principle operates in the development of Graves' disease – not too much TSHR A-subunit (which promotes intra-thymic self-tolerance) and not too little, namely insufficient TSHR A-subunit protein in wild-type NOD.$H2^{h4}$ mice to stimulate antibody development (21).

It is possible that our unexpected results (antibody exacerbation rather than tolerance) could relate to physical differences between the human TSHR A-subunit protein expressed by the transgene and the purified protein. This possibility seems unlikely because the protein expressed by the TSHR A-subunit transgene is coded for by the identical cDNA used for eukaryotic cell generated protein for coupling to nanoparticles or subcutaneous injection. However, we cannot exclude the possibility that our findings relate to variable processing of human TSHR A-subunit protein into peptides when provided to the immune system as purified protein or on the surface of nanoparticles.

Injecting mice with TSHR A-subunit protein, either soluble (20) or coated on nanoparticles (present study) overcomes the lack of sufficient A-subunit protein to stimulate the immune system, inducing non-pathogenic TSHR antibodies in wild-type NOD.$H2^{h4}$ mice and enhancing pathogenic TSHR antibodies in hTSHR/NOD.$H2^{h4}$ mice. Importantly, both approaches in the hTSHR/NOD.$H2^{h4}$ mice used in the present study result in greater availability of human TSHR A-subunit protein without increased central tolerance as occurs in the same strain with much higher intrathymic human A-subunit expression (Table 1).

In NOD.$H2^{h4}$ and BALB/c mice not expressing the human TSHR A-subunit, xenogeneic human TSHR A-subunit is immunogenic in that subcutaneous injection of the protein alone (without adjuvant) induces non-pathogenic (but not pathogenic) TSHR antibodies (20). However, when provided to hTSHR/NOD.NOD.$H2^{h4}$ mice, TSHR A-subunit protein enhanced the levels of pathogenic TSHR antibodies. As mentioned above, the 'inactive' (though native) form of TSHR A-subunits is not recognized by pathogenic TSHR antibodies (10, 39). The ability of this material to stimulate B cells specific for pathogenic antibodies reflects the role of "original antigenic sin" (as discussed previously (40). The enhancement by A-subunit protein coated nanoparticles in the present study is reminiscent of the transient increase in TSHR antibodies observed in Graves' patients after ^{131} iodine therapy [for example (41, 42) which follows the release of thyroid autoantigens as exhibited by thyroglobulin (43). However, in our mouse model, TgAb and TPOAb levels are unchanged, and enhancement is specific for TSHR antibodies.

In conclusion, in transgenic hTSHR/NOD.$H2^{h4}$ mice, injecting nanoparticles coated with a tolerogenic molecule together with human TSHR A-subunit protein (a 'self' antigen) failed to induce tolerance but, instead, accelerated the development of pathogenic TSHRAb. Similarly, injecting these mice with soluble human TSHR A-subunit protein (without adjuvant) also enhanced the development of pathogenic TSAb. These observations suggest that the amount of free TSHR A-subunit generated by the transgene in the thyroid is limiting in its ability to induce or enhance the development of pathogenic autoantibodies to the TSHR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations:

hTSHR	*human* thyrotropin receptor
ITE	ligand for the endogenous aryl-hydrocarbon receptor
hTSHR	*human* thyrotropin receptor
hTSHR/NOD.$H2^{h4}$	NOD.$H2^{h4}$ mice expressing the human TSHR A-subunit in the thyroid
TBI	Inhibition of TSH binding to the TSHR
Tg	thyroglobulin
TPO	thyroid peroxidase

REFERENCES

1. Shakya AK, and Nandakumar KS. 2018 Antigen-Specific Tolerization and Targeted Delivery as Therapeutic Strategies for Autoimmune Diseases. Trends Biotechnol 36: 686–699. [PubMed: 29588069]
2. Mikecz K, Glant TT, Markovics A, Rosenthal KS, Kurko J, Carambula RE, Cress S, Steiner HLR, and Zimmerman DH. 2017 An epitope-specific DerG-PG70 LEAPS vaccine modulates T cell responses and suppresses arthritis progression in two related murine models of rheumatoid arthritis. Vaccine 35: 4048–4056. [PubMed: 28583308]
3. Kong YM, Brown NK, Morris GP, and Flynn JC. 2015 The Essential Role of Circulating Thyroglobulin in Maintaining Dominance of Natural Regulatory T Cell Function to Prevent Autoimmune Thyroiditis. Horm. Metab Res 47: 711–720. [PubMed: 26158397]
4. Braley-Mullen H, Tompson JG, Sharp GC, and Kyriakos M. 1980 Suppression of experimental autoimmune thyroiditis in guinea pigs by pretreatment with thyroglobulin-coupled spleen cells. Cell Immunol 51: 408–413. [PubMed: 6154539]
5. Yeste A, Takenaka MC, Mascanfroni ID, Nadeau M, Kenison JE, Patel B, Tukpah AM, Babon JA, DeNicola M, Kent SC, Pozo D, and Quintana FJ. 2016 Tolerogenic nanoparticles inhibit T cell-mediated autoimmunity through SOCS2. Sci. Signal 9: ra61. [PubMed: 27330188]
6. Yeste A, Nadeau M, Burns EJ, Weiner HL, and Quintana FJ. 2012 Nanoparticle-mediated codelivery of myelin antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 109: 11270–11275. [PubMed: 22745170]

7. Rapoport B, and McLachlan SM. 2016 TSH receptor cleavage into subunits and shedding of the A-470 subunit; a molecular and clinical perspective. Endocr. Rev 37: 114–134. [PubMed: 26799472]
8. Chazenbalk GD, Pichurin P, Chen CR, Latrofa F, Johnstone AP, McLachlan SM, and Rapoport B. 2002 Thyroid-stimulating autoantibodies in Graves disease preferentially recognize the free A subunit, not the thyrotropin holoreceptor. J. Clin. Invest 110: 209–217. [PubMed: 12122113]
9. Chen C-R, Pichurin P, Nagayama Y, Latrofa F, Rapoport B, and McLachlan SM. 2003 The thyrotropin receptor autoantigen in Graves' disease is the culprit as well as the victim. Journal of Clinical Investigation 111: 1897–1904. [PubMed: 12813025]
10. Chazenbalk G, McLachlan S, Pichurin P, and Rapoport B. 2001 A prion-like shift between two conformational forms of a recombinant thyrotropin receptor A-subunit module: purification and stabilization using chemical chaperones of the form reactive with Graves' autoantibodies. Journal of Clinical Endocrinology and Metabolism 86: 1287–1293. [PubMed: 11238522]
11. Rapoport B, Aliesky HA, Banuelos B, Chen CR, and McLachlan SM. 2015 A unique mouse strain that develops spontaneous, iodine-accelerated, pathogenic antibodies to the human thyrotrophin receptor. J Immunol 194: 4154–4161. [PubMed: 25825442]
12. Rasooly L, Burek CL, and Rose NR. 1996 Iodine-induced autoimmune thyroiditis in NOD-H2h4 mice. Clin. Immunol. Immunopathol 81: 287–292. [PubMed: 8938107]
13. Braley-Mullen H, Sharp GC, Medling B, and Tang H. 1999 Spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. J. Autoimmun 12: 157–165. [PubMed: 10222025]
14. Hutchings PR, Verma S, Phillips JM, Harach SZ, Howlett S, and Cooke A. 1999 Both CD4(+) T cells and CD8(+) T cells are required for iodine accelerated thyroiditis in NOD mice. Cell Immunol 192: 113–121. [PubMed: 10087179]
15. Chen CR, Hamidi S, Braley-Mullen H, Nagayama Y, Bresee C, Aliesky HA, Rapoport B, and McLachlan SM. 2010 Antibodies to thyroid peroxidase arise spontaneously with age in NOD.H-2h4 mice and appear after thyroglobulin antibodies. Endocrinology 151: 4583–4593. [PubMed: 20573721]
16. Chazenbalk GD, Jaume JC, McLachlan SM, and Rapoport B. 1997 Engineering the human thyrotropin receptor ectodomain from a non-secreted form to a secreted, highly immunoreactive glycoprotein that neutralizes autoantibodies in Graves' patients' sera. J. Biol. Chem 272: 18959–18965. [PubMed: 9228077]
17. Chazenbalk GD, Wang Y, Guo J, Hutchison JS, Segal D, Jaume JC, McLachlan SM, and Rapoport B. 1999 A mouse monoclonal antibody to a thyrotropin receptor ectodomain variant provides insight into the exquisite antigenic conformational requirement, epitopes and in vivo concentration of human autoantibodies. J. Clin. Endocrinol. Metab 84: 702–710. [PubMed: 10022441]
18. McLachlan SM, Aliesky HA, Chen CR, Chong G, and Rapoport B. 2012 Breaking tolerance in transgenic mice expressing the human TSH receptor A-subunit: thyroiditis, epitope spreading and adjuvant as a 'double edged sword'. PLoS. One 7: e43517. [PubMed: 22970131]
19. McLachlan SM AH a. R. B. 2019 To reflect human autoimmune thyroiditis, thyroid peroxidase (not thyroglobulin) antibodies should be measured in female (not sex independent) NOD.H2h4 mice Clinical and Experimental Immunology.
20. Rapoport B, Banuelos B, Aliesky HA, Hartwig Trier N, and McLachlan SM. 2016 Critical differences between induced and spontaneous mouse models of Graves' disease with implications for antigen-specific immunotherapy in humans. J Immunol 197: 4560–4568. [PubMed: 27913646]
21. McLachlan SM, Aliesky HA, Banuelos B, Lesage S, Collin R, and Rapoport B. 2017 High-level intrathymic thyrotrophin receptor expression in thyroiditis-prone mice protects against the spontaneous generation of pathogenic thyrotrophin receptor autoantibodies. Clin. Exp Immunol 188: 243–253. [PubMed: 28099999]
22. McLachlan SM, Nagayama Y, Pichurin PN, Mizutori Y, Chen CR, Misharin A, Aliesky HA, and Rapoport B. 2007 The link between Graves' disease and Hashimoto's thyroiditis: A role for regulatory T cells. Endocrinology 148: 5724–5733. [PubMed: 17823263]
23. Costagliola S, Rodien P, Many M-C, Ludgate M, and Vassart G. 1998 Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. J. Immunol 160: 1458–1465. [PubMed: 9570567]

24. Nagayama Y, Kita-Furuyama M, Ando T, Nakao K, Mizuguchi H, Hayakawa T, Eguchi K, and Niwa M. 2002 A novel murine model of Graves' hyperthyroidism with intramuscular injection of adenovirus expressing the thyrotropin receptor. J. Immunol 168: 2789–2794. [PubMed: 11884447]
25. Gilbert JA, Gianoukakis AG, Salehi S, Moorhead J, Rao PV, Khan MZ, McGregor AM, Smith T, and Banga JP. 2006 Monoclonal pathogenic antibodies to the thyroid-stimulating hormone receptor in Graves' disease with potent thyroid-stimulating activity but differential blocking activity activate multiple signaling pathways. J. Immunol 176: 5084–5092. [PubMed: 16585606]
26. Holthoff HP, Li Z, Fassbender J, Reimann A, Adler K, Munch G, and Ungerer M. 2017 Cyclic Peptides for Effective Treatment in a Long-Term Model of Graves Disease and Orbitopathy in Female Mice. Endocrinology 158: 2376–2390. [PubMed: 28368444]
27. Fassbender J, Holthoff HP, Li Z, and Ungerer M. 2019 Therapeutic effects of short cyclic and combined epitope peptides in a long-term model of Graves disease and orbitopathy. Thyroid.
28. Jansson L, Vrolix K, Jahraus A, Martin KF, and Wraith DC. 2018 Immunotherapy With Apitopes Blocks the Immune Response to TSH Receptor in HLA-DR Transgenic Mice. Endocrinology 159: 3446–3457. [PubMed: 30099489]
29. 2016 In this issue: A Grave Warning from a Mouse Model of Spontaneous Autoimmunity. J Immunol 197: 4507–4508.
30. Podolin PL, Pressey A, DeLarato NH, Fischer PA, Peterson LB, and Wicker LS. 1993 I-E+ nonobese diabetic mice develop insulitis and diabetes. J. Exp. Med 178: 793–803. [PubMed: 8350054]
31. McLachlan SM, Lesage S, Collin R, Banuelos B, Aliesky HA, and Rapoport B. 2017 Genes outside the Major Histocompatibility Complex Locus are linked to the development of thyroid autoantibodies and thyroiditis in NOD.H2h4 mice. Endocrinology 158: 702–713. [PubMed: 28323998]
32. Miller SD, Wetzig RP, and Claman HN. 1979 The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells. J Exp Med 149: 758–773. [PubMed: 85683]
33. Luo X, Pothoven KL, McCarthy D, DeGutes M, Martin A, Getts DR, Xia G, He J, Zhang X, Kaufman DB, and Miller SD. 2008 ECDI-fixed allogeneic splenocytes induce donor-specific tolerance for long-term survival of islet transplants via two distinct mechanisms. Proc. Natl. Acad. Sci. U. S. A 105: 14527–14532. [PubMed: 18796615]
34. Smarr CB, Yap WT, Neef TP, Pearson RM, Hunter ZN, Ifergan I, Getts DR, Bryce PJ, Shea LD, and Miller SD. 2016 Biodegradable antigen-associated PLG nanoparticles tolerize Th2-mediated allergic airway inflammation pre- and postsensitization. Proc Natl Acad Sci U S A 113: 5059–5064. [PubMed: 27091976]
35. McCarthy DP, Yap JW, Harp CT, Song WK, Chen J, Pearson RM, Miller SD, and Shea LD. 2017 An antigen-encapsulating nanoparticle platform for TH1/17 immune tolerance therapy. Nanomedicine 13: 191–200.
36. McLachlan SM, Aliesky H, Banuelos B, Que Hee SS, and Rapoport B. 2017 Variable effects of dietary selenium in mice that spontaneously develop a spectrum of thyroid autoantibodies. Endocrinology 158: 3754–3764. [PubMed: 28938453]
37. Misharin AV, Rapoport B, and McLachlan SM. 2009 Thyroid antigens, not central tolerance, control responses to immunization in BALB/c versus C57BL/6 mice. Thyroid 19: 503–509. [PubMed: 19348579]
38. McLachlan SM, and Rapoport B. 2014 Breaking tolerance to thyroid antigens: changing concepts in thyroid autoimmunity. Endocr. Rev 35: 59–105. [PubMed: 24091783]
39. Miller-Gallacher J, Sanders P, Young S, Sullivan A, Baker S, Reddington SC, Clue M, Kabelis K, Clark J, Wilmot J, Thomas D, Chlebowska M, Cole F, Pearson E, Roberts E, Holly M, Evans M, Nunez Miguel R, Powell M, Sanders J, Furmaniak J, and Rees Smith B. 2019 Crystal Structure of a Ligand-free Stable TSH Receptor Leucine-Rich Repeat Domain. J Mol Endocrinol.
40. McLachlan SM, and Rapoport B. 2018 Thyroid autoantibodies display both "original antigenic sin" and epitope spreading. Front Immunol 8.

41. McGregor AM, Petersen MM, Capiferri R, Evered DC, Smith BR, and Hall R. 1979 Effects of radioiodine on thyrotrophin binding inhibiting immunoglobulins in Graves' disease. Clin. Endocrinol. (Oxf) 11: 437–444. [PubMed: 583262]

42. Laurberg P, Wallin G, Tallstedt L, Abraham-Nordling M, Lundell G, and Torring O. 2008 TSH-receptor autoimmunity in Graves' disease after therapy with anti-thyroid drugs, surgery, or radioiodine: a 5-year prospective randomized study. Eur. J Endocrinol 158: 69–75. [PubMed: 18166819]

43. Feldt-Rasmussen U, Bech K, Date J, Petersen PH, and Johansen K. 1982 A prospective study of the differential changes in serum thyroglobulin and its autoantibodies during propylthiouracil or radioiodine therapy of patients with Graves' disease. Acta Endocrinol (Copenh) 99: 379–385. [PubMed: 6896112]

44. McLachlan SM, Aliesky HA, and Rapoport B. 2017 Aberrant iodide autoregulation induces hypothyroidism in a mouse strain in the absence of thyroid autoimmunity. J Endocrine Society 29: 63–76.

Manuscript findings

- TSHR A-subunit NOD.$H2^{h4}$ mice spontaneously develop pathogenic TSHR antibodies
- TSHR+ITE+A-subunit nanoparticles accelerated pathogenic TSHR antibody development
- TSHR A-subunit stimulation is required to generate pathogenic TSHR antibodies

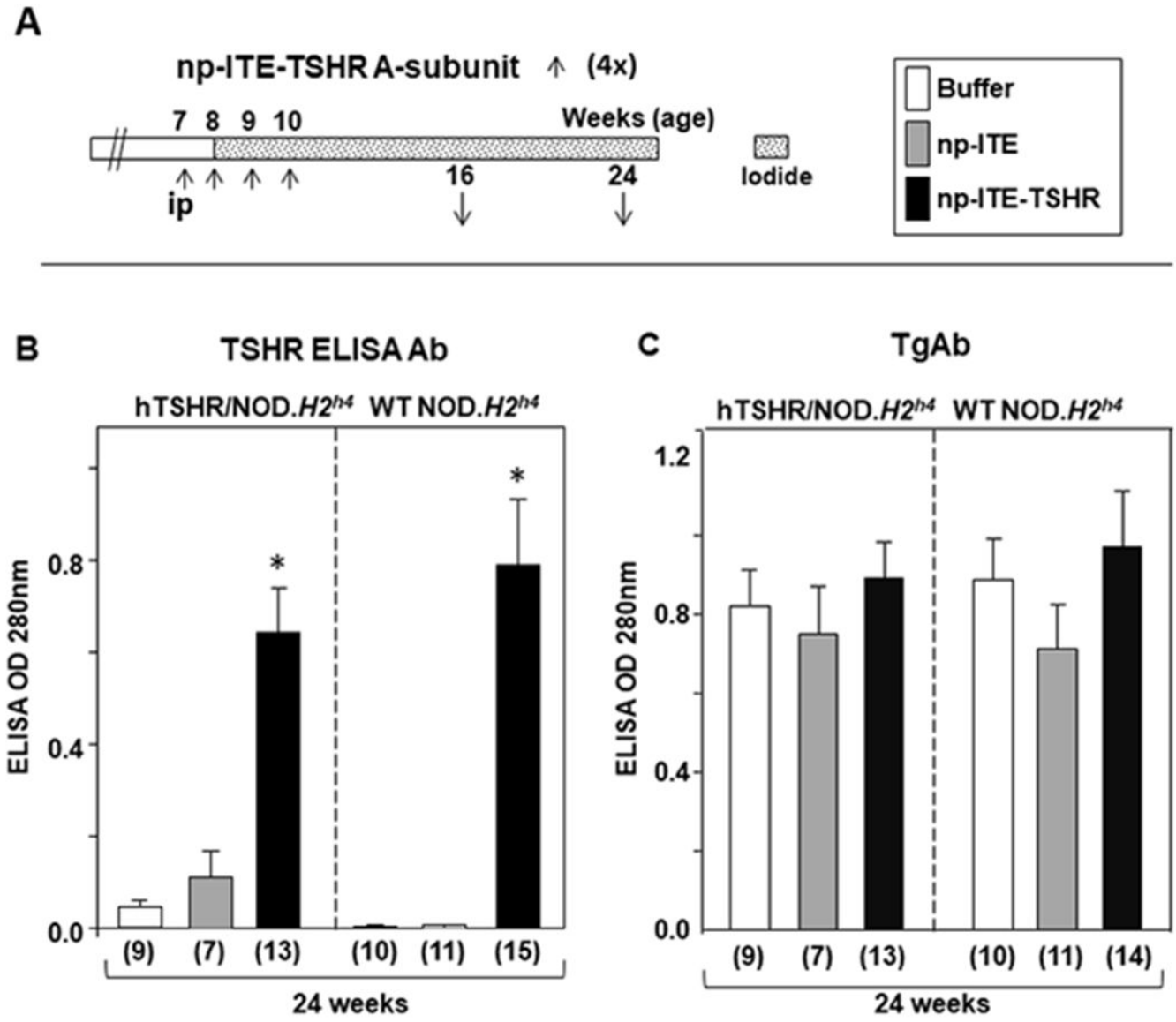

Figure 1.

Panel A, Protocol for intra-peritoneal (ip) injection of TSHR transgenic (Tgic) and wild-type (WT) NOD.$H2^{h4}$ mice with buffer, nanoparticles (np) coated with ITE, or np coated with both ITE and hTSHR A-subunit protein. In this and subsequent figures, the upward arrows indicate the times of injection and the downward arrows the time blood samples taken. As mentioned (Methods), 29 TSHR Tgic (10 males, 19 females) and 36 WT mice (15 males and 21 females) were studied (total 65 mice). More females than males were investigated because high levels of TSH in males on iodized water give spurious positives in TBI assays (see below) which precludes measuring pathogenic TSHR antibodies in this sex (11).

Panel B, Non-pathogenic (ELISA) TSHR antibody levels (Mean + SE) are enhanced in transgenic hTSHR/NOD.$H2^{h4}$ and wild-type (WT) NOD.$H2^{h4}$ mice injected ip with np coated with ITE-TSHR A- subunit compared with mice injected with buffer or np coated

with ITE only. Non-pathogenic TSHR antibodies develop similarly in males and females; therefore, data for males and females are combined. The numbers of mice are indicated in parentheses. * significantly higher in transgenics injected with np-ITE-TSHR than with buffer or np-ITE ($p<0.05$, ANOVA).

Panel C, TgAb levels for the mice studied in Panel B are not significantly different in hTSHR/NOD.$H2^{h4}$ or in WT mice injected with buffer, np-ITE, or np-ITE-TSHR A subunit. TgAb were measured by ELISA and data reported as the OD 490 nm (mean +SE). TgAb levels do not differ between males and females on iodide (12–14, 19) and therefore data are shown for males and females combined. The numbers of mice are indicated in parentheses.

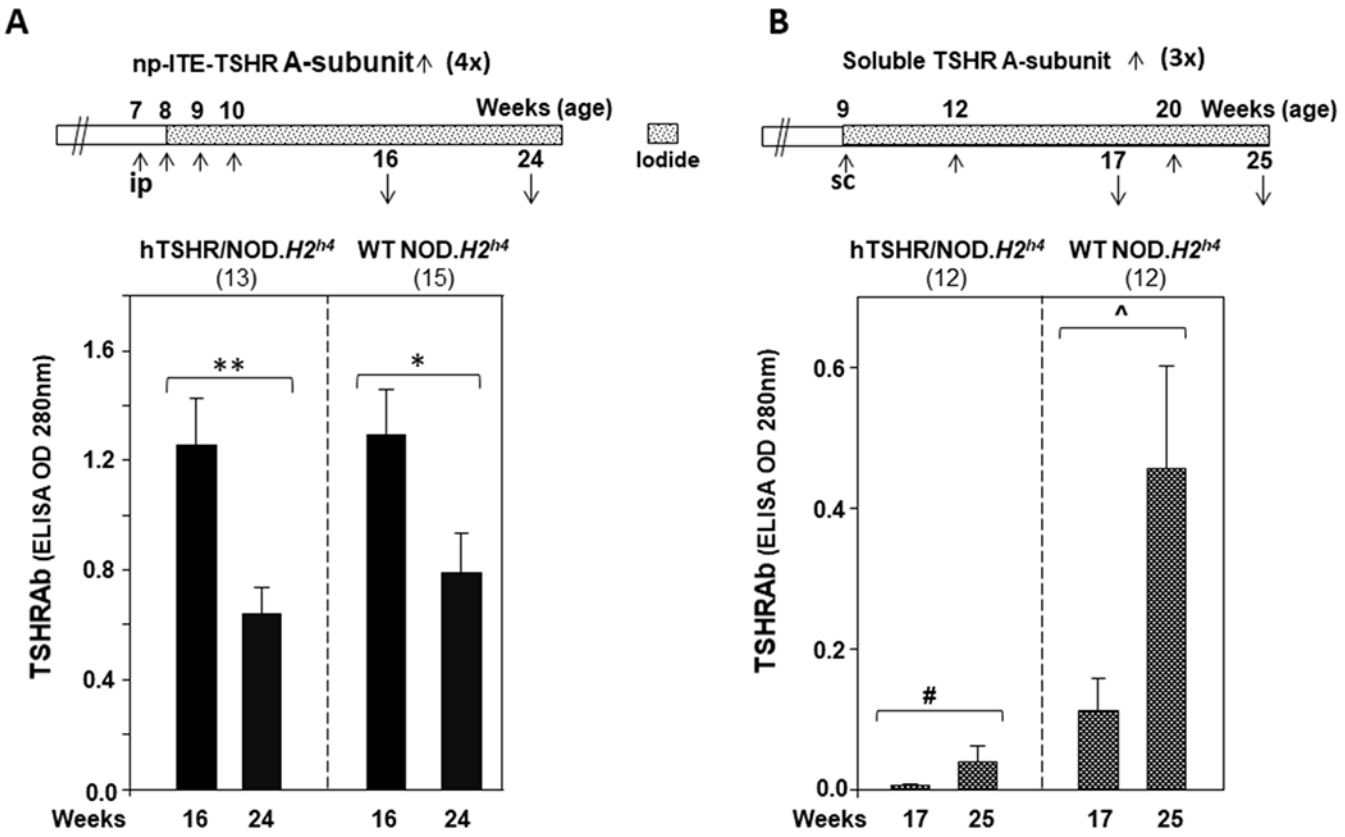

Figure 2.

Non-pathogenic TSHRAb (ELISA) develop at different times in hTSHR/NOD.$H2^{h4}$ and wild-type (WT) NOD.$H2^{h4}$ mice injected ip with np-TSHR A-subunit or soluble TSHR A-subunit protein injected subcutaneously (sc). Data are shown for males and females combined and the numbers of mice are indicated in parentheses.

Panel A: Accelerated development of TSHRAb after ip injections of np-TSHR A- subunits. TSHRAb levels were higher in both hTSHR/NOD.$H2^{h4}$ and WT NOD.$H2^{h4}$ mice at 16 weeks versus 24 weeks of age; * p=0.01 (t test); ** p=005 (t test).

Panel B: Slower development of TSHRAb in mice injected subcutaneously with TSHR A-subunit protein at 17 than at 25 weeks of age. Data for 25 weeks (16 weeks on iodide) replotted from reference (20). Previously untested sera obtained from the same mice at 17 weeks and stored at −80 C were assayed for the present study using the same standards; # p=0.004 (rank sum test); ^ p=0.024 (rank sum test).

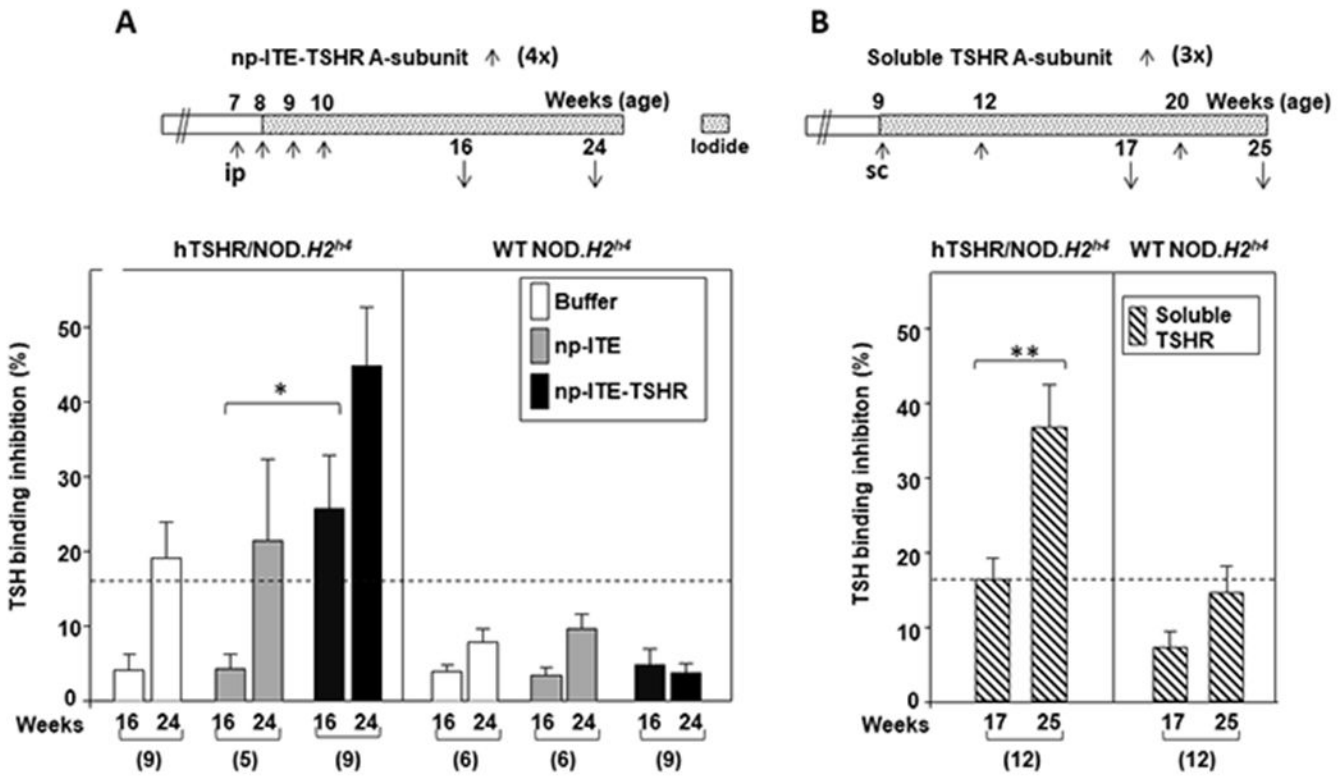

Figure 3.

Accelerated development of pathogenic TSHR antibodies measured by TSH binding inhibition (TBI) in **female** hTSHR/*H2*^{h4} transgenic (not WT) mice. As mentioned, TBI was not measured in males because they develop high TSH levels (11, 44) which give spurious positives in the assay. The numbers of mice are indicated in parentheses.

Panel A: TBI activity exceeded background values (horizontal dashed line) in 16 week old hTSHR/NOD.*H2*^{h4} females injected with np+ ITE+TSHR A-subunit protein and the levels increased in 24 week old mice. In contrast (and as expected), TBI activity was only positive in 24 week old hTSHR/NOD.*H2*^{h4} mice injected with buffer or np + ITE. In WT NOD.*H2*^{h4} females aged 16 and 24 weeks, TBI activity was below baseline levels regardless of injection with buffer, np + ITE or np +ITE+TSHR. Baseline TBI: mean +2SD for WT NOD. *H2*^{h4} aged 14 weeks after injection with saline and on iodide water (20). TBI higher at 16 weeks of age in transgenics injected with np +ITE+TSHR A-subunit versus np +ITE: * p= 0.05 (ANOVA).

Panel B: TBI values are only detectable in 25 week old (not at 17 weeks of age) female hTSHR/NOD.*H2*^{h4} mice injected subcutaneously with TSHR A-subunit protein. ** p=0.004 (rank sum test). As in panel A, TBI values in WT females lack TBI activity at both time points. Data for 125 weeks plotted from (20) and measured in stored 17 week sera for the present study.

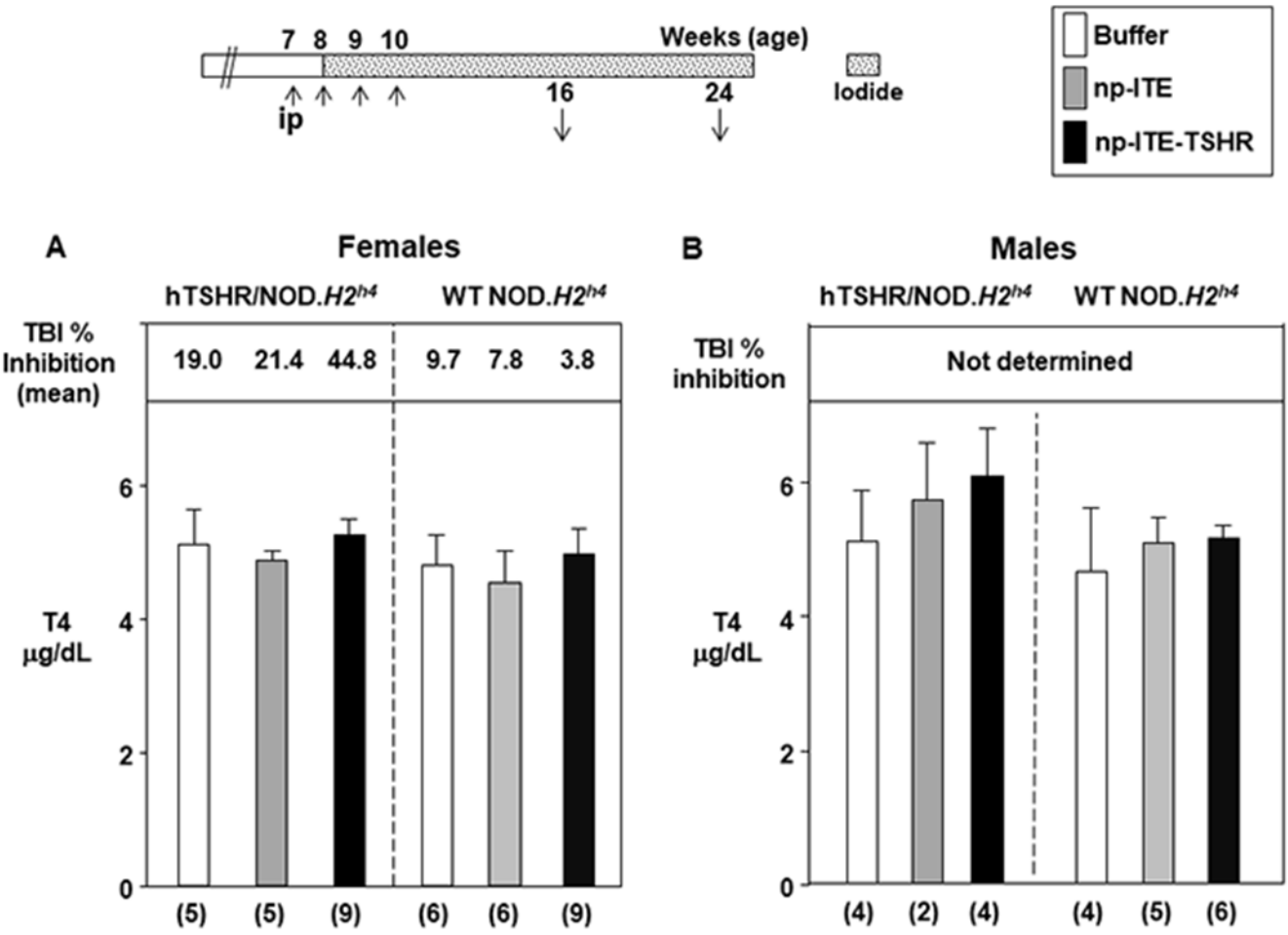

Figure 4.

Serum T4 values in hTSHR/NOD. *H2*^{h4} and wild-type (WT) NOD. *H2*^{h4} mice. Data (ug/dL) are indicated separately for females (panel A) and males (panel B). Also included are the mean TBI values for the female mice shown in Fig. 3. As mentioned previously, TBI activity cannot be measured in male mice because of high TSH levels. The numbers of mice are indicated in parentheses. Of note, pathogenic TSHRAb arise spontaneously to the human A-subunit generated by the transgene do not increase serum T4 levels because they do not cross-react with the endogenous mouse TSHR.

Table 1.

Ability of mouse strains to develop TSHRAb (non-pathogenic and pathogenic) in relation to the level of mRNA of the transgenic TSHR A-subunit expressed in the thyroid and thymus (21). Relative expression levels, indicated by +, ++ +++, from real time PCR data (21).

Mouse strain	Wild-type	Lo hTSHR/NOD.*H2*^{h4}	Hi hTSHR/NOD.*H2*^{h4}	Ref
TSHR A-subunit mRNA				
Intra-thymic	Un	+	++++	(21)
Intra-thyroid	Un	+	+++++	(21)
TSHR mRNA				
Intra-thymic	+	+	++	(21)
Intra-thyroid	+	+	++	(21)
TSHR A-subunit protein				
In the thyroid	Un	~0.1 ug/thyroid	~3 ug.thyroid	(22)
In serum	Un	Un	Un	(22)
TSHRAb				
Uninjected				
Non-pathogenic (24w)	None	++	+	(11)
Pathogenic (24w)	None	++	Un	
TSHR A protein sc				
Non-pathogenic (16w)	None	None	nd	(20)
Pathogenic (16 w)	None	None	nd	
Non-pathogenic (24w)	++	+++	nd	(20)
Pathogenic (24w)	None	None	nd	
TSHR A protein np				
Non-pathogenic (16w)	+++	+++	nd	Present
Pathogenic (16w)	None	++		study
Non-pathogenic (24w)	+++	+++	nd	Present
Pathogenic (24w)	None	+++	nd	study

Lo, Lo A-subunit transgenic expressor; Hi, Hi A-subunit transgenic expressor; Un: undetectable; nd, not done; Ref, Reference. Note; TSHR A-subunit mRNA is expressed by the transgene; it is undetectable in the thyroid and thymus of wild-type mice because it is cleaved from the full-length TSHR expressed on the cell surface