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REVIEW

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A transgenic mouse that spontaneously develops pathogenic TSH receptor antibodies will facilitate study of antigen-specific immunotherapy for human Graves' disease

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Abstract

Graves' hyperthyroidism can be treated but not cured. Antigen-specific immunotherapy would accomplish this goal, for which purpose an animal model is an invaluable tool. Two types of animal models are available. First, pathogenic TSHR antibodies (TSHRAb) can be induced by injecting mice with fibroblasts co-expressing the human TSHR (hTSHR) and MHC class II, or in mammals using plasmid or adenovirus vectors encoding the hTSHR or its A-subunit. Second, a mouse model that spontaneously develops pathogenic TSHRAb resembling those in human disease was recently described. This outcome was accomplished by transgenic intrathyroidal expression of the hTSHR A-subunit in NOD.$H2^{h4}$ mice that are genetically predisposed to develop thyroiditis but, without the transgene, do not generate TSHRAb. Recently, novel approaches to antigen-specific immunotherapy have been tested, primarily in the induced model, by injecting TSHR A-subunit protein or cyclic TSHR peptides. T-cell tolerance has also been induced in "humanized" HLA-DR3 mice by injecting synthetic peptides predicted in silico to mimic naturally processed TSHR T-cell epitopes. Indeed, a phase 1 study based on the latter approach has been conducted in humans. In the spontaneous model (hTSHR/NOD.$H2^{h}$ mice), injection of soluble or nanoparticle-bearing hTSHR A-subunits had the unwanted effect of exacerbating pathogenic TSHRAb levels. A promising avenue for tolerance induction, successful in other conditions and yet to be tested with the TSHR, involves encapsulating the antigen. In conclusion, these studies provide insight into the potential outcome of immunotherapeutic approaches and emphasize the importance of a spontaneous model to test future novel, antigen-specific immunotherapies for Graves' disease.

Keywords Animal models · TSHR · TSHR A-subunit · TBI · Tg · TPO

Abbreviations

hTSHR	*human* thyrotropin receptor
hTSHR/NOD.$H2^{h4}$	NOD.$H2^{h4}$ mice expressing the human TSHR A-subunit in the thyroid
ITE	ligand for the endogenous aryl-hydrocarbon receptor ($C_{14}H_{10}N_2O_3S$)
TBI	inhibition of TSH binding to the TSHR
Tg	thyroglobulin
TPO	thyroid peroxidase

Introduction

The development in the twentieth century of therapeutic modalities for Graves' disease, one of the most common autoimmune diseases in humans, represents a milestone in clinical medicine. Prior to their use, the great majority of Graves' disease patients remained debilitated with episodic or continual thyrotoxicosis [1]. However, these therapies can only treat, not cure, the disease. Thiourea derivatives (propylthiouracil or methimazole) that inhibit thyroid hormone synthesis can restore euthyroidism but their discontinuation, even after long-term therapy, is more likely to result in relapse rather than remission. Consequently, thyroid ablative approaches (radioiodine or surgery) are frequently elected and generally, especially with the former approach, result in permanent hypothyroidism requiring life-long thyroid hormone replacement. Therapy of the extrathyroidal manifestations of Graves' disease is an even

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more difficult challenge. Post-therapeutic quality of life surveys reveal suboptimal outcomes in many patients [2, 3]. Clearly, a cure, rather than treatment, for Graves' disease would represent a major advance.

Graves' disease is directly caused by autoantibodies to the thyrotropin receptor (TSHR) that mimic the stimulatory effect of TSH (reviewed in [4]). Given an autoimmune basis, a form of immunotherapy is the most logical investigative avenue to accomplish this goal. Effective antigen-specific immunotherapy requires a deep understanding of disease pathogenesis. For this purpose an animal model of Graves' disease is an invaluable, if not essential, tool. Animal models of disease can be either spontaneous or induced and can provide important clues into the basis for human diseases. Different models may focus on particular pathogenic aspects of disease including the genetic basis for the disorder, gender differences or environmental effects. A few animals spontaneously develop diseases like those in humans, such as type I diabetes mellitus in NOD mice (reviewed in [5]) whereas a widely used induced model is experimental autoimmune encephalomyelitis, a model for multiple sclerosis [6].

Until relatively recently, neither a spontaneous nor an induced Graves' disease animal model was available. Graves' disease has only been described in humans. Not even our closest relatives, non-human great apes, appear to develop the disease [7]. Prior to the molecular cloning of the TSHR in 1989 [8–10], neither could immunization with various thyroid antigenic preparation verifiably induce Graves' disease in animals. In the present review, we describe the progress made in the past 30 years in the development of induced models of Graves' disease, culminating in 2015 with a transgenic mouse that spontaneously develops pathogenic TSHR autoantibodies capable of activating the TSHR, the first animal to do so. We discuss the advantages and disadvantages of each model, finally offering a viewpoint towards future investigative approaches.

Structure, cleavage and shedding of the TSHR

Nearly 40 years ago, Rees-Smith and colleagues demonstrated that the TSHR contained two subunits (extracellular A and largely transmembrane B) linked by disulfide bonds [11]. Further evidence suggested that the TSHR receptor was synthesized as a single polypeptide chain with subsequent intramolecular cleavage into subunits [12] (Fig. 1). Subsequent molecular cloning of the TSHR, a member of the G-protein-coupled receptor family with seven membrane spanning helices, confirmed synthesis of the receptor as a single polypeptide chain.

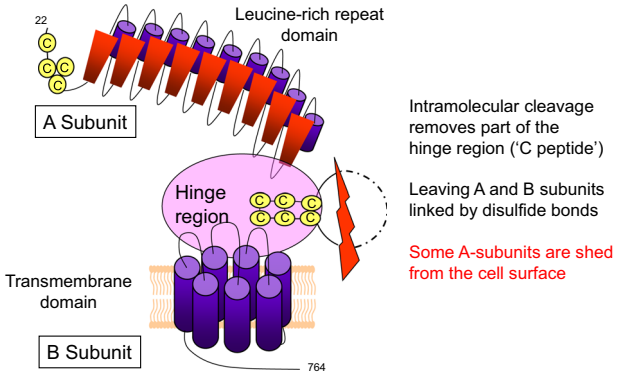

Fig. 1 TSHR structure, cleavage and A -subunit shedding. The TSHR comprises an extracellular leucine rich repeat domain linked by a hinge region to a heptahelical transmembrane domain. The hinge region folds on itself and forms a disulfide-bonded loop. TSHR on the thyrocyte cell surface undergo intramolecular cleavage within the hinge region deleting a C-peptide region thereby converting the single polypeptide chain into A- and B-subunits that remain attached by the disulfide bonds. Some A-subunits are subsequently shed from the cell surface into the extracellular fluid by an unclear mechanism, most likely following continued proteolytic digestion of the disulfide bonds [13]

After cleavage, some TSHR A-subunits are shed from the cell surface of living thyroid cells by a presently uncertain mechanism (reviewed in [13]).

Pathogenic TSHR antibodies

It is essential to understand, and it is commonly overlooked, that some TSHR antibodies are more equal than other (with apologies to George Orwell). TSHR antibodies are readily induced in animals (primarily mice or rabbits) using recombinant prokaryotic TSHR protein or synthetic peptides based on its primary amino acid sequence, but are not capable of activating the TSHR and are, therefore, nonpathogenic (for example [14]). Moreover, irrespective of how they arise in animals, TSHR antibodies detected directly by binding to TSHR protein or synthetic peptides coated on ELISA plates are nonpathogenic in having neither thyroid stimulatory or TSH blocking activity. Therefore, in studying animal models of Graves' disease it has proven necessary to utilize assays that detect pathogenic TSHR antibodies, namely ligand binding inhibition assays and bioassays. A recently introduced TSHR bridge clinical assay [15] would also be suitable; however, there is presently no report of its use for detecting TSHR antibodies in mouse serum. Regardless of its type, the very small volume of serum obtained in mouse studies requires custom modification of the assay in the research laboratory.

There is presently an attempt to stress the clinical importance in humans of using a bioassay rather than a ligand binding or bridge assay to measure TSHR antibodies,

a point that could also be applied to studying these antibodies in mice. The thesis for this argument (with a strong commercial undertone) is that binding inhibition or bridge assays do not distinguish between thyroid stimulating antibodies (TSAb) and TSH blocking antibodies (TBAb). However, in our opinion this thesis is incorrect. As is evident from clinical experience over many decades, the individual patient's thyroid provides the ultimate bioassay. In vivo, TSHR antibodies in association with hyperthyroidism are primarily (if not exclusively) TSAb. Conversely, in the *absence* of hypothyroidism, TSHR antibodies detected are unlikely to be TBAb in a meaningful amount (if any). Moreover, detecting TBAb in sera containing TSAb is questionable given that a weak, or partial, agonist can function as an antagonist.

Similarities and differences in the properties of TSHR antibodies that are induced or arise spontaneously in mice are described below, after first outlining the relevant animal models.

TSAb induction in animals not susceptible to thyroid autoimmunity

Induction of pathogenic TSHR antibodies requires the availability of specific antigen and novel immunization procedures. Two advances led to the first reported success in this endeavor.

First, the molecular cloning of the TSHR enabled the generation of recombinant material. Previously, the very low level and the lability of the TSHR precluded its purification from thyroid tissue. Moreover, even with the advent of recombinant TSHR protein or synthetic peptides, conventional immunization studies employing a variety of TSHR preparations, adjuvants and mouse strains, the TSHR antibodies induced were nonpathogenic (reviewed in [16]). The second advance leading to success was discovery of aberrant MHC class II expression by thyrocytes in Graves' disease [17, 18]. In an ingenious approach using this information, Shimojo et al. generated a mouse fibroblast cell line that expressed both MHC class II and the recombinant TSH holoreceptor. Injecting intact cells of this line into syngeneic mice induced pathogenic TSAb with consequent hyperthyroidism [19]. Confirmation of these findings were reported using hamsters injected with syngeneic hamster fibroblasts [20] and mice using a syngeneic murine B-cell line [21], both coexpressing MHC class II and the TSHR (Fig. 2, left panel). Limitations to this major accomplishment were the need to propagate large numbers of transfected fibroblasts and restriction to syngeneic animals.

Recognition that effective immunization required in vivo TSHR antigen expression and presentation by endogenous antigen presenting cells (APC) led to the successful induction of TSAb by intramuscular injection of "naked" plasmid vectors (with antigen presentation enhanced by cardiotoxin or electroporation) [22–24] or adenoviruses, each bearing cDNA for the TSH holoreceptor [25] or its A-subunit [26] (Fig. 2, right panel). This approach has the advantage that it can be applied to any mammal, to date mice, hamsters (reviewed in [27]) and monkeys [28].

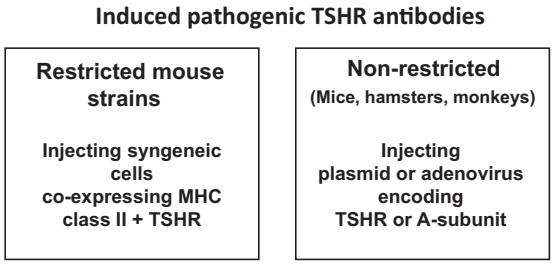

Fig. 2 Induced models of pathogenic TSHRAb generation. Left panel: Injecting cells expressing MHC class II and TSHR (fibroblasts or B cells [19, 21]); restricted to mouse strains with compatible MHC class II molecules. Right panel: Injecting plasmid or virus or DNA encoding the human TSHR or its A-subunit adenovirus (first described in [25, 26]). This model is not MHC restricted and can be applied to different mammalian strains, to date mice, hamsters and monkeys

Role of the TSHR A-subunit in mouse models of Graves' disease

Pathogenic TSAb in Graves' patients preferentially recognize the free TSHR A-subunit rather than the holoreceptor [29]. Moreover, substantial evidence supports the concept that the free TSHR A-subunit shed from thyrocytes is the primary antigen that initiates the autoimmune response to the receptor and/or drives affinity maturation of TSAb [26, 30]. Consequent to these studies, use of vectors expressing the isolated A-subunit has become standard practice in many laboratories. A possible factor contributing to A-subunit antigenicity is its heavy glycosylation (~40% of its mass), containing five of the six N-linked glycans present in the holoreceptor (reviewed in [31]). Glycosylation of proteins can be important for their uptake, processing and presentation to T cells (reviewed in [32]).

Spontaneous TSAb development in a mouse strain susceptible to thyroid autoimmunity

In studying potential approaches to immunotherapy in Graves' disease, a mouse that spontaneously develops TSAb holds a significant advantage over mouse models in which TSAb induction is necessary (as discussed further below). Recently, a mouse with this property was obtained

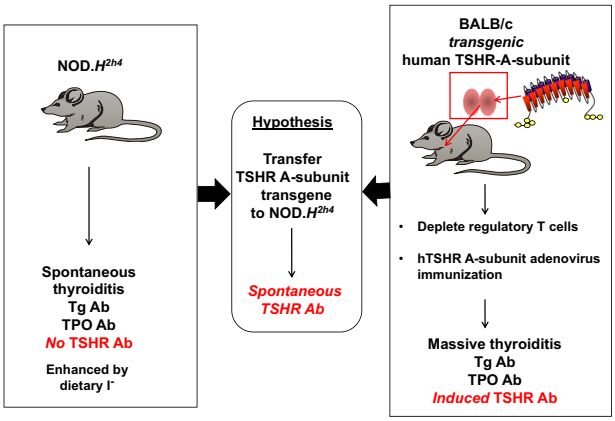

Fig. 3 Establishment of a mouse model that spontaneously develops pathogenic TSHR antibodies. The basis for this model was the hypothesis that the intrathyroidal expression of a human TSHR A-subunit transgene in thyroiditis-susceptible NOD.$H2^{h4}$ mice would lead to this outcome, producing a new mouse strain (human TSHR/ NOD.$H2^{h4}$) [33]. NOD.$H2^{h4}$ mice with or without the A-subunit transgene spontaneously develop TgAb and TPOAb

[33]. The underlying hypothesis for this development (Fig. 3) arose from studies in two mouse strains: (a) BALB/c mice expressing the human TSHR A-subunit transgene in the thyroid, after depletion of regulatory T cells and immunization with A-subunit adenovirus develop (in addition to induced TSHRAb) massive thyroiditis in association with antibodies to Tg and TPO [34]; (b) NOD.$H2^{h4}$ mice are susceptible to autoimmune thyroiditis and spontaneously develop autoantibodies to thyroglobulin (Tg) and thyroid peroxidase (TPO) [35–38], but *not* to the TSHR. Because the recombinant human TSHR A-subunit synthesized in mammalian cells, unlike the holoreceptor, is largely secreted rather than retained intracellularly [39], the same A-subunit expressed in the mouse thyroid would be expected to be secreted. Human A-subunit secretion by thyrocytes in NOD.$H2^{h4}$ mice, mimicking shedding from the holoreceptor, could then induce TSAb. In confirmation of this hypothesis, transgenic expression of the human TSHR A-subunit targeted to the NOD.$H2^{h4}$ mouse thyroid led to the spontaneous development of pathogenic TSHR autoantibodies (Fig. 3). Remarkably, NOD.$H2^{h4}$ mice with the *mouse* TSHR A-subunit transgene targeted to the thyroid do not develop TSHR antibodies [40]. This difference between the human and mouse A-subunits, involving both amino acid sequence and the degree of glycosylation, suggests that tolerance is more readily broken by the former, possibly explaining (at least in part) the observation that Graves' disease appears to be restricted to humans, not even involving our closest relatives, non-human great apes [7].

A serendipitous phenotype of these hTSHR/ NOD.$H2^{h4}$ mice, in our view a significant advantage, is that they do not become hyperthyroid. It is well-established that the thyroids of different mouse strains respond in a variable manner to TSAb induced in response to human TSHR (adenovirus) immunization. For example, TSAb induced against the human TSHR cross react well with the mouse TSHR in BALB/c mice but poorly or not at all with the mouse TSHR in C57BL/6 mice [25, 41, 42]. Indeed, wild-type NOD.$H2^{h4}$ were previously noted to be relatively unresponsive to TSHR antibodies induced by human A-subunit adenovirus immunization [43] despite antibody levels far higher than those arising spontaneously in hTSHR/ NOD.$H2^{h4}$ mice. For future antigen-specific immunotherapy, the goal is not to treat hyperthyroidism (for which effective therapies are presently available) but to blunt or prevent the development of pathogenic TSHR antibodies, the direct cause of hyperthyroidism. The absence of hyperthyroidism in studies on immunotherapeutic approaches in hTSHR/NOD.$H2^{h4}$ mice is an advantage in that it avoids the confounding influence of excess thyroid hormone levels on the adaptive and innate immune systems [44–46].

Similarities between TSHR antibodies in mouse models and human Graves' disease

Recognition of conformational TSHR protein

The failure to induce pathogenic TSHR antibodies by conventional immunization with recombinant TSHR protein in adjuvant versus success attained by in vivo expression of the TSH holoreceptor, or preferably its A-subunit [24, 26] (Figs. 2, 3), indicates the requirement for conformationally intact eukaryotic protein as the immune stimulus for B cells. Recognition of conformationally intact TSHR or its A-subunit, are characteristics of TSHR antibodies in Graves' disease (reviewed in [4]).

Linkage to the major histocompatibility (MHC) locus

Genetic factors predisposing to Graves' disease include MHC region genes (reviewed in [47]). As in humans, adenovirus-mediated induction of pathogenic TSHR antibodies in large panels of different strains of recombinant inbred female mice revealed linkage to a locus in the MHC region [48].

Th1 and Th2 cytokines

A common misperception is that, because humoral autoimmunity is the underlying cause of human Graves' disease, the latter is a T-helper cell type 2 ("Th2") disease. However, the majority of TSAb in humans are, with some exceptions [49], of subclass IgG1 [50] which reflects cytokines of Th1

type (reviewed in [51]). Therefore, human Graves' disease is primarily a Th1, but also in some instances, a Th2 disease.

Confusion arises because in mice, unlike in humans, IgG1 antibodies are of the Th2 type. Data from cytokine "knockout" mice and studies providing mice with cytokines demonstrated that TSHR antibodies induced by TSHR A-subunit adenovirus immunization involve both Th1 and Th2 cytokines [52, 53]. As in human disease, pathogenic TSHR antibodies induced in wild-type NOD.$H2^{h4}$ are both IgG2b (Th1-type) and IgG1 (Th2-type) [43]. The IgG subclasses of spontaneously arising TSHR antibodies in hTSHR/NOD.$H2^{h4}$ mice have not to date, been studied.

Intra-thymic TSHR expression and central tolerance

Immune responses to some self-antigens are controlled by expression of the autoantigen in the thymus during "T-cell education". T cells that express moderate binding for the autoantigenic peptide(s) are positively selected while those with high binding to autoantigenic peptides are deleted (for example [54]). One of the genes associated with Graves' disease in humans involves TSHR polymorphisms that control its expression levels in the thymus, high expression being associated with protection from, and low levels with susceptibility to, Graves' disease [55, 56].

Consistent with human disease, intra-thymic TSHR expression influences susceptibility to the development of autoantibodies to the TSHR in both the induced and spontaneous mouse models. As already mentioned, immunization of mice such as BALB/c with adenovirus expressing the *human* TSHR or its A-subunit (proteins not expressed in the mouse thymus) induces pathogenic TSHRAb (reviewed in [16]). In contrast, immunization of the same mouse strain with adenovirus expressing *mouse* TSHR fails to induce antibodies in wild-type mice but does induce TSHR antibodies in knockout mice that lack their endogenous TSHR [57] (Fig. 4a). Breaking central tolerance to the mouse TSHR can be attained by more potent immunization involving repeated electroporation of mouse TSHR A-subunit DNA [58]. In the reverse of TSHR knockout mice,

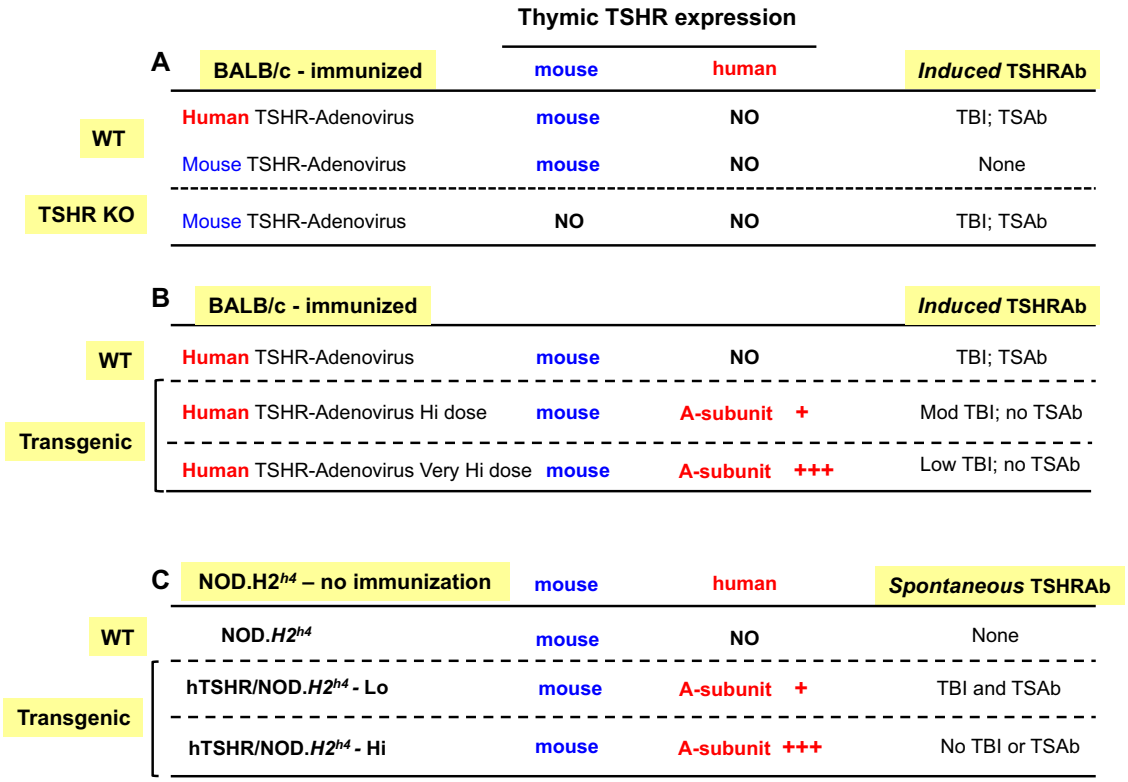

Fig. 4 Intra-thymic TSHR expression in relation to the induction or spontaneous development of TSHRAb in different mouse models. **a** BALB/c mice (like other strains) express the mouse, but not the human, TSHR. TSHRAb are induced in wild-type mice by immunization with adenovirus expressing the *human* TSHR or its A-subunit (reviewed in [16]). However, the same vector encoding the *mouse* TSHR A-subunit only induces TSHRAb in TSHR knockout (KO), not in wild-type mice expressing the endogenous mouse TSHR [57]. **b** Transgenic BALB/c mice express the *human* TSHR A-subunit at low or high levels in the thymus [59, 60]. Lo-expressors of the *human* TSHR A-subunit ("+") require higher doses of *human* TSHR A-subunit adenovirus to induce TSHR antibodies than wild-type mice. Extremely high levels of *human* TSHR A-subunit adenovirus are required to induce TSHRAb in Hi-expressor mice ("+++") [59]. **c** Wild-type NOD.$H2^{h4}$ mice do not develop TSHRAb. However, NOD.$H2^{h4}$ mice expressing the human TSHR A-subunit at a low level (Lo; "+") spontaneously develop TSHRAb (nonpathogenic ELISA type and pathogenic TBI/TSAb) [33]. A high expressor NOD.$H2^{h4}$ strain (Hi; "+++") develop very low levels of nonpathogenic TSHRAb and fail to develop pathogenic TBI and TSAb [60]

transgenic BALB/c mice expressing low levels ("Lo-expressors") of the *human* TSHR A-subunit (a self-antigen in these mice) in the thymus and the thyroid require higher adenovirus doses to induce TSHR antibodies [34] and even more so in animals expressing a much higher level of the human A-subunit transgene ("Hi-expressors") [59] (Fig. 4b)

Turning to the spontaneous model, transfer of the TSHR A-subunit transgenes from the Lo- and Hi-expressor BALB/c to the NOD.$H2^{h4}$ background recapitulated the findings for self-tolerance from the induced model. Thus, Lo-expressor hTSHR/NOD.$H2^{h4}$ mice spontaneously developed TSHRAb, both nonpathogenic (ELISA-type) and pathogenic (TBI, TSAb) [33] (Fig. 4c). In contrast, Hi-expressor hTSHR/NOD.$H2^{h4}$ mice had only low levels of ELISA-type TSHRAb and failed to develop pathogenic TSHRAb [60]. Obviously, the basis for high- and low-TSHR A-subunit expression in the thymus is not the same in the transgenic mice and humans. However, these findings in hTSHR/NOD.$H2^{h4}$ mice support their value as a model for human disease.

TSHR antibody levels

The levels of pathogenic TSHR antibodies that arise spontaneously in hTSHR/NOD.$H2^{h4}$ are similar to those in Graves' patients [33]. In contrast, TSHR- or A-subunit adenovirus immunization induces antibodies at far higher levels than those commonly observed in Graves' patients [25, 26]. To avoid inducing TSHR antibody levels exceeding those in Graves' patients, relatively low doses of A-subunit adenovirus need to be used [61].

Influence of gut microbiota

Risk factors for autoimmune thyroid disease included changes in the gut bacteria of Graves' patients compared with healthy controls [62], although no specific organisms have been identified. Similarly, immunization with TSHR antibodies using A-subunit DNA and electroporation induced differences in immune responses and TSHRAb associated with differences in gut microbiota in two mouse strains [63]. The availability of TSHR/NOD.$H2^{h4}$ that *spontaneously* develop pathogenic autoantibodies to the human TSHR provides a valuable tool for future investigations involving manipulation of their gut microbiota.

Differences between TSHR antibodies in mouse models and human Graves' disease

Lack of influence of female sex

In common with the female bias of many autoimmune diseases (reviewed in [64]), Graves' disease occurs predominantly in women (for example [65]). No sex difference was observed for TSHR antibodies induced by injecting fibroblasts co-expressing TSHR and MHC class II [66] or TSHR-adenovirus [25]. Despite this lack of sex difference, most studies inducing TSHRAb using plasmids or adenoviruses encoding the TSHR or its A-subunit are performed in female mice. It is presently unknown whether there is a female bias in hTSHR/NOD.$H2^{h4}$ mice that spontaneously develop pathogenic TSHR antibodies because only females have been studied to date. High TSH levels, present for unclear reasons in males, can spuriously provide false positives in TBI or TSAb assays [33]. Future studies to address this issue will require purification of IgG from very small volumes of individual sera, a difficult undertaking.

Nonpathogenic TSHR antibodies

Such antibodies, detected by direct binding to the TSHR A-subunit protein on ELISA plates, are absent in sera from Graves' patients [67]. On the other hand, in addition to pathogenic TSHR antibodies, nonpathogenic TSHRAb also develop in mice immunized with TSHR A-subunit adenovirus (for example [26]) and in hTSHR/NOD.$H2^{h4}$ mice that spontaneously develop pathogenic TSHRAb [33]. In silico docking of the crystal structure of a nonfunctional mouse monoclonal antibody [68] to that of the human TSHR A-subunit [69] provided evidence that affinity maturation of TSHR antibodies in Graves' disease involves recognition of shed TSHR A-subunit multimers, not monomers [67].

Antigen-specific immunotherapy for Graves' disease

As mentioned above, a major therapeutic goal in the present century is to develop a cure for Graves' disease, supplanting the less than satisfactory treatments developed during the last century. Attaining this goal requires some form of immunotherapy to prevent or reverse the autoimmune response leading to the development of pathogenic TSHR autoantibodies, the direct cause of disease. There are many molecules in the immune system that are potential targets for immunotherapy in an autoimmune disease. For example, induced TSHR antibody levels were reduced in some mice using decoy molecules of the tumor necrosis (TNF)-family ligand inhibitors (BAFF and APRIL) to target proliferation or survival of B cells [70]. However, without antigen specificity there is a heightened chance of untoward systemic consequences. In diseases for which one or more autoantigens have been identified, antigen-specific immunotherapy has been attempted in several

ways including, for example, altered peptide ligand and multi-peptide immunization (reviewed in [71]). Identification of a single autoantigen, the TSHR, in the pathogenesis of Graves' disease provides a major advantage relative to many other autoimmune diseases in the development of antigen-specific immunotherapy.

Immunotherapy can be applied to susceptible, presently normal, individuals or after the onset of disease. Both situations are amenable to testing in animals in which disease is induced, or in which it is known that disease will develop spontaneously. In humans, obviously, until susceptibility to disease can be predicted with high confidence the risk−benefit ratio (both medical and economic) will restrict immunotherapeutic trials to patients with existing Graves' disease. Experience to date in these endeavors is described below.

Mice with induced pathogenic TSHR autoantibodies

Injection of recombinant, eukaryotic (but not prokaryotic) human TSHR A-subunit protein into BALB/c mice *prior to* human A-subunit immunization attenuated the development of pathogenic TSHR antibodies (TBI, TSAb) and diverted the response to the nonpathogenic (ELISA) variety [14] (Fig. 5). However, this encouraging finding was not replicated when the same antigen was injected *after* the induction of pathogenic TSHR antibodies. Thus, in preexisting "disease" A-subunit protein administration did not reduce the level of pathogenic TSHR antibodies and augmented the level of nonpathogenic antibodies [14]. The timing of immunotherapy is also likely to be important. TSHR A-subunit antigen administered (via adenovirus) in neonatal mice, but not in older animals, induced tolerance to subsequent TSHR immunization with TSHR A-subunit adenovirus immunization [72].

In contrast to the intact human A-subunit protein, a number of synthetic cyclic peptides based on the amino acid sequence of this protein were reported to partially depress levels of pathogenic TSHR autoantibodies in BALB/c mice *following* their induction by A-subunit adenovirus (caution; the "ELISA" used in this study is actually a ligand binding inhibition assay, different to a direct ELISA that only detects nonpathogenic antibodies) [73, 74]. Tolerization to the TSHR A-subunit has also been tested in "humanized" HLA-DR3 transgenic mice by injection of human TSHR synthetic peptides predicted in silico to mimic naturally processed epitopes presented to MHC class II DR3 molecules on CD4+ T cells [75]. Unlike the study in BALB/c mice, peptide immunization of HLA-DR3 mice was performed *before* TSHR antibody induction by human A-subunit adenovirus injection. In both peptide immunization studies clinical features reflecting Graves' disease were partially ameliorated, but confirmatory data are awaited. For example, in the BALB/c mice TSAb levels did not decline significantly and the study with HLA-DR3 mice did not assay pathogenic TSHR antibodies, nor was tolerance induction attempted with TSHR antibodies already present. Nevertheless, it remains possible that some synthetic peptides (representing T-cell epitopes) are more tolerogenic than peptide epitopes naturally processed by antigen-presenting cells "fed" the intact TSHR. The variable route of antigen administration also needs to be examined; subcutaneously [14], intravenous [73, 74] and intradermal [75].

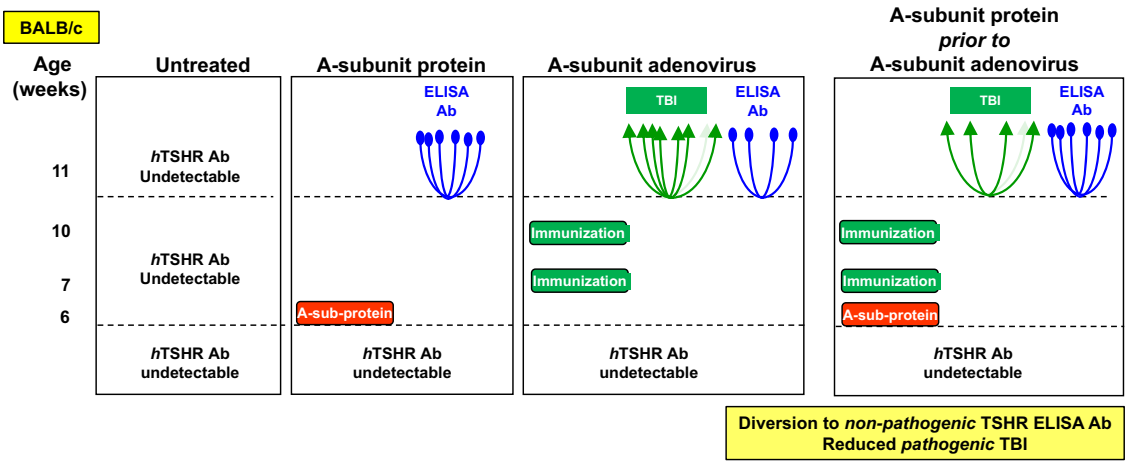

Fig. 5 Effect of TSHR A-subunit protein injection in BALB/c mice prior to immunization with hTSHR A-subunit-adenovirus (data based on [14, 86]). Blue: non-pathogenic TSHRAb measured by ELISA; Green: pathogenic TSHR antibodies (TBI). A-subunit protein injected alone induced ELISA-type antibodies, but not TBI. hTSHR A-subunit-adenovirus immunization alone induced both types of antibody. Injection of A-subunit protein diverted the proportion of TSHR antibodies induced by A-subunit-adenovirus immunization away from TBI towards ELISA-type antibodies

Thyroiditis-predisposed mice that spontaneously develop pathogenic TSHR autoantibodies

Unlike in BALB/c mice, injecting intact human TSHR A-subunit protein into TSHR/NOD.$H2^{h4}$ mice did not attenuate, but enhanced, levels of pathogenic TSHR antibodies (TBI and TSAb) [76] (Fig. 6, lower panel). This observation mimics the rise in autoantibodies to the TSHR [77], as well as to Tg and TPO (for example [78]) that occurs in humans after 131-I treatment for Graves' disease, presumably caused by the release of thyroid proteins from the damaged gland.

Because injection of gold nanoparticles bearing a tolerogenic molecule (ITE, a ligand for the endogenous aryl-hydrocarbon receptor; $C_{14}H_{10}N_2O_3S$) together with antigen prevented the spontaneous development of type I diabetes in NOD mice [79] and the induction of experimental encephalomyelitis in different mouse strains [80], the same approach was employed with TSHR/NOD.$H2^{h4}$ mice [81]. As with soluble A-subunits, this antigen coupled together with ITE to the same gold nanoparticles exacerbated the underlying autoimmune process, augmenting the levels of both pathogenic and nonpathogenic TSHR antibodies (Fig. 6, upper panel). In wild-type NOD.$H2^{h4}$ mice, both soluble and particle-bound TSHR A-subunits induced only nonpathogenic TSHR antibodies [76, 81].

Human Graves' disease

The first therapeutic trial in humans (phase I, open label) involved intradermal injection of two human TSHR synthetic peptides predicted in silico to mimic naturally processed T-cell epitopes [82]. As mentioned above, preinjection of these peptides had previously been found to tolerize T cells and blunt the induction of pathogenic TSHR antibodies in HLA-DR transgenic mice immunized with adenovirus encoding the human TSHR A-subunit [75]. Although potentially important, the efficacy of such therapy (7/10 responders and 3/10 worsening) requires evaluation in later phase clinical studies with appropriate controls. A number of concerns arise in this study. First, because conventional therapy was foregone for a long period of time (30 weeks), patients were selected for very mild hyperthyroidism, whose spontaneous remission rate without therapy cannot be gleaned from information on unselected patients with more severe disease. Second, the therapeutic approach in pre-existing disease may not be as effective as in the preceding mouse study in which immunotherapy was administered prophylactically. Finally, because the peptides were selected for optimal binding to MHC molecules DRB1*03, DRB1*04 DRB1*15 [75], therapy may only be effective in a limited number of patients.

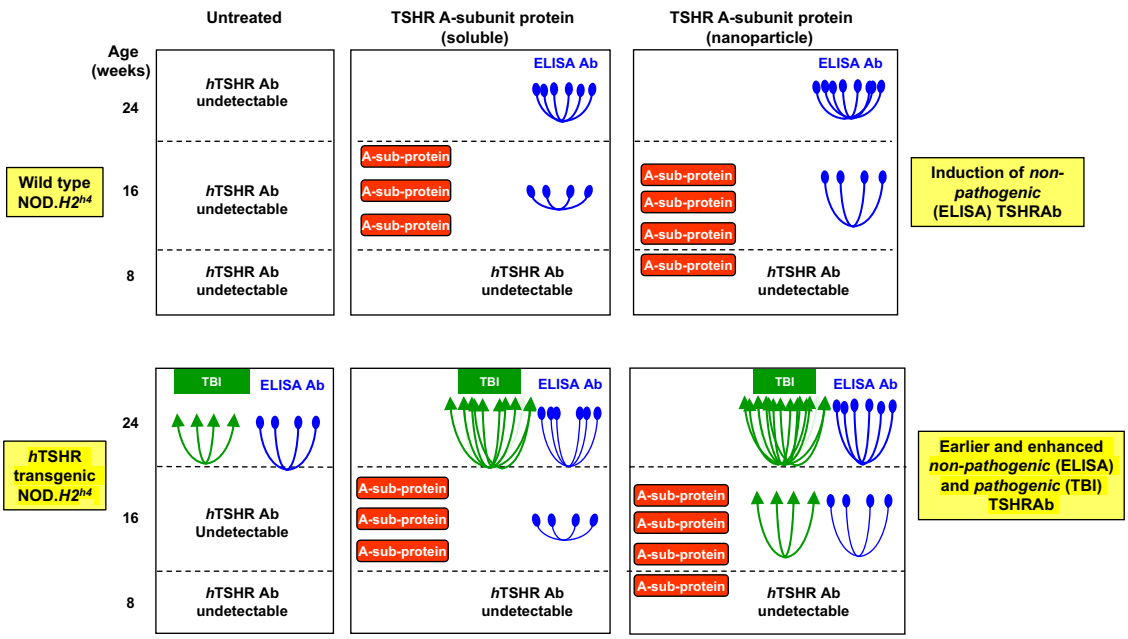

Fig. 6 In hTSHR/NOD.$H2^{h4}$ mice, injection of nanoparticle-bound A-subunit protein together with a tolerogenic molecule does not prevent, but exacerbates, the spontaneous development of pathogenic TSHRAb detected in a TBI assay. This effect is similar to, but is detectable earlier and at higher levels, than in mice injected with soluble A-subunit protein. Wild-type NOD.$H2^{h4}$ mice do not develop TSHRAb; however, injection with soluble or nanoparticle-bearing A-subunit protein induces only non-pathogenic ELISA-type TSHRAb. Blue: non-pathogenic TSHRAb measured by ELISA; Green: pathogenic TSHR antibodies (TBI). Data shown are qualitative and based on references [76, 81]

Insight into antigen-specific immunotherapy for Graves' disease

In our view, it is quite feasible, indeed likely, that effective immunotherapy for Graves' disease will become available in the twenty-first century. Although there is the temptation to conduct clinical trials in humans at an early stage, further studies in mouse models of disease will be necessary to assess the many potential immunotherapeutic approaches that will undoubtedly be developed in future years. For such studies, there are a number of considerations.

Mouse models for immunotherapeutic studies

Mice are available in which pathogenic TSHR antibodies can be induced and in which such antibodies arise spontaneously. In choosing which model to employ, present evidence suggests that immunotherapy is most effective when administered prior to antibody induction. However, as mentioned above, the onset of Graves' disease cannot presently be predicted with confidence. Possible exceptions to this situation are individuals at relatively high risk for developing Graves' disease in the recovery phase post lymphocyte depletion, such as multiple sclerosis patients treated with anti-CD52 (Alemtuzumab) [83]. These exceptions aside, present therapeutic options are limited to patients with active disease. For such patients, mice which spontaneously develop pathogenic TSHR antibodies are clearly more appropriate than the induced model for study.

TSHR antigen used to induce tolerance

Fortunately for Graves' disease, an antigen-specific immunotherapeutic approach need only consider a single autoantigen, the TSHR. Novel antigen-specific therapies are being developed that could prevent or block development of pathogenic TSHR antibodies and thereby cure (rather than treat) Graves' disease. For this purpose, different forms of antigen have been utilized, primarily recombinant TSHR A-subunits and various TSHR synthetic peptides. A potential problem in the application of these antigenic forms is that their efficacy may differ depending on the animal model in which they are used. For example, preimmunization with TSHR A-subunit protein deviates TSHR antibody generation from the pathogenic to nonpathogenic variety in the induced model but enhances both nonpathogenic and pathogenic TSHR antibodies in the spontaneous model, clearly an undesirable outcome. Whether synthetic TSHR peptide immunization in the spontaneous model will lead to a more favorable outcome remains to be established.

A most important recent development in immunotherapy for various conditions including autoimmunity, allergy and organ transplantation is the "package" of the antigen and route of administration. TSHR A-subunit bearing nanoparticles injected intraperitoneally did not induce tolerance but, rather, exacerbated pathogenic TSHR antibody generation in mice in which these antibodies arise spontaneously [81]. The antigen in this form is exposed, or "naked" on the nanoparticle. Miller and colleagues [84] emphasize that injecting unencapsulated antigen-coated nanoparticles can have adverse outcomes, such as anaphylaxis in presensitized mice. In contrast, the tolerogenic success of using encapsulated antigen-specific nanoparticles emphasizes the importance of initially concealing the specific autoantigen prior to uptake of the particles by antigen-presenting cells [85]. Unfortunately, we were unable to test the tolerogenic effect of encapsulated recombinant human TSHR A-subunits on the spontaneous development of pathogenic TSHR antibodies in TSHR/NOD.$H2^{h4}$ because of the involuntary closure of our laboratory. Currently these mice are heterozygous for the transgene. Future generation of homozygous hTSHR/NOD.$H2^{h4}$ mice might, perhaps, increase the proportion of animals developing pathogenic TSHR antibodies and possibly shorten the prolonged waiting period for TSHR antibody development. In the hope that other investigators will continue with this, or other potential lines of investigation, cryopreserved sperm and embryos from these mice are available on request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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