

Enhanced Response to Mouse Thyrotropin (TSH) Receptor Immunization in TSH Receptor  
Knockout Mice

Short title: anti-mouse TSHR immune response in TSHR KO mice

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## Abstract

Graves-like hyperthyroidism is induced in BALB/c mice by immunization with adenovirus expressing the *human* thyrotropin receptor (TSHR) A-subunit (amino acids 1-289). However, because non-identity between the *human* and *mouse* TSHR (~87 % amino acid homology), we compared the responses of mice immunized with adenoviruses expressing either the *mouse* or the *human* TSHR A-subunit. Wild-type (wt) BALB/c mice immunized with the *mouse* A-subunit developed neither TSHR antibodies (measured by flow cytometry) nor thyroid lymphocytic infiltration. However, wt C57BL/6 mice developed sparse intrathyroidal lymphocyte infiltration without antibody production. Depletion of naturally occurring regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells had little effect. These results indicate the inability to break tolerance to the *mouse* TSHR in wt mice. In contrast, TSHR knockout (KO) BALB/c mice generated *mouse* TSHR antibodies in response to *mouse* A-subunit immunization and augmented *human* TSHR antibody response to *human* A-subunit immunization. Thyroid stimulating antibody titers measured in a functional bioassay were comparable in *human* A-subunit immunized wt mice and in TSHR KO mice immunized with either the *mouse* or *human* A-subunit. In conclusion, immune response to the *mouse* TSHR is readily induced in TSHR KO but not in wt mice. Only in the former does immunization with adenovirus expressing the *mouse* A-subunit generate antibodies capable of activating the *mouse* TSHR. TSHR KO mice are, therefore, of value for future studies dissecting the autoimmune response to the *mouse* TSHR.

## **Introduction**

Graves' disease is a common organ-specific autoimmune disease that targets the thyroid gland. It is mediated by an abnormal humoral immune response against the thyrotropin receptor (TSHR), a thyroid-specific antigen. Thus, agonistic TSHR antibodies (thyroid stimulating antibodies; TSAb) cause overproduction of thyroid hormones from, and diffuse enlargement of the thyroid gland (reviewed in (1)). We previously generated a murine model of experimental Graves' disease by intra-muscular injection of recombinant adenovirus encoding the full-length *human* TSHR (764 amino acids) (*Ad-human* TSHR) (2) or the TSHR A-subunit (amino acids 1-289) (*Ad-human* TSHR289; hereafter *Ad-human* TSHR A-subunit) (3). This approach is highly efficient for inducing TSAb and Graves-like hyperthyroidism in a susceptible mouse strain (BALB/c). All other Graves' mouse models so far reported also involved immunization with the *human* TSHR cDNA (reviewed in (4) with one exception which used the *mouse* TSHR cDNA (5). In order to produce hyperthyroidism, TSAb induced by immunization with the *human* TSHR must cross-react with and stimulate the endogenous *mouse* TSHR. However, heterologous antigens are usually more antigenic than homologous self-antigens. Indeed homology between the *human* and *mouse* TSHRs is ~87 % at amino acid levels (6,7). Furthermore, different pathogenic mechanisms can be elicited by antigens from different species, as observed in a murine model of experimental autoimmune encephalitis (8). Therefore, the present study was performed to compare the immunogenicity of the *human* and *mouse* TSHR antigens in our murine model using wild type (wt) and TSHR knockout (KO) mice.

## **Materials and methods**

### **Mice**

Female wt BALB/c and C57BL/6 (B6) mice (6 weeks old) were purchased from Charles

River Japan Laboratory Inc. (Tokyo, Japan). B6/129 mice genetically deficient for the TSHR (9) were obtained from Jackson Laboratory Inc. (Bar Harbor, ME, USA), and were backcrossed to wt BALB/c mice for six successive generations. Genotyping was done using tail DNA as previously described (9). Expression of H-2<sup>d</sup> and not H-2<sup>b</sup> was confirmed by flow cytometry using FITC- or PE-conjugated anti-MHC class II antibodies (clone 7-16.17 and M5/114.15.2; from BD Pharmingen, San Jose, CA and eBioscience, San Diego, CA, respectively). Flow cytometry was performed using FACSCanto II (BD Biosciences, San Diego, CA). All the mice were kept in a specific pathogen free facility. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

### **Recombinant adenoviruses and immunization protocol**

Non-replicative recombinant human adenovirus expressing the *human* TSHR A-subunit (Ad-*human* TSHR A-subunit) was previously constructed (3). The same adenovirus vector expressing the *mouse* TSHR A-subunit (Ad-*mouse* TSHR A-subunit) was constructed as described previously for the *human* A-subunit (3). Briefly, the gene encoding the *mouse* TSHR A-subunit (amino acids 1-289), amplified by PCR from pcDNA5/FRT-*mouse* TSHR (10) as a template, was ligated into the adenovirus shuttle vector pHMCMV6. Following verification of the nucleotide sequence, the DNA fragment coding CMV promoter, the TSHR A-subunit and the poly A signal was inserted into pAdHM14, yielding pAd-HM14-TSHR A-subunit. Adenovirus was produced by transfecting Pac I-digested pAd-HM14-TSHR A-subunit into 293 human embryonal kidney cells. Amplification, purification and determination of the viral particle concentration were as described previously (2).

Mice were injected intramuscularly in the quadriceps with 100  $\mu$ l PBS containing  $10^{10}$  or  $10^8$  particles of adenovirus on two occasions at three-week-intervals. Groups of mice were also treated by intraperitoneal injection of 250  $\mu$ g/mouse anti-CD25 monoclonal antibody

(PC61) (11) four days prior to the first immunization. Blood, spleens and thyroid tissues were obtained two weeks after the final immunization.

### **Detection of TSHR A-subunits by immunoblotting**

Expression of TSHR was determined by immunoblotting with 40  $\mu$ g of total cell lysates prepared from COS-7 cells either mock-infected or infected with Ad-*human* TSHR A-subunit or Ad-*mouse* TSHR A-subunit (multiplicity of infection (MOI) of 10,000 particles/cell). The signal was developed with our monoclonal *human* TSHR antibody (3H9) (12) and horse radish peroxidase ABC method (Vectastatin ABC kit, Vector Laboratories, Burlingame, CA).

### **Thyroxine (T<sub>4</sub>) and TSHR antibody measurements**

Serum free T<sub>4</sub> concentrations were measured with a radioimmunoassay kit (DPC free T<sub>4</sub> kit; Diagnostic Products, Los Angeles, CA). The normal range was defined as the mean  $\pm$  3 S.D. of control untreated mice.

TSHR antibodies in mouse sera were determined using two different methods. The first was a TSAb functional bioassay that measures TSAb was performed with Chinese hamster ovary (CHO) cells stably expressing similar levels of either the *human* or the *mouse* TSHR (CHO-*human* TSHR and CHO-*mouse* TSHR) (10). Briefly, cells ( $3 \times 10^3$  cells/well in a 96-well culture plate) were incubated in 100  $\mu$ l hypotonic Hanks' Balanced Salt Solution containing 0.5 mM isobutyl-methylxanthine, 20 mM HEPES, 0.25 % BSA and 5  $\mu$ l sera (1:20 dilution) or  $10^{-7}$  M bovine (b) TSH (Sigma-Aldrich, St. Louis, MO) as positive control for two hrs at 37 C.

The second assay for TSHR antibodies to the native TSHR (irrespective of their function) used flow cytometry with CHO-*human* TSHR and CHO-*mouse* TSHR cells. Briefly, the cells were incubated for 60 min with PBS containing mouse sera (1:100 dilution) followed by FITC-conjugated anti-mouse IgG antibody (F2772, Sigma-Aldrich) for 60 min. Cells were

analyzed using FACSCanto II as previously described (11).

### **Thyroid histology**

Thyroid histology was examined with hematoxylin and eosin (H & E) staining of formalin-fixed tissue sections.

### **Statistical analysis**

Data on the titers of antibodies were analyzed by t-test. A 'p' value of less than 0.05 was considered statistically significant.

## **Results**

### **Adenovirus expression of the *mouse* TSHR A-subunit protein *in vitro***

To confirm *mouse* A-subunit expression by Ad-*mouse* TSHR A-subunit, COS-7 cells were infected with Ad-*mouse* TSHR A-subunit or, as a positive control, with Ad-*human* TSHR A-subunit at a MOI of 10,000 particles per cell. Two days later, the cells were harvested and analyzed by immunoblotting. TSHR A-subunit protein of both species (~50 kDa) was clearly detected using a monoclonal antibody to the human TSHR (Fig. 1A). The *human* TSHR A-subunit is approximately 5 kDa larger than the *mouse* TSHR A-subunit, as expected because of an additional N-linked glycan moiety and to a lesser extent the histidine-tagging at its C-terminus in the former (Fig. 1B). Our previous study (13) has shown that all the potential N-glycosylation sites are indeed glycosylated in the human TSHR.

### **Immunization of BALB/c and B6 mice with adenoviruses expressing the *mouse* or *human* TSHR A-subunit**

Wt BALB/c and B6 mice, which are susceptible and resistant respectively to induced hyperthyroidism (2) were immunized twice with Ad-*mouse* TSHR A-subunit or Ad-*human*

TSHR A-subunit. Serum T<sub>4</sub> and anti-TSHR antibody levels were measured two weeks after the second immunization. Although increased T<sub>4</sub> levels were observed in seven out of 10 BALB/c mice immunized with 10<sup>10</sup> particles of the *human* A-subunit (Fig. 2A), serum T<sub>4</sub> levels were unchanged relative to controls in BALB/c or B6 mice immunized with the *mouse* A-subunit (10<sup>10</sup> or even 10<sup>11</sup> particles) (Fig. 2D & G). Likewise, TSHR antibodies determined by flow cytometry using CHO-*human* TSHR cells were positive in most mice immunized with 10<sup>10</sup> particles of the *human* A-subunit (Fig. 2B) but those determined with CHO-*mouse* TSHR were negative in both BALB/c and B6 strains immunized with 10<sup>11</sup> particles of the *mouse* A-subunit (Fig. 2F & I). Naturally occurring regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells (Treg) are a T cell subset that negatively regulates the immune response (11). Treg depletion with anti-CD25 (PC61) had no detectable effect on T<sub>4</sub> or antibody levels (Fig. 2D to I).

We also evaluated cross-reactivity for the *mouse* TSHR of antibodies induced by immunization with the *human* A-subunit. Unlike their recognition of the *human* TSHR expressed on the surface of CHO cells (Fig. 2B), these antibodies exhibited minimal binding to CHO-*mouse* TSHR cells (Fig. 2C). However, these antibodies indeed induced hyperthyroidism *in vivo* as mentioned above (Fig. 2A) and showed comparable TSAb activities on CHO-*human* TSHR and CHO-*mouse* TSHR cells (Fig. 3A & B).

Histological examination revealed that the thyroid glands from *human* A-subunit immunized wt BALB/c mice showed thyroid hyperplasia typical of Graves' disease, but without lymphocyte infiltration (data not shown), as reported previously (2). However, a small proportion (20-33%) of the thyroids from wt B6 mice immunized with the *mouse* A-subunit had sparse intrathyroidal lymphocyte infiltration (Fig. 4 and Table 1). As for TSHR antibody induction, Treg depletion had little effect on thyroiditis.

There are two major findings in the foregoing experiments. First, it is extremely difficult to break tolerance and induce antibodies to the *mouse* TSHR on immunizing wt mice with the *mouse* A-subunit (even with Treg depletion). Second, although antibodies generated by *human* A-subunit immunization in BALB/c mice are functional in that they induce hyperthyroidism,

cross-reactivity with the *mouse* TSHR is poor, at least as detected by flow cytometry.

### **Immunization of TSHR KO BALB/c mice with adenovirus expressing the *mouse* or *human* A-subunit**

In principle, TSHR KO mice should not be tolerant to the *mouse* TSHR and, therefore, a stronger response could be anticipated to immunization with the *mouse* A-subunit. However, TSHR KO mice were originally on a B6/129 background that is resistant to induction of hyperthyroidism when immunized with the *human* A-subunit (2). Therefore, we back-crossed these mice for six generations onto a BALB/c background.

Indeed, unlike wt BALB/c mice (Fig. 2F), TSHR KO BALB/c mice immunized with the *mouse* A-subunit developed high titers of TSHR antibodies as detected by flow cytometry with CHO-*mouse* TSHR cells (Fig. 5A). In addition, TSHR KO mice responded vigorously to immunization with the *human* A-subunit as measured using CHO-*human* TSHR cells (Fig. 5D). The following comparisons provide insight into immune responses to the *mouse* vs. *human* TSHR:-

- 1) The levels of antibodies induced using the *human* A-subunit (and measured using the *human* TSHR) were higher in TSHR KO mice than in wt mice (Fig. 5D vs. Fig. 2B) ( $p < 0.01$ ).
- 2) Antibodies induced in TSHR KO mice to the *mouse* A-subunit bound well to the *mouse* TSHR but cross-reacted poorly with the *human* TSHR (Fig. 5A vs. 5B) ( $p < 0.05$ ).
- 3) In contrast, antibodies induced to the *human* A-subunit, which bound to the *human* TSHR, cross-reacted well with the *mouse* TSHR (Fig. 5D vs. 5C).

In terms of biological activity *in vivo*, induced TSHR antibodies will have no effect on thyroid function in TSHR KO mice because the thyroids in these mice lack the TSHR. However, functional TSHR antibodies can be assayed *in vitro* using CHO-*mouse* or -*human* TSHR cells. Regardless of which cell type was used, TSAb was detectable in sera from some of the mice whether immunized with the *mouse* or *human* A-subunits (Fig. 6). As with flow cytometry, TSAb activity was more readily detectable in the sera of mice immunized with the

*mouse* A-subunits when measured in a bioassay with CHO-*mouse* TSHR than in CHO-*human* TSHR cells (Fig. 6A & B), but a difference was not statistically significant, presumably because of variability of TSAb values. Finally, the thyroid glands from TSHR KO mice showed no histological change upon immunization (data not shown).

## **Discussion**

Despite our previous report showing efficient induction of TSAb and Graves-like hyperthyroidism in BALB/c mice by immunization with Ad-*human* TSHR A-subunit (3), we were unable to elicit TSHR antibodies by immunizing the same mouse strain with the *mouse* homolog (Ad-*mouse* TSHR A-subunit). B6 mice, which are resistant to developing hyperthyroidism after immunization with the *human* A-subunit, were also tolerant to the *mouse* A-subunit in terms of antibody production, and only a minority of animals developed sparse intra-thyroidal lymphocyte infiltration. Depletion of Treg had no effect on TSHR antibody generation or thyroiditis in either mouse strain. These results show that it is extremely difficult to break tolerance (particularly in terms of antibody) against the *mouse* TSHR in mice.

Central tolerance is a mechanism for preventing harmful immune responses to self-antigens. Ectopic expression of a large array of tissue-specific self-antigens (TSAs) in thymic medullary epithelial cells (mTECs) is found to be critical for this type of tolerance (14,15). Such promiscuous expression of TSAs in thymic medulla induces effective negative selection (e.g., clonal deletion) of TSA-specific self-reactive T cells. Thymic expression of some but not all self-antigens is controlled by the transcription factor autoimmune regulator (AIRE). For example, AIRE KO mice lack thymic expression of insulin and develop type 1 diabetes in their early life (16,17). Mutations in AIRE in mice cause an autoimmune disorder that affects a spectrum of organs and those in humans result in autoimmune disease APS-1 (autoimmune polyendocrine syndrome -1) (14,15). However, thymic expression of the *mouse* TSHR and the time course and magnitude of antibody responses to Ad-*human* TSHR A-subunit immunization

have recently been shown to be only moderately influenced by the absence of AIRE (18).

TSHR KO mice lack thymic expression of the TSHR and are unable to develop central tolerance to the TSHR. Importantly, TSHR antibodies were induced in TSHR KO BALB/c mice by immunization with the *mouse* A-subunit, consistent with the fact that the *mouse* TSHR is no longer an autoantigen. Although these mice can not develop hyperthyroidism because they lack TSHR expression in the thyroids, their sera contain strong TSAb activities, the levels being comparative, or even superior, to those in wt BALB/c mice immunized with Ad-*human* TSHR A-subunit, indicating that TSHR KO BALB/c mice can generate a potent immune response to the *mouse* TSHR. Thus thymic expression of this molecule likely sets the threshold of the immune response to the TSHR.

Incidentally, TSHR KO mice were previously tested for their immune response to the full-length *human* TSHR (19). However, immunization was performed by “naked” DNA vaccination, a protocol with a variable outcome involving minimal adjuvant which is much less effective than adenovirus (reviewed in (4)). Moreover, this earlier study was performed in a different mouse strain. Consequently, we cannot compare the lack of antibody differences between C57BL/6 /129 TSHR KO and wt mice vaccinated with the *human* TSHR DNA (19) with the findings in the present study, namely stronger responses in BALB/c TSHR KO vs. wt mice immunized with Ad-*human* TSHR A-subunit.

Mouse models of pemphigus and anti-neutrophil cytoplasmic autoantibody associated glomerulonephritis/vasculitis have recently been successfully generated using desmoglein 3 and myeloperoxidase, respectively, KO mice (20,21). Furthermore, stronger immune response to interphotoreceptor retinoid-binding protein (IRBP) and H/K APTase  $\beta$ -subunit, and development of exacerbated experimental autoimmune uveitis and gastritis by adoptive transfer of spleen cells have been demonstrated in the cognate antigen deficient mice as compared to wt mice (22,23). Additionally, in a uveitis model (23), transplantation of the thymus from wt to IRBP KO mice reverted enhanced anti-IRBP immune response and exacerbated uveitis, and wt

recipients of KO thymus developed stronger immune response to IRBP and severer uveitis.

Overall, KO mice appear to be very useful to elicit strong immune responses to self-antigens.

It is worth noting here that thymic expression of self-antigens is also crucial for generation of antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> Treg which are a main player for peripheral tolerance and control at the periphery self-reactive T cells escaped thymic negative selection (24). Thus thymic tolerance likely involves elimination of both self-reactive effector T cells and generation of Treg. However, the development of humoral immune responses to the *mouse* TSHR in TSHR KO mice, but not in Treg-depleted wt mice, indicates that central tolerance, rather than Treg-mediated peripheral tolerance, plays a major role in tolerance to the *mouse* TSHR. Nevertheless, we cannot completely exclude a possibility of peripheral tolerance mediated by expression of the *mouse* TSHR in the thyroids, which may involve presentation of the TSHR antigen (particularly the shed TSHR A-subunit) (25) by immature dendritic cells in the regional lymph nodes of the thyroids (26).

Another interesting finding in the present study is the relatively poor cross-reactivity of antibodies elicited by the *human* A-subunit immunization for the *mouse* TSHR when measured by flow cytometry. This finding was unexpected because such antibodies generated in wt BALB/c mice effectively induce hyperthyroidism in a majority of mice (2,3). However, TSHR antibodies induced by immunization with either the *human* or *-mouse* TSHR A-subunit were similarly effective in a TSAb bioassay using CHO cells expressing either of these two receptor species. Several amino acids on the *human* TSHR have been identified as contributing to the binding sites of stimulating (IRI-SAb2, IRI-SAb3 and M22, all of which also have TBI activities) and blocking (1H7 and RSR-B2) monoclonal anti-*human* TSHR antibodies (56T, 58K, 60I, etc) (27,28). All these amino acids are identical between the *human* and *mouse* TSHR (Fig. 1B), consistent with IRI-SAb2 and IRI-SAb3 also binding to the *mouse* TSHR (27). Overall, presently available data indicate that TSHR antibodies to the TSH binding site on the *human* TSHR can cross-react with the *mouse* TSHR in BALB/c mice.

Finally, although homology between *human* and *mouse* TSHR is relatively high, the Graves'

murine model using Ad-*human* TSHR A-subunit that we previously established may not be an ideal autoimmune disease model. Our present findings show that, rather than studying responses to a cross-reacting receptor (the *human* TSHR), immunization of TSHR KO mice with an adenovirus expressing the *mouse* TSHR A-subunit will be invaluable tools for dissecting responses to the autoantigen in *mice*, the *mouse* TSHR.

Table 1. Incidence of intrathyroidal lymphocyte infiltration in mice injected with Ad-*mouse* TSHR A-subunit with/without PC61.

mouse strains	immunization	no. of mice examined	no. of thyroiditis (% incidence)
BALB/c	control	3	0
	10 <sup>8</sup> Ad- <i>mouse</i> TSHR A-subunit	8	0
	10 <sup>9</sup> Ad- <i>mouse</i> TSHR A-subunit	5	0
	10 <sup>9</sup> Ad- <i>mouse</i> SHR A-subunit plus PC61	6	1 (17 %)
B6	control	10	0
	10 <sup>8</sup> Ad- <i>mouse</i> TSHR A-subunit	8	2 (25 %)
	10 <sup>9</sup> Ad- <i>mouse</i> TSHR A-subunit	10	2 (20 %)
	10 <sup>9</sup> Ad- <i>mouse</i> TSHR A-subunit plus PC61	6	2 (33 %)

## Figure legends

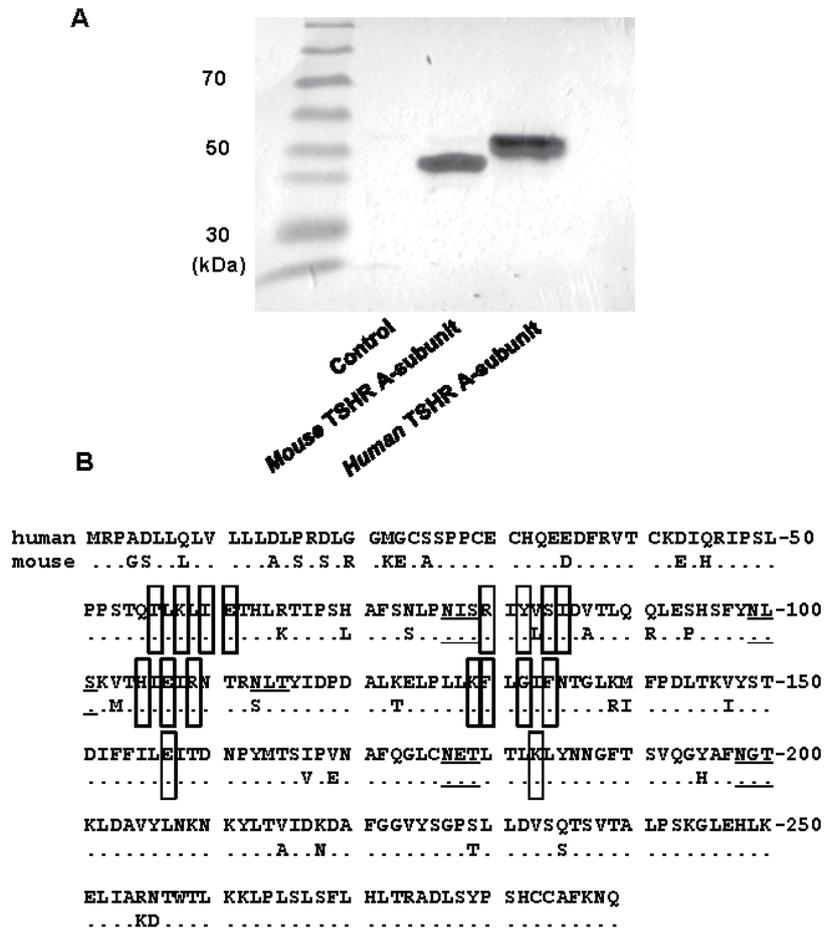


Figure 1A. (A) Western blot analysis of TSHR expression in COS cell infected with Ad-*human* TSHR A-subunit or Ad-*mouse* TSHR A-subunit, and (B) Amino acid sequence alignment of the *human* and *mouse* TSHR A-subunits (amino acids 1-289). A, The total cell lysates from control COS cells and those infected with Ad-*human* TSHR A-subunit or Ad-*mouse* TSHR A-subunit were subjected to western blotting as described in the *Materials and Methods*. B, Amino acid residues assigned for antibody binding sites are boxed (refs. 27 and 28). N-linked glycosylation sites are underlined.

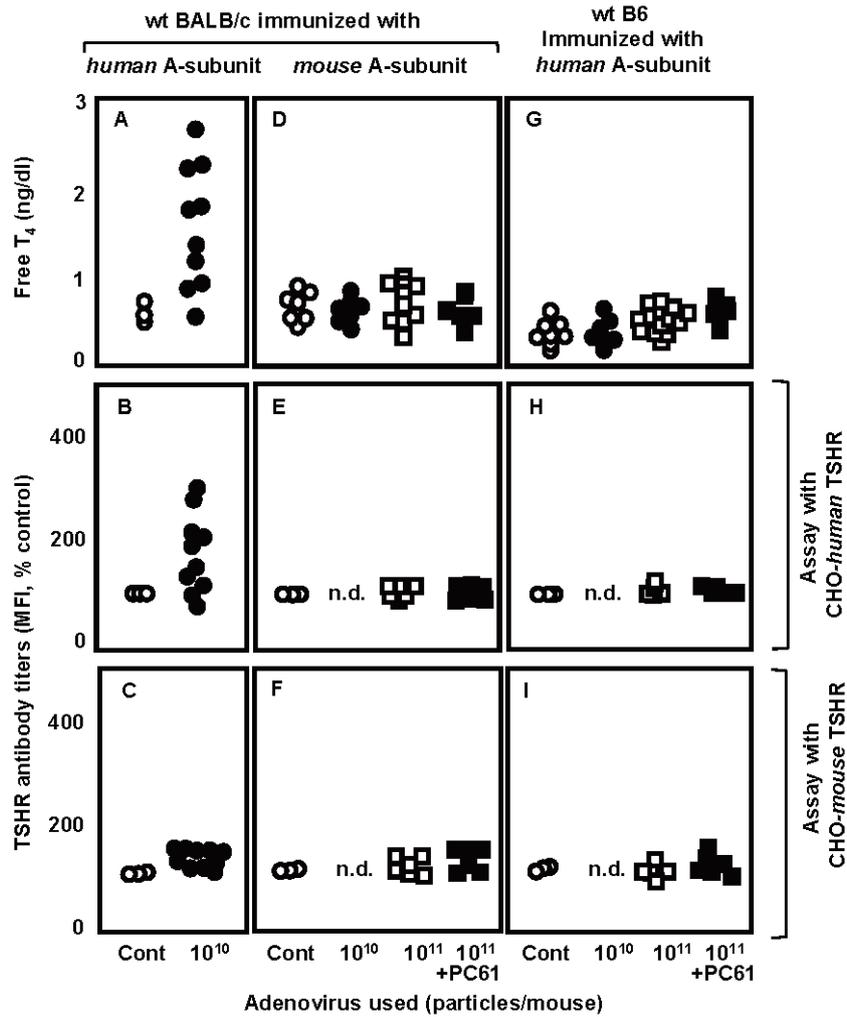


Figure 2. Free T<sub>4</sub> concentrations and TSHR antibody titers determined by flow cytometry in sera from control and immunized wt BALB/c and B6 mice. Mice were left untreated (cont) or immunized twice with Ad-*human* or *mouse* A-subunits and bled two weeks after second immunization. Some mice were depleted of Treg using anti-CD25 (PC61). Serum free T<sub>4</sub> was measured by RIA (A, D and G) and TSHR antibodies by flow cytometry using CHO-*human* TSHR (B, E and H) and CHO-*mouse* TSHR cells (C, F and I) as shown in the *Materials and Methods*. Data for individual mice are shown as ng/dl for free T<sub>4</sub> and as % control of the mean fluorescence index (MFI) for TSHR antibodies.

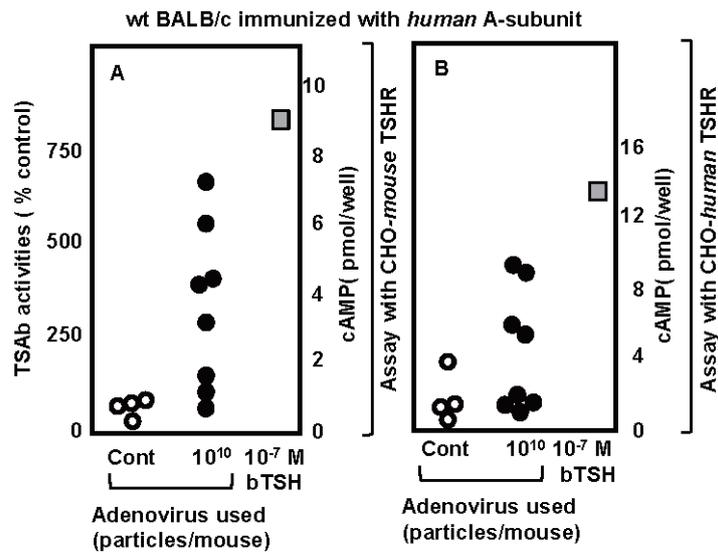


Figure 3. TSAb activities in sera from control and immunized wt BALB/c mice measured by a bioassay using CHO-*human* or -*mouse* TSHR cells. Sera used in Fig. 2 were subjected to a TSAb assay (described in *Materials and Methods*). TSAb values (means of duplicated assays, % control and pmol/well) are shown for individual mice. cAMP produced by 10<sup>-7</sup> M bTSH was also shown.

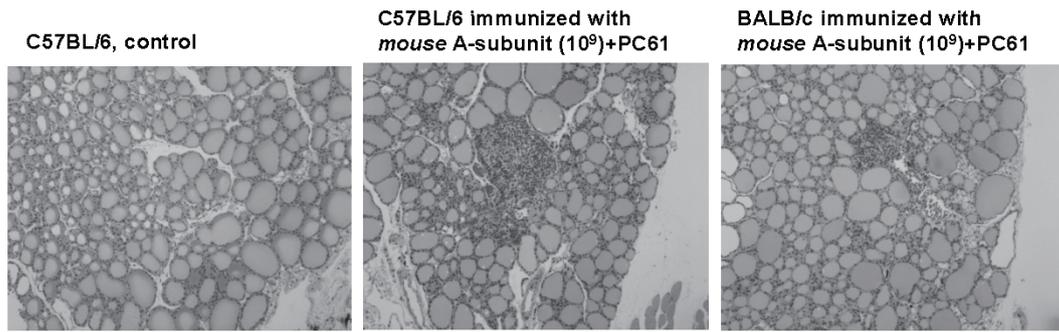


Figure 4. Histology (H and E staining) for representative thyroid glands from control B6 mice and B6 and BALB/c mice immunized with Ad-*mouse* TSHR A-subunit and depleted of Treg by PC61. Magnification, x100.

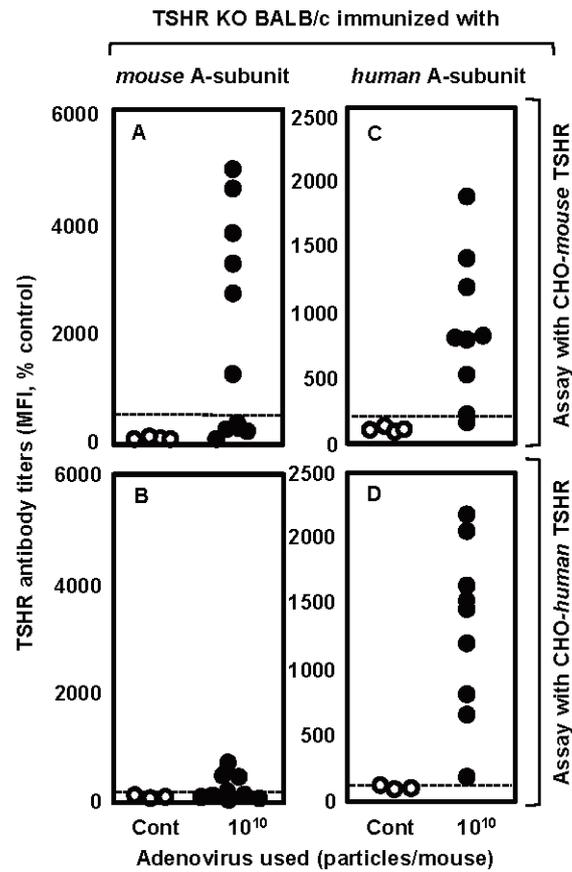


Figure 5. TSHR antibodies in sera from immunized TSHR KO BALB/c mice measured by flow cytometry using CHO-*mouse* or -*human* TSHR cells. TSHR KO mice were left untreated (cont) or immunized twice with Ad-*human* or -*mouse* TSHR A-subunit and bled two weeks after second immunization. TSHR antibodies were measured by flow cytometry (described in *Materials and Methods*) using CHO-*human* TSHR (A and C) and -*mouse* TSHR (B and D). Values are % control of the mean MFI for individual mice.

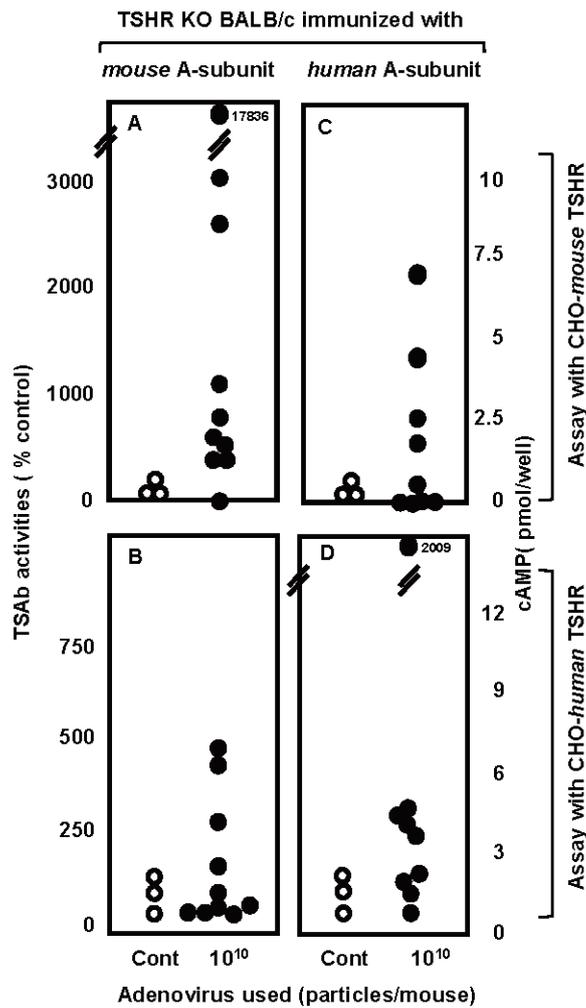


Figure 6. TSAb activities in sera from control and immunized TSHR KO BALB/c mice measured by a bioassay using *CHO-human* or *-mouse* TSHR cells. Sera used in Fig. 5 were subjected to a TSAb assay (described in *Materials and Methods*). TSAb values (means of duplicated assays, % control and pmol/well) are shown for individual mice.

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