Adoptive Transfer of Anti-Thyrotropin Receptor (TSHR) Autoimmunity from TSHR Knockout Mice to Athymic Nude Mice.

Running title: A Graves’ mouse model with mouse thyrotropin receptor

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Key words: thyrotropin receptor, autoimmunity, Graves’ disease, adoptive transfer

Disclosure summary: The authors have nothing to disclose.

The Word Count: 4572
Abstract

We have recently shown that wild type (wt) mice are highly tolerant, while thyrotropin receptor (TSHR) knockout (KO) mice are susceptible, to immunization with the mouse (m) TSHR, the autoantigen in Graves’ disease. However, because TSHR KO mice lack the endogenous TSHR, Graves-like hyperthyroidism cannot be expected to occur in these mice. We therefore performed adoptive transfer of splenocytes from TSHR KO mice into nude mice expressing the endogenous TSHR. Anti-TSHR autoantibodies were detected in ~50 % recipient mice 4 weeks after adoptive transfer of splenocytes (5x10^7/mouse) from TSHR KO mice immunized with adenovirus expressing mTSHR A-subunit and persisted for 24 weeks. Depletion of regulatory T cells by anti-CD25 antibody in the donor mice increased successful transfer rates without increasing antibody levels. Some recipient mice showed transient increases in thyroid-stimulating antibodies and T4 levels 4-8 weeks after transfer, but many became thyroid-blocking antibody-positive and hypothyroid 24 weeks later. Adoptive transfer of splenocytes from naïve TSHR KO mice transiently induced very low antibody titers when the recipient mice were treated with anti-cytotoxic lymphocyte antigen 4 and anti-programmed cell death 1 ligand 1 antibodies for 8 weeks after transfer. Histologically, macrophages infiltrated the retrobulbar adipose tissues and extraocular muscles in a small fraction of the recipients. Our findings demonstrate successful adoptive transfer of anti-TSHR immune response from TSHR KO mice to nude mice. Although the recipient mice developed only transient and infrequent hyperthyroidism, followed by eventual hypothyroidism, induction of orbital inflammation suggests the possible role of anti-TSHR immune response for Graves’ orbitopathy.
The Introduction

Graves’ disease is a thyroid-specific autoimmune disease characterized by agonistic anti-thyrotropin receptor (TSHR) autoantibody (thyroid stimulating antibody, TSAb) that mediates overproduction of thyroid hormones (hyperthyroidism) and diffuse enlargement of the thyroid glands, and is frequently accompanied by extrathyroidal manifestations such as orbitopathy and dermopathy (1-4). We have previously established novel mouse models of experimental Graves-like hyperthyroidism in which hyperthyroidism can be efficiently induced in susceptible mouse strains (e.g., BALB/c) by repetitive immunizations with recombinant adenovirus expressing the human (h) full-length thyrotropin receptor (5) or its A-subunit (amino acids 1-289) (6) (Ad-hTSHR or Ad-hTSHR A-subunit). In these models, however, hyperthyroidism was transient, and intrathyroidal lymphocyte infiltration or extrathyroidal lesions were not observed.

Human TSHR is not an authentic autoantigen in mice, because homology between the hTSHR and mouse (m) TSHR is ~87% at amino acid levels (7, 8). Thus, in our models mentioned above, hyperthyroidism is induced by anti-hTSHR antibodies that cross-react with endogenously expressed mTSHR in mouse thyroid glands (9). However, we have recently shown that immunization of wild type (wt) BALB/c mice with Ad-mTSHR A-subunit does not provoke an anti-TSHR immune response, whereas anti-mTSHR antibodies including TSAb can readily be induced in TSHR knockout (KO) mice (10). These data strongly indicate that tolerance to mTSHR, the authentic autoantigen, is extremely strong in wt mice, and that this tolerance is absent in TSHR KO mice. However, TSHR KO mice lack the endogenous TSHR in their thyroid glands thus preventing us from studying development of Graves’ hyperthyroidism/orbitopathy in these mice.

Because our goal is to establish an ideal Graves’ mouse model using mTSHR as an autoantigen, we performed adoptive transfer experiments of splenocytes from TSHR KO mice into immune-deficient athymic nude mice expressing the endogenous TSHR in their thyroids as
well and also likely in their orbital tissues. We now show successful adoptive transfer of anti-mTSHR autoimmune response from TSHR KO mice to nude mice. Despite transient and infrequent hyperthyroidism and eventual hypothyroidism, induction of orbital lesions indicates that anti-TSHR immune response appears to play at least partly a role for the development of Graves’ orbitopathy.

Materials and Methods

Mice

Female wt BALB/c mice (6 weeks old) and female athymic BALB/c nude mice (5 weeks old) were purchased from Charles River Japan Laboratory Inc. (Tokyo, Japan). TSHR KO BALB/c mice were previously generated by back-crossing TSHR KO B6/129 mice (11) (Jackson Laboratory Inc., Bar Harbor, ME, USA) with wt BALB/c mice for 6 successive generations (10). All 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

Construction, amplification and purification of non-replicative recombinant human adenovirus expressing the mTSHR A-subunit (Ad-mTSHR A-subunit) has been described previously (10). TSHR KO mice were injected intramuscularly in the quadriceps with 100 μl PBS containing $10^{10}$ particles of adenovirus on two occasions at three-week-intervals. For long-term follow-up studies (Figure 1), mice were bled regularly during 24 weeks after the second immunization.
Adoptive transfer of splenocytes

For adoptive transfer experiments (Figure 2), spleens were obtained from immunized mice 2 to 4 weeks after the final immunization or from naïve mice. For some studies, a group of mice was depleted of CD4⁺CD25⁺ regulatory T cells (Tregs) by intraperitoneal injection of 500 µg/mouse anti-CD25 monoclonal antibody (PC61) (12) 4 days prior to euthanization.

Splenocytes were injected intraperitoneally (i.p.) into nude BALB/c mice (5 x 10⁷ in 100 µl PBS/mouse). Groups of recipient mice were treated, after transfer, by i.p. injection of 100 µg/mouse anti-cytotoxic T-lymphocyte antigen 4 (CTLA4; clone #UC10-4F10-11) and/or anti-programmed cell death 1 ligand 1 (PD-L1; clone #10F.9G2) monoclonal antibodies (both from Bio X Cell, Inc., West Lebanon, NH, USA), alone or in combination, twice a week for 8 weeks. Mice were bled regularly during 24 weeks after transfer.

Thyroxine (T₄) and TSH measurements

Serum free T₄ concentrations were measured by radioimmunoassay (RIA) as previously described (10). Serum TSH was measured by a specific mouse TSH RIA as previously described (13) except that mouse TSH reference (AFP9090D) was used instead of mouse TSH/LH reference (AFP51718MP). The normal range was defined as the mean ± 3 S.D. of untreated control mice.

TSHR antibody measurements

TSHR antibodies in mouse sera were determined using 3 different methods. The first assay for binding of TSHR antibodies to the native TSHR (irrespective of their function) used flow cytometry with Chinese hamster ovary (CHO) cells stably expressing the mTSHR (CHO-mTSHR). 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
The second was a TSAb functional bioassay that measures antibodies that stimulates the TSHR expressed in the thyroids with CHO-mTSHR. Briefly, CHO-mTSHR cells (5 x 10⁴ cells/well in a 96-well culture plate) were incubated in 100 µl hypotonic Hanks’ Balanced Salt Solution containing 0.5 mM isobutyl-methylxanthine, 20 mM HEPES, 0.25 % BSA and PEG-precipitated IgG (30 µl equivalent) for 2 hrs at 37 C. cAMP in the supernatants was measured with LANCE cAMP kit (PerkinElmer, Boston, MA). TSAb activities were expressed as a percentage of cAMP levels attained with control sera.

The third was a functional bioassay for thyroid blocking antibody (TBAb), in which 100 µU/ml bovine (b) TSH (Sigma-Aldrich, St. Louis, MO) was added to the buffer used for TSAb assay. TBAb (percentage of inhibition of TSH-induced cAMP) was calculated as follows:

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[1-\text{(test serum with bTSH-test serum without bTSH)/(normal serum with bTSH-normal serum without bTSH)}] \times 100.
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Tg autoantibody measurements

Tg was purified from mouse thyroid glands as previously described (14). ELISA wells were coated overnight with 100 µl Tg protein (10 µg/ml) and incubated with mouse sera (1:100 dilutions). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (A3673, Sigma), color was developed using orthophenylene diamine and H₂O₂ as substrate and optical density (OD) read at 492 nm.

**Thyroid and orbital histology**

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

Immunohistochemistry was also performed to visualize macrophage infiltration in orbital tissues. Mice were anesthetized with pentobarbital and perfused via the left ventricle with cold 4%
paraformaldehyde solution. Whole orbits including the surrounding bones were excised, fixed in 4% PFA for at least 24 hrs, decalcified in EDTA for 24 hrs and, after dehydration, embedded in paraffin. The embedded issues were cut by a cryostat into 5-μm-thick sections, and incubated overnight at 4 C with rat anti-mouse F4/80 (1:100 dilution; Cl:A3-1, #MCA497, Abd Serotech, Dusseldorf, Germany). The biotin coupled secondary antibody (Biotin goat-anti rat, #sc-2041, Santa Cruz Biotech Inc., Santa Cruz, CA) was visualized using commercially available ABC and DAB-Kits (Vectastain ABC/ DAB, #PK-6100 and #SK-4100, Vector Labs, Burlingame, CA, USA). Endogenous peroxidase activity was inhibited with 3% H$_2$O$_2$. Slides were counterstained with hematoxylin.

Cells were counted at 400x magnifications. All counts were done blinded. To evaluate muscle involvement we counted cells per individual muscles for each per slide (usually 5 muscles). In addition, all cells within the adipose tissue and the muscle conus were counted. All counted slides were photographed using a digital microscope camera system (Nikon Digital Sights DS-U1, Nikon Instruments Europe, Kingston, UK). Since the available area of adipose tissue within the muscle conus varied in size from slide to slide, the area of adipose tissue was assessed using image editing software (Gimp 2.6.11, The Gimp Development Team) to calculate the amount of pixels (px) that are part of the adipose tissue in each slide. A quotient of positive cells per 1000 px was subsequently established to compare and proceed the results of each slide.

**Statistical analysis**

Data on the titers of antibodies were analyzed by $t$-test, and the successful transfer rates by chi-square. Statistical evaluation of cell counts was performed with Prism 5.01 (GraphPad software Inc, La Jolla, CA). Testing for significance was conducted using the Kruskal-Wallis one-way analysis of variance. Post testing was conducted with Bonferroni-Dunn’s multiple comparison test. A ‘p’ value of less than 0.05 was considered statistically significant.
Results

Immune response to Ad-mTSHR A-subunit in TSHR KO mice

We have recently shown that over 70% TSHR KO BALB/c mice developed anti-TSHR antibodies 2-4 weeks after 2 immunizations of Ad-mTSHR A-subunit (10). We have now monitored antibody responses over a longer time period. In TSHR KO mice that were antibody-positive by flow cytometry 4 weeks after the second immunization, TSHR antibodies remained positive for up to 24 weeks after second immunization (Figure 1A). The representative histogram for the flow cytometry was shown in Figure 1D. The antibody levels gradually declined but the difference was not statistically significant. These anti-TSHR antibody-positive mice were TSAb-dominant (TSAb being positive in all mice, but TBAb positive in some) 4 weeks after the second immunization, but most became TSAb-negative and TBAb-positive 20 weeks later (Figure 1B and C).

Anti-TSHR antibodies and thyroid function following adoptive transfer of splenocytes from immunized TSHR KO mice

The in vivo effect of functional anti-TSHR antibodies in TSHR KO mice following immunization with Ad-mTSHR A-subunit (Figure 1) cannot be examined because these mice lack expression of the target autoantigen TSHR. Therefore, we performed adoptive transfer of splenocytes from immunized TSHR KO BALB/c mice to immune deficient athymic nude BALB/c mice expressing the endogenous TSHR but lacking mature T or B cells. We anticipated that TSHR-reactive lymphocytes from TSHR KO mice would recognize the endogenously expressed TSHR in the recipient nude mice as an autoantigen and also that anti-TSHR antibodies produced would react with the endogenous TSHR in the recipient mice. Circulating anti-TSHR autoantibodies were detected in 6/13 (46%) of the recipient mice 4 weeks after adoptive transfer of splenocytes from the donor TSHR KO mice that were produced anti-TSHR antibodies after 2 immunizations with Ad-mTSHR A-subunit (Figure 2A; note that
most mice were sacrificed on 6th week, so that only 2 mice were followed up for 24 weeks). Interestingly, antibody production also occurred in 4/5 (80 %) of the recipient mice following adoptive transfer of splenocytes from immunized but antibody-negative donor TSHR KO mice (Figure 2C). Prior Treg depletion using anti-CD25 monoclonal antibody increased transfer efficiency 4 weeks after adoptive transfer of splenocytes from antibody-positive donor and antibody-negative donors, respectively (Figure B and D) (13/14, 93 % in Figure 2B and D versus (vs.) 10/18, 56 % in Figure 2A and C, p < 0.05) without increasing the antibody titers in the recipient mice. In all groups, antibody titers remained positive for 24 weeks. It may be worthy noted here that the titers of anti-TSHR antibodies are usually higher in the recipients (Figure 2) than the donors (Figure 1A). Although the exact reason(s) are unclear, lymphocyte proliferation by homeostatic proliferation in the recipients may augment antibody production (see Discussion).

Serum T₄ and TSH levels were subsequently determined. Despite the highly successful transfer of antibody production, the incidence of hyperthyroidism was disappointedly low in the recipient mice. Thus, only 5/24 (21 %) mice were hyperthyroid 4 weeks after transfer, 2/22 (9 %) 4 weeks later and none at 16 to 24 week time periods (Figure 3A). Conversely, 13/20 (65 %) mice became hypothyroid with sub-normal T₄ levels (Figure 3A) and increased TSH levels (Figure 3B). Consistent with these data, TSAb were dominant over TBAb 4 weeks after transfer, but finally became TBAb-dominant (Figure 4C and D). Five mice that were hyperthyroid 4 weeks after transfer (Figure 3A) deserve particular attention; 2 were sacrificed at 6th week; however, the remaining 3 hyperthyroid mice eventually developed hypothyroidism.

**Anti-TSHR antibodies and thyroid function following adoptive transfer of splenocytes from naïve TSHR KO mice**

We next examined whether anti-TSHR autoimmunity could be transferred from naïve TSHR KO mice to nude mice. Transfer of splenocytes from naïve TSHR KO mice transiently induced barely detectable antibodies in some mice (Figure 4A). We therefore attempted to
induce anti-TSHR antibodies by treating the donor mice before transfer with anti-CD25 antibody or by treating the recipient mice after splenocyte transfer with antibodies against negative co-stimulatory molecules such as CTLA4 and PD-L1. Although a single administration of anti-CD25, anti-CTLA4 or anti-PD-L1 antibodies was ineffective (Figure 4B to D), a combined injection of anti-CTLA4 and PD-L1 induced anti-TSHR antibodies at the 8 week time point, but this effect was transient (Figure 4E). Moreover, transiently induced low levels of anti-TSHR antibodies in the recipient mice treated with anti-CTLA4 and anti-PD-L1 antibodies (in Figure 4E) had no effect on thyroid function. Thus these mice remained euthyroid for 24 weeks (Figure 4F).

**Thyroid and retro-orbit histology**

H & E staining of the thyroid glands from hyperthyroid mice 4 weeks after transfer (see Figure 3A) showed the typical feature of Graves’ hyperthyroidism, namely diffuse enlargement of the glands and hypertrophy and hypercellularity of follicular epithelial cells (Figure 5C and D, compared to control mice in A and B). Three of these mice also showed small intrathyroidal lymphocyte infiltration (Figure 5E and F). In contrast, the thyroids of hypothyroid mice 24 weeks after transfer (Figure 4B) revealed a characteristic of TBAb-mediated hypothyroidism with thin epithelia without lymphocyte infiltration (Figure 5G and H). Of interest, extensive lymphocyte infiltration was observed in 2 recipient mice that did not produce anti-TSHR antibodies (one in Figure 2A, and the other in Figure 4E) (Figure 5I and J).

Orbital histology was also studied in 9 recipient mice adoptively transferred with splenocytes of TSHR KO mice that were immunized with Ad-mTSHR A-subunit and subsequently depleted of Tregs. Neither macroscopic findings of orbitopathy (swelling or redness) nor signs of production of extracellular matrix or adipogenesis were detected in H & E stained sections. However, in 2 mice that were anti-TSHR antibody-positive and euthyroid through the experiments (Figures 2A and 3A), we observed significantly increased numbers of infiltrating macrophages in both the adipose tissues and the interstitium of muscle tissues (no. 1
and 9 in Figure 6A and B). Representative photographs of control and macrophage-infiltrated orbits are provided in Figure 6C and D.

**Anti-Tg antibodies following adoptive transfer of splenocytes from immunized TSHR KO mice**

Development of extensive lymphocyte infiltration in the thyroids of 2 recipient mice indicates TSHR-induction of Hashimoto’s thyroiditis rather than Graves’ disease (15). Therefore, anti-Tg antibodies, an immunological hallmark of Hashimoto’s thyroiditis, were measured. Two mice with extensive lymphocyte infiltration, but not those with no or sparse lymphocyte infiltration, were positive for anti-Tg antibodies (Figure 7).

**Discussion**

We have recently demonstrated that immunization with Ad-mTSHR A-subunit readily induced anti-TSHR antibodies in TSHR KO mice, but not in wt mice (10). TSHR KO mice likely have T cells reactive with higher-affinity TSHR peptides than wt mice, because of lack of thymic expression of the TSHR that is crucial for negative selection (16). However, absence of the endogenous TSHR expression in the thyroids as well as orbital tissues of TSHR KO mice on the other hand hampered us from studying in vivo function of anti-TSHR immune response. Therefore, we here extended these studies and performed adoptive transfer of splenocytes from TSHR KO mice to immune deficient athymic nude mice harboring the endogenous TSHR. We found that splenocytes from Ad-mTSHR A-subunit-immunized TSHR KO mice adoptively transferred into nude mice produced readily detectable anti-TSHR antibodies for up to 24 weeks. However, sustained production of anti-TSHR antibodies was also observed for 24 weeks in immunized TSHR KO mice. Therefore, contrary to our expectation, these data do not necessarily imply that TSHR-reactive lymphocytes (primed by active immunization in the donor mice) were re-activated again by responding to the endogenous TSHR antigen in the
recipient mice. Instead, because T cells in lymphopenic hosts divide to reconstitute the peripheral lymphoid compartment (17), TSHR-reactive T cells primed in TSHR KO mice likely expanded in lymphopenic nude mice through homeostatic proliferation (17). However, the successful induction of anti-TSHR antibodies by transfer of splenocytes from TSHR KO mice that were immunized but did not produce antibodies indicate that T cells primed by active immunization and subsequently activated by homeostatic proliferation can fully activate anti-TSHR antibody-producing B cells. Since regulation of homeostatic proliferation by Tregs has been reported (18), increased successful transfer rates by depletion of Tregs support this idea.

In our original Graves’ model with Ad-hTSHR, although hyperthyroidism and TSAb production are transient and persists only for a few months, we have never measured anti-TSHR antibody titers by flow cytometry for a prolonged period of time. It is of interest to clarify whether or not persistent anti-TSHR antibody production can also be observed in the Graves’ model with wt mice and Ad-hTSHR. It may be noteworthy that a long-term persistence (8 months) of TSAb has been demonstrated in the Graves’ model using plasmid-TSHR and in vivo electroporation (19).

In contrast to the successful transfer of splenocytes from immunized donors, adoptive transfer of splenocytes from naïve TSHR KO mice failed to induce strong anti-TSHR immune response. Enhancement of immune response by antibodies against negative co-stimulatory molecules alone or in combination has previously been described to be very effective at enhancing immune responses (20, 21). However, applying this approach to adoptive transfer of splenocytes from native TSHR KO mice only transiently induced low levels of TSHR antibodies. Furthermore, our findings differ from the previous studies on a pemphigus model: adoptive transfer of splenocytes from naïve desmoglein KO mice (an autoantigen in this disease) successfully induced pemphigus in immune-deficient mice (22, 23). In that study, despite lower antibody titers in the recipients of splenocytes form naïve than in recipients of splenocytes from desmoglein-immunized donors, the degree of disease severity was comparable.
in the 2 groups. The explanation for the difference between our study and theirs is unclear.

The highly successful transfer of anti-TSHR immune response from immunized TSHR KO mice to the recipient nude mice did not, however, result in the efficient induction of Graves-like hyperthyroidism. Moreover, many mice developed hypothyroidism during a long-term follow-up. Based on thyroid histology, this outcome can be attributed to conversion of TSAb-dominant antibody repertoire to TBAb-dominance, rather than to the destruction of the thyroid glands by lymphocyte infiltration. Similar conversion of TSAb to TBAb was observed in TSHR KO mice immunized with Ad-mTSHR A-subunit and also has been demonstrated in some Grave’s patients previously (24, 25). It may be highly relevant to dissect the mechanisms underlying this phenomenon for developing new therapeutic strategies against Graves’ disease.

Although the incidence was very low, some mice developed variable levels of intrathyroidal lymphocyte infiltration. Such histological changes have never been observed in wt BALB/c mouse strain (used in this study) immunized with Ad-hTSHR (full-length or A-subunit) in most of our previous studies. Because there is limited cross-reactivity of anti-hTSHR antibodies with mTSHR (10), particularly in some strains (9), our present findings indicate similarly limited cross-reactivity of hTSHR-reactive T cells with mTSHR. An exception is massive intrathyroidal lymphocyte infiltration accompanied by hypothyroidism and autoantibodies to Tg and thyroid peroxidase in thyroid-specific hTSHR A-subunit transgenic BALB/c mice immunized with Ad-hTSHR A-subunit and depleted of Tregs (15), demonstrating destruction of hTSHR expressing mouse thyroids by hTSHR-reactive T cells.

Overall, our data demonstrate that naive TSHR-reactive lymphocytes from TSHR KO mice are not fully stimulated by the endogenous TSHR in the recipient mice. Thus, in the recipient nude mice, the endogenous TSHR did not stimulate either naïve TSHR-reactive T cells from TSHR KO mice or, at least in most cases, T cells primed by active immunization with Ad-mTSHR A-subunit. However, there were a few exceptions. Thus, a few nude recipients of transferred splenocytes from TSHR KO donors developed thyroid extensive lymphocytic infiltrates with anti-Tg antibodies, a process which reflects T cell recognition of mouse TSHR
peptides.

Graves’ orbitopathy is one of hallmarks of extrathyroidal manifestations accompanying Graves’ disease. Interestingly, many researchers have come to recognize that the TSHR is the primary autoantigen in this condition, although it is unclear whether anti-TSHR antibodies themselves are pathogenic or TSHR-reactive T lymphocytes are the primary mediators (3). It has also been reported that the receptor for insulin-like growth factor may be a second antigen (26). Furthermore, other candidate autoantigens have also been reported including calsequestrin, collagen XIII and G2s (27), which may however be secondary responses to tissue destruction (3).

Some mouse models of this condition have previously been reported (28-30), but have not fully been substantiated (30) or could not be reproduced (31). It may not be easy to induce the orbital lesions in mice, because of a difference in anatomy of orbits between humans and rodents: the enclosed bony orbit in humans, unlike connective tissue septa in the rodents, easily gives rise to increased pressure and mechanical trauma, leading to an aggravation of the immune response (3, 4). We observed significant increases in macrophage infiltration in the muscles and adipose tissues of the orbits in 2 out of 9 recipients of mTSHR-immunized donors, although clear muscle/fat tissue enlargement, lymphocyte infiltration or exophthalmos was not present. Similar macrophage infiltration has also been reported in the patients with Graves’ orbitopathy (32, 33). Since a profile dominated by Th1 type immune responses in the early disease stage followed by Th2 dominance in the later stage is proposed for Graves’ orbitopathy in humans (34), macrophage infiltration may represent the early stage of disease. Further progression may be hampered by the anatomical characteristics of mouse orbits mentioned above and/or lack of environmental factors such as smoking (1-3). Consequently, we prefer not like to make a definitive statement that our findings present an authentic mouse model of Graves’ orbitopathy. However, our data, as well as those from Banga’ group (35) showing connective tissue fibrosis in the orbits of BALB/c mice immunized with plasmid-TSHR by in vivo electroporation, are the first in vivo evidence to provide experimental evidence clearly
indicating a role for the TSHR in the pathogenesis of Graves’ orbitopathy. The observation that only some mice developed orbital changes resembles the human situation because only half of the patients with Graves’ disease develop orbitopathy.

In conclusion, we established a system for studying the responses to the mouse TSHR in mice. We believe that, rather than immunization with the receptor of human origin, use of mouse TSHR, as an autoantigen itself, will provide an optimal mouse model(s) harboring both Graves’ hyperthyroidism and orbitopathy.
Acknowledgements

We thank Drs. Sandra M. McLachlan and Basil Rapoport, Autoimmune Disease Unit, Cedars-Sinai Medical Center and University of California Los Angeles, for critical reading of the manuscript.
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Figure legends

Figure 1. Time-course of anti-TSHR antibodies (A), TSAb (B) and TBAb (C) in TSHR KO mice immunized with Ad-mTSHR A-subunit. TSHR KO mice were immunized twice with Ad-mTSHR A-subunit, and were bled regularly for 24 weeks. Serum anti-TSHR antibody levels, TSAb and TBAb were measured as described in the Materials and Methods. The broken lines indicate the upper limits of normal ranges. MFI, median fluorescence intensities. D, representative histogram for flow cytometry using CHO and CHO-mTSHR cells.

Figure 2. Time-course of anti-TSHR antibodies in nude mice adoptively transferred with splenocytes from TSHR KO mice immunized with Ad-mTSHR A-subunit with/without Treg-depletion by anti-CD25 antibody. TSHR KO mice were immunized twice with Ad-mTSHR A-subunit. Splenocytes (5 x 10^7/mouse) were prepared from immunized and antibody-producing donors (A and B) or immunized but antibody-nonproducing donors (C and D), and were adoptively transferred to nude mice. In B and D, Tregs were depleted 4 days before transfer. Anti-TSHR antibodies were measured by flow cytometry regularly for 24 weeks. The broken lines indicate the upper limits of normal ranges.

Figure 3. Time-course of Free T4, TSH, TSAb and TBAb in the recipient nude mice adoptively transferred with splenocytes from Ad-mTSHR A-subunit immunized TSHR KO mice. Some sera in Figure 2 were analyzed for T4, TSH, TSAb and TBAb levels as described in the Materials and Methods. The broken lines indicate the upper limits of normal ranges.

Figure 4. Time-course of anti-TSHR antibodies and free T4 in the recipient nude mice adoptively transferred with splenocytes of naïve TSHR KO mice and treated with antibodies against CD25, CTLA-4 and/or PD-L1. Mice were adoptively transferred with splenocytes of naïve TSHR KO mice, and left untreated (A) or treated with antibodies against CD25 (B),
CTLA-4 (C), PD-L1 (D) or CTLA-4/PD-L1 combination (E). Anti-TSHR antibodies were measured regularly for 24 weeks as in the legend for Figure 1. T4 (F) was measured in mice shown in C, D and E. The broken lines indicate the upper limits of normal ranges.

Figure 5. H & E staining of the thyroid glands. A & B, control mice; C & D, hyperthyroid mice (6 weeks after transfer); E & F, hyperthyroid mice with small lymphocyte infiltration (6 weeks after transfer); G & H, hypothyroid mice (24 weeks after transfer); I & J, mice with extensive lymphocyte infiltration (24 weeks after transfer). The magnifications, 40x for A, C, E, G and I and 100x for B, D, F, H and J.

Figure 6. Macrophage infiltration in the retrobulbar muscle and adipose tissues of the recipient nude mice. *, p<0.05; **, p<0.01. A and B, quantification of macrophage infiltration in the retrobulbar muscle (A) and adipose (B) tissues of nude mice in Figure 2B and D. The broken lines indicate the upper limits of normal ranges (Mean + 3S.D.). C and D, representatives of immunohistochemistry of the retrobulbar tissues from a control nude mouse (C) and a mouse showing macrophage infiltration (D). Positive stainings of macrophages were indicated by red circles in the insets (panel D). The magnifications, 25x for C and D, and 200x for the insets.

Figure 7. Anti-Tg autoantibody titers in mice with/without intrathyroidal lymphocyte infiltration. Anti-Tg autoantibody titers were determined by ELISA (see the Materials and Methods). Three sera from controls, 3 from hyperthyroid mice with no intrathyroidal lymphocyte infiltration at 4th week, 3 from hyperthyroid mice with sparse infiltration at 4th week and 2 from euthyroid mice with extensive infiltration at 24th week were used. Data are shown for individual mice. The broken lines indicate the upper limits of normal ranges.
A) TSHR Abs (MFI, % control)

B) TSAb (% control)

C) TBAb (% inhibition)

D) CHO vs. CHO-mTSHR

Nakahara et al. Figure 1
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Figure 7

- anti-Tg Abs (OD_{450})

- lymphocyte infiltration

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