

## Minor contribution of cytotoxic T lymphocyte antigen 4 and programmed cell death 1 ligand 1 in immune tolerance against mouse thyrotropin receptor in mice

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We have previously shown that wild type (wt) mice exhibit susceptibility to immunization with human (h) thyrotropin receptor (TSHR), but resistance to mouse (m) TSHR, while TSHR knockout (KO) mice are susceptible to mTSHR, indicating the existence of robust immune tolerance against the mTSHR in wt mice. This tolerance may be mediated by either centrally or peripherally. We here explored the contribution of a peripheral arm of immune tolerance against the mTSHR by using antibodies to deplete regulatory T cells (Tregs), to antagonize co-inhibitory molecules and/or to stimulate co-stimulatory molecules. Antagonistic anti-co-inhibitory molecules, cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death 1 ligand 1 (PD-L1), induced only low levels of anti-TSHR antibodies without induction of hyperthyroidism in a mouse Graves' model. In this experimental setting, antibody levels were significantly higher in TSHR<sup>-/-</sup> mice than wt mice. However, agonistic anti-co-stimulatory molecules, CD40 and CD137, and Treg-depleting anti-CD25 antibodies showed no effect. All these data suggest that peripheral immune tolerance against the mTSHR may play a minor role, and imply the importance of central tolerance, in immune tolerance against mTSHR in mice. Additional studies on central tolerance to the mTSHR will be necessary for completely delineating the mechanisms for immune tolerance against mTSHR in mice.

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### Introduction

Graves' disease is a B cell-mediated and T cell-dependent organ-specific autoimmune disease in which thyroid-stimulating antibodies (TSAb) stimulate the thyrotropin receptor (TSHR), the main autoantigen in the development of Graves' disease, leading to overproduction of thyroid hormones and diffuse enlargement of the thyroid glands.

We have previously established novel mouse models of Graves' hyperthyroidism by repeatedly injecting susceptible mouse strains (BALB/c) with adenovirus vector expressing the human (h) full-length TSHR (Ad-hTSHR) or the hTSHR A-subunit (Ad-hTSHR A-subunit)<sup>1,2</sup>, which have been

proved to be valuable for the studies on the pathogenesis and new treatment modalities for Graves' disease<sup>3-6</sup>. However, hTSHR is not strictly an autoantigen in these mouse models, because the hTSHR used in these mouse models and the mouse (m) TSHR endogenously expressed in mice is not completely identical with the amino acid homology being approximately 80%<sup>7</sup>. Indeed our recent study has revealed that wild type (wt) BALB/c mice are tolerant, while TSHR KO mice are responsive, to immunization with mTSHR<sup>7,8</sup>, indicating the existence of robust immune tolerance against mTSHR in mice.

Immune tolerance can largely be divided into 2 different categories, *e.g.*, central and peripheral. Central tolerance in-

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volves thymic deletion of auto-reactive T cells with high avidity to self-antigens<sup>9</sup>). Peripheral tolerance is exerted further by distinct mechanisms including ignorance of self-antigens, development of T cell unresponsiveness (anergy) and deletion of peripheral T cells, in which numerous molecules such as co-stimulatory and co-inhibitory molecules and numerous immune cells including regulatory T cells (Tregs) play roles<sup>10</sup>). Relative importance of central *versus* peripheral tolerance in immune tolerance against the mTSHR was however unknown. This study was therefore designed to explore the contribution of peripheral tolerance to immune tolerance against the mTSHR by utilizing monoclonal antibodies to deplete Tregs, to antagonize co-inhibitory molecules and/or to stimulate co-stimulatory molecules. Our data show that immune tolerance against the mTSHR can be only partially broken by antagonistic antibodies to co-inhibitory molecules, cytotoxic T lymphocyte antigen-4 (CTLA4, CD152) and programmed cell death 1 ligand 1 (PD-L1), suggesting that peripheral tolerance against the mTSHR may play a minor role in immune tolerance against mTSHR in mice.

## Materials and methods

### *Mice*

Wt (TSHR<sup>+/+</sup>) BALB/c mice (6 week-old) were purchased from Charles River Japan Laboratory Inc. (Tokyo, Japan). TSHR KO (TSHR<sup>-/-</sup>) BALB/c mice were previously generated by backcrossing TSHR KO B6/129 mice<sup>11</sup>) (Jackson Laboratory Inc., Bar Harbor, ME, USA) with wt BLAB/c mice for more than 6 successive generations<sup>7</sup>). Littermates of wt, TSHR<sup>+/-</sup> and TSHR KO mice were generated by crossing wt BALB/c mice with TSHR KO BALB/c mice. 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<sup>7</sup>).

Wt, TSHR<sup>+/-</sup> and TSHR KO BALB/c mice were injected intramuscularly in the quadriceps with 100  $\mu$ l PBS containing 10<sup>10</sup> particles of adenovirus on 2 occasions at 3-wk-in-

tervals. In addition, mice were also treated by intraperitoneal injection of 100  $\mu$ g/mouse anti-CTLA4 (clone no. UC10-4F10-11), anti-PD-L1 (10F.9G2), anti-CD40 (FGK45) and/or anti-CD137 (3H3) monoclonal antibodies (all from Bio X Cell, Inc., West Lebanon, NH) twice a week for 5 weeks after the first immunization with or without 500  $\mu$ g/mouse anti-CD25 monoclonal antibody (PC61)<sup>8, 12</sup>) once 4 days before the first immunization.

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

Blood samples were obtained 2-6 weeks after the second immunization. Serum free T<sub>4</sub> concentrations were measured by radioimmunoassay (RIA) as previously described<sup>7</sup>). The normal range was defined as the mean  $\pm$  3 S.D. of untreated control mice.

TSHR antibodies in mouse sera were determined using an 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 fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (F2772, Sigma-Aldrich, St. Louis, MO) for 60 min. Cells were analyzed using FACSCanto II (BD Biosciences, San Diego, CA) as previously described<sup>8</sup>). The data were expressed as % mean fluorescent index (MFI) as compared to the mean values from untreated control mice.

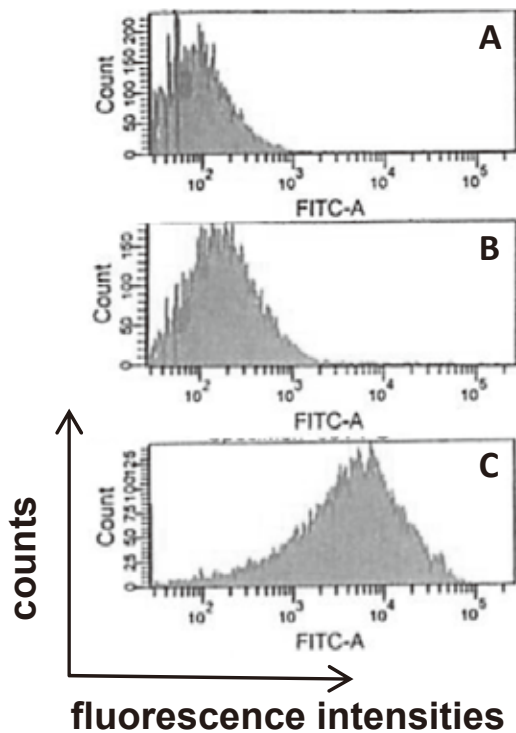
### *Statistical analysis*

Data on the levels of antibodies were analyzed by Mann-Whitney U test. A  $p < 0.05$  was considered statistically significant.

## Results

As we have previously reported<sup>7</sup>), TSHR KO mice were responsive to immunization with Ad-mTSHR A-subunit with an average antibody level being 1,039.8  $\pm$  829.6 % as compared to naïve mice (Fig. 1C *versus* A and Fig. 2A), while TSHR<sup>+/+</sup> (data not shown) and TSHR<sup>+/-</sup> (Fig. 2A) mice were tolerant.

We then examined whether anti-TSHR immune response could be induced in wt and TSHR<sup>+/-</sup> mice by combining immunization with Ad-mTSHR A-subunit and injection of monoclonal antibodies including antagonistic antibodies against co-inhibitory molecules (CTLA4 and PD-L1), ago-



**Figure 1.** Representative histograms of flow cytometric analyses of anti-TSHR antibodies in naïve mice (A), TSHR<sup>+/+</sup> mice immunized with Ad-mTSHR A-subunit in combination with anti-CTLA4 and anti-PD-L1 (B), and TSHR KO mice immunized with Ad-mTSHR A-subunit (C)

nistic antibodies to co-stimulatory molecules (CD40 and CD137) and/or Treg-depleting anti-CD25 antibody.

Combination of Ad-mTSHR with anti-CTLA4 and anti-PD-L1 plus/minus anti-CD25 induced relatively low levels of anti-TSHR antibodies in most of TSHR<sup>+/+</sup> and TSHR<sup>+/-</sup> mice with average antibody levels being ~200 to ~300 % at 2 and 6 weeks after the second immunization (Fig. 1B *versus* A and Fig. 2A). These antibody levels were significantly lower than those in TSHR KO mice ( $p < 0.01$ ). Addition of anti-CD25 to these 2 antibodies had no effect on antibody levels, an agreement with our previous study, showing no effect of anti-CD25 antibody on tolerance against the mTSHR in wt mice<sup>7,8</sup>). The antibody levels induced with Ad-mTSHR/anti-CTLA4/anti-PD-L1/anti-CD25 were higher in TSHR<sup>+/-</sup> than wt mice ( $314.8 \pm 119.7$  *versus*  $223.0 \pm 29.8$  %,  $p < 0.01$ ) 2 weeks after the final immunization, data consistent with our assumption that anti-TSHR immune response may be stronger in TSHR<sup>+/-</sup> mice as compared to TSHR<sup>+/+</sup> mice due to possibly lower expression levels of the TSHR in thymus and/or periphery in the former.

However, serum T<sub>4</sub> concentrations remained unchanged in all of wt and TSHR<sup>+/-</sup> mice, suggesting that the antibody levels induced were not high enough to induce hyperthy-

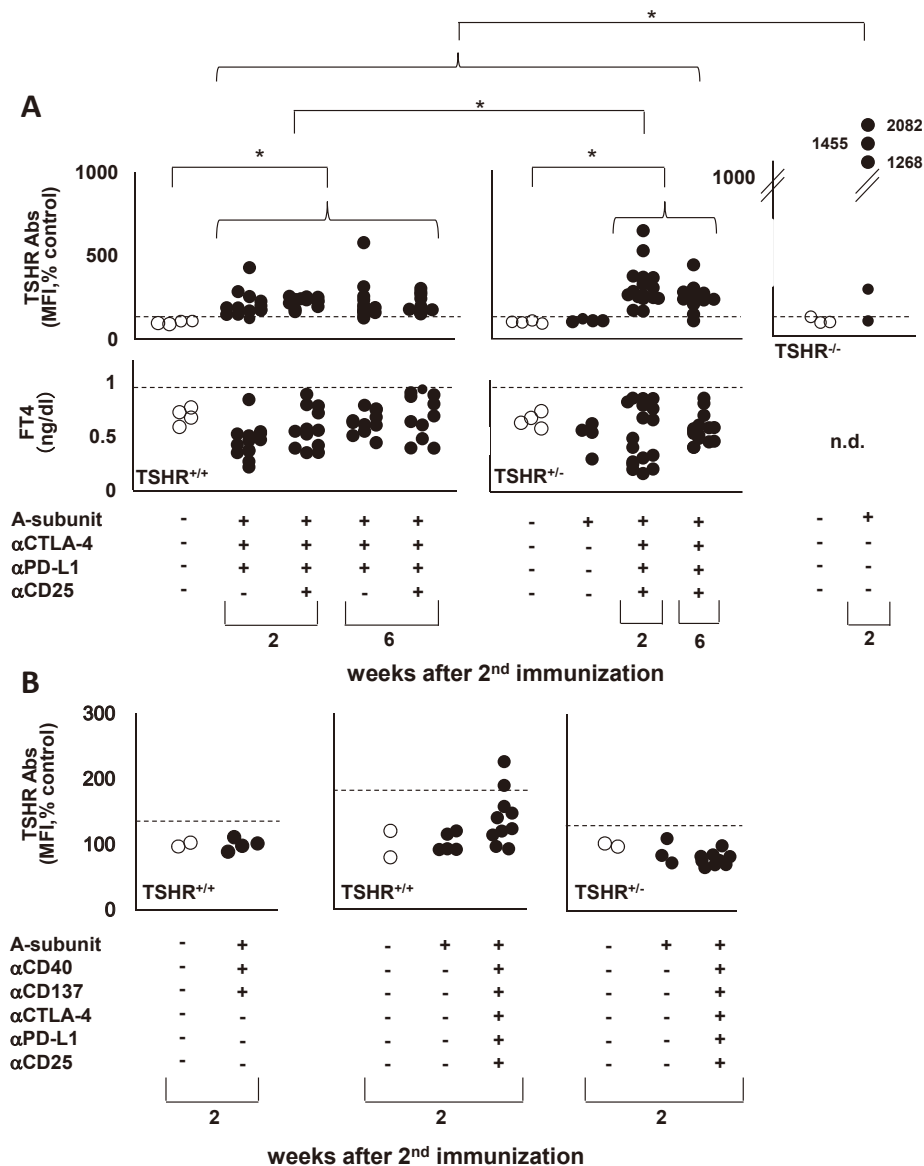
roidism.

Similarly, a combination of agonistic anti-CD40 and anti-CD137 antibodies showed no effect on anti-TSHR antibody levels, and did hardly enhance the effect of anti-CTLA4, anti-PD-L1 and anti-CD25 (Figure 2B).

## Discussion

We here attempted to break immune tolerance against the mTSHR in mice by using antagonistic anti-CTLA4 and anti-PD-L1, agonistic anti-CD40 and anti-CD137 and/or CD4<sup>+</sup>CD25<sup>+</sup> Treg-depleting anti-CD25 antibodies. CTLA4 and PD-L1 are co-inhibitory molecules expressed on activated T cells<sup>13</sup>. CTLA4 exerts its inhibitory effect during early phase of T cell activation, while PD-L1 functions during the effector phase of T cell activation<sup>13</sup>. On the other hand, CD40 and CD137 (4-1BB) are members of the tumor necrosis factor receptor superfamily and are well known co-stimulatory molecules. Activation of the former expressed on dendritic cells by CD40L expressed on major histocompatibility complex/antigen peptide complex-stimulated T cells is a second co-stimulatory signal required for full activation of T cells. CD137 is transiently expressed on T cells upon activation, and CD137 ligation ligand further co-stimulates CD137-expressing activated T cells<sup>14</sup>. The previous studies have demonstrated the efficacy of agonistic CD40 and/or CD137 antibodies and antagonistic CTLA4 and/or PD-L1 antibodies in enhancing tumor immunity and autoimmunity in humans and mice<sup>13-18</sup>). Furthermore, previous reports have identified an association of genetic polymorphism of CD40<sup>19</sup>), CTLA-4<sup>20</sup>) and PD-L1<sup>21</sup>) with development of Graves' disease.

Our results demonstrate that anti-mTSHR antibodies, but not hyperthyroidism, could be induced by Ad-mTSHR A-subunit immunization combined with blockade of co-inhibitory molecules CTLA4 and PD-L1 by antagonistic antibodies in wt and TSHR<sup>+/-</sup> mice, demonstrating that immune tolerance against the mTSHR can only partially be broken by these antibodies. Agonistic anti-CD40 and anti-CD137, and/or Treg-depleting anti-CD25 antibodies had no effect. These results are consistent with our recent data<sup>7,8</sup>). Thus, anti-TSHR antibodies are induced without induction of hyperthyroidism by adoptive transfer of splenocytes from naïve TSHR KO mice into nude mice injected with anti-CTLA4 and anti-PD-L1 antibodies not control nude mice<sup>8</sup>). Treg-depletion by anti-CD25 had no effect on tolerance of wt mice immunized with Ad-mTSHR A-subunit<sup>7</sup>) and on adoptive transfer of splenocytes from naïve TSHR KO mice



**Figure 2.** Anti-TSHR antibody levels and/or free T<sub>4</sub> concentrations in sera from wt, TSHR<sup>+/-</sup> and TSHR KO mice of untreated (open circle) or immunized (closed circle) with/without various combinations of antibody treatments. Antibodies used were anti-CTLA4, and anti-PD-L1 and anti-CD25 in *A* and additionally anti-CD40, anti-CD137 in *B*. Antibody levels and T<sub>4</sub> concentrations were measured as described in the Materials and Methods. Data for individual mice are shown as % mean fluorescent index (MFI) as compared to the mean values from untreated control mice for TSHR antibodies and as ng/dl for free T<sub>4</sub>. \*, p < 0.01

into nude mice<sup>8</sup>), although enhanced effect of Treg-depletion on anti-TSHR immune response is found in Ad-hTSHR A-subunit immunized, otherwise resistant C57/BL6 mice<sup>12</sup>). Thus, peripheral tolerance against the mTSHR seems to play a just minor role in immune tolerance against the mTSHR in wt mice. Particularly of interest is that CD4<sup>+</sup>CD25<sup>+</sup> Tregs, which is thought generally to be the main component of peripheral tolerance<sup>22</sup>), showed no if any little effect on tolerance against the mTSHR.

Anti-TSHR antibody levels induced in immunized TSHR<sup>+/-</sup>

mice treated with anti-CD25, anti-CTLA4 and anti-PD-L1 antibodies were significantly higher than those of wt mice treated with the same antibodies. The expression levels of TSHR in thymus or thyroid glands likely influenced this difference. As shown in experimental mouse models and several human autoimmune diseases<sup>23-25</sup>), even modest reductions in expression levels of a single tissue-specific self-antigen in the thymus, particularly in thymic medullary epithelial cells, increase the susceptibility to autoimmunity, because thymic expression of a certain self-antigen is es-

sential for negative selection of auto-reactive T cells<sup>9</sup>). This may also be the case for Graves' disease in humans, that is, there is an association between susceptibility to Graves' disease and TSHR gene variants that affect the intrathymic TSHR mRNA expression levels<sup>26</sup>). These data imply the importance of central tolerance in immune tolerance against the mTSHR. One of the reason(s) for lack of immune tolerance against mTSHR in TSHR KO mice may be absent thymic expression of the mTSHR<sup>27</sup>).

Our data indicate that peripheral tolerance against the mTSHR may play a minor role in immune tolerance against mTSHR in wt mice. Future studies on central tolerance to the mTSHR will be necessary for completely understanding the mechanisms for immune tolerance against mTSHR in mice.

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