Distinct Role of T Helper Type 17 Immune Response for Graves' Hyperthyroidism in Mice with Different Genetic Backgrounds

Running title: Th17 and Graves' disease

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Abstract

T helper type 17 (Th17) cells, a newly identified effector T cell subset, have recently been shown to play a role in numerous autoimmune diseases, including iodine-induced autoimmune thyroiditis in non-obese diabetic (NOD)-H2^{h4} mice, that had previously been thought Th1-dominant. We here studied the role of Th17 in Graves' hyperthyroidism, another thyroid-specific autoimmune disease, in a mouse model. Two genetically distinct BALB/c and NOD-H2^{h4} strains with intact or disrupted IL-17 genes (IL-17$^{+/+}$ or IL-17$^{-/-}$) were immunized with adenovirus expressing the thyrotropin receptor A-subunit (Ad-TSHR289). Both IL-17$^{+/+}$ and IL-17$^{-/-}$ mice developed anti-TSHR antibodies and hyperthyroidism at the equally high frequencies on the BALB/c genetic background. In contrast, some IL-17$^{+/+}$, but none of IL-17$^{-/-}$, mice became hyperthyroid on the NOD-H2^{h4} genetic background, indicating the crucial role of IL-17 for development of Graves' hyperthyroidism in non-susceptible NOD-H2^{h4}, but not in susceptible BALB/c mice. In the T cell recall assay, splenocytes and lymphocytes from the draining lymph nodes from either mouse strains, irrespective of IL-17 gene status, produced IFN-γ and IL-10 but not other cytokines including IL-17 in response to TSHR antigen. Thus, the functional significance of Th17 may not necessarily be predictable from cytokine expression patterns in splenocytes or inflammatory lesions. In conclusion, this is, to our knowledge, the first report showing that the role of Th17 cells for the pathogenesis of a certain autoimmune disease depends on the mouse genetic backgrounds.

Key words

autoimmunity, Th17, Graves' disease, thyrotropin receptor, mouse strain

Abbreviation

TSH, thyrotropin receptor; Th, T helper type; NOD, non-obese diabetic; CHO, Chinese hamster ovary; H & E , hematoxylin and eosin; MFI, fluorescein intensities; DDS, dextran sodium sulfate; Ad, adenovirus

Author Disclosure Statement

The authors have nothing to disclose.

Introduction

Graves' disease is an organ-specific autoimmune disease characterized by overstimulation of the thyroid glands by agonistic anti-thyrotropin receptor (TSHR) antibody (thyroid stimulating antibody; TSAb), leading to hyperthyroidism and thyroid enlargement [1,2].

Graves' disease has long been assumed to be T helper type 2 (Th2)-dominant, because the disease is autoantibody-mediated. However, this concept has been challenged both in humans and mouse models. TSAb in many [3], but not all [4], patients are IgG1, of Th1 subclass in humans. In all the mouse models of Graves' disease so far studied, both Th1 and Th2 immune responses are elicited against the TSHR, and the most monoclonal TSAb are of Th1 subclass [5]. In addition, the Th1 cytokine IFN-γ is secreted by splenocytes exposed to TSHR antigen in the T cell recall assay [6]. However, the outcomes of the immune manipulations to alter Th1/Th2 balance are different among distinct mouse models. Thus, Th2 immune response seems to be important in the mouse models using the fibroblasts or B cells expressing the TSHR and MHC class II, whereas Th1 is likely crucial in the models involving genetic immunization using plasmid or adenovirus, or dendritic cells expressing the TSHR [5]. Given all these controversial data, it is possible to speculate that Graves' disease may involve different type of immune response(s) rather than the classical Th1/Th2 immune responses.

IL-17 producing Th17 cells are another $CD4^+$ effecter T cell lineage recently identified [7,8]. Although the pathogenesis of most autoimmune diseases has long been argued on relative balance between Th1 *versus* (*vs.*) Th2, the recent studies revealed that Th17 immune responses play a major role in numerous autoimmune diseases, such as multiple sclerosis/experimental autoimmune encephalitis (EAE), uveitis, rheumatoid arthritis, Sjogren's syndrome, myasthenia gravis and psoriasis, all of which had previously been thought to be Th1-diseases [9]. Moreover we have recently demonstrated that Th17 cells as well as Th1 cells are critical for development of iodine-induced autoimmune thyroiditis in non-obese diabetic (NOD)-$H2^{h4}$ mice [9]. The number of Th17 cells has also been recently shown to be increased in Hashimoto's

thyroiditis in humans [10].

This study was therefore conducted to investigate the role for Th17 cells in the pathogenesis of Graves' hyperthyroidism, another thyroid-specific autoimmune disease, in a mouse model with adenovirus coding the TSHR A-subunit (Ad-TSHR289) [11,12]. Surprisingly, we observed that IL-17 cells are involved in the pathogenesis of Graves' hyperthyroidism in NOD-H2^{h4}, but not in BALB/c, mice. Thus, to our knowledge, this is the first report showing that the significance of Th17 in disease pathogenesis is dependent on the mouse genetic backgrounds.

Materials and Methods

Mice used

IL-17$^{-/-}$ NOD-H2^{h4} mice were previously generated [9]. IL-17$^{-/-}$ BALB/c mice [13] were crossed with wild type (wt) BALB/c mice (Charles River Japan Laboratory Inc. (Tokyo, Japan), and the resulting F1 mice were then intercrossed each other to produce F2 littermates of IL-17$^{+/+}$, IL-17$^{+/+}$ and IL-17$^{-/-}$ mice. Genotyping was performed by PCR analysis of tail DNA as previously reported [9,13]. All the mice were bred in the animal facility at Nagasaki University in a specific pathogen-free condition. 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. Both male and female mice were used for the current study.

Immunization protocols

Construction, amplification, and purification of non-replicative recombinant human adenovirus expressing the human TSHR-A subunit (Ad-TSHR289; kindly provided by Drs. McLachlan SM and Rapoport B at Cedars-Sinai Medical Center and University of California Los Angeles, CA), and determination of the viral particle concentration were described previously [11,12]. Six-week-old mice were injected intramuscularly in the quadriceps with

100 μl PBS containing different doses ($10^{8 \text{ to } 10}$ particles) of Ad-TSHR289 on two occasions at three-weekly intervals, as previously described [11,12]. Blood, spleens, and the thyroid glands were obtained two and six weeks after the second immunization. Through the whole experiments, the drinking water was not supplemented with iodide in NOD-$H2^{h4}$ mice.

Free thyroxine (T_4) and anti-TSHR antibody measurements

Serum free T_4 concentrations were measured with a RIA kit (DPC free T_4 kit; Diagnostic Products, Los Angeles, CA). The normal range was defined as the mean ± 3 S.D. of the control untreated mice.

Anti-TSHR antibodies were measured using two different methods. First is a flow cytometry assay as previously described [14]. Briefly, Chinese hamster ovary (CHO) cells stably expressing the TSHR [15] were incubated for 30 min at room temperature with mouse sera (1:100 dilution), followed by incubation for 30 min at 4 C with FITC-conjugated goat anti-mouse IgG (Sigma). Flow cytometry was performed using FACSCanto II (BD Biosciences, San Diego, CA). The normal range was also defined as the mean ± 3 S.D. of the control untreated mice. This assay does not discriminate between stimulating and non-stimulating antibodies. Second is a biological TSAb assay, which measures the stimulating antibodies responsible for hyperthyroidism [17]. Briefly, FRTL5 cells, a normal differentiated rat thyroid epithelial cell line expressing the rat TSHR, were incubated for 2 hrs at 37 C with mouse sera (1:10 dilution), and cAMP released into the medium was measured with a cAMP radioimmunoassay kit (Yamasa, Choshi, Japan) [11].

Thyroid histology

Thyroid tissues were fixed in 10 % formalin and embedded in paraffin. Five-μm-thick sections were prepared and stained with hematoxylin and eosin (H & E).

Cytokine assays

Splenocytes and lymphocytes from the draining lymph nodes were cultured (triplicate aliquots) at 5 x 10^5 cells per well in a 96-well round-bottomed culture plate in the presence or absence of 10 μg/ml TSHR289 protein, as previously described [6]. Four days later, the culture supernatants were collected. The concentrations of various cytokines in these culture supernatants were determined with a Bio-Plex™ Suspension Array System and 23-Plex Panel (IL-1α, 1β, 2-6, 9, 10, 12 (p40 and p70), 13, 17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α and 1β, Rantes and TNF-α) (Bio-Rad, Tokyo, Japan).

Statistical analysis

Levels of free T_4, antibodies and cytokines were analyzed by Student's *t*-test, and incidences of hyperthyroidism by chi-square test. A *p*-value less than 0.05 was considered statistically significant.

Results

Serum T_4 and anti-TSHR antibodies in wt and IL-17 gene-disrupted BALB/c and NOD-H2^{h4} mice to immunization with Ad-TSHR289

To clarify a role for Th17 cells in the pathogenesis of Graves' hyperthyroidism, IL-$17^{-/-}$ mice and their IL-$17^{+/+}$ and IL-$17^{+/-}$ littermates on the BALB/c or NOD-H2^{h4} backgrounds were immunized twice with different doses of Ad-TSHR289. Serum free T_4, anti-TSHR antibodies and antigen-specific secretion of cytokines were then compared two and/or six weeks after the second immunization. BALB/c and NOD-H2^{h4} mice are susceptible and intermediate, respectively, mouse strains to Graves' disease [11,17 and unpublished data].

In BALB/c mice immunized with high (10^{10} particle/mouse) or low doses (10^9 particles/mouse) of Ad-TSHR289 (Fig. 1 A and B), the mean serum free T_4 levels were increased to the comparable levels in IL-$17^{+/+}$, IL-$17^{+/-}$ and IL-$17^{-/-}$ mice (1.32 $\pm$ 0.56, 1.18 $\pm$

0.61 and 1.08 ± 0.48 ng/dl, respectively, in the high dose group, and 0.83 ± 0.48, 0.83 ± 0.39 and 0.66 ± 0.37 ng/dl in the low dose group; mean ± S.D., n = 9-11), as compared to the control untreated mice (0.52 ± 0.05 or 0.31 ± 0.023), two weeks after the second immunization. The incidences of hyperthyroidism were also similar in three groups (9/10 (90 %), 10/12 (83 %) and 9/11 (82 %), respectively, in the high dose group, and 100 % in the low dose group). To determine whether Th17 cells affect duration of hyperthyroidism, sera were also obtained six weeks after the second immunization in the low dose group. There were no differences in the mean free T_4 levels (0.86 ± 0.73, 1.52 ± 1.51and 0.74 ± 1.14 ng/dl in IL-17$^{+/+}$, IL-17$^{+/-}$ and IL-17$^{-/-}$ mice, respectively) or in the incidences of hyperthyroidism (33, 50 and 20 %) (Fig. 1 C). Immune responses were barely detectable in all the mice immunized with 10^8 particles/mouse of Ad-TSHR289 (data not shown).

Likewise, anti-TSHR antibody titers determined by flow cytometry were similar in IL-17$^{+/+}$ and IL-17$^{-/-}$ mice (1317 ± 1125 *vs.* 792 ± 485 mean fluorescein intensities (MFI), respectively, in the high dose group, and 1021 ± 1386 and 1386 ± 1580 MFI in the low dose group) (Fig. 2 A). These antibody titers were not significantly correlated with T_4 levels ($r = 0.356$ and $p > 0.05$). TSAb was determined in the high dose group and found to be increased in most hyperthyroid mice irrespective of IL-17 gene status, which was significantly and positively correlated with T_4 levels ($r = 0.62$ and $p < 0.01$, Fig. 2 B) as previously reported [14], confirming the causative role of TSAb for hyperthyroidism.

As previously reported [11,12], the thyroid glands from hyperthyroid BALB/c mice, irrespective of IL-17 gene status or the mouse genetic backgrounds, were diffusely enlarged with cuboidal thyroid epithelial cells, typical features for the hyperfunctioning thyroid glands (Fig. 3).

By contrast, in NOD-H2^{h4} mice, five out of 20 (25 %) IL-17$^{+/+}$ mice immunized with high dose (10^{10} particle/mouse) of Ad-TSHR289 developed hyperthyroidism *vs.* none (0 %) of IL-17$^{-/-}$ mice. The mean free T_4 levels were also significantly elevated in IL-17$^{+/+}$ mice compared to IL-17$^{-/-}$ mice (0.87 ± 0.27 *vs.* 0.70 ± 0.17 ng/dl, $p < 0.05$) (Fig. 4 A). The

anti-TSHR antibody titers in IL-$17^{+/+}$ mice were higher than those in IL-$17^{-/-}$ mice, but the difference was not significant (1729 $\pm$ 1660 *vs.* 1047 $\pm$ 876 MFI, $p > 0.05$, Fig. 4 B). Again these antibody titers were not significantly correlated with T_4 levels ($r = 0.118$ and $p > 0.05$). TSAb was not measured in these NOD-$H2^{h4}$ mice because we already showed increased T_4 was mediated by TSAb in this mouse Graves' model (Fig. 2B and ref. [14]). Again, the thyroid histology in hyperthyroid NOD-$H2^{h4}$ mice was compatible with that seen in hyperthyroid BALB/c mice (see above).

Comparison of cytokine expression profiles in BALB/c and NOD-$H2^{h4}$ mice

To seek possible explanation(s) for different responses between BALB/c and NOD-$H2^{h4}$ mice shown above, antigen-specific *in vitro* cytokine secretions of splenocytes were examined in the T cell recall assay.

Compared with splenocytes cultured in medium alone, TSHR289 antigen stimulated increased production of IFN-γ by splenocytes from IL-$17^{+/+}$ and IL-$17^{-/-}$ BALB/c mice (9.4 $\pm$ 1.6 and 13.0 $\pm$ 1.0 ng/ml, respectively) and from IL-$17^{+/+}$ and IL-$17^{-/-}$ NOD-$H2^{h4}$ mice (33.0 ± 6.71 and 27.0 ± 5.63) (Fig. 5 B and E). IL-10 tended to increase in response to TSHR289 antigen (Fig. 5 C and F). However, background levels of IL-17 secretion (absent from IL-$17^{-/-}$ mice) was not increased in response to TSHR289 antigen in either IL-$17^{+/+}$ BALB/c or NOD-$H2^{h4}$ mice (Fig. 5 A and D). Other 20 cytokines showed negligible response to antigen stimulation (data not shown). Thus, no noticeable differences were detected in the cytokine expression profiles between the two mouse strains. Essentially identical results were obtained with lymphocytes from the draining lymph nodes (data not shown).

Discussion

Th17 cells are newly identified effector T cells and are now well known to play a pivotal role in various autoimmune diseases [7, 8]. We have also recently found that, besides Th1

cells, Th17 cells are indispensable for iodine-induced autoimmune thyroiditis in thyroiditis-prone NOD-H2^{h4} mice [9]. We here extended our study to Graves' hyperthyroidism, another thyroid-specific autoimmune disease, using a mouse model with adenovirus expressing the autoantigen TSHR that we have previously established [11,12].

Our results clearly demonstrate that the consequence of IL-17 gene disruption for induced Graves' disease is dependent on the mouse genetic backgrounds. Thus, both IL-17$^{+/+}$ and IL-17$^{-/-}$ mice developed anti-TSHR antibodies and hyperthyroidism at the equally high frequencies on the susceptible BALB/c genetic background. In contrast, however, some IL-17$^{+/+}$, but not IL-17 $^{-/-}$, mice became hyperthyroid on the non-susceptible NOD-H2^{h4} genetic background.

Data reminiscent of our unexpected findings have been previously demonstrated in a mouse model of dextran sodium sulfate (DSS)-induced inflammatory bowel disease. Thus Th17 cells are pathogenic in C57BL/6 mice [18], but protective in BALB/c mice [19]. However, those two studies were performed in different ways: the former used IL-17$^{-/-}$ mice *vs*. the latter treatment with anti-IL-17 antibody. It is therefore possible that a trace amount of IL-17 may have aggravated colitis in the latter. In this regard, to our knowledge, we are the first to show a difference in the role of IL-17 in development of a certain autoimmune disease in mice with different genetic backgrounds using the same experimental approach.

What genetic factor(s) define the consequences of IL-17 deficiency? In the DSS-induced colitis model mentioned above, differences in the expression patterns of numerous cytokines, such as IL-1β, IL-12, IL-17, IFN-γ *etc*, in inflamed intestines have been described for these two mouse strains [20]. In contrast, we could not find any differences in cytokine expression profiles in splenocytes and lymphocytes from the draining lymph nodes from BALB/c and NOD-H2^{h4} mice irrespective of IL-17 gene status. We can only speculate that disruption of a single cytokine signaling may be much more crucial for disease development in resistant mice than in susceptible mice.

Absence of antigen-specific secretion of IL-17 from splenocytes in the T cell recall assay is reasonable in BALB/c mice, but is somewhat unexpected in NOD-H2^{h4} mice because of the critical role of Th17 cells in development of Graves' hyperthyroidism in this mouse strain as mentioned above. The exact reason(s) for these results are at present unclear. However, since Th17 cells are reported to be induced earlier than Th1 cells in some autoimmune diseases in mice [21,22], one possibility is that small numbers of Th17 cells participated the very early stage of elicitation of anti-TSHR immune response in NOD-H2^{h4} mice. Whatever the reasons, we have previously found the parallel results in the mouse Graves' model we used in this study. Thus splenocytes from immunized BALB/c mice produced IFN-γ, but not IL-4, in the response to TSHR289 antigen in the T cell recall assay [6], whereas both IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ BALB/c mice are resistant to hyperthyroidism [23]. The former implicates Th1, but the latter both Th1 and Th2, in the pathogenesis of Graves' disease. The opposite was also reported in NOD mice, namely that IL-17 deficiency did not affect the incidence of hyperglycemia, although IL-17 mRNA expression was increased upon development of diabetes [24]. Therefore, the importance of IL-17 can not necessarily be negated by the absence of antigen-specific splenocyte secretion of IL-17. Similarly, mere expression of IL-17 does not indicate the functional importance of IL-17 in disease pathogenesis.

Relative contribution of Th1 and Th17 immune responses for autoimmune disease pathogenesis varies in different autoimmune diseases. For example, although Th17 cells are the main effector T cells for numerous autoimmune diseases including EAE, uveitis, *etc*; Th1 cells are pathogenic while Th17 cells protective for the CD45RB^{hi} transfer model of colitis [25]; and both Th1 and Th17 cells are pathogenic for iodine-induced autoimmune thyroiditis [9]. Even in the same diseases, however, it is reported that the relative importance of Th17 is dependent on immunization protocols. Thus Th1 and Th17 cells play a dominant role in uveitis induced by immunization with interphotoreceptor retinoid-binding protein (IRBP) emulsified with Complete Freund's adjuvant or IRBP-pulsed mature dendritic cells, respectively

[26]. Transfer of *in vitro* generated antigen-specific Th1, Th2 and/or Th17 cells all induce EAE or uveitis [27,28]. More recently, Th17 cells were demonstrated to convert to Th1 cells in the mouse model of diabetes [29,30]. Furthermore, as mentioned in the Introduction, relative importance of Th1 or Th2 is different among the distinct Graves' models [5]. From these data, together with our present results, one should be cautious in interpreting the data on Th17 (and also other effector T cells) obtained from animal disease models. The results may be variable depending on immunization protocols, mouse strain, conventional or pathogen-free housing facilities, *etc*. This assumption also implies that the pathogenesis of a certain autoimmune disease may involve subtle difference in individual humans because each patient has different genetic and environmental backgrounds.

In conclusion, we demonstrate that the role of Th17 immune response in the pathogenesis of Graves' hyperthyroidism in a mouse model is dependent on the genetic background. Our findings and those of others [24] also suggest that the significance of Th17 in a certain disease can not be estimated from analysis of IL-17 expression in the inflamed tissues or splenocytes. Further studies will be necessary to clarify what genetic factors influence the relative significance of Th17 in autoimmune diseases. However, the outcomes for IL-17$^{-/-}$ mice on different genetic backgrounds may contribute to understanding heterogeneity in human Graves' disease.

Acknowledgments

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Figure legends

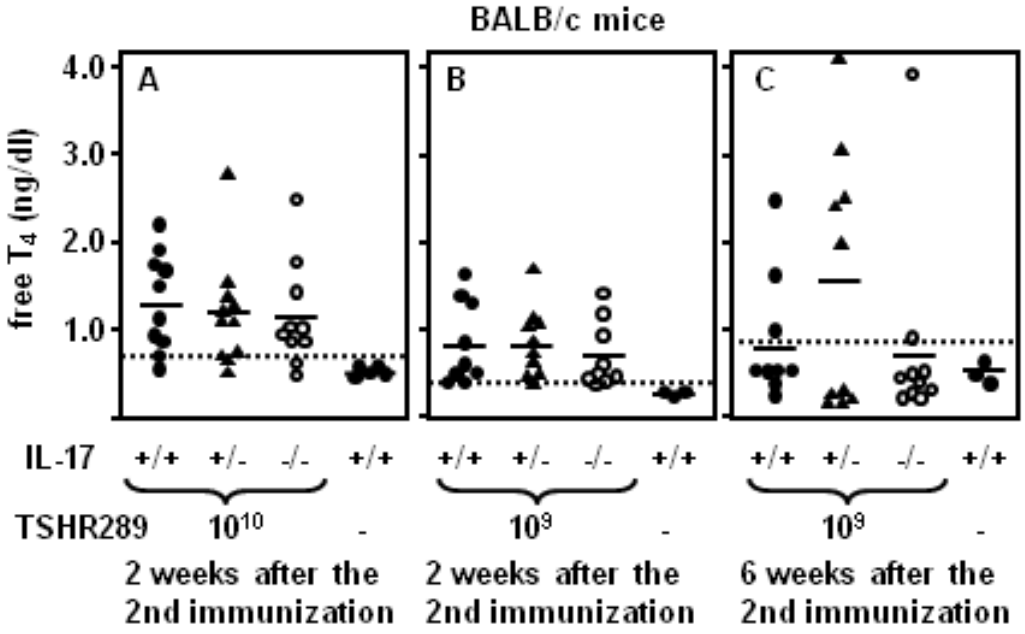

Figure 1. Serum free T_4 concentrations in IL-$17^{+/+}$, IL-$17^{+/-}$ and IL-$17^{-/-}$ BALB/c mice immunized with 10^{10} (*A*) or 10^9 (*B* and *C*) particles/mouse of Ad-TSHR289. Free T_4 levels were determined two (*A* and *B*) and six weeks (*C*) after the second immunization. Data are shown for individual mice. The horizontal solid lines indicate the mean values for each group. The horizontal broken lines designate the normal upper limits of free T_4 values. n.d., not determined.

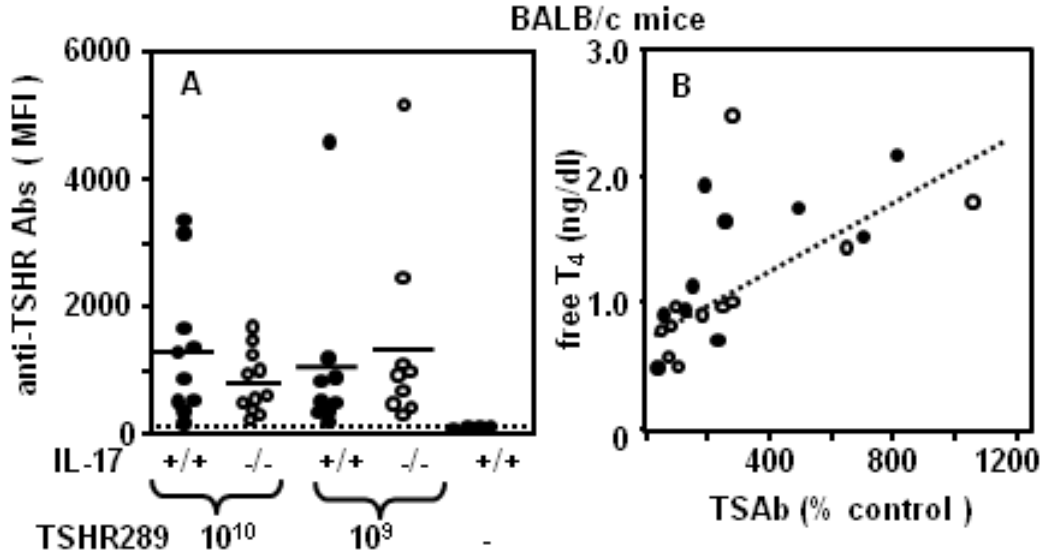

Figure 2. Anti-TSHR antibody titers and TSAb in sera from IL-$17^{+/+}$ and IL-$17^{-/-}$ BALB/c mice immunized with $10^{10 \text{ or } 9}$ particles/mouse of Ad-TSHR289. Sera were obtained from the mice two weeks after the second immunization. (*A*) anti-TSHR antibodies were determined by flow cytometry. Data are given as the mean fluorescence intensity (MFI). Values are shown for individual mice. The horizontal broken line designates the upper limit of anti-TSHR antibody values in control mice. (*B*) TSAb was measured in mice immunized with 10^{10} particles/mouse of Ad-TSHR289 by bioassay, and the correlation with T_4 values was shown.

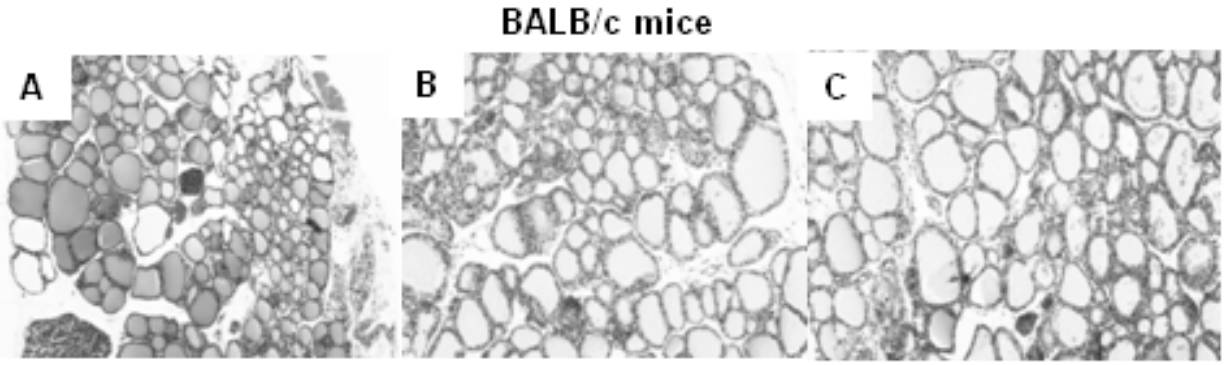

Figure 3. Representative histology of the thyroid glands in control mouse (*A*) and hyperthyroid IL-$17^{+/+}$ (*B*) and hyperthyroid IL-$17^{-/-}$ (*C*) BALB/c mice immunized with 10^{10} particles/mouse of Ad-TSHR289. Magnification, x100.

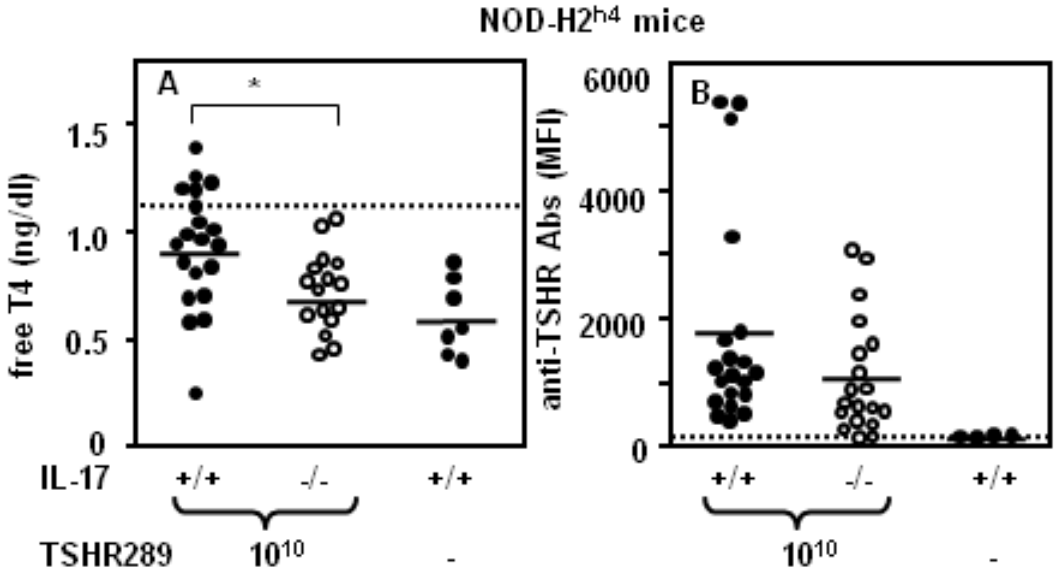

Figure 4. Serum free T_4 concentrations (*A*) and anti-TSHR antibody titers (*B*) in IL-$17^{+/+}$ and IL-$17^{-/-}$ NOD-$H2^{h4}$ mice immunized with 10^{10} particles/mouse of Ad-TSHR289. Free T_4 levels and TSHR antibody values were determined two weeks after second immunization. Data are shown for individual mice. The horizontal solid lines indicate the mean values for each group. The horizontal broken lines designate the upper limits for free T_4 and TSHR antibodies in normal mice. *, $p < 0.05$.

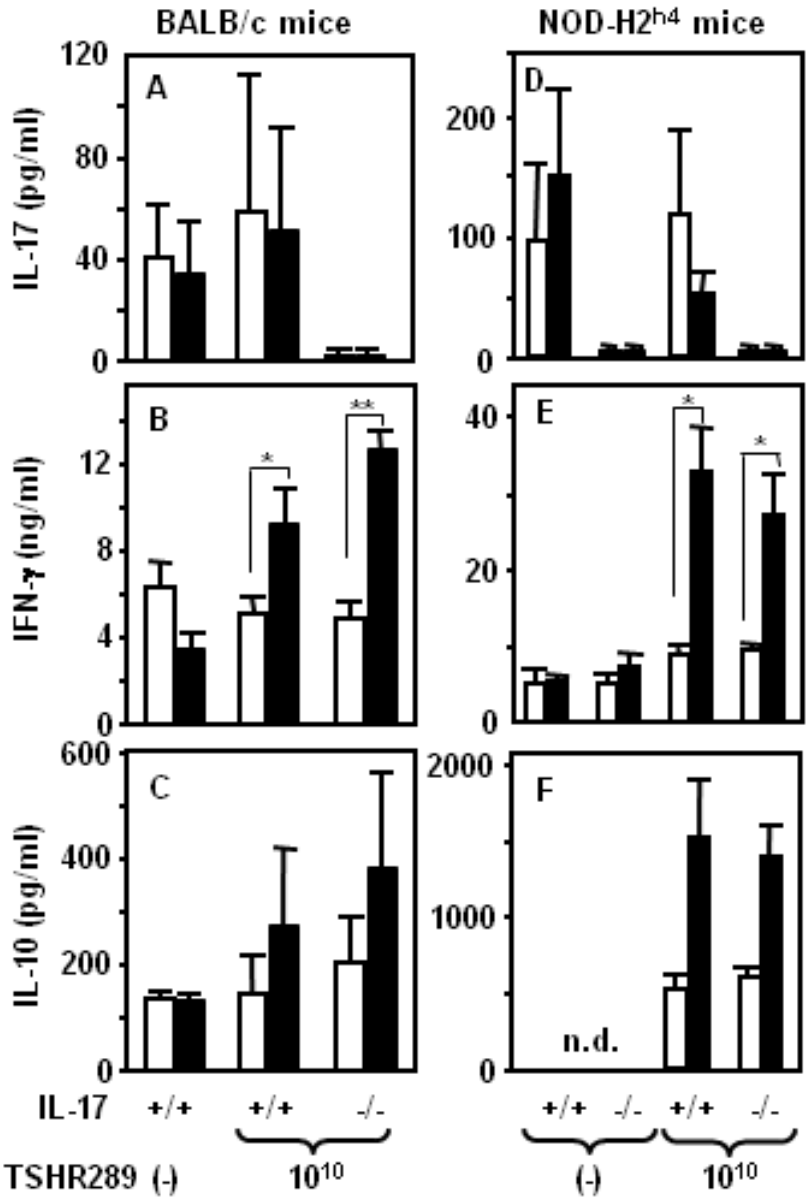

Figure 5. Cytokine production from splenocytes in IL-17$^{+/+}$ and IL-17$^{-/-}$ BALB/c and NOD-H2^{h4} mice of untreated and treated with 10^{10} particles/mouse of Ad-TSHR289. Splenocytes were cultured in the presence (solid bars) or absence (open bars) of 10 μg/ml TSHR289 protein for four days. IL-17 (*A* and *D*), IFN-γ (*B* and *E*) and IL-10 (*C* and *F*) were measured by ELISA (see the *Materials and Methods*). The data are means + S.E. in *A, B, D and E* (n = 4) or means + range in *C* and *F* (n=2) . *, $p < 0.05$; **, $p < 0.01$.