T Helper Type 17 Immune Response Plays an Indispensable Role for Development of Iodine-Induced Autoimmune Thyroiditis in Non-Obese Diabetic-H2^{h4} Mice.

Short title: Th17 in autoimmune thyroiditis

Precis: This manuscript demonstrates, by using interleukin-17 knockout mice, the significant role played by a newly identified T helper type 17 immune response in development of iodine-induced autoimmune thyroiditis in non-obese diabetic-H2^{h4} mice.

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Key words: iodine-induced autoimmune thyroiditis, IL-17, NOD-H2^{h4} mice, Th1, Th17

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Abstract

T helper type 1(Th1)/Th2 paradigm has been expanded by discovery of a novel effector T cell (T_{eff}) subset, Th17 cells, which produce a proinflammatory cytokine interleukin (IL)-17. Th17 cells have recently been shown to play a major role in numerous autoimmune diseases that had previously been thought to be Th1-dominant diseases. We here studied the significance of Th17 cells in iodine-induced autoimmune thyroiditis in non-obese diabetic (NOD)-$H2^{h4}$ mice, a mouse model of Hashimoto's thyroiditis in humans, which spontaneously develop anti-thyroglobulin (Tg) autoantibodies and intrathyroidal lymphocyte infiltration when supplied with iodine in the drinking water. We observed increased numbers of Th1 and Th17 cells in spleen and accumulation of both types of T_{eff} in the thyroid glands of iodine-fed wild-type (wt) mice, indicating that Th17 cells as well as Th1 cells constitute thyroid lesions. Furthermore, the incidence and severity of intrathyroidal lymphocyte infiltration, and the titers of anti-Tg autoantibodies were markedly reduced in iodine-treated IL-$17^{-/-}$ mice as compared with wt mice. Of interest, IL-$17^{+/-}$ mice showed an intermediate phenotype. Therefore, the present study, together with a previous report demonstrating the importance of Th1, not Th2, immune response for developing thyroiditis using mice deficient for IFN-γ or IL-4, clearly indicates that both Th1 and Th17 cells are critical T_{eff} subsets for the pathogenesis of spontaneous autoimmune thyroiditis in NOD-$H2^{h4}$ mice.

Introduction

Hashimoto's thyroiditis is the most common organ-specific autoimmune disease in humans (1). The disease is characterized by destruction of the thyroid glands by cytotoxic T lymphocytes and/or cytokine-induced thyroid cell apoptosis, resulting in hypothyroidism and appearance of autoantibodies against thyroid specific autoantigens such as thyroglobulin (Tg) and thyroid peroxidase in patients' sera. The etiopathogenesis still however remains to be elucidated.

Various animal models of Hashimoto's thyroiditis have been established. Non-obese diabetic (NOD)-$H2^{h4}$ mice, which were generated by crossing type 1 diabetes-prone NOD mice with the B10.A (4R) strain (2), are one of the well-characterized iodine-induced models. These mice develop anti-Tg autoantibodies and intrathyroidal lymphocyte infiltration when supplied with iodine in the drinking water (3, 4). Thus, this mouse model has long been used to dissect the pathogenesis of Hashimoto's disease. It has been demonstrated that disease is mediated by both $CD4^+$ and $CD8^+$ T cells as well as B cells, because antibody-mediated deletion of the respective cell populations abolished the development of anti-Tg autoantibodies and thyroiditis (4-6).

Regarding $CD4^+$ T cells, this T cell subpopulation can largely be divided into 2 different subsets with distinct differentiation profiles and functional characteristics; $CD25^-$ effector T cells (T_{eff}) and $CD25^+$ regulatory T cells (T_{reg}), which positively and negatively, respectively, regulate immune responses (7). The former subset has long been thought to be represented by T helper type 1 (Th1) and Th2 cells (8), but interleukin (IL)-17 producing T (Th17) cells have recently been identified as a novel T_{eff} subset (9, 10). Although the pathogenesis of most of autoimmune diseases has long been argued on relative balance between Th1 *versus* (*vs.*) Th2, recent studies revealed that Th17 immune responses play a major role in numerous autoimmune diseases such as multiple sclerosis/experimental autoimmune encephalitis (EAE) (10, 11), rheumatoid arthritis (12, 13), autoimmune uveitis (14-16), Sjogren's syndrome (17), myasthenia gravis (18) and psoriasis (19) all of which had previously been thought to be Th1-diseases.

The significance of Th1 and Th2 immune responses has previously been studied in NOD-$H2^{h4}$

mice genetically defective in interferon (IFN)-γ (IFN-$\gamma^{-/-}$) or IL-4 (20), which showed that Th1, not Th2, is indispensable for development of anti-Tg autoantibodies and thyroiditis, although both Th1 and Th2 cytokines are expressed in the thyroid glands (4, 21). Interestingly, it has also been shown that thyroiditis develops only in mice harboring thyrocytes able to respond to IFN-γ, that is, IFN-γ receptor knockout mice do not develop thyroiditis (22). However, a role for Th17 cells in thyroiditis in NOD-H2^{h4} mice has not been studied. Since IL-17 is a potent proinflammatory cytokine (23), involvement of Th17 in chronic inflammation observed in the thyroids of NOD-H2^{h4} mice might be anticipated. This study was therefore conducted to investigate the role for the newly identified Th17 cells in the pathogenesis of iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice. We first show differentiation of both Th1 and Th17 cells in spleen and accumulation of both types of T_{eff} in thyroid lesions in iodine-fed, wild type (wt, IL-$17^{+/+}$) mice, and then almost complete suppression of anti-Tg autoantibodies and thyroiditis in Il-17-deficient (IL-$17^{-/-}$) mice. These findings indicate a crucial role for Th17 as well as Th1 cells in the pathogenesis of iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice.

Materials and Methods

Mice used

NOD-H2^{h4} mice, obtained from Jackson Laboratory Inc. (Bar Harbor, ME, USA), are I-E^{-} and express H-2K^{k}, I-A^{k} and D^{b} on the NOD background (2). IL-$17^{-/-}$ mice previously generated (originally on the 129/Sv x C57BL/6 genetic background; ref. 24) were backcrossed to NOD mice for 7 successive generations. Analysis of the microsatellite markers for diabetes susceptibility (*Idd1-15*) loci by PCR of tail DNA as previously described (25) showed that the mice were fixed as homozygous for all NOD alleles. Development of type 1 diabetes in IL-$17^{+/+}$ and IL-$17^{-/-}$ NOD mice established was demonstrated (26). DNA was extracted with a REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO).

IL-$17^{-/-}$ NOD mice were then crossed with NOD-H2^{h4} mice and the resulting F1 mice were backcrossed with NOD-H2^{h4} mice to produce IL-$17^{+/-}$ NOD-H2^{h4} mice, which were selected by the

expression of the H-2K^{k} MHC class I molecule, not the H-2K^{d} MHC class I molecule, by flow cytometry (see below) and by PCR analysis of tail DNA. Antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-K^{k} (clone AF3-12.1) and phycoerythrin (PE)-conjugated anti-K^{d} (clone SF1-1.1; both from BD Bioscience, San Diego, CA), and primers used were described previously (24). IL-17$^{+/-}$ NOD-H2^{h4} mice were intercrossed to produce IL-17$^{+/+}$, IL-17$^{+/-}$ and IL-17$^{-/-}$ NOD-H2^{h4} littermate mice. Both male and female mice were used for the current study.

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.

Induction of thyroiditis

Six to 8 weeks old mice were supplied with 0.15 % sodium iodine (NaI) in the drinking water. Four and 8 weeks after NaI provision, mice were euthanized, and the thyroid glands, blood and the spleen were harvested to determine the extent of thyroiditis and cytokine mRNA expression, the titers of serum anti-Tg autoantibodies and cytokine expression, respectively.

Evaluation of thyroiditis

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). Thyroiditis was assessed for extent of lymphocyte infiltration as follows; grade 0, no lymphocytic infiltration; grade 1, less than 10 % lymphocytic infiltration of the thyroid; grade 2, 10 to 30 % lymphocytic infiltration; grade 3, 30 to 50 % lymphocytic infiltration; grade 4, greater than 50 % lymphocytic infiltration (27). The final thyroiditis scores were expressed as means of at least 3 noncontiguous sections from each thyroid gland.

ELISA assay for anti-Tg autoantibodies

Tg was purified as previously described (27) from thyroid glands of naïve mice and mice treated with NaI for 8 weeks (for iodinated Tg). ELISA wells were coated overnight with 100 μl Tg protein from naïve mice (10 μg/ml) and incubated with mouse sera (1:100 to 1:3,000 dilutions). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (A3673, Sigma), color was developed using orthophenylene diamine and H_2O_2 as substrate and optical density (OD) read at 492 nm.

Flow cytometric analysis of intracellular cytokines

Splenocytes were separated and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma) in the presence of 2 μM monensin for 5 hours. Thereafter, the cells were stained for extracellular CD4, followed by intracellular IFN-γ and IL-17 staining, and then analyzed by flow cytometry using FACSCant II (BD Biosciences). For this analysis, PE-Cy5 conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-IL-17 (clone eBio17B7) and FITC-conjugated anti-INF-γ (clone XMG1.2) antibodies (all from eBioscience, San Diego, CA) were used.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was extracted from spleen using Isogen (WAKO, Tokyo, Japan) and reverse-transcribed to generate cDNA with SuperScript III (Invitrogen, Carlsbad, CA) and oligo-dT. PCR was then performed with PrimeSTAR HS DNA polymerase (TaKaRa, Tokyo, Japan). The primer pairs used for IFN-γ and β-actin were previously described (27), and those for IL-17 were 5'-TCCAGAAGGCCCTCAGACTA-3' (forward) and 5'-CAGTTTGGGACCCCTTTACA-3' (reverse).

The intensity of specific bands on an agarose gel electrophoresis was quantified by NIH image J software. Expression levels of β-actin for each sample were used for data normalization.

Statistical analysis

All data were analyzed by either Student's *t*-test or chi-square test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Development of thyroiditis and anti-Tg autoantibodies in wt vs. *IL-17 deficient NOD-H2^{h4} mice given NaI in the drinking water*

To clarify a role for Th17/IL-17 in the pathogenesis of iodine-induced autoimmune thyroiditis, we generated NOD-H2^{h4} mice genetically deficient for IL-17. The extent of thyroiditis and the titers of anti-Tg autoantibodies were first compared in wt littermate control mice (IL-17$^{+/+}$) and heterozygous and homozygous knockouts (IL-17$^{+/-}$ and IL-17$^{-/-}$, respectively).

In IL-17$^{+/+}$ mice that received NaI in their drinking water for 4 weeks, 5 out of 14 (36 %) mice developed mild thyroiditis with thyroiditis scores of 0.61 $\pm$ 1.18 (mean $\pm$ S.D.) *vs.* 0.00 $\pm$ 0.00 in mice not exposed to NaI, hereafter termed "controls" (Fig.1 A). The incidence and extent of thyroiditis dramatically increased after 8 week-provision of NaI, the incidence being 14/16 (88 %) and mean thyroiditis scores 2.98 $\pm$ 1.47 (Fig. 1 B). Representative histology of normal thyroid gland (from a control mouse), and grades 2 and 4 thyroiditis (from NaI-treated mice) is shown in Fig. 2.

Likewise, the titers of anti-Tg autoantibodies were very low after 4 week-treatment with NaI (0.45 $\pm$ 0.39 *vs.* 0.31 $\pm$ 0.04 OD_{492} in control mice) and rose to 0.92 $\pm$ 0.42 (*vs.* 0.16 $\pm$ 0.28 in control mice) after 8 week-NaI treatment (Fig. 3 A and B). These data for wild-type NOD-H2^{h4} mice are largely consistent with those in the previous reports (3, 4, 27).

In IL-17$^{-/-}$ mice, after 4 weeks on NaI, the incidence of thyroiditis (6/17, 35 %) and mean thyroiditis scores (0.23 $\pm$ 0.42) were not significantly different from those in IL-17$^{+/+}$ mice (Fig. 1 A). However, unlike IL-17$^{+/+}$ mice, thyroiditis was not further exacerbated by the subsequent 4 week-exposure to NaI. Thus, the incidence of thyroiditis and thyroiditis scores after 8 week-treatment with NaI (7/16 (44 %) and 0.26 $\pm$ 0.40) were significantly lower than those in IL-17$^{+/+}$ mice treated with the same protocol (both $p < 0.01$) (Fig. 1 B). IL-17$^{+/-}$ mice showed

intermediate data.

As observed for thyroiditis, after treatment with NaI for 8 weeks, the titers of anti-Tg autoantibodies (0.18 $\pm$ 0.31) were significantly lower in IL-17$^{-/-}$ compared with IL-17$^{+/+}$ mice ($p < 0.01$) (Fig. 3 A and B). Concentration-dependency was clearly observed in this antibody titer assay with serially diluted sera (Fig. 3 C). Again, the titers were intermediate in IL-17$^{+/-}$ mice.

Expression of cytokine mRNAs in the thyroid glands of IL-17$^{+/+}$ and IL-17$^{-/-}$ NOD-H2^{h4} mice

Expression of a Th1 cytokine IFN-γ and a Th17 cytokine IL-17 in the thyroid glands were evaluated by RT-PCR. Representative examples of the RT-PCR products are shown in Fig. 4 A. In wt mice, whole thyroids expressed very low levels of both cytokine mRNAs, as demonstrated previously (21, 28). This is likely due to the presence of hemopoietic cells within the thyroids (28). However, their expression levels were significantly enhanced by providing NaI [from 0.17 $\pm$ 0.11 in control mice to 0.51 $\pm$ 0.38 and 0.80 $\pm$ 0.39 ($p < 0.01$) arbitrary units in mice treated with NaI for 4 and 8 weeks, respectively, for IL-17 mRNA; from 0.49 $\pm$ 0.34 to 1.0 $\pm$ 0.21 ($p < 0.05$) and 1.4 $\pm$ 0.52 ($p < 0.01$) for IFN-γ mRNA] (Fig. 4 B and D). These data indicate that both Th1 and Th17 cells are components of intrathyroidal lymphocyte infiltrates in NOD-H2^{h4} mice.

Expression of IL-17 in the thyroid glands from IL-17$^{-/-}$ mice with/without NaI was absent as expected (Fig. 4 A and C). Intrathyroidal expression of IFN-γ mRNA was significantly increased by NaI in IL-17$^{-/-}$ mice [from 0.25 $\pm$ 0.10 in control mice to 1.1 $\pm$ 0.19 ($p < 0.01$) and 0.92 $\pm$ 0.47 arbitrary units in mice treated with NaI for 4 and 8 weeks, respectively], levels comparable to those in IL-17$^{+/+}$ mice (Fig. 4 D and E).

Splenic Th1 and Th17 cells in IL-17$^{+/+}$ and IL-17$^{-/-}$ NOD-H2^{h4} mice

Cytokine production by splenocytes stimulated with PMA and ionomycin was evaluated by intracellular cytokine staining. As shown in Fig. 5 A, B and D, the numbers of IL-17-producing Th17 cells increased from 0.49 $\pm$ 0.11 in control mice to 0.86 $\pm$ 0.22 ($p < 0.05$) and 0.91 $\pm$ 0.66 % after treatment with NaI for 4 and 8 weeks, respectively; those of IFN-γ producing Th1 cells were

also increased from 1.67 ± 0.47 to 4.91 ± 0.26 ($p < 0.01$) and 4.84 ± 2.13 %. These results demonstrate that iodine administration induces differentiation and intrathyroidal accumulation of both Th1 and Th17 cells in NOD-H2^{h4} mice.

In IL-17$^{-/-}$ mice, Th17 cells were also absent in splenocytes as expected (Fig. 5 A and C). The numbers of IFN-γ producing Th1 cells were significantly elevated from 2.28 ± 0.77 in control mice to 4.15 ± 1.15 ($p < 0.05$) and 4.38 ± 0.54 % ($p < 0.01$) in mice treated with NaI for 4 and 8 weeks, respectively and were not significantly different from those in IL-17$^{+/+}$ mice (Fig. 5 D and E). Thus, the lack of IL-17 did not affect Th1 differentiation.

Discussion

We here studied the significance of Th17/IL-17 for the pathogenesis of iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice, an animal model of Hashimoto's thyroiditis in humans. We first observed increased numbers of Th1 and Th17 cells in spleen and accumulation of both types of T_{eff} in the thyroid glands of IL-17$^{+/+}$ mice supplied with NaI. Thus, Th17 cells as well as Th1 cells contribute to intrathyroidal lymphocytic infiltration, as previously shown in granulomatous autoimmune thyroiditis (29).

The functional significance of Th17 cells was then verified in subsequent studies with IL-17$^{-/-}$ mice. Our findings demonstrate that the incidence and severity of thyroiditis, and also the titers of anti-Tg autoantibodies, were markedly reduced in NaI-treated IL-17$^{-/-}$ mice compared to IL-17$^{+/+}$ mice.

Incidentally, we attempted to measure cytokine secretion induced by Tg simulation in splenocytes from mice treated with NaI for 8 weeks. However, cytokine productions were undetectable even if iodinated Tg was used (data not shown). This is presumably due to extremely low frequency of Tg-specific T_{eff} in NOD-H2^{h4} mice, although anti-Tg autoantibodies were readily detected as demonstrated above, but not due to a matter of antigenecity of Tg, although iodination of Tg has been reported to enhance its antigenecity (30). Fynn *et al*. (31) have also observed no T cell proliferation to Tg in DR3 transgenic class II-knock-out NOD mice. Further, similar data have

been reported in NOD mice; detection of insulin-specific T cell response has been reported to be difficult in the mice, despite clear elevation of anti-insulin autoantibodies in their sera (32).

Overall, the present study, together with a previous report demonstrating the importance of Th1, not Th2, immune response for developing thyroiditis using IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$mice (22), clearly demonstrate that Th17 as well as Th1 immune responses are crucial for the pathogenesis of iodine-induced autoimmune thyroiditis in NOD-$H2^{h4}$ mice.

Of interest, IL-$17^{+/-}$ mice showed an intermediate phenotype. These results of "haploinsufficiency" of IL-17, which we have first found, may be very important in a clinical point of view, because they imply the importance of a subtle difference in IL-17 expression in autoimmune reaction. Similar results are for examples demonstrated in studies on peroxisome proliferator-activated receptor-γ for B cell function (33) and on CD95 for T cell apoptosis (34).

The relationship and interaction between Th1 and Th17 subsets are controversial. Thus, it was originally thought that differentiation of these 2 T cell subsets is mutually exclusive (9, 10, 35). For example, Th17 cells are generally induced earlier than Th1 cells in some murine autoimmune diseases. In a mouse model of uveitis, Th17 cells are most abundant in the retina at early stage of the disease; however, Th1 cells are most abundant at a later stage associated with resolution of the disease (14). Similarly, T cells produced more IL-17 in the induction phase and more IFN-γ in the effector phase in response to glucose-6-phosphate isomerase (GPI) in a GPI-induced arthritis model (36). In these transient autoimmune disease models, diseases are induced by Th17 cells and then suppressed by Th1 cells through inhibiting the preceding Th17 immune response. Thus, Th17 cells are likely pathogenic and Th1 cells protective. Indeed IFN-$\gamma^{-/-}$ mice develop normal or even exaggerated diseases in most of inducible types of autoimmune disease models.

However, our findings are inconsistent with these reports. We observed that Th1 and Th17 cell are co-localized in the thyroid lesions. Moreover, the numbers of both Th1 and Th17 cells were increased during the first 4 weeks and then maintained at a constant level during the next 4 weeks in chronic, iodine-induced autoimmune thyroiditis, indicating similar kinetics of differentiation and accumulation of Th1 and Th17 cells. Because both IFN-$\gamma^{-/-}$ and IL-$17^{-/-}$ NOD-$H2^{h4}$ mice are

resistant to thyroiditis (ref. 20 and the present study), both Th1 and Th17 cells are likely pathogenic. We therefore hypothesize that:- (i) the relative importance of Th1 and Th17 cells may not be the same in different models of autoimmune diseases, particularly between transient *vs.* chronic autoimmune disease models; and (ii) that both types of T_{eff} appear to play a pathological role in a chronic, iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice. Recent studies also demonstrated co-localization of Th1 and Th17 cells in pathological environments of various autoimmune diseases in humans thus indicating cooperation of Th1 and Th17 cells (19, 37).

In this regard, it is of interest that Kryczek *et al*. (38) have recently challenged the dogma that IFN-γ suppresses Th17 and enhances Th1 development, by showing that Th1-derived IFN-γ attenuates Th1-mediated immune response and allows Th17 memory cells to expand. They concluded that IFN-γ prevents naïve T cells from Th17 cell differentiation, while promoting Th17 polarization of memory T cells.

However, the following reports make interpretation of relationship between different T_{eff} even more puzzling. First, either Th1 or Th17 cell lines generated *in vitro* from TCR-transgenic mice specific for hen egg lysozyme (HEL) can induce uveitis in eye-specific HEL transgenic mice (16). Second, the gastric parietal cell antigen (H^+K^+ ATPase)-specific, *in vitro*-expanded Th1, Th2 and Th17 cells from TCR-transgenic mice can all transfer autoimmune gastritis to nude mice (39). Third, antigen-primed, Th1- and Th17-polarized T cells can both induce EAE and uveitis in naïve mice (15, 40). These data indicate that any types of antigen-specific T_{eff} induced artificially *in vitro* are pathogenic. Furthermore, the relative importance of Th1/Th17 may also be dependent on immunization protocols. For example, Th1 and Th17 cells play a dominant role in uveitis induced by immunization with interphotoreceptor retinoid-binding protein (IRBP, an autoantigen in autoimmune eye diseases) emulsified with Complete Freund's adjuvant or IRBP-pulsed mature dendritic cells, respectively (15). The dominant T_{eff} phenotype may be determined by conditions of cytokine microenvironments and types of antigen-presenting cells. Further studies will be necessary to clarify these issues.

IL-17 may be critical for the initiation and/or effector phases depending on the particular

disease model studied. Thus, activation of T cells has been reported to be impaired in the induction phase of collagen-induces arthritis in IL-17$^{-/-}$ mice (12). It has also recently been reported that administration of IL-17 receptor-Fc fusion protein (41) or injection of anti-IL-17 antibody (15) during the effector phase suppresses collagen-induced arthritis or IRBP-induced uveitis, respectively. In the chronic EAE model, IL-17 receptor-Fc fusion protein or injection of anti-IL-17 antibody has been effective both in the initiation and effector phases (42). However, treatment with anit-IL-17 antibody was only effective in the initiation, not the effector, phase in a GPI-induced arthritis model (36). In any case, the effectiveness of anti-IL-17 treatment during the effector phase in the most studies suggests that Th17/IL-17 can potentially be a therapeutic target.

In conclusion, our present investigation together with previous studies demonstrates that both Th17 as well as Th1 cells are critical T_{eff} subsets for the development of iodine-induced autoimmune thyroiditis in NOD-H2^{h4} mice, although direct interaction between Th1 and Th17 cells in thyroiditis in these mice remains to be elucidated. Future studies regarding involvement of Th17 in the pathogenesis of Hashimoto's thyroiditis in humans will be of interest.

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Figure legends

Fig. 1. Thyroiditis scores in control and iodine-fed NOD-H2^{h4} mice. The mice were fed with the drinking water containing 0.15 % NaI for 4 (*A*) or 8 (*B*) weeks. Thyroid histology was examined with H & E staining to score the extent of intrathyroidal lymphocyte infiltration (see the *Materials and Methods*). Data are shown for individual mice. The horizontal bars indicate the mean values for each group. n.d., not determined. *, $p < 0.01$ (*t*-test).

Fig. 2. Representative histology of thyroid glands in control and iodine-fed NOD-H2^{h4} mice. *A*, grade 0 in a control mouse, *B* and *C*, grades 2 and 4, respectively, thyroiditis in NaI treated mice.

Fig. 3. Anti-Tg autoantibody titers in sera from control and iodine-fed NOD-H2^{h4} mice. Sera were obtained from the mice shown in Fig. 1, and anti-Tg autoantibody titers determined by ELISA (see the *Materials and Methods*). Sera were diluted at 1:100 (*A and B*) or serially (1:100 to 1:3,000) (*C*). Data are shown for individual mice. The horizontal bars indicate the mean values for each group. n.d., not determined. *, $p < 0.05$; **, $p < 0.01$ (*t*-test).

Fig. 4. Expression of IFN-γ and IL-17 mRNAs in the thyroid glands of control and iodine-fed NOD-H2^{h4} mice. Total RNA was extracted from the thyroid glands of mice in Fig. 1, and subjected to RT-PCR to amplify IL-17 and IFN-γ mRNAs (see the *Materials and Methods*). *A*, representative PCR products for IL-17 and IFN-γ. *B* and *D*, quantification of IL-17 and IFN-γ mRNAs in IL-17$^{+/+}$ mice. *C* and *E*, quantification of IL-17 and IFN-γ mRNAs in IL-17$^{-/-}$ mice. The data are means $\pm$ S.D ($n = 3$ to 6). *, $p < 0.05$; **, $p < 0.01$ (*t*-test).

Fig. 5. Flow cytometric analysis of Th1 ($CD4^+IFN\text{-}\gamma^+$) and Th17 ($CD4^+IL\text{-}17^+$) cells in spleens

of control and iodine-fed NOD-H2^{h4} mice. Splenocytes were prepared from spleens of mice shown in Fig. 1, stimulated with PMA and ionomycin for 5 hours, stained for cell surface CD4 and intracellular IFN-γ and IL-17 and analyzed with flow cytometry (see the *Materials and Methods*). *A*, representative staining of CD4^{+} splenocytes for intracellular IFN-γ and IL-17. *B* and *D*, numeration of Th1 and Th17 cells in IL-17$^{+/+}$ mice. *C* and *E*, numeration of Th1 and Th17 cells in IL-17$^{-/-}$ mice. The data are means $\pm$ S.D (n = 4). *, $p < 0.05$; **, $p < 0.01$ (*t*-test).

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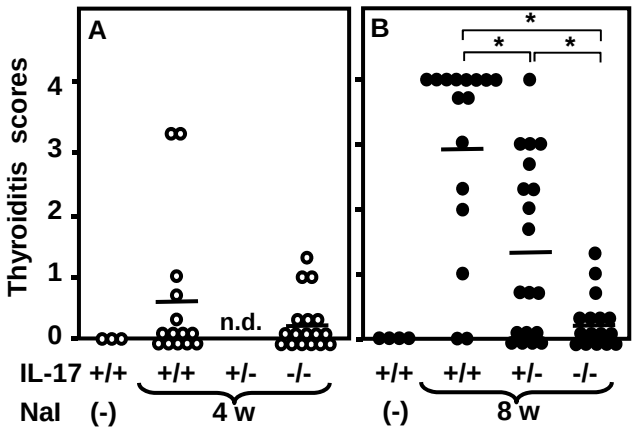
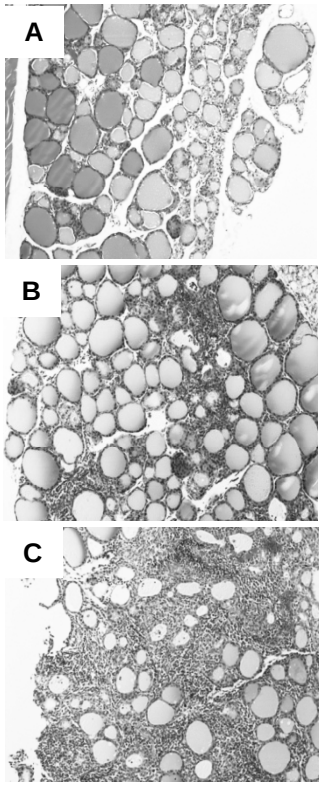


Horie et al. Fig. 1



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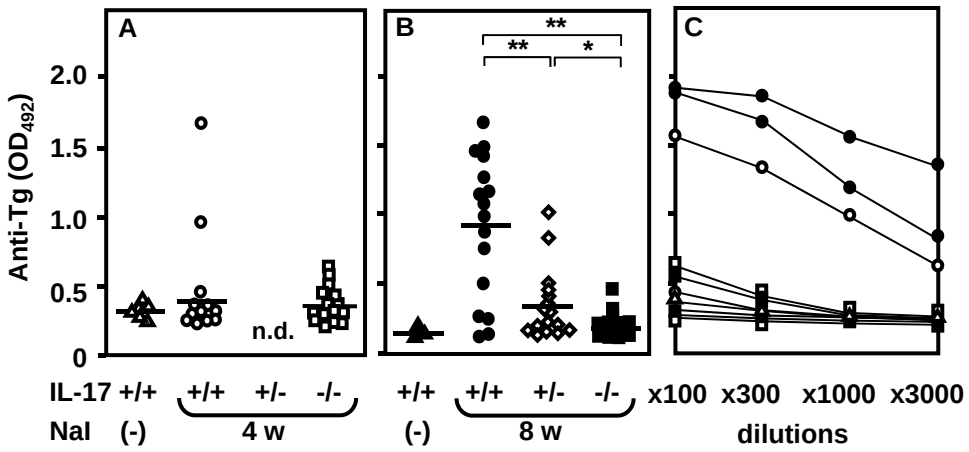

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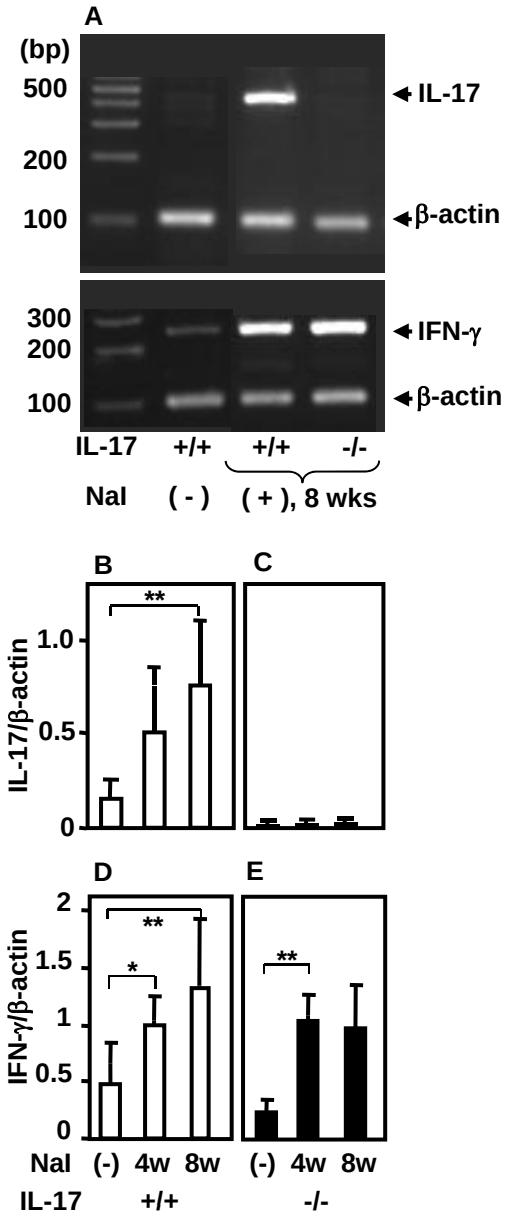

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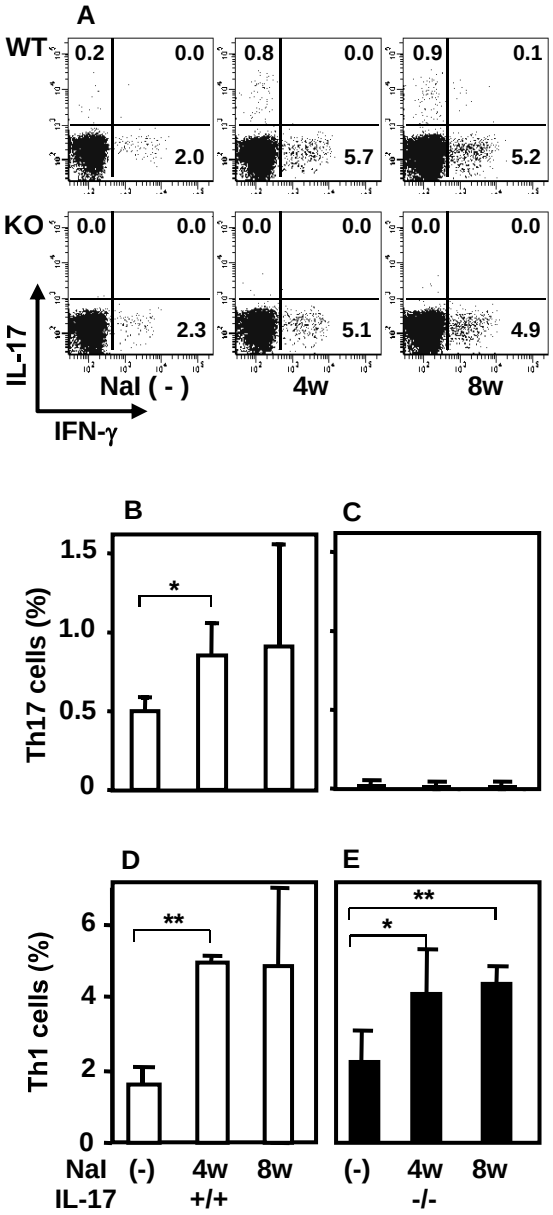

Horie et al. Fig. 5