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Evidence that MHC I-E dampens thyroid autoantibodies and prevents spreading to a second thyroid autoantigen in I-A^k NOD mice

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Abstract

NOD.*H2*^{k} and NOD.*H2*^{h4} mice carry the MHC class II molecule I-A^k associated with susceptibility to experimentally-induced thyroiditis. Dietary iodine enhanced spontaneous thyroid autoimmunity, well known in NOD.*H2*^{h4} mice, has not been investigated in NOD.*H2*^{k} mice. We compared NOD.*H2*^{h4} and NOD.*H2*^{k} strains for thyroiditis and autoantibodies to thyroglobulin (TgAb) and thyroid peroxidase (TPOAb) without or with dietary sodium iodide (NaI) for up to 32 weeks. TgAb levels were significantly higher in NOD.*H2*^{h4} than NOD.*H2*^{k} mice on NaI and TPOAb developed in NOD.*H2*^{h4} but not NOD.*H2*^{k} mice. DNA exome analysis revealed, in addition to the differences in the chromosome (Chr) 17 MHC regions, that NOD.*H2*^{k} and particularly NOD.*H2*^{h4} mice have substantial non-MHC parental DNA. KEGG pathway-analysis highlighted thyroid autoimmunity and immune-response genes on Chr 17 but not on Chr 7 and 15 parental B10.A4R DNA. Studies of parental strains provided no evidence for non-MHC gene contributions. The exon 10 thyroglobulin haplotype, associated with experimentally-induced thyroiditis, is absent in NOD.*H2*^{h4} and NOD.*H2*^{k} mice and is not a marker for spontaneous murine thyroid autoimmunity. In conclusion, the absence of I-E is a likely explanation for the difference between NOD.*H2*^{h4} and NOD.*H2*^{k} mice in TgAb levels and, as in humans, autoantibody spreading to TPO.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

Supplementary Information is available at Gene and Immunity's website.

INTRODUCTION

Lymphocytic infiltration of the thyroid gland and autoantibodies to thyroglobulin (Tg) and thyroid peroxidase (TPO) are the hallmarks of Hashimoto's thyroiditis as reflected by the presence of thyroid autoantibodies in 15% of the adult female population (1). The NOD.$H2^{h4}$ mouse strain, an invaluable mouse model of Hashimoto's disease, was derived from a cross between non-obese diabetic (NOD) mice and the non-diabetic B10.A4R strain (2). NOD.$H2^{h4}$ mice do not become diabetic but spontaneously develop thyroiditis together with autoantibodies to Tg (3–5) and, at a later stage, to TPO (6). Exposure to dietary iodide enhances development of thyroid autoimmunity in NOD.$H2^{h4}$ mice (3–6), as in humans (for example (7;8).

The development of diabetes in NOD mice is controlled, in part, by a unique MHC class II molecule (I-A^{g7}) together with the lack of expression of I-E, the second MHC class II protein in mice (for example 9;10). NOD.$H2^{h4}$ and NOD.$H2^{k}$ strains were among a panel of NOD strains developed to determine the impact of expressing a non-NOD MHC locus on the incidence of insulitis and autoimmune diabetes (2). Both NOD.$H2^{h4}$ and NOD.$H2^{k}$ strains carry the MHC class II molecule I-A^{k} which is associated with susceptibility to thyroiditis induced experimentally using Tg (for example (11;12). Mice of the NOD.$H2^{k}$ strain, unlike NOD.$H2^{h4}$ mice, express I-E molecules (2).

The effect of dietary iodide intake on thyroid autoimmunity has been extensively investigated in NOD.$H2^{h4}$ mice (for example (3–5;13–17) and the parent NOD strain (5;18;19). The NOD.$H2^{k}$ strain has been studied for spontaneous thyroid autoimmunity as well for thyroiditis induced by mouse Tg immunization, supporting a role for the H-2^{k} locus in increased susceptibility to thyroiditis relative to the H-2^{g7} MHC haplotype (20;21). However, there are no studies in NOD.$H2^{k}$ mice on the effects of increased dietary iodide.

Because of the critical role of MHC H-2^{k} in susceptibility to induced thyroiditis (for example (11;12), we considered that a comparative study of NOD.$H2^{k}$ and NOD.$H2^{h4}$ strains would allow us to determine the specific contributions of the MHC locus to thyroid autoimmunity following iodine intake. Therefore, we characterized NOD.$H2^{h4}$ and NOD.$H2^{k}$ mice for the development of autoantibodies to Tg and TPO and thyroid histology after long term exposure to dietary iodide. To provide further insight into the basis for murine thyroid autoimmunity, we performed exome analysis of DNA from NOD.$H2^{h4}$, NOD.$H2^{k}$ and NOD mice. We find that, despite the presence of I-A^{k}, a component of the MHC locus, likely the presence of I-E, dampens thyroid autoantibodies and particularly autoantibody spreading to TPO.

RESULTS

TgAb and thyroiditis in male NOD.$H2^{k}$, NOD.$H2^{h4}$ and NOD mice

From the age of 8 weeks, NOD.$H2^{k}$, NOD.$H2^{h4}$ and NOD mice were maintained on regular water or water supplemented with NaI and studied for thyroid autoantibodies and thyroiditis after 8, 16 or 32 weeks. TgAb developed at low and variable levels in NOD.$H2^{k}$ and NOD.$H2^{h4}$ mice on regular water with no significant difference between the strains (Fig.

1A). On NaI supplemented water, TgAb were significantly higher after 8 weeks, and reached a higher plateau after 16 weeks, in NOD.H^{h4} than in NOD.$H2^{k}$ mice (Fig. 1B).

Thyroiditis, measured as the percentage of the gland infiltrated by lymphocytes, was variable in individual mice (Supplemental Fig. S1). The extent of thyroiditis was significantly greater in NOD.$H2^{h4}$ than NOD.$H2^{k}$ mice on regular water at the earlier time point studied (8–16 weeks), despite variability as reflected in the standard error bars (Fig. 2A). The apparent decrease in thyroiditis in NOD.$H2^{h4}$ mice on regular water at 32 versus 8–16 weeks was not significantly different. Similarly, the apparent increase in thyroiditis at the later time point in NOD.$H2^{k}$ mice was also not significant. Turning to studies on NaI-water, a few NOD.$H2^{k}$ mice but none of the NOD.$H2^{h4}$ mice had very low levels of thyroid lymphocytic infiltration (less than 5%, Supplementary Fig. S1). Overall, however, there was no statistically significant difference in thyroiditis between the two strains after 8–16 and 32 weeks on NaI-water (Fig. 2B). Mice without TgAb had minimal thyroid lymphocytic infiltrates whereas moderate or extensive thyroiditis developed in TgAb positive NOD.$H2^{h4}$ or NOD.$H2^{k}$ mice (Supplementary Fig. S2).

The NOD.$H2^{k}$ and NOD.$H2^{h4}$ strains were maintained in Montreal and Los Angeles, respectively. To exclude possible environmental effects, NOD mice (purchased from The Jackson Laboratory) were studied in both Montreal and Los Angeles. Similar very low TgAb levels were observed in NOD mice maintained in either Montreal or Los Angeles (Fig. 3). In addition, the limited development of thyroiditis in NOD mice maintained in the two locations (Fig. 2C; Supplementary Fig. S1) confirmed the lack of an environmental effect.

Overall, we observed that NOD.$H2^{h4}$ mice are more susceptible than NOD.$H2^{k}$ mice to the development of TgAb enhanced by dietary NaI.

TPOAb in male NOD.$H2^{k}$, NOD.$H2^{h4}$ and NOD mice

Previously we found that TPOAb develop later than TgAb in NOD.$H2^{h4}$ mice, only being being detectable in older mice (aged about 7 months) (6). For this reason, we tested sera for TPOAb in mice exposed to NaI for 16 and 32 weeks (aged 6 and 10 months, respectively). At both time intervals, TPOAb levels were clearly present in NOD.$H2^{h4}$ mice and essentially absent in NOD.$H2^{k}$ mice (Fig. 4). Together with the above data for TgAb, these observations for TPOAb suggest that genetic variants between the two strains confer differences in the thyroid autoantibody profile.

Exome Analysis of NOD.H2^{h4} and NOD.$H2^{k}$ versus parental strains

The most obvious genetic difference between the two strains is the MHC locus. Indeed, NOD.$H2^{h4}$ and NOD.$H2^{k}$ mice were originally generated to investigate the impact of various MHC haplotypes, that do or do not bear a functional I-E molecule, on the incidence of diabetes (2). MHC class I and class II molecules expressed by NOD.$H2^{h4}$ and NOD.$H2^{k}$ mice are summarized in Fig. 5A. Both strains carry the genes for MHC class I K^{k} and class II I-A^{k} but they differ in two other respects. First, NOD.$H2^{h4}$ mice express MHC class I H2-D^{b} and NOD.$H2^{k}$ mice express D^{k}. Second, NOD.$H2^{h4}$ mice do not express I-E whereas

NOD.$H2^k$ mice are I-E positive. It is tempting to suggest that these differences in the MHC locus, specifically for genes between I-E and H-2D, dampen the susceptibility conferred by I-A^k to thyroid autoimmunity as reflected in TgAb and TPOAb.

However, in addition to the MHC locus, these two strains carry other genetic differences. NOD.$H2^{h4}$ and NOD.$H2^k$ mice were derived from NOD mice outcrossed to B10.A4R and B10.BR, respectively, and further backcrossed to NOD for 6 generations, while maintaining the $H2^{h4}$ or the $H2^k$ MHC loci, respectively (2). Subsequently, the NOD.$H2^{h4}$ strain was maintained by brother to sister mating. Theoretically, 0.6% of the genome from NOD.$H2^{h4}$ mice should be of B10.A4R origin. To determine the extent of genetic contamination from the parental strain, we undertook exome analysis of NOD.$H2^{h4}$ mice and of both NOD and B10.A4R parents. In addition to the B10.A4R MHC region on Chr 17 (2), NOD.$H2^{h4}$ mice have retained B10.A4R parental DNA from Chr 7 and Chr 15 amounting to 88.6 Mb (Fig. 5B).

The NOD.$H2^k$ strain was not simply maintained by brother to sister mating. Two transgenes, namely the 3A9 T cell receptor (TCR) and the ILK-3 insHEL transgenes in C57BL/6 mice, have been bred onto the NOD.$H2^k$ genetic background for more than 20 generations (22). Both transgenes are maintained as heterozygotes on the NOD.$H2^k$ background and only mice negative for both transgenes were used in the current study. Nevertheless, as expected, we find “carry over” alleles from C57BL/6 mice near the transgenic insertions on both Chr 5 and Chr 12 (23). In addition, NOD.$H2^k$ mice have the expected B10.BR Chr 17 DNA as well as non-NOD DNA (presumably from the B10.BR parent) on Chr 9 and 18 amounting to 14.2 Mb (Fig. 5B). Importantly, MHC class I H2-D^k lies at the border of the B10.BR region on Chr 17. For this reason, MHC-typing was performed in NOD.$H\text{-}2^k$ mice (and controls) and confirmed that mice of this strain carry the entire H2-k MHC haplotype including H2-D^k (Supplementary Fig. S3).

Analysis of B10.A4R gene segments in NOD.$H2^{h4}$ mice

DAVID analysis was used for functional classification of the genes found within the B10.A4R regions in NOD.$H2^{h4}$ mice into “Biological Process (BP) Gene Ontology (GO) Terms” and “KEGG pathways” (Supplemental Tables S1-S2). In addition to the expected MHC genes, Chr 17 harbors more genes related to immunological processes than the B10.A4R intervals on Chr 7 and Chr 15 of NOD.$H2^{h4}$ mice (Supplemental Table S1). Interestingly, only a few endocrine associated genes (such as *Pde3B, Ghr* and *Ppard)* are included within the B10.A4R intervals on Chr 7, 15 and 17 of NOD.$H2^{h4}$ mice (Supplemental Table S2. Based on KEGG pathway analysis, only genes encoded within the Chr 17 locus (and none of the genes encoded within the Chr 7 and 15 B10.A4R segments) have been previously associated with autoimmune thyroid disease or with antigen processing and presentation (Supplemental Table S3 and S4).

To further investigate whether the Chr 7 interval contributes to increased susceptibility to autoimmune thyroiditis in NOD.$H2^{h4}$ mice, we took advantage of the NOD.Lc7 strain (24). This NOD strain is congenic for a segment on the distal end of mouse Chr 7 which coincides with the B10.A4R interval retained in NOD.$H2^{h4}$ mice. As in the NOD.$H2^{h4}$ and NOD.$H2^k$ strains, the NOD.Lc7 strain is likely to include additional DNA from the donor strain. With

this proviso in mind, male mice of both NOD.Lc7 and B10.A4R strains were exposed to regular water or NaI water for 16 weeks. TgAb did not develop in NOD.Lc7 or B10.A4R mice and thyroiditis was minimal or absent (Table 1). These data suggest that the genes encoded in the B10.A4(4R) interval on Chr 7 retained in NOD.$H2^{h4}$ mice, as well as the background non-MHC genes in B10.A4R mice, are not sufficient to increase susceptibility to spontaneous or dietary iodine induced thyroiditis.

Exon 10 haplotypes of the Thyroglobulin gene (Chr 15)

Thyroglobulin (Tg) is a thyroid specific susceptibility gene for thyroid autoimmunity in humans (25). In mice, in addition to susceptibility conferred by MHC class II (namely I-A^k), susceptibility to experimentally-induced thyroiditis has been associated with the Tg haplotype Ser-Met-Thr and resistance by the haplotype Asn-Val-Ile (26). Tg is located on mouse Chr 15 at 66 Mb, upstream of the B10A.A4R interval in NOD.$H2^{h4}$ mice (Fig. 5B). Although Tg is found outside of the B10.A4R interval in NOD.$H\text{-}2^{h4}$ mice, we wished to establish the Tg haplotype of the mouse strains in this study. Sequencing of exon 10 showed that NOD.$H2^{h4}$, NOD and B10.A4R all have the Tg haplotype Asn-Val-Ile (Table 2). Because exome sequencing showed no differences on Chr 15 between NOD and NOD.$H2^k$ strains, NOD.$H2^k$ mice also have the Asn-Val-Ile Tg haplotype. Consequently, the Tg-haplotype, associated with experimentally-induced thyroiditis, is not a susceptibility marker for spontaneous thyroiditis in mice.

DISCUSSION

Our studies demonstrate that spontaneous thyroid autoimmunity is enhanced and sustained by dietary NaI in NOD.$H2^k$ as well as in NOD.$H2^{h4}$ mice but the magnitude of the response is much greater in the latter than in the former strain. In particular, TgAb are detectable earlier and reach higher plateau levels in NOD.$H2^{h4}$ mice than in the NOD.$H2^k$ strain. Antibodies to thyroid peroxidase (TPO) develop in the NOD.$H2^{h4}$ strain as we observed previously (6). Unexpectedly, TPOAb are virtually absent in NOD.$H2^k$ mice, reflecting a lack of autoantibody spreading to this other major thyroid autoantigen. These findings also contrast with our observations in transgenic BALB/c mice that express the human thyrotropin receptor (TSHR) A-subunit in the thyroid: after antibody-mediated depletion of regulatory T cells and human TSHR-A-subunit adenovirus immunization, these transgenics developed extensive thyroiditis together with autoantibodies to both Tg and TPO (27).

It should be emphasized that TgAb are associated with spontaneous thyroiditis and with the majority of experimentally induced thyroiditis models in mice. However, in patients with autoimmune thyroid disease, TPOAb are also present and are more common than TgAb (1). Consequently, because they spontaneously develop both TPOAb and TgAb, NOD.$H2^{h4}$ mice provide the mouse model that most closely represents human autoimmune thyroid disease (Hashimoto's thyroiditis).

We performed exome analysis to explore the observed differences in thyroid autoimmunity in NOD.$H2^k$ and NOD.$H2^{h4}$ mice. Both NOD.$H2^k$ and NOD.$H2^{h4}$ strains have the MHC regions (Chr 17) of the parental strains used in their generation, namely B10.BR and B10.A4R, respectively (2). In addition, NOD.$H2^{h4}$ and NOD.$H2^k$ strains have retained

segments of residual parental DNA. These findings raised questions about the contribution (if any) of the "left-over" DNA to the observed differences in spontaneous and NaI enhanced thyroid autoimmunity in these two strains.

DAVID analysis was used to explore possible contributions of the substantial residual B10.A4R chromosomal segments to the development of thyroid autoimmunity in NOD.$H2^{h4}$ mice. KEGG pathway analysis highlighted the presence of several genes associated with thyroid autoimmunity as well as multiple immune-associated genes on Chr 17. In contrast, in the residual B10.A4R segments from Chr 7 and Chr 15, there is a paucity of immune-response genes and no genes previously identified with thyroid autoimmunity.

Other potential "clues" for a role of these left-over segments were sought from previous studies. First, thyroiditis induced in the F2 offspring of NOD.$H2^{k}$ x CBA/J mice by immunization with Tg in Complete Freund's adjuvant was significantly associated with a Chr 17 locus distinct from the MHC named "Ceat1" (21) located at the same position as the plasminogen gene. Of two other loci associated with experimentally induced thyroiditis in the F2 offspring, the Chr 7 marker D7Mit20 (Cd79a) lies at 24.9 Mb, which is not within the residual B10.A4R segment on the same chromosome. Second, possible information regarding these gene segments could relate to enhanced apoptosis and impaired oxidative stress observed in NOD.$H2^{h4}$ thyrocytes which involves increased expression of the following genes: TPO, glutathione peroxidases, peroxiredoxins, superoxide dismutase 1, thioredoxin 1 and uncoupling proteins (28). Of these genes, none are located on Chr 15 and the genes for uncoupling proteins 2 and 3 are located on Chr 7 downstream of the residual B10.A4R segment (Fig. 5B). Overall, neither DAVID analysis nor earlier studies provided insight into a possible role for genes in the Chr 7 and 15 "left-over" B10.A4R segments in spontaneous or iodine enhanced thyroiditis in NOD.$H2^{h4}$ mice.

In a different approach, we sought potential clues to a role for the residual parental genes by undertaking studies in other NOD-related mice, including the parental strains B10.A4R and NOD, as well as NOD.Lc7 mice. The latter strain is congenic for a segment on the distal end of mouse Chr 7 which coincides with the B10.A4R interval retained in NOD.$H2^{h4}$ mice. Consequently, this strain could provide insight into the impact of B10.A4R Chr 7 DNA on thyroiditis in NOD mice with or without NaI intake. None of these three mouse strains (with or without NaI) developed TgAb and thyroiditis was minimal, consistent with data for NOD and B10.A4R versus NOD.$H2^{h4}$ mice summarized in a review (18). We found that sporadic NOD and NOD.$H2^{k}$ mice developed thyroiditis without NaI supplementation, also consistent with earlier findings (20) and with the established variability in diabetes and breeding in the NOD strain (for example (29).

What is the basis for the greater propensity of NOD.$H2^{h4}$ than NOD.$H2^{k}$ mice to develop TgAb and autoantibody spreading to TPO? Both strains bear I-A^{k}. Neither strain expresses the Tg haplotype previously associated with susceptibility to experimentally-induced thyroiditis (26) which we show herein is not a susceptibility marker for spontaneous thyroid autoimmunity in mice. In addition, the residual B10.A4R gene segment on Chr 7 on the NOD background is insufficient to produce the thyroid autoimmunity observed in NOD.$H2^{h4}$ mice in this study and in multiple other previous studies of the same strain (for

example (3–5). A major difference between these relatively closely related strains concerns the MHC region other than I-A^k. One discrepancy involves the H2-D region, D^b versus D^k for NOD.*H2*^{h4} versus NOD.*H2*^{k}, respectively). The presence of D^k has no adverse effect on thyroiditis induced in the "good responder" strain B10.BR (30) or in NOD.*H2*^{k} mice (20). We cannot exclude a contribution from variants in H2-D or other non-I-A^k MHC genes. However, the most conspicuous difference between the two strains concerns I-E: NOD.*H2*^{k} mice express I-E while NOD.*H2*^{h4} mice express I-Eβ but not I-Eα and are therefore I-E negative (2).

The importance of I-E expression for development of diabetes has been mentioned (Introduction). Lymphocytic infiltrates are present in the pancreatic islets and salivary glands in *unimmunized* mice of several strains with defective I-E (31). However, the lack of I-E expression in itself is insufficient to permit development of thyroiditis and thyroid-specific autoantibodies even after immunization, as exemplified by C57BL/6 mice (Table 2). I-A plays a role, as already mentioned for experimentally induced thyroiditis (for example (11;12) as well as for spontaneous thyroiditis (Table 3): I-A^k is the most permissive, I-A^{g7} next while I-A^q is not permissive for thyroiditis and thyroid autoantibodies. TgAb are absent, despite development of thyroiditis, in NOD mice and NOD transgenics that express I-E and a modified I-A (NOD-E and NOD-Asp, respectively) (5), likely because these strains lack I-A^k. As we show in the present study, thyroid autoimmunity develops in I-A^k mice that express I-E but, compared with I-E non-expressing NOD.*H2*^{h4} mice, TgAb levels are significantly lower and TPOAb are virtually absent on a NaI-diet. Consistent with our observations, a recent study observed that expressing additional I-E molecules in C57BL/6 mice limited autoimmunity (32).

In conclusion, exome sequencing demonstrated the anticipated MHC divergence between NOD.*H2*^{h4} and NOD.*H2*^{k} strains as well as differences in some non-MHC genes. These two strains differ in the quality and quality of thyroid autoantibody responses enhanced by increased dietary iodide. However, parallel studies of parental mouse strains did not suggest that non-MHC genes were responsible for the differences between NOD.*H2*^{h4} and NOD.*H2*^{k} strains. Instead, the lack of I-E expression, is a likely explanation for the marked difference between NOD.*H2*^{h4} and NOD.*H2*^{k} mice in terms of TgAb and, as in humans, autoantibody spreading to TPO.

MATERIALS AND METHODS

Mouse strains

All studies were performed in male mice to avoid the complications of early development of diabetes in female NOD mice. It should be noted that the incidence of autoimmune thyroiditis is reported to be similar in male and female NOD.*H2*^{h4} mice (4;20). The following mouse strains were investigated:-

1. NOD.*H2*^{h4} [NOD.Cg-*H*^{2h4}/DilTacUmmJ] and B10.A4R [B10.A-*H*^{2h4}/(4R) SgDvEgJ] from The Jackson Laboratory were bred at Cedars-Sinai Medical Center, Los Angeles.

2. NOD.*H2*^{k} mice (24) were bred in Montreal (Department of Immunology-Oncology, Maisonneuve-Rosemont Hospital).
3. NOD [NOD/ShiLtJ] and B10.BR [B10.BR-*H2*^{k2} H2-T18^{a}/SgSnJ] mice from The Jackson Laboratory and NOD.Lc7 mice (24) were bred at the Maisonneuve-Rosemont Hospital, Montréal.
4. NOD [NOD/ShiLtJ] mice from The Jackson Laboratory were purchased for studies in Los Angeles.
5. BALB/c mice (originally from The Jackson Laboratory) were bred in Los Angeles.

Exposure to regular water or iodide supplemented water

From the age of 8 weeks, mice were maintained on regular water ("Con") or water supplemented with 0.05% sodium iodide (NaI; Sigma-Aldrich, St Louis, MO) for 16 weeks (NOD, NOD.Lc7 and B10.A4R) and for up to 32 weeks (NOD.H2^{k} and NOD.*H2*^{h4}). Blood was drawn before treatment and after 8 or 16 weeks on regular water or NaI. At the 16 week time point, some mice (including all NOD, B10.A4R and NOD.Lc7) were euthanized to harvest blood and thyroid tissue. Additional NOD.*H2*^{k} and NOD.*H2*^{h4} mice were maintained on regular or NaI water for up to 32 weeks. NOD mice were tested for urinary glucose using Diastix, Reagent Strips for Urinalysis (Glucose Bayer HealthCare LLC, Mishawaka IN). Data for NOD mice after development of diabetes were excluded from analysis.

All mouse studies were performed in accordance with the guide-lines of the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center (Los Angeles) and Maisonneuve -Rosemont Hospital (Montréal) and performed with the highest standards of care.

Autoantibodies to murine thyroglobulin and thyroid peroxidase

Thyroid autoantibody assays were performed in Los Angeles. Tg was isolated from murine thyroid glands as previously described (6). ELISA wells (Immulon 4HBX, Thermo Scientific, Rochester NY) were coated with mouse Tg (1.5 μg/ml) and incubated with test sera (duplicate aliquots, 1:100 dilution). Antibody binding was detected with horse radish peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis MO), the signal developed with o-phenylenediamine and the reaction stopped using 20% H_2SO_4. The negative control was pooled sera from 8 week old NOD.*H2*^{h4} mice and the positive control was serum from a BALB/c mouse immunized with mouse Tg and complete Freund's adjuvant (33). Data for TgAb are presented as the optical density (OD) at 490 nm normalized against an appropriate dilution of the positive control serum included in each ELISA assay. TgAb were measured in separate assays for NOD.*H2*^{h4} and NOD.*H2*^{k} *mice*. To confirm that data from different assays were comparable, 20 sera from NOD.*H2*^{h4} and 20 sera from NOD.*H2*^{k} *mice* (obtained after the same time intervals on control or NaI water) were re-assayed on the **same** ELISA plate. These data confirmed that TgAb levels were significantly higher in NOD.*H2*^{h4} than in NOD.*H2*^{k} mice (Supplemental Table S5a; Rank Sum test, $p<0.001$). In addition, ELISA values obtained in the repeat ELISA were not

significantly different from the values in the original assays (Supplemental Table S5b; Wilcoxon Rank sum test, p=0.214).

TPOAb were measured by flow cytometry using Chinese hamster ovary (CHO) cells stably expressing mouse-thyroid peroxidase (mTPO) (6). Sera (1:50 dilution) were incubated with mTPO-CHO cells and binding was detected with fluorescein isothyocyanate-conjugated affinity purified goat anti-mouse IgG (Caltag Laboratories, Burlingame CA). Cells staining with propidium iodide (1mg/ml) were excluded from analysis. The negative controls for IgG class antibody binding to mTPO-CHO cells were sera from 8 week old BALB/c mice. Positive controls were (a) mouse monoclonal antibodies #15 and #64 generated against human TPO (34), provided to us by Dr. Jean Ruf that recognize mouse TPO (6); (b) sera from transgenic TSHR A-subunit BALB/c mice that developed TPOAb after regulatory T cell depletion and immunization with A-subunit adenovirus (33). Flow cytometry was performed (10,000 events) using a FACScan with CELLQUEST Software (Becton Dickinson, San Jose, CA). Data analyzed with FlowJo software (TreeStar, Ashland, OR, USA) are reported as the geometric mean (Geo Mean).

Thyroid histology

Thyroid glands were preserved in formaldehyde (Montreal) or zinc fixative (Los Angeles; IHC-zinc fixative, BD Pharmingen, San Diego CA), paraffin-embedded and serial sections stained with hematoxylin and eosin (IDEXX BioResearch Lab Animal and Biological Materials Diagnostic Testing, Columbia, MO). Scoring of thyroid lymphocytic infiltration was expressed as a percentage; thyroid sections with no infiltration were assigned the value 1%. All thyroid histology was examined in Los Angeles by the same two reviewers (SMM and BB) with limited information on each slide other than mouse strain; subsequently, the two scores were combined to provide a mean value for each mouse. Slides were evaluated in mixed groups of thyroids from several mouse strains.

Statistical analyses

Significant differences between responses in different groups were determined by Mann Whitney rank sum test, Wilcoxon signed rank test or, when normally distributed, by Student's t test. When necessary, data that failed to meet requirements for parametric analysis were log transformed. Multiple comparisons were made using analysis of variance (ANOVA or ANOVA on ranks). Tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

Exome analysis

DNA was obtained from The Jackson Laboratory (Bar Harbor ME) for the following mouse strains: NOD.$H2^{h4}$, NOD and B10.A4R. For NOD.$H2^{k}$ and B10.BR strains, DNA was prepared "in-house" (Department of Immunology-Oncology, Maisonneuve_Rosemont Hospital, Montreal). Exome analysis was performed by the Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati Ohio 45229. In addition, exon 10 on chromosome (Chr) 15 was sequenced and analyzed in DNA from NOD.$H2^{h4}$, B10.A4R and NOD mice by Dr. Yaron Tomer (Division of Endocrinology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA). This

study was undertaken to determine in these three strains the exon 10 thyroglobulin (Tg) haplotype previously associated with susceptibility to experimentally- induced thyroiditis (26).

DAVID analysis

Gene lists for intervals located on Chr 7, 15 and 17 (to be specified later) were obtained from the MGI database for the NCBI build m38 and processed into the DAVID Analysis Tool for Functional Classification (35). The list of Gene Ontology (GO) Terms for Biological Process, as well as KEGG pathways, were obtained for each gene contained within the intervals, and filtered on the basis of their biological relevance to autoimmune thyroiditis.

MHC typing

MHC typing was performed by flow cytometry. Spleens were pressed through a 70 μm cell strainer (Thermo Fisher Scientific, Ottawa, Ontario, Canada). Spleen cell suspensions were treated with NH_4Cl to lyse red blood cells. Single cell suspensions were stained with the following antibodies: I-A^k (10-3.6), I-A^k (11-5.2), I-A^b (AF6-120.1), I-E (14.4.4S), H-2K^k (36-7-5), H-2 D^k (15-5-5)(Biolegend, San Diego, CA, USA) and H-2 D^b (KH95; BD Biosciences, Mississauga, Ontario, Canada), Flow cytometry data were acquired on an FACSCalibur instrument (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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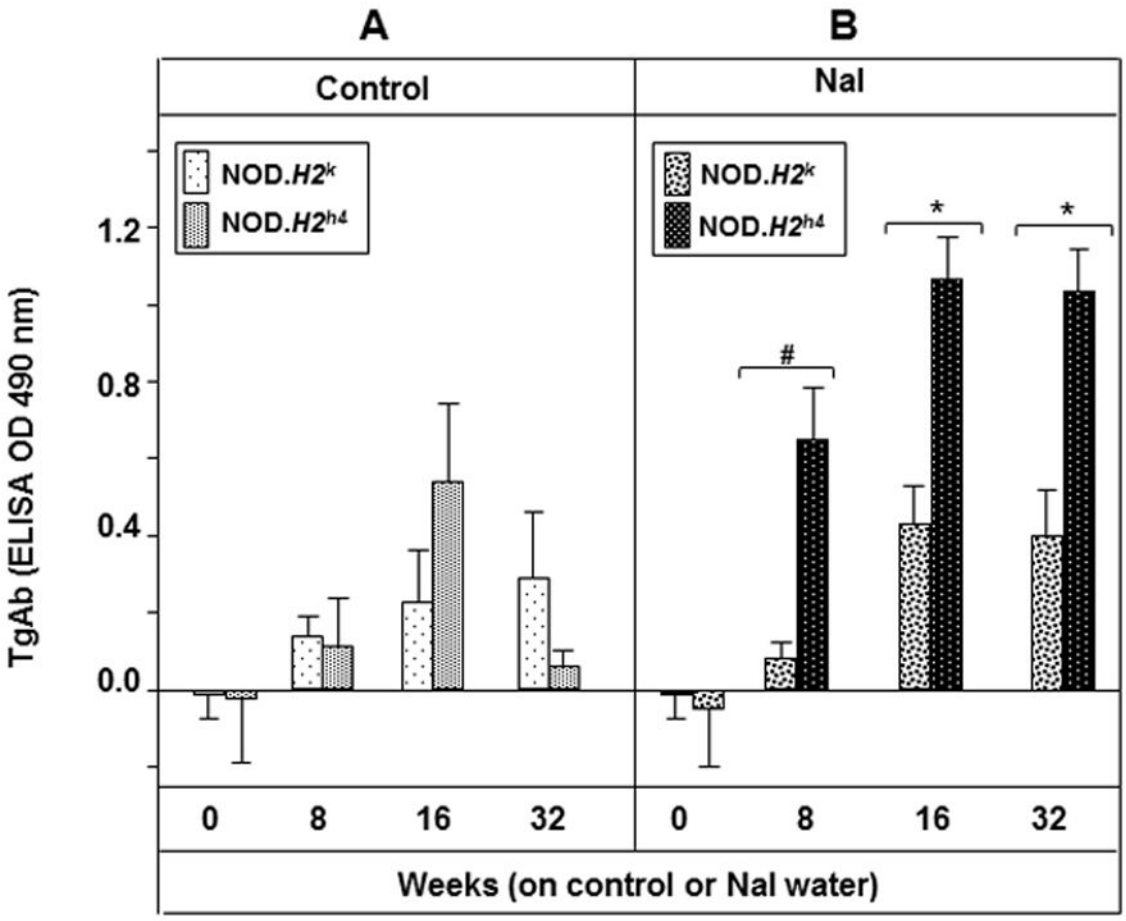

Figure 1.

TgAb in male NOD.$H2^{k}$ and NOD.$H2^{h4}$ mice exposed to regular water (Control, panel A) or water supplemented with NaI (panel B). Sera were studied in mice exposed for 0, 8, 16 and 32 weeks to control or NaI water. TgAb data are given as the optical density (OD490 nm; mean + SEM) using ELISA wells coated with mouse Tg and standardized against a positive control serum (appropriately diluted) from a BALB/c mouse immunized with Tg plus Complete Freund's adjuvant (33). The number of mice in each group ranged from 10 to 24 for NOD.$H2^{k}$ mice and from 13 to 31 for NOD.$H2^{h4}$mice. Values are significantly different between NOD.$H2^{k}$ and NOD.$H2^{h4}$ mice on NaI: after 8 weeks # $p<0.001$, rank sum test; after 16 and 32 weeks * $p<0.001$ (t test).

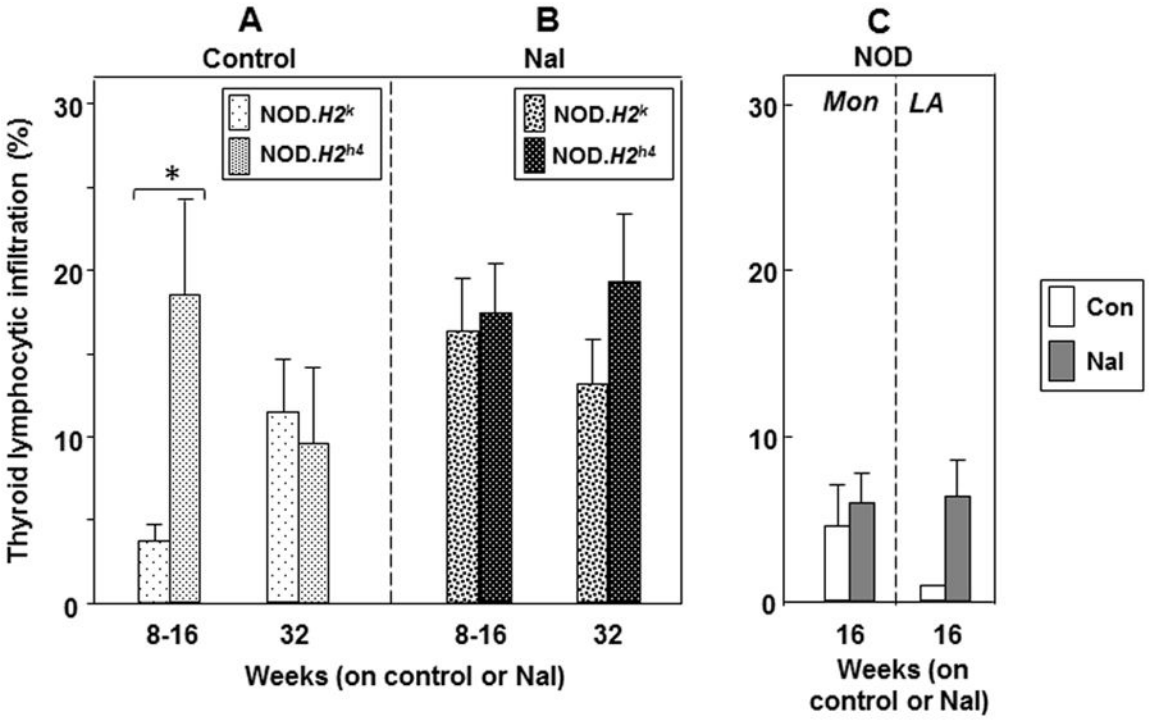

Figure 2.

Thyroiditis in male NOD.$H2^{h4}$ and NOD.$H2^{k}$ mice (panels A, B) and NOD mice (panel C) on control water or NaI-supplemented water. The extent of thyroiditis is given as the percentage (%) of the thyroid gland infiltrated by lymphocytes (mean + SE). NOD mice were maintained in Montreal (Mon) and Los Angeles (LA). The numbers of mice in each group ranged from 6 to 15 in panels A and B except NOD in Los Angeles on control water (where n=3, panel C). Values are significantly different between NOD.$H2^{k}$ and NOD.$H2^{h4}$ mice on control water for 8–16 weeks * p = 0.035 (t test).

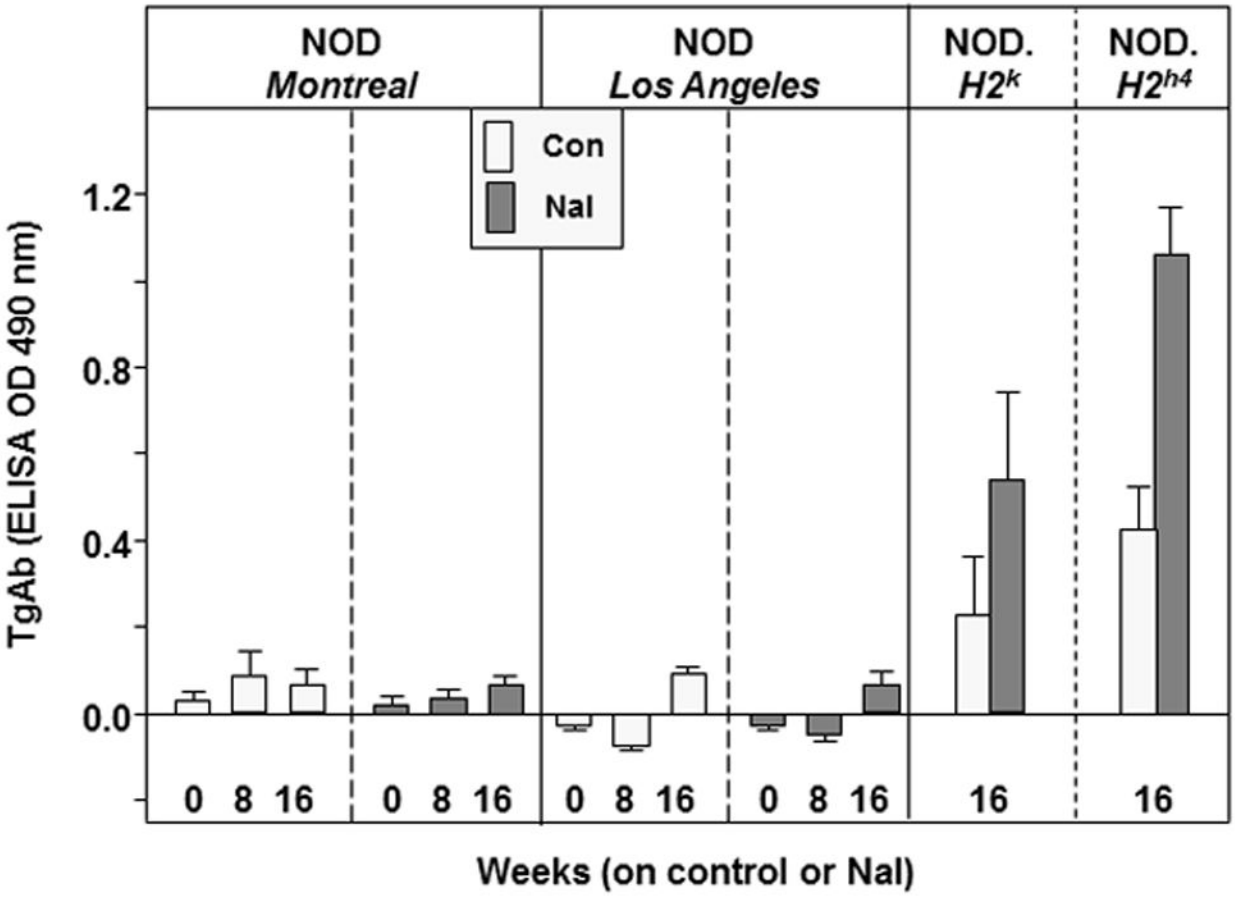

Figure 3.

TgAb in male NOD mice maintained in Montreal or Los Angeles on regular water (Con) or NaI water (NaI). Sera were studied in mice exposed for 0, 8 and 16 weeks to control or NaI water. Data are given as the optical density (OD490 nm, mean + SEM) in ELISA. The number of mice in each group ranged from 10 to 24 in NOD- Montreal and from 9 to 31 in NOD- Los Angeles. Included for comparison are the TgAb 16 week data for NOD.$H2^k$ and NOD.$H2^{h4}$ mice (from Fig. 1).

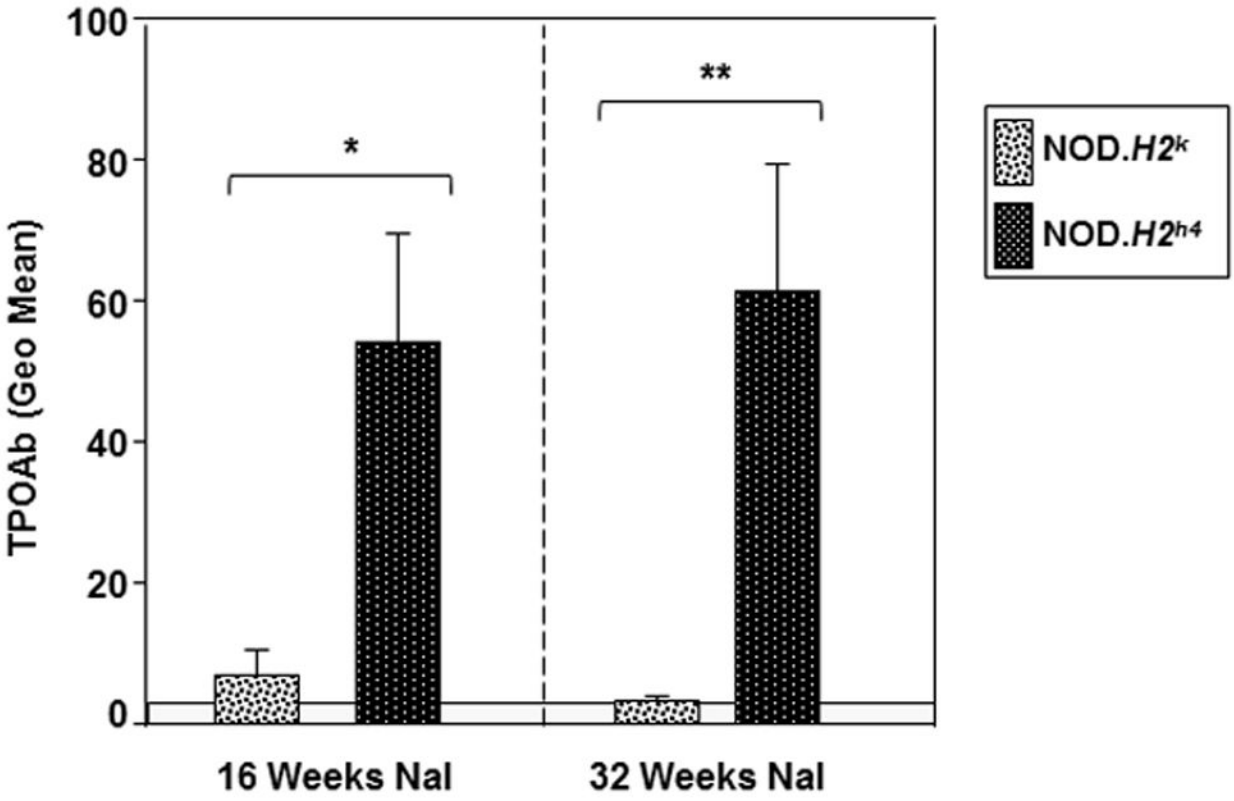

Figure 4.

TPOAb in male NOD.*H2*^{k} and NOD.*H2*^{h4} mice. Sera from mice exposed for 16 and 32 weeks to NaI were tested for IgG class antibody binding to CHO cells expressing mouse TPO. Data are the means (+ SEM) of Geometric mean values (Geo mean) obtained in flow cytometry. The number of mice in each group was 8 at 16 weeks and 14 mice at 32 weeks. The shaded area represents the baseline (mean ± 2SD) values for BALB/c mice exposed to NaI for 32 weeks. Values are significantly higher in NOD.*H2*^{h4} than in NOD.*H2*^{k} mice: * p=0.038 and ** p=0.004 (rank sum test).

A

	MHC Class I	MHC Class II		MHC Class I
	K	I-A	I-E	D
NOD	d	g7		b
B10.A(4R)	k	k	k/b	b
NOD.*H2*^{h4}	k	k		b
NOD.*H2*^{k}	k	k	k	k
B10.BR	k	k	k	k

B

	Unexpected	Chr	Location	Mb	Source	Expected Genes
NOD.*H2*^{h4}		Chr 17	27.3 to 44.8	17.5	B10.A(4R)	MHC locus
	88.6 Mb	Chr 7	111.9 to 145.5	33.7	B10.A(4R)	
		Chr 15	3.2 to 58.2	54.9	B10.A(4R)	
NOD.*H2*^{k}		Chr 17	29.0 to 34.7	5.7	B10.BR	MHC locus
		Chr 5	0 to 22.6	22.6		B6 alleles – TCR 3A9 site
		Chr 12	67.7 to 78.0	10.3		B6 alleles – insHEL site
	14.2 Mb	Chr 9	54.2 to 61.0	6.8	B10.BR	
		Chr 18	68.4 to 75.8	7.4	B10.BR	

Figure 5.

A) MHC class I and class II expressed in NOD.*H2*^{h4} and NOD.*H2*^{k} mice and for NOD, B10.A.4R and B10.BR mice, the parental strains from which they were derived (2). B) Exome analysis of NOD.*H2*^{h4} versus parental strains NOD and B10.A.4R and NOD.*H2*^{k} versus parental strains NOD and B10.BR. Exome regions other than the expected chromosome (Chr) 17 MHC locus from B10.A.4R (2) were observed for NOD.*H2*^{h4} on Chr 7 and Chr 15. The NOD.*H2*^{k} exome includes the B10.BR Chr 17 MHC locus (2), C57BL/6 (B6) alleles near the transgenic T cell receptor 3A-insertion site on Chr 5 and B6 alleles near the insulin HEL transgene insertion site on Chr 12 (24) as well as other DNA segments from B10.BR mice on Chr 9 and 18.

Table 1

TgAb do not develop and thyroiditis is minimal in B10.A.4R and NOD.Lc7 mice after 8 or 16 weeks on regular water (Con) or water supplemented with NaI.

Strain	Weeks	Con	n	NaI	n
TgAb (OD 490 nm; mean ± SEM)					
B10.A.4R	8	−0.02 ± 0.02	6	−0.01 ± 0.01	7
	16	−0.03 ± 0.02	7	−0.06 ± 0.01	7
NOD.Lc7	8	0.05 ± 0.01	9	−0.03 ± 0.02	9
	16	0.01 ± 0.03	9	0.00 ± 0.03	9
BALB/c	8	0.00 ± 0.02	5	−0.03 ± 0.01	15
	16	−0.28 ± 0.09	5	−0.16 ± 0.06	15
NOD.$H2^{h4}$	8	0.11 ± 0.13	31	**0.65 ± 0.05**	31
	16	**0.39 ± 0.14**	31	**1.07 ± 0.11**	31
Thyroiditis (% infiltration; mean ± SEM)					
B10.A.4R	16	1.0 ± 0.0	5	1.0 ± 0.0	6
NOD.Lc7	16	1.4 ± 0.2	9	1.9 ± 0.3	9
BALB/c	16	1.0 ± 0.0	3	1.0 ± 0.0	3
NOD.$H2^{h4}$	16	**18.6 ± 5.6**	9	**17.4 ± 3.0**	6

The NOD.Lc7 strain (24) carries a segment on the distal end of Chr 7 which corresponds to the B10.A.4R interval retained in NOD.$H2^{h4}$ mice (Fig. 5B). TgAb values are given as the standardized OD 490 nm in ELISA (see Methods). The extent of thyroiditis is given as the % lymphocytic infiltration of the gland; no infiltration is assigned the value 1.0 %. The number of mice (n) in each group is provided. Data for BALB/c and NOD.$H2^{h4}$ mice are included as negative and positive values, respectively; NOD.$H2^{h4}$ data are shown graphically in Fig. 1 and Fig. 2.

Table 2

The exon 10 haplotype of the thyroglobulin (Tg) gene (Chr 15) is not related to the development of spontaneous thyroid autoimmunity in NOD.*H2*^{h4} or NOD mice. Examples are included of previously described Tg haplotypes for mouse strains susceptible, or resistant, to thyroiditis induced experimentally by immunization with mouse Tg and adjuvant (26).

Strain	Thyroiditis	Exon 10 haplotype	MHC	I-A	I-E
	Spontaneous				
NOD.*H2*^{h4}	Yes	Asn-Val-Ile	H-2^{k}	I-A^{k}	ne
NOD	Yes (variable)	Asn-Val-Ile	H-2^{g7}	I-A^{g7}	ne
B10.A(4R)	No	Asn-Val-Ile	H-2^{k}	I-A^{k}	I-E^{b}
	Experimental				
CBAJ, C3H/HeJ, AKR/J	Susceptible	Ser-Met-Thr	H-2^{k}	I-A^{k}	I-E^{k}
C57BL/6, BALB/cJ, DBA/2J	Resistent	Asn-Val-Ile	H-2^{b}	I-A^{b}	ne

Amino acid positions for the exon 10 haplotype (from Ref. (26): Asn-757, Val-808, Ile-919; Ser-757, Met-808, Thr-919. Information is provided for MHC, I-A and I-E; ne, not expressed.

Table 3

Thyroiditis and autoantibodies to thyroglobulin (Tg) and thyroid peroxidase (TPO) enhanced by dietary iodide in mice on the NOD background differing at the MHC locus.

Strain	H2-K	I-A	I-E	H2-D	M/F	Thyroiditis	TgAb	TPOAb	Ref
NOD	d	g7	ne	b		minimal			(18;19)
	d	g7	ne	b	M	increased	no		(5)
NOD-E	d	g7	I-Eα^{d} *	b	M	increased	no		(5)
NOD-A-Asp	d	g7m	d	b	M	increased	no		(5)
NOD.*H2*^{h4}	k	k	ne	b		increased			(18;19)
	k	k	ne	b	M,F	increased	TgAb		(3–5;14)
	k	k	ne	b	M,F	increased	TgAb	TPOAb	(6)
	k	k	ne	b	M	increased	TgAb	TPOAb	Present study
NOD.*H2*^{k}	k	k	k	k	M	increased	TgAb	No TPOAb	Present study

ne, I-E not expressed;

* I-Eβ expressed endogenously; g7m: I-A beta chain mutated to express Asp instead of Pro at position 57 (36); blanks indicate no information