New Murine Model of Early Onset Autoimmune Thyroid Disease/Hypothyroidism and Autoimmune Exocrinopathy of the Salivary Gland

Timothy Daniel Kayes, Gary A. Weisman, Jean M. Camden, Lucas T. Woods, Cole Bredehoeft, Edward F. Downey, James Cole and Helen Braley-Mullen

*J Immunol* 2016; 197:2119-2130; Prepublished online 12 August 2016;
doi: 10.4049/jimmunol.1600133
http://www.jimmunol.org/content/197/6/2119

Supplementary Material
http://www.jimmunol.org/content/suppl/2016/08/12/jimmunol.1600133.DC1Supplemental

References
This article cites 63 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/197/6/2119.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
New Murine Model of Early Onset Autoimmune Thyroid Disease/Hypothyroidism and Autoimmune Exocrinopathy of the Salivary Gland

Timothy Daniel Kayes,* Gary A. Weisman,†‡ Jean M. Camden,†‡ Lucas T. Woods,†‡ Cole Bredehoeft,* Edward F. Downey,* James Cole,* and Helen Braley-Mullen*§

Sixty to seventy percent of IFN-γ−/− NOD.H-2h4 mice given sodium iodide (NaI)–supplemented water develop a slow onset autoimmune thyroid disease, characterized by thyrocyte epithelial cell (TEC) hyperplasia and proliferation (H/P). TEC H/P develops much earlier in CD28−/− mice and nearly 100% (both sexes) have severe TEC H/P at 4 mo of age. Without NaI supplementation, 50% of 5- to 6-mo-old CD28−/−IFN-γ−/− mice develop severe TEC H/P, and 2–3 wk of NaI is sufficient for optimal development of severe TEC H/P. Mice with severe TEC H/P are hypothyroid, and normalization of serum thyroxine levels does not reduce TEC H/P. Activated CD4+ T cells are sufficient to transfer TEC H/P to SCID recipients. Thyroids of mice with TEC H/P have infiltrating T cells and expanded numbers of proliferating thyrocytes that highly express CD40. CD40 facilitates, but is not required for, development of severe TEC H/P, as CD40−/−IFN-γ−/−CD28−/− mice develop severe TEC H/P. Accelerated development of TEC H/P in IFN-γ−/−CD28−/− mice is a result of reduced regulatory T cell (Treg) numbers, as CD28−/− mice have significantly fewer Tregs, and transfer of CD28+ Tregs inhibits TEC H/P. Essentially all female IFN-γ−/− mice develop much earlier in CD28−/− autoimmune thyroid disease, characterized by thyrocyte epithelial cell (TEC) hyperplasia and proliferation (H/P). TEC H/P have infiltrating T cells and expanded numbers of proliferating thyrocytes that highly express CD40. CD40 facilitates, but is not required for, development of severe TEC H/P, as CD40−/−IFN-γ−/− mice develop severe TEC H/P, and 2–3 wk of NaI is sufficient for optimal development of severe TEC H/P. Mice with severe TEC H/P are hypothyroid, and normalization of serum thyroxine levels does not reduce TEC H/P. Activated CD4+ T cells are sufficient to transfer TEC H/P to SCID recipients. Thyroids of mice with TEC H/P have infiltrating T cells and expanded numbers of proliferating thyrocytes that highly express CD40. CD40 facilitates, but is not required for, development of severe TEC H/P, as CD40−/−IFN-γ−/−CD28−/− mice develop severe TEC H/P. Accelerated development of TEC H/P in IFN-γ−/−CD28−/− mice is a result of reduced regulatory T cell (Treg) numbers, as CD28−/− mice have significantly fewer Tregs, and transfer of CD28+ Tregs inhibits TEC H/P. Essentially all female IFN-γ−/−CD28−/− NOD.H-2h4 mice have substantial lymphocytic infiltration of salivary glands and reduced salivary flow by 6 mo of age, thereby providing an excellent new model of autoimmune exocrinopathy of the salivary gland. This is one of very few models where autoimmune thyroid disease and hypothyroidism develop in most mice by 4 mo of age. This model will be useful for studying the effects of hypothyroidism on multiple organ systems. The Journal of Immunology, 2016, 197: 2119–2130.

Received for publication January 22, 2016. Accepted for publication July 7, 2016. This work was supported by National Institutes of Health Grant AI 074857 (to H.B.-M.), the Lottie Caroline Hardy Trust, and National Institutes of Health Grants R01 DE007389 and R01 DE02332 from the National Institute of Dental and Craniofacial Research (to G.A.W.).

Address correspondence and reprint requests to Dr. Helen Braley-Mullen, Department of Medicine, University of Missouri School of Medicine, M627 Medical Sciences Center, University of Missouri, Columbia, Missouri 65211, and Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, Missouri 65212.

The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; H/P, hyperplasia and proliferation; I-SAT, iodine-facilitated spontaneous autoimmune thyroiditis; MMRRC, Mutant Mouse and Rat Resource Center; NaI, sodium iodide; SS, Sjögren’s syndrome; T4, thyroxine; TEC, thyrocyte epithelial cell; Treg, regulatory T cell; TTF, thyroid transcription factor; WT, wild-type.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/$30.00

The Journal of Immunology

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1600133

Copyright 2015 by the Journal of Immunology, Inc.
and permanent reduction of Tregs would occur with genetic deletion of CD28, and would result in an increased incidence and/or earlier development of TEC H/P. To test this hypothesis, IFN-γ CD28−/− NOD.H-2h4 mice, generated in our laboratory for another project (8), were crossed with IFN-γ−/− NOD.H-2h4 mice to generate mice lacking both CD28 and IFN-γ. Genetic deletion of CD28 had profound effects on the development of severe TEC H/P such that severe TEC H/P developed in essentially 100% of IFN-γ−/− CD28−/− NOD.H-2h4 mice (both sexes) 6–8 weeks after NaI supplementation of the drinking water. TEC H/P in this strain was severe enough to result in low serum thyroid hormone levels, thus providing an important clinical correlate of Hashimoto’s thyroiditis that is lacking in many other mouse models of autoimmune thyroid disease. Because mice with severe TEC H/P become hypothyroid at a relatively young age, this mutant mouse strain will be useful for studying the effects of hypothyroidism and normalization of thyroid hormone levels on other organs/systems such as the cardiovascular system (16, 17).

The mutant mouse described in this study provides a robust experimental model to examine the role of CD4+ versus CD8+ T cells and CD40 expression in the thyroid in TEC H/P development and provide new information regarding the role of NaI in induction and maintenance of this autoimmune thyroid disease. These questions could not be addressed in CD28−/−IFN-γ−/− mice because of the much longer induction period and lower incidence of disease.

In addition to early development of severe TEC H/P, nearly all IFN-γ−/−/CD28−/− NOD.H-2h4 females spontaneously developed inflammation of the salivary gland by 4 months of age. Salivary gland inflammation greatly increases at 6 months of age and is accompanied by significant salivary hypofunction, a clinical manifestation of the human autoimmune disease Sjögren’s syndrome (SS). Some mice of both sexes also had inflammation of the pancreas that occasionally progressed to diabetes. IFN-γ−/−CD28−/− NOD.H-2h4 mice provide a robust model for studying mechanisms involved in development of autoimmune thyroid disease accompanied by hypothyroidism, and they may also be very useful as a new animal model of autoimmune exocrinopathy of the salivary gland.

Materials and Methods

Mice

IFN-γ−/− NOD.H-2h4 SCID and IFN-γ−/− NOD.H-2h4 mice (Mutant Mouse and Rat Resource Center [MMRRC] 037140) (18) were generated in the University of Missouri animal facility as previously described (1). CD28-deficient IFN-γ−/− NOD.H-2h4 mice (MMRRC 037411) were generated by crossing CD28−/− IFN-γ−/− NOD.H-2h4 mice (8) with IFN-γ−/− NOD.H-2h4 mice. The F1 mice were crossed and the resulting offspring were selected for homozygous expression of the IFN-γ and CD28 mutations by PCR analysis of tail DNA using primers designed on The Jackson Laboratory Web site. For some experiments, the IFN-γ−/−CD28−/− mice were crossed with IFN-γ−/−CD40−/− mice (MMRRC 037143) (18–20) to generate IFN-γ−/−CD28−/−CD40−/− mice (MMRRC 037143). Mice expressing enhanced GFP in Foxp3+ Tregs were generated in our animal facility by crossing previously generated wild-type (WT) Foxp3-GFP NOD.H-2h4 mice (8) with IFN-γ−/− NOD.H-2h4 mice. WT NOD.H-2h4 mice (The Jackson Laboratory 00447) and C57BL/6 (B6) mice were from the Weisman colony at the University of Missouri. All animal protocols were approved by the University of Missouri Animal Care and Use Committee. Mouse strains with MMRRC numbers are available to other investigators through the MMRRC.

Administration of NaI in the drinking water

Development of severe TEC H/P in 60–70% of IFN-γ−/−CD28−/− NOD.H-2h4 mice requires NaI supplementation of the drinking water for >60 days (1, 3). In this study, most mice were given 0.08% NaI in their drinking water beginning at 6–7 wk of age. For some experiments, mice were not given NaI water or were given NaI water for 2–4 wk and then maintained on plain water. In other experiments, mice were given NaI water for 6–7 wk and maintained on plain water for the duration of the experiment. Both male and female mice were used, but all mice in an individual experiment were the same sex.

Administration of T4 in the drinking water

In some experiments, mice were given NaI water for various periods of time. After they were determined to be hypothyroid (serum T4 of <3 μg/dl serum), they were given plain water (no added NaI) or plain water containing T4 (Sigma-Aldrich, St. Louis, MO) at a predetermined optimal concentration of 25 ng/ml water. They were maintained on water containing T4 or plain water without T4 for the duration of the experiment. Serum T4 concentrations were determined in all mice before addition of T4 to the water and again when the experiment was terminated.

Serum T4 assay

Blood was collected from the retro-orbital plexus immediately before collection of thyroid and, in some experiments, before addition of T4 to the drinking water. Serum T4 levels were measured by ELISA using a T4 test kit (Leinco, St. Louis, MO) according to the manufacturer’s protocol. Results are expressed as micrograms of T4 per decaliter of serum. Hypothyroid mice are defined as having <3 μg/dl T4 in serum. Values for normal mouse serum ranged from 4 to 8 μg/dl. As previously reported (20), serum T4 levels highly correlate with TEC H/P severity scores. Only mice with few or no residual normal thyroid follicles (severity scores of 5+ or 4+ scores with <20% residual normal thyroid follicles) have low serum T4.

TEC H/P severity scoring

Thyroids were removed and one thyroid lobe was fixed in formalin, sectioned, and stained with H&E. Thyroid histopathology was scored for the extent of thyroid follicular cell H/P using a scale of 0–5+ as previously described (1, 18). All slides were read in a blinded manner by two investigators, one of whom had no knowledge of the experimental details. Briefly, a score of 0 indicates a normal thyroid, and 0+ indicates mild follicular changes and/or a few inflammatory cells infiltrating the thyroid. A 1+ score indicates cellular infiltrates with at least 25 cells with hyperplastic changes sufficient to cause replacement of several follicles. A 2+ score represents 10–20 foci of cellular infiltration with hyperplastic changes causing replacement or destruction of up to one fourth of the gland, 3+ indicates that one fourth to one half of the gland has hyperplastic changes, and 4+ indicates that more than one half of the gland has hyperplasia. Thyroid tissue with a score of 5+ have few or no remaining normal follicles. Mice with TEC H/P graded 4–5+ had widespread clusters of proliferating thyrocytes with lymphocyte infiltration and areas of proliferating thyrocytes surrounded by collagen. All thyroids with mild or severe hyperplasia also had infiltrating lymphocytes.

Evaluation of salivary gland and pancreas infiltration

Submandibular salivary glands, pancreas, liver, and kidney were removed from some mice when thyroids were collected. They were fixed in formalin, sectioned, and stained with H&E. The extent of lymphocyte infiltration in the salivary glands was scored by counting the number of foci/lymphocyte aggregates consisting of >50 lymphocytes (9, 21, 22). All scoring was done in a blinded manner using the microscope and MetaMorph software indicated below. Focus scores are defined as the number of lymphocyte foci (>50 cells) per 4 mm2 of tissue (22). Images of whole submandibular gland sections were generated by stitching together multiple ×40 magnification images using a Zeiss Axiovert 200M microscope and MetaMorph software at the University of Missouri Molecular Cytology Core Resource. Pancreatitis in the renal (insulitis) was scored as previously described (9). Blood glucose levels were assessed using test strips and an Accu-Chek monitoring system. Mice with blood glucose levels >300 mg/dl were considered to be diabetic (9).

Saliva collection

Mice were anesthetized with tribromoethanol (Avertin) and an endotracheal tube (PE50 polyethylene tubing) was inserted through a 2-cm midventral incision to prevent aspiration. Saliva secretion was induced by i.p. injection of 0.25 mg/kg carbachol. Saliva was collected from the oral cavity for 15 min using a pipette tip and placed in a preweighed Eppendorf tube. Results are presented both as microliters of saliva per gram body weight and as microliters of saliva per 15 min.

Evaluation of fibrosis

The extent of fibrosis in thyroids of mice with TEC H/P was evaluated in a blinded manner by examination of H&E-stained thyroid sections and
confirmed in some mice by staining with Masson’s trichrome as previously described (1, 20).

In vivo depletion of CD4+ and/or CD8+ T cells

CD4+ and/or CD8+ T cells were depleted using anti-CD4 (GK1.5; Bio X Cell, West Lebanon, NH) or anti-CD8 (116-13.1; Harlan Laboratories, Indianapolis, IN). Abs were administered i.p. (250 μg/injection) beginning at 5–6 wk of age. Control mice were given rat IgG (250 μg i.p.) at the same intervals. Mice were given NaI water 1 wk later, and Ab injections were repeated every 10–12 d until termination of the experiment when mice were 15 wk old. When thyroids were removed, splenic CD4+ and CD8+ T cells were analyzed by flow cytometry. Only mice with few or no residual cells of the depleted subset are included in the figures.

Cell culture and adoptive transfer of TEC H/P

In some experiments, TEC H/P was induced by adoptive transfer of 72-h cultured splenocytes or purified T cells from mice with severe (4–5+) TEC H/P to IFN-γ−/− NOD.H-2h4 SCID mice as previously described in detail (1, 4, 23). Recipients were given NaI water, and thyroid histopathology was determined at various times as indicated in the figures. In some experiments, T cells in recipient spleens were depleted using anti-CD4 and/or anti-CD8 as indicated above. Abs or rat IgG was injected beginning 6 or 30 d after cell transfer and injections were repeated every 2 wk as indicated in the legend to Fig. 2. Thyroids were removed 28 or 60 d after cell transfer.

T cell purification

In some experiments, cultured splenocytes were separated into CD4+ and CD8+ subsets using CD4 or CD8 T cell isolation kits (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocols. Purity of isolated cells (always >95%) was determined by flow cytometry. Purified cells were transferred i.v. to SCID recipients (1.5 × 106 per recipient). Recipients of CD8+ T cells were given a single injection of anti-CD4 and recipients of CD4+ T cells were given a single injection of anti-CD8 the day after cell transfer to deplete any residual cells of the unwanted subset. Recipients were given NaI water and thyroids were removed 28 d later. Recipient spleens were analyzed by flow cytometry to ensure that only the transferred T cell subset was present.

Suppression of TEC H/P by adoptive transfer of Tregs

For Treg transfers, splenocytes from CD28−/IFN-γ−/− NOD.H-2h4 mice expressing Foxp3 enhanced GFP were sorted into GFP+ and GFP− fractions using a MoFlo XDP cell sorter (Beckman Coulter, Brea, CA). GFP+ Tregs (>98% pure) or control GFP− cells (1 × 106 per recipient) were transferred i.v. into IFN-γ−/− NOD.H-2h4 recipients that were irradiated (300 Gy) using an X-RAD 320 irradiator (Precision X-ray, New Branford, CT) 3–6 h before Treg transfer. Mice were given NaI water, and transfer of control GFP− cells was repeated 3 wk later without irradiation. TEC H/P severity was determined by histology 6–8 wk after transplantation. The percentages of donor (CD28−/+) and recipient (CD28−/−) Foxp3+ splenic T cells were determined at this time.

Flow cytometry

For analysis of sorted Foxp3+ Tregs, cells were stained with anti–CD28-FITC, anti–CD4-PercP Cy5.5, and anti–Foxp3-allophycocyanin (all from eBioscience). Intracellular staining was done after surface staining using a Foxp3 intracellular staining kit (eBioscience). To determine total numbers of Foxp3+ Tregs, the number of spleen cells was multiplied by the total percentage of CD4+Foxp3+ cells detected by flow cytometry. Flow cytometry was done using a CytoADP flow cytometer (Beckman Coulter) and data were analyzed using FlowJo (Tree Star). For assessment of CD4+ and CD8+ T cells in spleens of Ab-treated mice or separated T cell subsets, cells were stained with PE anti-CD4 (RMA-4; eBioscience or BioLegend) or PE anti-CD8 (53.6; eBioscience). Samples were analyzed on a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo.

Immunohistochemistry

Frozen or formaldehyde-fixed paraffin sections of thyroids were blocked with 5% BSA in PBS, and endogenous peroxidase was inhibited by incubation with 0.3% H2O2 for 30 min. Anti–thyroid transcription factor (TTF)-1 (H-190; Santa Cruz Biotechnology), anti-CD40 (1C10; eBioscience for frozen sections) or C-20 (sc975; Santa Cruz Biotechnology for paraffin sections), anti-human CD3 (rabbit polyclonal; Dako), anti-CD4 (clone GK1.5, supernatant), or anti-CD8 (clone 53.6, supernatant) was used as primary Ab. For staining of frozen sections (CD4, CD8, and CD40), biotinylated anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary Ab (1:500), followed by avidin-HRP binding using a Vectastain Elite PK-6100 kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using a Vector Nova Red substrate kit (Vector Laboratories). TTF-1, CD3, and CD40 staining of paraffin sections was done by IDEXX BioResearch (Columbia, MO). They were developed with biotinylated anti-rabbit or anti-goat IgG at previously determined optimal concentrations followed by avidin-HRP and visualized using diaminobenzidine tetrahydrochloride as the chromogen.

Statistical analysis

A nonparametric Mann–Whitney U test was used to determine significance of severity scores. A Student t test was used for all other data, including salivary flow and serum T4 analyses, using analysis software included with GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Groups were considered statistically significant when p < 0.05.

Results

Rapid development of severe TEC H/P in IFN-γ−/− CD28−/− NOD.H-2h4 mice

To test the hypothesis that genetic deletion of CD28 would lead to earlier development of severe TEC H/P, IFN-γ−/− CD28−/− NOD.H-2h4 mice (6–8 wk old) were given 0.08% NaI in their drinking water. Thyroids were removed 3, 5–6, and 7–8 wk later, and TEC H/P severity scores were determined by histology. IFN-γ−/− CD28−/− mice developed severe (4–5+) severity scores TEC H/P at a greatly accelerated rate compared with CD28+ mice (1, 3). After 5–6 wk on NaI-supplemented water, ~80% of CD28−/− mice had severe (4–5+) TEC H/P, and after 7–8 wk, nearly 100% of them had severe TEC H/P (Fig. 1A). After 7–8 wk, all mice with 5+ severity scores and those with strong 4+ severity scores (10–20% residual normal follicles) had low serum T4 (1–3 μg/dl), that is, they were hypothyroid. Mice with 4+ severity scores and 25–50% residual normal thyroid follicles and those with lower scores had normal serum T4 (≥3 μg/dl) (Fig. 1B). In comparison, only 60–70% of CD28−/IFN-γ−/− NOD.H-2h4 mice develop severe TEC H/P after prolonged (6–7 mo) administration of NaI water (1). More importantly, at 3–4 mo of age (age of mice in Fig. 1A after 6–8 wk on NaI water), ≤20% of CD28−IFN-γ−/− NOD.H-2h4 mice have severe TEC H/P (1, 3). Therefore, genetic deletion of CD28 greatly accelerates earlier development and increases the incidence of severe TEC H/P in IFN-γ−/− NOD.H-2h4 mice.

NaI supplementation promotes but is not required for development of severe TEC H/P

NaI supplementation of the drinking water is absolutely required for development of severe TEC H/P in CD28−IFN-γ−/− NOD.H-2h4 mice, but it clearly increases the incidence and promotes earlier development of severe TEC H/P in IFN-γ−/− CD28−/− NOD.H-2h4 mice, but it clearly increases the incidence and promotes earlier...
development of severe TEC H/P. As noted in our previous studies with CD28–/–IFN-γ−/− NOD.H-2h4 mice (1, 20), most mice have severe (4–5+) or no or mild (0–1+) TEC H/P, and this is also true for IFN-γ−/–CD28−/– NOD.H-2h4 mice. Only a few mice have intermediate (2–3+) TEC H/P severity scores even when thyroids are examined early (Fig. 1A). Thyroid histology of IFN-γ−/–CD28−/– NOD.H-2h4 mice with TEC H/P is indistinguishable from that of CD28–/–IFN-γ−/– mice (Fig. 1C–F), and histology is identical in mice given NaI or plain water (Fig. 1E–I). The primary pathologic feature is massive proliferation of thyrocytes and infiltration of inflammatory cells including CD4+ and CD8+ T cells, macrophages, and dendritic cells (data not shown). All thyroids have significant numbers of infiltrating eosinophils because IFN-γ is absent. Collagen deposition (fibrosis) is extensive in most thyroids with 5+ severity scores, including mice not given NaI water (Fig. 1G, 1J).

Iodine promotes initiation of TEC H/P, but lesions progress and are maintained in the absence of added iodine

Early development of severe TEC H/P in a high percentage of IFN-γ−/–CD28−/– mice requires NaI supplementation of the drinking water (Fig. 1B). Mice with very severe TEC H/P have low serum T4 (Fig. 1B). Thyroid lesions and hypothyroidism persist for life, and thyroid fibrosis becomes more extensive over time (H. Braley-Mullen, unpublished observations). We used multiple approaches in attempts to promote resolution of severe TEC H/P and reversal of hypothyroidism. No approaches were successful unless the intervention began before thyroid lesions became very severe (e.g., Fig. 2). It was suggested that removing excess iodine from the water and/or normalizing serum T4 levels might facilitate resolution of TEC H/P. Several approaches were used to address this question. To determine whether removing NaI from the water before severe TEC H/P developed limited progression of TEC H/P, mice were given NaI water for 2 wk and then given plain water for 6 wk. Most mice (12 of 14) developed severe TEC H/P when given NaI water for only 2 wk when they were maintained on plain water for an additional 6 wk (Table I). Similarly, 16 of 18 mice given NaI water for 3–4 wk developed severe TEC H/P when maintained an additional 3–4 wk on plain water (Table I, row 2). Disease severity scores remained constant after 15 wk on plain water (Table I, row 3). Therefore, after effector T cell activation is initiated (a process promoted by NaI supplementation), iodine has little or no influence on further progression of TEC H/P. Importantly, 4 wk of NaI water did not provide sufficient time for development of severe TEC H/P (Table I, row 4). After 4 wk, at least 3–4 wk on plain water was required for maximal disease development (Table I, row 3). Taken together, these results indicate that after T cell activation is initiated and facilitated by exposure to NaI, iodine supplementation is not required for further progression of thyroid lesions to maximal severity.

As shown above (Fig. 1B), mice with severe TEC H/P have low serum T4 levels. To determine whether normalization of serum T4...
levels and/or removal of excess iodine from the water would result in reduced TEC H/P severity, mice were given NaI water for 4–14 wk. Blood was collected to determine serum T4 levels, and groups of mice were maintained on plain water (no added NaI) or plain water to which 25 ng/ml T4 was added. Thyroids were removed 28 or 60 d later as indicated. Groups of recipients were given rat IgG (Con), anti-CD4, or anti-CD4 and anti-CD8 beginning 6 or 30 d after cell transfer as indicated. Ab injections were repeated at 12- to 14-d intervals. Thyroids were removed from all treated mice at day 60. Mice given anti-CD4 or anti-CD4 and anti-CD8 at day 6 (before recipients had severe disease) developed minimal TEC H/P at day 60 (p < 0.001), whereas delaying T cell depletion until day 30 when recipients had severe TEC H/P had no effect (p > 0.1); n = 9 (Con, day 30), 10 (Con, day 60), 5 (anti-CD4, day 6), 9 (anti-CD4 and anti-CD8, day 6), and 5 (anti-CD4 and anti-CD8, day 30). (C) Splenocytes from IFN-γ−/− CD28−/− females were cultured 72 h. Cells were separated into CD4− or CD8− subsets as described in Materials and Methods and transferred to SCID females. Recipients were given NaI water, and thyroids were removed 28 d later. Splenocytes and CD4+ T cells, but not CD8+ T cells, induced severe TEC H/P in recipient mice; n = 8 (Spl), 12 (CD4+), 11 (CD8+), and 8 (CD4 and CD8). Results are representative of two (C) or three (A and B) separate experiments. Thyroids and spleens of recipients of CD4+ T cells had no detectable CD8+ T cells (data not shown). Con, control; Iso, isotype control; Spl, splenocytes.

levels and/or removal of excess iodine from the water would result in reduced TEC H/P severity, mice were given NaI water for 4–14 wk. Blood was collected to determine serum T4 levels, and groups of mice were maintained on plain water (no added NaI) or plain water to which 25 ng/ml T4 was added. Thyroids were removed 28 or 60 d later as indicated. Groups of recipients were given rat IgG (Con), anti-CD4, or anti-CD4 and anti-CD8 beginning 6 or 30 d after cell transfer as indicated. Ab injections were repeated at 12- to 14-d intervals. Thyroids were removed from all treated mice at day 60. Mice given anti-CD4 or anti-CD4 and anti-CD8 at day 6 (before recipients had severe disease) developed minimal TEC H/P at day 60 (p < 0.001), whereas delaying T cell depletion until day 30 when recipients had severe TEC H/P had no effect (p > 0.1); n = 9 (Con, day 30), 10 (Con, day 60), 5 (anti-CD4, day 6), 9 (anti-CD4 and anti-CD8, day 6), and 5 (anti-CD4 and anti-CD8, day 30). (C) Splenocytes from IFN-γ−/− CD28−/− females were cultured 72 h. Cells were separated into CD4− or CD8− subsets as described in Materials and Methods and transferred to SCID females. Recipients were given NaI water, and thyroids were removed 28 d later. Splenocytes and CD4+ T cells, but not CD8+ T cells, induced severe TEC H/P in recipient mice; n = 8 (Spl), 12 (CD4+), 11 (CD8+), and 8 (CD4 and CD8). Results are representative of two (C) or three (A and B) separate experiments. Thyroids and spleens of recipients of CD4+ T cells had no detectable CD8+ T cells (data not shown). Con, control; Iso, isotype control; Spl, splenocytes.
injection, mice were given NaI water and thyroids were removed 7 wk later. TEC H/P severity was minimally affected by depletion of either subset alone, but development of severe TEC H/P was inhibited in most mice after depletion of both CD4+ and CD8+ T cell subsets (Fig. 2A). Depletion of the appropriate T cell subset with the single Ab or both Abs was essentially complete as determined by flow cytometry of splenocytes when thyroids were removed (data not shown). The two mice with 4+ TEC H/P in the group given both Abs did not have more residual splenic T cells, so it is unknown why their disease was minimally suppressed. These results indicate that severe TEC H/P in IFN-γ−/− CD28−/− mice is dependent on both CD4+ and CD8+ T cells for optimal development.

Severe TEC H/P can be transferred to SCID recipients by T cells from donors with severe TEC H/P but not by T cells from donors with no or mild TEC H/P (1) This is also true using donor T cells from IFN-γ−/−CD28−/− mice (T.D. Kayes and H. Braley-Mullen, unpublished observations). To determine whether T cell depletion inhibits TEC H/P induced by activated T cells, splenocytes from IFN-γ−/−CD28−/− donors with severe TEC H/P were transferred to SCID recipients, and CD4+ and/or CD8+ T cells were depleted by administration of anti-CD4 and/or anti-CD8 6 or 30 d later. Depletion of both T cell subsets starting 6 d after cell transfer inhibited development of severe TEC H/P in most mice up to 60 d after cell transfer (Fig. 2B). However, T cell depletion had no effect when it was delayed until 30 d after cell transfer, when recipients already had severe TEC H/P (Fig. 2B), suggesting that T cells are not required for maintenance of thyroid lesions. Unexpectedly, depletion of CD4+ T cells was as effective as depletion of both T cell subsets (Fig. 2C), suggesting that CD8+ T cells are not required when TEC H/P is induced by activated T cells. To address this directly, splenocytes from CD28−/− donors with severe TEC H/P were cultured as before and separated into purified CD4+ or CD8+ subsets (see Materials and Methods). Recipients of purified CD4+ T cells, unseparated splenocytes, or a mixture of CD4+ and CD8+ T cells all developed severe TEC H/P 4 wk later, whereas recipients of purified CD8+ T cells did not develop TEC H/P (Fig. 2C). The results were unexpected because CD8+ T cells are the major T cell subset in spleens and thyroids of SCID recipients of splenocytes from IFN-γ−/−CD28−/− donors (data not shown), and our previous results with CD28+IFN-γ−/− mice indicated that purified CD8+ T cells transferred TEC H/P more effectively than did CD4+ T cells (4, 18). We have no explanation as to why activated CD4+ and not CD8+ T cells transfer severe TEC H/P when CD28 is absent. However, these results were highly reproducible and are consistent with the Ab depletion results in Fig. 2B.

T cells and other cells in thyroids of IFN-γ−/−CD28−/− mice with severe TEC H/P

The characteristic thyroid pathology of TEC H/P in CD28−/− mice includes proliferation of thyrocytes (TECs), extensive fibrosis, infiltration of lymphocytes (1), and increased CD40 expression by thyrocytes (18). To determine whether thyroid pathology in CD28−/− mice is similar to TEC H/P in CD28+IFN-γ−/− NOD.H-2b mice, thyroids from IFN-γ−/−CD28−/− mice given NaI water were examined by immunohistochemistry. TTF-1 is a transcription factor expressed in the thyroid (24). When proliferating TECs are derived from epithelial cells, they will be TTF-1+. However, because they express p63 (5) and resemble thyroid solid cell nests

Table I. Nal supplementation of the water for 2–4 wk is sufficient for maximal development of severe TEC H/P

<table>
<thead>
<tr>
<th>Nal (wk)</th>
<th>Plain (wk)</th>
<th>TEC H/P Severity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6</td>
<td>0 1+ 2+ 3+ 4+ 5+</td>
</tr>
<tr>
<td>3–4</td>
<td>4</td>
<td>1 0 0 1 5 11</td>
</tr>
<tr>
<td>3–4</td>
<td>15</td>
<td>1 0 0 0 2 8</td>
</tr>
<tr>
<td>4+</td>
<td>0</td>
<td>2 2 0 1 0 0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0 1 0 0 2 8</td>
</tr>
</tbody>
</table>

*Groups of IFN-γ−/−CD28−/− NOD.H-2b mice (6 wk of age) were given Nal in their water for the indicated time. Mice in rows 1–3 were then maintained on plain water (no NaI) as indicated before thyroids were removed.

Table II. Normalization of serum T4 by administration of T4 does not influence the maintenance of severe TEC H/P

<table>
<thead>
<tr>
<th>Nal (wk)</th>
<th>Plain (wk)</th>
<th>T4 (wk)</th>
<th>4–5+ TEC H/P</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>0</td>
<td>5/5</td>
<td>ND</td>
<td>1.3 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>10</td>
<td>8/10</td>
<td>1.6 ± 0.1</td>
<td>8.2 ± 2.3</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>8/8</td>
<td>ND</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>0</td>
<td>5/6</td>
<td>3.9 ± 2.8d</td>
<td>2.1 ± 1.9d</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>6</td>
<td>12/12</td>
<td>1.1 ± 1.0</td>
<td>9.8 ± 3.3</td>
</tr>
</tbody>
</table>

*Groups of IFN-γ−/−CD28−/− mice were given NaI water for the indicated number of weeks. They were subsequently maintained as indicated on plain water (no NaI) or plain water containing T4 (25 ng/ml) before thyroids were removed.

Numbers of mice in each group with the indicated TEC H/P severity scores are shown.
histologically, they could be derived from ultimobrachial bodies and would be TTF-1(24). To distinguish between these possibilities, thyroids were stained with anti–TTF-1 (Supplemental Fig. 1A–D). TECs from normal mice (data not shown) and normal nonproliferating TECs express TTF-1 (data not shown). Similarly, proliferating TECs from mice with severe TEC H/P (Supplemental Fig. 1A–D) and nonproliferating TECs in their thyroids (Supplemental Fig. 1A, 1C, 1D, arrows) highly express TTF-1, indicating that proliferating TECs in mice with TEC H/P are derived from TECs and not from ultimobrachial bodies.

Thyroids from IFN-γ−/−CD28−/− mice with severe TEC H/P have infiltrating lymphocytes. Because TEC H/P is a T cell–dependent autoimmune disease, some thyroid-infiltrating cells should be T cells. Consistent with this expectation, thyroids from IFN-γ−/−CD28−/− mice had many CD3+ cells (Supplemental Fig. 1E–H), including both CD4+ and CD8+ T cells (Supplemental Fig. 1E–L). Macrophages, eosinophils, and dendritic cells are also present in thyroids of mice with TEC H/P (4, 20). Thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P are derived from TECs and not from ultimobrachial bodies. Thyroids from IFN-γ−/−CD28−/− mice with severe TEC H/P have infiltrating lymphocytes. Because TEC H/P is a T cell–dependent autoimmune disease, some thyroid-infiltrating cells should be T cells. Consistent with this expectation, thyroids from IFN-γ−/−CD28−/− mice had many CD3+ cells (Supplemental Fig. 1E–H), including both CD4+ and CD8+ T cells (Supplemental Fig. 1E–L). Macrophages, eosinophils, and dendritic cells are also present in thyroids of mice with TEC H/P (4, 20). Thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P are derived from TECs and not from ultimobrachial bodies.

Our previous studies indicated that CD40 expression by thyrocytes is important and may be essential for development of severe TEC H/P in CD28−/− mice. CD40+CD28−/− mice had milder lesions (Supplemental Fig. 2A, 2B). CD40−/−/CD28−/− mice were generated as described in Materials and Methods. Mice were given NaI water for 7–8 wk of age, and thyroids were removed 6 and 8 wk later (Supplemental Fig. 2A, 2B). CD40−/−/CD28−/− mice given NaI water for 6 wk did not have TEC H/P, but many that were given NaI water for 8 wk developed severe TEC H/P (19). Proliferating thyrocytes in IFN-γ−/−CD28−/− mice with severe TEC H/P have infiltrating lymphocytes. Because TEC H/P is a T cell–dependent autoimmune disease, some thyroid-infiltrating cells should be T cells. Consistent with this expectation, thyroids from IFN-γ−/−CD28−/− mice had many CD3+ cells (Supplemental Fig. 1E–H), including both CD4+ and CD8+ T cells (Supplemental Fig. 1E–L). Macrophages, eosinophils, and dendritic cells are also present in thyroids of mice with TEC H/P (4, 20). Thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P have very few B220+B cells (18), thyroids of mice with TEC H/P are derived from TECs and not from ultimobrachial bodies.

Development of severe TEC H/P in CD28−/− mice lacking CD40

Our previous studies indicated that CD40 expression by proliferating thyrocytes is important and may be essential for development of severe TEC H/P in CD28−/− mice. CD40−/−CD28−/− mice and bone marrow chimeras lacking CD40 expression in thyroids are resistant to TEC H/P (19). Therefore, we hypothesized that IFN-γ−/−CD40−/−/CD28−/− NOD.H-2h4 mice would not develop TEC H/P. To address this, CD40−/−CD28−/− mice were generated as described in Materials and Methods. Mice were given NaI water for 7–8 wk of age, and thyroids were removed 6 and 8 wk later (Supplemental Fig. 2A, 2B). CD40−/−CD28−/− mice given NaI water for 6 wk did not have TEC H/P, but many that were given NaI water for 8 wk developed severe TEC H/P. The incidence of severe TEC H/P in CD28−/−/CD28−/− mice given NaI water for 8 wk was not significantly different from that in a cohort of age- and sex-matched CD40+CD28−/− mice (p = 0.08), although more CD40−/− mice had milder lesions (Supplemental Fig. 2B). Histologically, thyroids of CD40−/−/CD28−/− mice with severe TEC H/P were indistinguishable from those in CD28−/−/CD40−/− mice (Fig. 3D, 3E), except they did not express CD40 (Supplemental Fig. 2F, 2G). They also had comparable infiltration of CD3+ T cells (Supplemental Fig. 2H). Splenocytes and CD4+ T cells, but not CD8+ T cells, from CD40−/−/CD28−/− donors with severe TEC H/P transferred severe TEC H/P to SCID recipients (Supplemental Fig. 2C), indicating that TEC H/P in CD40−/−/CD28−/− mice is induced by purified T cells and is likely autoimmune. CD40−/−/CD28−/− mice produce low levels of antithyroglobulin autoantibodies, the levels of which correlate with TEC H/P severity scores (H. Braley-Mullen, unpublished results), whereas antithyroglobulin Abs were not detectable in CD40−/−/CD28−/− mice (data not shown). Therefore when CD28 is absent, expression of CD40 by thyrocytes is not absolutely required for severe TEC H/P to develop, although CD40 clearly facilitates earlier development of and a higher incidence of severe TEC H/P, and it is required for development of detectable antithyroglobulin Ab.

IFN-γ−/−CD28−/− mice have fewer Tregs than do CD28−/−IFN-γ−/− mice, and Tregs from CD28−/− mice suppress TEC H/P development in CD28−/− mice

CD28 regulates Treg homeostasis, influencing both thymic development (14) and peripheral homeostasis (26, 27). Reduced Treg numbers in CD28−/− NOD mice account for their increased susceptibility to diabetes (26, 27), and reduced numbers of functional Tregs account for the increased severity of I-SAT in CD28−/− IFN-γ−/− NOD.H-2h4 mice (8). To test the hypothesis that reduced functional Tregs account for the increased severity of IFN-γ−/−CD28−/− mice to TEC H/P, splenic Foxp3+ T regs were enumerated by flow cytometry in naive mice 1–2 mo old and in mice with TEC H/P. Naive IFN-γ−/−CD28−/− mice had significantly fewer thymic and splenic Foxp3+ Tregs than did CD28−/− IFN-γ−/− mice (Fig. 3A). After being given NaI water to allow for development of TEC H/P, IFN-γ−/−CD28−/− mice had significantly fewer Foxp3+ Tregs compared with CD28−/− mice (Fig. 3A). Reduced numbers and/or function of Tregs in CD28−/− mice is largely responsible for their earlier development and increased incidence of severe TEC H/P, because transfer of sorted Tregs from Foxp3-GFP CD28−/−IFN-γ−/− mice significantly reduced the incidence and severity of TEC H/P induced by transferred CD28−/− effector T cells (Fig. 3B). Transfer of CD28−/− Tregs resulted in normalization of thyroid function, as Treg recipients had normal serum T4, whereas recipients of Foxp3+ T cells from CD28−/− mice were hypothyroid (Fig. 3C). The transferred CD28−/− Tregs expanded and persisted in the recipient spleens, comprising 50–80% of the total splenic Tregs 5 wk after transfer (Fig. 3D). These results indicate that the rapid development of severe TEC H/P and hypothyroidism in IFN-γ−/−CD28−/− mice is due, at least in part, to reduced numbers and/or function of Tregs.

IFN-γ−/−CD28−/− NOD.H-2h4 mice have robust lymphocytic infiltration of salivary glands

NOD mice that spontaneously develop diabetes commonly have inflammation in other organs, including the thyroid (28) and salivary gland (29, 30). WT NOD.H-2h4 mice, closely related to NOD mice, develop I-SAT when given NaI in their water (18), and many WT NOD.H-2h4 female mice have salivary gland infiltration at 10–12 mo of age (29) with ectopic follicles that increase in size over time (31). We previously reported that IFN-γ−/−CD28−/− NOD.H-2h4 mice have a higher incidence of salivary gland infiltration than do WT (CD28+) NOD.H-2h4 mice (8). CD28−/− IFN-γ−/− NOD.H-2h4 mice have a low incidence of lymphocyte infiltration in organs other than the thyroid (T.D. Kayes and H. Braley-Mullen, unpublished observations). Because IFN-γ−/−/CD28−/− NOD.H-2h4 mice have a high incidence of early and severe TEC H/P, it was important to determine whether other organs had lymphocyte infiltration. To address this, pancreas, submandibular salivary glands, liver, and kidney were examined in some 6–7-mo-old IFN-γ−/−CD28−/− mice. There were no infiltrates in liver or kidney (data not shown). Pancreas infiltration was present in 50–60% of 5–7-mo-old IFN-γ−/−CD28−/− mice of both sexes (data not shown), and a comparable percentage of retired breeders, 6–7 mo of age. Unexpectedly, ~10% of retired breeders, both sexes, had diabetes (blood glucose ∼300 mg/dl) despite absence of the MHC class II molecules associated with susceptibility to diabetes. Because the incidence was low, this was not addressed further.
Mice were given NaI water. Three weeks later, they received a second injection of sorted GFP+CD28+ Tregs or control GFP+ TEC H/P. Normal serum T4 levels whereas recipients of non-Tregs have low serum T4 levels. T4 results are from some of the mice in (Foxp3+ Tregs are significantly higher than for recipients of non-Tregs given NaI water, and GFP+ (Foxp3+) CD28+CD4+ T cells in the spleen were enumerated by flow cytometry 6–8 wk after transfer. Many Tregs in recipient 4–5 wk after the second injection of Tregs. Results are representative of three independent experiments. Recipients of CD28+ Tregs developed less severe transferred CD28+ Tregs in CD28−/− females (6 wk of age) were irradiated (300 Gy) and injected i.v. with control GFP− cells or sorted Foxp3+ Tregs from Foxp3-GFP CD28+ mice. Mice were given NaI water. Three weeks later, they received a second injection of sorted GFP+CD28+ Tregs or control GFP− cells. Tregs were removed 4–5 wk after the second injection of Tregs. Results are representative of three independent experiments. Recipients of CD28+ Tregs developed less severe TEC H/P (p < 0.0001) compared with recipients of Foxp3+ T cells; n = 11 (control) and 12 (Treg recipients). (C) CD28−/− recipients of CD28+ Tregs have normal serum T4 levels whereas recipients of non-Tregs have low serum T4 levels. T4 results are from some of the mice in (B). T4 values for recipients of Foxp3+ Tregs are significantly higher than for recipients of non-Tregs (p < 0.0001); n = 8 (control) and 9 (Tregs). (D) Persistence and expansion of transferred CD28−/− Tregs in CD28−/− mice. Sorted Foxp3+ cells from Foxp3-GFP CD28−/− mice were transferred to CD28−/− mice as in (B). Mice were given NaI water, and GFP− (Foxp3+) CD28−/−CD4+ T cells in the spleen were enumerated by flow cytometry 6–8 wk after transfer. Many Tregs in recipient spleens were of donor origin. No GFP− cells were detected in CD28−/− mice not given Tregs (data not shown). Results are representative of two separate experiments; n = 11.

Of particular interest, nearly 100% of female IFN−γ−/−CD28−/− NOD.H-2h4 mice had moderate to extensive lymphocyte infiltration in their submandibular salivary glands at 4 mo of age, and the number and size of lymphocytic foci increased substantially at 6 mo of age (Fig. 4A). Although TEC H/P develops equally in both males and females, salivary gland infiltration was more predominant in females (Fig. 4), as previously reported by others for WT NOD.H-2h4 mice (28, 30). Most males also had salivary gland lymphocyte infiltration, but the areas of infiltrate were predominant in females (Fig. 4), as previously reported by others. Of age-matched WT NOD.H-2h4 mice, suggesting that IFN−γ−/−CD28−/− mice may provide a more robust model of SS-like disease than do WT NOD.H-2h4 mice. Salivary gland function as determined by salivary flow was significantly reduced in female IFN−γ−/−CD28−/− NOD.H-2h4 mice compared with age-matched female WT NOD.H-2h4 mice or nonautoimmune B6 mice (Fig. 4B, 4C). This was true whether results were presented as total saliva collected in 15 min (Fig. 4B) or amount of saliva per 15 min relative to body weight (Fig. 4C). To further characterize this strain as an animal model of SS, sera from 4- and 6-mo-old female IFN−γ−/−CD28−/− mice (n = 10 of each age) were evaluated for anti-Ro and anti-La autoantibodies using ELISA kits from Alpha Diagnostic International. Autoantibody levels were low and often not detectable (data not shown). This is not surprising, as CD28/B7 interactions are important for T/B interactions (10), and IFN−γ− and IFN−γ−/−CD28−/− NOD.H-2h4 mice have much lower antithyroglobulin autoantibody responses than their do CD28+ counterparts, even though they have severe autoimmune thyroid disease (8, 18).

**Discussion**

This study describes and characterizes a new mouse model of autoimmune thyroid disease and SS. The model is unique because autoimmune thyroid disease is accompanied by hypothyroidism that develops spontaneously (no requirement for immunization) by 4 mo of age in most mice of both sexes. To our knowledge, this is one of very few murine models where hypothyroidism resulting from thyroid autoimmunity develops in most mice at a relatively young age. Therefore, this model will be useful for addressing basic mechanisms involved in thyroid autoimmunity accompanied by...
low thyroid hormone levels as occur in humans with Hashimoto’s thyroiditis. Perhaps more importantly, it will be useful for studying effects of low thyroid hormone levels on other organs and systems. Low thyroid hormone levels influence many other systems in the body, including the cardiovascular (17, 32), renal (33), reproductive (34), and immune systems (35). Autoimmunity is a major cause of hypothyroidism in humans. An animal model of autoimmune thyroid disease with low thyroid hormone levels at 4 mo of age provides a unique model for determining, for example, how thyroid hormone levels influence cardiovascular functions such as vascular stiffening and blood pressure abnormalities in models of diet-induced obesity (36–38). Autoimmunity and hypothyroidism are chronic, persisting for life, and provide a large window for testing effects of low thyroid hormone in other systems. Additionally, hypothyroidism can be normalized by adding T4 to the drinking water, comparable to treating humans with levothyroxine (Synthroid). Therefore, this model provides translational relevance by determining whether normalizing thyroid hormone levels can reverse conditions that are affected by hypothyroidism, such as increased vascular stiffness as a measure of cardiovascular disease.

The autoimmune thyroid disease TEC H/P develops very slowly in IFN-γ−/− NOD.H-2h4 mice that express CD28. NaI supplementation of the drinking water for 6 mo is required, and severe TEC H/P with hypothyroidism develops in only 60% of mice (1, 2). In contrast, when CD28 is absent, essentially all IFN-γ−/− NOD.H-2h4 mice of both sexes develop severe TEC H/P and hypothyroidism (low serum T4) by 4 mo of age (Fig. 1). The mechanism by which CD28 deficiency promotes development of spontaneous autoimmune diseases is primarily due to reduced Treg numbers and function in CD28−/− mice (8, 14, 39). Our results are consistent with those studies, as transfer of CD28+ Tregs reduced TEC H/P severity scores and normalized thyroid hormone levels (Fig. 3).

CD28− or low T cells are present in humans but not in mice. CD28+ T cells are present during T cell activation and are considered to be Ag-experienced highly differentiated cells that play significant roles in several human diseases (40–42). Intriguingly, patients with primary SS have increased percentages of CD8+CD28+ T cells that correlate with disease severity (43), and increases in circulating soluble CD28 have been reported in SS and other autoimmune diseases (44). Although the model used in this study

FIGURE 4. Female IFN-γ−/−CD28−/− mice develop SS-like infiltration of the submandibular salivary glands. (A) Mean submandibular gland focus scores of 4- and 6-mo-old IFN-γ−/−CD28−/−, 4- to 5-mo-old IFN-γ−/−CD40−/−, and 5-mo-old WT NOD.H-2h4 mice; n = 17 (6-mo-old females), 11 (4-mo-old females), 10 (6-mo-old males), 12 (4-mo-old males), 10 (CD40−/− females), and 9 (5-mo-old WT NOD.H-2h4 females). p < 0.001, 6-mo-old females versus 4-mo-old females, p < 0.001, 6-mo-old females versus 6-mo-old males, p = 0.06, 6-mo-old males versus 4-mo-old females, p = 0.08, 4-mo-old females versus 4-mo-old males, p < 0.001, 6-mo-old females versus CD40−/−/CD28−/−, p < 0.03, 6-mo-old females versus WT NOD.H-2h4, p = 0.05, 4-mo-old females versus WT NOD.H-2h4, (B and C) Mean salivary flow from the indicated strains (all females 5 mo old) expressed as saliva collected in 15 min (B) or microliter saliva collected in 15 min per gram of body weight; n = 6 (B6 mice), 8 (NOD.H-2h4 WT mice), and 14 (CD28−/− mice). Saliva production in CD28−/− mice was significantly reduced compared with both B6 (p < 0.001) and WT NOD.H-2h4 mice (p < 0.02 (B), p < 0.047 (C)). (D) Representative H&E-stained sections from salivary glands of 4- and 6-mo-old female and male CD28−/− and CD40−/− and WT NOD.H-2h4 females. Note the fewer and smaller infiltrates in CD28−/− males, CD28−/−CD40−/− females, and WT NOD.H-2h4 females compared with CD28−/− females. Original magnification ×40. Photos are representative of the mice in (A).
has a mutation that does not naturally occur in mice, studying CD28+ effector cells in a murine autoimmune disease could provide important information relevant for human medicine. This study provides new information regarding the requirement for NaI supplementation of the drinking water for development of autoimmune thyroid disease in NOD-H-2h4 mice. A common concern with studies of autoimmune thyroid diseases in NOD-H-2h4 mice is the requirement for supraphysiologic concentrations of iodine for early development of autoimmune disease (45–48). Although NaI supplementation facilitates early development of severe TEC H/P in CD28−/− mice, many mice develop severe TEC H/P without iodine supplementation (Fig. 1C), and 2–3 wk of NaI supplementation is sufficient for most mice to develop severe TEC H/P (Table I). Severe TEC H/P with low serum T4 levels then develop optimally with or without NaI supplementation. This is important because iodine administration can be shortened to 2–3 wk, thus helping to alleviate concerns that long-term exposure to excess iodine has adverse effects on the thyroid, or could result in the Wolff–Chaikoff effect, a mechanism that prevents thyroids from secreting normal amounts of thyroid hormones when iodine is in excess (46, 49). Our results show that facilitation of autoimmune thyroid disease by administration of iodine is an early event, suggesting that iodine facilitates autoreactive T cell activation, perhaps due, in part, to its ability to be incorporated into a target epitope recognized by autoreactive T cells (45, 50) and/or to facilitate upregulation of MHC or costimulatory molecules such as ICAM-1 on thyrocytes (45, 51). Although several studies have addressed possible underlying mechanisms by which iodine promotes thyroid autoimmunity in NOD-H-2h4 mice, the precise cellular events are poorly understood (reviewed in Ref. 45) and are beyond the scope of this study.

TEC H/P differs from another autoimmune thyroid disease, I-SAT, that develops in IFN-γ−/− NOD.H-2h4 mice following NaI supplementation of the drinking water (18, 45), because TEC H/P develops only when IFN-γ is absent (1, 2), raising concerns that this model lacks an appropriate human counterpart because humans are not IFN-γ deficient. However, a murine model of thyroid autoimmunity accompanied by hypothyroidism as occurs in humans can be an excellent model for determining how hypothyroidism developing as a consequence of autoimmunity affects various organs and organ systems. In most other mouse models of autoimmune thyroiditis, not requiring immunization, serum T4 levels are normal (8, 18, 45, 47), so those models lack the major biomarker of Hashimoto’s thyroiditis in humans, that is, low serum thyroid hormone levels. This biomarker also allows for assessment of disease severity without sacrificing the mouse, thus increasing the experimental usefulness of this model. WT NOD.H-2h4 mice with I-SAT do not have low serum T4 levels (18, 47) and cannot be used to study effects of low thyroid hormone levels on other organs or systems.

Several other murine models that spontaneously develop hypothyroidism have been described. Most comparable to ours are CCR7-deficient NOD mice (52) where both sexes spontaneously develop severe thyroid lesions that appear similar to those in CD28−/− NOD.H-2h4 mice. Mice also have thyroid fibrosis, low serum T4 levels, thyroid-infiltrating T cells, and antithyroglobulin Abs. The disease is autoimmune and transferable to SCID recipients with splenic T cells (52). Unlike IFN-γ−/− CD28−/− NOD.H-2h4 mice, CCR7-deficient NOD mice express IFN-γ, the thyroid infiltrate has a significant B cell component, and they have infiltrates in most organs. The infiltrate in our model is confined to the thyroid, salivary glands, and pancreas, and B cells are not detected in the thyroid. Thyroid disease in CCR7-deficient NOD mice develops somewhat later and has a lower incidence (~70%) compared with that in IFN-γ−/− CD28−/− NOD.H-2h4 mice. Other murine models that spontaneously develop goiter and hypothyroidism include mice expressing transgenes such as IFN-γ or IL-12 in the thyroid (53, 54) and iodine-induced hypothyroidism in SJL mice (55). Those models do not have an autoimmune basis.

The mutant mouse model described in the present study provides an excellent model for studying two organ-specific autoimmune diseases in the same animal, namely autoimmune thyroid disease and SS-like disease of the salivary gland. The coexistence of both diseases in the same mice is consistent with the relatively frequent coexistence of thyroiditis and SS in humans (56). Interestingly, autoimmune thyroid disease in IFN-γ−/− CD28−/− NOD.H-2h4 mice is equivalent in males and females, but females have a much greater incidence and severity of salivary gland infiltration than do males (Fig. 4). The female preponderance of SS-like lesions was also noted in other mouse models, including WT NOD.H-2h4 and NOD mice (29–31, 57) and is also true in humans (57). Essentially all female IFN-γ−/− CD28−/− NOD.H-2h4 mice have salivary gland infiltrates at 4 mo of age, and the number and size of the infiltrates greatly increases at 6 mo of age (Fig. 4D). NaI supplementation of the water, used to facilitate early development of thyroid lesions, has no influence on development of SS-like lesions, because the incidence and focus scores were comparable in experimental mice and retired breeders not given NaI (data not shown). Salivary gland infiltration is greater and develops earlier in female IFN-γ−/− CD28−/− NOD.H-2h4 mice than in WT NOD.H-2h4 mice, also used to study SS (8, 29, 31) (Fig. 4A–D). CD28−/− mice have significant loss of salivary function, a clinical manifestation of SS in humans (57, 58), that exceeds that in WT NOD.H-2h4 mice (Fig. 4B, 4C). WT NOD.H-2h4 mice have ectopic follicles in their salivary gland infiltrates, shown to be derived from splenic germinal centers (31). Because CD28−/− mice lack germinal centers (59), composition of the lymphocytic infiltrates is likely to differ in salivary glands of WT and CD28−/− mice. Our studies did not address this issue. The IFN-γ−/− CD28−/− mouse model is potentially a novel model of SS because it has an early robust onset of lymphocytic infiltration, correlating with loss of salivary gland function. CD28 deficiency also promotes salivary gland infiltration in WT NOD.H-2h4 mice (8), but infiltration is more extensive in IFN-γ−/− CD28−/− mice (H. Braley-Mullen, unpublished observations). CD28 deficiency has not been studied in other mouse models of SS. IFN-γ was shown to be crucial for development of the SS phenotype in other mouse models (60, 61), but IFN-γ is clearly not required when CD28 is absent. Studies on the role of Tregs in SS have been inconsistent and often contradictory (62). Therefore, the IFN-γ−/− CD28−/− mouse model provides a unique tool for investigating mechanisms involved in SS development. The extent to which this model compares to other mouse models of SS (30, 57, 58, 63) remains to be determined.

Finally, development of several spontaneous autoimmune diseases, including diabetes, pancreatitis, thyroiditis, and SS-like syndrome, is greatly promoted in mice lacking CD28, due primarily to reduced numbers of functional Tregs (7, 12, 13, 24). Whereas CD28 costimulation is required for activation of T cells responding to foreign Ags (6, 7, 10), self-reactive effector T cells are effectively activated in the Treg-deficient environment in CD28−/− mice. CD28 costimulation may be less critical when effector T cells are chronically stimulated by self-antigen, and other costimulatory molecules might be used for T cell activation in spontaneous autoimmune diseases (6, 14). Importantly, IFN-γ−/− CD28−/− NOD.H-2h4 mice represent a powerful new model for the study of autoimmune thyroid disease with hypothyroidism and...
for the study of SS-like lesions. Also, because SS and autoimmune thyroid disease both develop spontaneously in this model as in humans, this model will be useful for studying autoimmune thyroid disease by which hypothyroidism might provide salutary gland function.

Disclosures

The authors have no financial conflicts of interest.

References


