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*J Immunol* 2013; 191:4940-4949; Prepublished online 4 October 2013;
doi: 10.4049/jimmunol.1301253
http://www.jimmunol.org/content/191/10/4940

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Reduced Effectiveness of CD4⁺Foxp3⁺ Regulatory T Cells in CD28-Deficient NOD.H-2h4 Mice Leads to Increased Severity of Spontaneous Autoimmune Thyroiditis

Jason S. Ellis,* So-Hee Hong,* Habib Zaghouani,†‡ and Helen Braley-Mullen*,†

NOD.H-2h4 mice given NaI in their drinking water develop iodine-accelerated spontaneous autoimmune thyroiditis (ISAT) with chronic inflammation of the thyroid by T and B cells and production of anti-mouse thyroglobulin (MTg) autoantibody. CD28⁻/⁻ NOD.H-2h4 mice, which have reduced numbers of CD4⁺Foxp3⁺ regulatory T cells (Tregs), were developed to examine the role of Tregs in ISAT development. CD28⁻/⁻ NOD.H2-h4 mice develop more severe ISAT than do wild-type (WT) mice, with collagen deposition (fibrosis) and low serum T4. CD28⁻/⁻ mice have increased expression of proinflammatory cytokines IFN-γ and IL-6, consistent with increased mononuclear cell infiltration and tissue destruction in thyroids. Importantly, transferring purified CD4⁺ Foxp3⁺ Tregs from WT mice reduces ISAT severity in CD28⁻/⁻ mice without increasing the total number of Tregs, suggesting that endogenous Tregs in CD28⁻/⁻ mice are functionally ineffective. Endogenous CD28⁻/⁻ Tregs have reduced surface expression of CD27, TNFR2 p75, and glucocorticoid-induced TNFR–related protein compared with transferred CD28⁺/+ Tregs. Although anti-MTg autoantibody levels generally correlate with ISAT severity scores in WT mice, CD28⁻/⁻ mice have lower anti-MTg autoantibody responses than do WT mice. The percentages of follicular B cells are decreased and those of marginal zone B cells are increased in spleens of CD28⁻/⁻ mice, and they have fewer thyroid-infiltrating B cells than do WT mice. This suggests that CD28 deficiency has direct and indirect effects on the B cell compartment. B cell–deficient (B⁻/⁻) NOD.H-2h4 mice are resistant to ISAT, but CD28⁻/⁻B⁻/⁻ mice develop ISAT comparable to WT mice and have reduced numbers of Tregs compared with WT B⁻/⁻ mice. The Journal of Immunology, 2013, 191: 4940–4949.

Received for publication May 13, 2013. Accepted for publication September 2, 2013.
This work was supported by National Institutes of Health Grant RO1 AI 076395.
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Abbreviations used in this article: CLN, cervical lymph node; FO, follicular; GARP, gpa4 repetitions predominant; GITR, glucocorticoid-induced TNFR–related protein; ISAT, iodine-accelerated spontaneous autoimmune thyroiditis; LN, lymph node; MTg, mouse thyroglobulin; MZ, marginal zone; SAT, spontaneous autoimmune thyroiditis; Treg, regulatory T cell; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301253

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NOD.H-2h4 mice given NaI in their drinking water develop iodine-accelerated spontaneous autoimmune thyroiditis (ISAT) (1–4). ISAT is characterized by infiltration of the thyroid by T and B cells, with destruction of thyroid follicles and production of Abs to mouse thyroglobulin (MTg) (1, 4, 5). Although B cell–deficient (B⁻/⁻) mice are resistant to ISAT, they develop ISAT after transient depletion of CD4⁺CD25⁺ regulatory T cells (Tregs) (6, 7), suggesting an important role for Tregs in ISAT. Our earlier studies indicated that transient depletion of CD25⁺ cells in which CD4⁺CD25⁺ Tregs were depleted for 7–10 d had little effect on subsequent ISAT severity scores in wild-type (WT) NOD.H-2h4 mice (7), but Treg-depleted WT mice had increased anti-MTg autoantibody responses compared with controls (H. Braley-Mullen, unpublished observations). Other investigators showed that more prolonged Treg depletion, in which anti-CD25 Ab was administered repeatedly to maintain Treg depletion for ≥3 wk in WT NOD.H-2h4 mice, resulted in more severe ISAT and increased production of proinflammatory cytokines (8). In addition, Treg depletion for ≥3 wk in ISAT-resistant IL-17-deficient mice resulted in susceptibility to ISAT (9). These results suggest that Tregs play an important role in ISAT, but depletion for at least several weeks is needed to reveal their role.

CD28 signaling is important for the development and peripheral homeostasis of CD4⁺CD25⁺ Tregs (10). CD28 costimulation promotes IL-2 production by conventional T cells, and IL-2 is important for Treg survival (11). CD28-deficient mice have reduced numbers of CD4⁺CD25⁺ Tregs, and CD28⁻/⁻ NOD mice develop earlier and more severe diabetes than do WT NOD mice (12, 13). CD28 originally was described as an important costimulator of T cell activation (14, 15). CD28 signaling is important for activation of naive T cells following their interaction with APCs presenting foreign Ags (15), as well as for induction of most experimentally induced models of autoimmune disease, including thyroiditis (13, 16–18) (H. Braley-Mullen, unpublished observations). However, NOD mice lacking CD28 develop spontaneous autoimmune diseases, such as diabetes and autoimmune pancreatitis (10, 13, 15, 16, 19), indicating that CD28/B7 interactions are not required for activation of autoreactive T cells in a Treg-deficient environment and in mice with a genetic predisposition to develop autoimmune disease (13, 16). The reasons for the differences in requirements for development of experimentally induced versus spontaneous autoimmune diseases are not known, but they may be because CD28 costimulation is less critical when there is chronic stimulation by self-Ag, or because other costimulatory molecules are used in spontaneous autoimmune diseases (10, 13, 16, 20).

Because NOD.H-2h4 mice are closely related to NOD mice that develop diabetes, we hypothesized that an early permanent deficiency in Tregs, as in NOD mice (10, 13, 16), would lead to increased activation of autoreactive effector CD4⁺ T cells and increased ISAT severity in WT and B⁻/⁻ CD28⁻/⁻ NOD.H-2h4 mice. CD28⁻/⁻
NOD.H-2h4 mice were developed to test this hypothesis. The results presented in this article suggest that, in addition to having reduced Tregs compared with WT NOD.H-2h4 mice, CD28-deficient mice have Tregs that are less effective at suppressing autoimmune thyroiditis. Also of note, B cell function and/or the effectiveness of T cell help were affected by the lack of CD28.

Materials and Methods

Mice

NOD.H-2h4 mice express H-2K\(^b\), I-A\(^\alpha\), and D\(^\beta\) on the NOD background (21). Mice were bred and maintained in the animal facility at the University of Missouri. All animal protocols were approved by the University of Missouri Animal Care and Use Committee. CD28\(^+/−\) NOD male mice, obtained from The Jackson Laboratory (Bar Harbor, ME), were crossed with WT NOD.H-2h4 females. The F1 mice were crossed, and F2 mice were selected for expression of the NOD.H-2h4 MHC by flow cytometry and for deficiency of CD28 by PCR of tail DNA using the primer sequences and protocol provided on The Jackson Laboratory Web site. CD28\(^+/−\) NOD.H-2h4 WT mice were crossed with B\(^+/−\) NOD.H-2h4 mice to generate CD28\(^−/−\) NOD.H-2h4 mice. Foxp3-GFP NOD.H-2h4 mice were used for sorting and transfer of CD28\(^+\) Tregs. NOD.Foxp3:GFP mice (22) were used for detection of the gene in males by PCR of tail DNA and for homozygosity of the Foxp3:GFP reporter gene in females and the presence of the gene in males by PCR of tail DNA.

Assessment of thyroiditis

At 8 wk of age, mice were given 0.08% NaI in their drinking water. Thyroiditis were removed 8–9 wk later, and one thyroid lobe from each mouse was fixed in formalin, sectioned, and stained with H&E, as described previously (1, 5). Thyroid destruction and inflammatory cell infiltration were scored using a scale of 0 to 4+, as described previously (1, 4, 5). A score of 1+ is defined as having one or several foci consisting of ≥125 cells. Thyroids having 10–20 larger foci of cellular infiltration, with destruction of up to one fourth of the gland, are given a score of 2+. A 3+ score indicates that one fourth to one half of the thyroid follicles are destroyed or replaced by infiltrating inflammatory cells, and a 4+ score indicates that more than one half of the gland is replaced by inflammatory cells. Thyroid lesions in NOD.H-2h4 mice reach maximal severity 8 wk after mice are given NaI in their drinking water beginning at 2 mo of age (1, 5).

Autoantibody determination

MTg-specific IgG autoantibodies were determined by ELISA using serum from individual mice diluted 1/50 or 1/100, as previously described (23).

Serum T4 determination

Serum thyroxine (T4) levels were determined by ELISA, as previously described, using a Leinco T4 ELISA test kit (Leinco, St. Louis, MO) (24). Results are expressed as μg T4/dl serum. Values for normal mouse serum range from 4 to 8 μg T4/dl, and values ≥ 3 are considered normal (25).

Isolation and transfer of Tregs

Splenocytes from Foxp3-GFP NOD.H-2h4 mice were incubated with anti-CD4 (RM-4-5 allophycocyanin) (eBioscience, San Diego, CA) plus LPS (10 \(^\mu\)g/ml) for 45 min at 37°C. For intracellular analysis of cytokine production by flow cytometry, thyroid-infiltrating cells were isolated and stimulated with PMA (50 ng/ml) and ionomycin (1 μM) (Alexis Biochemicals, San Diego, CA) plus LPS (10 μg/ml) for 12 h. Brefeldin A (1 μg/ml) was added after 4 h of stimulation. Cells from WT or CD28\(^+\) NOD.H-2h4 mice were then incubated (1 × 10⁶ cells/100 μl) with Abs against CD45 (30-F11, FITC) and CD3-specific Ab (RA3-6B2, allophycocyanin) or with isotype control, as previously described (26). Cells were washed and then fixed and permeabilized using the eBioscience Foxp3 buffer set (eBioscience) and stained for IFN-γ (XMG1.2, PE). Cells were washed, and data were collected using the DAKO Cyan flow cytometer and analyzed using Summit software version 5.2 (Beckman Coulter). Abs for flow cytometry were purchased from eBioscience and BioLegend (San Diego, CA).

Cell surface and Foxp3 staining for flow cytometry

For determination of Treg numbers, cells from spleens or cervical lymph nodes (CLNs) of WT and CD28\(^−/−\) NOD.H-2h4 mice were incubated (1 × 10⁶ cells/100 μl) with Abs against CD4 (RM-4-5 PerCP-Cy5.5) and CD25 (PC61 FITC) or isotype control Ab for 30 min at 4°C and then stained for Foxp3 (FJK-16s allophycocyanin) using the eBioscience Mouse Regulatory T Cell Staining Kit (eBioscience). Cells were washed, and the data were collected using the DAKO Cyan flow cytometer and analyzed using Summit software version 5.2. For characterization of Tregs in recipients of sorted Tregs or control cells, splenocytes from Treg recipients and control mice were incubated (1 × 10⁶ cells/100 μl) with Abs against CD4 (RM-4-5 PerCP-Cy5.5), CD28 (E18 FITC), TNFR2 p75 (TR75-89 PE), gp40 peptides (DARP, F011-5 PE), glucocorticoid-induced TNFR-related protein (GITR; DTA-1 PE), or CD27 (LG.7.T9F6 PE) or isotype control Abs for 30 min at 4°C and then stained for Foxp3 (FJK-16s allophycocyanin) using the eBioscience Mouse Regulatory T Cell Staining Kit. Thyroid-infiltrating cells were isolated from single thyroid lobes of individual recipient mice by treating thyroids with Liberase (0.08 U/ml; Roche) for 45 min at 37°C. Cells were then incubated (1 × 10⁶ cells/100 μl) with Abs against CD4 (RM-4-5 PerCP-Cy5.5), CD28 (E18 FITC), and CD45 (30-F11, PE) or isotype control Ab for 30 min at 4°C and then stained for Foxp3 (FJK-16s APC) using the eBioscience Mouse Regulatory T Cell Staining Kit. Abs for flow cytometry were purchased from eBioscience and BioLegend. Data were collected and analyzed as above. For determination of plasma cell numbers, splenocytes or thyroid-infiltrating cells from WT or CD28\(^−/−\) NOD.H-2h4 mice were incubated (1 × 10⁶ cells/100 μl) with Abs against CD45 (30-F11, FITC), B220 (RA3-6B2, PerCP-Cy5.5), and CD138 (281.2, allophycocyanin). Cells were washed, and the data were collected using the DAKO Cyan flow cytometer. For determination of B cell subsets in the spleen, cells were stained with Abs against B220 (RA3-6B2, PerCP-Cy5.5), CD25 (PC61 FITC), CD138 (281.2, allophycocyanin) or B220 (RA3-6B2, FITC) and CD138 (281.2, allophycocyanin). Cells were washed, and the data were collected using the DAKO Cyan flow cytometer. For determination of B cell subsets in the thyroid, cells were stained with Abs against B220 (RA3-6B2, allophycocyanin), CD21 (eBioIgD9, PE), and CD23 (B13B4, FITC) or isotype control, as previously described (26). Cells were washed, and the data were collected using the BD FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo software version 8.8.6 (TreeStar, Ashland, OR).

Semiquantitative RT-PCR

Total RNA was isolated from thyroids using TRIzol reagent, and cDNA was generated as previously described (4, 27). Semiquantitative RT-PCR was performed, as previously described (4, 27–29), using β-actin to correct for sample-to-sample variations in the amounts of RNA. Samples were electrophoresed and stained with ethidium bromide, and densitometry analysis was performed. Densitometric units were normalized to the corresponding β-actin band (27). Results are expressed as ratios of gene of interest/the housekeeping gene β-actin. A value of 1.00 indicates a 1:1 ratio between a particular gene of interest and β-actin.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 4.0 (GraphPad, La Jolla, CA) with the Student t test or the nonparametric Mann–Whitney U test. A p value < 0.05 was considered statistically significant.

Results

CD28\(^−/−\) NOD.H-2h4 mice have reduced numbers of Tregs

CD28 is important for the generation and homeostasis of CD4\(^+\) CD25\(^+\) Tregs, and CD28\(^−/−\) NOD mice have reduced numbers of CD4\(^+\)CD25\(^+\) Tregs (11–13). To determine whether Treg numbers are reduced in CD28\(^−/−\) NOD.H-2h4 mice, spleen and CLN cells from adult WT or CD28\(^−/−\) NOD.H-2h4 mice were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry (Fig. 1). CD28\(^−/−\) mice had reduced numbers of CD4\(^+\)Foxp3\(^+\) Tregs in
features of ISAT in CD28-negative mice (data not shown). Many CD28+/− mice had increased numbers of thyroid-infiltrating CD4+CD28−T cells and reduced numbers of Foxp3+ Tregs compared with WT mice; therefore, they also had higher total numbers of CD45+CD3+IFN-γ−cells (data not shown). Intracellular IL-6 was not detected (data not shown).

CD28−/− NOD.H-2b4 WT mice develop severe ISAT and fibrosis and have reduced serum T4

Most WT NOD.H-2b4 mice given NaI in their drinking water develop ISAT, and transient (7–10 d) depletion of Tregs in WT NOD.H-2b4 mice has little effect on ISAT severity (6). To test the hypothesis that an earlier and sustained reduction in Tregs would have a greater effect on ISAT development, adult WT and CD28−/− NOD.H-2b4 mice were given NaI in the drinking water (Fig. 2D). SAT severity scores of CD28−/− mice were usually 3–4+ (i.e., very few of them developed the milder 1–2+ scores that are common in WT mice) (Fig. 2D). The histologic features of SAT in CD28−/− mice were indistinguishable from those of CD28−/− mice given NaI water in Fig. 2C (data not shown).

CD28−/− mice have increased expression of proinflammatory cytokines in the thyroid

Because CD28−/− mice develop more severe ISAT than do CD28+ mice, it was of interest to determine whether this was associated with alterations in the expression of particular cytokines in the thyroid. CD28−/− mice have increased expression of IFN-γ and IL-6 compared with WT mice (Fig. 3A). There is no difference in expression of the costimulatory molecule B7-1, which is a binding partner for CD28, and expression of IL-17 did not differ between WT and CD28−/− mice. The increased expression of IFN-γ and IL-6 mRNA is consistent with the increased inflammation and tissue damage in CD28−/− mice compared with WT mice, as well as with our previous studies indicating that IFN-γ is an essential cytokine for development of ISAT (27). Intracellular cytokine staining of thyroid-infiltrating cells also showed that more IFN-γ was produced, primarily by CD45+CD3+ cells, in thyroids of CD28−/− mice compared with WT mice (Fig. 3B). Thyroids of CD28−/− mice had higher numbers of total CD45+ infiltrating cells compared with WT mice; therefore, they also had higher total numbers of CD45+CD3+IFN-γ−cells (data not shown). Intracellular IL-6 was not detected (data not shown).

WT Tregs suppress development of ISAT in CD28−/− NOD.H-2b4 mice and differ phenotypically from Tregs in CD28−/− mice

Given that CD28−/− mice had reduced Treg numbers and percentages compared with WT mice, we hypothesized that the increased ISAT severity in CD28−/− mice was due, at least in part, to decreased function and/or numbers of Tregs in CD28−/− mice. If so, CD4+Foxp3+ Tregs from CD28−/− mice should reduce ISAT severity after transfer to CD28−/− mice. To test this, CD4+Foxp3+ Tregs were sorted from spleens of Foxp3-GFP CD28+/+ mice and transferred into 6–7-wk-old CD28−/− NOD.H-2b4 mice. All mice were given NaI in their drinking water, and recipient mice were given three i.v. injections of 106 sorted Tregs 2 wk apart. Controls included mice that did not receive Tregs or mice given the same number of CD4+Foxp3− T cells. When thyroids were removed 3 wk after the third injection of Tregs, recipients of CD28−/− Tregs had significantly reduced ISAT severity scores compared with controls (Fig. 4A) (p = 0.0005). CD28−/−CD4+Foxp3− cells had no effect on ISAT development (Fig. 4A), indicating that expression of Foxp3, and not simply expression of CD28, was important for suppression of ISAT.

Importantly, recipients of sorted CD28−/−CD4+Foxp3+ Tregs did not have significantly increased overall CD4+Foxp3+ Treg numbers compared with controls, but the CD28−/− donor Tregs accounted for more than half of the total CD4+Foxp3+ Tregs (Fig. 4B, 4C) (1.2 × 106 CD4+Foxp3+CD28−/− Tregs of 2.1 × 106 total Tregs). These results suggest that the transferred CD28−/− Tregs may have a survival advantage over CD28−/− Tregs, so that many of the endogenous Tregs are replaced by the transferred Tregs, as recently reported (32). As seen in Fig. 4D, the majority of CD45+...
CD4+Foxp3+ Tregs in the thyroids of the CD28−/− recipients of CD28+ Tregs express CD28, indicating that they can effectively migrate to the thyroid and, therefore, might exert some function at the site of inflammation. It is also of interest that recipients of CD28+CD4+Foxp3+ T cells had some splenic CD28+CD4+Foxp3+ cells in their spleens at the end of the experiment. These cells may have been present and undetectable in the transferred cells, or they may have been induced to express Foxp3 in the recipient environment. These cells were present in relatively low numbers and were unable to suppress ISAT. Also, ~40% of CD28+ T cells in recipients of CD28+CD4+Foxp3+ Tregs no longer expressed Foxp3 by 7–8 wk after the initial transfer (data not shown).

CD28−/− recipients of Tregs from CD28+/+ donors developed less severe ISAT, even though the total Treg numbers were similar to those of CD28−/+ mice not given CD28+ Tregs, suggesting that the transferred CD28+ Tregs differ functionally from the endogenous CD28− Tregs. To determine whether CD28+ and CD28− Tregs had phenotypic differences that might explain their functional differences, flow cytometry was used to compare the expression of various markers by CD28+ and CD28− Tregs in the CD28−/−

FIGURE 2. Comparison of ISAT severity, serum T4, and histology of thyroids from WT and CD28−/− NOD.H-2h4 mice. WT and CD28−/− NOD.H-2h4 mice were given NaI in their drinking water at 8 wk of age. After 8 wk, thyroids were removed, fixed, sectioned, and stained with H&E or for collagen by Trichrome staining. (A) ISAT severity scores 8 wk after NaI water (n = 35 WT mice and n = 21 CD28−/− mice). Results are pooled from four experiments and are representative of multiple experiments involving >100 mice, p < 0.001. (B) Serum T4 levels from individual mice (n = 11 WT mice and n = 21 CD28−/− mice from seven independent experiments). p = 0.0005. (C) H&E-stained thyroid sections demonstrating increased infiltration and follicle destruction in CD28−/− mice compared with WT mice. Note empty follicles (arrows) in thyroids of CD28−/− mice compared with colloid-filled follicles in WT thyroids. ISAT scores for WT mice = 2, 3, and 2; ISAT scores for CD28−/− mice = 4, 4, and 4. Trichrome staining shows collagen deposition (blue) in thyroids of CD28−/− mice with severe (4+) ISAT. Fibrosis is absent in thyroids of WT mice with ISAT scores of 3+. Results are representative of at least eight thyroids/group from five experiments. Scale bar, 0.01 mm (100×); scale bar, 0.005 mm (400×). (D) SAT severity scores of 4–8-mo-old WT and CD28−/− mice not given NaI water (n = 13 4–5-mo-old WT mice and n = 13 6–8-mo-old WT mice from four experiments; n = 15 4–5-mo-old CD28−/− mice from three experiments; and n = 21 6–8-mo-old CD28−/− mice from three experiments).
show expression of CD3 and IFN-γ separately. (b) represent the ratio of particular cytokine/CD28 expression did not differ for CD28+/+ and CD28−/− mice (H. Braley-Mullen, unpublished observations). Importantly, CD28−/− mice had significantly fewer thyroid-infiltrating B220+ B cells than did WT mice, as well as fewer thyroid-infiltrating CD138+ plasma cells (Fig. 5F, 5G). There were no differences in splenic plasma cell numbers between WT and CD28−/− mice (data not shown). These results are consistent with the reduced autoantibody responses in CD28−/− mice.

CD28−/− B−/− NOD.H-2b4 mice develop ISAT

The results presented above indicate that CD28−/− NOD.H-2b4 WT mice develop severe ISAT, even though they have greatly reduced anti-MTg autoantibody responses and reduced infiltration of B cells and plasma cells in their thyroids. We showed previously that B−/− NOD.H-2b4 mice are resistant to ISAT, but they...
develop ISAT when Tregs are transiently depleted (6). Because CD28−/− mice have fewer and functionally defective Tregs, we hypothesized that CD28−/− B−/− mice might develop ISAT without a requirement for Treg depletion. To test this hypothesis,
we first determined whether CD28−/−B−/− mice have fewer Tregs than do CD28+/+B−/− mice (Fig. 6A). Because total numbers of splenic and lymph node (LN) cells were similar in CD28+/+ and CD28−/− B−/− mice, CD28−/−B−/− mice also have significantly reduced numbers of CD4+Foxp3+ Tregs compared with CD28+/+B−/− mice. To determine whether CD28−/−B−/− mice develop ISAT, WT, CD28+/+B−/−, and CD28−/−B−/− mice were given NaI to transiently deplete Tregs from CD28−/−B−/− mice with ISAT differ phenotypically from WT B−/− Tregs with respect to the expression of CD27, GITR, and p75 (Fig. 6C), as shown above for B cell–sufficient mice (Fig. 4F). There is also an increased percentage of CD4+Foxp3+GARP+ Tregs in CD28−/−B−/− mice compared with WT B−/− mice.

**Discussion**

CD28−/− NOD.H-2h4 mice were developed to test the hypothesis that an early and permanent deficiency in Tregs would result in more severe ISAT in WT and B−/− NOD.H-2h4 mice. CD28 is known to be an important costimulator of TCR signaling (14, 15). However, a more important role for CD28 costimulation in the development of spontaneous autoimmune diseases may be in aiding the generation of Tregs that limit overactive immune responses and block autoimmunity (10, 13, 16, 20). Although CD28 costimulation is essential for the development of immune responses to most foreign Ags, as well as for experimentally induced autoimmune diseases (13, 15–18) (H. Braley-Mullen unpublished observations), it is not required for spontaneous development of diabetes or pancreatic exocrine disease in autoimmune-prone NOD mice (10, 13,
CD28-deficient B \textsuperscript{-/-} mice have reduced Treg numbers and develop ISAT similar to WT mice. (A) Splenocytes from WT B \textsuperscript{-/-} and CD28 \textsuperscript{-/-} B \textsuperscript{-/-} mice were analyzed by flow cytometry for the presence of CD4\textsuperscript{+}Foxp3\textsuperscript{+} Tregs. Dot plots of representative mice from each group show the percentages of splenic CD4\textsuperscript{+} cells that express Foxp3 (upper panels). Data are mean ± SEM (n = 6 spleen B \textsuperscript{-/-} and CD28 \textsuperscript{-/-} B \textsuperscript{-/-} mice and n = 3 LN B \textsuperscript{-/-} and CD28 \textsuperscript{-/-} mouse/group) and are pooled from two experiments. **p < 0.01, versus control CD28\textsuperscript{+} group. Student t test. (B) WT, CD28\textsuperscript{-/-}/B \textsuperscript{-/-}, or CD28 \textsuperscript{-/-} B \textsuperscript{-/-} mice were given NaI in their drinking water for 8 wk, and ISAT severity was determined (n = 19 WT, n = 13 WT B \textsuperscript{-/-}, and n = 16 CD28 \textsuperscript{-/-} B \textsuperscript{-/-} mice/group). *p < 0.05, **p < 0.01, Mann–Whitney nonparametric test. (C) Splenocytes from WT B \textsuperscript{-/-} or CD28 \textsuperscript{-/-} B \textsuperscript{-/-} mice given NaI water for 8 wk were stained for the presence of CD4, CD28, Foxp3 and CD27, GARP, GITR, or TNFR2 p75. Plots represent the percentage of CD4/Foxp3\textsuperscript{+} cells positive for the indicated marker (n = 7 mice/group combined from two experiments). ***p < 0.001, Student t test.

15, 19). NOD mice have reduced numbers of CD4\textsuperscript{+}CD28\textsuperscript{+} Tregs (11–13, 41), and they develop an early and aggressive form of diabetes compared with WT NOD mice (10, 13, 15) that is due to the reduction in Tregs (13).

In the current study, CD28-deficient WT and B \textsuperscript{-/-} mice on the autoimmune thyroiditis–prone NOD.H-2h4 background developed more severe ISAT than did their CD28\textsuperscript{+/+} counterparts (Figs. 2A, 6B). CD28 \textsuperscript{-/-} B \textsuperscript{-/-} NOD.H-2h4 mice almost uniformly developed severe ISAT with 4+ severity scores, which are relatively rare in WT NOD.H-2h4 mice (Fig. 2A). Moreover, administration of NaI in the drinking water is important for SAT development in WT NOD.H-2h4 mice (1), whereas many CD28 \textsuperscript{-/-} mice develop severe SAT if they are not given NaI in their drinking water (Fig. 2D). Importantly, thyroid lesions in CD28 \textsuperscript{-/-} mice differed histologically from those in WT CD28\textsuperscript{+/+} mice, and many CD28 \textsuperscript{-/-} mice were clinically hypothyroid (Fig. 2B, 2C). The more severe ISAT scores in CD28 \textsuperscript{-/-} mice were accompanied by increased infiltration of the thyroid by CD4\textsuperscript{+} T cells and increased expression of proinflammatory cytokines, including IFN-\textgamma and IL-6 in thyroids (Fig. 3), as determined by semiquantitative PCR. Intracellular cytokine staining confirmed that IFN-\textgamma–producing CD4\textsuperscript{+} CD3\textsuperscript{+} T cells are increased in thyroids of CD28 \textsuperscript{-/-} mice (Fig. 3B). IFN-\textgamma is required for the development of ISAT (27), and depletion of CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs results in increased expression of IFN-\textgamma in ISAT (8, 9). Diabetogenic effector T cells in CD28 \textsuperscript{-/-} NOD mice also were reported to have increased IFN-\textgamma responses to autoantigen (40), whereas Th2 and Th17 responses were reported to be reduced in CD28 \textsuperscript{-/-} mice (40, 42). IL-6 and IFN-\textgamma may be upregulated as a result of the increased inflammation and tissue destruction in thyroids of CD28 \textsuperscript{-/-} mice that are due to their inherent Treg deficit.

As hypothesized, CD28 \textsuperscript{-/-} NOD.H-2h4 mice had fewer CD4\textsuperscript{+} Foxp3\textsuperscript{+} Tregs in CLNs, spleens, and thyroids compared with WT NOD.H-2h4 mice (Fig. 1, data not shown). Although CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs were reduced by 75–80% in CD28 \textsuperscript{-/-} NOD mice (10, 11, 13), Tregs were reduced by only ~50% in the spleen and 65–70% in the LNs in NOD.H-2h4 mice (Figs. 1, 6A). It is not known why Treg numbers were reduced to a lesser extent in CD28 \textsuperscript{-/-} NOD.H-2h4 mice compared with CD28 \textsuperscript{-/-} NOD mice, but the development of ISAT was profoundly affected, despite the relatively modest reduction in total Treg numbers. The diminished Treg numbers explain, at least in part, the increased ISAT severity in CD28 \textsuperscript{-/-} mice compared with WT NOD.H-2h4 mice, because transfer of Tregs from CD28\textsuperscript{+/+} mice suppresses ISAT development in CD28 \textsuperscript{-/-} mice (Fig. 4A).

The results of our Treg-transfer experiments are consistent with those reported by other investigators in the NOD diabetes model (13, 19), and they provide new information regarding the function of Tregs in CD28 \textsuperscript{-/-} mice. First, the endogenous Tregs in CD28 \textsuperscript{-/-} NOD.H-2h4 mice presumably differ functionally from those in CD28\textsuperscript{+/+} mice, because transfer of WT Tregs into CD28 \textsuperscript{-/-} mice significantly reduced ISAT severity without increasing the overall Treg pool (Fig. 4A–C). The transferred CD28\textsuperscript{+/+}Foxp3\textsuperscript{+} Tregs make up more than half of the total Treg pool 7 wk after the first transfer (Fig. 4B, 4C), suggesting that Tregs in CD28 \textsuperscript{-/-} mice could have a survival defect that allows for their replacement by CD28\textsuperscript{+} Tregs.

The idea that CD28\textsuperscript{+} Tregs differ functionally from those in CD28 \textsuperscript{-/-} mice is supported by the finding that CD28\textsuperscript{+} Tregs transferred to CD28 \textsuperscript{-/-} recipients differ from the recipients’ endogenous Tregs with regard to the expression of several surface TNFR superfamily member markers that were shown to identify functionally distinct subsets of Tregs in other models. TNFR2 p75 was reported to identify highly suppressive Tregs by several groups (33, 43, 44), and CD27 plays a role in T cell differentiation and is reported to be expressed on effective Tregs in humans (35, 36). GITR can play a role in Treg suppression of effector responses, and CD4\textsuperscript{+}Foxp3\textsuperscript{+}GITR\textsuperscript{+} cells were reduced when CD28/ B7 interactions were blocked (11, 34). All three of these TNFR superfamily members are expressed on significantly more CD28\textsuperscript{+} WT donor Tregs than on recipient CD28\textsuperscript{-/-} Tregs (Fig. 4E), consistent with the hypothesis that CD28 \textsuperscript{-/-} Tregs are inherently less effective suppressors than are CD28\textsuperscript{+} Tregs. Tregs lacking CD28 were recently reported to have phenotypic differences and a survival disadvantage compared with WT Tregs in a model in which CD28 was specifically deleted from Foxp3-expressing cells (32). Tregs in these mice had markedly reduced CTLA4, CCR6, and PD-1 expression (32), and expression of CTLA4 on Tregs was also reduced when CD28 deletion was induced in adult mice (45). The phe-
notic differences between WT and CD28+/− Tregs are not an artifact of the transfer system, because splenic Tregs from WT and CD28+/− mice with ISAT have similar differences in CD27, GITR, and p75 (Fig. 4F), and Tregs in B−/−CD28− and CD28− mice also had differential expression of these molecules (Fig. 6C). Splenic Tregs in naïve WT and CD28−/− mice also differ in their expression of CD27, GITR, and p75 (data not shown), although the differences are less pronounced than in mice with ISAT (Fig. 4F). This suggests that natural Tregs in WT and CD28−/− mice are inherently different. It is interesting that recipients of CD4+ Foxp3+ T cells had some CD28−/CD4+Foxp3+ Tregs 7 wk after transfer (Fig. 4C), but they had no effect on ISAT severity (Fig. 4A). These CD28−/CD4+Foxp3+ Tregs could have expanded as the result of CD4+Foxp3+ Treg contamination in the sorted CD4+ Foxp3+ pool, or they could be induced Tregs that upregulated Foxp3 expression after transfer. Combined with the transfer experiments, the phenotyping results suggest that Tregs in CD28+/+ and CD28−/− mice differ from one another both phenotypically and functionally. The transfer experiments did not provide a direct comparison of the function of CD28−/− and CD28+/+ Tregs; they indicate that Tregs from CD28+/+ mice are capable of suppressing ISAT development, whereas the endogenous Tregs in CD28−/− mice permit the development of severe ISAT.

Although anti-MtG autoantibody responses generally correlate with ISAT severity scores in WT NOD.H-2h4 mice (1, 27), CD28−/− NOD.H-2h4 mice have greatly reduced serum anti-MtG autoantibodies compared with WT mice (Fig. 5A). There are several possible explanations for the reduced autoantibody responses in CD28−/− NOD.H-2h4 mice. First, CD28/B7 interactions are important for activation of B cells by CD4+ T cells (9, 46), as well as for the development of germinal centers (38). CD28−/− mice have reduced Ab responses following immunization with foreign Ags (40, 46), and CD28−/− NOD.H-2h4 mice produce minimal anti-MtG autoantibody following immunization with MtG and LPS (H. Braley-Mullen, unpublished observations). CD28+/+ and CD28−/− mice had comparable numbers of CD19+ B cells, but CD28−/− mice had more splenic MZ B cells and fewer FO B cells compared with WT mice (Fig. 6B–E). MZ B cells are found primarily in the splenic marginal sinus (47), and they have been implicated as the primary autoantibody producers in mouse models of lupus (48–50). They also increase in number and are important APCs in pancreatic LNs of NOD mice developing diabetes (51). However, FO B cells, the major subset of splenic B cells and the major circulating B cell population, are the primary B cell subset inflicting thyroids of mice with ISAT, and when FO B cells are depleted by anti-CD20, ISAT is inhibited (S.H. Hong and H. Braley-Mullen, submitted for publication). These results suggest that FO B cells are the major B cell subset in this model, and it is evident that the modest reduction in FO B cells in CD28−/− mice was not sufficient to reduce ISAT severity. B220+ B cells in thyroids of CD28−/− mice are diffuse and reduced in number compared with those in thyroids of WT mice (Fig. 5F, data not shown). Although WT and CD28−/− NOD.H-2h4 mice had comparable numbers of splenic CD138+ cells (S.H. Hong, J.S. Ellis, and H. Braley-Mullen, unpublished observations), CD28−/− NOD.H-2h4 mice have fewer thyroid-infiltrating CD138+ plasma cells (Fig. 5G). These results may be consistent with those reported recently by another group indicating that long-term survival of bone marrow plasma cells and subsequent humoral immunity were reduced in CD28−/− mice (46). However another group reported that both short- and long-lived plasma cells produced more Ab in CD28−/− mice compared with WT mice (52). Therefore, further studies are needed to determine how CD28 regulates B cell and plasma cell numbers and function.

The results of this study demonstrate that lack of CD28 in NOD.H-2h4 mice leads to more severe ISAT than that seen in WT mice, with increased thyroid follicle destruction, low serum T4, and collagen deposition (fibrosis) in thyroids (Fig. 2). The increased thyroid destruction and inflammation were shown to be due, at least in part, to the fact that CD28−/− mice have fewer Tregs compared with WT mice. Importantly, CD28−/− Tregs appear to differ both functionally and phenotypically from those in WT NOD.H-2h4 mice, and WT CD4+Foxp3+ Tregs can suppress ISAT after transfer to CD28−/− mice, although the overall numbers of Tregs are not increased.

Disclosures

The authors have no financial conflicts of interest.

References


