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Divergent Effects of T Cell Costimulation and Inflammatory Cytokine Production on Autoimmune Peripheral Neuropathy Provoked by Aire Deficiency

Xiaopei L. Zeng,*1 Anil Nagavalli,*1 Colin-Jamal Smith,* James F. Howard,† and Maureen A. Su*§‡

Chronic inflammatory demyelinating polyneuropathy results from autoimmune destruction of the peripheral nervous system and is a component of the multiorgan autoimmunity syndrome that results from Aire gene mutations in humans. In parallel, peripheral nervous system autoimmunity resembling chronic inflammatory demyelinating polyneuropathy develops spontaneously in NOD mice with a partial loss of Aire function (NOD.AireGW/+ mice) and is a T cell–mediated disease. In this study, we analyze how key aspects of T cell activation and function modulate disease development in Aire-deficient mice. We show that genetic ablation of the Th1 cytokine IFN-γ completely prevents clinical and electrophysiological evidence of neuropathy in NOD.AireGW/+ mice. IFN-γ deficiency is associated with absence of immune infiltration and decreased expression of the T cell chemoattractant IP-10 in sciatic nerves. Thus, IFN-γ is absolutely required for the development of autoimmune peripheral neuropathy in NOD.AireGW/+ mice. Because IFN-γ secretion is enhanced by B7-CD28 costimulation of T cells, we sought to determine the effects of these costimulatory molecules on neuropathy development. Surprisingly, B7-2 deficiency accelerated neuropathy development in NOD.AireGW/+ mice, and Ab blockade of both B7-1 and B7-2 resulted in fulminant, early-onset neuropathy. Thus, in contrast to IFN-γ, B7-2 alone and B7-1/B7-2 in combination function to ameliorate neuropathy development in NOD.AireGW/+ mice. Together, these findings reveal distinct and opposing effects of the T cell costimulatory pathway and IFN-γ production on the pathogenesis of autoimmune peripheral neuropathy. The Journal of Immunology, 2013, 190: 3895–3904.

Chronic inflammatory demyelinating polyneuropathy (CIDP) is characterized by long-term sensory and motor dysfunction resulting from autoimmune attack of the peripheral nervous system (PNS) (1). Two reports of CIDP in unrelated patients with autoimmune polyendocrinopathy syndrome type 1 (APS1), a disorder linked to mutations in the Aire gene, suggest a relationship between Aire and CIDP (2, 3). In addition, we recently showed that a strain of NOD mice with a dominant G228W mutation (NOD.AireGW/+ mice) develops spontaneous autoimmune peripheral neuropathy resembling CIDP (4, 5). Thus, Aire dysfunction has been linked to PNS autoimmunity in both mice and humans.

In the thymus, Aire promotes ectopic expression of peripheral tissue Ags, which mediates the negative selection of self-reactive thymocytes (6, 7). The dominant G228W mutation results in partial loss of Aire function, reducing expression levels of self-antigens to ~10% of wild-type levels (5). This decreased expression allows escape of self-antigen–recognizing T cells from thymic negative selection, which predisposes to autoimmune disease. A major self-antigens recognized by T cells in NOD.AireGW/+ mice and APS1 patients with autoimmune peripheral neuropathy is myelin protein zero (P0), a PNS-specific protein (4). NOD.AireGW/+ mice express P0 in the thymus at greatly reduced levels, suggesting that ectopic P0 expression in the thymus is regulated by Aire (5). Consistent with a defect in the negative selection of P0-specific T cells, increased peripheral T cell responses to P0 are seen in NOD.AireGW/+ mice (4).

The role of Aire in T cell negative selection suggests that T cell dysregulation underlies PNS autoimmunity in Aire deficiency. In addition, there is ample evidence that T cell dysregulation is a key component of PNS autoimmunity. For instance, in experimental allergic neuritis (EAN), an induced model of inflammatory demyelinating disease of the PNS, T cell–deficient mice are clinically and histologically unaffected by EAN compared with wild-type mice (8). Also, in spontaneous models of PNS autoimmunity, T cells are sufficient to transfer neuropathy to immunodeficient recipients (4, 9). Although the evidence for an important role of T cells in PNS autoimmunity is strong, how T cell costimulation impinges on PNS-specific T cells and how T cell inflammatory cytokine production directs neuropathy development require further clarification.

In addition to engagement of the TCR by Ag and MHC on the APC, costimulation is necessary for either the activation of naive T cells or immunoregulation in different disease settings. A prominent costimulatory interaction is between CD28 on CD4+ T cells and B7-1/B7-2 (CD80/CD86) on APCs (10, 11). In certain autoimmune diseases, this interaction promotes autoimmune dis-
ease development. For instance, in the adoptive-transfer model of experimental autoimmune encephalitis (EAE), blocking costimulation attenuates clinical outcomes of disease (12), and genetic ablation of CD28 or B7-1/B7-2 confers resistance to disease (13). Also, CD28 deficiency prevents the development of neuropathy in EAN, suggesting a pathogenic role for this costimulatory pathway in autoimmune peripheral neuropathy (14). In addition to a pro-inflammatory role, this costimulatory interaction can dampen autoimmune disease development. For example, the same deficiencies of B7-1/B7-2 and CD28 in NOD mice cause an accelerated development of autoimmune diabetes (15). Interestingly, genetic ablation of only B7-2 redirects autoimmunity from the pancreas to the peripheral nerves in NOD mice (9). Thus, the way in which B7-1/B7-2 costimulation of T cells affects autoimmune disease development is not straightforward.

B7-CD28 costimulation of T cells has multiple effects on naïve T cells, including modulation of T cell inflammatory cytokine production. CD28 stimulation acts through the AKT intracellular signaling pathway to increase production of IFN-γ (16, 17), and it enhances IFN-γ promoter activity by 3–6-fold (18). At the same time, T cells from NOD mice deficient in B7-1/B7-2 and T cells from EAN mice deficient in CD28 demonstrated decreased IFN-γ production (14, 15). Therefore, IFN-γ production in these two autoimmune disease settings is promoted by T cell costimulatory activity.

Whether IFN-γ exacerbates or protects from autoimmune peripheral neuropathy development is contentious. IFN-γ was shown to be required for development of disease in both induced and spontaneous models of autoimmune peripheral neuropathy (19–21). Conversely, Zhang et al. (22) recently reported that IFN-γ-deficient mice developed exacerbated clinical symptoms of EAN, indicating a protective function of IFN-γ. Although inconsistent with previous reports in autoimmune peripheral neuropathy, this protective role of IFN-γ falls in line with evidence from other autoimmune diseases that are aggravated in the absence of IFN-γ, such as EAE (23), collagen-induced arthritis (24), and rheumatoid arthritis (25).

In this study, we focus on delineating how T cell IFN-γ production and B7-CD28 costimulation affect development of spontaneous autoimmune peripheral neuropathy in NOD.AireGW/+ mice. We demonstrate that genetic ablation of IFN-γ prevents clinical, histological, and electrophysiological evidence of neuropathy in NOD.AireGW/+ mice, which indicates that IFN-γ is absolutely required for disease development. In contrast, deficiency of B7-2 alone and blockade of B7-1/B7-2 in combination significantly accelerate neuropathy development, pointing to a protective role for T cell costimulation in this model. Together, these results suggest that IFN-γ production and B7-CD28 costimulatory pathways have distinct and potentially opposing effects on T cells in the development of autoimmune peripheral neuropathy.

**Materials and Methods**

**Mice**

NOD.AireGW/+ mice were generated as previously described (5). NOD.AireGW/+ mice are from the Type-1 Diabetes Mouse Resource (The Jackson Laboratory). Because Aire and IFN-γ are both on chromosome 10, NOD.AireGW/+IFNγ−/− mice were generated from a founder mouse with crossover between these two loci. The line was continued by maintaining the mutant IFN-γ locus in the homozygous state. NOD.B7-1/B7-2−/− mice were a gift from Dr. Jeffrey Bluestone (University of California, San Francisco [UCSF]). Mice in these experiments were backcrossed onto the NOD/ShiLtJ background for 10 generations. NOD.scid mice were purchased from The Jackson Laboratory. Mice were housed in a pathogen-free barrier facility at UCSF and University of North Carolina Chapel Hill. Animals were assessed for clinical neuropathy and diabetes, as described (4). Mice were assessed at least once per week. Experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research.

**Histology**

Harvested organs were fixed in 10% formalin overnight, changed into 30% ethanol for 30 min, and then stored in 70% ethanol. Organs were embedded in paraffin, sectioned, and stained with H&E. Immune infiltration was scored in a blinded fashion using a system that was described previously (5). Scores of 0, 1, 2, 3, and 4 indicate 0%, 25%, 50%, 75%, and >75% infiltration, respectively.

**Intracellular cytokine staining**

Intracellular cytokine staining and flow cytometry were performed as described (26). Splenocytes were stained with anti-mouse CD3e (clone 145.2C11; eBioscience) and anti-mouse IFN-γ (BD Biosciences) Abs and analyzed on a Cyan ADP Analyzer (Beckman Coulter).

**Immunohistochemistry**

Staining for immune cells in OCT-embedded sciatic nerves were performed with anti-CD4 (clone GK1.5; Bio X Cell) and anti-F4/80 (clone BM8; eBioscience) Abs, as described (26). Staining for IP-10 was performed with anti-IP-10 Ab (R&D Systems), as described (27).

**Electrophysiology**

Sciatic nerve conduction studies were performed as described (28), using a Teca Synergy EMG system.

**Adoptive transfer**

Adoptive transfer of CD4+ T cells from spleen and lymph nodes of female neuropathic NOD.AireGW/+ mice or age-matched IFN-γ-deficient NOD.AireGW/+ mice was performed, as described (4). CD4+ T cells were isolated using anti-CD4 MicroBeads (Miltenyi Biotec) on a MS or LS column. CD4+ T cells were activated in the presence of anti-CD3 and anti-CD28 (1 µg/ml in PBS) for 96 h. A total of 7 × 10⁶ CD4 T cells was transferred retro-orbitally into female NOD.scid recipients.

**Ab treatment**

Anti-B7-1 Ab (clone 16.10A1) and anti-B7-2 Ab (clone GL1) were generous gifts of Greg Sotz (UCSF) or purchased from the UCSF Hybridoma Core. Fourteen-day-old mice were treated with 50 µg Ab or isotype control every other day for 10 treatments. For flow cytometry analysis of regulatory T cells (Tregs), mice were sacrificed 8 d after the last treatment.

**Foxp3 staining**

Thymocytes and lymphocytes were prepared for flow analysis, as described (26). Cells were stained according to the manufacturer’s instructions (eBioscience) with fluorochrome-labeled Ab specific for mouse CD4 (clone RM4-5), CD8a (clone 53-6.7), CD25 (clone PC61.5), and Foxp3 (clone FJK-16a).

**Real time RT-PCR**

Fresh sciatic nerves were placed immediately into cell lysis buffer, and mRNA was isolated with the RNeasy Miniprep Kit (QIAGEN). The SuperScript VILO cDNA Synthesis Kit (Invitrogen) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for cDNA synthesis and PCR, respectively. Commercially available TaqMan primer-probe set (Applied Biosystems) for CXCL10 was used. Cyclophilin A was used as an internal control and detected with the primer-probe set reported by Su et al. (5). Reactions were run on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed, as described (5).

**Statistics**

Data were analyzed with Prism software (GraphPad) using unpaired t tests. Log-rank tests were used for comparison of survival curves; p ≤ 0.05 was considered significant.

**Results**

IFN-γ is required for development of spontaneous autoimmune peripheral neuropathy in NOD.AireGW/+ mice

The specific cytokines critical for the pathogenesis of autoimmune peripheral neuropathy are not clear. Multiple cytokines, including IFN-γ, IL-17, and TNF-α, have been implicated in disease development (20, 22, 29–32). In NOD.AireGW/+ mice, IFN-γ is a pre-
dominant cytokine produced by sciatic nerve-infiltrating CD4+ T cells (4). However, it is not known whether IFN-γ production is required for the development of autoimmune peripheral neuropathy in NOD.AireGW/+ mice. To test this, we genetically ablated IFN-γ from NOD.AireGW/+ mice to produce doubly-deficient NOD.AireGW/+IFNγ−/− mice. Lack of IFN-γ production by T cells in these mice was confirmed by intracellular IFN-γ staining and flow cytometry of CD3+ splenocytes (Fig. 1A). To determine the effect of IFN-γ deficiency on the development of neuropathy, we monitored a female cohort of NOD.AireGW/+IFNγ−/− mice and NOD.AireGW/+IFNγ−/− or NOD.AireGW/+IFNγ−/− controls for a total of 38 wk. Consistent with our previous reports, 80% of IFN-γ-sufficient NOD.AireGW/+ mice developed neuropathy by 22 wk of age (Fig. 1B) (4). Strikingly, however, none of the IFN-γ-deficient NOD.AireGW/+ mice developed clinical signs of neuropathy. Protection from clinical neuropathy was associated with absence of cellular immune infiltration within the sciatic nerves on H&E staining (Fig. 1C, 1D). We previously reported that the immune infiltrates found in sciatic nerves of NOD.AireGW/+ mice predominantly consist of CD4+ T cells and F4/80+ macrophages (4). Minimal CD4 and F4/80 expression could be detected by immunohistochemical staining within the sciatic nerves of IFN-γ-deficient NOD.AireGW/+ mice (Fig. 1E). Together, these data clearly demonstrate that IFN-γ is necessary for the development of autoimmune peripheral neuropathy in NOD.AireGW/+ mice.

In addition to neuropathy, NOD.AireGW/+ mice develop other autoimmune manifestations, including diabetes and sialitis (5), allowing us to simultaneously access the role of IFN-γ in the development of these multiple autoimmune manifestations. In contrast to neuropathy, autoimmune diabetes developed at the same incidence in IFN-γ-deficient NOD.AireGW/+ mice as in IFN-γ-sufficient NOD.AireGW/+ controls (Fig. 1F). In addition, the severity of insulinitis within the pancreas was unchanged, regardless of IFN-γ status (Supplemental Fig. 1A, 1B). Therefore, IFN-γ is dispensable for the development of autoimmune diabetes in NOD.AireGW/+ mice. This finding is consistent with reports that genetic deficiency of IFN-γ or IFN-γR in NOD.WT mice does not significantly affect diabetes incidence (33–36). Furthermore, no changes in the development of sialitis, dacroadenitis, or uveitis were detected by histological analysis in IFN-γ-deficient versus IFN-γ-sufficient NOD.AireGW/+ mice (Supplemental Fig. 1C, data not shown). Thus, among the autoimmune manifestations in NOD.AireGW/+ mice, autoimmune peripheral neuropathy appears to be uniquely dependent on IFN-γ.

**Lack of IFN-γ prevents development of electrophysiological abnormalities on nerve conduction studies in NOD.AireGW/+ mice**

Nerve conduction studies of CIDP patients demonstrate characteristic findings of chronic demyelination (37). These abnormalities include slowed conduction velocity and dispersion of compound muscle action potentials. To determine whether autoimmune peripheral neuropathy in NOD.AireGW/+ mice results in electrophysiological changes, we performed nerve conduction studies, as described (28), on the sciatic nerves of >15-wk-old neuropathic NOD.AireGW/+ mice and age-matched IFN-γ-deficient NOD.AireGW/+ mice. Studies on neuropathic NOD.AireGW/+ mice demonstrated abnormalities indicative of predominantly demyelinating neuropathy, including reduced amplitude, slowed conduction velocity, and increased duration (Fig. 2A, 2B, 2D). In contrast, NOD.AireGW/+IFN−/− sciatic nerves were indistinguishable from wild-type sciatic nerves (Fig. 2C, 2D). Therefore, IFN-γ deficiency prevents the development of the electrophysiological abnormalities seen in NOD.AireGW/+ sciatic nerves.

**FIGURE 1.** IFN-γ deficiency prevents neuropathy and neural infiltration, but not diabetes, in NOD.AireGW/+ mice. (A) Representative flow cytometry plots of CD3+ splenocytes from IFN-γ-sufficient (IFNγ+/+) and IFN-γ-deficient (IFNγ−/−) NOD.AireGW/+ (AireGW/+ mice) stained for intracellular IFN-γ. (B) Neuropathy incidence curve of female IFN-γ-sufficient NOD.AireGW/+ mice (AireGW/+IFNγ+/+, n = 11) and IFN-γ-deficient NOD.AireGW/+ mice (AireGW/+IFNγ−/−, n = 13). (C) Representative H&E-stained, formalin-fixed, longitudinal sections of sciatic nerves from NOD wild-type (Aire+/+IFNγ+/+), NOD.AireGW/+ (AireGW/+IFNγ−/−), and IFN-γ-deficient NOD.AireGW/+IFNγ−/− mice (original magnification ×200). (D) Cumulative infiltration scores of sciatic nerves from IFN-γ-sufficient and IFN-γ-deficient NOD.AireGW/+ mice. Each symbol represents an individual mouse. (E) Immunohistochemical stains of sciatic nerve from either neuropathic IFN-γ-sufficient NOD.AireGW/+ mouse or age-matched IFN-γ-deficient NOD.AireGW/+ mouse for CD4 and F4/80. Images shown are representative of two separate experiments (original magnification ×200). (F) Diabetes incidence curve of female IFN-γ-sufficient and IFN-γ-deficient NOD.AireGW/+ mice. *p < 0.0001, n.s., Not significant.
Lack of IFN-γ production by CD4+ T cells from NOD.AireGW/+ mice prevents transfer of peripheral neuropathy

IFN-γ is produced mainly by T cells and NK cells. We showed previously that CD4+ T cells from neuropathic NOD.AireGW/+ mice are sufficient to transfer neuropathy to immunodeficient SCID mice on the NOD background (NOD.scid) (4). To determine whether IFN-γ production by CD4+ T cells is necessary for neuropathy transfer, we performed adoptive-transfer experiments with CD4+ cells from 20-wk-old NOD.AireGW/+ IFNγ−/− females into NOD.scid females. In this setting, IFN-γ production was lacking in T cells but present in NK cells of reconstituted mice. None of the recipients of IFN-γ-deficient CD4+ T cells developed neuropathy, even 12 wk posttransfer, whereas all recipients of IFN-γ–sufficient CD4+ T cells developed neuropathy by 6 wk posttransfer (Fig. 3A). Consistent with this finding, dense immune infiltrates were seen on histologic sections of all sciatic nerves from recipients of IFN-γ–producing CD4+ T cells, whereas no immune infiltration was seen in any of the sciatic nerves from recipients of IFN-γ–deficient CD4+ T cells (Fig. 3B, 3C). Thus, lack of IFN-γ production by CD4+ T cells prevents transfer of neuropathy.

Decreased IP-10 expression in sciatic nerves of NOD.AireGW/+ mice lacking IFN-γ

IFN-γ has a variety of effects on the immune response (38–41), one of which is the induction of chemokines required for immune cell targeting to tissues (42–44). Of particular interest is the IFN-γ–inducible chemokine CXCL10/IP-10, which is highly expressed on endoneurial endothelial cells in inflammatory demyelinating neuropathies (45, 46). Additionally, increased expression of IP-10 in nerves is associated with CIDP in humans (46) and with clinical neuropathy in NOD.AireGW/+ mice (4). IP-10 promotes the transendothelial migration of macrophages and activated CD4+ T cells to sites of inflammation by binding to its receptor CXCR3 expressed on these immune cells (47–49). In an EAE model, blocking IP-10 reduced the accumulation of immune cells within the CNS while increasing absolute splenocyte counts (47). In NOD.AireGW/+ mice, we also observed increased total splenocyte (Fig. 4A) and CD4+ T cell (Fig. 4B) numbers in the setting of decreased immune infiltration within the sciatic nerves (Fig. 1C, 1E). These similarities led us to hypothesize that IFN-γ deficiency in NOD.AireGW/+ mice may be associated with reduced IP-10 expression in sciatic nerves.

To test this hypothesis, we analyzed the relative expression of IP-10 in the sciatic nerves of neuropathic NOD.AireGW/+ mice versus age-matched IFN-γ–deficient NOD.AireGW/+ mice. Immunohistochemical staining showed abundant IP-10 within the sciatic nerves of neuropathic NOD.AireGW/+ mice and decreased IP-10 within the sciatic nerves of age-matched IFN-γ–deficient NOD.AireGW/+ mice (Fig. 4C). Consistent with this finding, real time RT-PCR analysis of IP-10 mRNA expression in the sciatic nerve demonstrated increased expression in neuropathic NOD.AireGW/+ mice compared with age-matched IFN-γ–deficient NOD.AireGW/+ mice (Fig. 4D). As expected from our previously published data (4), IP-10 expression was low in young NOD.AireGW/+ and young NOD.AireGW/+IFNγ−/− mice. Increased IP-10 was not merely a baseline feature of the NOD strain, because aged IFN-γ–sufficient NOD.AireGW/+ mice did not demonstrate increased IP-10 levels (Fig. 4D). Interestingly, the IP-10 expression of aged NOD.AireGW/+IFNγ−/− mice was not zero, suggesting a basal level expression that is not dependent on IFN-γ. This is consistent...
with studies showing that other factors, including IFN-α, IFN-β, and LPS, also induce IP-10 expression (42–44).

**Blockade of B7-1/B7-2 in NOD.AireGW/+ mice results in fulminant, early-onset autoimmune peripheral neuropathy**

Previous work demonstrated that T cell IFN-γ production is controlled, in part, by B7-1/B7-2 costimulation. In NOD mice, genetic ablation of both B7-1 and B7-2 resulted in decreased T cell IFN-γ production (15). Given our finding that IFN-γ is absolutely required for neuropathy development, we sought to determine the effect of B7-1 and B7-2 blockade on neuropathy development in NOD.AireGW/+ mice. Surprisingly, treatment of NOD.AireGW/+ mice between 2 and 4 wk of age with anti–B7-1 and anti–B7-2 (anti–B7-1/B7-2) Abs resulted in fulminant early-onset autoimmune peripheral neuropathy.

**FIGURE 3.** IFN-γ expression by CD4+ T cells is required for neuropathy transfer. (A) Neuropathy incidence curve of NOD.scid mice receiving 7 × 10^6 CD4+ T cells from either neuropathic IFN-γ-sufficient NOD.AireGW/+ or age-matched IFN-γ-deficient NOD.AireGW/+ mice (n = 4 per group). 

*p < 0.0084. (B) Representative H&E-stained, formalin-fixed, longitudinal sections of sciatic nerve from recipient mice receiving CD4+ T cells from either IFN-γ-sufficient or IFN-γ-deficient NOD.AireGW/+ mice at time of sacrifice. Original magnification ×200.

(C) Infiltration scores of sciatic nerves from recipient mice at the time of sacrifice. Each symbol represents an individual mouse. *p < 0.0001.

**FIGURE 4.** Decreased expression of IP-10 chemokine in sciatic nerves of IFN-γ-deficient NOD.AireGW/+ mice. Absolute number of total splenocytes (A) and CD4+ T cells (B) in NOD.AireGW/+ (n = 3) and IFN-γ-deficient NOD.AireGW/+ mice (n = 6), as determined by flow cytometry. *p < 0.01 and *p < 0.02, respectively. (C) Representative immunohistochemical stains of sciatic nerve from either neuropathic IFN-γ-sufficient or age-matched IFN-γ-deficient NOD.AireGW/+ mice for IP-10 (original magnification ×200). Images shown are representative of two separate experiments. (D) Real-time RT-PCR assay for relative transcriptional expression of IP-10 (normalized to cyclophilin A) in sciatic nerves of IFN-γ-sufficient and IFN-γ-deficient NOD.AireGW/+ mice at either >15 wk of age or <10 wk of age and IFN-γ-sufficient NOD.AireGW/+ mice at >15 wk of age (n = 3–5 per group). Data are from two separate experiments. *p < 0.03 between aged IFN-γ-sufficient and IFN-γ-deficient NOD.AireGW/+ mice, *p < 0.002 between aged IFN-γ-sufficient NOD.AireGW/+ and NOD.WT mice.
onset neuropathy (Fig. 5A). NOD.Aire<sub>GW</sub>/<sup>+</sup> mice became neuropathic at 8 wk of age, and 100% of mice were neuropathic by 11 wk. Treatment with anti–B7-1/B7-2 Abs accelerated neuropathy to a greater degree in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice than in wild-type mice, suggesting an additive effect of Aire deficiency and lack of costimulation in the development of autoimmune peripheral neuropathy. Manifestation of neuropathy was associated with severe immune infiltration of sciatic nerves (Fig. 5B, 5C). Thus, despite decreased IFN-γ production in NOD mice lacking B7-1/ B7-2 (15), blockade of B7-CD28 costimulation accelerated neuropathy development in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice.

In addition to assessing neuropathy, we simultaneously monitored diabetes development in anti–B7-1/B7-2 Ab–treated mice. Genetic ablation of B7-1 and B7-2 in a mixed NOD-C57BL/6 strain was shown to result in early-onset autoimmune diabetes (15). Consistent with this finding, treatment of wild-type NOD mice with anti–B7-1/B7-2 Abs resulted in early-onset autoimmune diabetes (Supplemental Fig. 2), as previously reported (50). In contrast, diabetes was not seen in any NOD.Aire<sub>GW</sub>/<sup>+</sup> mice treated with anti–B7-1/B7-2 Abs. Thus, B7-1 and B7-2 blockade specifically accelerates neuropathy development without accelerating diabetes development in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice.

Blockade of B7-1/B7-2 in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice is associated with decreased Tregs

B7-CD28 costimulation is required for the development and peripheral maintenance of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (15, 51, 52). Treg population size was reported to be reduced by as much as 80% in NOD mice deficient in B7-1/B7-2 (10) and CD28 (15). Therefore, we hypothesized that the acceleration of neuropathy seen in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice in the setting of B7-1 and B7-2 costimulation blockade may reflect a dependence on Tregs to delay neuropathy onset in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice.

To test this possibility, NOD.Aire<sub>GW</sub>/<sup>+</sup> mice were treated with anti–B7-1/B7-2 Abs, and Treg levels were measured 1 wk after the treatment course. Similar to NOD.WT mice, NOD.Aire<sub>GW</sub>/<sup>+</sup> mice treated with anti–B7-1/B7-2 Abs demonstrated significantly decreased frequencies of Tregs in both the thymus and spleen compared with the isotype control–treated groups (Fig. 6A, 6B). These findings suggest that the effect of anti–B7-1/B7-2 Abs on Treg frequency is not dependent on the presence of Aire. Additionally, the absolute number of Tregs in the thymus was significantly reduced in the anti–B7-1/B7-2 Ab–treated Aire<sub>GW</sub>/<sup>+</sup> group (Fig. 6C). In the spleen, there was ~70% decrease in absolute numbers of Tregs in both the anti–B7-1/B7-2 Ab–treated NOD.WT and NOD.Aire<sub>GW</sub>/<sup>+</sup> groups (Fig. 6C).

Although previous reports of Tregs in Aire-deficient mice indicate no change in Treg numbers (6, 7, 53, 54), Lei et al. (55) recently reported that Aire-deficient mice have a reduction in the number of thymic Tregs. Consistent with this latter report, we also noticed a decrease in the absolute numbers of Tregs in the thymus of isotype control–treated NOD.Aire<sub>GW</sub>/<sup>+</sup> mice compared with NOD.WT mice (Fig. 6C). To investigate this further, we compared Treg numbers in untreated 8-wk-old NOD.WT and NOD.Aire<sub>GW</sub>/<sup>+</sup> mice. Treg numbers were significantly lower in untreated NOD.Aire<sub>GW</sub>/<sup>+</sup> thymi compared with NOD.WT thymi (Fig. 6D). Thus, in line with the report of Lei et al. (55), NOD.Aire<sub>GW</sub>/<sup>+</sup> mice demonstrate decreased numbers of thymic Tregs.

B7-2 deficiency and loss of Aire function have additive effects on the development of peripheral neuropathy

By itself, B7-2 deficiency in NOD mice (NOD.B7-2<sup>−/−</sup> mice) protects against autoimmune diabetes but redirects the autoimmune response toward the PNS (9). Lack of B7-2 may promote neuropathy development by upregulating B7-1 expression on CD11b<sup>+</sup> and CD11c<sup>+</sup> APCs within sciatic nerves (9). Onset of spontaneous neuropathy in NOD.B7-2<sup>−/−</sup> mice occurs ~7–10 wk later than in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice. To determine how B7-2 deficiency alone impinges on Aire-mediated spontaneous neuropathy development, we crossed NOD.B7-2<sup>−/−</sup> mice with NOD.Aire<sub>GW</sub>/<sup>+</sup> mice to create NOD mice doubly deficient for Aire and B7-2. These mice developed neuropathy at an accelerated rate compared with singly Aire-deficient controls, with 100% of mice developing neuropathy by 19 wk of age (Fig. 7A, 7B). This effect was more pronounced in male mice (Fig. 7B) than in female mice (Fig. 7A), although the difference was significant in both genders. Neuropathy was associated with sciatic nerve infiltration on histological analysis (Fig. 7C, 7D). NOD.Aire<sub>GW</sub>/<sup>+</sup> mice treated with an anti–B7-2–blocking Ab (GL-1) also displayed accelerated clinical neuropathy compared with those treated with isotype control (data not shown). The additive effects of these two immune tolerance defects suggest that Aire and B7-2 may function in two distinct pathways to prevent the development of autoimmune peripheral neuropathy.

Discussion

Autoimmune peripheral neuropathy in NOD.Aire<sub>GW</sub>/<sup>+</sup> mice shares a number of features with human CIDP, including demyelination of peripheral nerves, immune infiltration consisting largely of CD4<sup>+</sup> T cells and macrophages, and the clinical manifestation of chronic, bilaterally symmetrical weakness (4). Although the exact
mechanism underlying autoimmune peripheral neuropathy is not fully understood, there is strong evidence for a critical role for T cells in disease development. Thus, understanding T cell activation and function in autoimmune peripheral neuropathy is key to understanding pathogenesis. In this study, we examine the roles of T cell inflammatory cytokine production and the B7-CD28 co-stimulatory pathway in the development of autoimmune peripheral neuropathy. We show that IFN-\(\gamma\) is indispensable for immune infiltration of peripheral nerves and clinical neuropathy development in NOD.AireGW/+ mice. Furthermore, transfer of disease by CD4+ T cells requires IFN-\(\gamma\) production by T cells. In contrast, T cell costimulation by either B7-2 alone or B7-1/B7-2 in combination has immunoregulatory effects in autoimmune peripheral neuropathy, as indicated by the acceleration of autoimmune peripheral neuropathy in NOD.AireGW/+ mice lacking these co-stimulatory signals. Thus, despite evidence that pathogenic IFN-\(\gamma\) production is controlled, in part, by B7-1/B7-2 costimulatory signaling, IFN-\(\gamma\) production and B7-CD28 costimulation have opposing effects on the development of autoimmune peripheral neuropathy.

Levels of inflammatory cytokines have been correlated with active disease in CIDP patients and animal models (32, 56). However, the precise role of individual cytokines is not clear. A critical role for Th1 cytokines was demonstrated in both induced and spontaneous mouse models by delayed onset and reduced severity of neuropathy in mice deficient for IFN-\(\gamma\)R (19), IFN-\(\gamma\) (20, 57), and TNF-\(\alpha\) (30). At the same time, other reports suggest that production of the Th17 cytokine IL-17, and not IFN-\(\gamma\), is important in disease pathogenesis. Increased IL-17 production was associated with exacerbated EAN in the setting of IFN-\(\gamma\) deficiency (22), and IL-17 is the predominant cytokine in a spontaneous ICAM-1–deficient model of neuropathy (31). Our study

**FIGURE 6.** Decreased frequency and number of Tregs with blockade of B7-1 and B7-2 in NOD.AireGW/+ mice. (A) Representative flow cytometry plots of CD4+ single-positive thymocytes and CD4+ splenocytes from anti–B7-1/B7-2 Ab–treated and isotype control Ab–treated 4-wk-old NOD.WT (WT) and NOD.AireGW/+ (AireGW/) mice stained for CD25 and intracellular Foxp3. (B) Frequency of CD4+CD25+Foxp3+ Tregs from thymocytes and splenocytes of anti–B7-1/B7-2 Ab–treated and isotype control Ab–treated WT and AireGW/+ mice (n = 3 Ab or 4 per group). Data are from two separate experiments. (C) Absolute number of CD4+CD25+Foxp3+ Tregs from thymocytes and splenocytes of anti–B7-1/B7-2 Ab–treated and isotype control Ab–treated WT and AireGW/+ mice. (D) Absolute numbers of CD4+CD25+Foxp3+ Tregs from thymocytes of untreated 8-wk-old WT (n = 6) and AireGW/+ mice (n = 6). Data are from two separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
clearly shows that IFN-γ is required for the development of autoimmune peripheral neuropathy in Aire-deficient NOD mice. The lack of neuropathy in IFN-γ–deficient mice suggests that IL-17 cannot substitute for IFN-γ in this model. Differences between our findings and other reports identifying IL-17 as a critical cytokine may lie in differences inherent to the models used in these studies.

Chemokines play an important role in immune cell trafficking, and IFN-γ induces the production of a variety of chemokines, including IP-10 (49, 58). Importantly, mice deficient in the RANTES receptor CCR5 are not protected from EAN (27), whereas IP-10–deficient mice are protected from demyelination and inflammatory cell infiltration (59). Together, these findings suggest a critical role for IP-10, and not RANTES, in this model. Differences between our IP-10 levels were significantly reduced in the sciatic nerves of IFN-γ–deficient NOD.AireGW/+ mice compared with age-matched, neuropathic NOD.AireGW/+ mice. Taken together, these data suggest that the lack of IP-10 in IFN-γ–deficient NOD.AireGW/+ mice may prevent immune infiltration of the sciatic nerves.

Absence of CD28 signaling confers protection from EAN development (14). In contrast, NOD mice deficient in B7-2 are protected from diabetes but develop spontaneous autoimmune peripheral neuropathy (9). Thus, there is evidence that B7-CD28 costimulation has both a pathogenic and protective effect in autoimmune peripheral neuropathy. In this study, we show that lack of B7-1 and B7-2 accelerates disease, indicating a protective effect for B7-CD28 costimulation signaling in NOD.AireGW/+ mice. This protective effect may be mediated by the B7-1/B7-2 signaling promotion of Treg development, because significantly fewer Tregs were observed in anti–B7-1/B7-2 Ab–treated NOD.AireGW/+ mice compared with isotype control–treated NOD.AireGW/+ mice. Moreover, the decrease in Tregs seen in NOD.AireGW/+ mice compared with NOD.WT mice suggests that the more severe pathology observed in anti–B7-1/B7-2 Ab–treated NOD.AireGW/+ mice compared with anti–B7-1/2 Ab–treated NOD.WT mice may be a result of the combined action on Tregs by costimulation blockade and Aire deficiency. In addition to its effects on Tregs, another possible mechanism by which blockade of B7-1/B7-2 signaling may accelerate neuropathy is by altering clonal deletion of autoreactive T cells in the thymus. Although some studies did not reveal a role for B7-1/B7-2 in thymic negative selection (60–62), others showed that blocking B7-1CD28 costimulation can result in the accumulation of self-reactive T cells in the thymus. Some studies have also shown that blocking B7-1CD28 costimulation can result in the accumulation of self-reactive T cells in the thymus and that release of these T cells into the periphery can contribute to autoimmune disease pathogenesis (63–67).

Multiple autoimmune diseases, including autoimmune neuropathy, diabetes, and sialitis, occur with high penetrance in NOD.AireGW/+ mice, which allow us the unique opportunity to evaluate the effects of interventions on multiple diseases in parallel. Remarkably, the development of these diseases appears to be differentially regulated by T cell IFN-γ production and costimulation blockade. Our data demonstrate that, although IFN-γ production is absolutely required for neuropathy development, it is dispensable for autoimmune diabetes and sialitis development in NOD.AireGW/+ mice. This is in line with evidence demonstrating that IFN-γ knockout (33) and IFN-γR knockout (35, 36) NOD mice are not
protected from the development of autoimmune diabetes. Additionally, our data reveal distinct effects of B7-CD28 costimulation on diabetes and neuropathy development in NOD.Aire\(^{GW/}\) mice. Although lack of B7-1/B7-2 accelerates neuropathy development in NOD.Aire\(^{GW/}\) mice, this deficiency does not accelerate diabetes development in these same mice. Together, these findings suggest that the multiple autoimmune manifestations in NOD.Aire\(^{GW/}\) mice appear to be governed by distinct effector mechanisms.

Findings from this study have important implications for the potential treatment of patients with multiorgan autoimmunity, including APS1 patients. Modulating specific aspects of T cell function may have diverse effects on each individual autoimmune disease. For example, IFN-\(\gamma\) blockade may have therapeutic efficacy in treating autoimmune peripheral neuropathy, but it is unlikely to have efficacy in other manifestations, such as sialitis. Indeed, in a case report of an APS1 patient treated with the immunosuppressant cyclosporine A, improvement in autoimmune gastrointestinal disease, keratoconjunctivitis, and alopecia universalis was seen. However, cyclosporine A treatment did not have an effect on autoimmune ovarian failure or adrenal disease (68).

Furthermore, some treatments may have opposite effects on different diseases. For instance, blockade of B7-2 in the B7-CD28 costimulatory pathway may ameliorate autoimmune diabetes, but accelerate autoimmune peripheral neuropathy development. Thus, the distinct and potentially opposing effects of T cell immunomodulatory agents on each individual affected organ need to be considered when treating patients with multiorgan autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. IFNγ deficiency does not affect autoimmune diabetes or sialitis in NOD.AireGW/+ mice. A) H/E stained sections through pancreas of IFNγ deficient NOD.AireGW/+ mice (AireGW/+ IFNγ−/− mice) versus IFNγ sufficient controls. B) Comparison of islet infiltration between IFNγ deficient NOD.AireGW/+ mice (AireGW/+ IFNγ−/− mice) versus IFNγ sufficient controls. C) Comparison of salivary infiltration scores in IFNγ deficient NOD.AireGW/+ mice (AireGW/+ IFNγ−/− mice) versus IFNγ sufficient controls.
Supplemental Figure 2. Diabetes incidence curves of NOD wildtype (Aire\textsuperscript{+/-}) or NOD.Aire\textsuperscript{GW/+} (Aire\textsuperscript{GW/+}) mice treated with a combination of anti-B7-1 and B7-2 antibodies.