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Targeting of Myelin Protein Zero in a Spontaneous Autoimmune Polyneuropathy[1]

Hye-Jung Kim,[2] Cha-Gyun Jung,[2] Mark A. Jensen, Danuta Dukala, and Betty Soliven[3]

Elimination of the costimulatory molecule B7-2 prevents autoimmune diabetes in NOD mice, but leads to the development of a spontaneous autoimmune polyneuropathy (SAP), which resembles the human disease chronic inflammatory demyelinating polyneuropathy (CIDP). In this study, we examined the immunopathogenic mechanisms in this model, including identification of SAP Ags. We found that B7-2-deficient NOD mice exhibit changes in cytokine and chemokine gene expression in spleens over time. There was an increase in IL-17 and a decrease in IL-10 transcript levels at 4 mo (preclinical phase), whereas IFN-γ expression peaked at 8 mo (clinical phase). There was also an increase in transcript levels of Th1 cytokines, CXCL10, and RANTES in sciatic nerves of mice that developed SAP. Splenocytes from SAP mice exhibited proliferative and Th1 cytokine responses to myelin P0 (180–199), but not to other P0 peptides or P2 (53–78). Adoptive transfer of P0-reactive T cells generated from SAP mice induced neuropathy in four of six NOD.SCID mice. Data from i.v. tolerance studies indicate that myelin P0 is one of the autoantigens targeted by T cells in SAP in this model. The expression of P0 by peri-islet Schwann cells provides a potential mechanism linking islet autoimmunity and inflammatory neuropathy. *The Journal of Immunology,* **2008, 181: 8753–8760.**

The NOD mouse spontaneously develops polyendocrine autoimmunity and is used as a model for type 1 diabetes, thyroiditis, and Sjögren's syndrome, as reviewed previously (1). Manifestations of autoimmunity in NOD mice are regulated by the cytokine milieu, and by costimulatory molecules such as B7-1 and B7-2. Neutralization or deficiency of B7-1 causes exacerbation of diabetes. In contrast, elimination of B7-2 leads to protection against diabetes, although peri-insulitis is present in some mice (2–4). Interestingly, B7-2 elimination triggers the development of a spontaneous autoimmune polyneuropathy (SAP)[4] that mimics human chronic inflammatory demyelinating polyneuropathy (CIDP) clinically, histologically and electrophysiologically, albeit not in all aspects (3). Compared with SAP, CIDP is more heterogenous with regard to disease onset, and the course can be relapsing-remitting or chronic progressive. Histologically, CIDP in humans is characterized by the presence of segmental demyelination in peripheral nerves and nerve roots, axonal loss of variable degree, and an immune-mediated pathophysiology (5). NOD mice can develop both type 1 diabetes and SAP, or exclusively one but not the other, thought to depend on the balance between effector T cells and regulatory T cells (Tregs) (6).

Department of Neurology, The University of Chicago, Chicago, IL 60637

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[2] H.-J.K. and C.-G.J. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Betty Soliven, Department of Neurology, MC2030, The University of Chicago; 5841 South Maryland Avenue, Chicago, IL 60637. E-mail address: bsoliven@neurology.bsd.uchicago.edu

[4] Abbreviations used in this paper: SAP, spontaneous autoimmune polyneuropathy; CIDP, chronic inflammatory demyelinating polyneuropathy; PNS, peripheral nervous system; EAN, experimental autoimmune neuritis; GFAP, glial fibrillary acid protein; KO, knockout; P0, myelin protein zero; Treg, regulatory T cell; WT, wild type.

Based on the cytokine profile, $CD4^+$ effector T cells are classified into IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, and IL-17-producing Th17 cells (7, 8). Both Th1 and Th17 cells are capable of inducing autoimmunity (9). The recruitment of effector T cells and macrophages into the target organ of autoimmunity is directed by chemokines and their receptors. For example, CXCL10 (IP-10), a chemoattractant for T cells, has been implicated in the pathogenesis of CIDP (10, 11). The goal of this study was to elucidate the molecular mechanisms underlying the pathogenesis of SAP in B7-2 knockout (KO) NOD mice, focusing on the characterization of cytokine and chemokine profile, and the identification of potential SAP Ags. Candidate SAP Ags include but are not limited to known proteins of peripheral nerve myelin such as P0, P2 that can induce experimental autoimmune neuritis (EAN), a model of human Guillain-Barré syndrome (12–15). We chose to focus on myelin protein zero (Mpz), or P0, in this study. It should be noted that $P0^{+/-}$ mice develop an inflammatory neuropathy spontaneously that is attributed to impaired central tolerance to P0 (16–18). In CIDP, proliferative responses to P0 or P2 as well as autoantibodies against P0, P2, and glycolipids have been reported in some patients (15.6–46%) but none has been shown to be highly sensitive and specific (19–21). This finding may reflect epitope spreading of variable extent or more likely the heterogeneity of this disease. Given the development of inflammatory neuropathy in NOD mice lacking B7-2, it is plausible that CIDP-like illness occurring on the background of type 1 diabetes is a unique subset characterized by autoreactivity to a specific peripheral nervous system (PNS) Ag that is shared by pancreatic islets.

Materials and Methods

Clinical and electrophysiology assessment

All animal use procedures were conducted in strict accordance to the National Institutes of Health and University of Chicago institutional guidelines. For clinical assessment, the following nominal scale was used: 0—normal; 1—flaccid tail; 2—mild paraparesis; 3—severe paraparesis; 4—tetraparesis; 5—death. Grip strength testing consisted of five separate measurements in each of two trials per session with a grip strength meter (Columbus Instruments). Results of two trials were averaged for each mouse per session. After the last grip strength measurement, electrophysiologic studies of sciatic nerves were performed as described in our previous publications (3, 22). Latency, conduction velocity, and peak-to-peak amplitudes were measured.

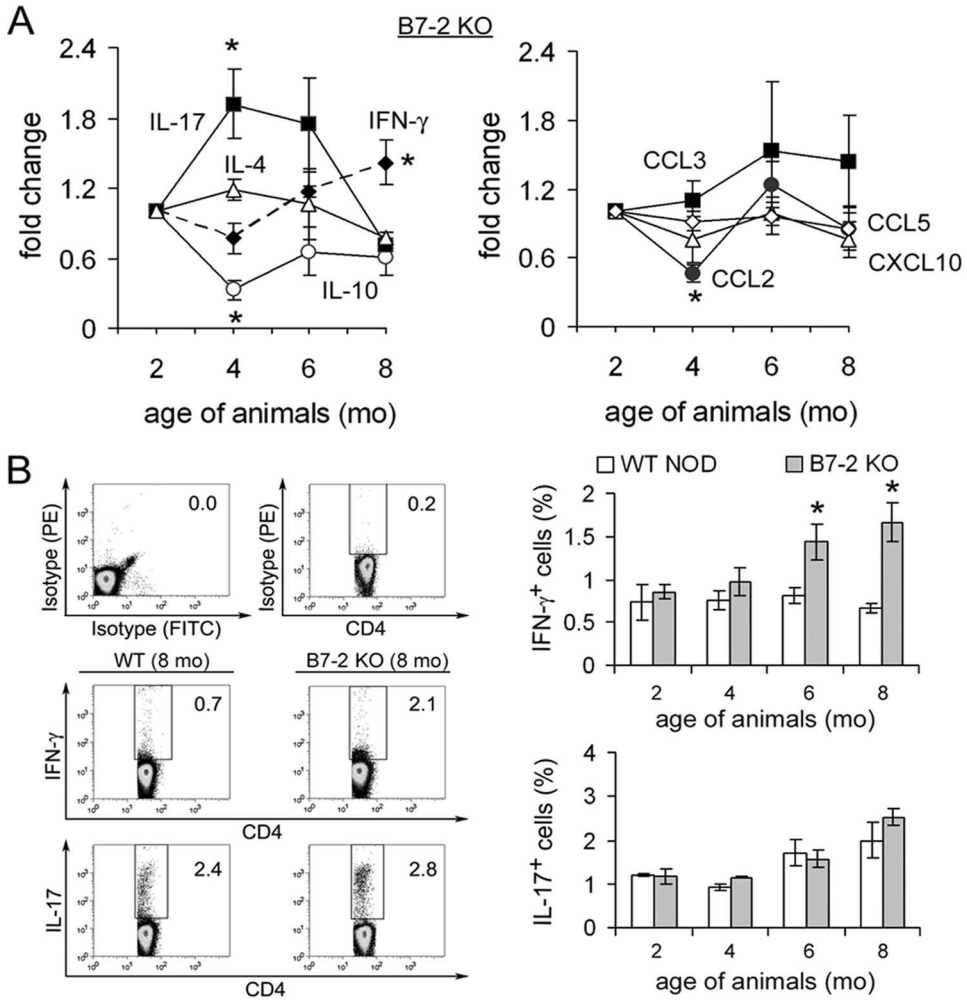

FIGURE 1. Cytokine and chemokine perturbations in the spleens of B7-2 KO NOD mice. *A*, Time course of cytokine and chemokine transcripts in the spleens of B7-2 KO NOD. At 4 mo (preclinical phase), there was an increase in IL-17, a decrease in IL-10 and no change in IFN-γ transcript levels. *, $p < 0.04$ for IL-17 vs IL-10; *, $p < 0.037$ for IL-17 vs IFN-γ; *, $p < 0.002$ for IL-10 vs IFN-γ. At 8 mo (clinical phase), there was evidence for Th1 bias. *, $p < 0.003$ for IFN-γ vs IL-17 and *, $p < 0.01$ for IFN-γ vs IL-10. There was a decrease in CCL2 transcript levels at 4 mo. *, $p < 0.004$ for CCL2 vs CCL3. Data shown represent an average from three independent experiments. The relative amount of each product was calculated by the threshold cycle method ($C_T = 2^{-\Delta\Delta CT}$). Data were normalized to that of 2-mo-old B7-2 KO NOD mice. *B*, Examples of intracellular cytokine analysis (*left*) and summary (*right*) showing an increase in IFN-γ-producing, but not in IL-17-producing $CD4^+$ T cells in spleens of 6- and 8-mo-old B7-2 KO mice when compared with age-matched WT NOD mice ($n = 3$ each). *, $p < 0.026$ in 8-mo data for $CD4^+$ IFN-γ^+ T cells and *, $p < 0.036$ for 6-mo data, B7-2 KO vs WT NOD. Splenocytes were incubated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 h.

Histologic studies of sciatic nerves

Segments of the sciatic nerves were fixed in 0.5–4% paraformaldehyde, embedded in OCT compound, and snap frozen. Longitudinal sections (10 μm slices) of sciatic nerves were stained with H&E for evaluation of mononuclear cell infiltration, and used for immunohistochemistry with rat anti-CD3 Ab (1/100; Southern Biotechnology Associates) using the peroxidase method.

Splenocyte culture, proliferation, and cytokine production assays

For proliferation assay, splenocytes were cultured at a density of 5×10^5 cells/well in HL-1 medium plus nonessential amino acids (BioWhittaker), 2 mM L-glutamine, 1 mM sodium pyruvate, 55 μM 2-ME in flat-bottom 96-well plates. Cells were then stimulated with Con A (2.5 μg/ml), PNS myelin (100 μg/ml), P0 peptide (20 μg/ml), P2 peptide (20 μg/ml), or Schwann cell lysates (100 μg/ml). On day 3, these cultures were pulsed for 16 h with 1 μCi [*methyl*-^{3}H]thymidine, and then harvested on glass fiber filter. The amount of incorporated [*methyl*-^{3}H]thymidine was measured using Beckman liquid scintillation counter. A stimulation index was defined by cpm in the presence of Ag divided by cpm in the absence of the Ag. Supernatants collected at 48 h from replicate cultures were assayed for IFN-γ (Endogen), IL-2, IL-10 (BD Biosciences), and IL-17 (eBioscience) using ELISA Minikits following the manufacturer's instructions. The binding of peroxidase-conjugated secondary Ab was detected by TMB Substrate Reagent set (BD Biosciences).

Ags used in in vitro T cells activation include P0 and P2 peptides, which were synthesized at the University of Chicago Protein-Peptide Core Facility. The following peptides were used: P0 (180–199) SSKRGRQTPVLY AMLDHSRS; P0 (41–60) PEGGRDAISIFHYAKGQPYI; P0 (106–125) IVGKTSQVTLYVFEKVPTRY; P2 (53–78) TESPFKNTEISFKLGQEFEE TTADNR; OVA (323–339) ISQAVHAAHAEINEAGR (GenScript). Myelin was prepared from mouse sciatic nerves as described by other investigators (23, 24). Schwann cell lysates were prepared by five cycles of rapid

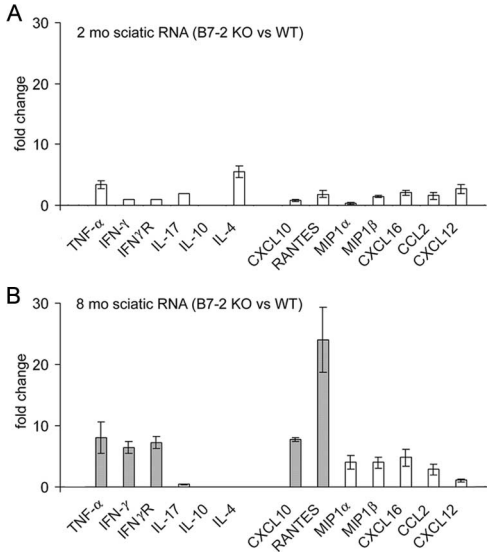

FIGURE 2. Comparison of cytokine and chemokine transcripts in sciatic nerves of B7-2 KO NOD mice vs WT NOD mice at 2 mo (*A*) and 8 mo (*B*). Data shown represent an average from three independent experiments except for 2-mo data on IFN-γ, IFN-γ receptor, and IL-17 ($n = 2$ each). There was a significant increase in the expression of TNF-α, IFN-γ, IFN-γ receptor, CXCL10, and RANTES in sciatic nerves of B7-2 KO NOD mice at 8 mo, when compared with age-matched WT NOD mice or 2-mo-old B7-2 KO NOD mice. Other chemokines were increased to a lesser extent. There was no increase in IL-17 transcripts in sciatic nerves at 8 mo. IL-10 and IL-4 transcripts were not detected.

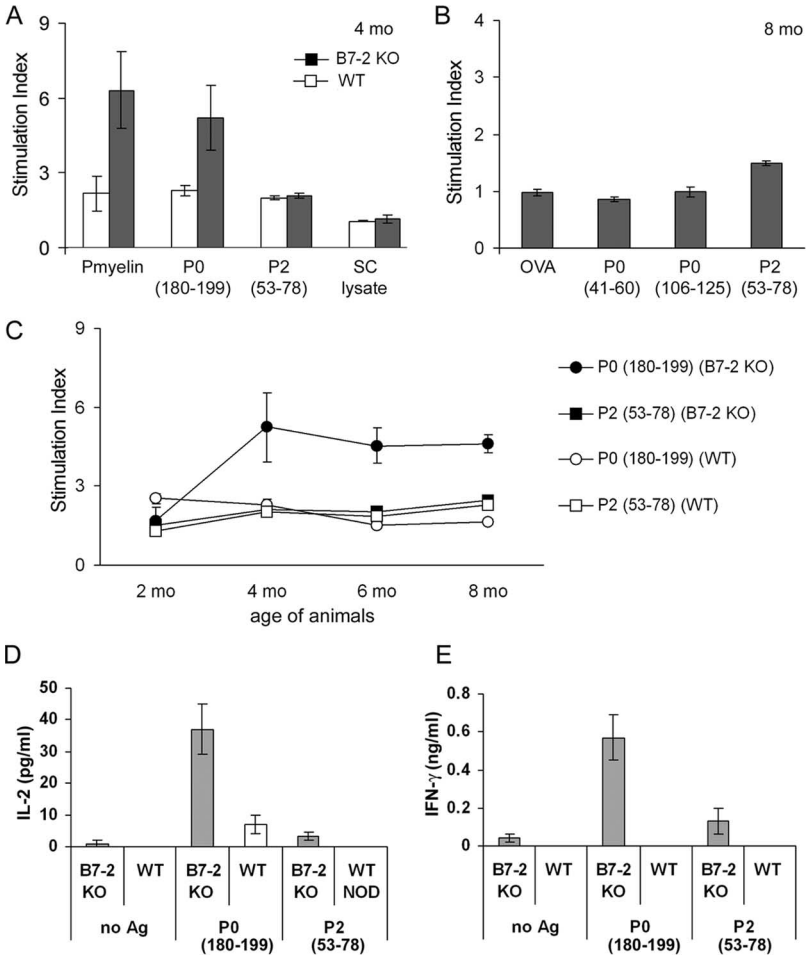

FIGURE 3. Splenocyte proliferative and cytokine responses to candidate SAP Ags. *A* and *B*, Splenocyte proliferation induced by PNS myelin (100 μg/ml), various P0 peptides (20 μg/ml), P2 (53–78) (20 μg/ml), SC lysate (100 μg/ml), and OVA (20 μg/ml). WT NOD (□) and B7-2 KO NOD (■) mice (n = 3–4 each in *A*, n = 3–5 in *B*) are shown. Results are expressed as stimulation index, with values >2.2 considered positive. Untreated at 1110–2468 cpm. *C*, Time course of splenocyte proliferative response to P0 (180–199) and P2 (53–78) in mice (n = 3–4). *D*, P0 (180–199) but not P2 (53–78) stimulates the secretion of IL-2 and IFN-γ by splenocytes from B7-2 KO mice (n = 3–4). Treatment duration was 72 h for proliferative response and 48 h for cytokine assays.

freeze (−80°C) and thaw (37°C) without detergents. Cultured neonatal rat Schwann cells were established as previously described (25).

Preparation of T cell lines and adoptive transfer studies

Lymphocytes were isolated from spleens, axillary and inguinal lymph nodes of B7-2 KO and wild-type (WT) NOD mice using the Lympholyte-M gradient (Cedarlane Laboratoruies). Purified $CD4^+$ T cells were obtained using Dynal Mouse CD4 negative isolation kit (Invitrogen). To generate P0-specific T cell lines, $CD4^+$ T cells (1.5×10^6/well) were cultured with 3×10^6 irradiated (3000 rad) syngeneic splenic APCs in the presence of P0 (180–199) (20 μg/ml) plus rIL-2 (25 U/ml) for 5 days followed by a 5-day resting period without Ag but with rIL-2 (25 U/ml). After three cycles of stimulation and rest, the specificity of the cell line was examined by proliferation assay in response to P0 (180–199) and control peptides. For adoptive transfer studies, P0-specific T cell lines were activated in vitro with P0 (180–199) for 3 days before injection of 6.5×10^6 cells into the tail vein of NOD.SCID mice. OVA-reactive T cell lines were generated from pooled splenocytes and lymph node cells of mice immunized with OVA (100 μg) in CFA by s.c. injections over four sites.

Real-time PCR

The total RNA was isolated using a TRIzol reagent (Invitrogen), followed by Qiagen column purification. Reverse transcription was performed from 1 μg total RNA and the cDNA was used for SYBR green real-time PCR. Amplification was performed with forward and reverse primers for transcripts of interest, which was designed using Primer3 software. The expression of each cytokine or chemokine gene was normalized by corresponding amount of GAPDH mRNA for each condition. The relative amounts of each product were calculated by the comparative threshold cycle method ($C_T = 2^{-\Delta\Delta C_T}$) as described for the ABI Prism 7900 Sequence Detection System (user manual no. 2; Applied Biosystems). Primer sequences for real-time PCR studies are listed in Table I (supplementary data).[5]

Intracellular cytokine analysis

Splenocytes (10×10^6/well) in 6-well plates were stimulated at 37°C in a humidified CO_2 incubator for 4 h with Leukocyte Activation Cocktail containing PMA, ionomycin, brefeldin A, and BD Golgiplug. After culture, cells (1×10^6) were stained for cell surface CD4 and intracellular IFN-γ and IL-17, using the Intracellular Cytokine Staining Starter kit (BD Pharmingen). The number of IFN-γ- and IL-17-producing $CD4^+$ T cells was analyzed by FACScan (BD Biosciences) and FlowJo software.

Western blot analysis and immunofluorescence studies

Samples (equal amount per lane) were resolved by 12% SDS-PAGE and electroblotted to PVDF membranes. Blots were blocked with 5% nonfat milk in 10 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20 for 1 h at room temperature. Blots were incubated with mouse Ab α P0 (1/3000; Astexx) or sera (1/200) overnight at 4°C, washed three times and then incubated for 1 h with goat anti-mouse IgG HRP-conjugated secondary Abs (1/5000; Calbiochem). In some experiments, isotype-specific HRP-conjugated secondary Abs (Santa Cruz Biotechnology) were used at 1/2000 to 1/10,000. Abs used for immunofluoresence studies include: mAb α P0 (1/500; Astexx); rabbit Ab α glial fibrillary acid protein (GFAP) (1/100–1/200; DakoCytomation); Alexa Fluor 488- and Alexa Fluor 598-conjugated secondary Abs (1/500; Molecular Probes).

Data analysis

Results from real-time PCR experiments, immunologic studies, grip strength measurements and electrophysiology are expressed as mean ± SEM.

[5] The online version of this article contains supplemental material.

Statistical significance was determined by ANOVA followed by Student's *t* test and the Bonferroni method for multiple group experiments. Significance levels were set at $p < 0.05$.

Results

Cytokine and chemokine profile in spleens and sciatic nerves

B7-2 KO NOD mice begin to exhibit hindlimb weakness at 6–7 mo, progressing to generalized paralysis with time (3). To examine the cytokine and chemokine profile in spleens, real-time PCR studies were performed on splenic RNA from female B7-2 KO NOD mice at age 2, 4, 6, and 8 mo ($n = 3$ mice each). Results are expressed as fold change in gene expression compared with 2-mo data (calculated by the $2^{-\Delta\Delta C_T}$ method). As shown in Fig. 1*A*, there was a decrease in IL-10 transcript levels in spleens of female B7-2 KO NOD mice starting at 4 mo of age (preclinical phase). IL-17 transcripts peaked at 4 mo but declined toward baseline at 8 mo, whereas IFN-γ transcripts peaked at 8 mo (clinical phase) (At 4 mo, $p < 0.04$ for IL-17 vs IL-10; $p < 0.037$ for IL-17 vs IFN-γ, $p < 0.002$ for IL-10 vs IFN-γ. At 8 mo, $p < 0.003$ for IFN-γ vs IL-17 and $p < 0.01$ for IFN-γ vs IL-10). With regard to chemokines, there was a decrease in CCL2 transcript levels at 4 mo ($p < 0.004$ for CCL2 vs CCL3). There was no change in the levels of TNF-α, LT-β, IL-4, CCL3, CCL5 (RANTES), CXCL10 (IP-10), CXCL16 transcripts at any time points. For clarity, only four graphs are shown for each experiment (Fig. 1*A*). To determine whether changes in IFN-γ and IL-17 transcript levels correlate with functional polarization of Th cells, we examined the percentage of IFN-γ- and IL-17-producing $CD4^+$ T cells from the spleens of these mice by flow cytometry. Upon stimulation with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 h, there was an increase in the percentage of $CD4^+$ IFN-γ^+ cells but not in $CD4^+$ IL-17^+ cells in splenocytes from B7-2 KO mice when compared with those from WT NOD mice at 6 and 8 mo (Fig. 1*B*).

For cytokine and chemokine profile in sciatic nerves, real-time PCR studies were performed focusing on two time points only (2 and 8 mo). Three independent experiments were conducted, and each sample consisted of pooled sciatic nerve RNA from three mice. TNF-α, IFN-γ, IFN-γR, CXCL10, and RANTES mRNAs were highly up-regulated in sciatic nerves of B7-2 KO mice at 8 mo compared with those from age-matched WT NOD mice, or to those from 2-mo-old B7-2 KO NOD mice (Fig. 2). A more modest increase in MIP1α, MIPβ, CXCL16, and CCL2 was also observed at 8 mo. There was no increase in IL-17 transcript levels in sciatic nerves of 8-mo-old B7-2 KO NOD mice when compared with WT NOD mice. These data suggest that Th1 cytokines, CXCL10 and RANTES are important mediators during the effector phase of SAP.

Autoreactivity to PNS Ags in SAP

We characterized the autoreactive T cell repertoire responsible for SAP by examining the proliferative response of B7-2 KO NOD splenocytes to PNS myelin (100 μg/ml), lysates of cultured neonatal Schwann cells (100 μg/ml), and two known neuritogens myelin P0 (180–199) (20 μg/ml) and P2 (57–81) (20 μg/ml). A stimulation index of >2.2 in the thymidine incorporation assay was considered positive. We observed a proliferative response to PNS myelin but not to lysates of neonatal Schwann cells (nonmyelinating), indicating that an autoantigen resides in peripheral nerve myelin or myelinating Schwann cells. Autoreactivity to P0 (180–199) was not detected at 2 mo, but was detected at 4, 6, and 8 mo (Fig. 3, *A–C*). Each data point represents the average from three to four independent experiments, each in triplicate wells. P2 (53–78), P0 (41–60), and P0 (106–125) did not induce a proliferative response ($n = 3–5$). Similarly, P0 (180–199) but not P2 (53–78) stimulates the secretion of IL-2 and IFN-γ by SAP splenocytes, as measured by ELISA ($n = 3–4$) (Fig. 3, *D* and *E*). There was no significant change in IL-10 or IL-17 secretion (data not shown). In contrast to B7-2 KO NOD mice, splenocytes from WT NOD mice exhibit minimal proliferative and cytokine responses to P0 (180–199) or P2 (53–78).

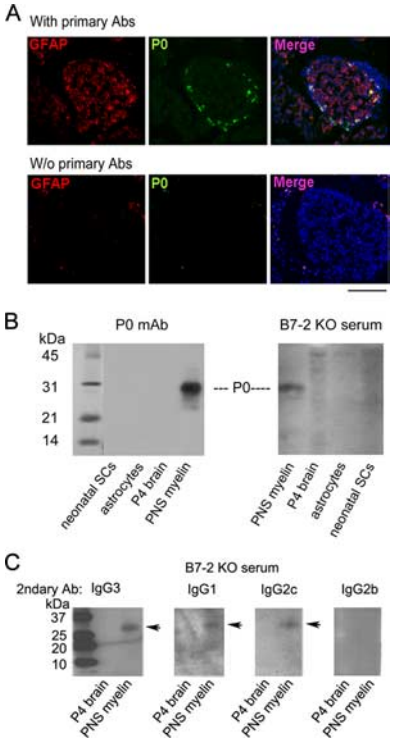

FIGURE 4. Expression of P0 by peri-islet Schwann cells and PNS, and autoantibodies to P0 in SAP sera. *A*, Immunofluorescence studies demonstrating that P0 expression was restricted to peri-islet Schwann cells, whereas GFAP was expressed by both peri-islet Schwann cells and Schwann cells infiltrating the islets. Scale bar represents 100 μm. *B*, Western blot analysis showing that the mAb α P0 used in *A* labeled a band at 28 kDa, the expected molecular mass of P0, in the lane loaded with PNS myelin. A similar band was labeled by B7-2 KO serum. Serum dilution was 1/200. *C*, Isotyping of anti-P0 Abs present in SAP sera. Serum Abs α P0 were of either Th1 or Th2 predominant IgG isotypes. Serum dilution was 1/200.

Myelin P0-reactive T cells are involved in the pathogenesis of SAP

Given that peri-islet Schwann cells, which express GFAP and S100β, are target of autoimmune attack in early insulitis (26), we examined whether these cells express P0 protein. Whereas the immunoreactivity against GFAP was observed in both peri-islet Schwann cells and Schwann cells infiltrating the islets of WT NOD mice, P0-reactive Schwann cells were restricted to the peri-islet region (Fig. 4*A*). Western blot analysis using a mAb α P0 identified a band at 28 kDa in the lane loaded with PNS myelin extract, but not in lanes loaded with lysates of cultured Schwann cells (nonmyelinating), cultured astrocytes, or postnatal day 4 brains (Fig. 4*B*). The same band was labeled by sera from B7-2 KO mice, albeit more frequently by sera obtained from 6- to 8-mo-old mice than those from 2- to 4-mo-old mice (8/8 (100%) vs 1/6 (16.7%)). Immunoreactivity was also detected occasionally by WT NOD sera (1/7 (14.3%)). Further studies revealed that serum Abs α P0 were of either Th1 or Th2-predominant IgG isotypes, with the frequency of IgG3 > IgG1 > IgG2c > IgG2b (Fig. 4*C*). It should be noted that IgG2a gene is deleted in NOD mice (27).

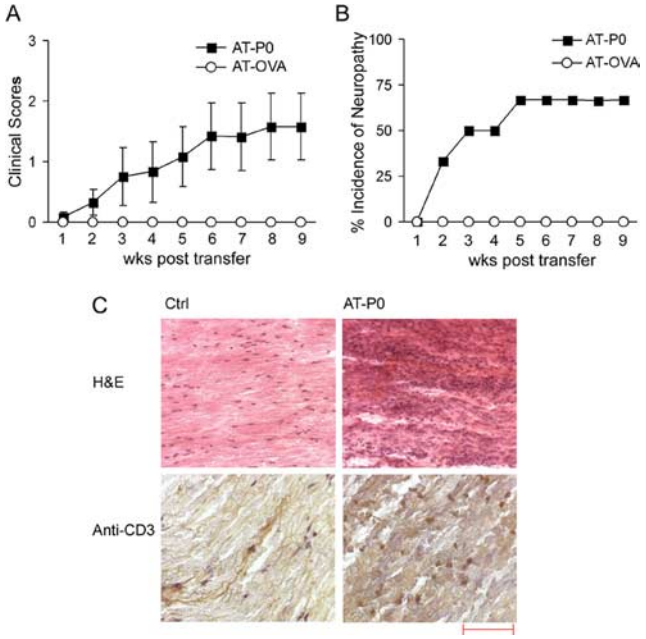

FIGURE 5. Adoptive transfer (AT) of P0 (180–199)-specific and OVA-reactive $CD4^+$ T cell lines into 8- to 10-wk-old female NOD.SCID mice. *A*, Summary of clinical scores ($n = 6$ for AT-P0; $n = 3$ for AT-OVA). P0-specific T cell lines were generated from SAP mice, whereas OVA-specific T cells lines were generated from WT NOD mice immunized with OVA. *B*, Incidence of neuropathy in AT-P0 mice and AT-OVA mice. *C*, Histologic examples of sciatic nerve sections showing T cell infiltrates ($CD3^+$ cells) in nerves from AT-P0 mice. Hematoxylin was used for counterstaining in immunohistochemical studies. Scale bar represents 100 μm for H&E and 50 μm for anti-CD3.

$CD4^+$ T cells but not sera from SAP mice are sufficient to transfer disease to NOD.SCID mice (3). To further examine whether P0-reactive T cells are pathogenic in SAP, we generated P0-reactive T cell lines from pooled splenocytes and lymph node cells isolated from inguinal and axillary lymph nodes of SAP mice (7- to 8-mo-old). These cell lines secrete greater amounts of IFN-γ (14.7–15.4 ng/ml) than IL-17 (0.5–0.67 ng/ml) upon exposure to P0 (180–199), but only IFN-γ secretion was inhibited by Ab α MHC class II (I-A/I-E) (10 μg/ml) (data not shown). Adoptive transfer of 6.5×10^6 P0-reactive $CD4^+$ T cell lines (AT-P0) led to the development of peripheral neuropathy in four of six (66.7%) NOD.SCID mice, as shown in Fig. 5, *A* and *B*. By comparison, transfer of purified $CD4^+$ T cells from WT NOD mice or OVA-reactive T cell lines (AT-OVA) did not induce neuropathy ($n = 3$ each). Because we were not able to generate OVA-specific T cell lines from SAP mice, these cell lines were derived from WT NOD mice immunized with OVA. Results were confirmed with grip strength measurements at peak disease or at the end of 9 wk. Forelimb grip strength (in Newtons) was 2.37 ± 0.26 and 3.2 ± 0.01 in AT-P0 and AT-OVA mice, respectively ($p < 0.01$), whereas hindlimb grip strength was 0.38 ± 0.11, and 1.02 ± 0.01, respectively ($p < 0.0003$) (data not shown). Histologic studies revealed the presence of T cell infiltrates in sciatic nerves of symptomatic AT-P0 mice (Fig. 5*C*).

Lastly, we investigated whether SAP can be prevented in B7-2 KO NOD mice by a single i.v. injection of 200 μg P0 (180–199) at 2–4 mo of age. As depicted in Fig. 6, *A* and *B*, a very mild neuropathy was observed at 8 mo in only two of nine (22.2%) B7-2 KO mice injected with P0 (180–199), as compared with a more severe neuropathy in 10/10 (100%) mice receiving PBS, and in 5/6 (83.3%) animals given other peptides (OVA (323–339) or P2 (53–78)). In P0-injected mice, there was no decline in grip strength over time, in contrast to grip strength measurements in other groups of animals (Fig. 6*C*). Furthermore, sciatic nerve conduction studies revealed a significant difference in distal latency, conduction velocity and amplitude of the motor response in P0-injected mice compared with those from other groups of animals (Fig. 6*D*). No improvement in clinical severity was observed when P0 (180–199) was given at 8 mo (after disease onset) ($n = 2$, data not shown). Immunologic studies done at 8 mo revealed that there was a significant attenuation of the proliferative response to P0 (180–199), and a decline in IL-2 and IFN-γ secretion in splenocytes from animals injected with P0 at 2–4 mo. No significant effect on IL-10 or IL-17 levels was noted (Fig. 7). Taken together, these

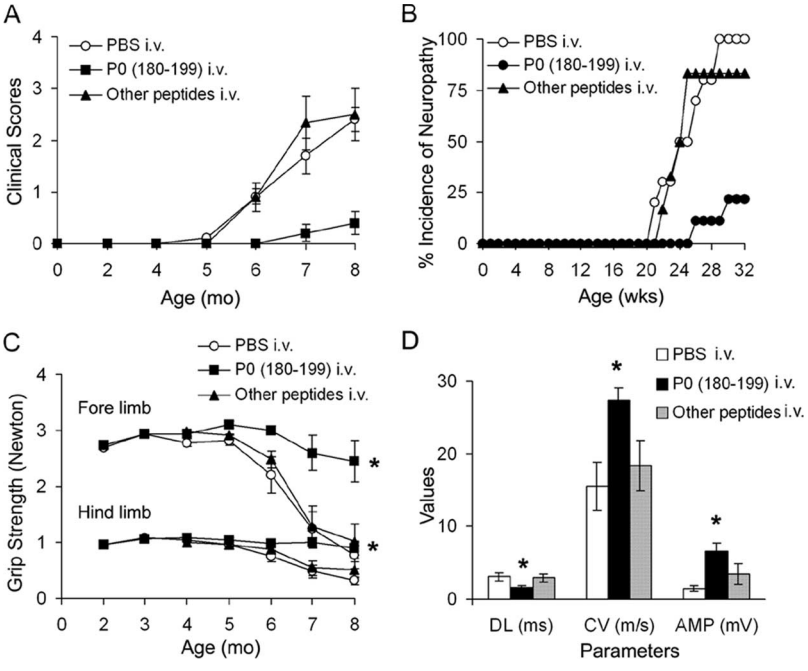

FIGURE 6. Prevention of SAP by a single i.v. injection of P0 (180–199), but not by injection of PBS or other peptides in B7-2 KO NOD mice at 2 or 4 mo. *A*, Clinical scores. Three groups of animals were compared ($n = 9$ for P0 (180–199), $n = 10$ for PBS, $n = 3$ each for other peptides including P2 (53–78) and OVA (323–339)). $p < 0.0001$ for P0 (180–199) vs PBS or other peptides at 8 mo. Peptide dose injected was 200 μg. *B*, Incidence of neuropathy in these three groups of mice. *C*, Grip strength measurements. $*$, $p < 0.0006$ for P0 (180–199) vs PBS and other peptides in forelimb at 8 mo, and $*$, $p < 0.0001$ in hindlimb grip strength. *D*, Sciatic nerve electrophysiology at 8 mo. Distal latency (DL), conduction velocity (CV), and amplitude (AMP) of sciatic motor responses were measured. $*$, $p < 0.009$ in distal latency measure for P0 (180–199) vs PBS or other peptides; $*$, $p < 0.003$ in conduction velocity; $*$, $p < 0.001$ in amplitude measure.

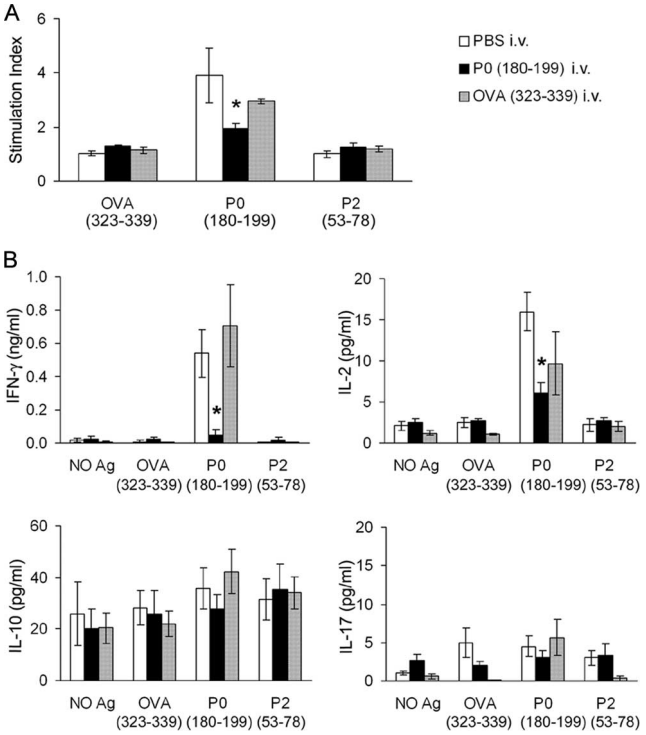

FIGURE 7. Suppression of splenocyte proliferation and Th1 cytokine secretion in B7-2 KO NOD mice given i.v. injection of P0 (180–199) at 2 or 4 mo. Three groups of mice were sacrificed at 8 mo (PBS group, $n = 4$; P0 group, $n = 6$; OVA group, $n = 3$). *A*, Proliferative responses of splenocytes to 20 μg/ml OVA (323–339), P0 (180–199), and P2 (53–78). Treatment duration was 72 h. The proliferative response to P0 (180–199) was decreased in splenocytes from P0-tolerized animals. *, $p < 0.004$ for P0 vs PBS group, and *, $p < 0.008$ for P0 vs OVA group). *B*, Cytokine responses of splenocytes to the same Ags in replicate cultures. Treatment duration was 48 h. *, $p < 0.03$ for P0 vs PBS group and $p < 0.003$ for P0 vs OVA group for IFN-γ; *, $p < 0.002$ for P0 vs PBS group for IL-2, but $p > 0.05$ for P0 vs OVA group. There was no significant difference in IL-10 and IL-17 production in response to P0 (180–199) among three groups of animals.

results indicate that myelin P0 (180–199) is one of the autoantigens involved in the development of SAP.

Discussion

NOD mice exhibit a number of immune system defects that contribute to their susceptibility to develop multiorgan autoimmunity (1). Similar to other models of autoimmunity, functional polarization of Th subsets is one of the crucial factors in the pathogenesis of type 1 diabetes. Th1 effector response (high IFN-γ to IL-4 ratio) is associated with destructive insulitis, although a dual pathogenic and protective role for IFN-γ has been demonstrated by other investigators (28–31). Recently, evidence has been introduced to indicate that IL-17 may contribute to the development of type 1 diabetes (31, 32).

We observed changes in cytokine and chemokine microenvironment in the spleens of B7-2 KO NOD mice, with IL-17 and IFN-γ predominating during the preclinical phase and clinical phase, respectively. That IFN-γ is a key effector mediating nerve damage in SAP mice is supported by the following: 1) IFN-γ and not IL-17 transcripts predominate in sciatic nerves of symptomatic mice, 2) splenocytes from symptomatic mice produce IFN-γ and not IL-17 upon stimulation with P0 (180–199); and 3) there was an increase in IFN-γ-producing, but not in IL-17-producing $CD4^+$ T cells in spleens of SAP mice at 6–8 mo when compared with age-matched WT NOD mice. Furthermore, disease severity in both SAP and EAN is attenuated in mice deficient in IFN-γ or its receptor, respectively (33, 34). Although there is evidence supporting a Th1 bias in SAP, the role of Th17 cells and the source of increased IL-17 transcripts in spleens of 4–6 mo old B7-2 KO mice remain to be clarified. Both Th1 and Th17 cells have been implicated in experimental autoimmune encephalomyelitis, though recent data indicate that they induce distinct types of CNS inflammatory disease characterized by macrophage-rich infiltrates and neutrophil-rich infiltrates, respectively (8, 35–37). It has also been reported that increased Th17 to Th1 ratio is more critical in the induction of CNS inflammation than in spinal cord inflammation (38).

The decline in IL-10 expression in spleens and its absence in sciatic nerves may contribute to disease development and progression in SAP. IL-10 expression correlates with spontaneous recovery in EAN, and exogenous IL-10 suppresses EAN by down-regulating Th1 responses (39, 40). The role of TNF-α in inflammatory neuropathies is more complex, as both anti-inflammatory and proinflammatory actions of TNF-α have been reported (41, 42). With regards to chemokines, the transient dip in CCL2 transcript levels in spleens is interesting. CCL2 KO mice have diminished Th2 cytokines supporting a role for CCL2 in the regulation of Th2 polarization, in addition to its role as a monocyte chemoattractant (43). Cytokines and chemokines that are up-regulated in sciatic nerves of SAP mice are the same ones found in EAN and CIDP nerves with prominent increase in TNF-α, IFN-γ, CXCL10, and RANTES expression (10, 44–46).

Aside from cytokine perturbations, another crucial determinant in the development of autoimmune diseases is the delicate balance between autoreactive T cells and Tregs, which is controlled by B7-2 and other costimulatory molecules (4, 6, 47). Though both B7-1 and B7-2 bind to their receptors CD28 and CTLA-4, signaling through CD28 promotes T cell activation, whereas signaling through CTLA-4 down-regulates T cell responses (48, 49). $CD28^{-/-}$ C57BL/6 mice are resistant to EAN induction and CTLA-4 blockade enhances the severity of EAN (50, 51). The requirement for B7-1 vs B7-2 in autoimmunity is more complex and is influenced by genetic background, and the target organ involved. Absence of either B7-1 or B7-2 leads to attenuation of experimental autoimmune encephalomyelitis induced in NOD mice but not in C57BL/6 mice (52, 53). B7-2 elimination triggers the development of SAP in NOD mice but not in C57BL/6 mice. In SAP nerves, high levels of B7-1 expression were observed in infiltrating $CD11b^+$ and $CD11c^+$ cells (3). Similarly, there is a preferential up-regulation of B7-1 in CIDP nerves (54). Yet, neuropathy is accelerated by treatment with anti-B7-1 Ab in B7-2 KO NOD mice (6, 33). Collectively, these findings suggest a dominant role of B7-1 in the development of autoimmune neuropathies, but its requirement can be bypassed in the absence of functional Treg compartment. It is possible that other costimulatory molecules also play a role in inflammatory neuropathies. ICOS and its unique ligand (ICOS-L) mRNA are up-regulated in infiltrating T cells and macrophages, respectively, in nerve samples from CIDP and other inflammatory neuropathies (55). Other investigators found up-regulation of a costimulatory molecule in Schwann cells of CIDP nerves that was detected by anti-BB-1 Ab, but not by anti-B7-1 or anti-B7-2 Abs (56).

It is intriguing that elimination of a costimulatory molecule such as B7-2 would shift the autoimmunity from pancreatic islets to peripheral nervous tissue. Due to a generalized defect in central tolerance induction, NOD mice contain a diverse repertoire of T cells reactive against multiple tissue Ags including several nervous system Ags such as myelin basic protein and GFAP (26, 57). Yet, B7-2 KO NOD mice develop neuropathy spontaneously but not encephalomyelitis. Perhaps, the above dichotomy is partially due

to a lesser ability of the blood nerve barrier than the blood brain barrier to impede lymphocytes access to the tissues. A second explanation could be derived from our data. Splenocyte proliferative responses indicate that an autoantigen resides in peripheral myelin or myelinating Schwann cells. Further studies revealed that P0 (180–199) but not P0 (106–125) or P0 (41–60) elicited splenocyte proliferative and Th1 cytokine responses. Two P0 peptides used in the current study, P0 (180–199) and P0 (106–125), have been used to induce EAN in resistant C57BL/6 mice (34, 58, 59). Mice are generally more resistant to EAN induction than Lewis rats. The third peptide, P0 (41–60), has been reported to elicit a strong immune response in P0 KO mice but not in WT mice immunized with peripheral myelin (60). Interestingly, P0 is expressed by peri-islet Schwann cells, which would not be expected of typical nonmyelinating Schwann cells with rare exceptions such as perisynaptic Schwann cells (61). These findings imply that the initiation and amplification of autoreactivity to P0 can occur either in pancreatic islets or in peripheral nerves, and taken together with the imperfect blood nerve barrier, would account for the propensity of NOD mice to develop SAP rather than encephalomyelitis.

We found that P0 (180–199) reactive T cells are pathogenic, based on our data from adoptive transfer and i.v. P0 experiments. The i.v. injection of soluble proteins such as myelin basic protein induces tolerance in experimental autoimmune encephalomyelitis by clonal deletion, anergy or induction of Th2 Tregs depending on the timing of administration (62–64). The effect of soluble Ag treatment on effector T cell mobility and cytokine up-regulation can be extremely rapid (within minutes) associated with trapping of these cells within lymphoid organs and later activation-induced cell death (65). In our study, a single i.v. injection of P0 peptide at 2 or 4 mo was sufficient to prevent SAP, which was associated with decreased activation of Th1 cells in response to P0 but was not accompanied by a shift to Th2 polarization. That myelin P0 is one of the SAP Ags has been demonstrated recently by Bour-Jourdan and colleagues using a different approach. Oligoclonal Ab responses to a 30-kDa protein were detected in accelerated models of autoimmune neuropathy, and the targeted protein was subsequently identified as myelin P0 by mass spectrometry (66). We found that Ab responses to P0 are more frequent in 6- to 8-mo-old than in younger B7-2 KO NOD mice or WT NOD mice. Further studies are required to determine whether P0 Abs contribute to the pathogenesis of SAP, or simply act as markers of peripheral nerve damage.

That a CIDP-like illness can develop on a background of diabetes is interesting, given similar observations in humans; however, not all patients reported in the literature had type 1 diabetes (67–70). There are other findings demonstrating a link between islet and nervous system autoimmunity, or supporting a role of neurons and glial cells in type 1 diabetes. Islet inflammation and insulin resistance are controlled by $TRPV^+$ PNS (71). Furthermore, GFAP-reactive T cell lines can transfer insulitis to NOD. SCID mice (26). It would be interesting to determine whether induction of tolerance to P0 would lead to suppression of islet autoimmunity in future studies. It is also recognized that some islet autoantigens are constituents of the nervous system such as glutamic acid decarboxylases GAD65 and GAD67; Ab α glutamic acid decarboxylase is associated with specific neurologic syndromes such as stiff person syndrome (72).

In summary, we found that 1) elimination of B7-2 leads to altered cytokine and chemokine milieu in lymphoid organs; 2) SAP is primarily a Th1-mediated disease; the cytokine and chemokine profile mimics that observed in CIDP nerves; and 3) myelin P0 is one of the autoantigens in SAP. These findings do not exclude the possibility of intramolecular spreading involving other P0 epitopes, intermolecular spreading to other PNS Ags, or a possible role for autoantibodies/B cells in the progression of SAP. The expression of P0 by peri-islet Schwann cells provides a potential mechanism linking islet autoimmunity and inflammatory neuropathy.

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Disclosures

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