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Thymic Epithelium Determines a Spontaneous Chronic Neuritis in Icam1\textsuperscript{tm1Jcgr} NOD Mice

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The NOD mouse strain spontaneously develops autoimmune diabetes. A deficiency in costimulatory molecules, such as B7-2, on the NOD genetic background prevents diabetes but instead triggers an inflammatory peripheral neuropathy. This constitutes a shift in the target of autoimmunity, but the underlying mechanism remains unknown. In this study, we demonstrate that NOD mice deficient for isoforms of ICAM-1, which comediately costimulatory functions, spontaneously develop a chronic autoimmune peripheral neuritis instead of diabetes. The disease is transferred by CD4\textsuperscript{T} T cells, which infiltrate peripheral nerves together with macrophages and B cells and are autoreactive against peripheral myelin protein zero. These Icam1\textsuperscript{tm1Jcgr} NOD mice exhibit unaltered numbers of regulatory T cells, but increased IL-17–producing T cells, which determine the severity, but not the target specificity of autoimmunity. Ab-mediated ICAM-1 blockade triggers neuritis only in young NOD mice. Thymic epithelium from Icam1\textsuperscript{tm1Jcgr} NOD mice features an altered expression of costimulatory molecules and induces neuritis and myelin autoreactivity after transplantation into nude mice in vivo. Icam1\textsuperscript{tm1Jcgr} NOD mice exhibit a specifically altered TCR repertoire. Our findings introduce a novel animal model of chronic inflammatory neuropathies and indicate that altered expression of ICAM-1 on thymic epithelium shifts autoimmunity specifically toward peripheral nerves. This improves our understanding of autoimmunity in the peripheral nervous system with potential relevance for human diseases. The Journal of Immunology, 2014, 193: 2678–2690.

The NOD mouse strain is prone to develop various autoimmune manifestations (1). Most prominently, NOD mice spontaneously develop autoimmune destruction of the insulin-producing β cells of the endocrine pancreatic islets and, thus, serve as a well-established animal model of human type 1 diabetes (2). Costimulatory molecules are known to modulate autoimmunity in NOD mice.

Deficiency of both B7-1/B7-2 costimulatory molecules in NOD mice exacerbates diabetes (3), whereas, surprisingly, B7-2 single-deficient NOD mice are protected from diabetes but develop an inflammatory neuropathy (4). IFN-γ was identified as a dominant mediator of this spontaneous neuritis (5), and myelin protein zero (MPZ) is an important antigenic target (6, 7). Subsequent studies reported that the absence of PD-1 in NOD mice carrying the antiabetogenetic H-2b haplotype triggered inflammation in peripheral nerves, stomach, and exocrine tissues (8). NOD mice deficient in ICOS ligand (ICOS-L) develop spontaneous inflammation of muscle, sensory nerve fibers, and the CNS (9). Thus, autoimmunity of the peripheral nervous system (PNS) is a common phenotype in NOD mice with alterations in costimulatory molecules. Although the determinants of autoimmune susceptibility in NOD mice have been studied extensively, the mechanisms by which costimulatory molecules determine the target specificity of spontaneous autoimmunity in NOD mice remain unknown. Thymic-selection mechanisms may contribute to the observed deviation of autoimmune targets in the absence of costimulation in NOD mice.

ICAM-1 is expressed by vascular endothelium and APCs, and it participates in leukocyte adhesion (10) and costimulatory function (11). Three mouse strains carrying a targeted mutation of the ICAM-1 locus have been developed: Icam1\textsuperscript{tm1Bay} (12), Icam1\textsuperscript{tm1Lcr} (13), and Icam1\textsuperscript{tm1Alb} (14). Both Icam1\textsuperscript{tm1Bay} and Icam1\textsuperscript{tm1Lcr} mice show residual expression of alternative ICAM-1 splice isoforms (15, 16), whereas only the targeting strategy of the Icam1\textsuperscript{tm1Alb} strain generates a true ICAM-1–null allele (14). Despite these limitations, Icam1\textsuperscript{tm1Bay} and Icam1\textsuperscript{tm1Lcr} mice have been widely used to study the function of ICAM-1. We previously demonstrated that the Icam1\textsuperscript{tm1Lcr} allele on the NOD genetic background abrogates autoimmune diabetes (17). We report in this article that, instead of developing diabetes, these Icam1\textsuperscript{tm1Lcr} NOD mice develop a chronic inflammatory demyelinating neuropathy that targets MPZ. The disease is mediated by CD4\textsuperscript{T} cells whose IL-17 production determines the severity, but not the target specificity, of the neuritis. Instead, we identify that thymic epithelial cells from Icam1\textsuperscript{tm1Lcr} NOD mice are sufficient to trigger peripheral nerve myelin autoreactivity and spontaneous neuritis in nude mice.

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first time that costimulatory signals expressed by thymic epithelium determine the target specificity of autoimmunity in NOD mice, in part by generating a neurotrophic, instead of a diabetogenic, T cell repertoire.

Materials and Methods

Animals, phenotyping, and treatment

Icam1tm1Jcgr mice on the C57BL/6 background (13) had been backcrossed to NOD/Bom background (MHC haplotype H-2q7; Bomholt Breeding Centre, Ry, Denmark) for eight generations, as previously described (17), and these homozygous Icam1tm1Jcgr/NOD mice were subsequently inbred. Homozygosity was confirmed by routine PCR from tail biopsies, as previously described (13). Young prediabetic NOD/Bom mice were used as controls. All animals were maintained under specific pathogen-free conditions at the German Diabetes Center. SCID mice on the NOD background (NOD-SCID) and conventional SCID mice were purchased from Tac onc. Nude mice (BALB/cByJ-HiH1nu) aged 6–8 wk were purchased from The Jackson Laboratory. All mice were checked weekly for glucosuria (Combur10; Roche) and analyzed for clinical signs of neuropathy using a modified clinical score (18): 0, no impairments; 1, reduced tone of the tail; 2, limp tail; 3, absent righting reflex; 4, gait ataxia; 5, mild paraparesis; 6, moderate paraparesis or paraplegia; 7, severe paraparesis or paraplegia; 8, tetraparesis; 9, moribund; and 10, death due to neuropathy. Age at onset of neuropathy (score > 2) was recorded and nonaffected (score < 1) and clinically affected (score ≥ 5) animals were selected for subsequent analyses. Intermediate animals (score 1–4) were excluded. Animals reaching a score of 7 and diabetic animals were sacrificed for ethical reasons. Mice were placed on a round horizontal bar (diameter 20 mm; bar-test), and their average holding time (maximum 60 s) in three independent trials was recorded. All phenotyping was performed in a blinded fashion. Animal experimentation was approved by the responsible state authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) under the approval reference number 8.87-50.10.34.0833.

Anti–ICAM-1 treatment

In contrast to all other experiments, anti–ICAM-1 treatment was performed in conventionally housed NOD/MtTac mice (Tac onc) to provide sufficiently large cohorts of equal age. Animals either were aged 4 wk at purchase (n = 20) or derived from purchased pregnant mothers (female offspring; n = 33). Animals were treated for 7 wk—from 5 wk of age (adult) or from 10 d of age (juvenile)—with i.p. injections (100 ng/kg) of rat anti–mouse ICAM-1 Ab (clone 1N1/1.7.4, rat IgG2b; low endotoxin azide-free; BioLegend) or saline only. The anti–ICAM-1 Ab amount was adjusted to the animals’ body weight for each injection (2.5 μg/g body weight), and treatment was given three times/week in the first 3 wk and once a week in the subsequent 4 wk. Phenotyping, glucosuria checks, and sacrificing were performed weekly, as described above, for 35 wk. Nerve electrophysiology and histology were performed when the experiment was terminated in the respective animal.

Adaptive-transfer experiments

All transfer experiments were performed by i.v. injections. Initially, splenocytes from prediabetic NOD mice (n = 5) were transferred into NOD-SCID mice. In three independent experiments, nonskewed cells were adaptively transferred from 1-μg-old, clinically affected Icam1tm1Jcgr/NOD mice (n = 5 [experiment and group] into different recipient strains. Total splenocytes were transferred in all three experiments (n = 15). CD4+–enriched (n = 10) or CD4+–depleted cells were maintained under specific pathogen-free conditions at the German Diabetes Center. CD4+–enriched T cells were generated by MACS and were transferred in two separate experiments. Positive CD4+ sorting (clone L3T4) and indirect non–CD4+ cell depletion (unlabeled CD4+ T Cell Isolation Kit; both from Miltenyi Biotec) were used in one experiment. Total splenocytes and CD4+–enriched T cells were maintained in culture with the presence of Abs against CD3 (1 μg/ml, 145-2C11) and CD28 (1 μg/ml, 37.51; both from BD Biosciences), as previously described (14). CD4+–depleted cells were maintained in the presence of CD3 and CD28 Abs and IL-2 (20 U/ml; R&D Systems). After 48 h in culture, cells were washed three times with PBS, counted, and injected i.v. in 200 μl into 6–8-wk-old SCID or NOD-SCID immunodeficient hosts. A total of 9–20 × 106 unselected splenocytes, 4–8 × 106 CD4+ T cells, or 7–16 × 106 CD4+–depleted T cells was injected per animal.

For the transfer of cytokine-skewed cells, CD4+ T cells were MACS sorted from affected NOD mice and were transferred into NOD or NOD-SCID animals. CD3 and CD28 Abs (1 μg/ml each) together with either TGF-B1 (2 ng/ml), IL-6 (20 ng/ml), and IL-23 (20 ng/ml; Th17) or IL-12 (40 ng/ml; Th1) (all from R&D Systems) or without further cytokines for 4 d in 35-mm bacterial-grade petri dishes at 2 × 106/ml. Before transfer, intracellular cytokine staining and flow cytometry were performed as described above. Cell numbers were adjusted and, after washing three times, 5–6 × 106 cells were transferred to recipient NOD-SCID animals (n = 4 per Icam1tm1Jcgr/NOD group, n = 3 per NOD group). When terminating the experiments, lymphocytes were analyzed for intracellular cytokine production, and sciatric nerve histology was performed. Two independent experiments (n = 21 total recipients/experiment) were performed. Recipient mice in all transfer experiments were maintained under specific pathogen-free conditions for up to 21 wk and were analyzed for phenotypic signs of neuropathy and glucosuria twice a week in a blinded fashion.

Nerve-conduction studies

Mouse sciatric nerve conduction was measured as previously described (19). Briefly, mice were anesthetized using an i.p. injection of ketamine (100 mg/kg) and xylazine (5 mg/kg), while constant body temperature was maintained using a heating plate connected to a rectal temperature sensor (CMA). Two recording electrodes were inserted into the small foot muscles to assess motor response. Two monopolar stimulating electrodes were placed dorsal of the ankle and at the sciatric notch enclosing the sciatic nerve for distal and proximal stimulation, respectively. Stimulation was performed with increasing current until supramaximal stimulation was achieved. Maximum compound muscle action potential voltage (mV) was recorded. Nerve conduction velocity (NCV; m/s) was calculated from differences between distance and motor latency differences between proximal and distal stimulation. Average values were calculated from two independent recordings/animal.

Histology

Animals were sacrificed by cervical dislocation and immediately intracardially perfused with PBS, followed by 4% paraformaldehyde. Tissue samples from brain, spinal cord, spinal nerve, and brachial plexus were embedded in paraffin, cut into 5-μm sections on a standard microtome, and stained with H&E following standard protocols. For fluorescent staining, sections were incubated with anti–mouse primary Abs (CD3 [ab5690] Abcam; CD68 [FA-11] AbD Serotec; n = 30, 1:20; [RA3-8B5] in-house; S100 [Dako]; clone names in brackets) and corresponding FITC- or PE-labeled goat anti-rabbit secondary Abs (Invitrogen), as previously described (20). Sections were incubated with DAPI for 5 s, mounted in 80% glycerol in PBS, and photodocumented on the subsequent day. For plastic resin embedding, sciatric nerves were dissected from perfused animals, postfixed in 4% paraformaldehyde and 2.5% glutaraldehyde for ≥24 h (21), and embedded in epoxy resin, as described (22). Semi-thin sections (0.5 μm) were cut using an ultramicrotome (Leica Ultracut S) and stained with toluidine blue.

For the detection of autoantibodies, sciatric nerves were dissected from NOD-SCID mice, frozen in mounting medium (Tissue-Tek), and cut into 7-μm sections using a cryostat (Leica CM3050S). Sections were fixed with acetone (−20°C, 10 min), blocked with 5% normal horse serum (1 h), and incubated with NOD and Icam1tm1Jcgr/NOD mouse serum dilutions (1:200 in PBS, 1 h), followed by washing and incubation with biotinylated horse anti-mouse IgG (H+L) secondary Ab (1:200), hematoxylin counterstain, and DAB-based visualization. For fluorescent staining, sections were fixed using methanol (25°C, 10 min), blocked with normal goat serum, and incubated with a Alexa Fluor 594–labeled goat anti-mouse IgG secondary Ab (1:200, 1 h; Invitrogen) and DAPI (1.5 μg/ml). Photo documentation was done using an Axioskop 2 microscope (Zeiss). All cryosection-related reagents were purchased from Vector Labs.

Intracellular cytokine staining and ELISA

Splenocytes were extracted by passing tissue through a 40-μm cell strainer, followed by ammonium chloride–based erythrocyte lysis (BD Biosciences). Intracellular cytokine profiles were assessed using intracellular cytokine staining buffers (BD Biosciences), following the manufacturer’s protocol. Briefly, splenocytes (1 × 106/well) in six-well plates were stimulated at 37°C in a humidified 10% CO2 incubator for 4 h with PMA (10 ng/ml), ionomycin (1 μg/ml), and BD GolgiStop. Cells were subse- quently stained for cell surface CD3 and CD4 and intracellular IFN-γ (XMG1.2), IL-17 (TC11-18H10), and IL-10 (JES-519F1) (all from BD Biosciences; clone names in parentheses). Splenocytes were stained for cell surface CD4 and CD25, as well as intracellular Foxp3 (MF23) using the Foxp3 Buffer Set (all from BD Biosciences). Flow cytometry was performed using a FACSCan and II flow cytometer. For assessing cytokine production, splenocytes were cultured for 72 h in 96-well flat-bottom plates in the presence of soluble anti-CD3 Ab (1 μg/ml), and concentrations of IFN-γ, IL-2, and IL-17 were measured in the culture supernatants using colorimetric sandwich ELISA (R&D Systems), following the manufacturer’s protocol.
**Proliferation assays**

Sciatic nerves, forebrain, pancreas of NOD mice, and nonmyelinating mouse Schwann cells, cultured as described (23), were homogenized in PBS with an ULTRA-TURRAX. The homogenate were centrifuged, and the remaining tissue pellet was discarded. Sciatic nerve myelin fractions of C57BL/6 mice were purified by gradient centrifugation, as previously described (24). Tissue and cell homogenates were adjusted to equal total protein content by BCA protein assay (Thermo Fischer). Dilutions of tissue samples and of peptides of MPZ (PO06-125, PO180-196; JPT Peptide Technologies), myelin protein 2 (P235-786; JPT Peptide Technologies), and the recombinant proteins Hsp60 (BIOTREND) and GAD65 (Diamyd Diagnostics) were added to spleenocyte cultures. The final concentration of tissue samples ranged from 1–50 mg/ml and the final concentration of recombinant proteins and peptides ranged from 10–40 mg/ml as indicated in the figure legends. Optimal proliferation was observed at 6.25 and 40 µg/ml peptide content, and these concentrations were used in subsequent experiments. A total of 1.5 × 10^5 responder spleenocytes from NOD and Icam1^tm1Jcgr^NOD mice and 1 × 10^5 irradiated (10 Gy) NOD splenocytes were maintained in 96-well plates for 96 h. [^3H]thymidine was added for the last 24 h, and proliferation was assessed in quadruplicate wells by measuring [^3H]thymidine incorporation. Stimulatory indices were calculated by dividing the cpm of each well by the average cpm of nonstimulated wells.

**Thymus culture and transplantation**

Thymic lobes were dissected from female neonatal Icam1^tm1Jcgr^NOD and NOD mice and maintained at the liquid/air interface in 24-well 0.4-µm Transwells over 1 ml medium (DMEM supplemented with 10% FCS, 2 nM glutamine, nonessential amino acids, 10 mM HEPES, 0.05 mM 2-ME, penicillin/streptomycin) at 37°C and 10% CO₂, as previously described (25, 26). For flow cytometric analysis, thymus were digested in trypsin and mechanically disrupted, and thymic cells were stained with Abs against CD45 (30-F11), IA-IE (2G9), Ly51 (6C3), and either CD80 (16-10A1), CD86 (GL1), CD274 (MIH1; all from BD Biosciences) or CD275 (HK5.3; eBioscience). To deplete hematopoietic cells, 1.35 mM deoxyguanosine (Sigma-Aldrich) was added to the culture medium for 6 d before transplantation. In some experiments, flow cytometry was performed using a FACSCanto II flow cytometer (all from BD Biosciences; clone names in parentheses). Optimal proliferation was observed at 6.25 and 40 µg/ml peptide content, and these concentrations were used in subsequent experiments. A total of 1.5 × 10^5 responder spleenocytes from NOD and Icam1^tm1Jcgr^NOD mice and 1 × 10^5 irradiated (10 Gy) NOD splenocytes were maintained in 96-well plates for 96 h. [^3H]thymidine was added for the last 24 h, and proliferation was assessed in quadruplicate wells by measuring [^3H]thymidine incorporation. Stimulatory indices were calculated by dividing the cpm of each well by the average cpm of nonstimulated wells.

**Cell extraction from peripheral nerves**

After intracardial perfusion with PBS, both sciatic nerves from two animals were dissected and pooled. For extracellulae staining, six to eight animals/group were used in each experiment. For intracellular cytokine staining, both sciatic nerves from four animals were pooled for each analysis, and 12–16 animals/group were used in each experiment. Nerves were homogenized using a scalpel in a petri dish and incubated with 1 ml collagenase/dispase (0.5 mg/ml; Roche) for 1 h at 37°C. After further homogenization with a pipette tip, 1 ml DNase I (0.1 mg/ml; Roche) was added and incubated for 1 h at 37°C. Tissue samples were passed through a 40-µm cell strainer, washed, resuspended in 70% Percoll (GE Healthcare) diluted with RPMI 1640 medium, and covered with 30% Percoll diluted with PBS. After centrifugation for 30 min (1000 g), mononuclear cells were collected from the interface and washed with medium, and cell numbers were assessed. Cells were subsequently stained using two different six-fluorophore combinations of Abs (all from BD Biosciences) recognizing CD3 (500A2), CD4 (L3T4), CD8 (53-67), CD25 (3C7), CD45 (30-F11), B220 (RA3-6B2), Ly6G (1A8), CD11b (M1/70), or CD11c (HL3) and analyzed on a FACSCanto II flow cytometer (all from BD Biosciences; clone names in parentheses). Intracellular staining was performed as described below.

**Serum Ab detection**

Sera were obtained from sacrificed NOD and Icam1^tm1Jcgr^NOD mice. The detection of autoreactive Abs was modified from a previous description injected under the left kidney capsule using a Hamilton syringe. Abdominal wall and skin were sutured, and mice were kept in a warmed cage until awakening. Four weeks after thymic transplantation, 35 µl blood was taken from transplanted nude mice by tail vein bleeding and, after erythrocyte lysis, blood samples were stained for CD3 (500A2), CD4 (L3T4), and CD8 (53-67) (all from BD Biosciences; clone names in parentheses) and analyzed by flow cytometry. Transplantation was considered successful if a CD3⁺CD4⁻T compartment defined by >5% of the leukocyte gate being CD3⁺ was detectable. Four independent transplantation experiments were performed that included a total of 16 Icam1^tm1Jcgr^NOD (NudeIcam1^tm1Jcgr^NOD) and 6 NOD (Nude^NOD) nude recipients. Blood samples for Ab detection were taken before and 9 and 16 wk after transplantation. Recipient nude mice were tested weekly for glucosuria and clinical signs of neuropathy for up to 180 d. Experiments were terminated, and nerve-conduction studies and histology were performed when severe clinical neuropathy occurred or the maximum observation period was reached.

**FIGURE 1.** Spontaneous chronic progressive inflammatory peripheral neuritis in Icam1^tm1Jcgr^NOD mice. (A) Icam1^tm1Jcgr^NOD mice spontaneously develop slowly progressive hind limb paresis and fail to grip a round horizontal bar (arrows). (B) Female (n = 106) and male (n = 18) Icam1^tm1Jcgr^NOD mice, nondiabetic female (n = 18) and male (n = 20) NOD mice, and female Icam1^tm1Jcgr^C57BL/6 mice (n = 12) were checked for clinical impairment every other week until 18 mo of age. Sciatic nerve sections were generated from NOD mice (C) and affected Icam1^tm1Jcgr^NOD mice (D). Subperineurial (arrow) and endoneurial (arrowhead) cell infiltration are observed in Icam1^tm1Jcgr^NOD mice. Representative sections of six animals analyzed per group in two independent experiments. Scale bars, 50 µm. Semi-thin (0.5 µm) sections of epox resin–embedded sciatic nerves from NOD (E) and Icam1^tm1Jcgr^NOD (F) mice were stained with toluidine blue. (F) Demyelination (white arrows), inflammatory cell infiltration (arrow), and completely demyelinated axons (arrowhead) are marked in an affected Icam1^tm1Jcgr^NOD mouse. Scale bars, 10 µm.
PNS tissue from female NOD-SCID mice was homogenized using an ULTRA-TURRAX for 30 s on ice in a buffer containing 50 mM Tris (pH 8), 2% SDS, 100 mM 2-ME, 10% glycerol, and protease inhibitors (Roche). Homogenates were boiled for 5 min and centrifuged at 13,000 rpm for 5 min; after protein quantification, the supernatants (200 µg) were separated on a 12% SDS-PAGE gel with a single continuous lane and transferred onto a nitrocellulose membrane (Bio-Rad). The Odyssey two-color protein size marker was used (7.5–250 kDa; LI-COR Biosciences).

**FIGURE 2.** Preclinical infiltration of T cells, B cells, and macrophages into peripheral nerves. Paraffin-embedded sciatic nerve sections from Icam1<sup>tm1Jcgr</sup>NOD mice were fluorescently immunostained against CD3 (A; green), CD68 (B; green) and B220 (C; green). (D) Costaining against Schwann cell marker S100 (red) was performed together with CD68. Nuclei were visualized using DAPI. Samples from five animals were stained. Scale bars, 20 µm. (D) Leukocytes were extracted from peripheral nerves of NOD and Icam1<sup>tm1Jcgr</sup>NOD mice by enzymatic digestion and gradient centrifugation, costained, and analyzed by flow cytometry. The number of CD45<sup>+</sup> cells extractable from a single NOD sciatic nerve (white) is plotted against the number extractable from nonaffected (−, light gray) and affected (+, dark gray) Icam1<sup>tm1Jcgr</sup>NOD mice. (E) The proportion of nerve-derived CD45<sup>+</sup> cells staining positive for B20, CD3, CD11b, Ly6G, or CD11c in six-color flow cytometry was compared in nonaffected (light gray) and affected (dark gray) Icam1<sup>tm1Jcgr</sup>NOD mice. (F) In an independent staining, the proportion of nerve-derived CD4<sup>+</sup> and CD8<sup>+</sup> cells of CD45<sup>+</sup>CD3<sup>+</sup> cells was quantified. In (E)–(G), sciatic nerve tissue from two animals was pooled for each extraction, and average values from three independent experiments (n = 6–10 animals/group/experiment) are shown. (G) Representative flow cytometry plots illustrate the gating strategy for nerve-derived cells of two affected Icam1<sup>tm1Jcgr</sup>NOD mice. Gating of CD45 against CD3 (upper left panel), CD4 against CD8 (lower left panel), B20 against CD3 (upper middle panel), B20 against CD11b (lower middle panel), CD11b against CD11c (myeloid dendritic cells; upper right panel), and B20 against CD11c<sup>+</sup> (plasmacytoid dendritic cells; lower right panel). *p < 0.05, **p < 0.01, ***p < 0.005.
Membranes were blocked (PBS, 0.1% Tween 20, 5% nonfat milk) overnight at 4°C and incubated for 2 h at room temperature with sera diluted in blocking buffer in a multiscreen apparatus (Bio-Rad). Serial arrays from 1:12 to 1:600 were tested, and 1:100 dilutions of sera were used for subsequent experiments. Monoclonal anti-MPZ (provided by Dr. J. Archelos, Medical University of Graz, Graz, Austria) and anti-tubulin (Abcam) Abs were used as positive controls. After washing five times with PBS, 0.1% Tween, membranes were incubated with fluorescently labeled goat antimouse Ab in blocking buffer (1:15,000; LI-COR IRDye 800) for 1 h after removing the membrane from the multiscreen apparatus. After washing, membranes were visualized on an Odyssey imaging system (LI-COR Biosciences).

CDR3 spectratyping

Total RNA was extracted from spleen, pancreas, and sciatic nerves of prediabetic NOD mice aged 12 wk and clinically affected nonaffected Icam1tm1jcgr NOD mice aged 1 y (n = 4 animals/group) using the RNeasy Lipid Tissue Kit (QIAGEN). RNA was photometrically quantified and tested for purity (NanoDrop; Thermo Scientific). cDNA was synthesized using a TCR β-chain–specific primer (sequence: 5'-GAAGAAGCCCGCTGCGGCTGCGC-3'), and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer’s protocol. CDR3-spectratyping PCR was performed using standard reagents (Roche) and Vβ element–specific forward primers and FAM-labeled reverse primers (MWG Biotech), as previously described (28, 29). Primers were newly designed for elements Vβ1 (5'-CCAGGCTCATTAACCTCGAGATGC-3'), Vβ3 (5'-CAAATGACATGACCTGGAAACG-3'), Vβ12 (5'-CCAGGCTAGTCTCTTTTG-3'), Vβ18 (5'-CTGAACCAATACGAGAGTGA-3'), and Vβ20 (5'-GGTCAAGGGAGATTCTCAGCTG-3'). The length of the resulting fluorescence-labeled PCR products was determined using an ABI310 genetic analyzer and 400HD ROX as size standard. Distribution of fragment length was analyzed using Peak Scanner Software (all from Applied Biosystems). The size distribution of the individual Vβ elements was rated as nondetectable, normal, shifted, or skewed by a blinded observer, as previously described (30). The most prominent size shift was identified and compared between tissues and genotypes. For expression analysis of previously described (30). The most prominent size shift was identified and compared between tissues and genotypes.

RESULTS

Spontaneous and specific inflammation of peripheral nerves in Icam1tm1jcgr NOD mice

As previously reported (17), Icam1tm1jcgr NOD mice are completely protected from insulitis and autoimmune diabetes. The absence of a full-length ICAM-1 transcript confers protection from NOD diabetes, despite the known expression of alternative splice isoforms of ICAM-1 in this specific mouse line (Supplemental Fig. 1A) (15, 16). Instead of diabetes, we surprisingly observed that Icam1tm1jcgr NOD mice spontaneously developed slowly progressive tetraparesis and muscle atrophy of all extremities (Fig. 1A).

Impairments occurred stochastically from 7 mo of age and progressed from hind to fore limbs, and the incidence reached ~50 and 80% in the predominantly affected females at 12 and 18 mo of age, respectively (Fig. 1B). In our colonies, neither NOD nor Icam1tm1jcgr mice on a C57BL/6 background developed any impairments during 18 mo of observation (Fig. 1B). All subsequent analyses were restricted to nonaffected (score ≤ 1) and clinically affected (score ≥ 5) female Icam1tm1jcgr NOD mice and prediabetic NOD controls aged 2–3 mo.

We performed histological analyses to further characterize the neuritis in Icam1tm1jcgr NOD mice. As expected, NOD mice showed inflammatory destruction of endocrine pancreatic islets but no abnormalities of peripheral nerve (Fig. 1C). In Icam1tm1jcgr NOD mice, in contrast, pancreatic islets were free of infiltration, whereas peripheral nerves showed severe mononuclear cell infiltration (Fig. 1D). Spinal roots and CNS tissue did not show any inflammation and no further pathology was identified in other

FIGURE 3. CD4+ T cells adoptively transfer neuritis to NOD-SCID mice. (A) Incidence of neuropathy (left y-axis, black graphs) and diabetes (right y-axis, gray graph) after adoptive cell transfer. Splenic lymphocytes (○), sorted CD4+ T cells (□), or non-CD4+ lymphocytes (△) from Icam1tm1jcgr NOD mice were transferred into NOD-SCID mice. As controls, lymphocytes from Icam1tm1jcgr NOD mice were transferred into conventional SCID mice (○), and lymphocytes from NOD mice were transferred into NOD-SCID mice (△). (B) A NOD-SCID recipient mouse with severe forelimb and hindlimb paresis (arrows) 8 wk after transfer of Icam1tm1jcgr NOD-derived splenocytes. (C-F) Representative paraffin-embedded sciatic nerve sections (upper panels) and electrophysiology plots (lower panels) from different donor/recipient combinations. (C) Icam1tm1jcgr NOD splenocytes (sp) transferred into NOD-SCID mice. (D) Sorted Icam1tm1jcgr NOD CD4+ T cells transferred into NOD-SCID mice. (E) Sorted Icam1tm1jcgr NOD non-CD4+ T cells transferred into NOD-SCID mice. (F) Icam1tm1jcgr NOD splenocytes transferred into conventional SCID mice. Scale bars, 100 μm. Results are summed from three independent experiments including different experimental groups (n = 5 recipients/group/experiment).
organs of Icam1tm1JcgrNOD mice (data not shown). Semi-thin sections of sciatic nerves confirmed normal PNS myelination in NOD mice (Fig. 1E), but severe demyelination, hypomyelination, and formation of “onion-bulb”–like structures, indicative of chronic demyelination and remyelination, were noted in the PNS of impaired Icam1tm1JcgrNOD mice (Fig. 1F). Again, widespread mononuclear cell infiltrates were observed (Fig. 1F). Thus, Icam1tm1JcgrNOD mice spontaneously develop a severe peripheral neuropathy that features a demyelinating and inflammatory phenotype, and inflammation in Icam1tm1JcgrNOD mice is restricted to peripheral nerves.

Preclinical infiltration of T and B cells and macrophages into peripheral nerves

We further characterized the cell types infiltrating the PNS in Icam1tm1JcgrNOD mice. Using an immunohistochemical approach, we detected abundant CD3+ T cells (Fig. 2A) and CD68+ macrophages (Fig. 2B) in Icam1tm1JcgrNOD peripheral nerve sections. B220+ B cells were observed at a lower frequency (Fig. 2C). With the exception of rare tissue-resident macrophages (31), no inflammatory cells were detected in the PNS of NOD mice (data not shown). To characterize the local effector cells in greater detail, we extracted leukocytes from the PNS and analyzed their composition by flow cytometry. Cell numbers extractable from NOD peripheral nerves were negligible (Fig. 2D). In contrast, compared with NOD mice, ~50- and ~100-fold higher numbers of CD45+ hematopoietic cells were retrievable from the PNS of nonaffected and affected Icam1tm1JcgrNOD mice, respectively (Fig. 2D). This confirms that PNS cell infiltration is specific to Icam1tm1JcgrNOD mice and indicates that infiltration precedes, and most probably triggers, clinical impairments.

By flow cytometry, PNS-derived CD45+ cells in Icam1tm1JcgrNOD mice were composed primarily (~60%) of T cells and B220+ B cells (Fig. 2E). T cells were mainly CD4+ (Fig. 2F). Also, CD11b+ macrophages were frequently present, and CD11b+ CD11c+ myeloid dendritic cells were detectable (Fig. 2E, 2G). B220+ cells rarely expressed CD1c, arguing against a relevance of plasmacytoid dendritic cells. Low numbers of Ly6G+ granulocytes were present. In comparison with nonaffected animals, nerve infiltrates in impaired Icam1tm1JcgrNOD mice exhibited significantly higher percentages of CD3+ T cells and lower amounts of B220+ B cells (Fig. 2E). Thus, we identified T cells, macrophages, and B cells as the predominant effector cell populations in the inflammatory neuropathy of Icam1tm1JcgrNOD mice.

CD4+ T cell–dependent neuritis and proinflammatory Th17 cell skew in Icam1tm1JcgrNOD mice

We performed adoptive-transfer studies to identify the contribution of individual leukocyte subsets in Icam1tm1JcgrNOD neuritis. Transfer of NOD splenocytes into immunodeficient NOD-SCID mice triggered insulitis and diabetes with 80% penetrance, as

FIGURE 4. Increased IL-17 and IFN-γ production in spleen and peripheral nerves. (A) Representative flow cytometry dot plots of intracellular IL-17 and IFN-γ staining of splenocytes derived from NOD mice (left panel) and nonaffected (middle panel) and affected (right panel) Icam1tm1JcgrNOD mice. Gating included CD3+CD4+ cells. (B) Average proportions of IFN-γ–, IL-17–, and IL-10–producing CD4+ T cells in NOD and nonaffected and affected Icam1tm1JcgrNOD mice. (C) Splenocyte-derived cytokine production was measured by ELISA. In (A)–(C), one of three independent experiments (n = 3–5 mice/group) is depicted. (D) Leukocytes were extracted from sciatic nerves of NOD mice (left panel) and nonaffected (middle panel) and affected (right panel) Icam1tm1JcgrNOD mice and stained for the intracellular production of IL-17 and IFN-γ. One representative of two experiments using 12–16 animals/group is shown. (E) The expression of immune-relevant mRNA transcripts in sciatic nerve tissue from NOD (n = 7) and Icam1tm1JcgrNOD (n = 9) mice was analyzed using a predefined mouse immune TaqMan array. All differentially (p, 0.05) regulated transcripts are depicted on a logarithmic scale and sorted by context and level of regulation. Labels represent official murine gene symbols. *p < 0.05, **p < 0.01.
expected (Fig. 3A). Transfer of splenocytes from affected Icam1<sup>tm1Jcgr</sup>NOD mice into NOD-SCID mice resulted in clinically obvious tetraparesis (Fig. 3B) that was paralleled by reduced NCV and mononuclear cell infiltration into peripheral nerves (Fig. 3C). Neuropathy first occurred between 8 and 12 wk after transfer, progressed slowly, and reached a cumulative incidence ~85% (Fig. 3A). Transfer of purified CD<sup>4+</sup> T cells equally resulted in clinical signs of neuropathy (Fig. 3A), reduction of NCV, and inflammatory infiltrates in recipient NOD-SCID mice (Fig. 3D). Results did not differ between positive (anti-CD4 Ab) and negative (CD4<sup>+</sup> untouched) CD4<sup>+</sup> sorting (data not shown). In contrast, transfer of the CD4<sup>+</sup>-depleted fraction (non-CD4 cells) into NOD-SCID mice did not elicit clinical, histological, or electrophysiological signs of neuropathy in recipients during 21 wk of follow-up (Fig. 3A, 3E). Thus, CD4<sup>+</sup> T cells are required and essential mediators of the inflammatory neuropathy in Icam1<sup>tm1Jcgr</sup>NOD mice. Transfer of splenocytes from Icam1<sup>tm1Jcgr</sup>NOD mice into conventional SCID mice—without NOD genetic background—did not result in either neuropathy or diabetes (Fig. 3F). This indicates that, in addition to autoreactive T cells, a permissive host environment—most probably the NOD-specific H-2<sup>g7</sup>—is required for the generation of the inflammatory neuropathy.

We further characterized effector mechanisms in Icam1<sup>tm1Jcgr</sup>NOD mice by intracellular cytokine staining, cytokine ELISA, and gene expression analysis. Frequencies of IFN-γ– and IL-17–producing splenic lymphocytes were higher in affected Icam1<sup>tm1Jcgr</sup>NOD mice than in NOD mice (Fig. 4A, 4B), both in representative (Fig. 4A) and averaged (Fig. 4B) results from intracellular cytokine staining. The percentage of IL-10–producing T cells did not differ (Fig. 4B). Cultured splenocytes from affected Icam1<sup>tm1Jcgr</sup>NOD mice produced significantly higher amounts of soluble IL-17 (Fig. 4C), whereas the amount of secreted IFN-γ was not different (Fig. 4C). We next performed intracellular cytokine staining of lymphocytes extracted from the PNS and, in the

**FIGURE 5.** Myelin-targeted serum response and lymphocyte autoactivity. (A) Unfixed sciatic nerve cryostat sections generated from NOD-SCID tissue were incubated with serum dilutions (1:200) from prediabetic NOD mice (left panel) and affected Icam1<sup>tm1Jcgr</sup>NOD mice (right panel), followed by DAB-based staining with an anti-mouse–directed secondary Ab. (B) NOD-SCID nerve tissue was stained with NOD (left panel) and Icam1<sup>tm1Jcgr</sup>NOD (right panel) sera using a red fluorescently labeled secondary Ab and DAPI nuclear stain. Two independent experiments with sera from five animals/group were performed. Scale bars, 100 μm. (C) NOD-SCID nerve tissue was separated on a single-lane SDS-PAGE gel and stained with serial dilutions (1:12–1:600) of sera from two Icam1<sup>tm1Jcgr</sup>NOD mice in a multiscreen apparatus. (D) Serum dilutions (1:100) from eight individual Icam1<sup>tm1Jcgr</sup>NOD mice and six NOD mice were tested for nerve tissue reactivity. Staining with anti-MPZ (P0) and anti-tubulin (T) Ab was used as control in (C) and (D). One representative of four independent experiments is shown. (E) Splenocytes from either NOD mice or nonaffected or affected Icam1<sup>tm1Jcgr</sup>NOD mice, together with irradiated APCs from NOD mice, were cocultured in the presence of Ag. Homogenates of mouse peripheral nerve (PNS), purified peripheral nerve myelin (Myelin), cultured nonmyelinating Schwann cells (SC), forebrain (CNS), and pancreas (Pancr) were added to the splenocyte cultures. Data are expressed as stimulatory indices calculated as fold incorporation of [3H]thymidine into unstimulated wells. (F) Proliferatory responses against myelin protein peptides P0<sub>106-125</sub>, P0<sub>180-199</sub>, P2<sub>53-78</sub>, GAD65, and Hsp60. Please note the different scale used in the Hsp60 plot (right panel). One representative of four independent experiments with quadruplicate wells of six to eight animals/group is shown. *p < 0.05, **p < 0.01, versus NOD samples.
target tissue, again found that the proportion of IL-17– and IFN-γ–producing CD4+ T cells was increased in affected Icam1tm1JcgrNOD mice (Fig. 4D). The proportion of splenic Foxp3+ regulatory T cells did not differ between groups (data not shown). Thus, the CD4+ T cell–mediated inflammatory neuropathy in Icam1tm1JcgrNOD mice features a proinflammatory Th17 phenotype.

To further characterize the Icam1tm1JcgrNOD immune response in the PNS, we screened for differentially regulated immune-relevant mRNA transcripts in the sciatic nerves of Icam1tm1JcgrNOD mice using a predefined 96-gene serum immune array. Molecules expressed by T cells (CD4, CD8, CD3), B cells (CD19, CD45R), and macrophages (CD68, H2-Eb1 [i.e., MHC class II]) were significantly more abundant in Icam1tm1JcgrNOD sciatic nerves after normalization against 18S (Fig. 4E). Among the cytokines analyzed, Ccl5 and Cxcl10 were most prominently upregulated (Fig. 4E). Various molecules related to costimulation, expressed by either T cells (CTLA-4, CD80, ICOS) or APCs (CD80, CD86, CD40), were increasingly expressed in the Icam1tm1JcgrNOD PNS (Fig. 4E), which may indicate a compensatory upregulation of other costimulatory molecules or the infiltration of T and B cells and macrophages into the PNS.

Peripheral nerve myelin-reactive Abs and T cells

We next tested how the Icam1tm1Jcgr allele alters the target specificity of NOD-defined autoimmunity. We stained sciatic nerve sections with sera from NOD and Icam1tm1JcgrNOD mice using a DAB-based (Fig. 5A) and a fluorescent (Fig. 5B) staining approach. NOD-derived sera stained only small perinuclear areas of a DAB-based (Fig. 5A) and a fluorescent (Fig. 5B) staining approach. The staining was more diffuse, but it clearly retraced the ring-shaped morphology of myelinated fibers. This indicated that Abs in Icam1tm1JcgrNOD PNS myelin directed serum autoreactivity in Icam1tm1JcgrNOD mice.

To identify potential target Ags, we next adopted an immunoblotting approach (27). Serum dilutions from Icam1tm1JcgrNOD mice were tested for reactivity against whole-nerve homogenates from NOD-SCID PNS tissue and resulted in a concentration-dependent single band at ∼28 kDa, which appeared to be identical in size to the signal of MPZ mAb (MPZ synonymously termed P0; Fig. 5C). This 28-kDa reactivity was reproducibly observed in individual Icam1tm1JcgrNOD mice, although at varying intensities, whereas this band was always absent in NOD mice (Fig. 5D). Thus, we considered MPZ an interesting antigenic candidate in Icam1tm1JcgrNOD-defined neuritis.

To confirm this antigenic target in Icam1tm1JcgrNOD mice, we tested the lymphocyte proliferation response to different stimuli. Lymphocytes from Icam1tm1JcgrNOD mice responded against murine peripheral nerve homogenates and purified PNS myelin but not against lysates of cultured nonmyelinating Schwann cells (Fig. 5E). In accordance with the serum-derived data, this indicates a target Ag localized within PNS myelin. CNS tissue did not induce relevant proliferation; rather, it was mildly suppressive (Fig. 5E), potentially as the result of the high content of lipids and nonantigenic proteins. Pancreas homogenates did not induce proliferatory responses in either NOD or Icam1tm1JcgrNOD mice (Fig. 5E), most likely because of the very low abundance of NOD target Ags in whole-tissue samples.

Consequently, we tested PNS myelin protein peptides with known antigenic capacity in rodent-induced neuritis models (32, 33). Both MPZ (P0) peptide P0106–125 and myelin protein two (P2) peptide P253–78 induced a proliferation response in Icam1tm1JcgrNOD mice, whereas P0106–125 did not induce proliferation (Fig. 5F). The proliferation induced by Hsp60, a known pancreatic Ag in NOD mice (2), was significantly lower in Icam1tm1JcgrNOD mice (Fig. 5F). The NOD late-stage Ag GAD65 did not elicit proliferatory responses in the young prediabetic NOD mice that we used. In accordance with previous reports (6, 7, 33), myelin protein P0 represents an important Ag in Icam1tm1JcgrNOD neuritis. Overall, Ag-specific proliferation was low, which may reflect the chronic and slowly progressive autoimmunity in Icam1tm1JcgrNOD mice. The inverse correlation between the degree of clinical impairment and proliferatory responses may indicate that autoreactivity has “burned out” in severely disabled animals.

FIGURE 6. Th17 differentiation determines the pathogenicity, not the target specificity, of Icam1tm1JcgrNOD CD4+ T cells. (A) Purified CD4+ T cells from affected Icam1tm1JcgrNOD mice were differentiated in vitro. Representative intracellular IL-17 and IFN-γ staining of undifferentiated cells (left panel), Th1-differentiated T cells (middle panel), and Th17-differentiated T cells (right panel). Gating was on CD3+CD4+ cells. (B) Differentiated cells were injected i.v. into NOD-SCID mice, and recipients of undifferentiated cells (○), Th1 cells (∆), or Th17 cells (□) were tested weekly for clinical signs of neuropathy. (C) The average clinical score in each experimental group was calculated. One of two independent experiments (n = 21 recipients/experiment) with four recipient animals in each NOD group is shown in (A) through (C). (D) Splenic lymphocytes were extracted from recipient NOD-SCID mice at the end of the observation period and stained for intracellular cytokines. Representative IL-17 and IFN-γ staining after transfer of undifferentiated cells (left panel), Th1 cells (middle panel), or Th17 cells (right panel). Gating was on CD3+CD4+ cells. (E) Paraffin-embedded sciatic nerve sections of NOD-SCID mice were stained with H&E after receiving undifferentiated cells (left panel), Th1 cells (middle panel), or Th17 cells (right panel). Scale bars, 100 μm. *p < 0.05
In summary, the Icam1\(^{tm1Jcgr}\) allele on the NOD genetic background decreases autoreactivity against endocrine pancreas and, instead, increases autoreactivity against PNS myelin. Specifically, MPZ/P0 constitutes a dominant autoantigen, which is in accordance with data from other PNS autoimmunity models (6, 7). This indicates that deficiency of full-length ICAM-1 specifically shifts the target of autoimmunity in NOD mice; however, a contribution of other alternative splice isoforms of ICAM-1 cannot be excluded using the Icam1\(^{tm1Jcgr}\) NOD mouse strain.

**Th17 differentiation determines the pathogenicity, not the target specificity, of autoreactive CD4\(^+\) T cells in Icam1\(^{tm1Jcgr}\) NOD mice**

We next tested whether the T cell differentiation observed in Icam1\(^{tm1Jcgr}\) NOD mice determines whether autoimmune diabetes or neuritis evolves. Therefore, we sorted CD4\(^+\) T cells from clinically affected Icam1\(^{tm1Jcgr}\) NOD mice and differentiated these into predominantly IFN-\(\gamma\)–producing Th1 cells or IL-17–producing Th17 cells (Fig. 6A). After injection into NOD-SCID mice, we screened recipient mice for glucosuria and clinical signs of neuropathy. Within 11 wk, all mice receiving Th1 or Th17 cells and 75% of mice receiving undifferentiated cells developed clinically overt neuropathy (Fig. 6B). Th17 cells generated a significantly earlier onset and greater average severity of neuropathy than did unstimulated cells (Fig. 6B, 6C). This also was reflected by a greater reduction in NCV after transfer of either type of cytokine-stimulated cells (Supplemental Fig. 1A). We did not observe diabetes in any recipient of Icam1\(^{tm1Jcgr}\) NOD-derived lymphocytes (Supplemental Fig. 1B). Inversely, control transfer of NOD-derived donor cells consistently caused diabetes (Supplemental Fig. 1D) but no signs of neuropathy, irrespective of the cytokine prestimulation (Supplemental Fig. 1C). The phenotype of the transferred T cells converted in vivo to Th1 in all recipient groups (Fig. 6D), in accordance with previous reports (34). Thus, our findings indicate that Th17 differentiation determines the pathogenicity, but not the target specificity, of autoreactive CD4\(^+\) T cells from Icam1\(^{tm1Jcgr}\) NOD mice.

**ICAM-1 blockade triggers neuritis only in young NOD mice**

To identify at which stage of development ICAM-1 deficiency takes effect, we applied blocking anti–ICAM-1 Abs to cohorts of young juvenile (10 d) and young adult (4 wk) female NOD mice for 7 wk and compared the occurrence of diabetes and neuritis during 35 wk of follow-up. Saline-treated adult NOD mice developed diabetes with 72% penetrance (Fig. 7A), which was reduced to 50% by ICAM-1 blockade, but neither of the adult groups developed clinical signs of neuropathy (Fig. 7B). In contrast, saline-treated juvenile NOD mice developed diabetes with 40% penetrance, which was reduced to 13% in ICAM-1 Ab–treated mice (Fig. 7C). ICAM-1 blockade resulted in clinical signs of neuropathy in 33% of juvenile treated NOD mice at 10 mo of age (Fig. 7D). This incidence is comparable to that seen in Icam1\(^{tm1Jcgr}\) NOD mice.
then transplanted these thymic rudiments under the kidney capsule of female athymic nude (Foxn1<sup>−/−</sup>) mice on BALB/c background. Successful engraftment was tested in all transplanted mice by staining peripheral blood to determine whether ≥5% of leukocytes were CD3<sup>+</sup> T cells 4 wk after transplantation (data not shown). Transplantation was successful in 87.5% (14/16) and 83.3% (5/6) of nude mice receiving Icam1<sup>tm1Jcgr</sup>NOD (Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD) thyhi or NOD (Nude<sup>NOD</sup>) thyhi, respectively. All animals were tested weekly for the occurrence of diabetes and neuropathy. Within 180 d of follow-up, 6 of 14 (42.9%) Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice developed slowly progressing tetraparesis (Fig. 8A) with a continuously increasing incidence (Fig. 8B). Affected Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice showed electrophysiological signs of peripheral nerve demyelination and reduced NCV (Fig. 8C), whereas electrophysiological abnormalities were absent in Nude<sup>NOD</sup> mice (Fig. 8C). Paraffin-embedded sections of sciatic nerve revealed mild, but consistently occurring, infiltration of mononuclear cells in affected Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD animals (Fig. 8D), whereas nude mice (Nude<sup>NOD</sup>) receiving NOD thymus did not develop clinical or histological signs of neuropathy (Fig. 8D) or diabetes (data not shown). Accordingly, semi-thin

**FIGURE 8.** Transplantation of Icam1<sup>tm1Jcgr</sup>NOD thymus induces neuritis in athymic nude mice. (A) Thymi from female neonatal Icam1<sup>tm1Jcgr</sup>NOD (n = 16) and NOD (n = 5) mice were depleted of hematopoietic cells and transplanted under the kidney capsule of female athymic nude mice. Nude recipient of Icam1<sup>tm1Jcgr</sup>NOD thymus (Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD) with hind limb paresis (arrow). (B) All recipients were tested weekly for glucosuria and clinical impairments for up to 180 d. Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice (○), but none of the recipients of NOD thymus (Nude<sup>NOD</sup>, □), developed signs of neuropathy. (C) Sciatic nerve electrophysiology was performed in Nude<sup>NOD</sup> mice (upper panel) and Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice (lower panel), and the average NCV was calculated. (D) Paraffin-embedded sciatic nerve sections were generated from Nude<sup>NOD</sup> mice (left panel) and neuropathic Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice (right panel). Scale bars, 100 μm. (E) Semi-thin sciatic nerve sections were generated from neuropathic Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice. Demyelination (white arrow) and axonal degeneration (black arrow) are indicated. Scale bar, 10 μm. (F) Cryosections of NOD-SCID sciatic nerves were incubated with serum dilutions (1:200) of Nude<sup>NOD</sup> mice (left panel) or neuropathic Nude<sup>Icam1<sup>tm1Jcgr</sup></sup>NOD mice (right panel) and stained using red fluorescently labeled anti-mouse Ab. Scale bars, 50 μm. One representative of three independent stainings is shown.
sections showed signs of demyelination (Fig. 8E). We next tested sera of thymic recipients for myelin autoreactivity. We found that, similar to Icam1\textsuperscript{tm1Jcgr}NOD mice, sera from affected Nude\textsuperscript{Icam1tm1Jcgr}NOD mice, but not Nude\textsuperscript{NOD} mice, stained PNS myelin in cryosections (Fig. 8F). This indicates that Nude mice develop myelin autoreactivity similar to Icam1\textsuperscript{tm1Jcgr}NOD mice.

Given the dominant role of thymic epithelium in the disease process, we next assessed the expression of different molecules with known costimulatory function on thymic CD45-IA-IE+ cells (Fig. 9). The average CD80-staining intensity and the proportion of CD80-expressing thymic epithelial cells were significantly increased in Icam1\textsuperscript{tm1Jcgr}NOD mice (Fig. 9A, 9B). CD274 (programmed cell death ligand 1) staining intensity was significantly reduced (Fig. 9C, 9D). The expression of CD86 and CD275 (ICOS-L) were unchanged (data not shown). Results did not differ between directly ex vivo–analyzed neonatal thymi and hematopoietic cell–depleted thymi (data not shown). Despite the potential expression of alternative ICAM-1 splice isoforms, we speculate that deficiency of full-length ICAM-1 in Icam1\textsuperscript{tm1Jcgr}NOD mice may act by altering the composition of costimulatory molecules on thymic epithelial cells and by subsequently altering selection of the T cell repertoire.

Altered TCR repertoire in Icam1\textsuperscript{tm1Jcgr}NOD mice

To address this, we next performed CDR3 spectratyping using spleen, sciatic nerve, and pancreas tissue of NOD and Icam1\textsuperscript{tm1Jcgr}NOD mice and tested for the generation of a differential V\(\beta\) element repertoire. A proportion of V\(\beta\) elements was nondetectable (V\(\beta\)3, 13, 17, 18, 19), equally expanded in both genotypes (V\(\beta\)8.2, 9, 10, 11, 14, 20), or showed a nonconsistent pattern of expansion in either tissue or genotype (V\(\beta\)1, 4, 6) (Supplemental Tables I–III). The remaining V\(\beta\) elements (V\(\beta\)2, 5, 8.1, 8.3, 12, 15, 16) showed a genotype-specific pattern of expansion. Specifically, V\(\beta\)2, V\(\beta\)12, and V\(\beta\)16 were only skewed in the PNS of affected Icam1\textsuperscript{tm1Jcgr}NOD mice but generally were not detectable in NOD mice (Fig. 9E, Supplemental Table II). The distribution of V\(\beta\)5 showed greater abnormalities across all tissues in NOD mice compared with Icam1\textsuperscript{tm1Jcgr}NOD mice (Fig. 9E, Supplemental Tables I–III). V\(\beta\)8.1 was shifted predominantly in the spleen of NOD mice (Supplemental Table I), whereas V\(\beta\)8.3 and V\(\beta\)15 were shifted predominantly in the spleen of Icam1\textsuperscript{tm1Jcgr}NOD mice (Fig. 9F, Supplemental Table I). This indicates that diabetes in NOD mice and neuritis in Icam1\textsuperscript{tm1Jcgr}NOD mice do not share T cell specificity and that ICAM-1 determines the peripheral TCR repertoire in NOD mice.

Discussion

In this study, we demonstrate that Icam1\textsuperscript{tm1Jcgr}NOD mice, which lack expression of full-length ICAM-1 and are protected from autoimmune diabetes (17), instead spontaneously develop a chronic, demyelinating, inflammatory peripheral neuropathy, whereas all other organs are spared. Autoimmunity targets peripheral nerve myelin and, more specifically, MPZ. The neuritis is mediated by CD4\(^+\) T cells, which exhibit a predominant Th17 differentiation, which determines the disease severity, but not the target specificity,
of the T cells. Rather, their specificity is instructed in developing T cells by thymic epithelial cells. Thymic epithelial cells from Icam1 tm1Jcgr NOD mice suffice to trigger neuropathy. To our knowledge, this demonstrates for the first time that ICAM-1, which bears costimulatory and adhesive functions, shifts autoimmune specifically from pancreas to the PNS by a thymic selection–dependent mechanism.

Our study has technical limitations. The Icam1 tm1Alb mouse strain used in this study was generated targeting exon 4 of the ICAM-1 gene (13) and still allows expression of alternative, nonmembrane-bound splice isoforms of ICAM-1 (16). Other splice isoforms were reported in the Icam1 tm1Bay mouse line (15), whereas only the Icam1 tm1Alb strain carries a true null allele (14). Thus, we are not analyzing ICAM-1 deficiency per se but rather an altered expression of ICAM-1 on the NOD genetic background. Expression of alternative ICAM-1 transcripts is another potential explanation for the phenotype that we observed. Future studies are directed toward generating and examining Icam1 tm1Alb NOD mice carrying a true ICAM-1 null allele. Of note, Ab-mediated blockade also triggered neuritis and reduced the incidence of diabetes in juvenile NOD mice (Fig. 7), demonstrating that ICAM-1 blockade by nongenetic techniques also can shift autoimmunity from endocrine pancreas to peripheral nerves. This argues for a wider applicability of the identified mechanism beyond the use of the Icam1 tm1Jcgr mouse strain.

Previous studies reported that deficiency of costimulatory molecules on the autoimmune-prone NOD background prevents diabetes but instead triggers spontaneous autoimmune syndromes with special disposition for peripheral nerves. B7-2−/− NOD mice develop peripheral neuritis (4), PD-1−/− NOD-H-2 b mice develop neuritis together with inflammation of stomach and exocrine tissues (8), and ICOS-L−/− NOD mice develop nonspecific inflammation of muscle and nervous system (9). We report an autoimmune-mediated peripheral neuritis that is somewhat similar to the disease observed in B7-2−/− NOD mice. Based on this novel phenotype, we pursued different hypotheses of how deficiency of ICAM-1 may shift autoimmunity in NOD mice.

First, altered adhesion in an Icam1 tm1Jcgr NOD environment may direct T cells to other target organs. ICAM-1 is expressed by APCs and vascular endothelium; thus, a T cell transferred from an Icam1 tm1Jcgr NOD mouse into a NOD-SCID mouse encounters an intact ICAM-I environment. This argues against an adhesion-dependent mechanism. Second, altered T cell differentiation changes the phenotype of experimental autoimmune encephalomyelitis due to a change in integrin expression (36, 37). Our observations argue against similar mechanisms in Icam1 tm1Jcgr NOD neuritis (Fig. 6). The fact that disease severity determines Th17 cells in Icam1 tm1Jcgr NOD mice contrasts with the Th1-mediated diabetes in NOD mice (2). For example, NOD mice deficient in the Th1 transcription factor T-bet are protected from diabetes (38), and diabetes severity in IFN-γ−deficient NOD mice is reduced (39), whereas reconstitution of Th17 to Th1 cells was observed in NOD-SCID mice in vivo (34). For unknown reasons, in contrast to diabetes, Th17 cells may have relevance for neuritis, as reported in patients with chronic neuritis (40).

Third, we speculated that thymic-selection processes may be altered in the absence of full-length ICAM-1. Indeed, the age-restricted effects of ICAM-1–mediated blockade, the differential distortion of the TCR repertoire in Icam1 tm1Jcgr NOD mice (Fig. 9), and the induction of neuritis by Icam1 tm1Jcgr thymic epithelium alone (Fig. 8) support that an altered expression of ICAM-1 on thymic epithelium educates a neuritogenic, instead of a diabetogenic, T cell repertoire in NOD mice and identifies a novel mechanism determining target specificity of autoimmunity. In accordance, a recent study confirmed the relevance of CD28 costimulation for clonal deletion of thymocytes in vivo (41). It is intriguing to speculate whether related mechanisms are also involved in the pathogenesis of chronic inflammatory neuropathies in humans.

A specific propensity for NOD autoimmunity to target the PNS has been well documented in different settings. Deficiency of B7-2 in NOD mice (4) and of PD-1 in NOD.H-2 b mice (8) results in spontaneous neuritis. Anti–IL-2 treatment of NOD mice depletes regulatory T cells and, in addition to diabetes, triggers widespread autoimmunity, including a CD4+ T cell–mediated neuritis (42).

Indeed, the tissue specificity of autoimmunity in the NOD environment may be determined by the composition of T cell subsets (43). However, we provide evidence in this study for a different, selection-dependent mechanism. We did not observe differences in the proportion of Foxp3+ regulatory T cells in Icam1 tm1Jcgr NOD mice and observe modulation of target specificity by Th cell differentiation. Instead, we provide evidence that the selection of the TCR repertoire is altered by Icam1 tm1Jcgr thymic epithelium, which determines the phenotype of autoimmunity in NOD mice.

The protein Aire is essential for the expression of otherwise tissue-restricted protein Ags by medullary thymic epithelial cells (44), as well as for the maintenance of central tolerance to individual Ags (27). Previous studies demonstrated that thymic negative selection is functionally intact in NOD mice (45). NOD mice carrying one allele of a hypomorphic Aire mutant develop a distinct autoimmune syndrome characterized by spontaneous inflammation in eye, lacrimal and salivary glands, and, especially, peripheral nerves (46). In this setting, NOD autoimmunity targets MPZ (47). In accordance with our findings, these studies confirm that thymic selection defines the pattern of autoimmunity in NOD mice and that the PNS is an important secondary target organ if thymic selection is modulated.

In conclusion, we identify a novel mechanism by which co-stimulation defines the target specificity of autoimmunity in the PNS that has potential relevance for human inflammatory neuropathies.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1: Th1 and Th17 differentiation do not trigger diabetes in Icam<sup>1<sup>tm1Jcgr</sup></sup> NOD mice.

(A) cDNA generated from thymus served as template for RT-PCR amplifying full-length ICAM-1 (arrow) and alternative splice isoforms of ICAM-1. (B) Purified CD4+ T cells from affected Icam<sup>1<sup>tm1Jcgr</sup></sup>NOD mice were differentiated in vitro and adoptively transferred into NOD-SCID mice as described in figure 7. Average nerve conduction velocities (NCV) of sciatic nerve electrophysiology recordings were calculated for recipients of unstimulated (no) and Th1 and Th17 skewed T cells. (C) The proportion of diabetic recipient mice after transfer of skewed Icam<sup>1<sup>tm1Jcgr</sup></sup>NOD donor cells is depicted. (D-E) Purified CD4+ T cells from NOD mice were differentiated in vitro and adoptively transferred into NOD-SCID mice and recipients were tested for glucosuria and clinical signs of neuropathy. The proportion of impaired (D) and diabetic (E) mice following transfer of skewed NOD cells is depicted.
CDR3 spectratyping was performed using mRNA preparations of spleens from affected lcam1<sup>tm1Jcgr</sup>NOD (Icam1NOD aff, n = 4), non-affected lcam1<sup>tm1Jcgr</sup>NOD (Icam1NOD non aff, n = 4) and NOD (n = 4) mice. The presence and extent of a shift or – if more pronounced – skew of the Vβ element length distribution from a Gaussian distribution was assessed. The numbers in the lower part of each row represent the direction (- to shorter length, + to larger length) and extent (measured in amount of length iterations) of the respective shift. Genotype specific expansions are highlighted. nd not detected, n normally distributed, s shifted, w skewed, aff affected, bp base pairs.
### Supplementary Table 2: Summarized results of CDR3 spectratyping in sciatic nerve.

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CDR3 spectratyping was performed using mRNA preparations of sciatic nerves from affected *Icam1tm1Jcgr* NOD (Icam1NOD aff, n = 4), non-affected *Icam1tm1Jcgr* NOD (Icam1NOD non-aff, n = 4) and NOD (n = 4) mice. The presence and extent of a shift or skew of the Vβ element length distribution from a Gaussian distribution was assessed. The numbers in the lower part of each row represent the direction (- to shorter length, + to larger length) and extent (measured in amount of length iterations) of the respective shift. Genotype specific expansions are highlighted. nd not detected, n normally distributed, s shifted, w skewed, aff affected, bp base pairs.
Supplementary Table 3: Summarized results of CDR3 spectratyping in pancreas.

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CDR3 spectratyping was performed using mRNA preparations of pancreas from affected Icam1\textsuperscript{tm1Jcgr}\textsuperscript{NOD} (Icam1NOD aff, n = 4) and NOD (n = 4) mice. The presence and extent of a shift or skew of the Vβ element length distribution from a Gaussian distribution was assessed. The numbers in the lower part of each row represent the direction (- to shorter length, + to larger length) and extent (measured in amount of length iterations) of the respective shift. A genotype specific expansion is highlighted. nd not detected, n normally distributed, s shifted, w skewed, aff affected, bp base pairs.