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\textit{J Immunol} 2014; 193:2678-2690; Prepublished online 8 August 2014; doi: 10.4049/jimmunol.1400367

http://www.jimmunol.org/content/193/6/2678

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/08/08/jimmunol.1400367.DCSupplemental

References

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Thymic Epithelium Determines a Spontaneous Chronic Neuritis in Icam1<sup>tm1Jcgr</sup>NOD Mice

Gerd Meyer zu Horste,*1 Anne K. Mausberg,* Steffen Cordes,* Houda El-Haddad,* Hans-Joachim Partke,† Verena I. Leussink,* Michael Roden,‡‡ Stephan Martin,§ Lawrence Steinmann,¶ Hans-Peter Hartung,* and Bernd C. Kieseier*

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 mediate costimulatory functions, spontaneously develop a chronic autoimmune peripheral neuritis instead of diabetes. The disease is transferred by CD4<sup>+</sup> T cells, which infiltrate peripheral nerves together with macrophages and B cells and are autoreactive against peripheral myelin protein zero. These Icam1<sup>tm1Jcgr</sup>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 the neuritis. Blockade 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 anti-diabetogenic 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<sup>tm1Bay</sup> (12), Icam1<sup>tm1Jcgr</sup> (13), and Icam1<sup>tm1Alb</sup> (14). Both Icam1<sup>tm1Bay</sup> and Icam1<sup>tm1Jcgr</sup> mice show residual expression of alternative ICAM-1 splice isoforms (15, 16), whereas only the targeting strategy of the Icam1<sup>tm1Alb</sup> strain generates a true ICAM-1–null allele (14). Despite these limitations, Icam1<sup>tm1Bay</sup> and Icam1<sup>tm1Jcgr</sup> mice have been widely used to study the function of ICAM-1. We previously demonstrated that the Icam1<sup>tm1Jcgr</sup> allele on the NOD genetic background abrogates autoimmune diabetes (17). We report in this article that, instead of developing diabetes, these Icam1<sup>tm1Jcgr</sup>NOD mice develop a chronic inflammatory demyelinating neuropathy that targets MPZ. The disease is mediated by CD4<sup>+</sup> 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<sup>tm1Jcgr</sup>NOD mice are sufficient to trigger peripheral nerve myelin autoreactivity and spontaneous neuritis in nude mice. To our knowledge, this demonstrates for the
first time that costimulatory signals expressed by thymic epithelium determine the target specificity of autoimmune 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 C57BL6 background (13) had been backcrossed to NOD/Bom background (MHC haplotype H-2d; 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 Taconic. Nude mice (BALB/cByJ-Hhi1nu) 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, tetraparesis; 8, 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 8.87-50.10.34.08.339.

Anti–ICAM-1 treatment

In contrast to all other experiments, anti–ICAM-1 treatment was performed in conventionally housed NOD/MkTic mice (Taconic) 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 µl) of rat anti-mouse ICAM-1 Ab (clone YM1/1.7.4, 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.

Adoptive-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 adoptively transferred from 1-y-old, clinically affected Icam1tm1Jcgr/NOD mice (n = 5/experiment and group) into different recipient strains. Total splenocytes were transferred in three experiments (n = 15). CD4+ or CD4− enriched T cells were isolated by using MACS and were transferred in two separate experiments. Positive CD4+ sorting (clone L3T4) and indirect non–CD4+ cell depletion (unaffected 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 in 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 Abs against 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 maintained in the presence of CD3 and CD28 Abs (1 µg/ml each) together with either TGF-β1 (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 × 105/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 scatic notch eliciting the sciatric 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 root, skin, lung, heart, small intestine, liver, kidney, skeletal muscle, sciatric 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 [al5690] Abcam; CD68 [FA-11] AbD Serotec; CD31 [RA3-8B2] AbD Serotec; CD100 [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 autointoabodies, 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 Axioplan 2 microscope (Zeiss). All crossection-related reagents were purchased from Vector Labs.

Intracerebral 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). Intracerebral cytokine profiles were assessed using intracerebral cytokine staining buffers (BD Biosciences), following the manufacturer’s protocol. Briefly, splenocytes (1 × 107/well) in six-well plates were stained 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 CD4 and CD41 and intracellular IFN-γ (XMGL1.2), IL-17 (TC11-181H10), and IL-10 (JES3-19F1) (all from BD Biosciences; clone names in parentheses). Splenocytes were stained for cell surface CD4 and CD25, as well as intracerebral Foxp3 (MF23) using the Foxp3 Buffer Set (all from BD Biosciences). Flow cytometry was performed using a FACSCanto II flow cytometer. For assessing cytokine production, splenocytes were cultured for 72 h in 96-well flat-bottom plates with the presence of soluble anti-CD3 Ab (1 µg/ml) and concentra- tions 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 homogenates 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 protein content by BCA protein assay (Thermo Fischer). Dilutions of tissue samples and of peptides of MPZ (P0106-125, P0180-190; JPT Peptide Technologies), myelin protein 2 (P235-736; JPT Peptide Technologies), and the recombinant proteins Hsp60 (BIOTREND) and GAD65 (Diamyd Diagnostics) were added to spleenocyte cultures. The final concentration of tissue 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<sup>tm1Jcgr</sup>NOD mice and 1 × 10^5 irradiated (10 Gy) NOD spleenocytes 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 Icam<sup>tm1Jcgr</sup>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 mM glutamine, nonessential amino acids, 10 mM HEPES, 0.05 mM 2-ME, and recombinant proteins Hsp60 (BIOTREND) and GAD65 (Diamyd Diagnostics)) 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 (MH1; 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 transduction. In some experiments, flow cytometry was performed using a FACSCanto II flow cytometer (all from BD Biosciences; clone names in 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 Icam<sup>tm1Jcgr</sup>NOD mice and 1 × 10^5 irradiated (10 Gy) NOD spleenocytes 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 extracellular 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/dispace (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 x 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-color fluorophore combinations of Abs (all from BD Biosciences) recognizing CD3 (500A2), CD4 (L3T4), CD8 (53-6.7), CD25 (3C7), CD20 (J5-814), CD22 (RA3-6B2), Ly6G (1A8), CD11b (M1/70), or CD11c (HL3) and analyzed on a FACS Canto 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 Icam<sup>tm1Jcgr</sup>NOD mice. The detection of autoreactive Abs was modified from a previous description

FIGURE 1. Spontaneous chronic progressive inflammatory peripheral neuritis in Icam<sup>tm1Jcgr</sup>NOD mice. (A) Icam<sup>tm1Jcgr</sup>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) Icam<sup>tm1Jcgr</sup>NOD mice, nondiabetic female (n = 18) and male (n = 20) NOD mice, and female Icam<sup>tm1Jcgr</sup>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 Icam<sup>tm1Jcgr</sup>NOD mice (D). Subperineurial (arrow) and endoneurial (arrowhead) cell infiltration are observed in Icam<sup>tm1Jcgr</sup>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 epoxy resin–embedded sciatic nerves from NOD (E) and Icam<sup>tm1Jcgr</sup>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 Icam<sup>tm1Jcgr</sup>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). (B) 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 B220, 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), B220 against CD3 (upper middle panel), B220 against CD11b (lower middle panel), CD11b against CD11c (myeloid dendritic cells; upper right panel), and B220 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 dilutions 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 or nonaffected Icam1tm1Jcgr NOD mice aged 1 y (n = 4 animals/group) using the RNeasy Lipid Tissue Kit (QIAGEN). The size distribution of the individual VB elements length was analyzed using Peak Scanner Software (all from Applied Biosystems). The size distribution of the individual VB elements was rated by a blinded observer, as nondetectable, normal, shifted, or skewed by a blinded observer, according to the manufacturer’s protocol. CDR3-spectratyping PCR was performed using standard reagents (Roche) and VB element–specific forward primers and FAM-labeled reverse primers (MWG Biotech), as previously described (28, 29). Primers were newly designed for elements VB1 (5’-CCAGGTGGTTTATACCTGAATGC-3’), VB3 (5’-CAATAGACATGCTGCAAAACG-3’), VB12 (5’-CCGGCAGCAAGTCTTATGG-3’), VB18 (5’-CTGAAAGCCACATACGAGAGTGGA-3’), and VB20 (5’-GGTCAAGAGAGAGTCTCAGCTGT-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 VB 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 ICAM-1 isoforms, RNA was extracted from thymus of wild-type NOD and Icam1tm1Jcgr NOD mice and transcribed into cDNA as described above, which was used as a template in a standard PCR reaction using primers that were described previously (Fwd, 5’-TGCCCCGCCCCGCTGCAAT-3’, Rev 5’-GGGTCAGCCAGGAGGCTCACA-3’) (15).

**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 prediabetic 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). Spinous roots and CNS tissue did not show any inflammation and no further pathology was identified in other
organs of Icam1<sup>tm1Jcgr</sup>NOD 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, were noted in the PNS of impaired Icam1<sup>tm1Jcgr</sup>NOD mice (Fig. 1F). Again, widespread mononuclear cell infiltrates were observed (Fig. 1F). Thus, Icam1<sup>tm1Jcgr</sup>NOD mice spontaneously develop a severe peripheral neuropathy that features a demyelinating and inflammatory phenotype, and inflammation in Icam1<sup>tm1Jcgr</sup>NOD 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 Icam1<sup>tm1Jcgr</sup>NOD mice. Using an immunohistochemical approach, we detected abundant CD3<sup>+</sup> T cells (Fig. 2A) and CD68<sup>+</sup> macrophages (Fig. 2B) in Icam1<sup>tm1Jcgr</sup>NOD peripheral nerve sections. B220<sup>+</sup> 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<sup>+</sup> hematopoietic cells were retrievable from the PNS of nonaffected and affected Icam1<sup>tm1Jcgr</sup>NOD mice, respectively (Fig. 2D). This confirms that PNS cell infiltration is specific to Icam1<sup>tm1Jcgr</sup>NOD mice and indicates that infiltration precedes, and most probably triggers, clinical impairments.

By flow cytometry, PNS-derived CD45<sup>+</sup> cells in Icam1<sup>tm1Jcgr</sup>NOD mice were composed primarily (∼60%) of T cells and B220<sup>+</sup> B cells (Fig. 2E). T cells were mainly CD4<sup>+</sup> (Fig. 2F). Also, CD11b<sup>+</sup> macrophages were frequently present, and CD11b<sup>+</sup>CD11c<sup>+</sup> myeloid dendritic cells were detectable (Fig. 2E, 2G). B220<sup>+</sup> cells rarely expressed CD11c, arguing against a relevance of plasmacytoid dendritic cells. Low numbers of Ly6G<sup>+</sup> granulocytes were present. In comparison with nonaffected animals, nerve infiltrates in impaired Icam1<sup>tm1Jcgr</sup>NOD mice exhibited significantly higher percentages of CD3<sup>+</sup> T cells and lower amounts of B220<sup>+</sup> B cells (Fig. 2E). Thus, we identified T cells, macrophages, and B cells as the predominant effector cell populations in the inflammatory neuropathy of Icam1<sup>tm1Jcgr</sup>NOD mice.

**CD4<sup>+</sup> T cell–dependent neuritis and proinflammatory Th17 cell skew in Icam1<sup>tm1Jcgr</sup>NOD mice**

We performed adoptive-transfer studies to identify the contribution of individual leukocyte subsets in Icam1<sup>tm1Jcgr</sup>NOD neuritis. Transfer of NOD splenocytes into immunodeficient NOD-SCID mice triggered insulitis and diabetes with 80% penetrance, as
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>+</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](http://www.jimmunol.org/)

**FIGURE 5.** Myelin-targeted serum response and lymphocyte autoreactivity. (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 Icam1tm1Jcgr NOD mice (Fig. 4D). The expression of splenic Foxp3+ regulatory T cells did not differ between groups (data not shown). Thus, the CD4+ T cell–mediated inflammatory neuropathy in Icam1tm1Jcgr NOD mice features a proinflammatory Th17 phenotype.

To further characterize the Icam1tm1Jcgr NOD immune response in the PNS, we screened for differentially regulated immune-relevant mRNA transcripts in the sciatic nerves of Icam1tm1Jcgr NOD mice using a predefined 96-gene murine 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 Icam1tm1Jcgr NOD sciatic nerves after normalization against 18S (Fig. 4E). Among the cytokines analyzed, Cc15 and Cxcl10 were most prominently upregulated (Fig. 4E). Various molecules related to costimulation, expressed by either T cells (CTLA-4, CD28, ICOS) or APCs (CD80, CD86, CD40), were increasingly expressed in the Icam1tm1Jcgr NOD 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-determined autoimmunity. We stained sciatic nerve sections with sera from NOD and Icam1tm1Jcgr NOD mice using a DAB-based (Fig. 5A) and a fluorescent (Fig. 5B) staining approach (27). Serum dilutions from Icam1 CD4+ T cell–mediated inflammatory neuropathy in Icam1tm1Jcgr NOD mice features a proinflammatory Th17 phenotype.

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To identify potential target Ags, we next adopted an immunoblotting approach (27). Serum dilutions from Icam1tm1Jcgr NOD 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). This 28-kDa reactivity was reproducibly observed in individual Icam1tm1Jcgr NOD 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 Icam1tm1Jcgr NOD-defined neuritis.

To confirm this antigenic target in Icam1tm1Jcgr NOD mice, we tested the lymphocyte proliferation response to different stimuli. Lymphocytes from Icam1tm1Jcgr NOD 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 Icam1tm1Jcgr NOD 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 P0180–199 and myelin protein two (P2) peptide P253–73 induced a proliferation response in Icam1tm1Jcgr NOD 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 Icam1tm1Jcgr NOD 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 Icam1tm1Jcgr NOD neuritis. Overall, Ag-specific proliferation was low, which may reflect the chronic and slowly progressive autoimmunity in Icam1tm1Jcgr NOD 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](http://www.jimmunol.org/)

**FIGURE 6.** Th17 differentiation determines the pathogenicity, not the target specificity, of Icam1tm1Jcgr NOD CD4+ T cells. (A) Purified CD4+ T cells from affected Icam1tm1Jcgr NOD 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 (C), Th1 cells (D), or Th17 (E) 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<sup>tm1Jcgr</sup> 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<sup>tm1Jcgr</sup>NOD mouse strain.

**Th17 differentiation determines the pathogenicity, not the target specificity, of autoreactive CD4<sup>+</sup> T cells in Icam1<sup>tm1Jcgr</sup>NOD mice**

We next tested whether the T cell differentiation observed in Icam1<sup>tm1Jcgr</sup>NOD mice determines whether autoimmune diabetes or neuritis evolves. Therefore, we sorted CD4<sup>+</sup> T cells from clinically affected Icam1<sup>tm1Jcgr</sup>NOD mice and differentiated these into predominantly IFN-γ–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<sup>tm1Jcgr</sup>-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<sup>+</sup> T cells from Icam1<sup>tm1Jcgr</sup>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<sup>tm1Jcgr</sup>NOD mice.

![FIGURE 7.](http://www.jimmunol.org/)

**A** and **B** Female conventional NOD mice aged 4 wk (adult) received i.p. injections of anti–ICAM-1 Ab (2.5 μg/g of body weight in 100 μl saline; n = 10, □) or saline only (n = 10, ○) three times/wk for 3 wk and then once weekly for an additional 4 wk. Subsequently, animals were tested weekly 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<sup>tm1Jcgr</sup>-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<sup>+</sup> T cells from Icam1<sup>tm1Jcgr</sup>NOD mice.

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**FIGURE 7.** Anti–ICAM-1 Ab treatment triggers neuritis only in juvenile NOD mice. (A and B) Female conventional NOD mice aged 4 wk (adult) received i.p. injections of anti–ICAM-1 Ab (2.5 μg/g of body weight in 100 μl saline; n = 10, □) or saline only (n = 10, ○) three times/wk for 3 wk and then once weekly for an additional 4 wk. Subsequently, animals were tested weekly for glucosuria (A) and clinical signs of neuropathy (B) for 35 weeks. (C and D) Female NOD mice aged 10 d (juvenile) received anti–ICAM-1 Ab (n = 15, □) or saline (n = 18, ○) for 7 wk following the identical protocol as for adult mice. The bracketed lines below the x-axis in (A)–(D) indicate the duration of ICAM-1 Ab treatment. Animals were tested weekly for glucosuria (C) and for clinical signs of neuropathy (D) for 35 wk. (E) Sciatic nerve electrophysiology was performed at the end of the experiment in PBS-treated (upper panel) and anti–ICAM-1 Ab–treated (lower panel) juvenile animals, and NCVs were calculated. (F) H&E-stained paraffin sections of pancreas (upper panels) and sciatic nerves (lower panels) were prepared from saline-treated (left panels) and anti–ICAM-1 Ab–treated (right panels) juvenile NOD mice. Scale bars, 100 μm.
Nerve conduction studies were normal in saline-treated NOD mice and were abnormal in clinically affected anti-ICAM-1 Ab–treated NOD mice (Fig. 7E); average NCV was reduced to 63% of normal in affected animals. Corresponding to the clinical and electrophysiological phenotype, histology demonstrated inflammatory infiltrates predominately in pancreas sections of saline-treated NOD mice and only in peripheral nerve sections of affected anti–ICAM-1–treated NOD mice (Fig. 7F). Of note, this experiment was performed in conventional nonspecific pathogen–free housing, which may explain the lower and delayed incidence of diabetes in juvenile NOD mice, because the incidence of NOD diabetes decreases with viral (35) and other infections. Thus, ICAM-1 blockade was required early postnatally for neuritis to occur. Therefore, we hypothesized that the specificity of T cells selected by a NOD thymus is altered in Icam1tm1JcgrNOD mice.

Icam1tm1JcgrNOD thymus induces peripheral neuritis in athymic nude mice

To test this hypothesis in vivo, we dissected and cultured thymi from neonatal female NOD and Icam1tm1JcgrNOD mice in the presence of deoxyguanosine to deplete hematopoietic cells. We then transplanted these thymic rudiments under the kidney capsule of female athymic nude (Foxn1−/−) 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+ 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 Icam1tm1JcgrNOD (NudeIcam1tm1JcgrNOD mice) thymi or NOD (NudeNOD mice) thymi, respectively. All animals were tested weekly for the occurrence of diabetes and neuropathy. Within 180 d of follow-up, 6 of 14 (42.9%) NudeIcam1tm1JcgrNOD mice developed slowly progressing tetraparesis (Fig. 8A) with a continuously increasing incidence (Fig. 8B). Affected NudeIcam1tm1JcgrNOD mice showed electrophysiological signs of peripheral nerve demyelination and reduced NCV (Fig. 8C), whereas electrophysiological abnormalities were absent in NudeNOD mice (Fig. 8C). Paraffin-embedded sections of sciatic nerve revealed mild, but consistently occurring, infiltration of mononuclear cells in affected NudeIcam1tm1JcgrNOD animals (Fig. 8D), whereas nude mice (NudeNOD) 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 Icam1tm1JcgrNOD thymus induces neuritis in athymic nude mice. (A) Thymi from female neonatal Icam1tm1JcgrNOD (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 Icam1tm1JcgrNOD thymus (NudeIcam1tm1JcgrNOD) with hind limb paresis (arrow). (B) All recipients were tested weekly for glucosuria and clinical impairments for up to 180 d. NudeNOD mice (○), but none of the recipients of NOD thymus (NudeNOD, □), developed signs of neuropathy. (C) Sciatic nerve electrophysiology was performed in NudeNOD mice (upper panel) and NudeIcam1tm1JcgrNOD mice (lower panel), and the average NCV was calculated. (D) Paraffin-embedded sciatic nerve sections were generated from NudeNOD mice (left panel) and neuropathic NudeIcam1tm1JcgrNOD mice (right panel). Scale bars, 100 μm. (E) Semi-thin sciatic nerve sections were generated from neuropathic NudeIcam1tm1JcgrNOD 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 NudeNOD mice (left panel) or neuropathic NudeIcam1tm1JcgrNOD 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 lcam1<sup>tm1Jcgr</sup>NOD mice, sera from affected Nude<sup>lcam1<sup>tm1Jcgr</sup>NOD</sup> mice, but not Nude<sup>lcam1<sup>tm1Jcgr</sup></sup>NOD mice, stained PNS myelin in cryosections (Fig. 8F). This indicates that Nude<sup>lcam1<sup>tm1Jcgr</sup>NOD</sup> mice develop myelin autoreactivity similar to lcam1<sup>tm1Jcgr</sup>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<sup>+</sup> cells (Fig. 9). The average CD80-staining intensity and the proportion of CD80-expressing thymic epithelial cells were significantly increased in lcam1<sup>tm1Jcgr</sup>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 lcam1<sup>tm1Jcgr</sup>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 lcam1<sup>tm1Jcgr</sup>NOD mice**

To address this, we next performed CDR3 spectratyping using spleen, sciatic nerve, and pancreas tissue of NOD and lcam1<sup>tm1Jcgr</sup>NOD mice and tested for the generation of a differential V<sub>β</sub> element repertoire. A proportion of Vβ elements was nondetectable (Vβ3, 13, 17, 18, 19), equally expanded in both genotypes (Vβ8.2, 9, 10, 11, 14, 20), or showed a nonconsistent pattern of expansion in either tissue or genotype (Vβ1, 4, 6) (Supplemental Tables I–III). The remaining Vβ elements (Vβ2, 5, 8.1, 8.3, 12, 15, 16) showed a genotype-specific pattern of expansion. Specifically, Vβ2, Vβ12, and Vβ16 were only skewed in the PNS of affected lcam1<sup>tm1Jcgr</sup>NOD mice but generally were not detectable in NOD mice (Fig. 9E, Supplemental Table II). The distribution of Vβ5 showed greater abnormalities across all tissues in NOD mice compared with lcam1<sup>tm1Jcgr</sup>NOD mice (Fig. 9E, Supplemental Tables I–III). Vβ8.1 was shifted predominantly in the spleen of NOD mice (Supplemental Table I), whereas Vβ8.3 and Vβ15 were shifted predominantly in the spleen of lcam1<sup>tm1Jcgr</sup>NOD mice (Fig. 9F, Supplemental Table I). This indicates that diabetes in NOD mice and neuritis in lcam1<sup>tm1Jcgr</sup>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 lcam1<sup>tm1Jcgr</sup>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<sup>+</sup> 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<sup>tm1Jcgr</sup> 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 autoimmunity specifically from pancreas to the PNS by a thymic selection–dependent mechanism.

Our study has technical limitations. The Icam1<sup>tm1Jcgr</sup> 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<sup>tm8Aab</sup> mouse line (15), whereas only the Icam1<sup>tm1Alb</sup> 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<sup>tm1Alb</sup>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<sup>tm1Jcgr</sup> 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<sup>−/−</sup> NOD mice develop peripheral neuritis (4), PD-1<sup>−/−</sup> NOD-H<sup>2<sup>b</sup></sup> mice develop neuritis together with inflammation of stomach and exocrine tissues (8), and ICOS-L<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>tm1Jcgr</sup>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<sup>tm1Jcgr</sup>NOD mouse into a NOD-SCID mouse encounters an intact ICAM-1 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<sup>tm1Jcgr</sup>NOD neuritis (Fig. 6). The fact that disease severity determines Th17 cells in Icam1<sup>tm1Jcgr</sup>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<sup>tm1Jcgr</sup>NOD mice (Fig. 9), and the induction of neuritis by Icam1<sup>tm1Jcgr</sup>NOD thymic epithelium alone (Fig. 8) support that an altered expression of ICAM-1 on thymic epithelium educates a neuropigenic, 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<sup>2<sup>b</sup></sup> 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<sup>+</sup> 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<sup>+</sup> regulatory T cells in Icam1<sup>tm1Jcgr</sup>NOD mice and or observe modulation of target specificity by Th cell differentiation. Instead, we provide evidence that the selection of the TCR repertoire is altered by Icam1<sup>tm1Jcgr</sup>NOD 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 autoimmune 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 costimulation defines the target specificity of autoimmunity in the PNS that has potential relevance for human inflammatory neuropathies.

Acknowledgments

We thank Zippora Kohne, Bianca Wolff, and Tatjana Males for excellent technical assistance; Ruth Stassart (Department of Neurogenetics, Max-Planck-Institute for Experimental Medicine, Göttingen, Germany) for help with epoxy resin embedding; and Dr. J. Archelos for providing MPZ Ab.

Disclosures

The authors have no financial conflicts of interest.

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