Mediation of Protection and Recovery From Experimental Autoimmune Encephalomyelitis by Macrophages Expressing the Human Voltage-Gated Sodium Channel NaV1.5

Kusha Rahgozar, BS, Erik Wright, BS, Lisette M. Carrithers, BA, and Michael D. Carrithers, MD, PhD

Abstract

Multiple sclerosis (MS) is the most common nontraumatic cause of neurologic disability in young adults. Despite treatment, progressive tissue injury leads to accumulation of disability in many patients. Here, our goal was to develop an immune-mediated strategy to promote tissue repair and clinical recovery in an MS animal model. We previously demonstrated that a variant of the voltage-gated sodium channel NaV1.5 is expressed intracellularly in human macrophages, and that it regulates cellular signaling. This channel is not expressed in mouse macrophages, which has limited the study of its functions. To overcome this obstacle, we developed a novel transgenic mouse model (C57BL6/N-Tg(Human NaV1.5)SCV5), in which the human macrophage NaV1.5 splice variant is expressed in vivo in mouse macrophages. These mice were protected from experimental autoimmune encephalomyelitis, the mouse model of MS. During active inflammatory disease, NaV1.5-positive macrophages were found in spinal cord lesions where they formed phagocytic cell clusters; they expressed markers of alternative activation during recovery. NaV1.5-positive macrophages that were adoptively transferred into wild-type recipients with established experimental autoimmune encephalomyelitis homed to lesions and promoted recovery. These results suggest that NaV1.5-positive macrophages enhance recovery from CNS inflammatory disease and could potentially be developed as a cell-based therapy for the treatment of MS.

Key Words: Alternative activation, Experimental autoimmune encephalomyelitis, Macrophage, Multiple sclerosis, Sodium channel.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory presumed autoimmune disease of the CNS and is a common cause of neurologic disability (1). Although several immune-modulating therapies are available to treat MS patients, there is very limited efficacy on measures of disease progression and accumulation of neurologic deficits (2). Some investigators have hypothesized that innate immune mechanisms mediate chronic tissue injury in MS lesions (3). This hypothesis is based in part on the pathology of lesions, which demonstrate the presence of macrophages and activated microglia (4). Although the lineage of microglia has been controversial, recent studies indicate that they are resident mononuclear phagocytic cells of the brain and are derived from primitive macrophages (5).

Mononuclear phagocytes are highly motile cells that mediate host defense and tissue homeostasis. Metchnikoff was the first to describe the crucial role of phagocytes in the clearance of infectious pathogens and injured cells (6). The biochemical mechanisms and physiologic relevance of macrophage phagocytosis of pathogens have been areas of intense study since then, but their role in what Metchnikoff termed “physiological inflammation” has until recently been largely ignored. Consistent with the emerging role of alternatively activated macrophages in tissue repair (7), he hypothesized that phagocytes not only mediated host defense but were also necessary for recovery from tissue injury.

We have focused on how novel sodium channel variants regulate human macrophage function (8–10). Intracellular expression and activity of NaV1.5 and NaV1.6 variants in macrophages regulate basic cellular functions that are necessary for optimal innate immune responses. The initial description of sodium channel expression in phagocytes occurred during studies of the neuroprotective effects of sodium channel blockers in the murine MS model experimental autoimmune encephalomyelitis (EAE). In this work, the expression of the voltage-gated sodium channel, NaV1.6, was demonstrated in microglia from brains of mice with EAE and humans with MS (11). We subsequently characterized NaV1.5 and NaV1.6 expression and function in macrophages (8–10). These studies demonstrated that NaV1.5 regulates phagocytosis in human macrophages, and that NaV1.6 regulates cellular movement through its association with the F-actin cytoskeleton and regulation of podosome formation. Black et al (12) demonstrated NaV1.5 expression in phagocytes within MS lesions, but this variant is not expressed in mouse macrophages. Unlike sodium channels in excitable tissues in which they are present on the plasma membrane, macrophages NaV1.5 and NaV1.6 are expressed on intracellular organelles where they regulate intracellular signaling in part through mitochondrial sodium-calcium exchange (8, 10).
Despite the in vitro findings, the relevance of macrophage sodium channels to immune-mediated disease and regulation of cellular phenotype remains unclear. This issue is particularly pertinent to the functional significance of human macrophage NaV1.5 because it is not expressed in murine macrophages and could not be readily studied in mouse EAE models. For example, enhanced phagocytosis by macrophages might enhance recovery from tissue injury through clearance of extracellular debris and apoptotic cells (13). Alternatively, inflammatory macrophages could enhance tissue injury through local release of reactive oxygen and nitrogen species. In view of the in vivo complexity of macrophage phenotype and function, the phenotype of human NaV1.5-positive macrophages needs to be assessed in an in vivo model.

To examine in vivo function of macrophage NaV1.5, we developed a knock-in mouse in which the human macrophage NaV1.5 splice variant is expressed selectively in monocytomacrophage lineage cells (C57BL/c-fms-hSCN5A mice). These mice developed much less severe EAE despite the presence of CNS inflammatory infiltrates. The macrophages in these mice demonstrated an unexpected polarization to an arginase-positive phenotype during EAE; and, when adoptively transferred to mice with clinical disease, the cells entered the CNS and enhanced recovery.

**MATERIALS AND METHODS**

**Cells**

Primary mouse bone marrow cells were obtained from femurs and/or tibia of transgenic and wild-type (WT) mice and were differentiated to bone marrow–derived macrophages (BMDMs) in RPMI media supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, and macrophage colony-stimulating factor (20 ng/mL) for 5 to 7 days. For adoptive transfer experiments, cells were dissociated with TrypLE (Invitrogen, Carlsbad, CA), washed, resuspended in PBS, and then injected intraperitoneally into recipient mice (5 × 10^5 cells per mouse).

THP-1 cells, a human monocytic cell line, were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, sodium pyruvate, and nonessential amino acids. Differentiation to a macrophage phenotype was induced by treatment with 12-O-tetradecanoylphorbol-13-acetate (10 ng/mL) for 72 hours.

cDNA Cloning

SCN5A is the gene that encodes NaV1.5. A polymerase chain reaction (PCR)–based approach was used to sequence SCN5A cDNA generated from differentiated THP-1 cells (9). Messenger RNA was isolated and reverse transcribed. To obtain the full-length coding region of SCN5A, 2 separate PCR products were generated and then stitched together. The first PCR product was generated using exon 3 forward and exon 27 reverse primers. The second one used exon 2 forward and exon 3 reverse primers (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A453). These 2 PCR products were gel purified and used as a template for another PCR reaction using exon 2 forward and exon 27 reverse primers to obtain the full-length cDNA (6 kb). Polymerase chain reaction was performed using platinum Taq DNA polymerase high fidelity (Invitrogen) according to the recommendations of the supplier. Cycle conditions were as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 7 minutes for the first PCR product and 1 minute for the second one (40 cycles). The full-length PCR product was sequenced in the forward and reverse directions at the Keck Biotechnology core facility at Yale University School of Medicine using the listed primers. DNASTAR (Lasergene) was used for the analysis of sequence data.

**Human Macrophage SCNSa Transgene Construct**

The cDNA for the full-length coding region of the SCN5A splice variant was spliced downstream of a mouse c-fms promoter. This promoter contains a 3.5-kb 5′ flanking sequence of the c-fms gene and the downstream intron 2 (14). Pronuclear injection, generation of founder mice, and Southern blot analysis were performed at inGenious Laboratories, Stony Brook, NY. This construct specifically targets expression of the human macrophage SCN5A splice variant to the monocyte-macrophage lineage.

**Quantitative PCR**

RNA purification, reverse transcription, quantitative PCR, and data analysis were performed as described previously (10). Data were acquired on a Cepheid SmartCycler and analyzed by the ΔΔCt method. The following TaqMan primers were obtained from Applied Biosystems: dHs00165693_m1 (human SCN5A), Mm99999915_g1 (mouse gapdh), Mm99999915_g1 (mouse cdhl), and Mm00475988_m1 (mouse argl).

**Mice**

Experimental mouse strains were bred at our on-campus breeding facility (Biotron facility), where our transgenic colony is maintained. Experimental mice were transferred to an approved University of Wisconsin animal facility for the performance and monitoring of all in vivo experiments. Age (12–16 weeks) and sex-matched transgene-positive and negative (WT) littermates were used for EAE experiments, and transgene-positive mice were used as bone marrow donors. Seven to 13 mice were used for each EAE condition, and 3 to 4 mice for each histologic and flow cytometry condition (same age and sex ratios as for EAE experiments).

**EAE Induction**

A commercially available kit (Hooke Laboratories, Lawrence, MA) was used to induce EAE. Mice were immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35-55) emulsified in complete Freund adjuvant by injecting them subcutaneously at 2 sites on the back (0.1 mL of emulsion per site; 1 mg/mL MOG35-55, and 2.5 mg/mL killed H37Ra Mycobacterium tuberculosis). On the same and subsequent days, an intraperitoneal injection of pertussis toxin in PBS, at 100 ng per mouse per dose (0.1 mL), was performed. Mice were examined daily and were given additional soft food (Dough Diet) and a hydration source (Napa Nectar) in the...
were stained for immunofluorescence with anti-neurofilament mice immunized with MOG35-55 were analyzed (onset of peak 6-diamidino-2-phenylindole (DAPI). Whole spinal cords from ical signs.

Flow Cytometry

Cell preparation, staining, and analysis for flow cytometry were performed as described previously (15, 18). For analysis of CNS mononuclear cells, whole spinal cord was used (19). Data acquisition was performed on an LSRII flow cytometer (Becton-Dickinson) at the University of Wisconsin Carbone Cancer Center core facility at the Wisconsin Institute for Medical Research. The following antibodies were used: rabbit anti-NaV1.5 (Alomone); sheep anti–arginase 1 (fluorescein; R&D Systems, Minneapolis, MN); APC-labeled anti-F4/80 (clone BM8), APC-Fluor 780 CD4 (GK1.5), PE-CD8, eFluor 450 CD11b (M1/70), PerCP-eFluor 710 CD324 (DECA-1), and fluorescein isothiocyanate– interleukin 10 (IL10) were from eBioscience. Secondary antibodies for anti-NaV1.5 were either Alexa 488 goat–anti-rabbit (Invitrogen) or PE-labeled goat anti-rabbit Fab fragments (eBioscience). Similar results were obtained for both approaches. Isotype controls were obtained from eBioscience.

Data Analysis

Data were analyzed using Axiovision 4.8 (Zeiss) and FlowJo (Treestar, Ashland, OR) software. Statistical analysis (SEM calculation, analysis of variance, and \( t \) test) was performed using Kaleidagraph 4.1 (Synergy).

RESULTS

cDNA Cloning of Macrophage SCN5A Reveals That It Is a Novel Splice Variant

Complementary DNA cloning of human macrophage SCN5A (hSCN5A) demonstrated that it was a novel splice variant that lacked exon 25, contained exon 7, and was most homologous to a predicted splice variant identified as SCN5A transcript variant 4 (Table 1; Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A453). Exons 6 and 7 are alternate, duplicate coding exons; however, exon 25 is an in-frame coding region that encodes an extracellular portion of the channel. Deletion of exon 25 results in an 18–amino acid deletion in NaV1.5 (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A454).

Generation of C57BL\( ^{c-fms-hSCN5A}\) Mice

Because NaV1.5 is not expressed in murine macrophages, it was necessary to develop a transgenic model to study its in vivo function (Fig. 1). We selected the \( c-fms \) promoter, which encodes the macrophage colony-stimulating factor receptor, to direct expression of the human SCN5A channel variant in all macrophage subsets, including monocytes, macrophages, and phagocytic bone marrow–derived microglia (14). As indicated in a recent review, it is not intrinsically possible to limit transgene expression solely to classic macrophages with any of the macrophage-specific promoters that have been used, particularly for cell ablation studies (20). The advantage of the \( c-fms \) promoter construct

Histology

Perfusions, tissue preparation for frozen sections, sectioning, and staining for immunofluorescence were performed as described (8, 15). The following antibodies were used for immunohistochemistry: rabbit anti-human NaV1.5 (Alomone Labs, Jerusalem, Israel); anti–arginase 1 (clone 19/Arginase 1; BD Biosciences, San Jose, CA); anti-F4/80 (Cl:A3-1), rabbit anti–E cadherin, mouse (MBP101) and rabbit anti–myelin basic protein, and rabbit anti–200-kd neurofilament were from Abcam (Cambridge, MA); anti–phospho-PHF-tau (paired helical filaments tau, pSer202/Thr205, clone AT8) was from Thermo Fisher; and anti-CD11b (M1/70) and anti-CD4 (GK1.5) were from eBioscience (San Diego, CA). Alexa dye (488 or 555) labeled secondary antibodies (donkey anti-rabbit, rat, or mouse) were from Invitrogen. Isotype controls were obtained from eBioscience. Processing and staining for hematoxylin and eosin staining were performed at the University of Wisconsin Department of Pathology core facility.

Fluorescence Microscopy

Fluorescence images were acquired and analyzed using a Zeiss Axiocert 200 fluorescent microscope equipped with Axiovision version 4.8 software. Quantitative analysis of hyperphosphorylated tau staining was performed as a modification of our previous approach (16, 17). Frozen longitudinal sections (8 \( \mu \)m) of mouse spinal cord samples were separated by more than 20 \( \mu \)m to avoid staining the same cell twice. Sections were stained for immunofluorescence with anti-neurofilament and anti–phospho tau and subsequently with the nuclear stain 4, 6-diamidino-2-phenylindole (DAPI). Whole spinal cords from mice immunized with MOG35-55 were analyzed (onset of peak disease, Day 19), and regions of inflammatory infiltrates, as defined by DAPI staining and analysis of serial sections stained for immune cell markers, were imaged using a 20× objective (Zeiss) within a 400 \( \times \) 400 \( \mu \)m2 microscopic field. Three complete sections from 3 separate spinal cords were analyzed for each condition. The approximate area of infiltrate per longitudinal cross section in the WT spinal cord was 31.2 ± 3.7 \( \mu \)m2 versus 8.2 ± 1.3 \( \mu \)m2 in the transgensics. Images were analyzed using the AutoMeasurement component of Axiovision software; areas of phospho tau staining were expressed as square micrometers per square millimeter tissue.

bottom of their cage if needed. Mice were followed for up to 40 days. Animals were observed on a daily basis for signs of clinical EAE. The animals were graded by a blinded examiner as follows: 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, hind and front limb paralysis; and 5, moribund. Mice were killed if they developed a score of 4. Animals were cared for in accordance with the University of Wisconsin-Madison IACUC guidelines and the National Re-
TABLE 1. Macrophage SCN5A Is a Splice Variant Homologous to Transcript Variant 4

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<td>6</td>
<td>99.3</td>
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<td>NM_001099404</td>
<td>c</td>
<td>3</td>
<td>7</td>
<td>99.4</td>
</tr>
<tr>
<td>NM_001099405</td>
<td>d</td>
<td>4</td>
<td>7 and 25</td>
<td>99.5</td>
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</table>

*KC858891 (Macrophage variant)

Total RNA was isolated from differentiated and primed THP-1 cells and reverse-transcribed to cDNA. PCR amplification of SCN5A was performed using gene specific primers to generate a 6-kb fragment that was purified and cloned into a TA cloning vector. Nucleotide sequencing was performed at the Keck facility at the Yale University School of Medicine. Sequencing was performed from both the forward and reverse ends to provide sequence confirmation. Sequence comparisons were performed using DNAStar software (Lasergene).

for gene knock-in studies is that it results in reliable expression of genes in F4/80-positive macrophages with a lower level of expression in other myeloid cells. Mouse founder lines were screened by Southern blotting (inGenious Laboratories). All founder lines that screened positive by Southern blotting were screened by Southern blotting (inGenious Labora-
tories). All founder lines that screened positive by Southern blotting were healthy and viable; the line that best demonstrated human SCN5A expression in BMDMs was used for subsequent experiments.

Human Macrophage NaV1.5 Is Expressed in Monocyte-Macrophages From C57BL6-c-fms-hSCN5A Mice

Heterozygous transgenic mice had a normal appearance, life span, thymic and splenic development, and cell number (data not shown). Analysis of peripheral blood C11b-positive, F4/80-positive cells and BMDMs by flow cytometry and immunohistochemistry demonstrated expression of human macrophage NaV1.5 (hNaV1.5), the protein encoded by hSCN5A (Fig. 2A–C).

Bone marrow–derived macrophages from C57BL6-c-fms-hSCN5A mice also demonstrated expression of hSCN5A mRNA. Human SCN5A mRNA expression in transgenic BMDM was similar to that previously observed in primary monocyte-derived human macrophages (8). The expression of SCN5A in transgenic cells was 10.1 ± 0.3 copies/GAPDH × 10⁻⁵. hSCN5A expression was not detected threshold cycle >50 in WT cells. In addition, one unexpected result was that, in the absence of any polarizing cytokines, quantitative PCR analysis demonstrated an approximately 3-fold increase in arginase (arg1) and E-cadherin (cdh1) mRNA expression (Fig. 3A). Increased expression of these molecules serves as a marker for alternative macrophage activation (7, 21). In addition, E-cadherin is expressed within phagocytic nodules and early granulomas to facilitate cell-cell adhesion (22). To assess markers of alternative activation further, we also performed intracellular staining for arg1 and IL10 (Fig. 3B). As expected, the percent of arg1-positive BMDMs was increased in the transgenic condition as compared with that in WT controls. In addition, hNaV1.5-positive BMDMs expressed intermediate and high levels of IL10, whereas control cells demonstrated a low expression.

Decreased EAE Severity in C57BL6-c-fms-hSCN5A Mice

We used the MOG135–155 immunization model to assess disease course in age- and sex-matched transgenic mice and WT littersmates. C57BL6-c-fms-hSCN5A mice demonstrated reduced peak disease severity, cumulative disease score, and disease incidence (Fig. 4; Table 2). These results suggested that the transgenic mice have either an impaired immune response or enhanced anti-inflammatory regulatory mechanisms.

Reduced CD4 T-Lymphocyte Infiltration and Presence of Mononuclear Phagocytic Cell Clusters During EAE in C57BL6-c-fms-hSCN5A Mice

To assess the possible mechanisms of disease protection in C57BL6-c-fms-hSCN5A mice, we analyzed inflammatory cell infiltrates from transgenic mice and sex-matched WT littermates by flow cytometry and histologic analysis. At the approximate onset of peak disease (~Day 19), transgenic and WT mice demonstrated similar numbers of total mononuclear cells but an altered ratio of CD4/CD11b cells (Fig. 5). Spinal cord infiltrates from transgenic mice demonstrated a reduced but detectable frequency of CD4-positive T lymphocytes and an increased frequency of CD11b-positive immune cells.

Total mononuclear cell counts per spinal cord were 65,338 ± 4,686 cells in the transgenic and 55,778 ± 5,271 cells in the WT mice (n = 3, not significant). The cell frequencies were CD11b, 79.3% ± 1.2% in the transgenic versus 52.5% ± 5.4% in the WT (n = 3, p < 0.01); CD4, 5.6% ± 0.5% in the transgenic versus 18.7% ± 1.2% in the WT (n = 3, p < 0.01); there were no significant differences in CD8 frequencies. These results suggested that immune cells from C57BL6-c-fms-hSCN5A mice can respond to immunization and home to inflamed spinal cord, but that there is a reduced CD4 T cell–mediated injury response in the target organ.
Histologic analysis of spinal cord infiltrates at the same time point demonstrated extensive inflammation and tissue injury in WT mice but more limited, and clearly demarcated, demyelinating lesions in C57BL6c-fms-hSCN5A mice (Fig. 6). In the transgenic mice, the lesions had a more “punched-out” appearance and consisted predominantly of F4/80-positive phagocytic cells (Fig. 6C), and many of these were immunopositive for hNaV1.5 (Fig. 7). Figures 6C and 7 show higher power views of the lesions shown in Figure 6B. Cells within these clusters also expressed CD11b.

Wild-type lesions demonstrated large linear regions of injury and showed a greater number of CD4-positive cells, with fewer F4/80-positive cells (Fig. 6B, C). These observations not only confirmed the results from flow cytometry but suggest that hNaV1.5-positive phagocytes have enhanced capacity to form discrete clusters within lesions. In a general sense, phagocytic clusters might be a precursor to granuloma formation and contain and limit local inflammatory responses (23). Such clusters within the CNS might represent a restricted host response to injury that prevents dissemination inflammation and resolves without residual injury (24, 25).

**Decreased Axonal Injury in C57BL6c-fms-hSCN5A Mice**

We further examined lesional tissue injury by staining for phospho-PHF-tau and neurofilament to assess axonal injury at onset of peak disease (Day 19) (16).

In spinal cord lesions from WT littermate controls, there was diffuse axonal injury, as determined by axonal swelling and the presence of phospho-PHF tau that colocalized with neurofilament staining; whereas in transgenic mice, only a minimal degree of phospho-PHF tau staining was detected and was found primarily in phagocytic cells within clusters (Fig. 8).

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**FIGURE 2.** The human macrophage NaV1.5 variant (hNaV1.5) is expressed in mouse peripheral blood mononuclear cells (PBMCs) and bone marrow–derived macrophages (BMDMs) from C57BL6c-fms-hSCN5A mice. (A) PBMCs were isolated and stained for surface markers and intracellular hNaV1.5 expression. Rabbit isotype control staining for NaV1.5 is also shown in the lower tracing. (B) BMDMs from mature mice (hSCN5A) were differentiated for 10 days in the presence of macrophage colony-stimulating factor and then analyzed by immunohistochemistry. Low-power views show coexpression of hNaV1.5 and the mouse macrophage marker F4/80 in BMDMs. The higher power view (bottom right) demonstrates a vesicular-type staining pattern for hNaV1.5, similar to that observed in primary human macrophages and cell lines. Scale bars = 50 μm (low power) and 10 μm (high power). (C) Flow cytometry analysis confirmed hNaV1.5 expression in most F4/80-positive BMDMs. Staining for hNaV1.5 was assessed using a rabbit anti-human NaV1.5; rabbit isotype control staining is shown in the right tracing.
Figure 3. Quantitative PCR (qPCR) and fluorescence-activated cell sorting analysis of macrophage markers. (A) The hSCN5A encodes the human NaV1.5 protein. Bone marrow–derived macrophages (BMDMs) were differentiated in macrophage colony-stimulating factor for 5 to 7 days and then analyzed for mRNA expression by qPCR. Threshold cycle (Ct) values were normalized using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a control for each experiment; the ΔΔCt method was used to calculate copy number relative to GAPDH expression. Arginase (arg1) and E-cadherin (cdh1) expression in macrophages derived from C57BL/6 c-fms-hSCN5A mice were increased approximately 3-fold versus wild-type mice. The measured mRNA levels were arg1, 12.03 ± 0.69 copies/GAPDH × 10^-5 in the transgenic group and 4.5 ± 0.17 copies/GAPDH × 10^-5 (n = 4, p < 0.001); cdh1, 1.27 ± 0.13 copies/GAPDH × 10^-5 in the transgenic group and 0.45 ± 0.03 copies/GAPDH × 10^-5 in the wild-type group (n = 4, p < 0.05). (B) The percent of arginase protein-positive cells was increased in hSCN5A-positive mouse BMDMs as determined by intracellular staining and flow cytometry (left tracing). In addition, the percent of high and intermediate interleukin 10 (IL10)–expressing cells was greater in the transgenic cells as compared with that in littermate controls (right tracing).
Within regions that contained inflammatory infiltrates, the area of phospho-PHF tau staining was $17,484 \pm 2,868 \mu m^2/mm^2$ tissue in the WT and $223 \pm 75 \mu m^2/mm^2$ tissue in the transgenics ($p<0.01$). These results suggested that there was decreased axonal injury in the C57BL6 c-fms-hSCN5A mice.

Arginase-1 and E-Cadherin Expression in Phagocytic Cell Clusters

Increased expressions of arginase and E-cadherin serve as markers for alternative macrophage activation (7, 21), and E-cadherin (cdh1) is also expressed within phagocytic clusters and early granulomas to facilitate cell-cell adhesion (22). This analysis was performed on sections from mice from a slightly later period (recovery or stabilization, 10 days from disease onset, approximately Day 24), so that sufficient numbers of myeloid cells would be present within WT lesions.

Consistent with our hypothesis that macrophage NaV1.5 expression facilitates alternative activation, phagocytic cell clusters within lesions from C57BL6 c-fms-hSCN5A mice demonstrated high levels of expression of cdh1 and arginase (arg1) in CD11b-positive cells. In contrast, in WT littermate controls, arg1 expression was only detected in nerve fibers, and cdh1 was expressed at low levels in only a few CD11b-positive cells. These results suggested that lesional phagocytes have an altered phenotype in C57BL6 c-fms-hSCN5A mice.

**FIGURE 4.** C57BL6 c-fms-hSCN5A (hSCN5A) mice demonstrate decreased severity of experimental autoimmune encephalomyelitis (EAE) as compared with wild-type mice. EAE was induced, and the mice were evaluated clinically as described in the Materials and Methods section. Clinical data are shown Table 2.

**FIGURE 5.** Flow cytometry analysis of CNS mononuclear cells during experimental autoimmune encephalomyelitis (EAE) in C57BL6 c-fms-hSCN5A (hSCN5A) and wild-type (WT) mice. Single-cell preparations from mouse spinal cord were performed at onset of peak disease (5 days after disease onset, approximately Day 19), stained for cell markers, and analyzed. (A) A representative plot is shown that demonstrates an increased ratio of CD11b-positive/CD4-positive cells in hSCN5A mice versus WT littermate controls. (B) Quantitative analysis of cell counts and frequencies revealed that there were similar numbers of cells within the mononuclear immune cell gate; there were altered cell frequencies of CD11b-positive and CD4-positive but not CD8-positive populations. The cell counts for total mononuclear cells per spinal cord were $65,538 \pm 4,686$ for the hSCN5A transgenic and $55,778 \pm 5,271$ for the WT ($n=3$, not significant). The percent CD11b positive was $79.3 \pm 1.2$ for the transgenic and $52.5 \pm 5.4$ for the WT ($p<0.01$). The percent CD4 positive was $5.6 \pm 0.5$ for the transgenic and $18.7 \pm 1.2$ for the WT ($p<0.01$).

CD11b-positive cells (Fig. 9). In contrast, in WT littermate controls, arg1 expression was only detected in nerve fibers, and cdh1 was expressed at low levels in only a few CD11b-positive cells. These results suggested that lesional phagocytes have an altered phenotype in C57BL6 c-fms-hSCN5A mice.

**TABLE 2.** Experimental Autoimmune Encephalomyelitis Clinical Data

<table>
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<tr>
<th>Strain</th>
<th>No. Mice (male/female)</th>
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<td>13/13</td>
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<td>39.1 ± 7.8</td>
</tr>
<tr>
<td>C57BL6 c-fms-hSCN5A</td>
<td>7/4</td>
<td>3/11</td>
<td>0.9 ± 0.6*</td>
<td>10.9 ± 7.1*</td>
</tr>
</tbody>
</table>

All recipient and donor mice were 12 to 16 weeks of age and were age- and sex-matched littermates.

* $p \leq 0.05$
FIGURE 7. Human NaV1.5 expression in spinal cord demyelinating lesions in C57BL6-c-fms-hSCN5A (hSCN5A) mice with experimental autoimmune encephalomyelitis. Many of the cells within the phagocytic nodular lesions in transgenic mice are NaV1.5 positive (red, middle panel). Myelin basic protein (MBP) immunostaining is shown in green. Scale bar = 20 μm.

FIGURE 8. Analysis of spinal cord lesions during experimental autoimmune encephalomyelitis reveals decreased axonal injury in C57BL6-c-fms-hSCN5A (hSCN5A) mice. Inflammatory spinal cord lesions were analyzed for evidence of axonal injury using phospho-PHF tau (AT8; green) and neurofilament (NF; red) immunohistochemistry. Wild-type (WT) mice (upper micrographs) demonstrated more severe axonal injury as compared with C57BL6-c-fms-hSCN5A mice (lower micrographs). Lesions in WT mice show increased numbers of swollen axons and hyperphosphorylated NF-positive axons. Quantitative analysis (bar graph, right) shows that the area of phospho-PHF tau immunoreactivity was $17,484 \pm 2,868 \mu \text{m}^2/\text{mm}^2$ tissue in the WT and $223 \pm 75 \mu \text{m}^2/\text{mm}^2$ tissue in the transgenics ($p < 0.01$). Scale bar = 50 μm.

FIGURE 6. Histologic analysis of spinal cord inflammatory infiltrates showed a reduction in lesional CD4-positive T lymphocytes in C57BL6-c-fms-hSCN5A (hSCN5A) mice and reveals the presence of phagocytic cell clusters. Mice were sacrificed for tissue analysis at onset of peak disease (5 days after disease onset). (A) Spinal cord white matter lesions were analyzed in longitudinal sections to maximize the area analyzed; the lesions often had an elongated oval shape that extended over multiple segments. Representative lesions from a WT mouse are shown at low power. The hematoxylin and eosin stain (left) demonstrates a moderately sized ovoid lesion containing mononuclear cells; the lesion is outlined in black. The immunofluorescent stains for myelin basic protein ([MBP] green) and the nuclear DAPI stain (blue) in frozen sections (right) show more extensive inflammation. (B, C) Higher power images of immunofluorescent staining of frozen sections for MBP (green in [B] and [C]), CD4 (red in [B]), and F4/80 (red in [C]). There are discrete well-defined regions of demyelination in hSCN5A mice that are associated with dense clusters of F4/80-positive phagocytic cells ([C] lower micrographs) and very few CD4-positive cells ([B] lower micrographs, white arrow). In contrast, wild-type mice have more diffuse, linear lesions associated with more numerous CD4-positive cells ([B] upper micrographs) and fewer F4/80-positive cells ([C] upper micrographs). Areas of detail in (B) are designated by the white outline in the adjacent micrograph. Scale bar = (A) 100 μm; (B) 50 μm; (C) 20 μm.
We also observed increased expression of arg1 in adjacent resident cells in some lesions (Fig. 9, lower micrographs). Arginase 1 has been shown previously to be expressed in neurons and can enhance axonal regeneration after injury through increased polyamine synthesis (26, 27). Arginase 1 also inhibits the synthesis of nitric oxide by the inducible nitric oxide synthase pathway and, through this mechanism, can decrease local tissue injury in EAE and other disease models (28).

Recovery From EAE After Adoptive Transfer of hNaV1.5-Positive BMDMs From C57BL6 c-fms-hSCN5A Mice

Based on analysis of the disease course and phenotype of inflammatory cell infiltrates, we hypothesized that hNaV1.5-positive macrophages from our transgenic mice mediated protection from EAE. Alternative hypotheses included transgene-mediated alterations in peripheral antigen presentation and cell movement. However, in our experiments, C57BL6 c-fms-hSCN5A mice still developed inflammatory lesions associated with milder or absent clinical disease. These results suggested that the presence of the transgene did not prevent the development of a peripheral immune response or inhibit trafficking of immune cells to the CNS. Second, based on our in vitro analysis of hNaV1.5-positive BMDMs, we hypothesized that peripherally derived alternatively activated cells would be sufficient to mediate disease protection, and that resident tissue NaV1.5-positive macrophages, activated microglia, would not be necessary to initiate these protective mechanisms.

To test our hypotheses, we adoptively transferred hNaV1.5-positive BMDM (C57BL6 c-fms-hSCN5A age- and sex-matched donors) into WT littermates with early signs of EAE (EAE score 1–2 at disease onset, Days 14–17). Disease onset
was selected as the time for adoptive transfer to demonstrate the potential therapeutic efficacy of these cells during a treatment paradigm and to avoid any potential effects on peripheral immune responses after immunization.

Mice that received hNaV1.5-positive BMDMs demonstrated improved recovery from EAE as compared with the control group. Significant differences between the hNaV1.5-positive BMDMs and the vehicle group were observed during the recovery period, starting at Day 26, and continued through the end of the observation period (Fig. 10A; Table 3). The differences between groups at peak disease onset (5 days after disease onset) were not statistically significant (3.7 ± 0.5 in the vehicle group and 2.6 ± 0.4 in the adoptive transfer group). At this peak severity level of disease in MOG-induced EAE, few animals show substantial recovery and usually develop a chronic stable disease course (Fig. 4; Table 2). It is noteworthy, therefore, that a relatively small number of adoptively transferred cells could mediate clinical recovery during experimental conditions where the mice develop relatively severe disease (12 of 15 mice, 6 per group, reached a score of 3).

By definition, in these experiments, there was 100% disease incidence. Male mice were included in these experiments and those shown in Figure 4 because they tend to develop more severe disease in this EAE model. Twelve of 15 total mice (6 in each group) and 9 of 9 males reached a score of 3. There were 4 males and 3 females in the vehicle group and 5 males and 3 females in the treatment group. Improvement of at least 1.0 in disease score occurred in 1 of 7 of control mice and 7 of 8 of treated animals. Cumulative disease score through 40 days was 86.1 ± 11.9* in the adoptive transfer group. There were no statistically significant differences which represented approximately 0.5% of adoptively transferred cells. There were no statistically significant differences between the treated and untreated conditions in the CD4-positive, CD11b-positive or total mononuclear cell number. The total mononuclear cell counts at this time point were 57,836 ± 11,258 cells per spinal cord in the adoptive transfer condition and 51,358 ± 9,176 cells per spinal cord in the vehicle-treated condition (n = 4; p = not significant).

Immunofluorescent staining for hNaV1.5 also demonstrated the presence of high-expressing cells within spinal cord phagocytic clusters in mice that received adoptively transferred cells (Fig. 12). The clusters that contained hNaV1.5-positive cells appeared to have a single positive cell associated with other CD11b-positive cells and with extensive cellular processes. These results confirmed the results from flow cytometry, demonstrated that phagocytic clusters form after adoptive transfer, and revealed that hNaV1.5-positive, CD11b-positive cells are present within the clusters. The combined flow cytometry and histologic data demonstrated that adoptively transferred hNaV1.5-positive BMDMs can home to the inflamed CNS and reside within phagocytic clusters that are similar in appearance to those observed in C57BL6-c-fms-hSCN5A mice (Figs. 6, 7, 9). Given the high cadherin expression (Fig. 11) and extensive cellular processes within these cell clusters (Fig. 12), the results also suggested that hNaV1.5-positive BMDMs may enhance the formation of phagocytic clusters.

### Increased Arginase-1 Expression in CNS and Peripheral Myeloid Cells After Adoptive Transfer of hNaV1.5-Positive BMDMs

We also examined the expression of Arg1 in isolated CNS mononuclear cells (EAE Days 28–30) after adoptive transfer of hNaV1.5-positive BMDMs from C57BL6-c-fms-hSCN5A mice at disease onset. There was an approximately 2-fold increase in percent CD11b-positive, Arg1-positive cells in CNS mononuclear spinal cord cells (Fig. 13). The percent of CD11b-positive, Arg1-positive cells in the adoptive

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**TABLE 3. Adoptive Transfer Treatment Paradigm**

<table>
<thead>
<tr>
<th>Strain/Treatment</th>
<th>No. Mice (male/female)</th>
<th>Incidence of Peak Clinical Score ≥3.0</th>
<th>Incidence of Recovery ≥1.0 in Clinical Score</th>
<th>Cumulative Disease Score Through 40 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6/Vehicle</td>
<td>4/3</td>
<td>6/7</td>
<td>1/7</td>
<td>86.1 ± 11.9*</td>
</tr>
<tr>
<td>C57BL6/hSCN5A AT</td>
<td>5/3</td>
<td>6/8</td>
<td>7/8</td>
<td>52.9 ± 9.6</td>
</tr>
<tr>
<td>C57BL6/WT AT</td>
<td>4/3</td>
<td>7/7</td>
<td>0/7</td>
<td>122 ± 12.3*</td>
</tr>
</tbody>
</table>

Bone marrow–derived macrophages (hSCN5A transgenic [hSCN5A] or wild-type [WT] cells) or vehicle (PBS) was transferred at disease onset to WT recipients. All recipient and donor mice were 12 to 16 weeks of age and were age- and sex-matched littermates.

*p ≤ 0.05 vs hSCN5A adoptive transfer group.

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transfer group was 23.2% ± 1.1% and 12.2% ± 1.2% in the vehicle-treated control group (n = 4; p < 0.01). This increase in CD11b-positive, Arg1-positive cells cannot be explained entirely by the presence of adoptively transferred cells because of the relatively small number of CD11b-positive, hNaV1.5-positive cells within the CNS after adoptive transfer. Although the mechanism is unclear, these results suggest that hNaV1.5-positive macrophages may not only directly regulate recovery from tissue injury but also might mediate a bystander effect and induce an arginase-positive phenotype in host cells.

**DISCUSSION**

We here demonstrate that the human NaV1.5 macrophage variant is a novel splice variant that regulates cellular

![FIGURE 11. Detection of hNaV1.5-positive macrophages in the spinal cord after adoptive transfer. Approximately 2 weeks after adoptive transfer at onset of experimental autoimmune encephalomyelitis, spinal cord mononuclear cells were analyzed by flow cytometry. Within the mononuclear cell gate, myeloid (R1) and lymphocyte (R2) populations could be identified based on cell size (left). Within the myeloid gate, approximately 5% to 10% of cells demonstrated a high level of hNaV1.5 expression in the adoptive transfer group (middle). These high-expressing cells also expressed higher levels of E-cadherin (cdh1, CD324) as compared with the total CD11b-positive population, consistent with an alternatively activated phenotype (right). hNaV1.5-positive, CD11b-positive cells were not observed in untreated controls (middle). Similar numbers of CD4 T lymphocytes were observed in the treated and untreated groups (lower middle). FSC, forward scatter; SSC, side scatter.](http://jnen.oxfordjournals.org/DownloadedFrom)
phenotype and disease pathogenesis in vivo. This channel variant is not expressed in mouse macrophages. To study its in vivo function, we cloned the human macrophage variant of SCN5A, the gene that encodes the NaV1.5 channel, and developed a novel transgenic mouse (C57BL6/c-fms-hSCN5A) that selectively expresses the channel variant in phagocytic cells, particularly CD11b-positive, F4/80-positive macrophages. These transgenic mice developed much less severe EAE and had smaller inflammatory CNS lesions associated with a reduction in CD4 T-lymphocyte infiltration and tissue injury as compared with littermate WT controls. The lesions in transgene-positive mice were characterized by small phagocytic cell clusters and expression of arginase, a marker of alternatively activated macrophages. Based on these results, we hypothesized that human macrophage NaV1.5 expressed in peripheral and central mononuclear phagocytes mediates anti-inflammatory mechanisms that ameliorate CNS inflammation and promote tissue repair. However, we could not rule out an effect of the gene knock-in on peripheral and central antigen presentation. To address that issue in subsequent EAE experiments, we adoptively transferred BMDMs from C57BL6/c-fms-hSCN5A mice into WT recipients at the time of disease onset. Analysis of these mice demonstrated enhanced clinical recovery, the presence of transplanted cells in recipient spinal cord, and increased numbers of arginase-positive cells in mononuclear CNS infiltrates. Based on these results, we hypothesize that hNaV1.5-positive macrophage cell therapy might be an effective treatment for patients with MS.

Our hypothesis contrasts with many earlier models of inflammatory pathogenesis in MS. For example, a common model of disease pathogenesis in MS posits that lymphocyte-initiated inflammation leads to recruitment of monocytes, differentiation into macrophages within the CNS microenvironment, local activation and recruitment of microglia, and subsequent phagocyte-mediated demyelination and axonal injury (1). This theory is based, in part, on the observation that macrophages and activated microglia are abundant within MS lesions (4). However, the presence of mononuclear phagocytes within brain lesions does not necessarily demonstrate that they have only a pathogenic role. In addition, other immune cell subtypes can directly mediate tissue injury during autoimmunity. These subtypes include cytotoxic CD8 T lymphocytes and B lymphocyte–dependent autoantibody production coupled with complement fixation. An unsupported component of this model was that it postulated that macrophages were not only scavengers of debris and apoptotic cells, but that they somehow directly engulfed myelin from healthy axons. A more likely pathogenic mechanism in MS would be

**FIGURE 12.** hNaV1.5-positive bone marrow–derived macrophages can be detected within spinal cord phagocytic clusters after adoptive transfer. Histologic analysis of spinal cord was performed approximately 2 weeks after adoptive transfer (killed on Days 28–30). Longitudinal sections were stained for expression of CD11b and hNaV1.5. Small CD11b-positive (green) phagocytic cell clusters were observed in the white matter (the white line demarcates the meningeal border). A single high-expressing hNaV1.5-positive, CD11b-positive cell was observed within 2 different representative cell clusters (upper and lower panels). Scale bar = 20 μm.
phagocytic mechanisms mediated by a variant of the cation on their own because of increased weakness, and there

not meet its primary outcome measure, that is, reduced clini-

lamotrigine, for treatment of secondary progressive MS did

studies of phagocytic cell function in chronic CNS diseases.

positive transgenic mice are consistent with these related
disease model (12), our results in macrophage hNaV1.5-
creased disease severity in EAE (30), neurodegenerative (31),
pletion or inhibition of mononuclear phagocytes leads to in-
ing peripherally recruited cells and tissue resident cells such as
microglia (5), mediate recovery from injury. In the CNS, de-
pletion or inhibition of mononuclear phagocytes leads to in-
creased disease severity in EAE (30), neurodegenerative (31),
and stroke (32) disease models. A recent genetic study in
families with Alzheimer disease also suggests that defective
phagocytic mechanisms mediated by a variant of the TREM2
(triggering receptor expressed on myeloid cells 2) gene in-
crease disease risk (33). Although it might be considered
counterintuitive that the knock-in of a human gene that en-
hances macrophage phagocytic function could result in re-
duced disease severity in EAE or other CNS inflammatory
disease model (12), our results in macrophage hNaV1.5-
positive transgenic mice are consistent with these related
studies of phagocytic cell function in chronic CNS diseases.

In addition, a large study of the sodium channel blocker,
lamotrigine, for treatment of secondary progressive MS did
not meet its primary outcome measure, that is, reduced clin-
cal progression (34). Many of the patients stopped the medica-
tion on their own because of increased weakness, and there

was increased brain volume loss in the treatment group that
was only partially reversible after cessation of treatment. The
rationale for that trial is that sodium channel blockers may
have a neuroprotective effect on demyelinated axons. How-
ever, any potential benefit could have been negated by the
inhibition of protective macrophage-microglia responses.

Macrophages can mediate recovery from tissue injury by at least 2 mechanisms: phagocytosis of debris and apo-

totic cells within lesions (13) and differentiation to an al-
ternatively activated phenotype (35). In a simplistic sense,
macrophages have been classified as M1 inflammatory or M2
alternatively activated cells. Interferon-γ mediates polariza-
tion to an M1 phenotype and significantly enhances phago-
cytic activity (36), whereas interleukin 4 and interleukin 13
polarize cells to alternatively activated macrophages. Alter-
atively activated macrophages demonstrate anti-inflammatory
and regenerative properties in mouse EAE models and ex-
press high levels of arginase (30, 37). Alternatively activated
macrophages within lesions mediate these effects through
local release of cytokines and regulation of tissue repair (38).

Although this classification scheme has been useful in deter-
mring the role of cell subsets in cellular function, macro-
phage phenotype in vivo is more complex and a range of
intermediate phenotypes exist that are clinically relevant (39).

It is unclear how NaV1.5 regulates macrophage pheno-
type at a cellular and molecular level. Persistent expression
of NaV1.5 and alterations in intracellular calcium signaling
(10) may enhance polarization to an arginase-positive pheno-
type. In addition, modeling of mouse macrophase differentia-
tion suggests that conversion of arginase-positive cells to an
M1 phenotype readily occurs in inflammatory condition, but
M1 polarization appears to be a more stable phenotype (40).

Macrophage NaV1.5 may enhance the maintenance of the
arginase-positive phenotype within inflammatory lesions and
overcome some of the limitations of immune regulatory cell
plasticity (41). Although additional characterization of these
molecular mechanisms is required before clinical translation,
therapeutic use of macrophages requires the identification of
specific cell subtypes that are therapeutically relevant. Use of
nonselected macrophage populations could potentially lead
to macrophage activation syndrome (42) or a sepsis-like process;
the development of severe EAE in mice treated with unselected
WT BMDMs in our experiments demonstrates some of the
challenges of cell therapy (Fig. 10B). Stability of phenotype in
vivo will be a critical criterion in the development of this
therapeutic approach.

Despite protective endogenous mechanisms, chronic pro-
gressive injury continues in many MS patients and pathologic
inflammation persists within existing lesions. From a clinical
perspective, 2 unanswered questions are “Why are these re-
pair mechanisms defective in many patients with neurologic
disease?” and “How can these repair mechanisms be en-
hanced?” Novel strategies are required to deliver sufficient
numbers of therapeutically effective cells to disseminated lesion
sites, maintain their pro-repair and anti-inflammatory pheno-
type, minimize adverse events, and avoid the use of long-term
immune suppressive treatments. Our long-term goal is to de-
velop human NaV1.5-positive macrophages as a cell-based
therapy that meets these criteria.

![FIGURE 13. Increased arginase-1 expression in CD11b-positive cells after adoptive transfer of NaV1.5-positive bone marrow-derived macrophages. (A) Mononuclear cell populations were analyzed by flow cytometry approximately 2 weeks after adoptive transfer. (B) There was an increase in the percent of CD11b-positive, Arg1-positive cells in spinal cords in the adoptive transfer group as compared with untreated controls (left). The total mononuclear cells counts at this time point were not significantly different (right).](http://jnen.oxfordjournals.org/)

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ACKNOWLEDGMENTS

We thank Gouri Chatterjee for assistance with cDNA cloning and Toshi Kinoshita for help with hematoxylin and eosin staining. We acknowledge the assistance of Khen Macllvay and the University of Wisconsin Carbone Cancer Center Flow Cytometry Laboratory with acquisition of flow cytometry data.

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