Subcutaneous Transplantation of Neural Precursor Cells in Experimental Autoimmune Encephalomyelitis Reduces Chemotactic Signals in the Central Nervous System

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ABSTRACT

Neural precursor cell (NPC) transplantation has been proposed as a therapy for multiple sclerosis (MS) and other degenerative disorders of the central nervous system (CNS). NPCs are suggested to exert immune modulation when they are transplanted in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). Herein, we explore whether the effect of NPC transplantation on the clinical course and the pathological features of EAE is combined with the modulation of chemokines levels expressed in the inflamed CNS. NPCs were isolated from brains of neonatal C57/Bl6 mice and were subcutaneously administered in female mice with myelin oligodendrocyte glycoprotein (MOG)-induced EAE. Clinical signs of the disease and transcript analysis of the CNS in the acute phase were performed. In addition, the presence of inflammatory components in the spinal cord was evaluated and ex vivo proliferation of lymphocytes was measured. NPC recipients exhibited ameliorated clinical outcome and less pronounced pathological features in their spinal cord. Downregulation of chemokine mRNA levels throughout the CNS was correlated with diminished Mac-3-, CD3-, and CD4-positive cells and reduced expression levels of antigen-presenting molecules in the spinal cord. Moreover, NPC transplantation resulted in lymphocyte-related, although not splenocyte-related, peripheral immunosuppression. We conclude that NPCs ameliorated EAE potentially by modulating the levels of chemokines expressed in the inflamed CNS, thus resulting in the impaired recruitment of immune cells. These findings further contribute to the better understanding of NPCs’ immunomodulatory properties in neuroinflammatory disorders, and may lead to faster translation into potential clinical use.

SIGNIFICANCE

Endogenous neural precursor cells of the central nervous system are able to migrate and differentiate toward mature cells to repair an injury. There is increasing evidence that autologous transplantation of these cells in experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis, may have a beneficial effect on the disease process. Several mechanisms have been proposed—among them, the potentiation of endogenous precursor cell differentiation of the central nervous system and the modulation of demyelinating and neurodegenerative immune-mediated processes. This article provides evidence of interference in immune signaling within the central nervous system as a potential mechanism underlying the immunomodulatory properties of transplanted neural precursor cells.

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS). MS is a T cell-mediated disease in which autoreactive cells against myelin epitopes cross the blood-brain barrier (BBB) and attack myelin by releasing a variety of immune substances and molecules [1]. One of the approaches being explored in MS animal models is neural precursor cell (NPC) transplantation. NPCs are located mainly at the subventricular zone and the dentate gyrus in the brain, and they are considered a heterogeneous population of mitotically
active, self-renewing, multipotent, immature progenitor cells [2]. They exist both in the developing and the adult nervous system, and are mobilized in conditions of tissue injury in the CNS. In the case of MS, they can differentiate into oligodendrocytes to remyelinate demyelinated axons [3, 4]. NPC transplantation was initially proposed as a cell replacement therapy, since intracerebroventricular transplantation in MS murine model, experimental autoimmune encephalomyelitis (EAE), has shown that they can differentiate into oligodendrocytes or trigger the differentiation of the endogenous NPCs toward oligodendrocytes, enhancing the remyelination of damaged axons due to the neuro-inflammatory pathogenesis and reducing inflammation and demyelination overall [5, 6]. Although this theory was supported by several studies, more recent data indicate the immunomodulatory mechanisms of NPCs [7, 8]. Following transplantation via routes other than those directly targeted in the CNS, such as intravenous or subcutaneous routes, NPCs induced the same amelioration in clinical outcome of EAE animals, as well as in the pathological features of the CNS, even without entering the CNS. Additionally, reduced activation and proliferation of autoimmune CD4+ T lymphocytes [8, 9] and restrained dendritic function were some of the main in vivo effects [9]. Furthermore, in vitro cocultures of NPCs with myelin epitope-specific lymphocytes resulted in severe apoptosis of the latter [8–11].

Similar findings are also valid for bone marrow mesenchymal stem cell (BMSC) transplantation [12, 13], although some potential complications of their use have been reported, particularly if intraventricular administration is considered [14]. Moreover, although not among the primary endpoints of the study, NPCs exhibited better clinical outcome when compared with BMSC transplantation [14]. However, future comparison studies are warranted in which either cell type will be administered via the same route and at the same time points following EAE induction.

Chemokines consist of a family of small cytokines that control the migration of lymphocytes and are involved in MS and EAE pathogenesis [15]. Particularly, C-C motif ligand 2 or monocyte chemotactic protein-1 (CCL2/MCP-1) expression was elevated in brains of patients suffering with MS [16], whereas blocking of the signaling axis CCL2-chemokine (C-C motif) receptor 2 (CCR2; CCL2 receptor) almost abolishes subsequent EAE symptoms [17]. Activated T cells produce several chemokines, such as CCL3/MIP-1α (macrophage inflammatory protein-1α) within CNS lesions in the EAE model, resulting in recruitment of other inflammatory cells into the CNS [18], a finding in accordance with the elevated levels of CCL3, CCL5, and other chemokines throughout the acute phase of EAE [19]. The expression of CXCL10/IP-10 (interferon γ-induced protein 10 kDa) has been reported to be elevated in the CSF of MS patients and correlated significantly with increased number of T lymphocytes expressing the corresponding receptor, chemokine (C-X-C motif) receptor 3 (CXCR3), in the lesions [20]. Moreover, CXCL13/BLC, a B-lymphocyte chemottractant, was expressed in active demyelinating lesions, while invasive B cells within the CSF expressed the CXCL13 receptor, CXCR5. CXCL12/SDF-1 (stromal cell-derived factor-1) levels were also elevated in inactive lesions and, with CXCL13, are suspected of being responsible for the B-cell trafficking within the CNS [21]. Uprogation of chemokines in EAE/MS seems to be a part of the innate immune response, which is predominant in neuroinflammation [22]. Chemokines mediate their signal through G protein-coupled receptors, which are part of a greater family of receptors implicated in signaling through molecules such as neurotransmitters, hormones, and inflammatory mediators. Chemokine receptors also seem to be important for the development of EAE, as blockade or genetic silencing of CXCR2, the receptor of CXCL1 and CXCL2, abrogates BBB breakdown, CNS infiltration by leukocytes, and typical clinical pathological features of EAE [23, 24].

Herein, we investigate the effects of subcutaneous NPC transplantation in terms of modulation of chemokine gene expression in the CNS of C57Bl/6 mice with acute myelin oligodendrocyte glycoprotein (MOG) EAE, based on the general immunomodulation proposed for this administration route [9]. We examined whether transplanted NPCs inhibit inflammatory progress and subsequent demyelination, and whether these phenomena are associated with downregulation of chemokines’ mRNA profile in the CNS, resulting in reduced migration of autoreactive inflammatory components [25].

**Materials and Methods**

**Animal Handling**

Female C57Bl/6 mice (n = 18), 8–10 weeks old, were purchased from the Hellenic Pasteur Institute (Athens, Greece, http://www.pasteur.gr) and housed in the animal facility of the B’ Neurology Department, AHEPA University Hospital, Thessaloniki, Greece (EL54 BIO29). Animals were fed a normal diet and given free water without antibiotics ad libitum. All experimental procedures were conducted according to institutional guidelines and in compliance with Greek regulations and the European Communities Council Directive of November 24, 1986 (86/609/EEC).

**Neural Precursor Cells Isolation, Culture, and Characterization**

NPCs were cultured using a previously described protocol [5, 8, 10, 26]. Briefly, cerebral hemispheres were dissected from newborn C57Bl/6 mice and meninges were removed. Brain tissue was minced, digested in 0.025% trypsin (Invitrogen, Carlsbad, CA, http://www.thermofisher.com) for 20 minutes, and mechanically dissociated to create a single-cell suspension. The cells were suspended in serum-free F12/Dulbecco’s modified Eagle’s medium supplemented with 10 mg/ml human apo-transferrin, 1 mM sodium pyruvate, 0.05% bovine serum albumin, 10 ng/ml D-biotin, 30 mM sodium selenite, 20 mM progestosterone, 60 μM putrescine, 2 mM l-glutamine, 25 μg/ml gentamycin, and 250 ng/ml bovine insulin (all from Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). The cells were plated at 10 × 10⁶ cells per T75 uncoated flask (Corning, Corning, NY, http://www.corning.com) and supplemented daily with 10 mg/ml basic fibroblast growth factor (bFGF2; R&D Systems, Minneapolis, MN, http://www.rndsystems.com) and 20 ng/ml epidermal growth factor (EGF; R&D Systems). These conditions allowed multipotential NPCs to survive and proliferate into clusters of small, round cells that grew into floating spheres (neurospheres).

Characterization of NPCs was performed by immunocytochemical staining of the neurospheres with mouse anti-gliarial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark, http://www.dako.com), mouse anti-nestin (Chemicon International, Billerica, MA, http://www.chemicon.com), rabbit anti-neuron-glial antigen 2 (NG2) (Chemicon International), mouse anti-oligodendrocyte marker O4 (Chemicon International), rabbit anti-galactocerebroside (GaLC; Chemicon International), mouse anti-neuronal nuclei (NeuN) (Chemicon International) and mouse anti-polyasaclic acid neuronal cell adhesion molecule (PSA-NCAM; Chemicon International). Free-floating neurospheres were attached to poly-o-lysine- and
fibronectin-coated slides (Sigma-Aldrich) at a density of 150–300 neurospheres per slide and were derived from bFGF and EGF. The cells were fixed in acid alcohol at −20°C, blocked with 1% normal goat serum (Invitrogen), and stained for the indicated markers on days 1 and 5. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

**EAE Induction and Clinical Evaluation**

EAE was induced as previously described [5, 14, 27–29]. Briefly, on day 0 (disease induction), each mouse was injected subcutaneously (s.c.) in the left paralumbar region with 150 μg of MOG epitope: MEVGWYRSPFSRVVHLYRNGK; Sigma-Aldrich) emulsified in 200 μl of 4% complete Freund’s adjuvant in phosphate-buffered saline (PBS). Additionally, mice were injected intraperitoneously (i.p.) with 400 ng of pertussis toxin (Sigma-Aldrich) dissolved in 500 μl of PBS. On day 2, animals received an i.p. booster of 0.5 ml of pertussis toxin solution at a concentration of 400 ng/ml. Finally, on day 7, an s.c. booster of MOG emulsion in the right paralumbar region was performed.

Animals were weighed daily throughout the observation period and were clinically evaluated using the following 0–6 clinical scale: 0 = asymptomatic animal, 1 = inability to elevate the tail above the horizontal level, 2 = flail tail, 3 = score 2 plus inability to turn from supine to prone position, 4 = score 3 plus paresis or paralysis of hind limbs, 5 = score 4 plus paresis of front limbs, and 6 = death due to EAE.

**Experimental Groups and Preparation of Injected Emulsions**

Animals were randomized according to their initial body weight (day 0, disease induction) into 2 groups (PBS-treated control group and NPC group) and they were all inoculated with MOG for the induction of EAE. On day 7 postinduction, animals were injected s.c. in their hindlimb flanks with different emulsions according to their assigned group: the PBS control group (n = 7) or the bromodeoxyuridine (BrdU, Sigma Aldrich)-labeled NPC group (n = 8). BrdU labeling was performed following to a previously described protocol [5]. The control group received 150 μl of PBS per flank and the NPC group received 1 × 10⁶ single cell-disassociated NPCs emulsified in 150 μl of PBS in each flank.

Neurospheres were single-cell dissociated before transplantation [30]. Briefly, neurospheres were centrifuged and the pellet was reconstituted in 2 ml of an enzyme mix (Accutase cell dissociation reagent, Chemicon International) to disrupt the bonds between cells in the neurospheres. The cells were mixed twice and were incubated at 37°C. After confirming the absence of neurospheres microscopically, the cells were collected and diluted in PBS for s.c. transplantation.

**Tissue Collection**

All animals were humanely euthanized with intraperitoneal injections of anesthetics on day 24 (acute phase) postinduction. Animals from each group were distributed in two subgroups, with both subgroups having the same mean clinical score. Half of the animals in each group were selected for in vitro assays (gene expression analysis and lymphocyte proliferation assays). For real-time polymerase chain reaction (PCR) analysis, CNS tissue (brain, spinal cord, splenocytes [SPCs], and lymph node cells [LNCs]) were rapidly removed and emulsified in RNA extraction lysis buffer (Mini RNA II; Zymo Research, Irvine, CA, http://www.zymoresearch.com) and stored at −80°C. Spleens and lymph nodes were used for the lymphocyte ex vivo proliferation assay. The rest of the animals were killed with transcardial perfusion of 4% paraformaldehyde in ice-cold PBS (pH 7.2), and brain, spinal cord, lymph node, and footpad tissues were removed, postfixed for 20 hours in the same fixative at 4°C, and routinely processed for paraffin embedding and sagittal sectioning.

**Histopathology, Immunohistochemistry, and Double Immunofluorescence**

All tissues were processed for 6-μm paraffin sections. The inflammatory and demyelination burdens were evaluated on sections stained with hematoxylin and eosin and Luxol fast-blue (LFB), respectively, using standard protocol. Histochemistry (HC) was performed as follows. Briefly, sections were deparaffinized, hydrated, rinsed with Tris-buffered saline (TBS) and endogenous peroxidase was blocked with H₂O₂ in TBS. Endogenous biotin was blocked for lectin HC using an Avidin/Biotin blocking Kit (Vector Laboratories, Burlingame, CA, http://vectorlabs.com). Citrate buffer (pH 6) or EDTA (pH 9) was used for antigen retrieval for the other stains. Sections were incubated in blocking buffer (10% fetal bovine serum [FBS]) and treated with primary antibodies against GFAP (rabbit, Dako); β-amyloid precursor protein (mouse, clone:22c11; Chemicon); CD3 (rabbit; Thermo Fisher Scientific, Waltham, MA, http://www.thermoscientific.com); CD4 (mouse; Spring Bioscience, http://www.springbio.com); CD25 (rabbit; Santa Cruz Biotechnology, http://www.scbt.com); Mac-3 (rat; Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com); and B220 (rat; Becton Dickinson). Immunoreactions were visualized with the EnVision+ System-HP HRP Kit (Dako) and the LSAB-2 System-HP; 3,3′-diaminobenzidine (DAB; Dako), while anti-rat secondary antibody (AbDSerotec, Oxford, U.K., https://www.abdserotec.com), and anti-mouse secondary antibody (Vector Laboratories) were used as chromogens, and sections were counterstained with hematoxylin.

For double immunofluorescence, proteinase K treatment was used for antigen retrieval and 2N HCl for DNA denaturation. Slides were further washed and blocked with 10% FBS supplemented with 0.3% TritonX. The first antibodies used were BrdU (rat; Abcam, Cambridge, U.K., http://www.abcam.com) along with caspase-3 (rabbit; R&D Systems), brain-derived neurotrophic factor (BDNF) (rabbit; Santa Cruz), nestin (mouse; EMD Millipore, Billerica, MA, http://www.emdmillipore.com), NG2 (rabbit; EMD Millipore), NeuN (mouse; EMD Millipore), and GFAP (rabbit; Dako). Following washing steps, the slides were incubated with respective secondary antibodies (Biotium, Hayward, CA, https://biotium.com) and they were counterstained with DAPI (Biotium).

**Evaluation of CNS Pathology**

Quantification of pathology was performed under a light microscope (Zeiss Axiosplan-2; Carl Zeiss, Oberkochen, Germany, http://www.zeiss.com) with the aid of a charge-coupled device camera (Nikon, Tokyo, Japan, http://www.nikon.com), by two independent investigators. Depending on the staining, 5–6 randomly selected spinal cord sections (each spaced at least 100 μm apart) were evaluated for each animal, under ×20 or ×40 magnification (depending on the object of study) using a prefrontal grid. The histopathological findings were evaluated on spinal cord sections, since EAE pathogenesis is most intense there.
The following evaluating protocols were performed. Inflammatory processes were studied under ×20 magnification and the number of infiltrating cells (perivascular or parenchymal) were expressed as cells per mm² with respect to the size of inflammatory foci and defined as the number of infiltrating cells per infiltration. Demyelination was evaluated on LFB sections using a prefrontal grid under ×40 magnification; data were expressed as percentage of demyelinating white matter area. Axonopathy was evaluated on APP immunohistochemically stained sections under ×40 magnification; APP-positive (APP⁺) axons within the white matter were counted per prefrontal grid area and data were expressed as the number of APP⁺ per mm². Astrocytes and microglial cells were counted under ×40 magnification and evaluated as GFAP-positive (GFAP⁺) cells (astrocytes per mm²) and lectin-positive cells (microglia per mm²), respectively. Inflammatory cells (i.e., CD3, CD4, B220, and Mac-3) were counted under ×40 magnification and evaluated as percentage of positive cells in the inflammation sites.

**Lymphocyte Culture**

Inguinal, intestinal, and axillary lymph nodes, as well as spleens, were removed and cells were extracted after passing the tissues through a nylon mesh after repeated suspensions with a 19-gauge needle. LNCs and SPCs from each group were pooled in full growth medium that contained 0.3% 2-mercaptoethanol solution (Sigma-Aldrich), 1 mM sodium pyruvate, 200 mM glutamine, 5% fetal calf serum, 1% penicillin/streptomycin, 1% minimum essential medium nonessential amino acids, and Roswell Park Memorial Institute 1640 medium with glutamine (all from Thermo Fisher Scientific).

Isolated cells were centrifuged, washed three times, and the pellet was reconstituted in fresh full growth medium. The collected LNCs were resuspended in a concentration of 1 × 10⁶ cells per milliliter for a proliferation assay and incubated at 37°C in 5% CO₂.

**In Vitro Proliferation Assay**

LNCs and SPCs were plated in 96-well micropatter plates (10⁶ cells per milliliter). Cells from each group were seeded in 3 conditions: cells incubated with 100 μg/ml linear MOG, cells incubated with 1 μg/ml concanavalin A (ConA; Sigma-Aldrich) as a nonspecific mitogen, and plain cells, which served as negative controls. The proliferation assay was performed by using TACs MTT cell proliferation assay kit (R&D Systems) following the manufacturer’s instructions. Briefly, after a 48-hour incubation in 5% CO₂ at 37°C, 10 μl of MTT was added in each well, followed by a 4-hour incubation at 37°C. Formazan crystals were dissolved in MTT detergent for 1 hour at 37°C. Absorbance was determined with a spectrophotometer at 570 nm.

**RNA Extraction and cDNA Synthesis**

RNA was extracted from tissues (brain, spinal cord) and cells (LNCs and SPCs), using the Mini RNA II kit (ZymoResearch) and following the manufacturer’s instructions. cDNA was prepared from 1 μg of total RNA using the Iscript cDNA synthesis kit (BioRad, Hercules, CA, http://www.bio-rad.com) according to the manufacturer’s instructions, in a final volume of 20 μl.

**Real-Time PCR**

Quantification experiments performed in the IQ5 (BioRad) real-time detection system. The IQ SYBR Green kit (BioRad) was used for the quantification of the following genes: CCL2, CCL3, CCL5, CCL20, CXCL1, CXCL10, CXCL12, CXCL13, CXCR2, CXCR4, and IL17A. The PCR reaction mixtures contained 1 μl of cDNA along with 12.5 μl of SYBR Green buffer (BioRad) and 300 nM of each primer in a total reaction volume of 25 μl. Melting curve analysis was performed at the end of each PCR assay. PCR amplification products were visualized on a 1.5% Tris-borate EDTA agarose gel. The comparative threshold cycle ΔΔCT was used for the relative quantification data analysis. For all genes, expression was normalized against the expression of the β-actin gene. The relative expression of genes was estimated using the relative expression software tool [31]. The sequences of primers can be found in supplemental online Table 1.

**Statistical Analysis**

Statistical analyses of the data were performed using the SPSS 17.0 software (IBM, Armonk, NY, http://www-01.ibm.com) and GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, http://www.graphpad.com). For scale, data normality was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests. The parametric Student’s t test and the equivalent nonparametric Mann-Whitney U test were used for comparison of two groups. Values of all scale data are expressed as mean ± SEM.

For nominal or ordinal data, we used the Pearson chi-square or Fisher exact tests for comparison of two groups, depending on the tables’ properties. Kaplan-Meier survival analysis was performed for “disease onset” (score 1). Clinical EAE was evaluated using the following: (a) the mean maximal scores (MMS) for each group, calculated as the average maximal scores of each corresponding group; (b) the mean day of disease onset, calculated as the average day that each animal initially reached score 1; and (c) mean area under the curve (mAUC), calculated for each animal using the following equation [32]:

$$\text{AUC} = \left( \frac{\text{score}_1}{2} \right) + \sum_{i=2}^{k-1} \text{score}_i + \left( \frac{\text{score}_k}{2} \right)$$

**RESULTS**

**Expansion and Immunocytochemical Characterization of NPCs**

NPCs were characterized in vitro to verify the immature and multipotent nature of the NPCs [5, 8, 33, 34] and their potency to differentiate into neuronal lineages. To identify their ability to differentiate into the three cell types of neural lineage (neurons, astrocytes, oligodendrocytes), NPCs were attached in vitro on fibronectin and poly-α-lysine-coated plates after withdrawal of mitogen factors. On day 1, the population was homogenous with most of the cells being nestin and PSA-NCAM positive (92% ± 1.3% and 86% ± 2.6%).

Within 1 day (day 2), multiple NG2-positive oligodendrocyte progenitor cells (19% ± 1.5%) were observed. On day 5, the spheres differentiated into 52% ± 2.4% GFAP⁺ astrocytes, 32% ± 2.5% O4-positive oligodendrocytes, 34% ± 4.1% GalC-positive oligodendrocytes, and 3% ± 1.2% NeuN-positive neurons (data not shown) [28].

**Subcutaneous Injection of NPCs Ameliorates the Clinical Course of EAE**

The clinical courses of acute EAE for the control group and the NPC group are displayed in Figure 1A. The NPC group showed...
significant amelioration in clinical severity of EAE in comparison with the control group (days 14, 17–18, 21–24; \(p < .05\)). Amelioration of clinical course was also confirmed by the difference in the mAUC (control group: 35.33 ± 14.10; NPC group: 16.58 ± 17.31; \(p < .05\)). The NPC group also showed delayed disease onset (control group: 14.3 ± 4.1 days; NPC group: 19.0 ± 4.8 days; \(p < .05\)) and smaller MMS (control group: 3.5 ± 1.1; NPC group: 2.0 ± 1.9; \(p < .05\)) (Fig. 1C–E). In addition, animals in the NPC group did not experience the same reduction in body weight as those in the control group (days 11, 16–20, and 24; \(p < .05\)) (Fig. 1B).

NPCs Reduce Acute EAE Pathology

Inflammatory burden tended to be reduced within the spinal cord of NPC-treated animals. The total number of infiltrating cells within the spinal cord of the NPC group tended to be lower than that in controls (NPC group: 74.1 ± 105.12 cells per mm\(^2\); control group: 168.42 ± 237.75 cells per mm\(^2\); \(p > .05\)) (Fig. 2A, 2B, 2I), with similar size of inflammatory foci (cells per infiltration: 29.46 ± 33.35 and 35.95 ± 29.63 for the NPC and control groups, respectively; \(p > .05\)). On the other hand, the NPC group showed significantly reduced demyelination (4.56% ± 9.33%) within the CNS compared with those in the control group (demyelination: 19.96% ± 24.09%; \(p < .0001\)) (Fig. 2C, 2D, 2J). Moreover, we observed a reduced number of CD4 cells (5.841 ± 2.158 and 2.165 ± 0.729, respectively; \(p < .0001\)) (Fig. 3J–L). The reduced infiltration of T cells and monocytes was also supported by the spinal cord mRNA levels of interleukin 17A (IL-17A), expressed specifically from Th17 cells, and the antigen-presenting molecules (CD80, CD86) (Fig. 3P–R). They were both reduced in the NPC group, although IL-17A levels were not reduced in a significant way (IL-17A: \(p = .1406\); CD80: \(p = .0215\), 1.66-fold reduction; CD86: \(p = .0204\), 2.06-fold reduction).

Immune Cell Recruitment Within the Spinal Cord Is Modulated by NPC Transplantation

We studied the consistency of infiltrated immune cells within the spinal cord of the NPC and control groups. The number of Mac-3-positive cells was higher in the control group compared with the NPC group (33.30 ± 2.649 and 10.08 ± 1.265, respectively; \(p < .0001\)) (Fig. 3A–C). On the contrary, the proportion of B cells within the spinal cord of the 2 experimental groups was not significantly altered (2.193 ± 0.399 and 1.973 ± 0.456, respectively; \(p = .3522\)), as seen by the B220 staining (Fig. 3D–F). Three markers were used to identify T cells. CD25-positive cells in control mice were also more abundant than in NPC-transplanted mice, although marginally and nonsignificantly (7.759 ± 1.925 and 5.892 ± 2.397, respectively; \(p = .0588\)) (Fig. 3G–I). Moreover, we observed a reduced number of CD3-positive cells (27.18 ± 4.213 and 19.17 ± 4.765; \(p = .0014\)) (Fig. 3M–O). The reduced infiltration of T cells and monocytes was also supported by the spinal cord mRNA levels of interleukin 17A (IL-17A), expressed specifically from Th17 cells, and the antigen-presenting molecules (CD80, CD86) (Fig. 3P–R). They were both reduced in the NPC group, although IL-17A levels were not reduced in a significant way (IL-17A: \(p = .1406\); CD80: \(p = .0215\), 1.66-fold reduction; CD86: \(p = .0204\), 2.06-fold reduction).
NPCs Remain in an Undifferentiated State and Are Localized Mainly in the Site of Injection

We located the transplanted cells within the host’s skin at the site of injection and approximate areas (Fig. 4A). Cells were not present in either the lymphoid organs or the CNS. Transplanted cells were viable; they were not positive for caspase-3 (data not shown). Furthermore, we examined whether the transplanted cells had differentiated toward a specific neuronal lineage. The cells were negative for typical glial and neuronal markers, namely GFAP, NeuN, and NG2 (data not shown). On the contrary, they were positive for nestin, which is a prototypical marker for immature NPCs (Fig. 4A–D). Additionally, NPCs were double positive for the neurotrophic factor BDNF (Fig. 4E–H).

NPC Transplantation Modulates Chemokine Gene Expression in the CNS

To further study the reduced recruitment of immune cells within the spinal cord, we explored the chemokine gene expression profile of the CNS. CCL2 expression levels were elevated in non-treated animals in comparison with the NPC group, both in brain and spinal cord, although not in a statistically significant manner (brain: \( p = 0.3513, \) 2.4-fold elevation; spinal cord: \( p = 0.1924, \) 2.5-fold elevation) (Fig. 5A, 5B). This was in contrast to the statistically significantly reduced levels of CCL5 in the whole CNS of the NPC group compared with control group (brain: \( p = 0.0164, \) 2.7-fold downregulation; spinal cord: \( p = 0.003, \) 5-fold downregulation) (Fig. 5C, 5D). CXCL10 was significantly downregulated only in the spinal cord, not in the brain (spinal cord: \( p = 0.007, \) 5-fold downregulation).
Figure 3. NPC transplantation resulted in reduced presence of immune cells within the spinal cord. Presence of macrophages (Mac-3) (A–C), B cells (B220) (D–F), T regulatory cells (CD25) (G–I), and T cells (CD4, CD3) (J–O) was evaluated. Gene expression levels of CD80 (P), CD86 (Q), and IL-17a (R) were measured. *p < .05, **p < .001. Error bars: mean ± SEM. Scale bars = 100 μm. Original magnification of all images, ×40. Abbreviations: EAE, experimental autoimmune encephalomyelitis; NPC, neural precursor cell.
Figure 4. Neural precursor cells remained in an immature state at the injection site. Transplanted bromodeoxyuridine-positive cells (A, E), located at the skin at the site of injection, were positive for nestin (B) (images merged in (D)) and brain-derived neurotrophic factor (F) (images merged in [H]). (C, G): Images counterstained with 4',6-diamidino-2-phenylindole. Original magnification of all images, ×40. Scale bars = 100 μm.

downregulation; brain: \( p = .1831 \) (Fig. 5E, 5F). NPCs seemed to downregulate the recruitment of macrophages and B cells, since significant changes were evident only in the brain for CCL3 (brain: \( p = .0381, 4.7\)-fold downregulation; spinal cord: \( p = .7873 \)) (Fig. 5G, 5H) and CXCL13 (brain: \( p = .0397, 12\)-fold downregulation; spinal cord: \( p = .1409 \)) (Fig. 5I, 5J). Similar results were noticed for dendritic- and Th17-cell recruitment, as observed from the significantly reduced levels of CCL20 (brain: \( p = .044, 1.8\)-fold downregulation; spinal cord: \( p = .0288, 3.1\)-fold downregulation) (Fig. 5K, 5L) [21, 35–37].

The receptor CXCR2 was reduced both in brain and spinal cord (brain: \( p = .0428, 1.95\)-fold downregulation; spinal cord: \( p = .0157, 5\)-fold downregulation) (Fig. 6A, 6B). CXCL1, which is the ligand of CXCR2, was also significantly downregulated in the brain (\( p = .0412, 2.3\)-fold downregulation), a result that agrees with the CXCR2 expression profile. CXCL1 was not significantly reduced in the spinal cord (\( p = .1247 \)) (Fig. 6C, 6D).

Finally, CXCR4 expression levels were significantly downregulated in the CNS of the NPC group (brain: \( p = .0035, 2.4\)-fold downregulation; spinal cord: \( p = .0295, 4\)-fold downregulation) (Fig. 6E, 6F). In the spinal cord, CXCL12 was significantly upregulated in the NPC group (\( p = .0083, 2.93\)-fold upregulation), but the opposite effect was observed in the brain (\( p = .0016, 1.7\)-fold downregulation) (Fig. 6G, 6H).

Inhibition of T-cell Proliferation Against Self Antigens by NPCs Ex Vivo

Isolated lymphocytes from spleens and lymph nodes were restimulated ex vivo with the antigen of immunization. Upon addition of linear MOG, there was significantly reduced proliferation of LNCs derived from the NPC group compared with those derived from the control group—a finding supported by other studies, as well [5, 7, 11]. The proliferation of ConA-treated LNCs did not differ significantly between groups, thus indicating that the reduction in the proliferation rate was MOG specific. The observed downregulation was not observed in the SPCs, since, despite the trend, the differences were not statistically significant (Fig. 7A, 7B).

To elaborate further on the downregulation of ex vivo proliferation of lymphocytes, we examined the gene expression levels of chemokine receptors that characterize their Th1 phenotype predominantly acquired during EAE and MS. We saw that isolated cells from the NPC group expressed significantly reduced levels of CCR1 and CCR5, receptors of Th1 T cells. Interestingly, the expression levels of CXCR3, another receptor characterizing Th1 polarized cells, were significantly upregulated in the NPC group (Fig. 7C).

DISCUSSION

Targeting the immune response in patients suffering from MS is the rationale of development of suitable immunotherapies. We transplanted NPCs s.c. in mice before the onset of EAE symptoms and observed significantly improved clinical outcome. This effect was accompanied by the diminution of the basic underlying pathologic features observed during EAE: The number of inflammatory infiltrates, the extent of demyelination and axonopathy, and glial activation were all reduced in NPC recipients [5, 6, 8]. The novel finding of this study is the partial elucidation of the effects of NPCs in the downregulation of the chemotactic signals expressed by the inflamed CNS aiming to recruit a plethora of inflammatory cells.

Chemokines are very important molecules in neuroinflammation occurring in MS; their secretion from CNS-resident endothelial cells and infiltrating leukocytes (through the BBB) leads to additional recruitment of other inflammatory components. Particularly, T and B lymphocytes, dendritic cells, and Th17 cells, among others, are recruited within the CNS [38]. Reduced levels of CCL5 and CCL20 expression present an altered recruitment status of inflammatory components in NPC recipients, specifically for autoimmune Th1 cells, Th17 cells, and dendritic cells. This effect was in accordance with the reduced number of infiltrating CD3-positive cells within the spinal cord as well as the reduced expression levels of IL-17α, although these were not statistically significant. Levels of CCL2, which is a crucial chemokine for migration of T cells within the CNS, also tended to be reduced, although not significantly. CXCL10 expression levels were downregulated.
only in the spinal cord. CXCL10 is responsible for the recruitment of encephalitogenic CD4+ T cells within the CNS [39]. Regarding the proinflammatory role of CXCL10, it has been suggested that enhanced expression in the subventricular zone of EAE mice leads to the recruitment of inflammatory blood cells from the periphery toward the CNS, contributing to the local inflamed environment [40]. Additionally, CXCL10 signaling through its receptor, CXCR3, seems to have a beneficial effect in EAE by controlling the parenchymal distribution of effector and regulatory cells [41]. CXCL10 is expressed mainly by astrocytes in actively demyelinating lesions [20]. The interrelationship between CXCL10 expression levels and active demyelinating lesions may explain the selective location of these chemokine changes in the spinal cord, since the latter is the predominant CNS compartment in which the underlying pathology takes place in MOG-EAE [42, 43]. Therefore, the levels of this specific chemokine are much more crucial in the context of spinal cord inflammation, the core target of NPCs’ immunomodulatory effects in MOG-EAE. NPCs affected astroglial activation in the context of GFAP+ cells surrounding the inflamed spinal cord, being in concordance with the modulation of CXCL10 expression.

CXCR2 expression has been linked to enhanced neutrophil migration during EAE [44] and to inhibition of migration and optimal localization of oligodendrocyte progenitors (OPCs) in demyelinating lesions, thus contributing to enhanced remyelination [24, 45].

Figure 5. NPCs modulate the expression of chemokines within the CNS. (A–L): Relative mRNA expression of chemokine genes in the brain (left column) and spinal cord (right column). Normalized expression of CCL2 (A, B), CCL5 (C, D), CXCL10 (E, F), CCL3 (G, H), CXCL13 (I, J), and CCL20 (K, L) is shown. Expression of each gene was normalized against β-actin as the reference gene. *, p < .05; **, p < .01. Error bars: mean ± SEM. Abbreviations: EAE, experimental autoimmune encephalomyelitis; NPC, neural precursor cell.
Downregulation of CXCR2 expression toward enhanced remyelination may be accompanied by the unaltered levels of CXCL1 in the spinal cord, which has a neuroprotective effect during autoimmune demyelination [46]. Reduced expression of CXCR2 further indicates disturbed IL-8 signaling (IL-8 is a proinflammatory cytokine secreted by activated macrophages recruiting neutrophils to the inflammation site and is part of the innate immunity response) [47, 48].

CXCR4 expression is downregulated in the CNS of transplanted animals. This is extremely important, since CXCR4 expression is upregulated in parenchymal cells of the spinal cord both in acute and chronic EAE relapses, thus playing a crucial role in the initial recruitment of inflammatory leukocytes [49]. CXCR4 directs the migration of transplanted OPCs into the white matter in the context of neuroinflammatory diseases such as EAE [50]. Another reason that NPC recipients express lower levels of CXCR4 may be the lack of stimuli that drive the host to mobilize its OPCs to repair the destructed myelin, since, in the transplanted mice, the demyelination is much reduced as revealed by the Luxol fast-blue staining [51]. Expression levels of CXCL12, a CXCR4 ligand, present a contradictory profile between the spinal cord and the brain. CXCL12 is involved in the modulation of polarization of effector Th1 cells, directing them toward CD4+CD25−Foxp3−IL-10high antigen-specific regulatory T cells [52]. It also downregulates the inflammation caused by the autoimmune attack by limiting the intraparenchymal migration of mononuclear cells [53]. In EAE, immune infiltrates in the spinal cord are present in greater amounts than in the brain, and possible anti-inflammatory action of CXCL12 generated by NPC transplantation is beneficial because it enhances the population of T regulatory cells within the CNS [42, 43]. This notion is supported by the unaltered levels of CD25-positive cells in the spinal cord [54]. A neuroprotective role has also been attributed to CXCL12, explaining at least partially the elevated levels in the spinal cord [55–57].

CD80 and CD86 are surface proteins expressed in B cells and monocytes, and contribute to the ongoing process of immunopathogenesis.

Figure 6. NPCs modulate the expression of chemokines and their receptors within CNS. (A–H): Relative mRNA expression of chemokine receptors and their ligands genes in the brain (left column) and spinal cord (right column). Normalized expression of CXCR2 (A, B), CXCL1 (C, D), CXCR4 (E, F), and CXCL12 (G, H) is shown. Expression of each gene was normalized against β-actin as the reference gene. *, p ≤ .05, **, p < .01. Error bars: mean ± SEM. Abbreviations: EAE, experimental autoimmune encephalomyelitis; NPC, neural precursor cell.
by restimulating infiltrating T cells [58]. CD80 and CD86 expression levels were downregulated both in the brain and spinal cord, indicating a decrease in the restimulation signals that support the continuation of pathogenesis. CD80 and CD86 expression levels correlated with the reduced number of macrophages and microglia accumulations that we demonstrate in this study. Although myeloid cells were reduced within the CNS, the correspondent chemokine that drives their attraction was downregulated only in the brain. Although CCL3 is strongly correlated with the exacerbation of symptoms in EAE and the presence of immune cells within the CNS, Subileau et al. suggested that CCL3 is not responsible for the recruitment of monocytes; rather, it is expressed in astrocytes during pathogenesis of MS [38]. Since EAE exhibits a pathogenesis different from, although relevant to, MS, with main inflammatory effects or a direct preference for lymphocytes rather than splenocytes, for unidentified reasons [9].

Moreover, CXCL13 is strongly connected to the exacerbation of symptoms in EAE and the presence of immune cells within the CNS, Subileau et al. suggested that CCL3 is not responsible for the recruitment of monocytes; rather, it is expressed in astrocytes during pathogenesis of MS [38]. Since EAE exhibits a pathogenesis different from, although relevant to, MS, with main inflammatory infiltrates being located in the spinal cord, CCL3 seems to increase rather early in the course of EAE pathogenesis and is not a target of NPC therapy.

The number of B220-positive cells was not downregulated in the spinal cord, at least not significantly. The number of B220 cells was not reduced in NPC recipients and this may be related to the unaltered levels of CXCL13. Moreover, CXCL13 is strongly connected to the presence of immune cells and increased immunoglobulin levels in the CSF [21]. We observed reduced ex vivo proliferation of lymphocytes in the presence of MOG in the NPC group compared with the control group. This observation agrees with previous reports [5, 7–9, 11]. On the contrary, this effect was not observed in the monocytes isolated from the spleen, thus complicating more the understanding of mechanisms that NPCs use in favor of the host. Since NPCs were injected subcutaneously, they were being expected to home to the neighboring lymph nodes as, reported by Pluchino et al. [9]. However, no similar observation was noticed in our current study. This controversy may be attributed to a number of reasons, including the different source of NPCs (newborn vs. adult) and the different EAE model (MOG-EAE vs. proteolipid protein-EAE) [9]. Nevertheless, similarly to Pluchino et al., transplanted NPCs remained undifferentiated [9]. Moreover, the cells remained alive at the injection site and the approximate areas until 24 days after injection while being able to express BDNF, thus providing a potential neuroprotective effect [59]. Presumably, their overall effect on the EAE process may easily be considered as bystander; nevertheless, it is of interest that lymph node proliferation, although not splenocyte proliferation, was affected. This finding further indicates that transplanted NPCs have either a bystander effect or a direct preference for lymphocytes rather than splenocytes, for unidentified reasons [9].

Modulation of Th1 phenotype on T cells extracted from lymph nodes was confirmed by the reduced expression of all chemokine receptors but CXCR3. This assumption, together with the selective modulation of lymphocyte sensitization toward MOG, indicates a selective mechanism of action.

Our findings show that injected NPCs can modulate the inflammatory environment within the CNS and thus have an impact on the clinical score of the host. Confirming previous studies, we saw that NPCs modulate the activation of T cells and downregulate chemotaxis, the latter being the novel finding of this study. This reduction in chemotactic signals derived by the CNS concerns not only the principal mediators of the disease, which are the T cells, but also other inflammatory components.

Last but not least, an in vivo immune response to the transplanted cells may not be totally ruled out. Our study was based on previous reports in which either paraformaldehyde-fixed cells or heat-killed cells were also administered in EAE, although exhibiting no significant results [8, 9]. However, there is a possibility that either the route of administration (subcutaneous or intracerebral) or the protocol for the inactivation of cells with or without concomitant preservation of their antigen epitopes plays a role [8, 9]. Therefore, it would be interesting in future studies to examine such a possibility. In our laboratory, we are running a relevant project with some interesting results that are under analysis.

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Figure 7. NPCs modulate peripheral immune responses. Ex vivo proliferation of lymph node cells (A) and splenocytes (B). The experimental conditions were cells without restimulation (unstimulated), cells incubated with +ConA, and cells incubated with restimulation agent (+MOG). (C): Relative mRNA expression of chemokine receptors in cell extracts isolated from lymph nodes. Normalized expression of CCR1, CCR5, and CXCR3 is shown. Expression of each gene was normalized against β-actin as the reference gene. **p < .01; ***p < .001. Error bars: mean ± SEM. Abbreviations: +ConA, concanavalin A; +MOG, myelin oligodendrocyte glycoprotein; EAE, experimental autoimmune encephalomyelitis; NPC, neural precursor cell; ns, not significant.
CONCLUSION

Our findings on the differential expression of various chemokines may further support the already identified anti-inflammatory and/or immunomodulatory effects induced by the transplanted NPCs. Moreover, our current study supports previous reports of the beneficial bystander effect of transplanted NPCs (i.e., the cells do not migrate within the CNS or to the peripheral lymphatic system). It is also of interest that transplanted NPCs may at least partially contribute to neuroprotection throughout the spinal cord via CXCL12 overexpression and BDNF expression.

In this study, we have demonstrated that overall, NPCs modulate events of innate immunity, which is activated early during EAE onset [60]. The accumulation of macrophages and microglia is significantly reduced within spinal cord, as is expression of an enhanced panel of chemokines; this results in reduced demyelination and recruitment of CD3⁺ autoreactive T cells [61, 62]. It remains to be clarified whether the chemokine kinetics are an epiphenomenon and/or concomitant to changes in other critical factors such as toll-like receptors and protective versus destructive components of inflammation, and lead to a fine balance of immune response [63–66].

Following NPC transplantation, chemokine levels throughout the CNS at successive time points and the ability of protein extracts from the CNS to recruit inflammatory components should be further explored. This knowledge would clarify whether the reduction of chemokine levels is due to the directed action of NPCs toward their cellular sources or if it is part of general downregulation of inflammation. Finally, NPCs showed signs of high potency in reducing clinical symptoms in EAE, although their optimal source for clinical use remains to be clarified. Further experiments aiming at transducing somatic cells like fibroblasts or induced pluripotent stem cells into NPCs with the same immunomodulatory properties could potentially lead to their introduction into clinical routine [67].

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AUTHOR CONTRIBUTIONS

S.R.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; K.N.P.: conception and design, collection and/or assembly of data, manuscript review; R.L.: conception and design, collection and/or assembly of data, P.A. and A.L.: conception and design, data analysis and interpretation; E.N., P.T., and E.K.: collection and/or assembly of data; D.T.: statistics, manuscript review; D.K.: manuscript review; M.G. and K.C.: data analysis and interpretation; N.G.: conception and design, data analysis and interpretation, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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