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Accelerated and enhanced effect of CCR5-transduced bone marrow neural stem cells on autoimmune encephalomyelitis

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Abstract

The suppressive effect of neural stem cells (NSCs) on experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), has been reported. However, the migration of NSCs to inflammatory sites was relatively slow as was the onset of rather limited clinical benefit. Lack of, or low expression of particular chemokine receptors on NSCs could be an important factor underlying the slow migration of NSCs. To enhance the therapeutic effect of NSCs, in the present study we transduced bone marrow (BM)-derived NSCs with CCR5, a receptor for CCL3, CCL4, and CCL5, chemokines that are abundantly produced in CNS-inflamed foci of MS/EAE. After i.v. injection, CCR5-NSCs rapidly reached EAE foci in larger numbers, and more effectively suppressed CNS inflammatory infiltration, myelin damage, and clinical EAE than GFP-NSCs used as controls. CCR5-NSC-treated mice also exhibited augmented remyelination and neuron/oligodendrocyte repopulation compared to PBS- or GFP-NSC-treated mice. We inferred that the critical mechanism underlying enhanced effect of CCR5-transduced NSCs on EAE is the early migration of chemokine receptor-transduced NSCs into the inflamed foci. Such migration at an earlier stage of inflammation enables NSCs to exert more effective immunomodulation, to reduce the extent of early myelin/neuron damage by creating a less hostile environment for remyelinating cells, and possibly to participate in the remyelination/neural re-population process. These features of BM-derived transduced NSCs, combined with their easy availability (the subject's own BM) and autologous properties, may lay the groundwork for an innovative approach to rapid and highly effective MS therapy.

Keywords

Neural stem cells; Chemokine receptor; MS/EAE

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease resulting from an autoimmune reaction against CNS myelin [20, 28]. MS begins when peripherally activated myelin-reactive T cells infiltrate the CNS, predominantly in the white matter. Once in the CNS, myelin-reactive T cells are presented with myelin antigens and become activated (or likely reactivated after a first peripheral activation), thereby triggering an inflammatory cascade that results in lesions with extensive demyelination and neuron/axonal loss [19, 22]. The vascular permeability of acute lesions resolves within 4–6 weeks, and the lesions then enter into a more chronic phase, where oligodendrocytes are absent and myelin is lost [32]. Spontaneous remyelination in MS is often incomplete or lacking, probably due to the hostile microenvironment for oligodendrocyte precursor cells (OPCs) created by proinflammatory cells and soluble mediators, which limit or block the migration of OPCs into the demyelinated foci and their maturation into mature, myelinating oligodendrocytes [8].

Recent studies have shown the therapeutic potential of neural stem cells (NSCs) in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, due to their capacity for neural repopulation and their weak immunomodulation [7, 17, 42, 43]. We have shown that adult NSCs can be trans-differentiated from bone marrow (BM) mesenchymal cells *in vitro* and that, after transplantation, these BM-derived NSCs (BM-NSCs) possess neural cell differentiation capacity *in vivo* and therapeutic effect on clinical EAE comparable to those derived from the subventricular zone (SVZ) of the brain [44]. However, migration of both types of NSCs to inflammatory sites was relatively slow: a significant clinical improvement did not occur until 20 days after NSC injection; and the presence of NSCs in the parenchyma was not directly demonstrated until day 30 after injection [29, 42, 44]. A low level of expression of particular chemokine receptors on NSCs could be an important factor underlying these therapeutic limitations [29, 31].

Chemokines are a family of proteins with a variety of physiological functions, including regulation of cellular migration and inflammatory responses, and of cell development and growth, all mediated by interaction with chemokine receptors [15, 16, 36, 38, 46]. At the onset of acute EAE, the CNS exhibits high levels of CCL3, CCL4, CCL5 and CCL2, chemokines that are involved in the accumulation and activation of leukocytes bearing CCR5 and CCR2 receptors; by contrast, in the CNS of chronic EAE/MS, increased levels of CCL19 and CCL21, which bind CCR7, are observed [15, 16, 36, 38, 46]. Recently it has been found that mouse NSCs express detectable levels of CCR1, CCR2, CCR5, CXCR3 and CXCR4 [13, 18, 21, 31], but do not express CCR3 or CCR7 [31]. As a result, NSCs have no chemotactic response to CCL2, CCL3 or CCL4 in spite of a weak response to CCL5 and CXCL12/SDF-1a [13, 21].

To enhance and accelerate the migration capacity of NSCs into CNS inflammatory sites, which contain high levels of chemokines [15, 16, 36, 46], in the present study we expressed CCR5, a receptor for CCL3, CCL4, and CCL5, on BM-NSCs. These cells possess a “guided missile”-like property (i.e., rapid and targeted NSC migration to EAE inflammatory foci) after transplantation, resulting in decreased local inflammation, reduced neural damage, and earlier and more efficient remyelination. This property, combined with the easy accessibility and autologous nature of BM-NSCs, could make this a novel, attractive approach to rapid and effective immunotherapy for MS.

MATERIALS AND METHODS

Generation of BM-NSCs and CCR5 transduction

BM-NSCs were generated from BM mesenchymal cells of adult C57BL/6 mice (Jackson Laboratory), 8–10 wk of age, as described in our laboratory [44]. Cells at 5th–10th passages were used in our experiments. To induce BM-NSC differentiation, dissociated single cells or small neurospheres were incubated in stem cell differentiation medium for 10 to 14 days and processed for immunocytochemistry staining.

To modify BM-NSCs with CCR5 genes, a lentiviral vector that had been successfully used for IL-10 transduction of NSCs [42], was applied. Briefly, dissociated neurospheres were infected with lentiviral vectors Lv.CCR5 encoding both CCR5 and GFP, or Lv.GFP encoding GFP alone as a control. Transduction efficiency was evaluated by flow cytometry and GFP positive cells were sorted by FACS to a purity of >99%. These cells were then re-seeded in growth medium for further expansion.

Chemotaxis, proliferation and neural differentiation of CCR5 transduced BM-NSCs in vitro

We then determined the biological function of CCR5 transduced on BM-NSCs. The migratory response of transduced NSCs to CCL3, CCL4 and CCL5, all ligands of CCR5 [15, 16, 36], was evaluated using a microchemotaxis Boyden chamber system (Neuro Probe) as described [25]. Concentrations of these chemokines were chosen based on previous reports [14, 25], and cells that migrated into the lower well were stained with Diff-Quik dye (blue) and counted [14, 25]. The proliferation potential of CCR5-transduced NSCs was assessed by bromodeoxyuridine (BrdU) incorporation and by serial passage growth curves at 1st to 6th passages as described [34]. Cell survival was assessed by measuring release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium as described [12].

EAE induction and BM-NSC treatment

To induce active EAE, female C57BL/6 mice at 8–10 wks of age were injected subcutaneously (s.c.) with 150 µg MOG_{35–55} in CFA containing 5 mg/ml Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) at 2 sites on the back. Pertussis toxin at 200 ng was injected i.p. on days 0 and 2 post immunization (p.i.). To induce adoptive transfer EAE, draining lymph nodes of immunized mice were harvested at day 11 p.i. and stimulated at a density of 2×10^7 cells/ml in Click's EHAA medium (Irvine Scientific, Santa Ana, CA) supplemented with 15% FCS, 20 ng/ml recombinant mouse IL-12, and 50 µg/ml MOG peptide for 4 days. Cells were harvested, washed and 3×10^7 cells/200 µl PBS were injected i.v. into each recipient mouse as described [10]. Clinical score was checked daily by two researchers blindly according to a 0–5 scale, as described [10]. Single dissociated CCR5- or GFP-transduced NSCs (1.5×10^6 cells in 200 µl PBS/each mouse) were intravenously (i.v.) injected via the tail vein at the peak of disease (day 22 p.i.). Age-, sex- and strain-matched mice injected i.v. with PBS served as PBS-treated controls. All work was performed in accordance with the guidelines for animal use and care at Thomas Jefferson University.

Immunomodulation of CCR5-NSCs in the periphery

To address the influence of CCR5-NSCs vs. GFP-NSCs on peripheral immune responses, splenocytes of NSC-treated or PBS-treated mice with active EAE were prepared at week 2 post-transplantation (p.t.). These cells were cultured in the presence of autoantigen MOG_{35–55} (10 µg/ml) and mitogen Con A (5 µg/ml) for 3 days. Proliferative responses were determined by incorporation of ³H-thymidine and cytokine production in culture supernatants as determined by ELISA.

Histopathology and immunohistochemical staining

All groups of animals were sacrificed 64 days p.i. Brains and spinal cords were harvested for pathological and immunological analysis. All pathology/immunohistochemistry studies were performed on eight predefined white matter regions as described [23] in cervical, thoracic and lumbar sections for mice of all groups, and an average value from each mouse was obtained. Seven- μ m sections were stained with H&E for inflammation and Luxol fast blue (LFB) for demyelination respectively. Slides were assessed in a blinded fashion for inflammation and demyelination using a 0–3 scale as described [4]. For inflammation, the following scale was used: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. For demyelination, the following scale was used: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination. Immunohistochemical staining was performed using antibodies for neural cells, NSCs, infiltrating CD45⁺ cells, and anti-von Willebrand factor (vWF; for blood vessels) [6]. Immunofluorescence controls were routinely performed with incubations in which isotype-specific antibodies were used as the 1st antibodies. Results were visualized by fluorescent microscopy (Nikon Eclipse E600) or confocal microscopy (Zeiss LSM 510).

Electron microscopy

Lumbar spinal cords were fixed and processed by electron microscopic analysis for assessing de-/re-myelination as described [42]. Remyelinated axons were defined by the g ratio, a fraction of axon diameter divided by the entire fiber diameter of axon plus myelin sheath (measured by ImageJ software). Identification of abnormally thin myelin sheaths (greater than normal g ratio) remains the most reliable means of identifying remyelination [8, 40]. At least 50 myelinated axons on the electron micrographs were measured for each mouse; 5 mice per group were evaluated.

Statistical analysis

Clinical and pathological (H & E and Luxol fast blue) scores were analyzed by the Kruskal–Wallis test. Differences for other parameters were evaluated by the ANOVA (for multiple groups) or Student's t test (for two groups). Differences were considered significant at a value of $p < 0.05$.

RESULTS

Transduction of GFP and CCR5 into NSCs

Cells at 4th–10th passages were used in our experiments. To identify neurospheres, double immunostaining was performed to test the expression of neural-specific markers nestin and SOX2. Both BM-neurospheres (Fig. S1A) and single BM-NSCs (Fig. S1B) were double-positive for nestin and SOX2. BM-NSCs exhibited morphological and functional properties similar to those of SVZ-NSCs, as previously described by our group and by others [3, 37, 44].

We then transfected these cells at the 4th passage with either bicistronic lentiviral vector Lv.CCR5 encoding both CCR5 and GFP, or with Lv.GFP encoding GFP alone, to serve as control (Fig. S1C). At day 3 post-transduction, strong CCR5 staining was visible in NSCs transduced with Lv.CCR5, but not with Lv.GFP, while strong GFP expression was visible in cells transduced with both vectors (Fig. S1D). 82.5% of NSCs among those transduced with Lv. CCR5 expressed CCR5 compared with 7.1% of those transduced with Lv. GFP (Fig. S1E), demonstrating the high efficiency of CCR5-transduction into NSCs. GFP⁺ cells were sorted by FACS to a purity of >99%, and re-seeded in growth medium for further expansion.

GFP and CCR5 were consistently expressed on these cells up to the 10th passage (data not shown).

Chemotaxis, proliferation, differentiation, and survival of transduced NSCs

To determine the function of CCR5 transduced to NSCs, migratory response of NSCs to chemokines was evaluated using a microchemotaxis Boyden chamber system (Neuro Probe) as previously described [31]. CCR5-transduced NSCs exhibited significantly enhanced chemotactic migratory responses to CCL5 in a dose-dependent manner (Fig. 1A, B), indicating that the transduced chemokine receptor is biologically functional. On the other hand, NSCs transduced with CCR5 exhibited similar patterns as GFP-transduced NSCs and non-transduced NSCs in BrdU⁺ incorporation, neural cell differentiation and LDH release (Fig. S2A-E). These cells also exhibited a similar growth rate, as shown by their absolute numbers counted in 1–6 passages (Fig. S2F). These results indicate that CCR5 transduction does not influence NSC proliferation, differentiation, or survival.

Accelerated and enhanced suppression of clinical symptoms of EAE by CCR5-NSCs

While both CCR5-NSCs and GFP-NSCs significantly suppressed disease severity in adoptive transfer EAE mice compared to sham-treated mice ($p < 0.05$ – 0.01), CCR5-NSCs exhibited a significantly more effective suppression of disease severity than GFP-NSCs (Fig. 2A; $p < 0.05$). A more rapid reduction of EAE severity was also obtained in mice treated with CCR5-NSCs than with GFP-NSCs; thus, the time between NSC transplantation and the onset of statistically significant protection was day 7 ± 1.5 in CCR5-NSC-treated mice vs. 18 ± 2.2 in GFP-NSC-treated mice ($p < 0.05$). Mice that were treated with CCR5-NSCs also exhibited a more profound decrease in disease severity than those treated with GFP-NSCs ($p < 0.05$). Consistent with clinical observation, mice treated with CCR5-NSCs exhibited a significant reduction in inflammatory infiltrates in spinal cord white matter as compared with control GFP-NSC treated mice and PBS-treated mice (Fig. 2B, C).

To test the effect of CCR5-NSCs on actively induced EAE, NSCs were i.v. injected at day 22 p.i. Similar to results in the adoptive transfer EAE model, CCR5-NSCs suppressed actively induced EAE more effectively than GFP-NSCs (Fig. 3A), with significantly reduced CNS infiltration (Fig. 3B, C). These results indicate that CCR5-NSCs have an enhanced therapeutic effect on EAE regardless of the method of disease induction.

CCR5- and GFP-NSCs modulate peripheral immune responses to a comparable extent

We then tested whether transduction of CCR5 altered the immunoregulatory effect of NSCs. As shown in Fig. 3D, splenocytes from mice with actively induced EAE that were treated with either GFP- or CCR5-NSCs displayed lower levels of proliferative responses to MOG_{35–55} peptide and Con A compared to those from PBS-treated EAE mice (all $p < 0.05$). Further, suppression of IFN- γ /IL-17 production and a slight upregulation of IL-10 production in these splenocytes were comparable in the two groups (Fig. 3E).

Given that similar results were obtained in adoptively transferred EAE and actively-induced EAE for CNS pathological and immunohistochemical analyses (data not shown), we only present data derived from the former (mice in Fig. 2A) as representative in this report.

Rapid migration of CCR5-NSCs into the CNS of EAE mice

Brains and spinal cords were harvested at weeks 2, 4 and 6 p.t. for immunohistology. Strong CCR5 expression (red) was observed in mice treated with CCR5-NSCs, but not with GFP-NSCs (Fig. 4A), demonstrating the high level of CCR5 expression in the CNS by CCR5-NSCs. The distribution of NSCs in the CNS is shown in Fig. 4B (week 4 p.t.), in which NSCs are seen around vessels and near adluminal endothelial cells (perivascular space) and

separate from the surrounding spinal cord parenchyma of inflamed foci [11]. At 2 weeks p.t., a number of CCR5-NSCs were observed in perivascular space, while only a few GFP-NSCs were found (Fig. 4C; $p<0.01$). By weeks 4 and 6 p.t., the majority of transplanted CCR5-NSCs (GFP⁺) had migrated into the parenchyma while a large proportion of GFP-NSCs was primarily confined to perivascular spaces (Fig. 4B, C), demonstrating the rapid and guided migration of CCR5-NSCs to EAE foci after transplantation.

As shown in Fig. 4D and E, CCR5-NSCs exhibited rapid and enhanced suppressive effect on infiltrating cells in the CNS. While there was no difference between GFP-NSC-treated mice and non-treated mice at an early time point (week 2 p.t.), significantly reduced numbers of CD45⁺ cells in inflamed CNS were observed in CCR5-NSC-treated mice at this time point. A more profound reduction of infiltrating CD45⁺ cells was also observed at weeks 4 and 6 p.t. in CCR5-NSC-treated mice compared to GFP-NSC-treated mice ($p<0.05$; Fig. 4E).

After i.v. injection, both CCR5- and GFP-transduced NSCs reached systemic organs (liver, heart, spleen, etc.) at days 2–3 after injection, and were then no longer detectable at days 18–20 for GFP-NSCs and days 13–15 for CCR5-NSCs ($p<0.05$) (data not shown). While cells that had migrated into the CNS were able to further proliferate and differentiate into neural cell lineages, those that remained in peripheral organs may finally have been cleared [29].

Differentiation of CCR5-NSCs in the CNS of EAE mice

At the end of the experiment (6 weeks p.t.), co-localization of GFP⁺ and neural specific markers⁺ in brain sections revealed that some of the transplanted cells differentiated into GalC⁺ mature oligodendrocytes, β -tubulin⁺ neurons and GFAP⁺ astrocytes, and a small proportion of GFP⁺ cells remained undifferentiated (nestin⁺) (Fig. 5A). Quantitative analysis showed that the numbers of differentiated and undifferentiated CCR5-NSCs in EAE foci were larger than those of control GFP-NSCs (Fig. 5B). The percentages of these neural cells among transplanted NSCs (GFP⁺) were similar between CCR5- and GFP-NSCs (Fig. 5C), indicating that CCR5-transduction does alter NSC differentiation, and that higher numbers of neural cells in CCR5-NSC-treated mice were due to increased CNS migration of these cells.

Enhanced remyelination by transplantation of CCR5-NSCs

Spinal cords of CCR5-NSC-treated mice exhibited rare demyelination foci and a significantly lower demyelination score than control GFP-NSC-treated and PBS-treated EAE mice (Fig. 6A, C). A significantly higher demyelination score was observed in PBS-treated than in EAE mice that were sacrificed at day 22 p.i. when NSC treatment started in other groups (EAE before i.v.), indicating that untreated EAE mice underwent progressive demyelination, which was blocked by NSC treatment (Fig. 6A, C). Electron microscopic analysis showed that CCR5-NSC-treatment increased the percentage of myelinated axons among total axons (Fig. 6B). Most of the myelinated axons in NSC-treated mice were encased in typical thin myelin sheaths, a well-established morphological hallmark of remyelination [8, 40], while a large number of demyelinated axons were found in PBS-injected sham-EAE and EAE mice before treatment (Fig. 6B; left panel). Greater g ratios (thinner myelin) were found in both CCR5- and GFP-NSC-treated mice than the normal g ratio in naïve mice ($p<0.05$, Fig. 6E), which is a characteristic of remyelination [8, 40]. There was a greater percentage of myelinated axons and a lower g ratio in CCR5-NSC-treated mice than in GFP-NSC-treated mice, indicating enhanced remyelination by CCR5 overexpression ($p<0.05$, Fig. 6E).

DISCUSSION

In the present study we provide evidence that, compared to conventional NSCs, CCR5-transduced NSCs rapidly migrated into EAE foci, more effectively suppressed CNS inflammation and accelerated endogenous and exogenous remyelination, thus significantly enhancing their therapeutic effect on CNS inflammatory demyelination.

Interaction of chemokines and their receptors mediates a variety of leukocyte responses, including chemotaxis and immune activation [15]. Active white matter lesions in MS patients and in the CNS of acute EAE mice exhibit high levels of CCL3, CCL4, CCL5, and CCL2, chemokines that are involved in the accumulation and activation of leukocytes bearing CCR5 and CCR2 receptors [15, 16, 38]. The fact that NSCs express a very low level (10^4 -fold lower than activated splenocytes) of CCR5 and CCR2 [13, 31] may explain why injection of NSCs, although effective, is relatively slow in suppressing EAE [29, 31]. Overexpression of chemokine receptor(s) on selected cells is potentially a useful approach to augment the migration of these cells into target sites containing high levels of corresponding chemokines. Indeed, transgenic mice expressing either CCR5 or CCR7 have been used as models of leukocyte migration *in vivo* [35, 41]. Dendritic cells transduced with CCR7 acquired strong chemotactic activity for its ligand, 6Ckine [27], and, when injected into mice, accumulated in the target organs approximately 5.5-fold more efficiently and elicited more effective antigen-specific immune response *in vivo* compared with control gene-transduced cells [27]. Consistent with these observations, our current *in vitro* and *in vivo* results indicate that transduction of CCR5 in NSCs is functional, without altering their proliferation and neural differentiation properties.

The therapeutic effect of NSCs on EAE has been attributed, at least in part, to restraining dendritic cell function [30] and immunosuppressing T-cell activation and proliferation [2, 7]. However, due to their non-specific properties, a systemic immunosuppression may also occur when these cells are present in the periphery before migration into the CNS (approx. 18–20 days post i.v. injection) [31, 42, 44]. Thus, accelerated migration of CCR5-transduced NSCs from the periphery into the CNS would provide at least two advantages in this regard: 1) shortening or minimizing systemic immunosuppression when these cells are in the periphery; and 2) enhanced suppression of inflammation in the target organ, where this effect is likely to be more effective than in the periphery. This would result from a greater density of transplanted NSCs in the inflamed foci, where they closely interact with and modulate the inflammatory effects of infiltrating, pathogenic immune cells, than in the periphery. Given that a few autoreactive T cells in an autoimmune infiltrate control a vast population of nonspecific cells, the suppression of these autoreactive T cells could lead to a significant reduction of other inflammatory infiltrates [39]. Similarly, Croxford et al. found that, while systemic administration of IL-10 failed to suppress EAE, local delivery of this cytokine had a significant effect [5]. Therefore, while systemic injection of NSCs exhibited a weak suppression of CNS inflammation in previous studies, accumulation of these cells in CNS foci would dramatically enhance this effect by a direct contact with autoreactive T cells, and would more effectively induce apoptosis of these cells [29, 42], thus leading to a rapid regression of the entire inflammatory cascade in the CNS. Further, reduced CNS inflammation would result in lower chemokine production, thus reducing the secondary waves of immune cell infiltration [15, 16, 36, 46]. These mechanisms, together, will result in an enhanced anti-inflammatory effect of CCR5-NSCs in the CNS in EAE.

At the chronic stage of MS/EAE, axonal damage and neuronal loss are considered the main mechanisms leading to irreversible deficits, and treatment at the earliest possible stage would be crucial to halting this process [20, 28]. In addition to their immunomodulatory capacity, the promotion of remyelination, neuronal repopulation and axonal growth are also

important mechanisms underlying NSC effect on EAE [29, 31, 42, 44]. Therefore, although CCR5-transduction *per se* does not drive NSCs to differentiate into neurons or oligodendrocytes more efficiently, the earlier availability of high numbers of NSCs in the demyelinated foci would be expected to enable more effective CNS recovery.

An important factor of regenerative failure is the presence of myelin debris from MS lesions and neuroregeneration inhibitors, which create a hostile environment for regeneration in the deteriorating and chronically inflamed CNS of MS patients [26, 45]. Although transplanted NSCs may not be able to neutralize these inhibitors of neuroregeneration or block their signaling (via LINGO-1/TROY system), an accelerated anti-inflammatory presence of NSCs in the CNS will impede further neuronal and myelin damage, thus reducing the production of such inhibitors. Further, reduced immune response in the NSC niche can significantly improve proliferation of NSCs and OPCs originating in the SVZ and their differentiation into mature oligodendrocytes [33]. In our study a significantly higher demyelination score was observed in control EAE mice that were treated with PBS and sacrificed at day 62 p.i. (sham-EAE) than in those sacrificed at day 22 p.i. (EAE before i.v.), when NSC-treatment started in other groups of mice, indicating that untreated EAE mice underwent progressive demyelination as expected in this chronic model (Fig. 6A, C). While this progression was significantly blocked in mice that received both CCR5-transduced NSCs and control NSCs, a more profound recovery was obtained by CCR5-transduced NSCs.

In summary (Fig. 7), expressing CCR5 on BM-NSCs endows these cells with the capacity for rapid and guided migration to EAE foci after transplantation, thus enabling them to directly exert their immunomodulatory effect at these foci, reducing further demyelination and promoting more efficient remyelination by a larger number of NSCs and at an earlier time period than with conventional NSCs. Based on these observations, we expect that transduction of CCR7 to NSCs may be beneficial for a later stage of chronic EAE/MS, at which increased levels of the CCL19 and CCL21 (ligands of CCR7) are expressed in the inflamed CNS [15, 16, 36, 46]. Similarly, transduction of CCR2 may be beneficial for MS relapses [24]. In future clinical trials, individualizing chemokine receptor expression in the transduction system could be done on a patient-by-patient basis by determining the chemokine profile in cerebrospinal fluid. Further, lentiviral vectors have been confirmed as a highly safe tool for gene therapy and have been widely used in clinical trials [1, 9]. These properties, combined with the availability and autologous nature of BM-NSCs (i.e., obtained from the patient's own BM), make this an attractive and promising approach for highly effective, cell-based immunotherapy of MS.

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Figure 1. Chemotaxis assay for CCR5-transduced NSCs

(A) NSCs at the 5th passage were transduced with CCR5. After 5 days, cells were plated into the upper well of a 48-well chemotaxis chamber, and different concentrations of CCL5 were added into the lower well for 5 hrs. Cells that migrated into the lower well were stained with Diff-Quik dye (blue) and counted as described in “Methods”. (B) Quantitative analysis of cell migration. Data represent mean values $\pm$ SE of three wells in Graph A. P values refer to comparisons between CCR5-transduced NSCs with GFP-transduced NSCs. *, $p < 0.05$; **, $p < 0.01$. One representative experiments of three is shown.

Fig. 2. Enhanced suppression of adoptive transfer EAE by CCR5-overexpressing NSCs
 CCR5-transduced NSCs were injected i.v. to EAE mice, 1.5×10^6 cells/mouse, at the peak of disease (day 22 post T-cell transfer). Mice receiving the same number of GFP-NSCs or PBS only served as controls. **(A)** Clinical scores were checked daily by two researchers blindly according to a 0–5 scale ($n = 8$ in each group). **(B)** H&E staining to detect CNS inflammation. EAE mice were sacrificed 2 weeks p.t., and spinal cords were harvested for H&E staining. A decrease in inflammatory infiltrates in the white matter of spinal cord and reduced mean score of inflammation **(C)** in H&E staining were found in mice treated with CCR5-NSCs as compared with control NSC-treated and sham-EAE (PBS-treated) mice; Scale bar: 50 μ m in B. * $p < 0.05$, ** $p < 0.01$, comparison between sham-EAE and other groups. # $p < 0.05$, comparison between GFP-NSC-injected and CCR5-NSC-injected mice. ($n = 8$ each group).

Figure 3. Effect of CCR5-transduced NSCs on actively induced EAE and peripheral immune system

(A) CCR5-transduced NSCs were injected i.v. to EAE mice, 1.5×10^6 cells / mouse, at disease peak of active EAE (day 22 p.i.). Mice receiving the same number of GFP-NSCs or PBS only served as controls. Clinical scores were checked daily by two researchers blindly according to a 0–5 scale. * $p < 0.05$, ** $p < 0.01$, comparison between sham-EAE and other groups. # $p < 0.05$, comparison between GFP-NSC-injected and CCR5-NSC-injected mice. (n = 6–8 each group). (B–C) H&E staining to determine CNS inflammation. EAE mice were sacrificed 2 weeks p.t., and spinal cords were harvested for H&E staining as described in Fig. 2B and C (n = 6–8 each group). (D–E) Splenocytes from each group of EAE mice

described in Fig. 2B were harvested two weeks p.t., cultured at $1.5 \times 10^6/\text{ml}$ and stimulated with MOG_{35–55} (10 $\mu\text{g}/\text{ml}$) or Con A (5 $\mu\text{g}/\text{ml}$) for 3 days. **(D)** Proliferative responses were determined by ^3H -thymidine incorporation and **(E)** cytokine production in culture supernatants analyzed by ELISA. *, $p < 0.05$, comparisons between sham-EAE and other groups ($n = 6–8$ each group).

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Figure 4. Localization and migration of transplanted CCR5-NSCs in the CNS

Mice treated with NSCs i.v. at day 22 post T cell transfer were sacrificed 2, 4 and 6 weeks p.t.; brains and spinal cords were harvested for immunohistology. (A) Brain sections were immunostained with anti-CCR5 antibody and examined by confocal microscopy. Strong CCR5 expression (red) was observed in mice treated with CCR5-NSCs but not in GFP-NSCs, both of which were GFP⁺. Nuclei were stained with DAPI (blue). Scale bar: 10 μ m. (B) The majority of transplanted CCR5-NSCs (green) had migrated to the parenchyma (examples indicated by arrow) by 4 weeks p.t., while most of the GFP-NSCs (green) were primarily confined to perivascular spaces (examples indicated by arrowhead). Blood vessels were stained with anti-vWF antibody (red). Nuclei were stained with DAPI (blue). (C) Quantitative analysis of GFP⁺ NSCs in spinal cord of EAE mice at 2, 4 and 6 weeks p.t. Symbols represent mean values and SD of 6–8 mice each group. * $p < 0.05$, ** $p < 0.01$, comparisons between CCR5-NSC- and GFP-NSC-treated groups. (D) GFP⁺ NSCs remained

in inflammatory areas, where blood-borne CD45⁺ immune cells (red) that form the CNS inflammatory infiltrate persisted. Nuclei were stained with DAPI (blue). Scale bar: 10 μ m in A, 50 μ m in B, 25 μ m in D. **(E)** Quantitative analysis of CD45⁺ T cells in spinal cord of EAE mice at 2, 4 and 6 weeks p.t. * $p < 0.05$, ** $p < 0.01$, comparisons between sham-EAE mice and mice treated with CCR5-NSCs or GFP-NSCs. # $p < 0.05$, comparisons between CCR5-NSC- and GFP-NSC-treated groups.

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Figure 5. Differentiation of transplanted NSCs in the CNS

Brains of EAE mice treated with GFP- and CCR5-NSCs were harvested at week 6 p.t., 7 μ m cryosections were immunostained with anti-neural specific antibodies. **a** Cells co-labeled with GFP and neural specific markers (red) were identified as differentiated cells derived from transplanted NSCs (arrows); cells positive only for neural specific markers (red) were endogenous cells (dashed arrows). Co-localization of GFP⁺ and neural markers⁺ (red) in the CNS indicated that both transplanted GFP- and CCR5-NSCs had differentiated into β -tubulin⁺ neurons, GalC⁺ mature oligodendrocytes, GFAP⁺ astrocytes, and a small proportion of GFP⁺ cells remained undifferentiated (nestin⁺). A similar pattern was observed in both groups, suggesting a comparable differentiation potential in vivo of these

two types of NSCs. Scale bar: 20 μm . **b** Absolute number and **c** percentage of differentiation of transplanted NSCs (GFP⁺) in the CNS at week 6 post NSC injection. (n = 8 each group).
* p<0.05.

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Figure 6. Transplanted CCR5-NSCs more efficiently promote remyelination

Spinal cords were harvested 6 weeks p.t. (A) Luxol fast blue (LFB) staining of spinal cord sections for detection of demyelination. Magnification $\times 50$. (B) Electron microscopic analysis. The presence of thin myelin sheaths (remyelination; arrows) had been shown in the demyelinated lesions of spinal cords in both CCR5-NSC- and control-NSC-treated mice, while a large number of demyelinated axons (dashed arrows) were found in PBS-treated sham-EAE and EAE mice that were sacrificed at day 22 post disease induction, when NSC treatment was started in other groups (EAE before i.v.). Normal thick myelin sheaths (arrowheads) in naïve mice serve as control. Magnification $\times 500$, insets $\times 2000$. (C) Mean scores of demyelination. Symbols represent mean values and SD of 6–8 mice each group. (D) Quantification of percentage of myelinated axons among total axons as shown in electron micrographs. # $p < 0.05$, ## $p < 0.01$, comparisons between EAE before NSCs i.v. (day 22 p.i.) and other groups; ** $p < 0.01$, comparisons between sham-EAE and other groups; @ $p < 0.05$, comparison between GFP-NSCs i.v. and CCR5-NSCs i.v. (E) Mean g ratio (axon diameter divided by entire myelinated fiber diameter) was determined using ImageJ software. * $p < 0.05$, comparisons between naïve group and other groups, # $p < 0.05$, comparison between GFP-NSC-i.v. and CCR5-NSC-i.v. Symbols represent mean values and

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Figure 7. Schema: Mechanisms of action of CCR5-NSCs in EAE

Expressing CCR5 on BM-NSCs endows these cells with the capacity for rapid and guided migration to EAE foci after transplantation, thus reducing further demyelination and promoting more efficient remyelination at an earlier stage than control NSCs. Increased exogenous NSCs in demyelinated foci may also participate in the remyelination/neural re-population process. BBB: blood-brain barrier; NSCs: neural stem cells. Thick line/arrows: major pathways; thin line/arrows: secondary pathways.