The CCR2/CCL2 Interaction Mediates the Transendothelial Recruitment of Intravascularly Delivered Neural Stem Cells to the Ischemic Brain

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Background and Purpose—The inflammatory response is a critical component of ischemic stroke. In addition to its physiological role, the mechanisms behind transendothelial recruitment of immune cells also offer a unique therapeutic opportunity for translational stem cell therapies. Recent reports have demonstrated homing of neural stem cells (NSC) into the injured brain areas after intravascular delivery. However, the mechanisms underlying the process of transendothelial recruitment remain largely unknown. Here we describe the critical role of the chemokine CCL2 and its receptor CCR2 in targeted homing of NSC after ischemia.

Methods—Twenty-four hours after induction of stroke using the hypoxia-ischemia model in mice CCR2+/+ and CCR2−/− reporter NSC were intra-arterially delivered. Histology and bioluminescence imaging were used to investigate NSC homing to the ischemic brain. Functional outcome was assessed with the horizontal ladder test.

Results—Using NSC isolated from CCR2+/+ and CCR2−/− mice, we show that receptor deficiency significantly impaired transendothelial diapedesis specifically in response to CCL2. Accordingly, wild-type NSC injected into CCL2−/− mice exhibited significantly decreased homing. Bioluminescence imaging showed robust recruitment of CCR2+/+ cells within 6 hours after transplantation in contrast to CCR2−/− cells. Mice receiving CCR2+/+ grafts after ischemic injury showed a significantly improved recovery of neurological deficits as compared to animals with transplantation of CCR2−/− NSC.

Conclusions—The CCL2/CCR2 interaction is critical for transendothelial recruitment of intravascularly delivered NSC in response to ischemic injury. This finding could have significant implications in advancing minimally invasive intravascular therapeutics for regenerative medicine or cell-based drug delivery systems for central nervous system diseases. (Stroke. 2011;42:00-00.)

Key Words: chemokines ■ intravascular transplantation ■ neural stem cells ■ regenerative medicine ■ stroke ■ transendothelial recruitment

A common characteristic of many central nervous system pathologies, including ischemic stroke, is the inflammatory response with recruitment of leukocytes to the site of injury.1,2 Although this physiological response has important consequences for further brain damage, it also offers a unique therapeutic opportunity. It has been shown that molecules that guide immune cell trafficking after brain damage stimulate homing of neural stem cells (NSC) into the brain parenchyma after intravascular delivery.3 A major obstacle in the context of intravascular stem cell-based therapies is the low number of NSC entering the brain.4,5 A better understanding of the mechanisms governing transendothelial stem cell recruitment will be essential in improving this approach.

There is evidence suggesting that different types of stem cells, including NSC, may utilize similar extravasation mechanisms to immune cells, such as chemoattraction, adhesion, and transendothelial migration.4,6 Ischemia results in activation of the endothelium, leading to expression of adhesion molecules including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and selectins on the endothelial cell surface.7 NSC have been shown to express integrins α2, α6, and β1,7,8 as well as CD49d.3,4 We recently have demonstrated that the vascular cell adhesion molecule-1/CD49d interaction is a crucial step for NSC recruitment to the ischemic brain.8

After ischemia, astrocytes and activated microglia produce chemokines, such as C-C chemokine ligand (CCL) 2, CCL3, CCL4, CCL5, C-X-C motif ligand (CXCL) 12a and C-X3-C motif ligand 1.9 NSC, like leukocytes, express the chemokine receptors CCR1, CCR2, CCR5, CXCR3, and CXCR4.10 Recent evidence suggests that CCR2 and CXCR4-dependent mechanisms are important for migration of endogenous NSC.
toward areas of central nervous system injury. However, it remains unclear whether these factors are involved in guiding transendothelial migration after exogenous delivery of NSC. We hypothesize that the CCL2/CCR2 interaction might play a central role in the therapeutic homing of intravascularly delivered NSC.

**Materials and Methods**

**Experimental Animals**

CCR2<sup>−/−</sup> mice (B6.129S4-Ccr2<sup>tm1Ifc</sup>/J), CCL2<sup>−/−</sup> mice (B6.129S4-Ccl2<sup>tm1Kdr</sup>/J), and wild-type (WT) controls (C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, ME). Both CCR2<sup>−/−</sup> and CCL2<sup>−/−</sup> animals were backcrossed to the C57BL/6 background for 10 generations. Nu/Nu mice (Crl:NU-Foxn1<sup>nu</sup>) for bioluminescence imaging (BLI) studies were from Charles River Laboratory (Wilmington, MA). All experiments used males 10 to 14 weeks of age (body weight, 26–30 g). The Institutional Animal Care and Use Committee at Stanford University approved all procedures.

**Isolation and Culture of NSC**

NSC were isolated from postnatal P0 CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> mouse pups and cultured as described in the Supplemental Information (SI; http://stroke.ahajournals.org).

**Genotyping**

A segment of the WT CCR2 gene or the neomycin resistance gene used to make the knockout mice was amplified. The primer sequences were as follows: WT forward, CCA CAG AAT CAA AGG AGA TC; and neomycin reverse, AGG TGA GAT GAC AGG AGA TC. Amplification of the WT CCR2 gene or the neomycin resistance gene used to make the knockout mice was amplified. The primer sequences were as follows: WT forward, CCA CAG AAT CAA AGG AGA TC; and neomycin reverse, AGG TGA GAT GAC AGG AGA TC. Amplification was confirmed by polymerase chain reaction was 424 bp for the WT and 280 bp for the neomycin gene.

**Analysis of CCR2 Expression by Flow Cytometry**

Immediately after passaging, NSC were analyzed for CCR2 expression using flow cytometry (anti-CCR2, 1:200; Epitomics) as described elsewhere in detail and in the SI.

**In Vitro Migration Assay**

A modified Boyden chamber assay was used to investigate the migratory response of NSC to CCL2 (5, 10, 40, and 70 ng/mL; R&D Systems) and CXCL12a (100, 500, and 1000 ng/mL; R&D Systems). Assays utilized a 96-well cell migration assay kit (CellSelect, Cell Biolabs); 100 000 CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> NSC were seeded into polycarbonate membrane chambers (8-μm pore size) and incubated at 37°C (n=8; 2 independent experiments). Migration was stimulated by addition of CCL2 and/or CXCL12a to the lower compartment. After 1 or 2 hours, respectively, migrated cells were detached from the membrane, lysed, and incubated with CyQuant GR dye (Molecular Probes) for 20 minutes. Migrated NSC were quantified by measuring the fluorescence at 480 nm/520 nm using a scanning fluorometer (FLEXstation II/384; Molecular Devices).

**Differentiation Assay**

CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> neurospheres were dissociated and seeded in 24-well plates (n=8; 2 independent experiments). After 7 days under differentiation conditions, cultures were fixed and immunocytochemistry was performed for double cortin and glial fibrillary acidic protein (GFAP) as described in the SI.

**Proliferation and Differentiation Assays**

To investigate possible effects of CCR2 on proliferation of NSC, we used a BrdUrd incorporation assay. CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> neurospheres were pulse-labeled with 0.2 mmol/L BrdUrd (Sigma) in culture medium for 12 hours, dissociated, and seeded on glass plates (n=8 per group; 2 independent experiments). BrdUrd-positive cell numbers were then analyzed using immunocytochemistry, and the percentage of BrdUrd-labeled proliferating cells was calculated as described in the SI.

For analysis of neural differentiation capability, CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> neurospheres were dissociated and seeded in 24-well plates (n=8; 2 independent experiments). After 7 days under differentiation conditions, cultures were fixed and immunocytochemistry was performed for double cortin and GFAP as described in the SI.

**Stroke Surgery**

Transendothelial recruitment of intra-arterially delivered NSC was studied in a hypoxia-ischemia (HI) model as described previously.8 WT (n=40) and CCL2<sup>−/−</sup> (n=8) C57/Bl6 mice and Nu/Nu nude mice (n=10) were anesthetized with 1.5% to 2% isoflurane (Baxter) in 20% O<sub>2</sub>/80% air. Mice underwent temporary unilateral common carotid artery occlusion and exposure to hypoxia at 8% O<sub>2</sub> for 20 minutes at 37°C, followed by reperfusion 30 minutes later.

**Plasmid Transfection**

Dissociated CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> NSC were transfected 72 hours before transplantation with either a green fluorescent protein (GFP) expression vector (pWiz-GFP; Genlantis) or a human cytomegalovirus (CMV) promoter-driven reporter construct encoding for firefly luciferase and GFP (a kind gift from Dr S. S. Gambhir, Stanford University) using the Neuromporter system (Gene Therapy Systems) according to the manufacturer’s instructions. No significant difference in transfection rates was observed for either plasmid between CCR2<sup>−/−</sup> and CCR2<sup>−/−</sup> NSC on the day of transplantation (P>0.05).

**Cell Transplantation**

NSC were delivered into the internal carotid artery 24 hours after ischemia as previously described.8 Briefly, after induction of anesthesia as described, a custom-made 33-G microneedle was introduced through the proximal external carotid artery into the internal carotid artery. Then, 5 μL of single-cell suspension containing 5×10<sup>5</sup> (for BLI experiments) or 3×10<sup>5</sup> (for functional study and histological analysis) CCR2<sup>−/−</sup> or CCR2<sup>−/−</sup> NSC, respectively, were slowly infused over 2 minutes using a 10-μL microsyringe.

**Behavioral Testing**

Animals were trained to run across a 90-cm-long horizontal ladder with evenly spaced bars once per week for 3 weeks before treatment, as described previously.15 Before stroke surgery, animals were given a baseline score. Scores were based on the function of the contralateral forepaw and quantified by number of errors divided by number of total steps. At 24 hours after ischemia, mice were selected based on poststroke deficit. We excluded animals that did not increase in score after stroke by at least 10%. Animals were then randomized to 3 groups receiving CCR2<sup>−/−</sup>, CCR2<sup>−/−</sup>, NSC, or saline (n=6 each). Animals were tested 1 and 3 days and 1, 2, 3, and 4 weeks after stroke by 2 blinded investigators.

**BLI and Analysis**

Imaging for luciferase activity was performed at 6, 24, and 48 hours after transplantation of CCR2<sup>−/−</sup> or CCR2<sup>−/−</sup> NSC into HI stroke Nu/Nu mice and controls without stroke (n=3–4 per group). In vivo BLI was conducted on the IVIS Spectrum system (Xenogen) as described previously.5 Photon flux was quantified in regions of interest including the whole brain and the individual right and left hemispheres.

**Tissue Processing and Immunohistochemistry**

At experimental end points, animals were transcardially perfused and the brains were sectioned at 30 μm. Immunohistochemistry was performed for neuronal nuclear antigen (NeuN), neuronal class III b-tubulin (TuJ1), GFAP, NG2 chondroitin sulfate proteoglycan, activated microglia (CD68), ionized calcium-binding adapter molecule 1, platelet-derived growth factor-b (PDGF-β), platelet endothe-
Results

The Chemokine Receptor CCR2 Is Expressed on NSC

Live staining with an anti-CCR2 antibody demonstrated that CCR2 is expressed on the membrane of CCR2\(^{+/+}\), but not CCR2\(^{-/-}\), NSC (Supplemental Figure IA). Flow cytometry revealed that 77.1% of NSC derived from CCR2\(^{+/+}\) mice expressed CCR2 (Supplemental Figure IB). Knockout status of CCR2\(^{-/-}\) NSC was additionally confirmed by polymerase chain reaction (Supplemental Figure IC).

The CCR2 Receptor Does Not Influence In Vitro Proliferation and Differentiation

To examine whether proliferation of NSC in vitro was influenced by the CCR2 receptor status, neurospheres derived from CCR2\(^{+/+}\) and CCR2\(^{-/-}\) mice were pulse-labeled with BrdUrd to determine the number of proliferating cells. The percentage of dividing cells was not different between CCR2\(^{+/+}\) and CCR2\(^{-/-}\) cultures (9.30%±0.52% and 9.74%±0.44%; \(P>0.05\)). Next, we addressed whether knocking out CCR2 might influence cell differentiation in vitro. After 7 days growing under differentiation conditions, the CCR2\(^{+/+}\) and CCR2\(^{-/-}\) NSC showed a similar differentiation pattern toward double cortin-expressing neuronal progenitors (25.24%±2.90% and 22.78%±2.90%; \(P>0.05\)) and GFAP-expressing astrocytes (17.83%±3.16% and 16.92%±3.16%; \(P>0.05\)). These findings indicate that knocking out CCR2 does not affect the proliferation rate of NSC and their ability to differentiate toward the neuronal and glial lineage.

CCR2 Expression on NSC Is Needed to Respond to a CCL2 Gradient In Vitro

First, we investigated in vitro whether the migratory response to CCL2 depends on a specific CCL2/CCR2 ligand-receptor interaction (Figure 1A). After stimulation for 1 hour (Figure 1B) and 2 hours (Figure 1C), CCR2\(^{+/+}\) NSC showed a dose-dependent migratory response to CCL2, with a peak at 10 ng/ml that was significantly different from CCR2\(^{-/-}\) NSC. No significant differences in the number of transmigrated CCR2\(^{+/+}\) and CCR2\(^{-/-}\) cells were found after stimulation with CCL2 at 1 hour (D) (Student's unpaired \(t\) test as appropriate. Differences were considered statistically significant at \(P<0.05\). Values are presented as mean±standard error of the mean. (n=8). *\(P<0.05\).
10 ng/mL. CCR2−/− NSC showed no migratory response to CCL2 at all concentrations (Figure 1B). Similar findings were observed after 2 hours (Figure 1C). There was no significant difference in the migratory behavior to CXCL12a between CCR2+/+ and CCR2−/− NSC (Figure 1D). Combined exposure with the maximal efficient doses of CCL2 and CXCL12a had no additive effect (data not shown).

**CCR2 Expression on NSC Is Required for Efficient Transendothelial Recruitment**

We investigated the recruitment of CCR2+/+ and CCR2−/− NSC after delivery into the ipsilesional internal carotid artery at 24 hours after transient cerebral ischemia (Figure 2A). At 3 days after transplantation, GFP-labeled NSC were detected predominantly in the ischemic penumbra of the cortex and striatum (Figure 2B). Higher numbers of GFP-expressing cells were found in mice that received CCR2+/+ grafts than in those transplanted with CCR2−/− cells in the ipsilateral hemisphere (Figure 2C, D). In both groups, significantly more NSC were found on the ipsilateral side. No significant difference in the number of GFP-expressing cells was observed in the contralateral hemisphere between animals treated with CCR2+/+ and CCR2−/− NSC. The same is found in CCL2 knockout animals (SI and Supplemental Figure II).

**Dynamics of NSC Homing to the Ischemic Brain After Intravascular Delivery**

In vivo BLI was used to noninvasively demonstrate the influence of the CCR2 receptor status on the dynamics of NSC recruitment to the ischemic brain. Equal luciferase activity in CCR2+/+ and CCR2−/− NSC was validated by measuring 5×10^6 cells from each group in vitro immediately before transplantation (1.23×10^7 and 1.28×10^7 photons/s/cm²/sr). At 6 hours after intra-arterial delivery, BLI revealed a significant difference in luciferase activity between animals grafted with CCR2+/+ and CCR2−/− cells in a region of interest, including both hemispheres (Figure 3A). This effect persisted at 24 hours (Figure 3B) and 48 hours after transplantation (Figure 3C). The intracranial distribution was assessed by measuring region of interest over the left and right hemispheres. In the CCR2+/+ group, we observed significantly increased luciferase activity in the ipsilateral side at 6, 24, and 48 hours after cell injection. In contrast, the hemispheric predominance observed at 6 hours was lost after 24 hours in animals grafted with CCR2−/− cells. The difference in left/right distribution ratio comparing CCR2+/+ and CCR2−/− groups was significant at 6 (Figure 3A) and 24 (Figure 3B) hours but not at 48 hours (Figure 3C). These results demonstrate that the CCL2/CCR2 interaction is crucial for the acute (within 6 hours) concentration of transplanted cells in the hemisphere with lesions. Luciferase activity in nonstroke controls receiving CCR2+/+ NSC was significantly lower than in mice with lesion at 6, 24, and 48 hours (P<0.05), indicating that NSC homing is limited in the uninjured brain.

**Intravascularly Delivered NSC Survive and Differentiate Into Neuronal and Glial Progeny**

CCR2+/+ and CCR2−/− cell fate was compared at 14 days after intra-arterial delivery (Figure 4A). Significantly more CCR2+/+ cells were found in the stroke brain as compared to CCR2−/− cells (Figure 4B). Independent of the NSC receptor status, significantly higher numbers of GFP-positive cells were counted in the ipsilateral as compared to the contralateral hemisphere (Figure 4B). No significant difference in the percentage of GFP-positive cells colocalizing with the neuronal markers NeuN and TuJ1 and the astrocyte marker GFAP were found between mice receiving CCR2+/+ or CCR2−/− NSC.
CCR2−/− NSC (Figure 4C). Interestingly, there was a non-significant tendency toward lower numbers of NeuN-coexpressing, TuJ1−coexpressing, and GFAP-coexpressing cells on the contralateral as compared to the ipsilateral side (Figure 4C). GFP-positive cells immunoreactive to NG2 chondroitin sulfate proteoglycan were not detected. Representative confocal microscopy images from the ipsilateral and contralateral hemisphere are shown in Figure 4D.

To investigate if transmigrated NSC integrate in the vessel wall and express endothelial or pericyte antigens, we performed double staining for GFP-positive cells and lectin, platelet endothelial cell adhesion molecule-1 (CD31), or PDGFR-β. Although there was no overlap of GFP-positive cells with lectin or platelet endothelial cell adhesion molecule-1, we found a 12% colocalization with PDGFR-β.

Mice Receiving CCR2+/+ Grafts Exhibited Significantly Improved Neurological Recovery

Analysis of skilled limb movements was performed using the horizontal ladder test. HI resulted in a significant (≈3-fold) increase in the number of stepping errors in the horizontal ladder test in mice transplanted with CCR2+/+ and CCR2−/− NSC and saline controls. Animals that received CCR2+/+, but not CCR2−/−, grafts or saline showed significantly improved recovery from neurological impairment at 3 days and at 2, 3, and 4 weeks after stroke (Figure 5A).

When investigating the survival of NSC, time-course analysis of ipsilateral GFP-positive cell numbers revealed a decrease of 94.3% in the CCR2+/+ group and 94.4% in the CCR2−/− group between day 3 and day 14 after grafting. There was only a nonsignificant decrease in numbers of surviving cells thereafter up to 4 weeks after transplantation (Figure 5B). This is consistent with the previously described reduction in cell number as shown by in vivo imaging.5 We used MRI to analyze stroke size and immunohistochemistry to characterize stroke outcome parameters at the cellular level. The percent reduction in hemisphere size compared to the uninjured side was quantified. We found a significant difference between saline-injected (84%) and cell-treated animals (98%). There was no significant difference between CCR2+/+ and CCR2−/− animals.

At a cellular level, we investigated the effect of cell transplantation on the immune response (ionized calcium-binding adapter molecule 1 and CD68) and on the number of degenerating neurons using Fluoro-Jade staining. There was a nonsignificant trend of less ionized calcium-binding adapter molecule 1–positive cells in the animals undergoing

Figure 3. Bioluminescence imaging demonstrating the recruitment dynamics of CCR2+/+ and CCR2−/− neural stem cells (NSC) to the postischemic brain at 6 (A), 24 (B), and 48 hours (C) after intra-arterial delivery. Representative images of transplanted animals with wild-type (CCR2+/+) and knockout (CCR2−/−) NSC, photon flux measurements (photon flux), and calculated left-right (L/R) distribution ratios (left/right ratio). At all time points, luciferase activity was significantly higher in mice transplanted with CCR2+/+ NSC (A–C). In both groups, the differences in the L/R ratio decreased during the given time period. Pseudocolor scale bars represent luminescence photon output in photons/s/cm²/sr. Data are mean±standard error of the mean (n=3 per group). *P<0.05. n.s., not significant.
Intraarterial Delivery: CCR2** and CCR2* NSCs

Figure 4. Number of recruited cells and differentiation of intra-arterially delivered neural stem cells (NSC) in the postischemic brain at 14 days after transplantation. Experimental set-up (A). Significantly higher numbers of cells were found in the ipsilateral hemisphere in the CCR2** group (B). Colocalization studies with the neuronal (NeuN, neuronal class III b-tubulin [TuJ1]) and astrocytic (GFAP) markers did not show any significant differences in NSC differentiation in vivo between the CCR2** and the CCR2* group (C). Representative confocal photomicrographs from the ipsilateral and contralateral hemispheres demonstrating colocalization of NeuN (red, upper panel) and GFAP (red, lower panel) with green fluorescent protein (GFP)-positive (green) NSC (D). Data are mean±standard error of the mean (n=12 per group, 2 independent experiments). *P<0.05, **P<0.01. n.s. indicates not significant. Scale bars: 200 μm (split color panel), 20 μm (high-magnification orthogonal images).

transplantation with CCR2** (126.41±14.07 cells/mm² versus 164.97±18.24 cells/mm², P>0.05), but there was a significant reduction of activated CD68-positive microglia in animals undergoing transplantation with CCR2** (Figure 5C). There was a nonsignificant trend toward less GFAP-positive cells in animals transplanted with CCR2** than CCR2* cells (218.42±9.32 cells/mm² versus 254.79±15.59 cells/mm²; P>0.05). Animals treated with CCR2** cells did have significantly less Fluoro-Jade-positive degenerating neurons than animals treated with CCR2* cells (Figure 5D).

Discussion

Intravascular delivery of NSC is a promising experimental treatment strategy for ischemic stroke. We have previously shown that NSC express the integrin CD49d, potentially allowing them to actively enter the ischemic brain from the
vascular compartment. Enrichment of NSC for CD49d improved this process and resulted in better functional recovery.8

Here, we suggest that CCL2 is a crucial molecule governing transendothelial recruitment of NSC in the central nervous system. CCL2, a member of the C-C motif subfamily of chemokines,13,28,29 is known to promote chemotaxis of monocytes and hematopoietic progenitors18–21 to sites of inflammation in the brain. In experimental models of stroke, CCL2 is upregulated in the ischemic areas22–24 in stroke patients, elevated CCL2 levels have been found in the systemic circulation,25,26 CCL2 accumulates in the perivascular space of the cerebral microvasculature, is actively relayed across the blood–brain barrier.8 These lines of evidence suggest that CCL2 may be significantly involved in the recruitment of intra-arterially delivered CCR2-expressing NSC across the blood–brain barrier.

Intraparenchymal migration of NSC in response to CCL2 has been described.13,28,29 However, CCL2 may act through receptors other than CCR2.30 Using an in vitro migration assay, we found that CCR2+/+ NSC exhibited a dose-dependent migratory response to CCL2 whereas CCR2−/− NSC did not. To rule out that CCR2−/− NSC may have lost their general migratory capacity, we demonstrated chemotaxis in response to CXCR12a, which acts on the receptor CXCR4.31 These data support the hypothesis that the observed effects are specifically mediated by the CCL2/CCR2 interaction.

In response to ischemic stroke in rodents, CCL2 is significantly upregulated in the brain after 6 hours, peaks at 24 hours, and gradually declines to baseline levels over the next 5 days.13 Thus, we chose the time point of 24 hours after stroke for transplantation. After intracarotid delivery, we observed significantly higher numbers of CCR2+/+ NSC recruited into the ischemic brain areas as compared to CCR2−/− cells, proving the importance of CCR2 for active homing of NSC across the blood–brain barrier (Figure 2A–D). Accordingly, we observed improved locomotor recovery in animals that received CCR2+/+ grafts as compared to mice transplanted with CCR2−/− NSC (Figure 5A). To demonstrate dependence on CCL2 in a converse experiment, we transplanted CCR2-expressing NSC into WT and CCL2 knockout animals. This experiment further confirmed the necessity of the CCL2/CCR2 interaction for transendothelial recruitment of NSC to the injured brain (Supplemental Figure II).

Previous studies have reported that CCL2 deficiency attenuates infarct volumes in focal rodent stroke models,32,33 which could represent a confounding factor. However, we found only a minor difference in lesion size on T2-weighted MRI 72 hours after stroke in CCL2+/+ and CCL2−/− animals using the HI model (Supplemental Figure IIB), which would not be sufficient to explain the difference in the number of recruited WT NSC between CCL2+/+ and CCL2−/− animals.

To overcome the limitations of histology in assessing whole brain distribution and dynamic changes over time in the same subject, we monitored the dynamics of NSC recruitment to the stroke brain areas during the early period after intra-arterial delivery using BLI of luciferase-transfected CCR2+/+ and CCR2−/− NSC. We found that the majority of the CCR2+/+ cells were specifically recruited to the hemisphere with lesions during the first few hours, whereas CCR2−/− NSC showed almost no homing (Figure 3). Analysis of the left–right redistribution of the BLI signal in both CCR2+/+ and CCR2−/− animals (Figure 3, left/right ratio) revealed a possible secondary recruitment of NSC to the brain from the systemic circulation. Cell death on the ipsilateral side could also account for the observed changes in left/right ratio. Whereas the CCR2/CCL2 interaction is cru-
cial for recruitment of NSC, the lineage determination fate was not dependent on the CCR2 receptor status of the transplanted NSC in our experiments (Figure 4B–D).

Because it allows reperfusion after ischemia, the HI model of stroke offers advantages for the study of intravascular stem cell delivery techniques as compared to focal ischemia models based on permanent vessel occlusion. In the present study, HI resulted in reproducible cortico-striatal infarction on the ipsilateral side of the brain. Because differentiated oligodendrocytes are less susceptible to ischemic damage than oligodendrocyte progenitors, white matter affecion is less pronounced in adult mice than in the neonatal HI model.

We further investigated the relation of transmigrated NSC and the cerebral vasculature. NSC that undergo transendothelial migration also may directly participate in angiogenesis. A recent article demonstrated that PDGFR-β–positive pericytes were required for blood–brain barrier integrity during embryogenesis.8 It also has been shown that circulating cells expressing PDGFR-β were crucial for the maturation of brain vessels after stroke.5 We found that 12% of our transplanted GFP-positive NPC were positive for PDGFR-β.

Histology and BLI data suggest that intra-arterially delivered NSC need to cross the blood–brain barrier to improve behavioral recovery. This finding is in line with our previous study investigating the effects of vascular cell adhesion molecule-1/CD49d.8 Other groups, however, have demonstrated that mesenchymal stem cells do not necessarily need to enter the brain for improving recovery from neurological deficits.36,37 and immunomodulatory and/or neuroprotective mechanisms have been postulated to mediate the observed effects on behavioral outcome. However, to date, no such data are available for NSC or neural progenitors, and it remains to be elucidated whether homing to the ischemic brain, enabling neural differentiation and facilitating delivery of secreted growth factors and cytokines, also might have additional beneficial effects for mesenchymal stem cells. Several mechanisms have been implicated in cell-mediated functional recovery. The proangiogenic vascular endothelial growth factor-mediated role of transplanted stem cells has been extensively studied.38 Here, we demonstrate an immunomodulatory mechanism with reduction in CD68–positive cells and a neuroprotective effect with reduction in Fluoro-Jade–positive cells (Figure 5C, D) and reduction in lesion size. All these mechanisms are potentially involved in stem cells-mediated recovery.

Conclusions

Our data indicate that the CCL2/CCR2 interaction plays a central role for transendothelial recruitment of intra-arterially delivered NSC and has important implications for functional recovery. We have previously shown that the vascular cell adhesion molecule-1/CD49d interaction is another important factor for therapeutic homing of intravascularly delivered NSC and selecting CD49d–expressing NSC for transplantation improved neurological outcome.8 CXCL12a and its receptor CXCR4 mediate NSC chemotaxis after intraparenchymal transplantation.11 Whereas vascular cell adhesion molecule-1 and its receptor CD49d play a crucial role for the cellular attachment to the endothelium, the CCL2/CCR2 and CXCL12a/CXCR4 interactions are critical for transendothelial recruitment and further guide intraparenchymal migration of NSC. Influencing expression of CCR2 on NSC and CCL2S2 in the host brain may offer new ways to improve the efficiency of intra-arterial stem cell therapy in the future.

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Disclosure

None.

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SUPPLEMENTAL MATERIAL

The CCR2/CCL2 interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain

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Supplemental Methods

Isolation and Culture of Neural Stem Cells

Whole brains were isolated from postnatal (P0) CCR2+/+ and CCR2−/− mouse pups, rinsed in 0.1 M phosphate-buffered saline (PBS), finely minced, and enzymatically digested in a cocktail of 2.5 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ), 1 U/ml neutral protease (Roche Applied Science, Indianapolis, IN), and 250 U/ml DNase-I (Worthington) in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5% glucose (Mediatech Cellgro, Herndon, VA). Dissociated cells were resuspended in growth medium containing Neurobasal-A (Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen), 1% antibiotics/antimycotics (No. 061-05240D; Gibco-BRL, Grand Island, NY), 2% B-27 supplement without vitamin A (Invitrogen), 20 ng/ml fibroblast growth factor (FGF)-2 (Peprotech, Rocky Hill, NJ), and 20 ng/ml epidermal growth factor (EGF) (Sigma, St. Louis, MO), and cultured as spherical aggregates (neurospheres) at 37°C in an atmosphere of humidified air and 5% CO₂. Half of the culture medium was replaced every second day. Antibiotics/antimycotics were omitted after 10 days in vitro. Every 5-7 days neurospheres were passaged using Hanks’-based Enzyme-free Cell Dissociation Buffer (Gibco). All experiments used cells from passages 5-10.

Proliferation Assay

To identify newly dividing cells within cultured neurospheres, a cumulative 5-bromo-2′-deoxyuridine (BrdU) labeling method was used as described before (M. Suzuki et al., Proc Natl Acad Sci USA 2004;101:3202-3207). Briefly, neurospheres derived from CCR2+/+ and CCR2−/− mice were incubated with 0.2 mM BrdU (Sigma) in culture medium for 12 hours, dissociated into a single-cell suspension, seeded on poly-L-lysine/laminin (Sigma) precoated glass plates and cultured in growth medium for 60 minutes at 37°C. Cultures were then fixed in ice-cold 4% paraformaldehyde (Sigma) and incubated in 2 N HCl for 10 minutes at 37°C, followed by a borate buffer wash for 10 minutes at room temperature. Cultures were blocked, labeled with a rat anti-BrdU antibody (1:250; Abcam, Cambridge, MA) overnight at 4°C, and subsequently incubated with a secondary antibody (Alexa Fluor 546, Molecular Probes, Eugene, OR) for 1 hour at room temperature. Nuclear counterstaining was performed with 4′,6-diamidino-2′-phenylindole-dihydrochloride (DAPI; 1 mg/ml, AnaSpec, San Jose, CA) and plates were mounted on glass slides with FluorSave reagent (Calbiochem, San Diego, CA). Cell numbers were analyzed using an epifluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan) connected to a digital image processing system (StereoInvestigator; Microbrightfield, Williston,
Cells with distinct nuclear immunoreactivity for BrdU- and DAPI-positive nuclei, representing the total cell number, were counted in six randomly chosen areas per well and the percentage of BrdU-labeled proliferating cells was calculated.

**Differentiation Assay**

Dissociated CCR2+/+ and CCR2-/- neural stem cells (NSCs) were seeded in 24-well plates and cultured in differentiation medium consisting of Neurobasal-A, 1% fetal calf serum (Hyclone, Logan, UT), 100 ng/ml all-trans retinoic acid (Sigma), 1 ng/ml FGF-2, 10 ng/ml neurotrophin-3 (Peprotech), and 10 ng/ml brain-derived neurotrophic factor (Peprotech) (A. Keravala et al., J Neurosci Methods 2008;173:299-305). After 7 days, cultures were processed for immunocytochemistry as described above, using either a goat anti-doublecortin (DCX) antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) or a guinea pig anti-glial fibrillary acidic protein (GFAP) antibody (1:500; Advanced Immunochemical, Long Beach, CA) and corresponding secondary antibodies (Alexa Fluor 488 and 546, Molecular Probes). For each marker, cells with distinct immunoreactivity and a nucleus were counted in six randomly chosen areas per well, and the percentage of DCX- or GFAP-immunoreactive cells was calculated.

**Analysis of CCR2 expression by Flow Cytometry**

Immediately after passage, dissociated NSCs were labeled with a primary rabbit anti-CCR2 monoclonal antibody (1:200; Epitomics, Burlingame, CA) for 30 minutes on ice and subsequently incubated with a FITC-conjugated secondary antibody (1:250; Jackson Immunoresearch, West Grove, PA). Cells were stained using the Live/Dead Aqua Stain Kit (Invitrogen) and analyzed for CCR2 expression by flow cytometry (FACSAria; Becton Dickinson, San Jose, CA). Appropriate cells were selected using forward scatter, side scatter, and live/dead staining. Compensation beads and secondary only controls were used to confirm the results.

**Tissue Processing and Immunohistochemistry**

Brain sections were blocked and incubated overnight with primary antibodies (mouse anti-neuronal nuclear antigen (NeuN), 1:250 (Chemicon, Temecula, CA); chicken anti-neuronal class III b-Tubulin (TuJ1), 1:1000 (Chemicon); guinea pig anti-GFAP, 1:500 (Advanced Immunochemical); rabbit anti-Ng2 chondroitin sulfate proteoglycan (Ng2), 1:500 (Chemicon); rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1), 1:1000 (WAKO, Richmond, VA); rabbit anti-GFP, 1:500 (Invitrogen); rat anti-CD31, 1:500 (BD Bioscience, San Diego, CA); and rabbit anti-PDGFR-beta, 1:50 (Abcam) and subsequently incubated with corresponding secondary antibodies (Alexa Fluor 488 and 546, Molecular Probes), counterstained with 4′-6-diamidino-2-phenylindole (DAPI; 1 mg/ml, AnaSpec, San Jose, CA) and mounted on glass slides. For the Fluoro-Jade C staining, sections were immersed in 1% sodium hydroxide and 80% ethanol for 5 minutes and were rinsed for 2 minutes in 70% ethanol, 2 minutes in distilled water and then incubated in a 0.06% potassium permanganate solution for 20 minutes. Slides were then transferred into a 0.0001% solution of Fluoro-Jade C (Histo-Chem Inc., Jefferson, AR, USA) and dissolved in 0.1% acetic acid. This was followed by three 1-minute rinses of distilled water and mounting.

Unbiased stereology was used to quantify intraparenchymal NSC migration. Cell numbers were analyzed in 3 regions of interest, including a circular area measuring 1.4 mm² centered on the striatal transplantation site (bolus), the cortical area surrounding the ischemic lesion, approximately 1.5 mm around the borders of the demarcated lesion (penumbra) and in the interjacent area (intermediate zone). Six sections per animal that contained the injured area were evaluated and the results expressed as cells per mm². Cell numbers were corrected
according to Abercrombie’s correction factor, based on a mean nuclear diameter of 5.81 µm, as determined from measurements of 25 NSC nuclei in our samples.

Supplemental Results

Transendothelial recruitment of NSCs is dependent on the presence of CCL2
To further demonstrate that the CCL2/CCR2 interaction was responsible for the observed effects after grafting of CCR2−/− and CCR2+/+ cells, GFP-transfected WT NSCs were injected into WT and CCL2-deficient recipients 24 hours after stroke (Fig. S2A). There was no significant difference in infarct size between the CCL2+/+ and the CCL2−/− group (Fig. S2B). At 3 days after intracarotid delivery, the distribution pattern of transplanted cells did not differ from the findings in the previous experiment investigating the recruitment of CCR2+/+ and CCR2−/− in WT mice. Significantly higher numbers of GFP-expressing cells were found in the ipsilateral hemisphere of WT mice than in CCL2−/− recipients (Fig. S2C-D). GFP positive NSC numbers were significantly lower in the contralateral hemisphere without a significant difference between CCL2+/+ and CCL2−/− mice.
Supplemental Figures and Legends

Figure S1

Characterization of CCR2+/+ and CCR2−/− NSCs in culture. Immunostaining shows prominent receptor expression on CCR2+/+ cells and absence on CCR2−/− cells (A). Flow cytometry showed that 77.1% of NSCs expressed the CCR2 receptor (B). Knockout status of CCR2−/− NSCs was confirmed by PCR (C). Results are representative of 2 independent experiments. Data are mean ± SEM. (n=8 per group). Scale bars: 100 µm (A).
Converse experiment to Fig. 2. CCR2+/+ NSCs were injected into CCL2+/+ and CCL2-/− recipients 24 h after stroke (A). MRI did not reveal a significant difference in stroke size between CCL2+/+ and CCL2-/− mice (B). Significantly higher cell numbers were found in the ipsilateral hemisphere in CCL2+/+ animals (C). Representative photomicrographs of NSCs in the ipsilateral hemisphere in brains from CCL2+/+ and CCL2-/− mice (D). Results are representative of 2 independent experiments. Data are mean ± SEM. (n=8 per group). * P < 0.05. n.s.: not significant. Scale bars: 100 µm.