Evidence That Ly6C\textsuperscript{hi} Monocytes Are Protective in Acute Ischemic Stroke by Promoting M2 Macrophage Polarization

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Background and Purpose—Ly6C\textsuperscript{hi} monocytes are generally thought to exert a proinflammatory role in acute tissue injury, although their impact after injuries to the central nervous system is poorly defined. CC chemokine receptor 2 is expressed on Ly6C\textsuperscript{hi} monocytes and plays an essential role in their extravasation and transmigration into the brain after cerebral ischemia. We used a selective CC chemokine receptor 2 antagonist, INCB3344, to assess the effect of Ly6C\textsuperscript{hi} monocytes recruited into the brain early after ischemic stroke.

Methods—Male C57Bl/6J mice underwent occlusion of the middle cerebral artery for 1 hour followed by 23 hours of reperfusion. Mice were administered either vehicle (dimethyl sulfoxide/carboxymethylcellulose) or INCB3344 (10, 30 or 100 mg/kg IP) 1 hour before ischemia and at 2 and 6 hours after ischemia. At 24 hours, we assessed functional outcomes, infarct volume, and quantified the immune cells in blood and brain by flow cytometry or immunofluorescence. Gene expression of selected inflammatory markers was assessed by quantitative polymerase chain reaction.

Results—Ly6C\textsuperscript{hi} monocytes were increased 3-fold in the blood and 10-fold in the brain after stroke, and these increases were selectively prevented by INCB3344 in a dose-dependent manner. Mice treated with INCB3344 exhibited markedly worsen functional outcomes and larger infarct volumes, in association with reduced M2 polarization and increased peroxynitrite production in macrophages, compared with vehicle-treated mice.

Conclusions—Our data suggest that Ly6C\textsuperscript{hi} monocytes exert an acute protective effect after ischemic stroke to limit brain injury and functional deficit that involves promotion of M2 macrophage polarization. (Stroke. 2015;46:1929-1937. DOI: 10.1161/STROKEAHA.115.009426.)

Key Words: ischemia ■ monocytes ■ receptors, CCR2 ■ stroke

After ischemic stroke there is an extensive inflammatory response in the brain, which involves both proliferation of resident microglia and recruitment of circulating leukocytes such as monocytes, neutrophils, lymphocytes, and macrophages.\textsuperscript{1,2} Generation of specific chemokines within a few hours at the site of injury facilitates and coordinates the selective recruitment, adhesion, and transmigration of leukocytes via interactions with their chemokine receptor(s) expressed on the circulating immune cells.\textsuperscript{3} As leukocyte activation in association with postischemic inflammation contributes to secondary brain injury,\textsuperscript{4,5} pharmacological targeting of chemokine receptors may be an attractive means to selectively exclude immune cells that might worsen acute damage in the initial period after a stroke.\textsuperscript{6,7}

The influence of monocytes/macrophages on brain infarct development and stroke outcome is not well defined. The 2 common types of murine monocytes are classified by their level of Ly6C expression: Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo}. Ly6C\textsuperscript{hi} monocytes are thought to typically exert a proinflammatory role in tissue injury, and they require their CC chemokine receptor 2 (CCR2) for mobilization and transmigration from the bone marrow into sites of inflammation.\textsuperscript{8-10} Indeed, CCR2 is predominantly expressed on Ly6C\textsuperscript{hi} monocytes,\textsuperscript{11} but is also on a small number of Ly6C\textsuperscript{lo} monocytes, activated T cells, natural killer cells, neutrophils, and dendritic cells.\textsuperscript{12} CCR2 is activated by its chemokine ligands, in particular chemokine (C-C motif) ligand (CCL2), but also CCL7, CCL8, and CCL13. CCL2 has been identified as an important chemokine involved in postischemic inflammation and disruption of the blood–brain barrier after stroke.\textsuperscript{6,13,14} There is, however, recent evidence for protective effects of Ly6C\textsuperscript{hi} monocytes recruited via CCR2 during inflammation, which challenges the dogma that Ly6C\textsuperscript{hi} monocytes are necessarily detrimental through promotion of secondary injury after myocardial infarction\textsuperscript{15} and in...
hemorrhagic infarct transformation after cerebral ischemia.\textsuperscript{16} Furthermore, there is evidence that CCR2/CCL2 mechanisms are important for the migration of neuroblasts from neurogenic regions to damaged regions of brain after cerebral ischemia in mice, suggesting a role in tissue recovery after stroke.\textsuperscript{17} Thus, for the development of future therapies to target detrimental elements of the poststroke acute inflammatory response, it is vital to clarify the role of CCR2\textsuperscript{+}/Ly6C\textsuperscript{hi} monocytes.

To our knowledge, no study has examined the effect of a selective CCR2 antagonist in ischemic stroke. INCB3344 is a novel CCR2 antagonist that inhibits the binding of CCL2 to monocytes with high potency and it is highly selective for the CCR2 receptor.\textsuperscript{18} It effectively reduces monocyte/macrophage accumulation in autoimmune encephalomyelitis in mice and in inflammatory arthritis in rats\textsuperscript{19} and in deoxycorticosterone acetate/salt-induced hypertension in mice.\textsuperscript{19} Its potency and selectivity for CCR2 inhibition have thus been used in this study to evaluate the effect of inhibiting Ly6C\textsuperscript{hi} monocyte migration to the brain early during poststroke inflammation.

Materials and Methods

Animals

This study was approved by the Monash University Animal Ethics Committee (Project MARP/2011/112) and performed in accordance with the National Health and Medical Research Council of Australia guidelines for the care and use of animals in research. A total of 102 male C57Bl/6 mice were studied (aged 8–12 weeks; 28±3 g). All mice had free access to water and food pellets and were kept in specific pathogen-free cages at 21°C.

Vehicle or INCB3344 Treatment

Mice were randomly assigned to receive intraperitoneal injections of vehicle (10% dimethyl sulfoxide/0.9% carboxymethylcellulose; n=40) or INCB3344 at doses of 10 mg/kg (n=6), 30 mg/kg (n=33), or 100 mg/kg (n=12) at 1 hour before stroke or sham surgery. Vehicle or INCB3344 administration was repeated at 2 and 6 hours after reperfusion or sham surgery. Total dosage of INCB3344 was therefore 30, 90, or 300 mg/kg, respectively. Investigators were blinded to the groups for which each animal was assigned. A further 11 sham-operated animals that did not receive any treatment were also studied.

Middle Cerebral Artery Occlusion

Mice underwent either sham surgery (n=25) or 1 hour of cerebral ischemia (n=77) as described previously.\textsuperscript{20,21} Briefly, mice were anesthetized and the right middle cerebral artery (MCA) was occluded with a nylon monofilament with silicone-coated tip, resulting in severe reduction (>70%) of blood flow to the MCA region, which was recorded by transcranial laser-Doppler flowmetry. The monofilament was retracted after 1 hour, allowing reperfusion to occur. Reperfusion was confirmed by an increase in regional cerebral blood flow, which was measured by transcranial laser-Doppler flowmetry. Concurrent sham-operated control animals underwent all procedures except ligation of arteries and insertion of the monofilament. Mice were monitored until they regained consciousness and were then returned to their cages (details of the MCA occlusion surgery is available in the online-only Data Supplement). Four mice were excluded from the study because of technical problems during surgery (n=2) or to death before the designated time for euthanasia (n=2). No evidence of hemorrhagic transformation was observed in the brain after cerebral ischemia.

Neurological Assessment

Neurological deficit scoring and hanging grip tests were performed 30 minutes before euthanasia, as described.\textsuperscript{1} Briefly, neurological deficit was assessed using a 5-point scoring system (0–4) in which a score of 0 represents normal motor function and 4 indicates no spontaneous motor activity. A hanging grip test was performed in which mice were suspended by their forelimbs for ≤60 s on a wire 60 cm above foam padding, and the average latency to fall was recorded from 3 trials.

Flow Cytometric Analysis

At 24 hours after stroke or sham surgery, mice were euthanized by isoflurane overdose. Blood was collected by cardiac puncture and mice were then perfused with PBS, followed by decapitation. The brain was removed from the skull and, after removing the cerebellum, the ischemic (right) hemisphere was collected for flow cytometry analysis. Leukocytes were isolated from blood and brain tissues for multicolor flow cytometry, as previously described\textsuperscript{21} (detailed protocol of flow cytometry is available in the online-only Data Supplement).

Infarct Analysis

Consecutive coronal brain sections (30 \(\mu\)m) were obtained every 420 \(\mu\)m throughout the brain and stained with 0.1% thionin to delineate the infarct. Images were captured using a CCD camera with a light source and analyzed using Image J software (NIH, Bethesda, MD). Infarct volume and hemispheric swelling were calculated as described previously.\textsuperscript{1}

Quantitative Polymerase Chain Reaction

At 24 hours after stroke or sham surgery, mice were euthanized by isoflurane overdose and perfused with RNAase-free PBS. After removing the cerebellum, the right hemisphere of the brain was snap frozen in liquid nitrogen. RNA extracted from whole ischemic hemispheres was reversed transcribed into cDNA. Levels of RNA expression of selected genes, including Arg1, CCL2, CCL7, CCL8, CCL12, interleukin (IL)-1\(\beta\), IL-6, IL-10, tumor necrosis factor-\(\alpha\), and Ym-1 were measured using pre-designed Taqman gene expression assays. Protocols for quantitative polymerase chain reaction are available in the online-only Data Supplement.

Immunofluorescence for 3-Nitrotyrosine and F4/80

Double-labeled immunofluorescence for 3-nitrotyrosine (3-NT; peroxynitrite marker) and F4/80 (macrophage/microglial cell marker) was performed on 10-\(\mu\)m coronal brain sections using methods similar to those reported previously.\textsuperscript{21} Detailed protocol of immunofluorescence studies is available in the online-only Data Supplement.

Statistical Analysis

Data are presented as mean±SEM, except for nonparametric neurological deficit scores for which individual data points and median values are indicated. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA). Infarct volume was compared by Student unpaired \(t\) test. Other data were analyzed by 1-way ANOVA with Bonferroni post hoc tests, except for neurological deficit scores, which were compared using a Mann–Whitney test. A robust regression and outlier test was used to identify statistical outliers. \(P<0.05\) was considered statistically significant.

Results

Effect of a CCR2 Antagonist on Infiltration of Immune Cells Into the Brain After Cerebral Ischemia

At 24 hours after cerebral ischemia, there was a 4-fold increase in the total number of leukocytes, and a 6-fold increase in the CCR2\textsuperscript{+} leukocyte subpopulation (ie, 10%–15% of total leukocytes), present in the ischemic hemisphere of vehicle-treated
mice compared with sham controls (Figure 1A). INCB3344 treatment, at a dose of 10, 30, or 100 mg/kg (3× daily), had no overall effect on total leukocytes but dose dependently reduced CCR2+ leukocytes in the ischemic brain (Figure 1A).

Among the ≈7000 total leukocytes (CD45hi) infiltrating the ischemic brain at 24 hours, monocytes (≈12%), neutrophils (≈35%), and T cells (≈10%) were all prominent (Figure 1A–1D). Of note, there was a ≈7-fold increase in total monocytes (ie, both Ly6C−CCR2+ and Ly6C+CCR2−) after stroke in vehicle-treated mice, and this increase was almost completely prevented by the 2 highest doses of INCB3344 (Figure 1B). By contrast, INCB3344 had no significant effect on total numbers of neutrophils or T cells in the ischemic brain after cerebral ischemia (Figure 1C and 1D).

The CCR2+ leukocytes entering the brain after ischemia consisted mainly of neutrophils (≈50%), monocytes (≈33%), and T cells (≈7%; Figure 1A–1D). INCB3344 treatment largely prevented the infiltration of CCR2+ monocytes and CCR2+ neutrophils but did not affect the number of CCR2+ T cells in the ischemic brain (Figure 1A–1D). Based on these data, 30 mg/kg was selected as an optimal dose of INCB3344 for study in the remaining experiments.

Effect of INCB3344 on Circulating Immune Cells After Cerebral Ischemia

Compared with sham animals, the total number of circulating leukocytes was not different at 24 hours after cerebral ischemia in vehicle-treated mice and was not affected by INCB3344.

![Figure 1. Leukocytes in the brain after stroke. Effect of the CC chemokine receptor 2 (CCR2) antagonist INCB3344 (10, 30, or 100 mg/kg IP, 3× daily) on postischemic infiltration of total and CCR2+ leukocytes (A), monocytes (B), neutrophils (C), and T cells (D) into the ischemic brain hemisphere at 24 hours after stroke. Data are compared with those obtained in sham-operated mice (Sham) or vehicle-treated mice (Veh) subjected to stroke. Data are presented as means±SEM, *P<0.05, 1-way ANOVA with Bonferroni post hoc test, n=6 to 12 per group.](http://stroke.ahajournals.org/doi/abs/10.1161/STROKEAHA.115.013793)
treatment (Figure 2A). Among individual cell types, total numbers of monocytes and neutrophils in the blood tended to increase after stroke, whereas T cells were reduced, but only circulating monocytes (≈5% of total leukocytes) were reduced by INCB3344 (Figure 2A–2D). Circulating CCR2⁺ leukocytes consisted of ≈85% monocytes and the ≈2-fold increase after cerebral ischemia was prevented by INCB3344 (Figure 2A and 2B). There was no effect of INCB3344 on circulating CCR2⁺ neutrophils or CCR2⁺ T cells (Figure 2C and 2D).

**INCB3344 Selectively Reduced Circulating and Infiltrating Ly6C⁺ Monocytes Into the Brain**

Most circulating CCR2⁺ monocytes express high levels of Ly6C, and these CCR2⁺Ly6C⁺ monocytes are presumed to be proinflammatory in that they are typically recruited to inflamed tissue in a CCR2-dependent manner, where they may produce Th1-type proinflammatory cytokines.²³ Consistent with this concept, a more detailed analysis of the monocyte subpopulations in blood revealed Ly6C⁺ monocytes to be selectively increased (whereas Ly6C⁻ monocytes were reduced) after stroke, and that INCB3344 prevented the increase in Ly6C⁺ cells (Figure 3A and 3B; Figure I in the online-only Data Supplement). Similarly, Ly6C⁺ monocytes represent the majority of monocytes in the brain after stroke, and this increase was effectively inhibited by INCB3344 (Figure 3C and 3D; Figure II in the online-only Data Supplement).

**Gene Expression of CCR2 Ligands**

Expression of CCR2 ligands, CCL2, CCL7, and CCL12, each markedly increased in the brain after stroke (by 25- to 180-fold; Figure 4), whereas expression of CCL8 was not altered. Treatment with INCB3344 had no effect on expression of any of these CCR2 ligands.
Effect of INCB3344 on Stroke Outcome

Cerebral blood flow to the cortical region supplied by the MCA was reduced by ≈80% in animals subjected to MCA occlusion in both vehicle and INCB treatment groups. Treatment with INCB3344 did not affect the levels of reperfusion compared with vehicle (Figure III in the online-only Data Supplement). In vehicle-treated mice, cerebral ischemia–reperfusion resulted in neurological deficit and reduced hanging grip time compared with sham-operated mice (Figure 5A and 5B; \( P < 0.05 \)). Treatment with INCB3344 had no effect on these parameters in sham mice, but markedly exacerbated the functional deficits after stroke (Figure 5A and 5B; \( P < 0.05 \)).

Representative coronal brain sections from vehicle- and INCB3344-treated animals at 24 hours after cerebral ischemia are shown in Figure 5C. There was a ≈50% larger infarct present in INCB3344-treated mice compared with vehicle controls (Figure 5D; \( P < 0.05 \)). This effect of INCB3344 was mostly because of a larger subcortical infarct, but there was also a trend for proportionally similar increases in cortical infarct volume and hemispheric swelling compared with vehicle-treated mice (Figure 5D).

Effect of INCB3344 on Macrophages and Microglia After Stroke

To better understand the mechanisms underlying the effect of INCB3344 treatment to exacerbate poststroke brain injury in association with fewer monocytes in the circulation and infiltrating the brain, we assessed the number of macrophages and microglia in the brain and expression levels of some inflammation-related genes associated with macrophage polarization.

Figure 3. Monocyte subsets in blood and brain after stroke. Effect of INCB3344 (30 mg/kg IP, 3× daily) on the number of Ly6C\(^{hi}\) and Ly6C\(^{lo}\) monocytes in the blood (A and B) and the ischemic brain hemisphere (C and D) at 24 hours after stroke. Data are compared with those obtained in sham-operated mice (Sham) or vehicle-treated mice (Veh) subjected to stroke. Data are presented as means±SEM, \(^*P<0.05\), 1-way ANOVA with Bonferroni post hoc test, \( n=5 \) to 12 per group.

Figure 4. CC chemokine receptor 2 (CCR2) ligand expression in the brain after stroke. Effect of INCB3344 (30 mg/kg IP, 3× daily) on expression of CCR2 chemokine (C-C motif) ligand (CCL2) (A), CCL7 (B), CCL12 (C), and CCL8 (D) in the ischemic brain hemisphere at 24 hours after stroke. Data are compared with those obtained in sham-operated mice (Sham) or vehicle-treated mice (Veh) subjected to stroke. Data are presented as means±SEM, \(^*P<0.05\) compared with levels in sham-operated mice (Sham), 1-way ANOVA with Bonferroni post hoc tests, \( n=7 \) to 9 per group.
First, neither the increased number of macrophages (CD45<sup>hi</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>) nor microglia (CD45<sup>med</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>) in the brain after stroke was significantly affected by INCB3344, although there was a trend for even more microglial cells (Figure 6A). As monocytes are a key influence on the polarization state of macrophages, we assessed the levels of genes associated with M1 (classically activated proinflammatory) or M2 (alternatively activated anti-inflammatory) cells. We found that cerebral ischemia resulted in marked upregulation of genes associated with both M1 (ie, tumor necrosis factor, IL-6, IL-1β; by 20- to 170-fold) and M2 (ie, Ym1, Arg1, IL-10; by 10- to 20-fold) polarization in the brain (Figure 6B and 6C). However, although INCB3344 had no effect on M1 gene expression (Figure 6B), it virtually prevented the increases in expression of the M2 markers Ym1 and Arg1 and also tended to reduce IL-10 expression (Figure 6C), reflecting much lower levels of M2-polarized macrophages in the poststroke brain.

**Effect of INB3344 on the Proportion of M1 Polarized Macrophages After Stroke**

Brain sections double-labeled using immunofluorescence against 3-NT (peroxynitrite/M1 marker; green) and F4/80 (macrophage marker; red) revealed a ≈3-fold increase in the proportion of F4/80<sup>+</sup> cells that also expressed 3-NT in the ischemic brain after cerebral ischemia, compared with sham brains (Figure 6D). Furthermore, despite having an equivalent total number of macrophages (Figure 6A; Figure IV in the online-only Data Supplement), brains from INCB3344-treated animals contained a 2-fold greater proportion of these cells that were 3-NT<sup>+</sup> (ie, M1-polarized, indicated by yellow arrow in representative image; Figure 6D) compared with vehicle-treated mice after stroke. The number and morphology of F4/80-stained cells in brain sections were consistent with those cells being macrophages and not microglia.
Discussion

There are several major findings of this study that provide novel insight into the roles of circulating CCR2+ leukocytes, particularly Ly6C<sup>hi</sup> monocytes, entering the blood and infiltrating the brain early after cerebral ischemia. First, we found that stroke markedly and selectively increases Ly6C<sup>hi</sup> monocyte numbers in the blood and in the ischemic brain within 24 hours. Second, treatment with a selective CCR2 antagonist (INCB3344) strikingly prevents these increases in monocyte numbers without affecting levels of CCR2 ligands in the brain. Third, the effect of INCB3344 after stroke involves a substantial reduction in the ratio of M2:M1 polarized macrophages in the poststroke brain and results in increased infarct volume and exacerbated functional deficits. The findings therefore indicate that the influx of circulating monocytes plays an important protective role via promoting M2 macrophage polarization to limit the development of inflammation-related brain injury in the early stages after ischemic stroke.

Murine monocytes are commonly classified into 2 subsets according to their level of Ly6C expression. Ly6C<sup>hi</sup> monocytes express high levels of CCR2 and are recruited rapidly to sites of infection or inflammation. By contrast, Ly6C<sup>lo</sup> monocytes express low levels of CCR2 and are thought to function as patrolling immune cells that migrate along the luminal surface of blood vessels and monitor endothelial cells. Using flow cytometry, we characterized the profile of major immune cells circulating in the blood and entering the brain early after stroke, and we identified an optimal dose of INCB3344 for inhibiting CCR2+ cell infiltration. Although there was no net effect of stroke on the total number of circulating leukocytes, there was a profound increase in the proportion of monocytes (from ≈20% to >40%), which were predominantly Ly6C<sup>hi</sup>CCR2+, whereas T cells were reduced by >50%. More than 10% of leukocytes infiltrating the ischemic brain were monocytes, and INCB3344 treatment selectively blocked this process as well as the stroke-induced monocytosis. This is consistent with previous findings indicating that CCR2 is critical for the extravasation of Ly6C<sup>hi</sup> monocytes from bone marrow and migration into inflamed tissue. Furthermore, treatment with INCB3344 had no effect on the gene expression of CCR2 ligands (CCL2, CCL7, CCL8, and CCL12) in the brain after stroke, suggesting that INCB3344 inhibited monocyte migration at the receptor level, without affecting the level of chemoattractant signaling.

Our study has clearly shown that pharmacological inhibition of CCR2+ monocyte infiltration into the brain is associated with worsened acute stroke outcome. Previous work has suggested that CCR2-dependent recruitment of Ly6C<sup>hi</sup> monocytes can promote M1-polarization of macrophages, which may then exacerbate ischemic damage in the myocardium.
However, there is recent evidence that Ly6C<sup>hi</sup> monocytes may contribute to both the inflammatory and the reparative phases occurring in the heart after myocardial infarction. Here, we found that CCR2 inhibition selectively reduced the number of Ly6C<sup>hi</sup> monocytes in the blood and brain after stroke in association with increased infarct development and exacerbated motor impairment, strongly suggesting a protective effect of these cells in limiting acute brain injury. Our finding is consistent with other evidence for protective effects of Ly6C<sup>hi</sup> monocytes in the brain during central nervous system injuries involving inflammation, such as spinal cord injury and hemorrhagic infarct transformation.

To better understand the mechanisms underlying the protective effects of Ly6C<sup>hi</sup> monocytes, we assessed postischemic changes in gene expression of several inflammatory markers involved in M1/M2 polarization of macrophages. Although ischemia promoted macrophage infiltration associated with increased expression of both M1 and M2 polarization markers, INCBl3344 treatment selectively blocked the increase in expression of M2 markers. Consistent with this finding, INCBl3344 treatment also markedly increased the proportion of brain macrophages expressing 3-NT (a marker of peroxynitrite), indicative of greater M1 polarization and phagocytic functions, as well as produce growth factors important for wound healing and repair.

It was recently demonstrated that newly recruited macrophages exhibit an M2 phenotype at early stages (1–3 days) after stroke in mice, and several studies have suggested that M2 macrophages can contribute to central nervous system repair by promoting neuronal survival and axon growth. Taken together, our data provide strong new evidence that Ly6C<sup>hi</sup> monocytes enter the circulation and then the ischemic brain during the acute poststroke period and serve to limit inflammatory and oxidative injury by promoting M2 macrophage polarization. Our results show that without the entry of these Ly6C<sup>hi</sup> monocytes into the poststroke brain, a much greater extent of M1 macrophage polarization ensues, resulting in a larger infarct and a more severe functional outcome.

It is noteworthy that our findings contrast conceptually with those made in CCR2<sup>−/−</sup> mice. Importantly, we found that acute CCR2 inhibition did not affect expression of CCR2 ligands, particularly its main ligand CCL2, in the brain after stroke. This is a key difference from mice with preexisting lifelong global CCR2 deletion, which had smaller infarcts, less hemispheric swelling, and fewer infiltrating neutrophils, but in which CCL2 expression in the postischemic brain was significantly lower than in wild-type controls. As CCL2 plays a powerful role in disruption of the blood–brain barrier during cerebral ischemia, it is possible that CCR2<sup>−/−</sup> mice are protected from postischemic brain injury as an indirect result of reduced CCL2-dependent blood–brain barrier permeability. Here, INCBl3344 treatment was initiated 1 hour before cerebral ischemia to eliminate early infiltration of monocytes into the brain after stroke. Although it had no effect on either CCL2 levels in the brain after stroke or on functional outcome of sham animals, we cannot rule out the possibility of a briefly altered baseline inflammatory profile immediately before ischemia. Any such effect would not, however, influence the interpretation of our data.

Our findings provide new insight into the role of CCR2<sup>−/−</sup> Ly6C<sup>hi</sup> monocytes infiltrating the posts ischemic brain. Although it is possible that these monocytes may develop into M2-polarized macrophages at early stages of ischemia–reperfusion, we postulate that they instead play a key role in promoting polarization of adjacent macrophages toward an M2 phenotype. The data clearly suggest that Ly6C<sup>hi</sup> monocytes do not always exert detrimental proinflammatory actions that contribute to secondary tissue injury, but seem to in fact modulate early posts ischemic brain inflammation to limit infarct development, resulting in milder functional impairment and potentially contributing to early stages of recovery. As a result, pharmacological targeting of CCR2<sup>−/−</sup> Ly6C<sup>hi</sup> monocytes is unlikely to provide benefits after cerebral ischemia–reperfusion.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Evidence that Ly6C^{hi} Monocytes are Protective In Acute Ischemic Stroke by Promoting M2 Macrophage Polarisation

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Supplemental Methods

Middle cerebral artery occlusion
Mice were anesthetised with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), and body temperature was maintained at 37.0 ± 0.5°C throughout the surgery using a heat lamp. A midline incision was made, and connective tissue was teased away to expose the common carotid artery (CCA). After cauterizing a branch of the external carotid artery, that artery was ligated distal to the bifurcation of the CCA. Focal ischemia was induced by occlusion of the right middle cerebral artery (MCA) using a nylon monofilament with silicone-coated tip (0.20-0.22 mm, Doccol Co., Redlands, CA, USA). Transcranial laser-Doppler flowmetry (Perimed, Jarfalla, Sweden) was used to confirm a severe reduction (>70%) in regional cerebral blood flow (rCBF) in the area of cerebral cortex supplied by the MCA. Mice in which there was <70% or >93% reduction in rCBF were excluded from the study (n=1). The filament was withdrawn after 1 h to allow reperfusion. Reperfusion did not occur in one animal which was excluded from the study. Wounds were sutured and covered with betadine and spray dressing (Smith and Nephew, Hull, UK). Concurrent sham-operated control animals underwent all procedures except ligation of arteries. Mice were monitored until they regained consciousness and were then returned to their cages.

Flow cytometric analysis
At 24 h after stroke or sham surgery, euthanasia was performed by isoflurane overdose. Blood was collected by cardiac puncture and mice were then perfused with phosphate buffered saline (PBS), followed by decapitation. Blood leukocytes were purified using red blood cell lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 3 mmol/L EDTA). The brain was removed from the skull and, after removing the cerebellum, the right (ischemic) hemisphere was collected for flow cytometry analysis. Leukocytes and microglia were isolated from brain tissue as described previously¹. Cells were then washed and resuspended in buffer (0.5% bovine serum albumin in PBS) containing a mixture of antibodies, including allophycocyanin (APC)-Cy7 anti-CD45, fluorescein isothiocyanate anti-Ly6C, phycoerythrin (PE)-Cy7 anti-Ly6G, V500 anti-CD3, PE-conjugated CD49b, PE-conjugated NK1.1, PE-conjugated CD3, PE conjugated CD49b, PE conjugated NK1.1, PE conjugated B220, PE conjugated CD90.2, brilliant violet 605-F4/80 (all from Biolegend, San Diego, CA, USA), Pacific Blue anti-CD11b (both from eBiosciences, San Diego, CA, USA) and APC-CCR2 (R&D systems, Minneapolis, MN, USA). After staining, cells were analysed by LSRII (BD Biosciences, San Diego, CA, USA) and FlowJo software (Tree Star Inc., Ashland, OR, USA). Countbright counting beads (Invitrogen, Carlsbad, CA, USA) were included to estimate the number of cells in the samples.

Quantitative PCR
At 24 h after stroke or sham surgery, mice were euthanised by isoflurane overdose, and perfused with RNAse-free PBS. After removing the cerebellum, the brain was separated into left and right hemispheres and whole ischemic (right) brain hemispheres were snap frozen in liquid nitrogen for RNA extraction. RNA was extracted using RNeasy Micro Kit-RNA (Qiagen, Hilden, Germany), quantified using the Nanodrop 1000D spectrophotometer (Thermo Scientific, Waltham, MA, USA), and followed by cDNA conversion using RT² First Strand Kit (Qiagen, Hilden, Germany). Pre-designed Taqman gene expression assays were used to measure RNA expression of selected genes, including Arg1, CCL2, CCL7, CCL8, CCL12, IL-1β, IL-6, IL-10, TNF-α, and Ym-1. GAPDH (Applied Biosystems, Carlsbad, CA, USA) expression was used as a house-keeping gene for reference. Expression data were thus
normalised to GAPDH and presented as fold-change compared to sham using the comparative CT method.

**Immunofluorescence for 3-nitrotyrosine (3-NT) and F4/80**

To determine if macrophages are colocalised with 3-NT, double-labelled immunofluorescence was performed using a 3-NT antibody and a macrophage-specific antibody, F4/80. Frozen brains were sectioned (10 µm) and thaw-mounted onto poly-L-lysine coated slides. Multiple serial coronal brain sections that spanned the infarct region were taken for analysis. Sections were fixed in 4 % paraformaldehyde for 15 min, washed in 0.01M PBS (3x 10 min) and blocked with 10 % goat serum and a Mouse on Mouse Ig blocking reagent for 1 h. Sections were then incubated with a mouse 3-NT antibody (1:50, Abcam, Cambridge, UK) and rat F4/80 antibody (1:100, AbD Serotec, Kidlington, UK) overnight in a humidified chamber. The following day, sections were washed in 0.01M PBS (3x 10 min) and incubated in goat anti-rat Alexa Fluor 594 (1:500, Invitrogen, Carlsbad, CA, USA) secondary antibody for 2 h. Brain sections were then washed in 0.01M PBS (3 x 10 min) and incubated in biotinylated anti-mouse IgG reagent (Vector Laboratories, CA, USA) for 10 min. After washing in 0.01M PBS (3 x 10 min), fluorescein avidin DCS (Vector Laboratories, CA, USA) was applied onto the sections for 5 min. Sections were washed in 0.01 M PBS (3 x 10 min), coverslipped, and examined using an Olympus fluorescence microscope. Numbers of 3-NT⁺ cells and F4/80⁺ cells in the right (ischemic) hemisphere were counted for each brain section. Data were presented as percentage of 3NT⁺ cells of total F4/80⁺ cells. Researchers were blinded during the experiment and all appropriate controls for primary and secondary antibodies were performed.

**Supplemental References**


Supplemental Figure I: Effect of INCB3344 on different subsets of monocytes, including CCR2^+ Ly6C^{hi} monocytes and CCR2^+ Ly6C^{lo} monocytes (A), CCR2^- Ly6C^{lo} monocytes and CCR2^- Ly6C^{hi} monocytes (B), and total Ly6C^{hi} monocytes and total Ly6C^{lo} monocytes (C), in the circulation at 24 h after stroke. Data are compared to those obtained in sham-operated mice (Sham) or vehicle-treated mice (Veh) subjected to stroke. Data are presented as mean ± SEM, *P<0.05, one-way ANOVA with Bonferroni post test, n=5-6 per group.
Supplemental Figure II: Effect of INCB3344 on different subsets of monocytes, including CCR2\(^+\)Ly6C\(^{hi}\) monocytes and CCR2\(^−\)Ly6C\(^{lo}\) monocytes (A), CCR2\(^+\)Ly6C\(^{lo}\) monocytes and CCR2\(^−\)Ly6C\(^{lo}\) monocytes (B), and total Ly6C\(^{hi}\) monocytes and total Ly6C\(^{lo}\) monocytes (C), in the ischemic brain hemisphere at 24 h after stroke. Data are compared to those obtained in sham-operated mice (Sham) or vehicle-treated mice (Veh) subjected to stroke. Data are presented as mean ± SEM, *P<0.05, one-way ANOVA with Bonferroni post test, n=6-12 per group.
Supplemental Figure III: Regional cerebral blood flow (rCBF) was recorded during and after 1 h of middle cerebral artery occlusion to induce focal cerebral ischemia in mice, treated either with vehicle or INCB3344 (30 mg/kg i.p., 3 times daily). Data are presented as mean ± SEM, n=20 per group.
Supplemental Figure IV: Immunohistochemical quantification of the number of F4/80$^+$ cells in brain sections of vehicle- (Veh) or INCB3344 (INCB)- treated animals at 24 h after stroke. Data are compared to those obtained in sham-operated mice (Sham), and presented as mean ± SEM, *P<0.05, one-way ANOVA with Bonferroni post test, n=4-7 animals per group.