Role of Neutrophils in Exacerbation of Brain Injury After Focal Cerebral Ischemia in Hyperlipidemic Mice

Josephine Herz, PhD; Pascal Sabellek, MD; Thomas E. Lane, PhD; Matthias Gunzer, PhD; Dirk M. Hermann, MD; Thorsten R. Doeppner, MD

Background and Purpose—Inflammation-related comorbidities contribute to stroke-induced immune responses and brain damage. We previously showed that hyperlipidemia exacerbates ischemic brain injury, which is associated with elevated peripheral and cerebral granulocyte numbers. Herein, we evaluate the contribution of neutrophils to the exacerbation of ischemic brain injury.

Methods—Wild-type mice fed with a normal chow and ApoE knockout mice fed with a high cholesterol diet were exposed to middle cerebral artery occlusion. CXCR2 was blocked using the selective antagonist SB225002 (2 mg/kg) or neutralizing CXCR2 antiserum. Neutrophils were depleted using an anti-Ly6G antibody. At 72 hours post ischemia, immunohistochemistry, flow cytometry, and real-time polymerase chain reaction were performed to determine cerebral tissue injury and immunologic changes in the blood, bone marrow, and brain. Functional outcome was assessed by accelerated rota rod and tight rope tests at 4, 7, and 14 days post ischemia.

Results—CXCR2 antagonization reduced neurological deficits and infarct volumes that were exacerbated in hyperlipidemic ApoE−/− mice. This effect was mimicked by neutrophil depletion. Cerebral neutrophil infiltration and peripheral neutrophilia, which were increased on ischemia in hyperlipidemia, were attenuated by CXCR2 antagonization. This downscaling of neutrophil responses was associated with increased neutrophil apoptosis and reduced levels of CXCR2, inducible nitric oxide synthase, and NADPH oxidase 2 expression on bone marrow neutrophils.

Conclusions—Our data demonstrate a role of neutrophils in the exacerbation of ischemic brain injury induced by hyperlipidemia. Accordingly, CXCR2 blockade, which prevents neutrophil recruitment into the brain, might be an effective option for stroke treatment in patients with hyperlipidemia. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.010620.)

Key Words: brain injury • CXCR2 • hyperlipidemia • inflammation • neutrophils • stroke

Inflammation is involved in stroke-induced brain damage.1 Besides local inflammatory processes in the ischemic brain, stroke also provokes peripheral immune responses, which influence secondary lesion growth and thus modulate long-term outcome. However, difficulties remain with regard to the translation of inflammation-targeting therapeutic approaches from preclinical to clinical studies,2 which might be because of a neglect of comorbidities such as hyperlipidemia that is frequently shown in patients with stroke. In fact, using dietary and genetically induced models of hyperlipidemia, previous studies demonstrated increased ischemic brain injury in hyperlipidemic mice.3–5 Underlying mechanisms involve alterations of the blood–brain barrier.5 However, in addition to vascular pathology, hyperlipidemia triggers granulocytosis6,9 and therefore adds another complexity to stroke-induced pathophysiology in hyperlipidemic mice hampering translation of experimental findings into clinical practice. Emerging experimental and clinical evidences suggest that inflammatory factors outside the brain markedly influence stroke susceptibility and outcome.7 Thus, we have previously shown that the combination of genetically and dietary-induced hyperlipidemia leads to increased cerebral ischemic tissue injury, which is accompanied by elevated levels of cerebral and circulating granulocytes, albeit the causal link and the functional significance of these observations still remained elusive.4 Early infiltration of polymorphonuclear neutrophils that is preceded by activation of endothelial cells and expression of chemokines and adhesion molecules is a major hallmark of posts ischemic inflammation.5 Several reports, including our own, suggest a detrimental role of brain infiltrating neutrophils in ischemic tissue damage, for example, by releasing oxygen radicals and inflammatory mediators.5,9–12 Besides adhesion
molecules, brain-derived chemokines facilitate immune cell transmigration into the inflamed brain tissue. Neutrophils are recruited through the specific interaction of CXCL1 and CXCL2/3, which are strongly upregulated in ischemic brains, with the neutrophil-specific receptor CXCR2. In addition to enrollment of neutrophils, CXCR2 is supposed to mediate neutrophil release from the bone marrow into the blood in response to inflammatory challenges.

In view of our earlier findings, we were interested whether neutrophils contribute to the exacerbation of ischemic brain injury induced by hyperlipidemia. Therefore, we investigated how the antagonization of the neutrophil-specific chemokine receptor CXCR2 by means of a selective pharmacological inhibitor or a neutralizing CXCR2 antisera influences ischemic brain injury and functional recovery in hyperlipidemic ApoE⁻/⁻ mice; furthermore, investigating consequences of CXCR2 antagonization on peripheral neutrophil homeostasis and phenotype, as well as on cerebral neutrophil infiltration.

**Materials and Methods**

**Animals and Group Allocation**

Experiments were performed in accordance to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals with local government approval. Seven- to 8-week old wild-type (C57BL/6J, Harlan) and ApoE⁻/⁻ male mice, which were generated on the same C57BL/6 background, were either fed with a normal chow or a cholesterol-rich chow (Western diet; TD.88137 Adjusted Calories Diet, Harlan Laboratories) for 6 weeks and submitted to 20 minutes of left-sided middle cerebral artery occlusion (MCAO) or sham surgery. Animals were randomly attributed to treatment paradigms, and experimenters were blinded at all stages of interventions and data analysis. The selective CXCR2 antagonist SB225002 (2 mg/kg, Merck, Darmstadt) or vehicle (1% dimethyl sulfoxide in PBS) was injected intraperitoneally at 0, 24, and 48 hours post ischemia. In other experiments, CXCR2 was specifically blocked by intraperitoneal injection of a neutralizing rabbit anti-CXCR2 serum (300 µL) at 0, 24, and 48 hours post ischemia. In the latter studies, normal rabbit serum served as control. In some experiments, neutrophils were depleted by intraperitoneal injection of 200 µg antimouse Ly6G (Clone 1A8, BioXcell) 24 hours before and 24 hours after ischemia. In these experiments, 200 µg of an isotype control antibody (Clone 2A3, BioXcell) was delivered as control. A total of 352 male mice (184 C57Bl/6J, 168 ApoE⁻/⁻) were examined. These mice were randomly assigned to the following groups: wild-type normolipidemic/sham/vehicle (n=18), wild-type normolipidemic/MCAO/vehicle (n=47), wild-type normolipidemic/MCAO/CXCR2 antagonist (n=18), wild-type normolipidemic/MCAO/CXCR2 antagonist (n=47), ApoE⁻/⁻ hyperlipidemic/sham/vehicle (n=18), ApoE⁻/⁻ hyperlipidemic/MCAO/vehicle (n=39), ApoE⁻/⁻ hyperlipidemic/sham/CXCR2 antagonist (n=18), ApoE⁻/⁻ hyperlipidemic/MCAO/CXCR2 antagonist (n=39), wild-type normolipidemic/MCAO/normal rabbit serum (n=9), wild-type normolipidemic/MCAO/anti-CXCR2 (n=9), ApoE⁻/⁻ hyperlipidemic/MCAO/anti-CXCR2 (n=9), wild-type normolipidemic/MCAO/anti-Ly6G/vehicle (n=9), wild-type normolipidemic/MCAO/anti-Ly6G/CXCR2 antagonist (n=9), ApoE⁻/⁻ hyperlipidemic/MCAO/anti-Ly6G/vehicle (n=9), and ApoE⁻/⁻ hyperlipidemic/MCAO/anti-Ly6G/CXCR2 antagonist (n=9). Numbers of animals to detect infarct size (n=9) and functional deficits (n=12) were determined via a priori sample size calculations (effect size f by ANOVA) of 0.6 for infarct size, and 0.5 for functional deficits (α=0.05, β=0.2). Results are presented as mean±SD. Differences between 2 groups were assessed by the 2-tailed Student t test. Differences across multiple groups were analyzed using 2- or 3-way ANOVA with phenotype (normolipidemic wild-type versus hyperlipidemic ApoE⁻/⁻), experimental intervention (sham versus MCAO), or treatment (vehicle versus CXCR2 antagonist) as independent factors followed by post hoc Bonferroni tests for pairwise comparisons. In all analyses, P<0.05 was considered statistically significant.

**Induction of Focal Cerebral Ischemia**

Induction of stroke was performed using the intraluminal monofilament occlusion model as described previously. A detailed description is given in the online-only Data Supplement.

**Analysis of Poststroke Motor Coordination Deficits**

Assessment of motor coordination deficits was performed on days 4, 7, and 14 using the rotarod and the tight rope test as previously described. Details are given in the online-only Data Supplement.

**Analysis of Postischemic Tissue Injury and Immunohistochemistry**

For infarct volume measurement and immunohistochemical analysis, mice were transcardially perfused with ice-cold PBS at 72 hours post ischemia. Brains were removed and fresh-frozen on dry ice. To determine infarct volume, coronal cryostat brain sections (20 µm thick) taken at 400-µm intervals between +2 mm up to −4.4 mm from bregma were stained with cresyl violet. For assessment of cell death, endothelial activation, neutrophil infiltration, and oxidative DNA damage, cryostat sections taken at the level of bregma were used for immunohistochemistry according to published protocols. A detailed description of stainings and quantifications is given in the online-only Data Supplement.

**Processing of Peripheral Blood, Bone Marrow, and Brain Tissues for Flow Cytometry Analysis**

Isolation of single-cell suspension for flow cytometry analysis was performed as previously described. Briefly, animals were euthanized by intraperitoneal injections of chloralhydrate (200 mg/kg body weight). Blood specimens were collected into EDTA-coated collection tubes by puncture of the inferior vena cava followed by transcardial perfusion with ice-cold PBS. Brains were dissected and hemispheres divided into ipsilesional and contralesional parts. Bone marrow from femurs and tibiae was flushed with PBS. A detailed description of further single-cell isolation, staining procedures, antibody cocktails, and gating strategies is given in the online-only Data Supplement (Methods and Table I in the online-only Data Supplement).

**Gene Expression Analysis of Sorted Neutrophils by Real-Time Polymerase Chain Reaction**

For gene expression studies, ex vivo isolated bone marrow cells were sorted for the neutrophil-specific antigen Ly6G using magnetic activated cell sorting (MACS). Total RNA was extracted from cell lysates followed by real-time polymerase chain reaction. Details on the experimental conditions and measurements, as well as primer sequences, are given in the online-only Data Supplement (Methods and Table II in the online-only Data Supplement).

**Statistics**

Numbers of animals to detect infarct size (n=9) and functional deficits (n=12) were determined via a priori sample size calculations (effect size f by ANOVA) of 0.6 for infarct size, and 0.5 for functional deficits (α=0.05, β=0.2). Results are presented as mean±SD. Differences between 2 groups were assessed by the 2-tailed Student t test. Differences across multiple groups were analyzed using 2- or 3-way ANOVA with phenotype (normolipidemic wild-type versus hyperlipidemic ApoE⁻/⁻), experimental intervention (sham versus MCAO), or treatment (vehicle versus CXCR2 antagonist) as independent factors followed by post hoc Bonferroni tests for pairwise comparisons. In all analyses, P<0.05 was considered statistically significant.
Results

CXCR2 Inhibition Promotes Functional Recovery and Reduces Ischemia-Induced Cerebral Tissue Injury in Hyperlipidemic ApoE−/− Mice

We and others have recently shown that induction of hyperlipidemia by means of a cholesterol-rich chow is associated with exacerbation of ischemic injury in ApoE−/− mice.4,5 Motor coordination deficits assessed in the rota rod and the tight rope test, which were aggravated by hyperlipidemia in ApoE−/− mice, were markedly improved by CXCR2 antagonization ≤14 days post ischemia (Figure 1A and 1B). Administration of the selective CXCR2 inhibitor SB225002 did not affect ischemic brain injury in normolipidemic wild-type mice, but reversed the increased brain injury in hyperlipidemic ApoE−/− mice (Figure 1C and 1D). This effect was mimicked by a neutralizing CXCR2 antiserum15 (Figure 2A). To exclude the possibility that 72 hours was too late to detect differences in brain injury of normolipidemic mice, we also analyzed infarct volume at 24 hours post ischemia. Again, infarct volume was not altered by CXCR2 deactivation (Figure I in the online-only Data Supplement).

**Figure 1.** The CXCR2 antagonist SB225002 promotes functional recovery and reduces brain injury in ischemic hyperlipidemic mice. The CXCR-2 antagonist SB225002 (2 mg/kg) or vehicle (1% dimethyl sulfoxide in PBS) was intraperitoneally injected at 0, 24, and 48 hours post ischemia in wild-type mice fed with normal chow (normolipidemic) or ApoE−/− mice fed with high cholesterol diet (hyperlipidemic). Poststroke functional recovery was analyzed on days 4, 7, and 14 using the rota rod (A) and the tight rope (B) tests (n=11–12). Maximal testing time was 300 s for the rota rod test (A). The tight rope test (B) was analyzed using a validated score from 0 (minimum) to 18 (maximum). Infarct volumes (C) and cellular degeneration (D) were assessed at 72 hours post stroke using cresyl violet staining (C, top) and TUNEL (TdT-mediated dUTP-biotin nick end labeling) staining (D, top; n=8–9). *P<0.05 and **P<0.01 ApoE−/− hyperlipidemic/vehicle vs wild-type normolipidemic/vehicle; #P<0.05 and ##P<0.01 ApoE−/− hyperlipidemic/vehicle vs ApoE−/− hyperlipidemic/CXCR2 antagonist. Scale bars: 1 mm in C; 500 μm (large scale images) and 50 μm (insets) in D.
Neuroprotective Effect of CXCR2 Antagonization Is Abrogated in Neutrophil-Depleted Hyperlipidemic ApoE−/− Mice

To test whether neuroprotective effects in hyperlipidemic mice were attributed to specific effects on neutrophils, we assessed infarct volume in ischemic mice, which had been neutrophil-depleted using a specific anti-Ly6G (1A8) antibody and in which CXCR2 was inhibited with SB225002 (Figure 2B). Neutrophil depletion led to a significant reduction of infarct volume in hyperlipidemic ApoE−/− mice but not in normolipidemic wild-type mice (Figure 2C). Of note, administration of the CXCR2 antagonist did not further reduce infarct volume in neutrophil-depleted mice (Figure 2C), indicating that neuroprotection by SB225002 can be assigned to a direct effect on CXCR2-expressing neutrophils.

Inhibition of CXCR2 Reduces Neutrophil Infiltration Without Affecting Endothelial Activation

To analyze whether brain neutrophil invasion was blocked by pharmacological CXCR2 antagonization, we quantified cerebral neutrophils infiltrated in ipsilateral and contralateral hemispheres by flow cytometry. The CXCR2 antagonist SB225002 decreased neutrophil counts in ischemic hemispheres of ApoE−/− mice on Western diet and wild-type mice on normal diet (Figure 3A). Recruitment of other immune cell subsets was not significantly modulated by CXCR2 inhibition (Figure II in the online-only Data Supplement), suggesting a selective interaction with neutrophils. To exclude potential confounders related to differences in lesion size, we quantified the regional densities of neutrophils within the lesion rim by Ly6G immunohistochemistry and furthermore evaluated their localization with respect to brain capillaries in costainings with the pan-endothelial marker CD31. Although previous studies had shown a predominant association of neutrophils with ischemic brain vessels, we observed a significant proportion of neutrophils in the brain parenchyma for all investigated groups (Figure 3B), closely in line with recent reports of others and us. The local distribution of neutrophils was not significantly modulated by hyperlipidemia or by administration of the CXCR2 antagonist SB225002 (Figure 3B, top middle). Distances of intraparenchymal neutrophils to the most adjacent vessel were also not changed (Figure 3B, top right). Therefore, we quantified the total number of neutrophils (vessel-associated and intraparenchymal) revealing significantly reduced numbers of neutrophils in the injured brain of ischemic hyperlipidemic ApoE−/− mice treated with SB225002 compared with vehicle-treated mice and ischemic normolipidemic wild-type mice (Figure 3B, bottom). To avoid false interpretation because of unspecific effects of SB225002 on blood–brain barrier characteristics, which might have contributed to inhibition of neutrophil infiltration and tissue injury, we also performed a detailed analysis of blood–brain barrier integrity and endothelial activation. Except an increased blood–brain barrier permeability (Figure IIIA in the online-only Data Supplement) and increased intracellular adhesion molecule-1 and vascular adhesion molecule-1 expression (Figure 3C and 3D) in ischemic hyperlipidemic ApoE−/− mice compared with normolipidemic wild-type mice, no significant changes were induced by systemic SB225002 administration. The overall number of vessels was affected neither by hyperlipidemia nor by CXCR2 antagonization (Figure IIIB in the online-only Data Supplement). Therefore, reduced neutrophil infiltration might be directly attributed to the CXCR2-inhibitory effect of SB225002.

Peripheral Neutrophilia in Ischemic Hyperlipidemic ApoE−/− Mice Is Reduced by CXCR2 Antagonization

In addition to chemotactic activity, we examined whether modulation of CXCR2 changes the number of peripheral neutrophils. Analysis of the absolute viable neutrophil counts demonstrated that focal cerebral ischemia induced an increase in circulating and bone marrow neutrophils in hyperlipidemic ApoE−/− mice, which was inhibited by SB225002 (Figure 4). These data suggest that neutrophil homeostasis is altered in the bone marrow,
Figure 3. CXCR2 antagonization reduces neutrophil infiltration without affecting endothelial activation. Wild-type mice fed a normal chow (normolipidemic) or ApoE<sup>−/−</sup> mice fed a Western diet for 6 weeks (hyperlipidemic) were exposed to middle cerebral artery occlusion followed by intraperitoneal administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% dimethyl sulfoxide in PBS) at 0, 24, and 48 hours post stroke. Analysis was performed at 72 hours post ischemia. The amount of brain infiltrated neutrophils was quantified in ipsilateral and contralateral hemispheres using flow cytometry by gating of PI<sup>−</sup>, CD45<sup>high</sup>, SSC<sup>high</sup>, and Ly6G<sup>+</sup> cells (A, 3 individual experiments, 3–4 brains pooled per group and experiment). Local neutrophil accumulation and distribution were assessed via immunohistochemical stainings for Ly6G on ischemic brain tissue sections at the level of bregma (B, n=6). The exact localization of neutrophils was determined through Ly6G/CD31 costainings that were evaluated by confocal microscopy (B, top left). Distances between single neutrophils (Ly6G, green) to the most adjacent vessel (CD31, red) were measured to differentiate between intravascular (0 µm, arrow), perivascular (0–10 µm, arrowhead), and intraparenchymal (>10 µm, asterisk) neutrophils. The percentage of neutrophils within the indicated regions was calculated (B, top middle), and distances for intraparenchymal cells were measured (B, top right). Finally, the total amount of neutrophils was quantified (B, bottom). ICAM-1 (C) and VCAM-1 (D) stainings combined with stainings for the pan-endothelial cell marker CD31 were performed on ischemic brain tissue sections. Positive ICAM-1, CD31, and VCAM-1 vessels were counted, and the percentage of ICAM-1 (C) and VCAM-1 (D) positive vessels was calculated (n=6). Scale bars: 50 µm (A, C, and D, bottom) and 25 µm (A, top). ICAM-1 indicates intracellular adhesion molecule-1; and VCAM-1, vascular adhesion molecule-1. *P<0.05, **P<0.01, ***P<0.001.
Mice Is Reversed by Antagonization of CXCR2

in addition to peripheral blood. Of note, neither focal cerebral ischemia nor CXCR2 antagonization by SB225002 influenced the number of blood and bone marrow neutrophils in normolipidemic wild-type mice (Figure 4). In addition to an ischemia-independent overall increase in myeloid cells in the blood and reduced lymphocyte numbers in the bone marrow of hyperlipidemic ApoE−/− mice, we observed an ischemia-induced reduction in lymphocyte subsets in all experimental groups (Figure IV in the online-only Data Supplement). However, none of these immune cell subsets were regulated by the CXCR2 inhibitor, suggesting a selective interaction with neutrophils (Figure IV in the online-only Data Supplement).

Stroke-Induced Reduction of Neutrophil Apoptosis and Increase in CXCR2 Expression on Bone Marrow Neutrophils of Hyperlipidemic ApoE−/− Mice Is Reversed by Antagonization of CXCR2

To explain the SB225002-mediated reduction in viable neutrophil counts in the periphery, we next evaluated neutrophil survival by quantifying the proportion of dead neutrophils by flow cytometry using propidium iodide. On CXCR2 antagonization, ischemic hyperlipidemic ApoE−/− mice revealed an increased proportion of dead neutrophils in the bone marrow (Figure 5A), suggesting that SB225002 counteracts the ischemia-induced increase of neutrophil survival in hyperlipidemic ApoE−/− mice. In normolipidemic wild-type mice, on the other hand, neither ischemia nor CXCR2 deactivation modulated the proportion of dead neutrophils (Figure 5A). To exclude any confounding effects by false-positive detection rarely associated with propidium iodide staining, we quantified the proportion of Annexin V positive bone marrow neutrophils in ischemic hyperlipidemic ApoE−/− mice either treated with vehicle or with the CXCR2 antagonist SB225002. Confirming propidium iodide measurements, increased numbers of apoptotic cells were noted in SB225002-treated mice (Figure 5B).

Next, we wondered whether CXCR2 expression on neutrophils was affected by hyperlipidemia or ischemia. Flow cytometry and real-time polymerase chain reaction revealed that CXCR2 protein and mRNA expression on bone marrow neutrophils were increased on ischemia in hyperlipidemic ApoE−/− mice, which were reversed through CXCR2 deactivation by SB225002 (Figure 5C and 5D). In normolipidemic wild-type mice, neither ischemia nor CXCR2 antagonization affected CXCR2 expression on bone marrow neutrophils (Figure 5C and 5D).

**CXCR2 Antagonist SB225002 Modulates Oxidative Stress–Related Enzyme Expression in Bone Marrow Neutrophils and Reduces Oxidative Damage in Ischemic Brains of Hyperlipidemic ApoE−/− Mice**

To further elucidate mechanisms for the differential effects of CXCR2 inhibition in normolipidemic and hyperlipidemic mice, we finally investigated whether oxidative stress–related enzyme expression was modulated by hyperlipidemia, ischemia, and CXCR2 antagonization. mRNA expression analysis of the inflammation-associated inducible nitric oxide synthase in sorted bone marrow neutrophils demonstrated that focal cerebral ischemia induces a strong increase in hyperlipidemic ApoE−/− but not in normolipidemic wild-type mice, which was reversed by CXCR2 blockade (Figure 6A). Moreover, a significant ischemia-induced decrease of NADPH oxidase 2 expression detected in normolipidemic wild-type mice was absent in hyperlipidemic ApoE−/− mice. However, CXCR2 deactivation by SB225002 reduced NADPH oxidase 2 expression in ischemic hyperlipidemic ApoE−/− mice to levels of ischemic normolipidemic wild-type mice (Figure 6B). We further observed an overall reduced expression of the antioxidative enzymes, catalase and superoxide dismutase 2, in ischemic and nonischemic hyperlipidemic ApoE−/− mice, which was not influenced by CXCR2 inhibition (Figure V in the online-only Data Supplement). Taken together, these data suggest that hyperlipidemia combined with focal cerebral ischemia promotes an inflammatory and reactive oxygen species producing phenotype in ischemic hyperlipidemic ApoE−/− mice, which is partially counterbalanced by CXCR2 deactivation. In light of the presented results, we further analyzed oxidative
DNA damage through immunohistochemical detection of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) as an indicator of oxidative stress. Quantification of 8-oxo-dG positive cells in ischemic brain tissue revealed that the CXCR2 antagonist SB225002 reduced increased levels of oxidative DNA damage in hyperlipidemic ApoE−/− mice, whereas normolipidemic wild-type mice exhibited an overall low density of 8-oxo-dG positive cells, which was not modulated by the CXCR2 antagonist (Figure 6C).

**Discussion**

Systemic inflammation is linked to stroke occurrence and severity. However, translation from bench-to-bedside targeting the immune system to prevent stroke or diminish damage has failed to date. One critical issue regarding the lack of successful translation is that underlying inflammation associated with comorbidity factors, such as hyperlipidemia, has been broadly neglected in most preclinical trials. We have previously shown that increased ischemic brain damage in hyperlipidemic mice coincides with increased cerebral and peripheral granulocyte numbers.4 In this study, we provide the causal link between these observations by showing that pharmacological or anti-serum-mediated antagonization of the neutrophil-specific chemokine receptor CXCR2 or anti-Ly6G antibody-induced neutrophil depletion restores functional outcome and reverses brain injury induced by hyperlipidemia, thereby unraveling the functional significance of neutrophils in the pathogenesis of ischemic brain injury. Interestingly, only hyperlipidemic mice were responsive to CXCR2 antagonization, cerebral neutrophil infiltration being strongly attenuated by CXCR2 inhibition in hyperlipidemic mice. This increased chemotactic activity of neutrophils in ischemic hyperlipidemic mice was most likely evoked by the increased CXCR2 expression on peripheral neutrophils, perhaps combined with the previously reported increase of its ligands CXCL1 and CXCL2 in ischemic brains of hyperlipidemic mice.5 Chemotactic receptors, such as CXCR2, are involved in interactions with endothelial cells and platelets, which play an important role in atherosclerosis development20 and which regulate neutrophil crawling in inflamed vessels.21 Thus, brain platelet–neutrophil interactions
might be associated with the initiation of neutrophil infiltration into the ischemic brain in hyperlipidemia.

In addition to enhanced chemoattraction to the brain, elevated cerebral neutrophil counts in hyperlipidemic stroke mice might partially result from the overall increased number of circulating neutrophils induced by ischemia. Importantly, CXCR2 antagonization by SB225002 reversed this peripheral neutrophilia. Because increased circulating neutrophils were paralleled by increased bone marrow neutrophils, elevation of blood neutrophils caused by a CXCR2-dependent release from the bone marrow to the circulation seems unlikely. Instead, stroke-induced survival of bone marrow neutrophils in hyperlipidemia was blocked by CXCR2 antagonization. Indeed, neutrophil viability has been shown to be regulated by chemokines in interaction with CXCR2. Furthermore, CXCR2 antagonization increases neutrophil apoptosis in a concentration-dependent manner, supporting our concept of induction of neutrophil apoptosis by the CXCR2 antagonist SB225002. The lack of apoptosis induction in sham-operated and ischemic normolipidemic mice might be explained by the lower CXCR2 expression. Thus, a certain chemokine receptor expression and activation level seems to be required for neutrophil survival.

Although CXCR2 blockade resulted in reduced cerebral neutrophil entry both in normolipidemic and hyperlipidemic mice, only hyperlipidemic mice benefited from CXCR2 inhibition, suggesting that in addition to the total number, the phenotype of neutrophils determines the impact on stroke outcome. We show that focal cerebral ischemia induces a strong increase of inducible nitric oxide synthase and NADPH oxidase in neutrophils of hyperlipidemic mice, which is downregulated by CXCR2 inhibition. Both enzymes are involved in the generation of reactive oxygen/nitrogen species, one of the main effector functions of neutrophils in stroke pathology. Alterations of the neutrophil phenotype in ischemic hyperlipidemic mice were associated with an increased level of cerebral oxidative DNA damage, which was similarly reversed by CXCR2 deactivation as inducible nitric oxide synthase. These associated findings may indicate that decreased neutrophil activation and reactive oxygen species production through CXCR2 antagonization might present a potential mechanism underlying the observed neuroprotective effects in hyperlipidemic ischemic mice. This is further supported by a previous study reporting reduced myeloperoxidase activity of neutrophils after CXCR2 antagonization. In light of previous

![Figure 6. Expression of oxidative stress–related enzymes in bone marrow neutrophils and cerebral oxidative DNA damage of ischemic hyperlipidemic mice are modulated by CXCR2 antagonization. mRNA expression analysis of inducible nitric oxide synthase (iNOS, A) and NADPH oxidase 2 (NOX2; B) was analyzed in magnetic activated cell sorting–sorted Ly6G+ bone marrow neutrophils at 72 hours post ischemia of wild-type mice fed a normal chow or ApoE−/− mice fed a Western diet for 6 weeks that were either sham operated or exposed to middle cerebral artery occlusion (MCAO) followed by intraperitoneal administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% dimethyl sulfoxide in PBS) at 0, 24, and 48 hours post stroke. Mean values of ∆∆CT values are presented (relative expression, n=4–6). C, Oxidative DNA damage was analyzed by immunohistochemical detection and quantification of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) positive cells in ischemic brain tissue (n=4–6). Scale bar: 50 µm. **P<0.01, ***P<0.001.
studies suggesting a certain neutrophil plasticity similar to macrophage plasticity distinguishing proinflammatory M1 and anti-inflammatory M2 macrophages. Increased levels of inducible nitric oxide synthase, a classical M1 marker, imply that the combination of brain ischemia and hyperlipidemia might induce a general switch of neutrophils to a proinflammatory N1 phenotype, which is reversed by CXCR2 antagonization. Additional studies will be required to characterize the whole repertoire of classical M1/N1 and M2/N2 markers in this specific experimental setting.

Despite reduced cerebral neutrophil infiltration, normolipidemic mice were not protected by CXCR2 inhibition. This observation appears to differ from recent studies, including our own, demonstrating neuroprotection by neutrophil depletion and blocking neutrophil invasion. The most likely explanation is the different severity of brain injury. Although the latter studies induced 45- and 60-minute MCAO, we used a mild ischemia model leading to much smaller infarcts and a lesser degree of neutrophil accumulation, suggesting that a certain threshold of cerebral neutrophil counts needs to be exceeded to uncover the cells’ pathogenic role. However, even if more severe injury models are used, several studies interfering with CXCR2 signaling and thereby reducing neutrophil infiltration failed to influence stroke outcome in normolipidemic wild-type mice. But most of these studies assessed stroke outcome at 24 hours post injury that might be too early to detect secondary neurodegeneration after the complex inflammatory cascade have been fully established. Although neutrophils are considered to be the first invaders of the ischemic brain, secondary recruitment of neutrophils through interleukin-17 producing γδ T cells peaking at 3 days post injury has been suggested. Therefore, it cannot be excluded to detect protection in a more severe stroke model at 3 to 7 days post ischemia. In fact, administration of neutralizing CXCR2 serum in a severe transient MCAO model in normolipidemic wild-type mice results in significantly smaller infarct sizes 3 days after stroke. However, Cuartero et al demonstrated that neutrophil infiltration is maximal after 24 hours and that modification of neutrophil phenotype by rosiglitazone is associated comorbidities, namely hyperlipidemia, which, as clinical studies should carefully consider inflammation-associated comorbidities, namely hyperlipidemia, which, as we show, may alter the responsiveness of the ischemic brain tissue.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Role of neutrophils in exacerbation of brain injury after focal cerebral ischemia in hyperlipidemic mice

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Supplemental Methods

Induction of focal cerebral ischemia
Animals were anesthetized with 1% isoflurane (30% O₂, remainder N₂O). Rectal temperature was maintained between 36.5 and 37.0°C using a feedback-controlled heating system. Cerebral blood flow was analyzed by laser Doppler flow (LDF) recordings and was monitored during ischemia for up to 10 min after reperfusion onset. For induction of cerebral ischemia, a midline neck incision was made; the left common carotid artery (CCA) and the external carotid artery were isolated and ligated. A nylon monofilament coated with silicon resin was introduced through a small incision into the CCA and advanced to the carotid bifurcation for induction of MCAO. Twenty minutes later, reperfusion was initiated by monofilament removal. In sham-operated animals, all procedures were performed exactly as for MCAO except for occlusion of the MCA. As such, the left CCA and the external carotid artery were isolated and ligated and a small incision was made in the CCA followed by immediate ligation without introducing a filament. After the surgery, wounds were carefully sutured, anesthesia was discontinued and animals were placed back into their cages.

Animal allocation and exclusion criteria for tMCAO experiments
A total of 352 male mice were enrolled into the study. In the first set of studies, 24 C57Bl/6J mice fed a normal chow and 24 ApoE−/− mice fed a western diet for 6 weeks underwent MCAO and were randomly assigned to 2 groups for each phenotype treated with the selective CXCR2 antagonist SB225002 (n=12/group) or vehicle (n=12/group). These mice were used for behavioral testing at 4, 7 and 14 days post-ischemia (dpi). A second cohort of mice (wildtype normolipidemic: n=34, ApoE−/− hyperlipidemic: n=18) that underwent MCAO and were either treated with SB225002 (n=8-9/group) or vehicle (n=8-9/group) was used for immunohistochemical analysis at 24 or 72 hours post-ischemia. A third set of mice (n=108) was used to determine infarct volumes of ischemic normolipidemic wildtype and hyperlipidemic ApoE−/− mice that were treated with normal rabbit serum (NRS) (n=9/group) or anti-CXCR2 (n=9/group) or anti-Ly6G + vehicle (n=9/group) or isotype + vehicle (n=9/group) or anti-Ly6G + CXCR2 antagonist (n=9/group) or isotype + CXCR2 antagonist (n=9/group). A fourth set of mice (n=96) was generated for analysis of leukocyte subsets in the blood, the brain and the bone marrow at 3 dpi. These mice were assigned to 8 experimental groups by balanced randomization: wildtype normolipidemic / MCAO / sham / vehicle (n=12), wildtype normolipidemic / MCAO / CXCR2 antagonist (n=12), ApoE−/− hyperlipidemic / sham / vehicle (n=12), ApoE−/− hyperlipidemic / MCAO / CXCR2 antagonist (n=12), ApoE−/− hyperlipidemic / sham / CXCR2 antagonist (n=12) and ApoE−/− hyperlipidemic / MCAO / CXCR2 antagonist(n=12). The same group assignment was used for the fifth set of mice to determine mRNA expression in MACS-sorted bone marrow neutrophils at 3 dpi (n=6/group).

Exclusion criteria were defined as follows: prolonged operation time > 15 min; no reperfusion after filament withdrawal; insufficient drop of blood flow (<70%); death within 72 hours after MCAO. Altogether, 28 mice (10.0%) met at least one of these criteria and had to be excluded from the study. Drop-out rates were not affected by SB225002, anti-Ly6G and anti-CXCR2-treatment albeit hyperlipidemicmice reveal slightly increased mortality rates (wildtype normolipidemic n=10, ApoE−/− hyperlipidemic n= 14).

Assessment of functional outcome
Before actual test performance at the time points given, mice were trained 1-2 days before induction of stroke. Both rota rod and tight rope test were performed twice per test point and means were calculated. As for the rota rod test, mice were put on an accelerating drum with a velocity range of 4-40 rpm. Maximal testing time was 300 s and the time until the animal fell
off was recorded and used for statistical analysis. Regarding the tight rope test, the animal was placed in the middle of 60 cm long rope with its forepaws. The rope itself was spun between two platforms with whole apparatus about 50 cm above the ground. Maximal testing time was 60 s and both whether or not the animal reached the platform and the time needed to achieve the latter was used applying a validated score from 0 (min) to 20 (max).

**Determination of infarct volumes**

Infarct volumes were analyzed on cresyl violet stained sections at a distance of 400 µm within the range of +2 mm up to -4.4 mm from bregma. Sections were scanned and analyzed using ImageJ (NIH, USA). Infarct volumes were determined by application of the indirect infarct measurement as previously described¹. As such, the non-lesioned volume of the ipsilateral hemisphere was subtracted from the total volume of the contralateral side.

**Immunohistochemistry**

Cellular degeneration was analyzed via staining of DNA fragmentation using terminal transferasedUTP nick end labeling (TUNEL) according to the manufacturers’ protocol (In situ Cell Death Detection Kit, Roche, Switzerland). For the remaining conventional immunohistochemistry, the following primary antibodies were used: biotinylated goat anti-mouse IgG (1:100, Santa Cruz Biotech, USA), biotinylated goat anti-mouse ICAM-1 (1:50, R&D Systems, USA), rat anti-mouse CD31 (1:500, BD Biosciences, Germany), goat anti-mouse VCAM-1 (1:100, R&D Systems), rat anti-mouse Ly6G (1:200, BD Biosciences). For co-stainings of Ly6G and CD31 a biotinylated rat-anti-mouse CD31 antibody was used (1:100, Biolegend, USA). For endothelial markers and evaluation of neutrophil infiltration and localization, slides were fixed with methanol/acetone followed by incubation with primary antibodies. Oxidative DNA damage was detected through staining of 8-hydroxy-2’-deoxyguanosine (8-oxo-dG, 1:100, Trevigen, USA) according to the manufacturers’ instructions. As secondary antibodies, Cy3-conjugated anti-rat (1:100, Jackson Lab., UK) antibody, streptavidin Alexa Fluor 488 (1:1000, Invitrogen, Germany) and Alexa Fluor 488 conjugated anti-goat antibody (1:500, Invitrogen, Germany) were used for CD31/ICAM-1 and CD31/VCAM-1 co-stainings, respectively. Primary antibody binding of Ly6G/CD31 co-stainings were detected with a goat anti rat Alexa Fluor 488 (1:500, Invitrogen, Germany) and streptavidin Alexa Fluor 594 (1:1000, Invitrogen, Germany), respectively. 8-oxo-dG was revealed by incubation with an anti-mouse Alexa Fluor 488 as secondary antibody. For detection of extravasated IgG, slides were fixed with 4% paraformaldehyde (PFA) followed by incubation with the primary antibody overnight. Antibody binding was visualized using the Vectastain®AB kit (Vector Laboratories, USA) according to the manufactures’ protocol. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 70% methanol in Tris-buffered saline (TBS) prior to the staining. Sections were evaluated under a bright light and fluorescence microscope (Axioplan; Zeiss, Germany) connected to a CCD camera (Microfire; AVT Horn, Germany) for quantification of cellular degeneration, endothelial activation, IgG extravasation and neutrophil accumulation. Cellular injury was analyzed by counting TUNEL⁺ cells in 6 different regions of interest (ROI, each 62,500 µm²) and mean values were calculated for individual animals. Confocal imaging (A1plus, Eclipse Ti, with NIS Elements AR software, Nikon, Germany) was used to generate large scale images of complete hemispheres on TUNEL-stained sections for representative image presentation (Fig. 1D). Using a 10x objective z-stacks of 30 µm thickness (2 µm focal plane distance) were acquired in 7 x 6 overlapping regions (15% overlap) covering the whole hemisphere. Maximal intensity projections were used for graphical presentation using the NIS Elements AR software. IgG extravasation was evaluated on photographs derived from 6 defined ROIs (each 125,000 µm²; 3 ROI striatum, 3 ROI cortex) that were converted into gray scale. Mean optical densities were measured using Image J software (NIH, USA). For analysis of CD31/ICAM-1 and CD31/VCAM-1 co-
stainings, positively stained CD31 vessels and ICAM-1 or VCAM-1 vessels were counted on the same photographs of 3 defined ROIs (125.00 µm² each) followed by calculation of percent values for ICAM-1 and VCAM-1 positive vessels of all CD31 positive vessels. Neutrophil infiltration was quantified by counting Ly6G⁺ cells in 8 regions of interest (each 25,000 µm²) within 2-3 sections per animal. For quantification of neutrophil-vessel-distances confocal analysis was performed as previously described². A total of 502 cells (wildtype normolipidemic / MCAO / vehicle (n=109), wildtype normolipidemic / MCAO / CXCR2 antagonist (n=65), ApoE⁻/⁻ hyperlipidemic / MCAO / vehicle (n=217), ApoE⁻/⁻ hyperlipidemic / MCAO / CXCR2 antagonist (n=111)) were analyzed. Histological analysis was performed while being blinded to the experimental groups.

Cell separation and flow cytometry

Animals were euthanized by i.p. injections of chloralhydrate (200 mg/kg body weight). Blood was taken from the vena cava and collected in ethylenediaminetetraacetate (EDTA) coated collection tubes followed by transcardial perfusion with ice-cold phosphate buffered saline (PBS). Flushed bone marrow from right and left femur and tibias were pooled and mechanically dissociated through a 70 µm cell strainer. For both, bone marrow and blood, erythrocytes were lysed by incubation with 155 mM NH₄Cl, 10 mM KHCO₃, 3 mM EDTA for 5 min followed by two washing steps with PBS. Brains were dissected and hemispheres divided into ipsilesional and contralesional parts. For each measurement, three to four hemispheres were pooled and homogenized through a 70 µm cell strainer (BD Biosciences) by continuous rinsing with 50 ml cold HEPES-buffered RPMI1640. Samples were centrifuged at 286 g for 5 min at 18°C. The supernatants were discarded and the pellets were resuspended in 15 ml of 37% Percoll in 0.01 M HCl/ PBS and centrifuged at 2800 g for 20 min. Myelin was removed and the remaining cell pellet was washed twice in PBS.

For multichannel flow cytometry isolated cell suspensions of the different organs were incubated with the blocking antibody rat anti-mouse CD16/CD32 (FC fragment) for 15 min at +4°C followed by incubation with the antibody cocktails listed in Supplemental Table I for further 30 min. Two panels of antibodies were used (Supplemental Table I): panel 1 enabled counting of myeloid-derived leukocytes and panel 2 divided lymphocyte subsets. For analysis of myeloid cells, CD45high cells were gated by propidium iodide and lymphocyte lineage markers (B220, CD3, NK1.1) to exclude contamination with dead cells and lymphoid subsets, which can partially express myeloid markers. Viable and lymphoid-depleted cells were subdivided by scatter characteristics and Ly6G expression to identify neutrophils. Macrophages/monocytes were defined by CD11b expression in the SSClow Ly6G⁺ cell population and macrophage/monocyte subsets were identified according to their Ly6C expression. dendritic cells were identified by CD11c expression. For analysis of lymphoid cells, CD45high cells were gated for viable lymphoid cells according to scatter characteristics and propidium iodide. B lymphocyte, NK cells and T lymphocyte subsets were defined by CD19, NK1.1, CD4 and CD8 antigen characteristics, respectively. For analysis of neutrophil apoptosis, CD45⁺, Ly6G⁺ cells were gated by propidium iodide in isolated blood and bone marrow cell suspensions. To confirm propidium iodide analysis a part of bone marrow samples (hyperlipidemic / MCAO / vehicle and hypelipidemic / MCAO / CXCR2 antagonist, n=6/group) was used for stainings of the apoptosis marker AnnexinV (BD Biosciences, Germany) according to the manufacturers’ protocol. Cells were analyzed using an LSR II and FACS Diva software (BD Biosciences, Germany). Absolute cell numbers for blood and CNS were analyzed by the use of BD TrueCount beads (BD Biosciences, Germany) according to the protocol on the basis of CD45 positive events. Bone marrow cell counts were determined by trypan blue exclusion and a haemocytometer.

Real time PCR
Total RNA was isolated from MACS-sorted neutrophils using an RNA purification kit (Promega) according to the manufacturers recommendations. First-strand cDNA was synthesized using 500 ng of total RNA and TaqMan reverse transcription reagents (Applied Biosystems). The PCR amplification was performed in 96-well optical reaction plates for 40 cycles with each cycle at 94°C for 15 s and 60°C for 1 min using FAST SYBR® Green Master Mix (Applied Biosystems). The PCR was conducted on the StepOne Real-Time PCR system (Applied Biosystems). CT values were normalized for the housekeeping gene β-actin [ΔCT=CT (target gene) - CT (β-actin)] and related to the mean of control samples (wildtype / sham / vehicle) using the ΔΔCT formula [ΔΔCT= ΔCT (sample) – ΔCT (control)]. Primer sequences are given in Supplemental Table 2.
## Supplemental Tables

### Supplemental Table I: Antibodies used for flow cytometry

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<th>antigen</th>
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<td><strong>Panel 1: myeloid</strong></td>
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<td><strong>Panel 2: lymphoid</strong></td>
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### Supplemental Table II: Sequences of primer used for real time PCR

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</tr>
<tr>
<td></td>
<td>Rev: GGTCCTTACGGATGTCAACGTC</td>
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Supplemental Figure 1: The CXCR2 antagonist SB225002 does not provide neuroprotection in normolipidemic wildtype mice 24 hours after mild focal cerebral ischemia. Wildtype mice fed with a normal chow were exposed to 20 minutes MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) during reperfusion. At 24 hours post-ischemia infarct volume was determined using cresyl violet staining.
Supplemental Figure II: CXCR2 antagonization does not modulate cerebral infiltration of macrophages/monocytes, dendritic cells and lymphocyte subsets. Wildtype mice fed a normal chow (normolipidemic) or ApoE\(^{-/-}\) mice fed a Western diet for 6 weeks (hyperlipidemic) were exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke. Analysis was performed at 72 hours post-ischemia. The amount of brain infiltrated macrophages/monocytes (A), dendritic cells (B) and lymphocyte subsets (C) was quantified in ipsilateral and contralateral hemispheres using flow cytometry by gating for PI\(^{-}\), CD45\(^{\text{high}}\), SSC\(^{\text{low}}\), Ly6G\(^{-}\), CD11b\(^{+}\) cells in (A); for PI\(^{-}\), CD45\(^{\text{high}}\), SSC\(^{\text{low}}\), Ly6G\(^{-}\), CD11c\(^{+}\) cells in (B) and for PI\(^{-}\), CD45\(^{\text{high}}\), SSC\(^{\text{low}}\), NK1.1\(^{+}\) (NK cells), CD19\(^{+}\) (B cells), CD4\(^{+}\) (CD4 T cells) and CD8\(^{+}\) (CD8 T cells) in (C). CD11b\(^{+}\) cells were further divided into Ly6C negative (resident) and Ly6C positive (inflammatory) macrophage/monocyte subsets (A). Data are derived from 3 individual experiments with 3-4 hemispheres pooled per group and experiment. *\(p < 0.05\) for Ly6C neg, § \(p < 0.05\) for Ly6C pos.
Supplemental Figure III: Blood brain barrier integrity and vessel densities are not altered by CXCR2 antagonization. The CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) were i.p. injected at 0 hours, 24 hours and 48 hours post-ischemia in wildtype mice fed with normal chow (normolipidemic) or ApoE−/− mice fed a high cholesterol diet (hyperlipidemic). Blood brain barrier integrity (A) and vessel density (B) were analyzed by immunohistochemical stainings of extravasated mouse IgG and CD31 on tissue sections of ischemic brains of the indicated groups (n=6/group). * p<0.05, ** p<0.01.
Supplemental Figure IV: The CXCR2 antagonist SB225002 does not influence bone marrow and blood derived macrophage/monocyte, dendritic cell and lymphocyte populations. White blood cells isolated from whole blood and bone marrow cells isolated from left and right femurs and tibiae of wildtype mice fed a normal chow (normolipidemic) or
ApoE<sup>−/−</sup> mice fed a Western diet for 6 weeks (hyperlipidemic) that were either sham operated or exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke were analyzed by flow cytometry (n=6-12). Absolute macrophage/monocyte (A,B), dendritic (C,D) and lymphocyte (E,F) counts were determined by quantification of PI, CD45<sup>+</sup>, SSC<sup>low</sup>, Ly6G<sup>−</sup>, CD11b<sup>+</sup> (A,B); PI, CD45<sup>+</sup>, SSC<sup>low</sup>, Ly6G<sup>−</sup>, CD11c<sup>+</sup> (C,D) and PI, CD45<sup>+</sup>, SSC<sup>low</sup>, NK1.1 (NK cells), CD19 (B cells), CD4 (CD4 T cells) and CD8 (CD8 T cells) (E,F), respectively. CD11b<sup>+</sup> cells were further divided into Ly6C negative (resident) and Ly6C positive (inflammatory) macrophage/monocyte subsets. ***p<0.001 for total macrophages/monocytes in (A), ***p<0.002 for dendritic cells in (D), **p<0.01 and ***p<0.001 for B cells in (E,F), §p<0.05 and §§p<0.01 for CD8 T cells in (E,F), ##p<0.01 for CD4 T cells in (E).
Supplemental Figure V: Expression of anti-oxidative enzymes in bone marrow neutrophils is not modulated by CXCR2 antagonization. mRNA expression analysis of superoxide dismutase 2 (SOD2) (A) and catalase (CAT) (B) was analyzed in (MACS)-sorted Ly6G⁺ bone marrow neutrophils at 72 hours post ischemia of wildtype mice fed a normal chow (normolipidemic) or ApoE⁻/⁻ mice fed a Western diet for 6 weeks (hyperlipidemic) that were either sham operated or exposed to MCAO followed by i.p. administration of the CXCR2 antagonist SB225002 (2 mg/kg) or vehicle (1% DMSO in PBS) at 0 hours, 24 hours and 48 hours post stroke (n=4-6). Mean values of \(2^{-\Delta\Delta Ct}\) values are presented (relative expression). **p<0.01; ***p<0.001.
Supplemental References
