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Endothelial CD36 Contributes to Postischemic Brain Injury by Promoting Neutrophil Activation via CSF3

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The scavenger receptor CD36 is a critical factor initiating ischemic brain injury, but the cell type(s) expressing CD36 and responsible for its harmful effects remain unknown. Using bone marrow (BM) chimeras subjected to transient middle cerebral artery occlusion, we found that CD36$^{-/-}$ mice transplanted with wild-type (WT) BM (WT→CD36$^{-/-}$) have smaller infarcts (-67%), comparable with those of mice lacking CD36 both in brain and hematogenous cells (CD36$^{-/-}$→CD36$^{-/-}$; -72%). Conversely, WT mice receiving CD36$^{-/-}$ BM (CD36$^{-/-}$→WT) have infarcts similar to WT→WT mice, suggesting that CD36 in the host brain (i.e., in microglia and endothelial cells), and not in hematogenous cells is involved in the damage. As anticipated, postischemic neutrophil infiltration in CD36$^{-/-}$→CD36$^{-/-}$ mice was attenuated. Surprisingly, however, in WT→CD36$^{-/-}$ mice, in which infarcts were small, neutrophil infiltration was large and similar to that of CD36$^{-/-}$→WT mice, in which infarcts were not reduced. Postischemic neutrophil free radical production was attenuated in WT→CD36$^{-/-}$ mice compared with CD36$^{-/-}$→WT mice, whereas expression of the neutrophil activator colony-stimulating factor 3 (CSF3) was suppressed in CD36$^{-/-}$ cerebral endothelial cells, but not microglia. In CD36$^{-/-}$ cerebral endothelial cultures exposed to extracts from stroke brains, the upregulation of CSF3, but not neutrophil attractant chemokines, was suppressed. Intracerebroventricular administration of CSF3, 24 h after stroke, reconstituted neutrophil radical production and increased infarct volume in WT→CD36$^{-/-}$ mice. The findings identify endothelial cells as a key player in the deleterious effects of CD36 in stroke, and unveil a novel role of endothelial CD36 in enabling neutrophil neurotoxicity through CSF3.

Key words: flow cytometry; free radicals; postischemic inflammation; scavenger receptor; stroke

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Significance Statement

Ischemic stroke is a leading cause of death and disability worldwide with limited therapeutic options. The inflammatory response initiated by cerebral ischemia-reperfusion contributes to ischemic brain injury and is a potential therapeutic target. Here we report that CD36, an innate immunity receptor involved in the initiation of postischemic inflammation, is a previously unrecognized regulator of neutrophil cytotoxicity. The effect is mediated by endothelial CD36 via upregulation of the neutrophil activator CSF3 in cerebral endothelial cells. Therefore, approaches to modulate cerebral endothelial CD36 signaling or to neutralize CSF3 may provide novel therapeutic opportunities to ameliorate postischemic inflammatory injury.

Introduction

Stroke remains the leading cause of disability worldwide with limited treatment options (Moskowitz et al., 2010). Whereas re-establishing cerebral perfusion with tissue plasminogen activator and/or clot-retrieval devices may improve the outcome of some patients (Campbell et al., 2015a), these interventions have a short therapeutic window or are not widely available, and, as such, can be used only in a minority of stroke patients. Consequently, the majority of stroke victims receive only supportive care, highlighting the need for more specific treatments targeting the ischemic cascade (Henninger et al., 2010; Campbell et al., 2015b).

There is increasing evidence that inflammation and immunity play a critical role in the acute phase of ischemic brain injury (Iadecola and Anrather, 2011). Ischemia and reperfusion lead to intravascular molecular events that promote leukocyte adhesion to the cerebral endothelium and their transmigration into the brain and its coverings (Iadecola and Anrather, 2011). At the same time, early brain injury caused by hypoxia generates danger signals or alarmins that activate innate immunity receptors (pattern recognition receptors) in brain and its vasculature, triggering a powerful immune response within the brain (Shichita et al., 2014). These inflammatory responses initiated by hematogenous and brain cells reinforce each other and contribute to the full expression of the tissue damage (An et al., 2014).

Because of their multiplicity, diverse sources, and redundant mechanisms of action, counteracting individual inflammatory mediators is not a feasible therapeutic strategy, and approaches targeting upstream pathways triggering the inflammatory response are likely to be more fruitful (Iadecola and Anrather, 2011). CD36, a multifunctional Class B scavenger receptor expressed mainly in monocytes/macrophages, microglia, and endothelial cells, has emerged as a critical factor in lipid transport, angiogenesis, as well as sterile inflammation (Silverstein, 2009). In addition to the diverse ligands binding CD36 (e.g., oxidized low-density lipoprotein, amyloid-β, thrombospondin), the signaling potential of CD36 is expanded by forming macromolecular complexes with other pattern recognition receptors, such as toll-like receptors (TLR), broadening further its biological impact (Hoebe et al., 2005; Abe et al., 2010; Stewart et al., 2010). Recent data implicate CD36 in the mechanism of ischemia-reperfusion. CD36 deletion ameliorates brain damage in models of transient focal cerebral ischemia, an effect linked to suppression of the transcription factor nuclear factor-kappaB, resulting in reduced postischemic inflammation, microglial activation, and free radical production (Cho et al., 2005; Kunz et al., 2008). Therefore, CD36 has emerged as an upstream "damage sensor" initiating and coordinating the expression of multiple inflammatory pathways mediating tissue damage. The cellular mechanisms by which CD36 regulates postischemic inflammation remain to be established. In particular, it remains unclear whether the expression of CD36 in infiltrating hematogenous cells or in resident brain cells is critical for the effects of this receptor in ischemic injury. Furthermore, the factors through which CD36-expressing cells contribute to postischemic inflammation have not been established.

In this study, we used CD36 bone marrow chimeras to provide insight into the cellular and molecular mechanisms by which this scavenger receptor participates in ischemic injury. We found that CD36 in brain cells is responsible for its deleterious effects in ischemic brain injury. Furthermore, using *in vivo* and *in vitro* approaches, we found that CD36 is required for the endothelial expression of CSF3 or granulocyte-colony stimulating factor (G-CSF), and that this cytokine is needed for postischemic neutrophil reactive oxygen species (ROS) production and toxicity. The findings establish endothelial CD36 as a critical link between parenchymal and blood-borne inflammatory responses and highlight a previously unrecognized contribution of endothelial CSF3 to the noxious effects of neutrophils in ischemic brain injury.

Materials and Methods

Mice. All procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College. Experiments were performed in 8- to 12-week-old male CD36$^{-/-}$ mice on a C57BL/6J genetic background obtained from in-house colonies (Abe et al., 2010). Age-matched wild-type (WT) mice (C57BL/6J, The Jackson Laboratory) served as controls. Five-week-old CD36$^{-/-}$ or WT mice were used for cerebral endothelial cell culture. All animal experiments were performed in accordance with the ARRIVE guidelines (Kilkenny et al., 2010).

Middle cerebral artery (MCA) occlusion (MCAo). Transient focal cerebral ischemia was induced using the intraluminal filament model of MCAo, as described previously (Jackman et al., 2011). Under isoflurane anesthesia (maintenance 1.5%–2%), a heat-blunted nylon suture (6/0) was inserted into the right external carotid artery of anesthetized mice and advanced until it obstructed the MCA. This was confirmed by cerebral blood flow measured using transcranial laser Doppler flowmetry (Periflux System 5010, Perimed) in the territory of the right MCA (2 mm posterior, 5 mm lateral to bregma). The filament was left in place for 35

min and then withdrawn. Only animals that exhibited a reduction in cerebral blood flow of >85% during MCAo and in which CBF recovered by >80% after 10 min of reperfusion were included in the study (Jackman et al., 2011). Inadequate ischemia-reperfusion was found to be <4% in the study and not different among groups. Rectal temperature was monitored and kept constant (37.0 ± 0.5°C) during the surgical procedure and in the recovery period until the animals regained full consciousness. No differences were found in mortality rate (<1%) among groups.

Intracerebroventricular injection of CSF3. Mice were anesthetized with isoflurane and placed in a stereotaxic apparatus 24 h after MCAo. After a small hole was drilled in the left parietal bone (coordinates: 0.5 mm posterior to bregma and 1.0 mm lateral from the midline), a glass micropipette was lowered 2.3 mm below the dural surface and 1 μg of CSF3 (0.1 μg/μl; Peprotech) or vehicle (0.1% BSA in saline) was injected. Mice were allowed to recover and returned to their cages.

Measurement of infarct volume. As described in detail previously (Jackman et al., 2011), infarct volume, corrected for swelling, was quantified by image analysis (MCID, Imaging Research) 72 h after ischemia in 30-μm-thick coronal brain sections stained with cresyl violet.

Bone marrow transplantation and chimerism. Whole-body irradiation was performed in 7-week-old male mice (CD36$^{+/+}$ and CD36$^{-/-}$) with a lethal dose of 9.5 Gy of γ radiation using a ^{137}Cs source (Nordion Gammacell 40 Exactor, Theratronics). Eighteen hours later, irradiated mice were transplanted with BM cells (2 × 10^6, i.v.) isolated from donor CD36$^{+/+}$ (WT) or CD36$^{-/-}$ (knock-out [KO]) mice. Therefore, four groups of chimeric mice were obtained: irradiated WT with WT-BM transplant or irradiated KO with KO-BM transplant, and irradiated chimeric mice, irradiated KO with WT-BM transplant and irradiated WT with KO-BM. Donor BM cells were flushed out with 2–3 ml of PBS from the femur and tibias, filtered through a 40 μm nylon mesh, and counted for viability before injection. Transplanted mice were housed in cages with sulfamethoxazole (0.12%; w/v) and trimethoprim (0.024%) antibiotic added to the drinking water for the first 2 weeks. For histological studies, GFP-transgenic mice (#006567 The Jackson Laboratory) were used as WT BM donor, and irradiated WT and KO mice were transplanted with GFP$^+$ (WT) BM. Reconstitution of BM cells was verified 5 weeks after irradiation by testing the percentage of positive CD36 genomic DNA in isolated blood leukocytes. To this end, 300 μl of blood was withdrawn by cardiac puncture from mouse under deep anesthesia, immediately before death. Erythrolysis was performed three times using 5 ml of erythrocyte lysis buffer (0.15 M NH$_4$Cl, 1 mM KHCO$_3$, 0.1 mM Na-EDTA; 5 min, room temperature). Afterward, DNA was purified from leukocytes using DNeasy Blood and Tissue Kit (QIAGEN #69504) according to the manufacturer's instructions. Reference primers sequences were as follows: m_*ICAM1*_prom.3, 5′-GGACTCACCTGCT GGTCTCT-3′ and m_ICAM1_prom.4, 5′-GAACGAGGGCTTCGGT ATTT-3′; target primers sequences were as follows: m_CD36_ gen.1, 5′-CAGTGCTCTCCCTTGATTCTG-3′ and m_*CD36*_gen.2, 5′-CAATAACAGCTCCAGCAATGAG-3′, all purchased from Invitrogen. qRT-PCR was conducted with 20 ng of DNA, in duplicate 15 μl reactions using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific). The reactions were incubated at 50°C for 2 min and then at 95°C for 10 min. A PCR cycling protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was used for quantification. CD36 relative expression levels were calculated by 2 ($-\Delta\Delta$ C$_T$) method (Livak and Schmittgen, 2001).

Brain cell isolation. Mice were transcardially perfused under deep anesthesia with 30 ml of heparinized saline to remove blood cells. The ischemic hemisphere was separated from the cerebellum and olfactory bulb and gently triturated in HEPES-Hanks buffered saline solution buffer (138 mM NaCl, 5 mM KCl, 0.4 mM Na$_2$HPO$_4$, 0.4 mM KH$_2$PO$_4$, 5 mM D-glucose, 10 mM HEPES) using Gentle MACS dissociator (Miltenyi Biotec) following the manufacturer's instructions. The suspension was digested with 62.5 μg/ml Liberase DH (Roche Diagnostics) and 50 U/ml DNase I (Worthington Biochemical) at 37°C for 45 min in an orbital shaker at 160 rpm. Cells were washed and subjected to discontinuous 70%/25% Percoll (GE Healthcare) density gradient centrifugation (25 min, 800 × g, 4°C). Enriched-mononuclear cells were collected from the interphase, washed with HEPES-Hanks buffered saline solution buffer,

and resuspended in 2% FBS in PBS FACS buffer for staining and FACS analysis.

Flow cytometry and cell sorting. Rat monoclonal antibodies used for flow cytometry analysis include the following: CD45-APC (clone 30F-11), CD11b-PE (clone M1/70), Ly6G-PerCP-Cy5.5 (clone 1A8), and Ly6C-FITC (clone HK1.4), from BioLegend. For blood leukocyte flow cytometry, 100 μl of blood was withdrawn by cardiac puncture from mice under deep anesthesia immediately before death. Erythrolysis was performed, as described above; and afterward, blood leukocytes were resuspended in FACS buffer. Isolated brain cells and blood leukocytes were stained at 4°C with predetermined optimal concentrations of antibodies for 20 min and analyzed on an Accuri C6 flow cytometer (BD Biosciences). Dead cells and debris were gated out by forward scatter and side scatter properties. Cell populations were separated based on CD45 and Ly6C expression and further sorted by CD11b and Ly6G expression. Leukocytes were identified as CD45^{hi} and further gated by CD11b and Ly6G markers. The cell markers used for cell type identification were as follows: neutrophils, CD45^{hi}/CD11b^{hi}/Ly6G^{hi}; monocytes/macrophages, CD45^{hi}/CD11b^{hi}/Ly6G^{lo}; lymphocytes, CD45^{hi}/CD11b^{lo}/Ly6G^{lo}; microglia, CD45^{int}/CD11b^{hi}/Ly6C^{lo}; and endothelial cells, CD45^{lo}/Ly6C^{hi} (Jutila et al., 1988). Blood leukocytes (CD45^{hi}) were classified as neutrophils (CD45^{hi}/CD11b^{hi}/Ly6G^{hi}), monocytes (CD45^{hi}/CD11b^{hi}/Ly6G^{lo}), and lymphocytes (CD45^{hi}/CD11b^{lo}/Ly6G^{lo}). Appropriate isotype control antibodies, "fluorescence minus one" staining, and staining of negative populations were used to establish sorting parameters. Absolute cell numbers and frequencies were recorded. Additionally, in other experiments, the cell fractions of neutrophils, monocytes/macrophages, microglia and endothelial cells, identified using the gating strategy detailed above, were sorted on a FACSVantage cytometer (BD Biosciences) for mRNA analysis. In our model of 30 min transient MCAo, the accumulation of leukocytes occurs at >24 h after ischemia-reperfusion (Garcia-Bonilla et al., 2014a), as shown by others as well (Gelderblom et al., 2009). However, accumulation at earlier times has been reported with longer ischemic periods (Pérez-de Puig et al., 2015).

In vivo cellular ROS measurement. Intracellular ROS production was detected with dihydroethidium (DHE; Molecular Probes, Invitrogen) staining and flow cytometry analysis. DHE was injected (10 mg/kg, i.v.) 4 h before animals were killed. DHE is a cell-permanent dye that is oxidized by superoxide (Robinson et al., 2006). The fluorescence signal attributable to DHE oxidation products reflects cumulative ROS production during the period between administration of DHE and killing the animal (Coleman et al., 2013). Isolated brain cells were further costained with CD45-APC, CD11b-APC/Cy7, and Ly6G-PercP/Cy5.5. DHE fluorescence was detected using MACSQuant analyzer (Miltenyi Biotec) with 488 nm laser and 585/40 nm bandpass filters. DHE-positive cells were identified in infiltrating leukocytes (CD45^{hi}), monocytes/macrophages (CD45^{hi}/CD11b^{hi}/Ly6G^{lo}), and neutrophils (CD45^{hi}/CD11b^{hi}/Ly6G^{hi}) using FlowJo software.

Histology of infiltrating GFP$^+$ leukocytes. WT and CD36 mice transplanted with GFP BM were subjected to MCAo and transcardially perfused with ice-cold PBS followed by 4% PFA in PBS 72 h after MCAo. Brains were removed, stored overnight in the same fixative at 4°C, and then submerged in 30% sucrose solution for 2 d and frozen. Then, brains were cut into serial coronal sections (thickness: 16 μm), 600 μm apart from each other, through the frontal cortex to the caudal hippocampus using a cryostat. The sections were permeabilized with 0.5% Triton X-100 (Sigma) in PBS (PBST) for 30 min at room temperature, blocked with 5% normal donkey serum in 0.1% in PBST, and incubated with the antibody against the blood vessel marker Collagen IV (1:500, rabbit polyclonal antibody; Abcam) in 1% normal donkey serum-0.1% PBST at 4°C overnight. After three 10 min washes with 0.1% PBST, brain slices were incubated with a Cy5-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories). Brain sections were then mounted on slides, and immunostaining was visualized by means of a Leica confocal microscope. The specificity of the immunofluorescence was verified in pilot studies by omission of the primary antibody. GFP$^+$ cells associated to vessels or parenchyma were quantified in the cortex and basal ganglia of 4 coronal sections within the ischemic lesion (1.0, 0.4, −0.2, and −0.8

bregma) using ImageJ software. GFP$^+$ cells within 10 μm of the vascular basement membrane (collagen IV) were considered vascular associated and not localized to the brain parenchyma.

Isolation and culture of mouse brain microvascular endothelial cells. For each cell culture, five mouse brains were aseptically collected, pooled and homogenized in isolation medium: 50 mM HEPES, pH 7.4, Medium 199 (Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) by Dounce homogenizer (KONTES Dounce Tissue Grinders, Kimble Chase, VWR). The homogenates were suspended in 15% dextran (average molecular weight 68,800; Fisher Scientific) and centrifuged at 3900 × g for 15 min at 4°C twice. The pellet was washed with isolation medium, centrifuged (1000 × g, 10 min, 4°C), and digested with 37.5 μg/ml Liberase DH (Roche Diagnostics) and 110 U/ml DNaseI in PBS at 37°C for 1 h in an orbital shaker at 75 rpm. After incubation, 1 ml of FBS was added to the sample and centrifuged (1000 × g, 10 min, 4°C). The pellet was resuspended in 4 ml of endothelial cell culture medium (DMEM high glucose; Lonza Walkersville), supplemented with 25 μg/ml endothelial mitogen (Bti, Biomedical Technologies-Alfa Aesar), 100 μg/ml heparin (Sigma), 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 μg/ml puromycin (Invitrogen). Resuspended cells were seeded on 60 mm Collagen I (Invitrogen) coated dishes and incubated at 37°C with 5% CO_2 in air. After overnight incubation, the medium was changed; and 2 d later, endothelial cell cultures were placed in cell culture medium without puromycin. Endothelial cells were cultured until the cells became subconfluent and subcultured by TrypLE (Invitrogen) at a 1:3 dilution. Cultures were fed with fresh endothelial cell culture medium every 3 d. Confluent endothelial cells were treated with 10% brain extract in endothelial cell culture medium (v/v) for 6 h.

Brain extract preparation. Mouse brains were aseptically collected 24 h after MCAo or sham surgery. Ischemic and nonischemic hemispheres were dissected and homogenized in 10% FBS-DMEM at a concentration of 150 mg tissue/ml with a Dounce homogenizer. The suspension was centrifuged (11,000 × g, 10 min, 4°C), and the supernatant was collected and frozen at −80°C until use.

qRT-PCR. The mRNA for chemokines, cytokines, and pro- and anti-inflammatory genes was examined by qRT-PCR in sham and ischemic brain hemisphere of chimeric mice 24 h after MCAo, as previously described (Kunz et al., 2008). The following genes were studied: interleukin (*Il*)-1β, chemokine (C-X-C motif) ligand (*Cxcl*) 1, 2, and 5, and colony stimulating factor (*Csf*) 2 and 3. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) was used to normalize gene expression. Data were expressed as mean fold change in the ischemic hemisphere relative to sham brain. Primer sequences, purchased from Invitrogen, were as follows: *Il-1β*, 5′-CTCTCCACCTCAATGGACAGA-3′ and 5′-TTTT GTCGTTGCTTGGTTCTC-3′; *Cxcl1*, 5′-GCGAAAAGAAGTGCAGA GAGA-3′ and 5′-AAACACAGCCTCCCACACAT-3′; *Cxcl2*, 5′-TG AACAAAGGCAAGGCTAACTG-3′ and 5′-GAGGCACATCAGGTAC GATCC-3′; *Cxcl5*, 5′-TGAGAAGGCAATGCTGTCAT-3′ and 5′-CC AGGCTCAGACGTAAGAACA-3′; *Csf2*, 5′-AGCTCTGAATCCAGCT TCTCA-3′ and 5′-TGGTCCCTTTAAGGCAGAAAT-3′; *Csf3*, 5′-AG TGTTCCCAAACTGGGTTCT-3′ and 5′-TTAGGGACTTCGTTCCT GTGA-3′; and *Hprt*, 5′-AGTGTTGGATACAGGCCAGAC-3′ and 5′-CGTGATTCAAATCCCTGAAGT-3′. Quantitative determination of gene expression from sorted cell fractions was examined as described previously (Garcia-Bonilla et al., 2014a). Briefly, sorted cells were collected in TRIzol LS (Invitrogen), and RNA was extracted as described by Tighe and Held (2010) (and treated with Rnase free DnaseI (Roche) to remove DNA contamination. cDNA was produced from mRNA samples by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). For isolation of RNA from endothelial cell cultures, RNeasy Plus Mini Kit (QIAGEN) was used. cDNA was produced from mRNA samples by using iScript reverse transcription supermix for RT-qPCR (Bio-Rad). Expression for *Cxcl1*, *Cxcl2*, *Csf2*, and *Csf3* genes was performed on a Chromo 4 Detector (Bio-Rad) using a two-step cycling protocol. HPRT was used to normalize gene expression. q-PCR was conducted with cDNA in duplicate 15 μl reactions using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific). The reactions were incubated at 50°C for 2 min and then at 95°C for 10 min. A PCR cycling
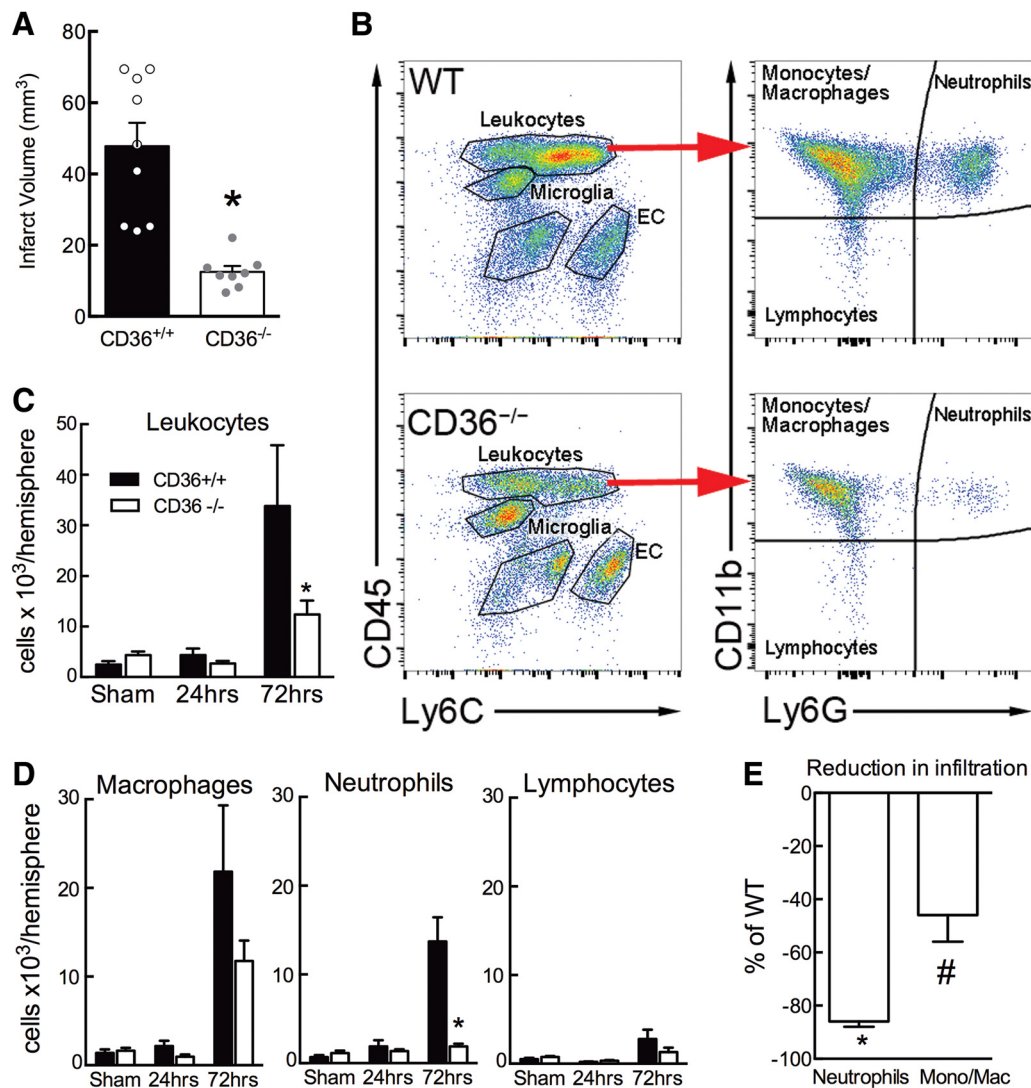
Figure 1. Ischemic injury and leukocyte infiltration are attenuated in CD36 $^{-/-}$ mice. **A**, CD36 $^{-/-}$ mice have smaller infarcts than CD36 $^{+/+}$ (WT) 72 h after MCAo; $n = 9$ or 10 mice/group. *$p < 0.001$. **B**, Gating strategy and flow cytometric analysis of infiltrating leukocytes stained for CD45, Ly6C, CD11b, and Ly6G cell surface markers. Six different populations are gated. Infiltrating leukocytes are identified as CD45 ^{hi} cells and further analyzed for expression of CD11b and Ly6G, identifying CD45 ^{hi}/CD11b ^{hi}/Ly6G ^{lo} monocytes/macrophages, CD45 ^{hi}/CD11b ^{hi}/Ly6G ^{hi} neutrophils, CD45 ^{hi}/CD11b ^{lo}/Ly6G ^{lo} lymphocytes, CD45 ^{int}/CD11b ^{hi} microglia, and CD45 ^{lo}/CD11b ^{lo} endothelial cells (EC). **C**, CD36 $^{-/-}$ mice show decreased leukocyte infiltration in the ischemic brain 72 h after MCAo; $n = 6$ mice/group. *$p < 0.05$ versus WT. **D**, A profound decrease of neutrophil infiltration accounts for the reduction in infiltrating leukocytes in the ischemic brain of CD36 $^{-/-}$ mice compared with WT mice 72 h after MCAo; $n = 6$ mice/group. *$p < 0.001$ versus WT. **E**, Neutrophil infiltration is reduced more than monocyte/macrophage infiltration in CD36 $^{-/-}$ mice relative to WT mice; $n = 6$/group. Values are mean ± SE. *$p < 0.05$ versus WT. #$p < 0.05$ versus neutrophils.

protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was used for quantification. *Cxcl1*, *Cxcl2*, and *Csf3* relative expression levels were calculated by the 2 ($-\Delta\Delta$ C$_T$) method (Livak and Schmittgen, 2001). *Csf2* was undetectable in all cell fractions. Data were expressed as mean fold change

Data analysis. Data are expressed as mean ± SE. Intergroup differences were analyzed using a Student unpaired *t* test, Kruskal–Wallis test, or one-way ANOVA with Dunnett's *post hoc* analysis, as appropriate. Differences were considered statistically significant for $p < 0.05$. Animals were randomly assigned to treatment and control groups, and analysis was performed in a blinded fashion.

Results
CD36 is required for the infiltration of BM-derived cells and for the full expression of ischemic brain injury
CD36 deletion reduces the size of the infarct produced by MCAo in mice and dampens postischemic inflammatory gene expression and glial activation (Cho et al., 2005; Kunz et al., 2008).

However, it remains to be established whether CD36 deletion confers protection by preventing the infiltration of BM-derived cells into the postischemic brain. To address this issue, we used flow cytometry to assess the accumulation of BM-derived cells at 24 and 72 h after ischemia in WT and CD36-null mice. CD36-null mice had markedly reduced infarct volumes 72 h after transient MCAo (Fig. 1A). Neutrophil infiltration was virtually abolished in the brain of CD36-null mice (Fig. 1B,D,E), whereas monocytes/macrophages were reduced to a lesser extent (Fig. 1B,D,E). Effects on lymphocytes were less pronounced, although the number of these cells was small relative to neutrophils and monocytes/macrophages (Fig. 1D). CD36-null mice have similar numbers of circulating neutrophils (WT: $17 \pm 2 \times 10^3$; CD36 $^{-/-}$: $13 \pm 1 \times 10^3$ cells/ml; $p < 0.05$) and monocytes (WT: $19 \pm 3 \times 10^3$; CD36 $^{-/-}$: $15 \pm 1 \times 10^3$ cells/ml; $p > 0.05$). Because the number of leukocytes decreased after ischemia both
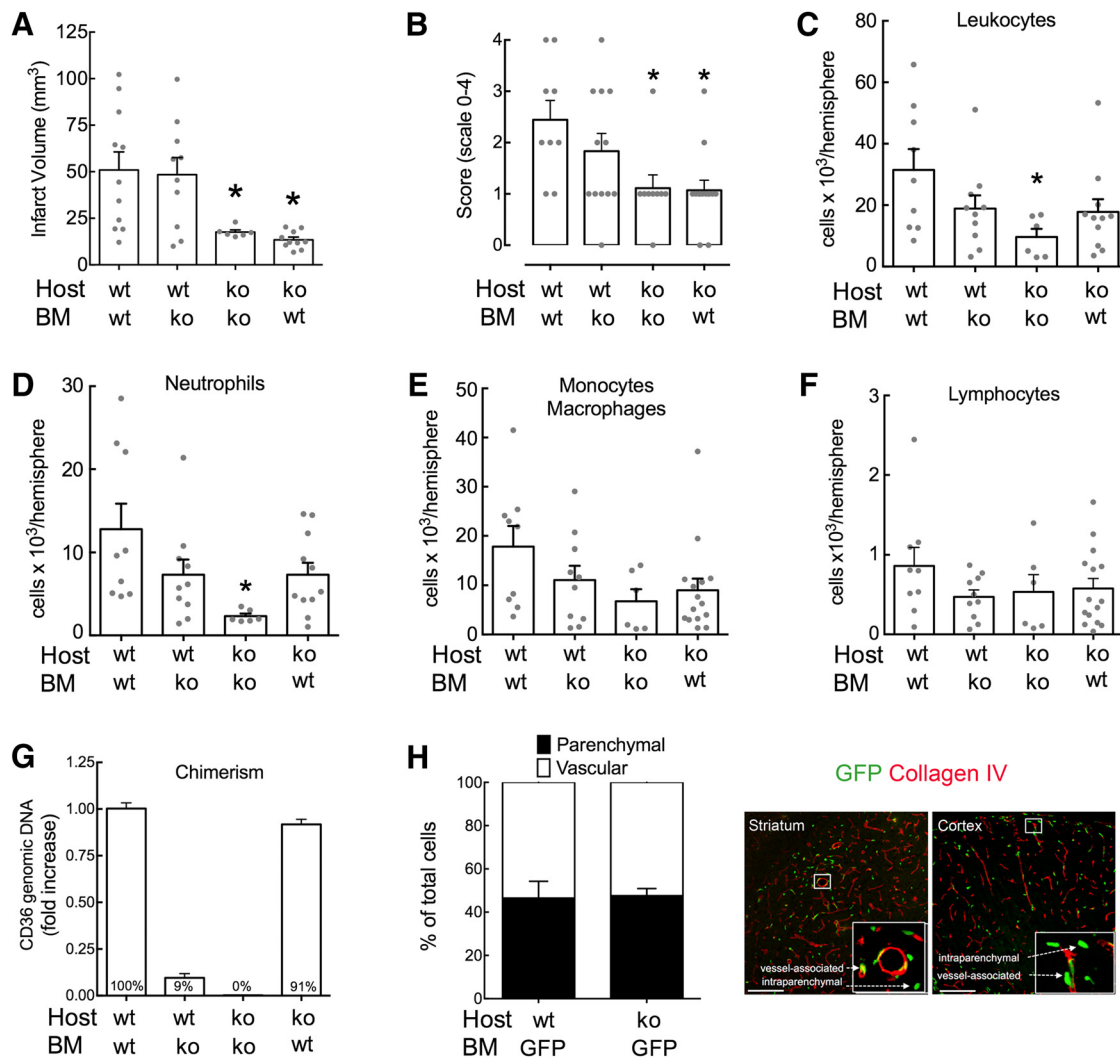
Figure 2. Effect of CD36 expression in brain cells on ischemic damage and leukocyte infiltration. ***A***, CD36-null mice (KO) transplanted with either WT or KO BM had smaller infarcts than WT mice transplanted with either WT or KO BM 72 h after MCAo (*n* = 9–11 mice/group). Values are mean ± SE. *p < 0.05 versus WT→WT. ***B***, CD36-null mice (KO) transplanted either with WT or KO BM had less severe neurological deficit than transplanted WT mice with either WT or KO BM 72 h after MCAo (*n* = 9–11 mice/group). Values are mean ± SE. *p < 0.05 versus WT→WT. ***C–F***, CD36 expression either in the brain or in hematogenous cells facilitates leukocytes (***C***), neutrophils (***D***), monocytes/macrophages (***E***), and lymphocytes (***F***) to enter the ischemic brain in CD36 chimera mice 72 h after MCAo (*n* = 9–11 mice/group). While leukocytes and neutrophils are decreased in CD36 KO mice receiving CD36 KO BM compared with WT mice receiving WT marrow, intermediate levels relative to these groups were found in either WT mice receiving CD36 KO BM or CD36 KO mice receiving WT BM. *p < 0.05 versus WT→WT. ***G***, Chimerism in blood leukocytes assayed by genomic qRT-PCR shows a >90% engraftment of KO BM cells in the WT host mice or WT BM cells in the KO host mice (*n* = 10–15 mice/group). Values are mean ± SE. *p < 0.05 versus WT→WT. ***H***, Percentage of GFP-infiltrating leukocytes associated with vessels or parenchyma in the brain of WT or CD36 KO host brain 72 h after MCAo; *n* = 6/group. Values are mean ± SE. Right, Infiltrating GFP ⁺ leukocytes in neocortex or striatum of WT mice 72 h after MCAo and their association or not with the vascular basement membrane, identified by collagen IV immunocytochemistry. Scale bar, 150 μm.

in WT (neutrophils: −29%; monocytes: −77%; at 72 h) and CD36⁻/⁻ mice (neutrophils: −13%; monocytes: −58%; at 72 h), it is unlikely that the reduced postischemic infiltration in CD36⁻/⁻ mice versus WT mice is due to differences in the number of these cells in the circulation. Therefore, CD36 deletion is associated with a reduction in BM-derived cells in the ischemic brain, neutrophils in particular.

CD36 in the host is responsible for the injury
Next, we sought to determine whether CD36 in hematogenous cells or brain resident cells was responsible for the injury. To this end, we performed BM chimera experiments in which CD36 KO BM was transplanted into WT mice and vice versa. Five weeks later, after full BM engraftment (chimerism >90%; Fig. 2), mice were subjected to transient MCAo for assessment of ischemic injury and neurological deficit 72 h later. CD36 KO mice receiv-

ing CD36 KO BM (KO→KO) still exhibited reduced infarcts and improved neurological deficits compared with WT mice receiving WT marrow (WT→WT) (Fig. 2*A*,*B*), attesting to the fact that BM transplant procedures did not alter the neuroprotective effect of CD36 deletion. However, WT mice transplanted with KO BM (KO→WT) had large infarcts comparable with WT→WT mice, whereas KO mice receiving WT BM (WT→KO) had small infarcts and improved neurological outcome like KO→KO mice (Fig. 2*A*,*B*). These findings indicate that CD36 in the host brain, and not in hematogenous cells, is critical for the outcome of cerebral ischemia.

Postischemic neutrophil infiltration in CD36-deficient hosts is not associated with brain injury
We used flow cytometry to determine whether the different outcomes of cerebral ischemia in CD36 BM chimeras could be at-
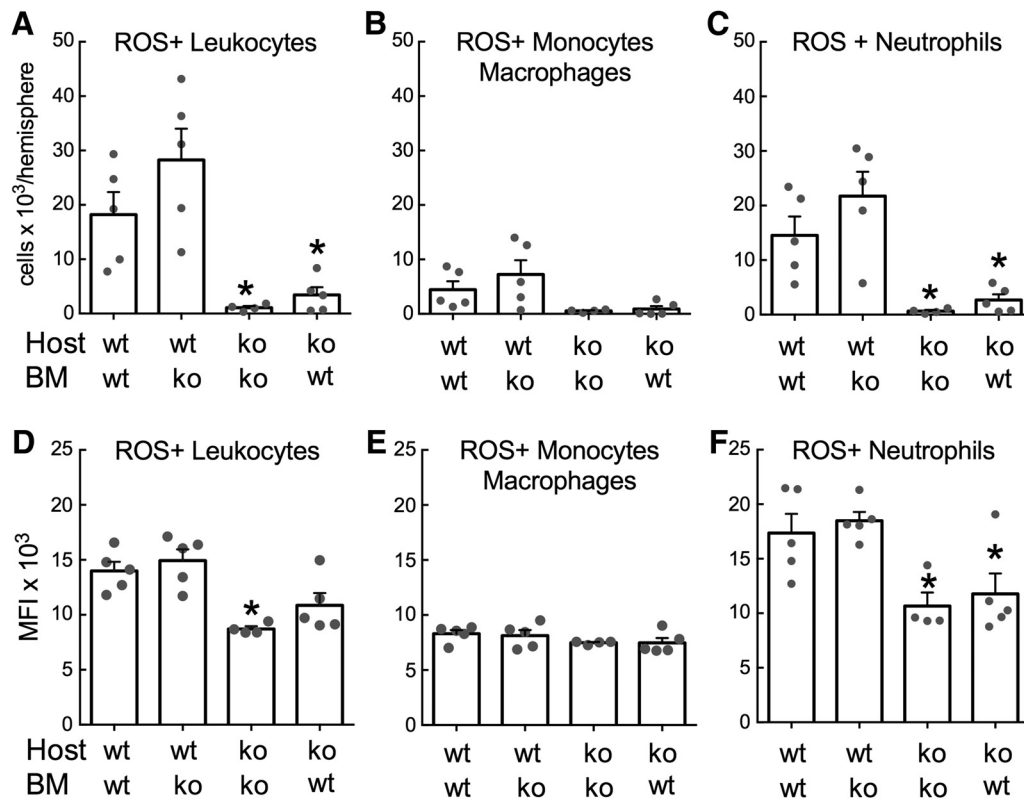
Figure 3. ROS production by infiltrating neutrophils is decreased in CD36-null hosts. *A–C*, Brain leukocytes (*A*), monocytes/macrophages (*B*), and neutrophils (*C*) producing ROS measured by *in vivo* DHE staining and flow cytometry 72 h after MCAo in CD36 chimeric mice (*n* = 4 or 5 mice/group). The number of neutrophils producing ROS is reduced in the CD36 KO host accounting for the decrease of the ROS-positive leukocytes. Values are mean ± SE. *$p < 0.05$ versus WT host→WT BM. *D, E*, Median fluorescence intensity (MFI) of DHE signals analyzed by flow cytometry of in brain leukocytes (*D*), monocytes/macrophages (*E*), and neutrophils (*F*) (*n* = 4 or 5 mice/group). DHE MFI is proportional to the amount of cellular ROS. Values are mean ± SE. *$p < 0.05$ versus WT host→WT BM.

tributed to changes in the brain infiltration of BM-derived cells 72 h after ischemia. As observed in CD36-null mice (Fig. 2*C*), leukocyte infiltration was attenuated in KO→KO mice compared with WT→WT mice, corresponding to the observed reduction in infarct volume (Fig. 2*A*). In WT mice receiving KO BM, there was a reduction in monocytes/macrophages and neutrophil infiltration (Fig. 2*D,E*), which, however, was not statistically significant or sufficient to reduce injury volume (Fig. 2*A*). Surprisingly, however, in KO mice receiving WT BM, in which the infarct was markedly reduced (Fig. 2*A*), the neutrophil infiltration was not different from that observed in WT mice receiving KO BM in which the infarct was not reduced (Fig. 2*D*). This observation attests to the fact that in these mice the accumulation of neutrophils in the postischemic brain is independent of the size of the infarct. No differences in monocytes/macrophages were observed among these groups (Fig. 2*E*).

To rule out that the neutrophils did not induce brain injury because they were sequestered in or around cerebral microvessels and did not reach the parenchyma, we determined histologically the proportion of vessel-associated and parenchymal leukocytes in WT and KO mice transplanted with GFP⁺ BM (WT for CD36). We found that, although their total number was reduced in the KO host, there were no differences in the proportion of leukocytes associated with vessels or with the parenchyma (Fig. 2*H*). Therefore, after ischemia, WT neutrophils and other white blood cells enter a CD36-deficient host but are not associated with increased injury.

Postischemic ROS production by WT neutrophils is attenuated in a CD36-deficient host

In cerebral ischemia, as in other inflammatory conditions, ROS production is a major mechanism by which neutrophils damage the brain (Nathan, 2006; Garcia-Bonilla et al., 2014a). Therefore, we sought to determine whether the lack of neutrophil neurotoxicity in a CD36-deficient host after stroke was related to their reduced ability to produce ROS. To this end, we examined ROS production in brain infiltrating leukocytes of CD36 BM chimeras 72 h after transient MCAo using the oxidative fluorescent dye DHE. In accordance with infarct volumes (Fig. 2*A*), the number of leukocyte-producing ROS was significantly reduced in KO→KO mice compared with WT→WT mice (Fig. 3*A*). Although the number of infiltrating leukocytes was similar in WT→KO and KO→WT chimeras (Fig. 2*C*), ROS-producing leukocytes were reduced in WT→KO mice to levels comparable with those observed in KO→KO mice (Fig. 3*A*). Analysis of leukocyte subpopulations revealed that neutrophils accounted for the majority of ROS-producing cells (Fig. 3*C*), whereas only few monocytes/macrophages showed ROS production (Fig. 3*B*). ROS-positive neutrophils were most abundant in WT mice receiving WT or KO BM and markedly reduced in KO mice receiving KO BM, corresponding to the large and small infarct size, respectively. However, ROS-positive neutrophils were also reduced in KO receiving WT BM, which also have small infarcts (Fig. 3*C*). Figure 3*D–F* illustrates the amount of ROS in infiltrating cell in the postischemic brain. Within ROS-producing leuko-
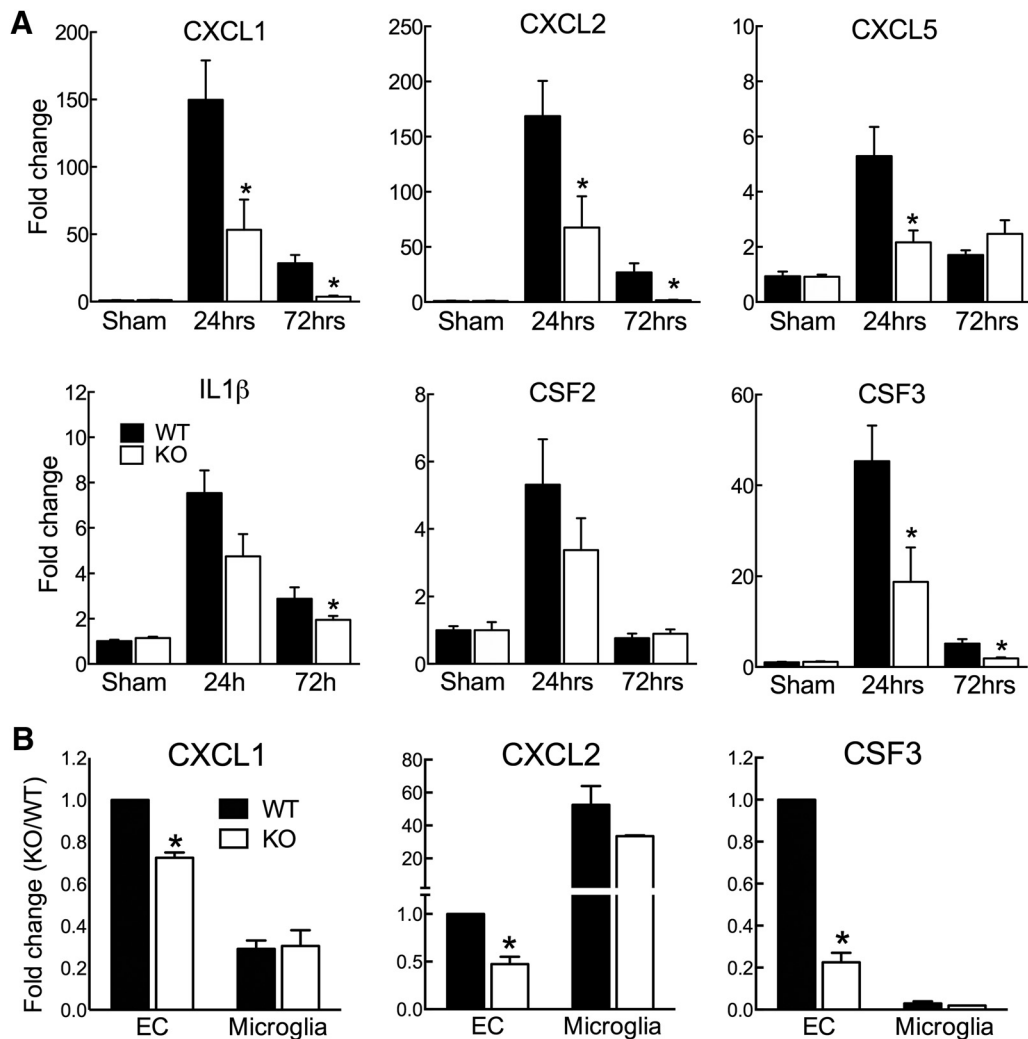
Figure 4. Neutrophil activating factors are attenuated in CD36-null hosts. ***A***, mRNA expression of *Cxcl1*, *Cxcl2*, *Cxcl5*, *Il-1β*, *Csf2*, and *Csf3*, in ischemic brain from WT and CD36 KO mice 24 and 72 h after MCAo or Sham surgery (*n* = 5–7 mice/group). Values are mean ± SE. *$p < 0.05$ versus WT. ***B***, *Cxcl1*, *Cxcl2*, and *Csf3* mRNA expression assessed in flow cytometric sorted microglia and endothelial cells (EC) from WT and CD36 KO mice 24 h after MCAo (*n* = 3 pool/group, with *n* = 3 mice/pool). *Cxcl1*, *Cxcl2*, and *Csf3* mRNA expression is reduced in endothelial cells (EC) of CD36 KO mice. No changes in the microglial expression are observed between WT and CD36 KO mice. *$p < 0.05$ versus WT. Values are mean ± SE.

cytes, the amount of cellular ROS production was also different among groups (Fig. 3D). While cellular ROS levels in infiltrating monocytes/macrophages were similar in the four groups (Fig. 3E), the cellular ROS production of WT and KO neutrophils infiltrating a WT host brain was increased compared with neutrophils infiltrating a KO host brain (Fig. 3F). These observations indicate that postischemic ROS production in WT neutrophils is dampened in a CD36-deficient brain.

Factors involved in neutrophil activation are suppressed in CD36-null mice after stroke

To gain insight into the molecular bases of the reduced neutrophil toxicity in CD36-null mice, we investigated the postischemic expression in brain of factors involved in neutrophil transmigration and activation (Nathan, 2006; Nauseef and Borregaard, 2014). We found that, although *Cxcl1*, *Cxcl2*, *Cxcl5*, *Il-1β*, *Csf2*, and *Csf3* mRNA were upregulated before neutrophil invasion (24 h after stroke), the expression of *Cxcl1*, *Cxcl2*, *Cxcl5*, and *Csf3* was reduced in CD36-null mice (Fig. 4A). Next, we sought to determine the cell type(s) in which these factors are expressed after stroke in a CD36-

dependent manner. Because in brain CD36 is present in endothelial cells and microglia (Cho et al., 2005; Park et al., 2011), we determined the expression of neutrophil attracting and activating factors in these cell types. We found that *Cxcl1*, *Cxcl2*, and particularly *Csf3* were reduced in endothelial cells sorted from CD36-null mice 24 h after stroke (Fig. 4B). No significant changes in these factors were observed in microglia (Fig. 4B).

CD36 is required for the full expression of endothelial CSF3

Based on these results, we sought to determine whether CD36 in endothelial cells is needed for the expression of factors involved in neutrophil attraction and activation after stroke. To this end, we exposed cerebral endothelial cells in culture to a brain extract prepared 24 h after stroke. An extract from the ischemic hemisphere, but not from the contralateral one, induced massive upregulation of *Cxcl1*, *Cxcl2*, and *Csf3* (Fig. 5A). However, only the *Csf3* induction was markedly suppressed in endothelial cells deficient in CD36 (Fig. 5A) (−50 ± 4%), attesting to the requirement of CD36 of its full expression.
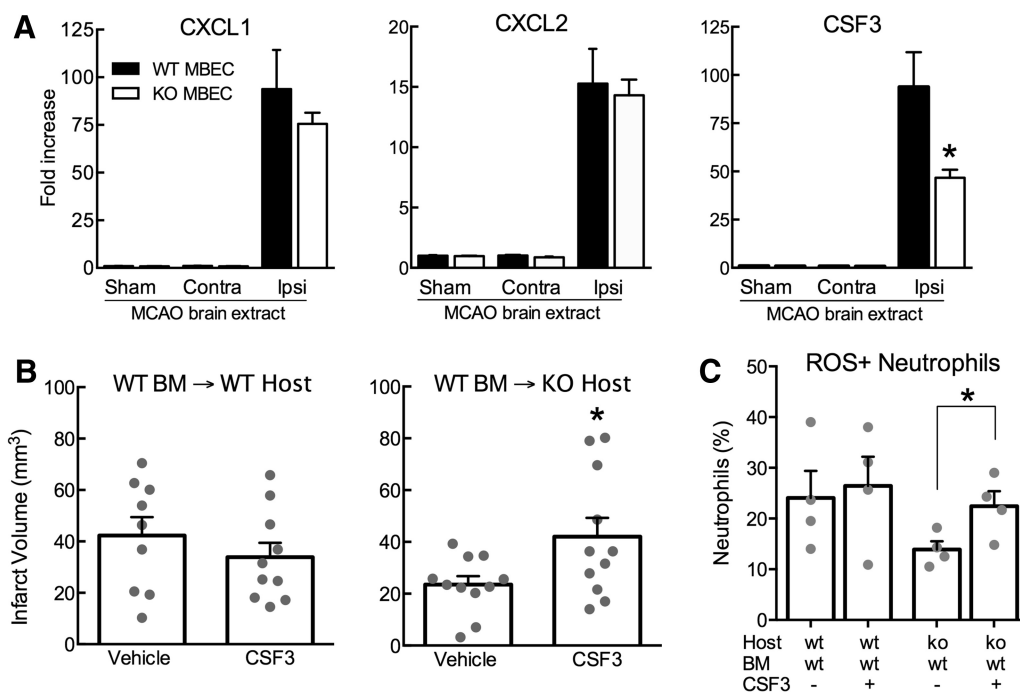
Figure 5. CSF3 is produced by cerebral endothelial cells in a CD36-dependent manner and increases neutrophil ROS and ischemic injury in CD36-null hosts receiving WT BM. *A*, *Cxcl1*, *Cxcl2*, and *Csf3* mRNA is upregulated in primary mouse brain endothelial cell (MBEC) cultures exposed for 6 h to brain extracts from ischemic tissue (Ipsi, ipsilateral hemisphere) but when exposed to brain extract from nonischemic tissue (Sham surgery or Contra, contralateral hemisphere). Reduced production of *Csf3* is observed in CD36 KO EC cultures exposed to ischemic brain extract (*n* = 6/group). *p < 0.05 versus WT. *B*, Intracerebroventricular administration of CSF3 (1 μg) 24 h after MCAo increases ischemic brain injury in CD36 KO mice receiving WT bone marrow (WT BM→KO Host), whereas CSF3 administration does not affect the injury in WT mice receiving WT marrow (WT BM→WT Host) (*n* = 9–11 mice/group). *p < 0.05 versus vehicle. *C*, CSF3-mediated reconstitution of ischemic brain injury is accompanied by an increase in the number of neutrophils producing ROS in WT BM→KO Host chimeras (*n* = 4 mice/group). *p < 0.05 versus vehicle. Values are mean ± SE.

CSF3 administrating increases ROS-positive neutrophils and counteracts the protective effect of CD36 deletion

Finally, we tested the effect of restoring cerebral CSF3 levels in chimeric mice in which WT BM was transplanted into a CD36-deficient host. In these mice, there was neutrophil infiltration (Fig. 2D), but stroke volume (Fig. 2A) and postischemic ROS production by neutrophils (Fig. 3C) were not increased. Administration of CSF3 into the cerebral ventricles did not increase stroke volume in WT mice receiving WT BM (Fig. 5B). However, in CD36-null mice receiving WT BM, CSF3 administration increased stroke volume and ROS-positive neutrophils (Fig. 5C).

Discussion

Major findings of the study

We investigated the cellular mechanism of the effect of CD36 in ischemic brain injury. BM chimera experiments demonstrated that CD36 in the host brain, not in infiltrating hematogenous cells, is required for the full expression of the damage. Thus, the injury was markedly reduced in CD36-null mice transplanted with WT BM. However, the damage was reduced despite significant infiltration of neutrophils, suggesting that these cells cannot fully exert their toxic effect in a CD36-deficient host. Accordingly, the capacity of WT neutrophils to produce postischemic ROS, a major effector of their toxicity, was impaired in a CD36-deficient host. Because CD36 in the host brain is present in cerebral endothelial cells and microglia, we examined the expression of factors involved in neutrophil trafficking and activation in these cells, which were sorted from WT and CD36-null brains after stroke. We found that CSF3, a cytokine involved in BM mobilization and neutrophil activation (McLemore et al., 2001; Semerad et al., 2002), is substantially upregulated after stroke

(Fig. 4A), an effect suppressed in cerebral endothelial cells of CD36-null mice. These observations indicated that CD36 is needed for the upregulation of this cytokine, key for neutrophil ROS production. In support of this hypothesis, brain extracts prepared 24 h after ischemia induced a profound upregulation of *Cxcl1* (>90-fold), *Cxcl2* (15-fold), and *Csf3* (>90-fold) in WT cerebral endothelial cells, but only the *Csf3* upregulation was attenuated in CD36-null endothelia. Administration of CSF3 (intracerebroventricular) to CD36-null mice transplanted with WT BM restored the ability of neutrophils to produce ROS and increased postischemic injury. These findings demonstrate a key role of endothelial CD36 in neutrophil trafficking and activation in the setting of cerebral ischemia, and the involvement of CD36-dependent CSF3 expression in postischemic neutrophil toxicity.

CD36 expression in the host is responsible for the damage

We found that transplantation of WT BM in CD36-null mice did not reconstitute the damage, whereas transplantation of CD36-null BM did not confer protection in WT mice, indicating that CD36 expression in the host is responsible for its deleterious effects on the outcome of cerebral ischemia. However, quantitative assessment of the postischemic infiltration of hematogenous cells by flow cytometry revealed that, although transplantation of CD36-null BM reduced the infiltration in a WT host, the damage was not reduced and was comparable with that of WT mice. These findings suggest that lack of CD36 in hematogenous cells reduces the ability of leukocytes to enter the postischemic brain, but this effect is not sufficient to influence brain damage. Indeed, lack of CD36 on infiltrating cells did not reduce the ability of neutrophils to produce ROS. Because blood neutrophils are not known to express CD36 (Ericson et al., 2014), their reduced

transmigration into the brain may involve interactions with other hematogenous cells expressing the receptors, such as monocytes or platelets (Ghasemzadeh and Hosseini, 2013; Schiwon et al., 2014). Furthermore, the absence of CD36 on neutrophil also rules out that their potential for ROS production is directly impaired by CD36 deletion. In addition, the observation that $CD36^{-/-}$ BM transplanted into WT hosts does not reduce damage argues against the protective effect of CD36 deletion being the result of reduced postischemic intravascular clotting due to a possible role of platelet CD36 (Ghosh et al., 2008).

On the other hand, in a CD36-null host receiving WT BM, brain damage was markedly reduced and not different from that of CD36-null mice. Such damage reduction occurred despite a leukocyte infiltration comparable with that of WT mice receiving CD36-null BM, in which the infarct was as large as in WT mice. In this instance, we found that the ability of infiltrating neutrophils to generate ROS was markedly reduced, indicating that CD36 in the host is needed for neutrophils to produce ROS in the postischemic brain. Collectively, these finding highlight a key role of CD36 in neutrophil infiltration and activation. However, the effect on neutrophil infiltration, which depends on the presence of CD36 in BM-derived cells as well as the host, has no impact on ischemic injury, whereas the effect on neutrophil toxicity, which depends on CD36 in the host, is the major determinant of stroke outcome.

CD36 and neutrophil ROS: role of endothelial CSF3

We found that CD36 in the host is required for the full production of ROS by neutrophils after stroke. Because ROS are produced by activated neutrophils (Nathan, 2006), we examined the impact of CD36 on factors involved in neutrophil chemoattraction and activation (Mantovani et al., 2011; Nauseef and Borregaard, 2014). We found that, whereas the chemokines CXCL1 and 2 were reduced by 20%–40% in CD36-null cerebral endothelial cells, CSF3, a cytokine critical for neutrophil mobilization and ROS production (Katsura et al., 1993; Sato et al., 1997), was suppressed by 70%. No changes in these factors were observed in CD36-null microglia. On these bases, we hypothesized that reduction of CSF3 in cerebral endothelial cells was related to the lack of neutrophil ROS in CD36-null mice receiving WT BM. To provide supportive evidence for this hypothesis, we exposed primary cerebral endothelial cell cultures to an extract prepared from brains after stroke. We found that this treatment induced substantial upregulation of CXCL1, CXCL2, and CSF3, but only the CSF3 upregulation was CD36-dependent. The observation that exogenous CSF3 restored the ability of neutrophils to produce ROS and reconstituted the damage in CD36-null mice receiving WT BM supports a critical role of this cytokine in neutrophil toxicity.

What is the cellular source of CSF3? Although in brain CD36 is expressed in endothelial cells and microglia (Park et al., 2011; Hickman et al., 2013), we found that the postischemic expression of CSF3 occurs only in endothelial cells. Endothelial cells have been reported to induce neutrophil activation and to potentiate ROS production through hematopoietic growth factors (Takahashi et al., 2001). Furthermore, our *in vitro* studies indicate that the supernatant of brains subjected to MCAo induced a massive CSF3 response in cerebral endothelial cells. Therefore, the cellular source of CSF3 is likely to be in endothelial cells.

CSF3 and stroke

CSF3 (G-CSF), best known for its involvement in the mobilization of BM progenitors, especially neutrophil precursors and

stem cells, is used extensively to expand BM cells in patients with hematological malignancies and BM transplantation (Beekman and Touw, 2010). In experimental stroke, CSF3 has been shown to be uniformly neuroprotective in the acute and chronic setting through multiple mechanisms, including enhancement of angiogenesis and neurogenesis, as well as suppression of inflammation and apoptosis (England et al., 2009). However, consistent with our results, a recent clinical trial (AXIS 2) has shown no efficacy of acute CSF3 administration after stroke (Ringelstein et al., 2013), highlighting an unanticipated complexity in its role in stroke. CSF3 was previously reported to enhance neutrophil ROS production (Katsura et al., 1993), but in the setting of stroke CSF3 was considered to be protective. Our studies unveil a previously unrecognized "dark side" of CSF3 in ischemic brain injury by demonstrating its involvement in the postischemic ROS production by neutrophils, a CD36-dependent effect. The mechanisms by which endothelial CD36 regulates CSF3 expression remain to be investigated. Oxidized low-density lipoprotein, a well-known CD36 activator, induces CSF3 expression in vascular cells (Kiyan et al., 2014). The effect requires the interaction of CD36 with other receptors, including TLR4 and the urokinase receptor uPAR, which may form a macromolecular complex with CD36 (Kiyan et al., 2014). Similarly, induction of neutrophil-activating genes, including CSF3, was found in endothelial cells exposed to the presumed CD36 ligand serum amyloid A (Lakota et al., 2013), which is upregulated after stroke (Rallidis et al., 2006). It is therefore conceivable that similar mechanisms are responsible for the CD36-mediated CSF3 expression after stroke. However, further studies are required to provide experimental evidence for this hypothesis.

CD36 and alarmins after stroke

Another questions concerns the ligands activating endothelial CD36 and their source. Brain-generated "danger signals" after stroke could reach endothelial cells either from the abluminal side or from the luminal side after leaking into the circulation (Liesz et al., 2015). CD36 is activated by numerous endogenous ligands, including amyloid-β, thrombospondin, oxidized low-density lipoprotein, surface fragments of apoptotic cells, advanced glycated proteins, cell derived microparticles, and others (Silverstein et al., 2010). The ligands engaging CD36 after stroke remain to be identified and, considering the diverse binding sites of the receptor and its ability to form macromolecular complexes with other pattern recognition receptors, are likely to be multiple. Indeed, we have previously demonstrated that CD36 signaling after stroke requires the TLR2/1 complex (Abe et al., 2010), and requirement for complexes with TLR2/6 and TLR4 have been described in other disease models (Hoebe et al., 2005; Kiyan et al., 2014). Using the endothelial *in vitro* system exposed to brain extracts after stroke, we are well equipped to identify potential CD36 ligands responsible for CSF3 expression in endothelial cells and ROS production in neutrophils. These experiments, which are beyond the scope of the present study, are underway.

Recent data have implicated CD36 also in Alzheimer's pathology, Parkinson disease, prion diseases, and spinal cord injury (Garcia-Bonilla et al., 2014b), suggesting that the deleterious effects of this receptor are not confined to ischemic brain injury. However, in models of neonatal cerebral ischemia (Woo et al., 2012) and intraparenchymal hemorrhage (Fang et al., 2014; Zhao et al., 2015), CD36 in activated microglia seems to be protective by facilitating the removal of apoptotic cells and the reabsorption of the hematoma, respectively. Therefore, the role of CD36 cannot be assumed to be uniformly beneficial or deleterious but may

depend on the nature of the injury, its state of evolution, and the age of the host.

In conclusion, we have demonstrated that CD36 has a critical role in the acute damage produced by the inflammatory component of ischemic brain injury. The cellular bases of the tissue damage are related to promoting the infiltration of neutrophil and enhancing their ability to produce ROS. The effect is attributable to CD36 expressed in host cells and not in hematogenous cells infiltrating the postischemic brain. In particular, CD36 in cerebral endothelial cells is essential for the full expression of CSF3, a potent activator of neutrophil ROS previously considered to be uniformly beneficial in experimental stroke. Collectively, the data identify CD36 in cerebral endothelial cells as a key initiator of innate immune responses after stroke, and highlight a previously unrecognized deleterious role of CSF3 in postischemic neutrophil activation and brain injury. Targeting the endothelial CD36-CSF3 axis may offer a potential new approach to dampen postischemic inflammation and counteract its harmful effects on the ischemic brain.

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