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The Spleen Contributes to Stroke-Induced Neurodegeneration

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Abstract

Stroke, a cerebrovascular injury, is the leading cause of disability and third leading cause of death in the world. Recent reports indicate that inhibiting the inflammatory response to stroke enhances neurosurvival and limits expansion of the infarction. The immune response that is initiated in the spleen has been linked to the systemic inflammatory response to stroke, contributing to neurodegeneration. Here we show that removal of the spleen significantly reduces neurodegeneration after ischemic insult. Rats splenectomized 2 weeks before permanent middle cerebral artery occlusion had a >80% decrease in infarction volume in the brain compared with those rats that were subjected to the stroke surgery alone. Splenectomy also resulted in decreased numbers of activated microglia, macrophages, and neutrophils present in the brain tissue. Our results demonstrate that the peripheral immune response as mediated by the spleen is a major contributor to the inflammation that enhances neurodegeneration after stroke.

Keywords

stroke; spleen; neurodegeneration; inflammation; ischemia

Protecting neurons and blunting the inflammatory response are key components for developing new treatments to alleviate the cerebral damage, disability, and ultimately death caused by stroke. Currently, the only U.S. Food and Drug Administration–approved treatment for stroke is tissue plasminogen activator, a clot buster, which has a limited 3-hr therapeutic window of administration after stroke and a success rate of only 33% (Marler and Goldstein, 2003). This treatment has no neuroprotective or anti-inflammatory properties outside of its clot-busting abilities, making it unsuitable for treating those key components of stroke that result in expanding ischemic damage. Therefore, it is necessary to develop new therapies for this injury that are based on a complete understanding of the mechanism of interaction between the body's immune response and the neurodegenerative processes.

After the initial ischemic injury, a compromised blood–brain barrier coupled with expression of adhesion molecules by the vascular endothelial cells permits an influx of peripheral immune cells including macrophages, neutrophils, leukocytes, T cells, and B cells (Emsley et al., 2003). This influx of peripheral immune cells into the brain exacerbates the local brain inflammatory response, leading to enhanced neurodegeneration. Previous studies have demonstrated altered splenic function after a stroke and increased circulating proinflammatory

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cytokines (Gendron et al., 2002; Offner et al., 2006a, Offner et al., 2006b; Vendrame et al., 2006). The infiltrating cells and increased proinflammatory cytokines negatively affect stroke outcome (Lucas et al., 2006). Therefore, immune/inflammatory processes within the spleen are potential targets for new therapies with the goal of decreasing stroke-induced inflammation in the brain.

Animal models of brain injury like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Benner et al., 2004), transient (Offner et al., 2006b), or permanent middle cerebral artery occlusion (MCAO) (Vendrame et al., 2006), and transient MCAO report that there is a reduction in the spleen weight, which may result from the release of splenocytes. Transfusion of human umbilical cord blood cells (HUCBC) 24 hr after permanent MCAO in rats results in retention of the spleen's mass as well as a reduction of infarct size by 60% (Vendrame et al., 2006). Further, there was an increase in the anti-inflammatory cytokine interleukin-10 with this treatment. These results are consistent with the hypothesis that the spleen responds to injury in the brain by releasing stored immune cells into the bloodstream, which then infiltrate the brain and promote a secondary inflammatory response that enhances neurodegeneration. Implicit in this hypothesis is the corollary that removal of the spleen or inhibition of splenic function should dampen the peripheral immune response, decreasing invasion of leukocytes into the central nervous system and preventing expansion of the stroke lesion. Indeed, in the current study, we show that splenectomy in the in vivo MCAO stroke model significantly reduces infarct volume. Furthermore, our findings imply that novel therapies that target the splenic immune response after stroke could ultimately produce a powerful treatment with an extended therapeutic window.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were housed in a climate-controlled room with water and laboratory chow available ad libitum. Animals were cared for according to the National Institutes of Health (NIH) guidelines for the Care and Use of Animals and overseen by the University of South Florida's Institutional Animal Care and Use Committee. Experimental groups for this experiment were as follows: sham operated ($n = 4$), MCAO ($n = 7$), and splenectomy-MCAO ($n = 12$).

Splenectomy

Rats were anesthetized with 3% to 4% isoflurane in 100% oxygen at a flow rate of 2 L/min. Splenectomy was performed by making a 2-cm dorsal midline skin incision at the caudal terminus at the level of the 13th rib. With blunt forceps, the spleen (with accompanying blood vessels and pancreatic tissue) was exteriorized through the incision. The blood vessels were tied off and ligated; the spleen was removed and stored at -80°C . The abdominal wall and incision were closed with sutures. Rats were allowed to recover 2 weeks before the MCAO surgery.

Laser Doppler Radar Blood Flow Measurement

Before MCAO surgery, anesthesia was induced with oxygen containing 5% isoflurane in an induction chamber, and the rat's head and neck were shaved. Before Doppler insertion, the rats were treated prophylactically with ketoprofen (10 mg/kg i.m.), atropine (0.25 mg/kg s.c.), and Baytril (20 mg/kg i.m.), which were approved according to IACUC guidelines. Ketoprofen injections were continued 3 days after MCAO to control pain. The rat was placed on the operating table dorsal side up anesthesia supplied through a nose cone (3% to 4% isoflurane in 100% oxygen, flow rate 2 L/min). An incision was made with a scalpel just lateral to the midline of the dorsal plates of the skull on the side that was the ipsilateral to the MCAO. Once

the incision was made, the skin was spread open and the membrane covering the skull pushed aside with a cotton-tipped applicator. A microdrill was used to drill a small hole into the skull at 1 mm posterior and 4 mm lateral to bregma. A hollow stainless steel guide screw was screwed into the hole in the skull and a fiber-optic cable (500 μ m) inserted through the screw guide and glued in with superglue. Blood perfusion in the brain was then detected by the Moor Instruments LTD laser Doppler with MoorLAB proprietary Windows-based software on a standard laptop computer. Once surgery was complete, the screw guide was removed and the scalp incision closed with surgical sutures. Rats that did not show $\geq 60\%$ reduction in perfusion during MCAO were excluded from the study.

Permanent Middle Cerebral Occlusion Model

Permanent focal ischemia was achieved during MCAO by using the intraluminal suture technique (Longa et al., 1989). After the Doppler probe was set, an incision was made in the neck and the right common carotid, external carotid, internal carotid, and pterygopalatine arteries were isolated by blunt dissection. The external carotid artery was ligated and cut, and a 4-cm-long 4-0 monofilament was advanced through the internal carotid artery into the middle cerebral artery. The embolus was then permanently anchored at the internal/external carotid junction to produce permanent occlusion. The incision was then sutured and the animal provided a 1-mL subcutaneous injection of saline.

Brain Extraction and Sectioning

The animals were killed at 96 hr after MCAO and per-fused with 0.9% saline and 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were harvested, fixed in paraformaldehyde, and immersed in serial solutions of 20% and 30% sucrose in phosphate-buffered saline (PBS). Brains were frozen and sliced into 30- μ m sections with a cryostat. Sections were either thaw mounted on slides or placed in Walter's Antifreeze cryopreservative and stored at -20°C .

Fluoro-Jade Histochemistry

Six coronal sections were chosen from each rat brain at specific intervals from 1.7 mm anterior to bregma through -3.3 mm posterior to bregma that included striatal and hippocampal regions of the infarct. These were stained with Fluoro-Jade (Histochem, Jefferson, AR), which labels degenerating neurons and is more sensitive than triphenyltetrazolium chloride in identifying neurodegeneration. This method was adapted from that originally described by Schmued et al. (1997) and has been detailed previously by Duckworth et al. (2005). Tissue was thaw mounted and dried to glass slides. Slides were then placed in absolute ethanol for 3 min followed by 70% ethanol and deionized water for 1 min each. Sections were then oxidized with a 0.06% KMnO_4 solution for 15 min, followed by three rinses of double-distilled water for 1 min each. Sections were stained in a 0.001% solution of Fluoro-Jade in 0.1% acetic acid for 30 min. Slides were again rinsed, allowed to dry at 45°C for 20 min, cleared with xylene, and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Nissl Staining With Thionin

Six coronal sections were chosen from each rat brain at specific intervals from 1.7 mm anterior to bregma through -3.3 mm posterior to bregma and were stained with thionin (Sigma-Aldrich, St. Louis, MO), which labels Nissl bodies in cells. Tissue was thaw mounted and dried to glass slides. Slides were then hydrated in alcohol series, stained in the thionin stock solution for 1.5 min, dehydrated, cleared with xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NY).

Infarct Volume Quantification

Infarct volume was calculated separately from the Fluoro-Jade-and thionin-labeled sections. Images of six 30- μ m-thick brain sections were taken at millimeter intervals from +1.7 to -3.3 mm from bregma to determine the volume of infarction. The stained tissue was digitally photographed with an Olympus IX71 microscope controlled by DP manager software (Olympus America Inc., Melville, NY) at a magnification of 1.25 $\times$. Each image was edited with Jasc Paintshop Pro to sharpen and enhance contrast to the same specifications. Volume of neurodegeneration was measured by NIH Image J software. The volume of the contralateral side of the brain tissue was also measured and used to compensate for possible edema in the ipsilateral hemispheres by dividing the infarction by the contralateral measurement. Infarct size was analyzed by ANOVA followed by Bonferroni's post hoc test.

Immunohistochemistry/Histology

We used immunohistochemistry and histology to determine whether there were changes in microglia and neutrophil infiltration into the brain with splenectomy and MCAO. We used isolectin IB4, which binds sugar residues found on activated microglia, infiltrating macrophages, and endothelial cells to identify myeloid cells. The morphology of the immune cells, however, is easily discerned from that of the endothelial cells (Abbott, 2000; Kim et al., 2006). We used an antibody to myeloperoxidase (MPO) to identify neutrophils. The tissue slides were thawed, rinsed with PBS (pH 7.2), and then placed in permeabilization buffer containing 10% goat serum, 3% 1 M lysine, and 0.3% Triton X-100 in PBS for 1 hr at room temperature. Next, they were stained with either isolectin IB4 (*Griffonia simplicifolia*) conjugated with Alexa-Fluor 488 (5 μ L/mL, Molecular Probes, Eugene, OR) or MPO rabbit polyclonal antibody (1:400, Affinity Bioreagents, Golden, CO) overnight at 4 $\times$ C in a PBS solution with 2% goat serum and 0.3% Triton X-100 in a foil-covered humidified chamber. The next morning, the slides were washed with PBS three times for 5 min. The MPO slides were then incubated with Alexa-Fluor 488 goat anti-rabbit secondary antibody (1:300, Molecular Probes, in PBS, 2% goat serum, 0.3% Triton X-100) in a foil-covered humidified chamber for 1 hr. The isolectin slides required no additional incubation step. After final washing, the slides were coverslipped with Vectashield hard-set mounting media with DAPI (Vector Laboratories, Burlingame, CA). The NIH Image J program was used to count individual MPO-positive cells in brain section images taken within the infarction at a magnification of 100 $\times$. MPO counts were then expressed as the linear regression of infarct volume and number of MPO-positive cells and the number of MPO-positive cells per infarct volume.

Blood Smear Preparation and Giemsa Staining

Blood smears were prepared at 0, 1, and 2 weeks after splenectomy and again at 0, 48, and 96 hr after MCAO. A small drop of blood was placed near the frosted end of a clean glass slide. A second slide was used as a spreader. Holding the spreader slide at a 30-degree angle, the spreader was drawn back against the drop of blood. The blood was then pulled to the opposite edge of the slide, producing a thin film on the slide. The smears were allowed to air dry and were then fixed in methanol for 5 min. For Giemsa (Sigma-Aldrich) staining, the slides were first washed three times for 2 min each with distilled water. Slides were laid flat and flooded with Giemsa stain (1 to 2 mL) for 4 min. Equal volumes of distilled water were added to the slides to stop staining. Slides were then dipped in a large container of water to remove excess stain followed by three rinses in distilled water for 3 min each. After air drying vertically overnight, the slides were coverslipped with Permount. For white blood cell percentages of a population, a total of 100 cells labeled with Giemsa were counted and separated on the basis of morphology of leukocytes, monocytes, neutrophils, basophils, and eosinophils. Platelets were counted as number of platelets per 100 white blood cells.

Hematocrit Preparation

For analysis of hematocrit, blood was obtained 0, 1, and 2 weeks after splenectomy and again at 0, 48, and 96 hr after MCAO. Free-flowing blood was collected into heparinized hematocrit tubes until they were approximately two-thirds full. Two samples were taken from each rat and sealed with sealing putty. The hematocrit tubes were spun for 2 min in a centrifuge, and hematocrit values were read on a microcapillary reader (International Equipment Company, Needham HTS, MA). Values for the two samples were averaged.

Statistical Analysis

Data are presented as means $\pm$ SEM. A value of $P < 0.05$ was considered significant. Significance of data were determined by ANOVA followed by Bonferroni's post hoc test, except for the correlation of infarct volume to MPO-positive cells, which was determined by linear regression.

RESULTS

Splenectomy Effects on MCAO-Induced Infarct

To determine the contribution of the spleen to the neurodegeneration occurring at the site of ischemic injury, we compared infarct size as determined with either Fluoro-Jade or thionin after MCAO in splenectomized vs. nonsplenectomized rats.

In rats subjected to MCAO only, the Fluoro-Jade extensively labeled degenerating cells in the cortex, striatum, and hippocampus of the hemisphere ipsilateral to the MCAO surgery (Fig. 1a, center). Brain sections from rats that underwent splenectomy before MCAO displayed considerably less Fluoro-Jade staining on the stroked side relative to brain sections of MCAO-only rats (Fig. 1a, center and right). Brain tissue from the sham-operated MCAO animals exhibited no Fluoro-Jade staining (Fig. 1a, left). Quantification of infarct volumes from Fluoro-Jade-labeled sections demonstrated that splenectomy before MCAO significantly decreased infarct volume by 82.3% compared with infarct volumes from the brains of rats subjected to MCAO-only ($*P < 0.001$) (Fig. 1c).

These results were confirmed with thionin labeling. The brain sections of the MCAO-only treated rats showed an absence of staining across the majority of the ipsilateral hemisphere, including the cortical, striatal, and hippocampal regions (Fig. 1b, center). The brain sections from animals subjected to splenectomy followed by stroke showed a marked increase in staining of intact tissue relative to rats with only MCAO. Brain sections from sham-operated rats showed no areas deficient in staining (Fig. 1b, left and right). On further analysis, there was a significant decrease in infarct volume of 80.8% in those animals whose spleens were removed before stroke ($*P < 0.001$) (Fig. 1d).

Inflammatory Cell Expression in Infarction After Splenectomy

Brain sections of MCAO-only rats showed extensive isolectin IB4-cell labeling in the ipsilateral hemisphere (Fig. 2b). In brain sections from splenectomized rats, the isolectin labeling of activated microglia and macrophages is localized to smaller regions within the cortex and striatum (Fig. 2c). In tissue sections from sham-operated rats, only endothelial cells of the blood vessels were labeled (Fig. 2a).

To detect neutrophils within the brain, immunohistochemistry for MPO was used. After MCAO only, many MPO-labeled neutrophils were observed in the infarct at the level of the striatum (Fig. 3b). Tissue sections from splenectomized rats that underwent MCAO showed few MPO-stained cells (Fig. 3c) compared with tissue sections from MCAO-only rats (Fig. 3b). Tissue sections from sham-operated rats contained no MPO staining (Fig. 3a). When the numbers of

MPO labeled neutrophils were quantified, there were significantly fewer neutrophils in the brain tissue of splenectomized rats (20.6 ± 8.9) that underwent stroke compared with counts from MCAO-only rats (206.7 ± 41.8 , $P < 0.001$). When the number of MPO-positive neutrophils was expressed as a function of infarct volume, there was still a tendency for there to be more neutrophils in the MCAO-only group compared with the splenectomized MCAO group, but this difference did not reach statistical significance ($P = 0.234$, Fig. 3d). There was, however, a significant correlation between infarct size and the number of MPO-labeled neutrophils showing that the larger the infarct size, the more neutrophils were present around the infarct ($R_2 = 0.6038$, $P < 0.0001$, Fig. 3e).

Blood Leukocyte, Platelet, and Hematocrit Profiles

Smears were obtained from all rats at 0, 48, and 96 hr after the stroke surgery. These blood smears were stained with Giemsa, and white blood cell populations were tabulated. There were no significant changes in leukocytes in the blood in any of the experimental groups (Fig. 4), and although there was a tendency for the lymphocyte population to decrease and the neutrophil population to increase in the splenectomized MCAO animals at 48 hr, both of these cell types returned to baseline levels by 96 hr (Fig. 4b). Similarly, there was no significant change in leukocyte populations as a result of splenectomy (Fig. 4e).

In addition to examining white blood cell counts, we also examined hematocrit and the number of platelets per 100 white blood cells. There was no significant difference between groups or across time after stroke in the hematocrit or platelets (data not shown).

DISCUSSION

We hypothesized that the spleen responds to stroke by releasing stored immune cells into the bloodstream, which then infiltrate the brain and promote a secondary inflammatory response that enhances neurodegeneration, and therefore that by removing the spleen before the stroke, the amount of damage in the brain would decrease. Indeed, our results show that splenectomy before stroke significantly reduces infarction size in the brain.

A proposed mechanism of action by which the spleen could be contributing to expansion of infarct size after a stroke is through activation of the sympathetic nervous system resulting in splenic contraction and a potential release of red and white blood cells (Stewart and McKenzie, 2002). This contraction causes a release of proinflammatory immune cells, which are attracted to the brain by chemokines induced by the stroke. These peripheral immune cells could then act to increase neuroinflammation and subsequently neurodegeneration (Hausmann et al., 1998; Abraham et al., 2002). This is consistent with other reports that show a splenic response in stroked rats (Offner et al., 2006b; Vendrame et al., 2006). Removal of the spleen before stroke would decrease the peripheral immune response by removing the largest pool of immune cells, resulting in decreased neuroinflammation.

These data also yield insight into the systemic nature of the body's response to a brain injury. Activated microglia have been thought to be the main cause of the second wave of neurodegeneration in the infarct area because these cells have been shown to outnumber the macrophages at the infarction (Liu and Hong, 2003; Schilling et al., 2003). However, systemic inhibition of macrophages by anti-CD11b, anti-CD18, and anti-Mac-1 monoclonal antibodies 1 hr after transient MCAO produced a significant reduction in infarction volume in the ischemic brain (Chen et al., 1994; Chopp et al., 1994; Zhang et al., 1995). These reports indicate that infiltrating monocytes/macrophages play a significant role in neuroinflammation, and may be necessary for the activation of microglia. This would be consistent with our data showing that removal of a population of infiltrating macrophages, neutrophils, B cells, and T cells through

splenectomy reduces cellular infiltration into the brain and interaction with the activated microglia at the site of ischemic injury resulting in decreased damage.

A hallmark of certain brain injury models is the infiltration of peripheral immune cells that work to remove damaged tissue from the central nervous system (Stoll et al., 1998). A high concentration of these cells will result in enhanced damage through the release of free radicals, inflammatory cytokines, chemokines, and cytotoxic substances (Raivich et al., 1999; Stoll et al., 2000) because these immune cells are responding to the ischemic injury as if it were invading pathogens. Microglia, infiltrating macrophages, and neutrophils all enhance neuroinflammation at the site of ischemia (Barone et al., 1995; Barone and Feuerstein, 1999; Allan and Rothwell, 2003; Lucas et al., 2006). The presence of immune cells from the periphery may be linked to the cells or to chemical signals that are possibly released from the spleen. Microglia, the resident macrophages of the brain (Lyons et al., 2000; Allan and Rothwell, 2003), and systemic macrophages, which invade through a compromised blood–brain barrier (Stoll et al., 2002; Kim et al., 2006), are not readily distinguishable as a result of lack of differential cellular markers and act in similar fashion in their expression of chemokines, inflammatory cytokines, and free radical production (Raivich et al., 1999). These cells are dramatically decreased in the infarct after removal of the spleen, suggesting that migrating monocytes/macrophages originate from the spleen and activate microglia at the site of the infarct. Treatment with HUCBC significantly reduces microglia/monocytes/macrophages in the injured brain, decreases infarct volume, and improves functional outcome after stroke (Vendrame et al., 2004). In this study, the HUCBC were only found within the infarcted hemisphere of the brain and in the spleen. Together, these data (decreased microglia/monocytes, improved outcome, and location of the intravenously injected cells) and our current study support the hypothesis of a splenic influence on these cells during stroke and infarct expansion.

Neutrophil granulocytes, cells of the innate immune system, are reported to be one of the first damaging cell types present in the infarction and exacerbate neurodegeneration resulting from stroke (Zhang et al., 1994; Akopov et al., 1996; Barone and Feuerstein, 1999; Emerich et al., 2002). Inhibition of neutrophils at the time of stroke has also shown a promising reduction in infarction (Chen et al., 1994; Chopp et al., 1994). These cells adhere to endothelium (Barone and Feuerstein, 1999), release inflammatory cytokines and chemoattractants (Barone and Feuerstein, 1999), and produce radicals resulting in tissue damage (Matsuo et al., 1995; Matsuo et al., 1996; Dirnagl et al., 1999; Iadecola and Alexander, 2001). Because these innate immune cells are found in the brain during ischemic injury and have been reported to reside in the spleen (Shi et al., 2001), we also examined whether splenectomy altered neutrophil infiltration into the injured brain after MCAO. Neutrophils were detected with immunolabeling for MPO, a peroxidase enzyme found in lysosomes of these cells (Feuerstein et al., 1998; Nauseef, 1998; Weston et al., 2007). These MPO-containing cells were significantly decreased in the brains of rats splenectomized before MCAO. These results suggest that neutrophils infiltrating in the stroke-damaged brain may originate from the spleen.

Preconditioning to injury is a response to a sublethal alteration of an organism's environment resulting in an acquired resistance against subsequent lethal injuries (Kirino et al., 1996; Schaller and Graf, 2002). However, the removal of the spleen is not a preconditioning stimulus that ultimately reduces infarction size in the rat. The brains from animals that had a sham splenectomy and then underwent MCAO 2 weeks later exhibited the same amount of brain damage as MCAO-only rats, demonstrating that the splenectomy did not cause preconditioning. Moreover, white blood cell populations were stable during the 2-week recovery period before stroke.

After stroke, there is a depression of the immune system, often resulting in infection and subsequent death. The implication that targeting the spleen after stroke may enhance poststroke infection is not well supported by studies in the field. In a mouse transient MCAO model, poststroke immunodeficiency was caused by activation of the sympathetic nervous system and a systemic apoptotic loss of interferon gamma-secreting T lymphocytes (Prass et al., 2003). Bacterial colonization of the lung and blood occurred after 3 days, resulting in extensive mortality. This loss was seen in both the thymus and the spleen. Treatment with the beta blocker propranolol greatly reduced this effect. Thus, the immunosuppression seen after stroke appears to be a systemic rather than a local response, and therefore it is unlikely that therapies that target the spleen will promote post-stroke infection. Furthermore, our data suggest that treatments with HUCBC at delayed time points do not impair immune function (Vendrame et al., 2004).

Although this study focused on the cellular immune response, a previous study indicated that the expression of proinflammatory cytokines was increased in the spleen after MCAO (Vendrame et al., 2005). The administration of HUCBC at 24 hr after MCAO not only inhibited the increase in the proinflammatory response but significantly increased levels of the antiinflammatory cytokine, interleukin-10. In this current study, removal of this proinflammatory organ causes a significant reduction in the damage to the brain. These results demonstrate that the spleen is reacting to the stroke-induced brain injury, ultimately promoting neurodegeneration. Therefore, any treatments that cause similar effects as HUCBC to block the splenic response would be excellent candidates to expand the therapeutic window for stroke and be an effective therapy.

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Fig. 1. Histological pathology of infarct in brain tissue of splenectomized rats 96 hr after MCAO
a: Fluoro-Jade staining shows a large area of damage in the ipsilateral hemisphere in the MCAO only group (**center**), whereas splenectomy reduces Fluoro-Jade-labeled degenerating neurons (**right**) and is similar in staining to tissue from sham-operated animals (**left**). **b:** Thionin staining of Nissl in living neurons in sections from sham-operated animals (**left**), MCAO-only animals (**center**), and splenectomized rats that underwent MCAO (**right**). Although there was extensive damage in the MCAO-only group as determined by thionin staining (**center**), splenectomized rats (**right**) that were subjected to stroke show reduced loss of tissue (scale bar = 5 mm, a,b). When infarct size was measured, there was significantly less damage in the brain of splenectomized rats after MCAO than in the MCAO-only group as determined with both Fluoro-Jade (**c**; $*P < 0.001$) and thionin (**d**; $*P < 0.001$) histological procedures. Significance was determined by ANOVA followed by Bonferroni's post hoc test.

Fig. 2. Immunohistochemical labeling of microglia and infiltrating macrophages at the level of infarction

a: There were few isolectin IB4-labeled microglia or macrophages in brain sections from sham-operated rats. Arrows point to intact fasciolae of the striatum. **b:** Box denotes area labeled with isolectin IB4 in which activated microglia and macrophages are found. The arrow points to endothelial cells of a blood vessel labeled by isolectin IB4. **c:** Isolectin IB4 labeling of brain sections from splenectomized shows reduced microglia and macrophage labeling localized to a smaller area, as seen inside the box. Scale bar = 400 μ m.

Fig. 3. MPO staining of neutrophils in the infarct zone

a: Immunohistochemical labeling of brain sections for MPO shows no neutrophils in the striatum of the sham-treated rats. **b:** MPO staining in brain sections from rats treated with MCAO only show many neutrophils in the striatum and a disappearance of fasciolae. Arrows denote labeled neutrophils. **c:** Brain sections from rats treated with splenectomy before stroke also had intact fasciolae similar to the sham-treated animals, but unlike the MCAO-only animals, there were few neutrophils within the striatum. Scale bars = 200 μ m. **d:** MPO-positive neutrophils expressed as a function of infarct volume show a tendency for there to be more neutrophils in the MCAO group compared with the splenectomy–MCAO group. ANOVA followed by Bonferroni's post hoc test showed no significant difference between these groups ($P = 0.234$). **e:** The linear regression of the correlation of the percentage of infarct volume to number of MPO-positive cells shows that the number of neutrophils significantly increases as infarction volume increases ($R^2 = 0.6038$, $*P < 0.001$).

Fig. 4.

White blood cell profiles during treatments. White blood cell profiles were determined at 0, 48, and 96 hr after stroke for rats that underwent (a) MCAO only; (b) splenectomy followed 2 weeks later by MCAO; (c) sham MCAO; or (d) splenectomy and sham MCAO. e: White blood cell profiles were also determined during the 2-week recovery period between splenectomy and stroke. ANOVA followed by the post hoc Bonferroni's post hoc test showed no significant differences between the groups.