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Selective Ablation of Proliferating Microglial Cells Exacerbates Ischemic Injury in the Brain

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Here we report *in vivo* evidence of a neuroprotective role of proliferating microglial cells in cerebral ischemia. Using transgenic mice expressing a mutant thymidine kinase form of herpes simplex virus driven by myeloid-specific CD11b promoter and ganciclovir treatment as a tool, we selectively ablated proliferating (Mac-2 positive) microglia after transient middle cerebral artery occlusion. The series of experiments using green fluorescent protein-chimeric mice demonstrated that within the first 72 h after ischemic injury, the Mac-2 marker [unlike Iba1 (ionized calcium-binding adapter molecule 1)] was preferentially expressed by the resident microglia. Selective ablation of proliferating resident microglia was associated with a marked alteration in the temporal dynamics of proinflammatory cytokine expression, a significant increase in the size of infarction associated with a 2.7-fold increase in the number of apoptotic cells, predominantly neurons, and a 1.8-fold decrease in the levels of IGF-1. A double-immunofluorescence analysis revealed a ~100% colocalization between IGF-1 positive cells and Mac-2, a marker of activated/proliferating resident microglia. Conversely, stimulation of microglial proliferation after cerebral ischemia by M-CSF (macrophage colony stimulating factor) resulted in a 1.9-fold increase in IGF-1 levels and a significant increase of Mac2$^+$ cells. Our findings suggest that a postischemic proliferation of the resident microglial cells may serve as an important modulator of a brain inflammatory response. More importantly, our results revealed a marked neuroprotective potential of proliferating microglia serving as an endogenous pool of neurotrophic molecules such as IGF-1, which may open new therapeutic avenues in the treatment of stroke and other neurological disorders.

Key words: **glia; growth factor; ischemia; mice; neuroinflammation; neuroprotection; transgenic**

Introduction

There is increasing evidence that inflammation plays an important role in CNS ischemia. Experimentally and clinically, stroke is followed by an acute and a prolonged inflammatory response characterized by the production of inflammatory cytokines, leukocyte, and monocyte infiltration in the brain as well as the activation of resident glial cells, events that may contribute to ischemic brain injury (Dirnagl et al., 1999; Kempermann and Neumann, 2003; Lo et al., 2003).

Microglial cells are the main effectors of the innate response after CNS injuries, including ischemia. However, whether microglial activation has beneficial or detrimental effects on adjacent ischemic neurons remains controversial (Nguyen et al., 2002; Wyss-Coray and Mucke, 2002; Nedergaard and Dirnagl, 2005). There is substantial evidence demonstrating that activated microglia have the potential of releasing cytotoxic molecules including nitric oxide, reactive oxygen species, and toxic prostanoids (Gibson et al., 2005), as well as proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), which can affects neuronal functions and promote neurotoxicity (Dirnagl et al., 1999; Raivich et al., 1999a; Allan and Rothwell, 2001; Hanisch, 2002; Rogove et al., 2002). In addition, attenuation of brain inflammatory response and microglial activation has conferred neuroprotection in various models of neurodegeneration (Yrjanheikki et al., 1999; Tikka et al., 2001; Kriz et al., 2002, 2003). However, there is a growing line of evidence suggesting a neuroprotective role for microglia in CNS pathologies (Streit, 2002; Neumann et al., 2006). Previous studies demonstrated that the exogenous application of microglia protects against different types of ischemic injury (Kitamura et al., 2004, 2005; Imai et al., 2006) and oxygen-glucose deprivation (Neumann et al., 2006). However, at present, the molecular mechanisms underlying neuroprotection by microglial cells remain elusive.

Reactive microgliosis after ischemic brain injury is characterized by a stereotypical and rather graded response including a number of distinct features such as microglial activation followed by a massive expansion and migration of the resident microglial cell population and the recruitment of bone marrow-derived cells, which migrate into neuronal parenchyma and differentiate into microglia (Raivich et al., 1999b; Stoll and Jander, 1999; Priller et al., 2001; Ladeby et al., 2005). At present, it is still unclear whether the above-mentioned different "grades" of the microglial response to brain injuries are associated with the distinct functional roles. Therefore, the specific aim of this study was to investigate the functional role of microglial proliferation in re-

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sponse to ischemic injury. Using a mouse model for selective ablation of proliferating microglia, we demonstrated that selective ablation of resident microglia markedly alters the proinflammatory brain response and exacerbates ischemic injury. Moreover, our results revealed an important neuroprotective potential of proliferating microglial cells serving as an endogenous pool of neurotrophic and antiapoptotic molecules such as IGF-1.

Materials and Methods

Transgenic mice

The *CD11b-thymidine kinase mutant-30* ($TK^{mt\text{-}30}$) transgenic mouse is unique mouse model in which the herpes simplex virus type 1 (HSV-1) $TK^{mt\text{-}30}$ gene is expressed under the control of the CD11b gene promoter (Gowing et al., 2006). The strategy here is to treat the transgenic mice with ganciclovir to conditionally eliminate proliferating microglia and macrophages at selected time points before and/or after stroke. The CD11b-$TK^{mt\text{-}30}$ transgenic mice were genotyped by PCR with *Thermus aquaticus* DNA polymerase (Amersham, Piscataway, NJ) in 20 mM $MgCl_2$ PCR buffer with the following primers: 5′-CCCCTGCCATCAACACGCGTCTGC and 5′ GCGTCGGTCACGGCATAAGGC (position 12–35 and 412–390, respectively). The PCR conditions were as follows: 94°C, 2 min, 34 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s); 72°C, 10 min. All experimental procedures were approved by the Laval University animal care ethics committee and are in accordance with *The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.*

Chimeric mice

As described previously (Simard et al., 2006), a group of wild-type (WT) mice were exposed to a total-body irradiation (10 gray) using a cobalt-60 source (Theratron-780 model; MDS Nordion, Ottawa, Ontario, Canada). A few hours later, the animals were injected via a tail vein with $\sim 5 \times 10^6$ bone marrow cells freshly collected from mice expressing green fluorescent protein (GFP) under control of β-actin promoter. The cells were aseptically harvested by flushing femurs with Dulbecco's PBS (DPBS) containing 2% fetal bovine serum. The samples were combined, filtered through a 40 mm nylon mesh, centrifuged, and passed through a 25 gauge needle. Recovered cells were resuspended in DPBS at a concentration of 5×10^6 viable nucleated cells per 200 ml. Irradiated mice transplanted with this suspension were housed in autoclaved cages and treated with antibiotics (0.2 mg of trimethoprim and 1 mg of sulfamethoxazole/ml of drinking water given for 7 d before and 2 weeks after irradiation). Intraluminal filament occlusion of the left middle cerebral artery occlusion (MCAO) surgery was performed surgery 2–4 months after transplantation bone marrow cells.

Surgical procedures

Unilateral transient focal cerebral ischemia was induced by MCAO during 1 h followed by 24 or 72 h reperfusion periods (Belayev et al., 1999; Beaulieu et al., 2002). The MCAO was performed on 2- to 3-month-old male CD11b-TK^{mt30} transgenic mice, their WT littermates, and chimeric mice (20–25 g). The animals were anesthetized with ketamine/xylazine 100/20 mg/kg (i.p.). To avoid cooling, the body temperature was regularly checked and maintained at 37°C with an infrared heating lamp and a heating pad. Under an operating microscope, the left common carotid artery and ipsilateral external carotid artery (ECA) were exposed through a midline neck incision and were carefully isolated from surrounding tissue. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent tissue and a 12-mm-long 6–0 silicon-coated monofilament suture was inserted via the proximal ECA into the ICA and then into the circle of Willis, thus, occluding the MCA. The size of the infarct was estimated using 2% solution of 2,3,5 triphenyltetrazolium chloride (TTC) (Sigma, Oakville, Ontario, Canada) vital die, dissolved in saline and stained for 20 min at 37°C in the dark. The relative size of the infarction was measured by using the Scion (Frederick, MD) Image-processing and analysis program, calculated in arbitrary units (pixels) and expressed as a percentage of the control, nonstroked area in the contralateral nonischemic hemisphere (100%) for each section (Weng and Kriz, 2007). All animals were allowed *ad libitum* access to water and food before and after surgery.

Treatment and experimental groups

To conditionally ablate early phase microglia/macrophage proliferation, ganciclovir (GCV; Cytovene; Roche, Mississauga, Ontario, Canada) was injected twice per day (100 mg/kg), intraperitoneally, starting 48 h before MCAO and mice were continuously treated with GCV for 24–72 h after surgery. Transgenic mice and their WT littermates ($n = 4$–9 per group) were injected with GCV for all experiments. Twenty-four hours after MCAO, all animals are tested for neurological deficits associated with the focal cerebral ischemia. Only the animal that expressed a "positive phenotype" such as circulating behavior, slight motor deficits of the contralateral front paw, reduced spontaneous activity, etc., were selected for the study. To stimulate proliferating responses of microglia in WT littermates, recombinant mouse macrophage colony stimulating factor (M-CSF) (Cedarlane, Hornby, ONT, Canada) was injected once per day (150 μg/kg/d, i.p.) for 3 d starting 8 h after transient MCAO. Controls were injected with saline ($n = 2$).

BrdU labeling

To visualize dividing cells, mice were injected intraperitoneally two times with bromodeoxyuridine (BrdU) (50 μg/g mouse weight in saline) 24 h before being killed. Mice were anesthetized and transcardially perfused with PBS and 4% paraformaldehyde (PFA). The brains were removed and postfixed overnight in 4% PFA. All brain samples were prepared for immunohistochemical manipulation.

Tissue collection

The animals were anesthetized via an intraperitoneal injection of chloral hydrate (10 mg/ml) and transcardially perfused with 30 ml of 0.9% NaCl, followed by ice-cold borax buffered 4% paraformaldehyde at pH 9.5 (for *in situ* hybridization) or PBS 1× buffered 4% PFA at pH 7.4 (for immunohistochemistry). Tissue samples were then postfixed overnight in 4% PFA and equilibrated in phosphate-buffered 20% sucrose/4% PFA for 48 h. Brains were embedded into Tissue-Tek (O.C.T. compound; Sakura, Torrance, CA), frozen at −80°C overnight, and cut into coronal sections (35 μm thick) with a Cryostat and stored at −20°C.

In situ *hybridization*

The expression and localization of mRNA encoding for IκBα, IL-1β, IL-6, and TNF-α was detected on every sixth section of the stroke area of the brain using ^{35}S-labeled riboprobes. The riboprobes used for this experiment were graciously provided by Dr. S. Rivest (Laval University, Quebec, Canada). Protocols for probe synthesis and *in situ* hybridization were described previously by Laflamme and Rivest (1999). Briefly, slides were dried out under vacuum overnight, postfixed in 4% paraformaldehyde, and digested by proteinase K (10 μg/ml in 0.1 M Tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0, at 37°C for 25 min), after which the brain sections were rinsed in water and by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated. The hybridization of the brains sections by the riboprobe involves 10^7 cpm/ml/slide of hybridization mixture and incubation at 60°C overnight in a slide warmer. Slides were rinsed in standard saline citrate (1× SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) and digested by RNase A at 37°C (20 μg/ml), rinsed in descending concentrations of SSC, washed in 0.1× SSC, and dehydrated through graded concentrations of ethanol. The sections were exposed to x-ray film (Kodak, Rochester, NY) for 18–24 h and dipped in NTB2 nuclear emulsion (diluted 1:1 with distilled water; Kodak). Slides were kept at 4°C for 2 weeks safe from light, developed in D19 developer (Kodak) at 14°C, washed in water and fixed in rapid fixer (Kodak). After exposition, tissues were rinsed in distilled water for 45 min and counterstained with thionin (0.25%).

Immunohistochemistry

Immunohistochemistry was performed according to the following procedures. Brain sections were blocked in TBS containing 5% goat serum and 0.25% Triton X-100. Attenuation of endogenous peroxidases was done by incubation in 0.6% hydrogen peroxide. Using the same buffer

solution, the sections were then incubated overnight at room temperature in primary antibody [rabbit polyclonal anti-Iba1 (ionized calcium-binding adapter molecule 1; Wako, Richmond, VA), rat anti-Mac-2 (American Type Culture Collection, Manassas, VA), rat polyclonal anti-BrdU (Axyll, Westbury, NY) and rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling, Danvers, MA)] (Kriz et al., 2002, 2003). Incubation for 2 h at room temperature in corresponding biotinylated goat secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA) was performed. For the amplification of the positive signal, incubation in a complex of avidin-biotine solution (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) was performed. Staining was developed for in nickel-DAB solution (0.3%) or in peroxidase substrate kit Vector SG (Vector Laboratories). Each of the above steps was followed by four 5 min rinses in TBS-0.25% Triton X-100. The sections were then dehydrated and coverslipped with DPX (a mixture of distyrene, tricresyl phosphate, and xylene; Electron Microscopy Sciences, Fort Washington, PA).

Double immunofluorescence

Brain sections were blocked in TBS containing 5% goat serum and 0.20% Triton X-100 for 30 min. Using the same buffer solution, the sections were then incubated overnight at room temperature in primary antibodies [rat polyclonal anti-BrdU (Axyll), mouse monoclonal anti-NeuN (Millipore, Temecula, CA), mouse monoclonal anti-GFAP (Sigma), rabbit polyclonal anti-Iba1 (Wako), rat polyclonal anti-Mac-2 (American Type Culture Collection), rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling), mouse anti-IGF-1 (Upstate, Lake Placid, NY), and chicken polyclonal anti-BDNF (Promega, Madison, WI)]. After wash in TBS, the sections were incubated in corresponding fluorescent goat or chicken secondary antiserum (Invitrogen, Eugene, OR). The sections were coverslipped with PVA/DABCO (a mixture of glycerol, alcohol polyvinyle, tris-HCl, diazabicyclooctane and H_2O) after wash in TBS.

Cytokine array

The protein expression analysis of inflammatory cytokines was performed with a mouse antibody array (RaybioMouse inflammation antibody array 1.1; catalog #AAM-INF-1L; RayBiotech, Norcross, GA). Protein lysates were obtained by homogenization of brains of control and transgenic mice in 1× cell lysis buffer (included in the RayBiotech kit) with protease-inhibitor mixture (#P8340; Sigma). The protein concentration was determined for each sample and diluted at 500 μg in 1× blocking buffer. Samples for each group (three mice/group) were pooled and incubated with the array membrane overnight at 4°C. After washes, the membranes were incubated with the biotin-conjugated antibodies overnight at 4°C. The membranes were then processed according to RayBiotech protocol. Membranes were exposed to x-ray film (Biomax MR1; #8701302; Kodak) and analyzed by Agfa (Kontich, Belgium) Arcus II system and ImageJ software.

Quantification and statistical analysis

Immunohistochemistry and immunofluorescence. Every fourth sample section through the brain was used for experiments. The density of labeled cells (brown–black staining) was estimated by the optical fractionator method using Stereo Investigator software. The ipsilateral (left) hemisphere was traced using a 2 Plan Apochromat objective and sampled using a 40 Plan Apochromat objective (Nikon, El Segundo, CA). The counting parameters were the distance between counting frames (100 μm), the counting frame size (400 × 400 μm), the disector height (10 μm), and the guard-zone thickness (1.5 μm). For the immunohistochemistry (Iba1, Mac-2, BrdU, and cleaved caspase-3) experiments on brain sections after MCAO, an area with a 300 μm radius was traced surrounding the wound region and cavities were excluded from the positive cell count. The data were averaged and analyzed by a two-tailed unpaired Student's *t* test for data in Figures 2 and 7. IGF-1 immunoreactivity was quantified with the MetaMorph Imaging System by measuring the intensity of fluorescence per unit of surface area (arbitrary units). Ten sections per mouse were used for this analysis. The data were averaged and analyzed by a Mann–Whitney test for the data Figure 8.

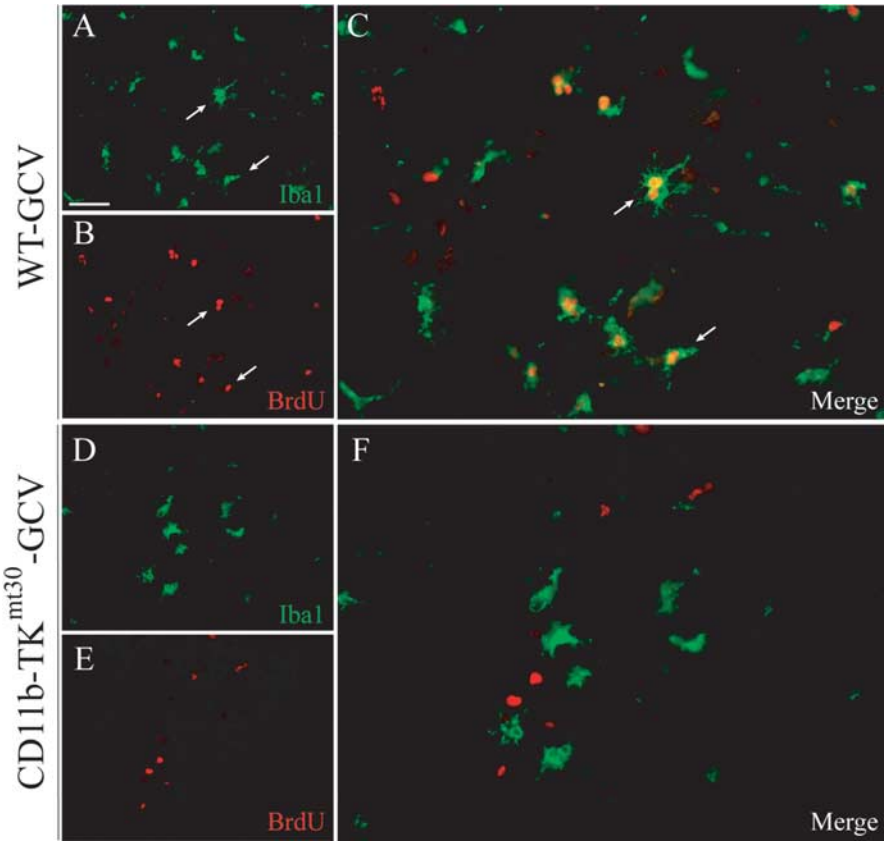

Figure 1. Conditional ablation of early microglia/macrophage proliferation in CD11b-TK ^{mt30} transgenic mice. ***A–F***, Representative double-immunofluorescence images of Iba1 (green) and BrdU (red) in WT (***A–C***) and transgenic mice (***D–F***) treated with GCV 72 h after transient MCAO. No colocalization was detected for BrdU and Iba1 staining in CD11b-TK ^{mt30}-GCV-treated mice. Scale bar, 50 μm.

In situ hybridization. Every sixth sample section through the brain was used for experiments. The density of positive cells (silver grain presence) was estimated by the optical fractionator method using Stereo Investigator software. The ipsilateral (left) hemisphere was traced using a 2 Plan Apochromat objective and sampled using a 40 Plan Apochromat objective. The counting parameters for the experiments with IL-1β, IL-6, and TNF-α were the distance between counting frames (120 μm), the counting frame size (300 × 300 μm), the disector height (10 μm), and the guard zone thickness (1.5 μm). The counting parameters for the experiments with IκBα were the distance between counting frames (120 μm), the counting frame size (500 × 500 μm), the disector height (10 μm), and the guard-zone thickness (1.5 μm). Counts were done on the entire ipsilateral hemisphere. Cells were counted only if their nuclei lay within the dissector area, did not intersect forbidden lines, and came into the focus as the optical plane moved through the height of the dissector. The data were averaged and analyzed by a two-tailed unpaired Student's *t* test for data in Figures 4–6.

Protein expression analysis. Cytokine expressions were obtained by measuring the optic density of each protein spot on the membrane with ImageJ software. Each cytokine was presented in duplicate and the anal-

ysis was performed twice. The background was subtracted from values of cytokines expression. The data were averaged and analyzed by a two-tailed unpaired Student's *t* test.

Results

Selective ablation of proliferating microglia by ganciclovir treatment

An important component of the postischemic inflammatory response in the brain is a marked expansion of the microglial cell population peaking a few days after injury. The functional role of such a cellular response is still not completely understood. To closer investigate the role of proliferating microglia in the pathobiology of ischemic injury, we took the advantage of the CD11b-TK^{mt-30} mouse model in which the selective elimination of proliferating myeloid cells can be achieved (Gowing et al., 2006). In this mouse model, HSV-1 TK is expressed under the control of CD11b, a myeloid-specific promoter, thus, treatment with GCV specifically ablates proliferating CD11b-positive cells (i.e., microglia/macrophages) without affecting the quiescent cell population. To test the efficiency of our model system in the context of ischemic injury, the GCV-treated transgenic mice and the WT littermates were injected with BrdU, a nucleotide analog. Double immunofluorescence for BrdU and Iba1, markers of cell proliferation and microglia/macrophages, respectively, 72 h after MCAO demonstrated colocalization of both markers in the brain sections of WT littermates (Fig. 1*A–C*). However, no colocalization between BrdU and Iba1was detected in the brains of GCV-treated CD11b-TK^{mt30} mice (Fig. 1*D–F*). Importantly, administration of GCV before MCAO did not affect the population of quiescent microglia in other brain regions. Because the administration of GCV effectively ablated proliferation of the Iba1-immunoreactive cells after brain ischemia in CD11b-TK^{mt30} mice, we decided to analyze glial responses in CD11b-TK^{mt30} mice by using two different markers: Iba1, a common marker for cells of myeloid origin, and Mac-2, a marker of microglia/macrophages activation (Dong and Hughes, 1997; Kriz et al., 2002, 2003). Because microglial proliferation induced by brain injuries normally peaks 48–72 h after injury, the administration of GCV in transgenic mice did not significantly affect the number of Iba1- and Mac-2-positive cells 24 h after injury (Fig. 2*B–E*). Immunohistological analysis of brain sections 72 h after transient MCAO revealed a marked increase in Iba1 and Mac-2 immunoreactivities in the ischemic, ipsilateral side of the brain in both experimental groups (Fig. 2*F–I*). Consistent with previous reports (Dirnagl et al., 1999; Stoll and Jander, 1999; Ladeby et al., 2005), microglial cells revealing the activated (amoeboid) phenotype were detected in the areas within the ischemic lesion and in large numbers in the peri-infarct zone (Fig. 2). In contrast, immunostaining of the contralateral, nonischemic hemisphere showed no immunoreactivity for Mac-2, whereas Iba1-positive cells displayed ramified, nonactivated morphology (data not shown). Quantitative analysis of Iba1 staining indicated a ~40% decrease in the number of Iba1 immunoreactive cells in transgenic mice treated with GCV compared with the WT littermates (WT, $10{,}220 \times 10^3 \pm 775.8 \times 10^3$, $n = 5$; CD11b-TK^{mt30}, $6134 \times 10^3 \pm 487.9 \times 10^3$, $n = 4$; $p = 0.0042$). To our surprise, quantification of Mac-2-immunoreactive cells indicated a reduction of ~65% (WT, $9404 \times 10^3 \pm 1060 \times 10^3$, $n = 4$; CD11b-TK^{mt30}, 3277 ± 491.9, $n = 4$; $p = 0.0019$) (Fig. 2*J*).

Mac-2 is preferentially expressed by resident microglia

Microglia are activated after cerebral ischemia and by transforming to round amoeboid cells they become indistinguishable from

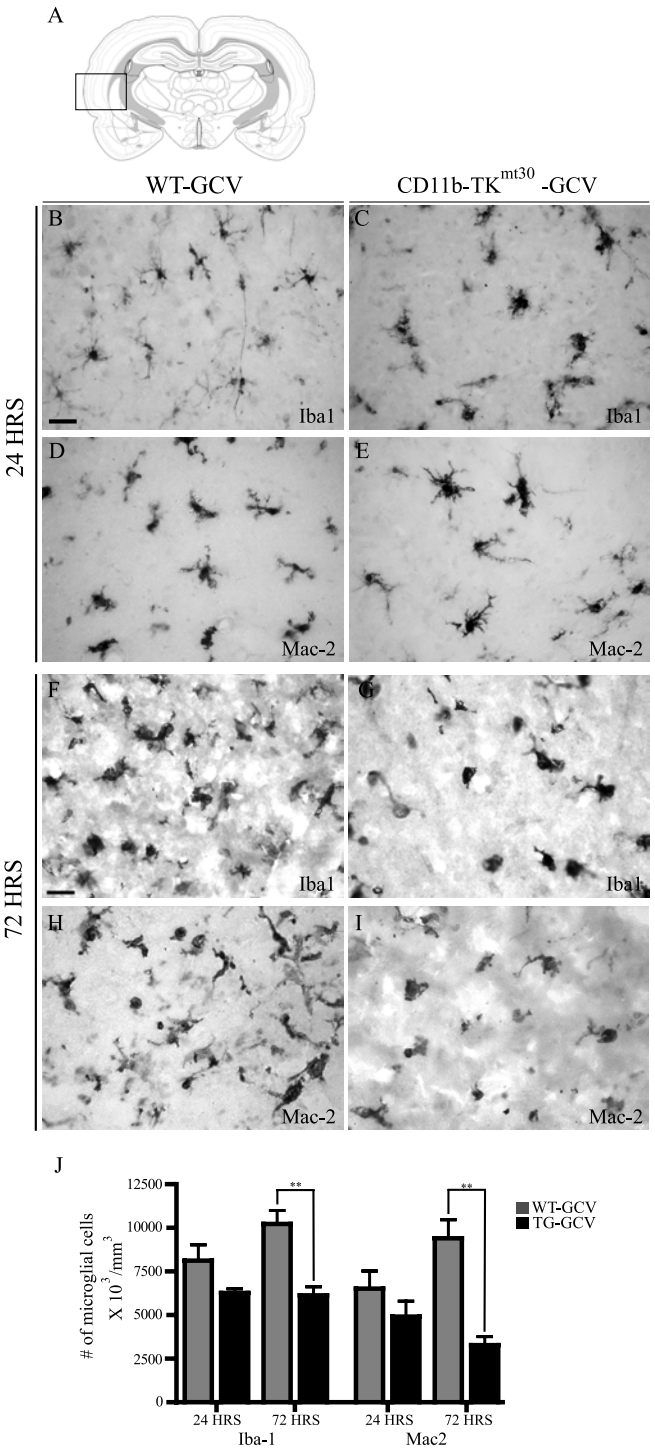

Figure 2. Microglia/macrophages in CD11b-TK^{mt30}-GCV-treated mice. ***A***, Schematic representation of the brain section used for the immunostaining experiment. Box represents the cortex area magnified in ***B–I***. ***B–E***, Photomicrographs of immunoreactivities for Iba1 (***B***, ***C***) and Mac-2 (***D***, ***E***) in WT and transgenic mice treated with GCV 24 h after transient MCAO. ***F–I***, Photomicrographs of immunoreactivities for Iba1 (***F***, ***G***) and Mac-2 (***H***, ***I***) in WT and transgenic mice treated with GCV 72 h after transient MCAO. ***J***, No significant change was detected in the total number of microglia/macrophages (Iba1 staining) and in the number of activated microglia/macrophages (Mac-2 staining) in CD11b-TK^{mt30}-GCV-treated mice compared with the WT-GCV control group (Iba1, $n = 4$, $p = 0.2507$; Mac-2, $n = 4$, $p = 0.0756$). A decrease of ~40% was detected in the total number of microglia/macrophages (Iba1 staining) in CD11b-TK^{mt30}-GCV-treated mice compared with the WT-GCV controls group ($n = 4–5$; $^{**}p = 0.0042$). A decrease of ~65% was observed in the number of activated microglia/macrophages (Mac-2 staining) in transgenic mice treated with GCV compared with the WT-GCV. Data are expressed as mean ± SEM ($n = 4$; $^{**}p = 0.0019$). Scale bars, 50 μm.

hematogenous macrophages morphologically based on the expression of immunocytochemical markers. Because in our experiments we were preferentially targeting Mac2$^+$ cells, the above results raised an important question: is Mac-2 a selective early marker for the subset of proliferating resident microglial cells or it is a nonselective marker for the proliferating cells of myeloid origin including peripheral macrophages? To address this question we generated a series of chimeric mice by lethal irradiation and transplantation of bone marrow cells expressing GFP under control of β-actin promoter (Simard et al., 2006). As described previously (Priller et al., 2001; Simard et al., 2006) in these mice, the peripheral bone-marrow-derived cells of myeloid origin would be labeled with GFP and therefore easily distinguishable from the resident microglial cell population. As demonstrated in Figure 3, a massive infiltration of GFP-positive cells occurs in the ischemic side of the brains of chimeric mice 72 h after transient MCAO. To determine whether the infiltrating cell population express Mac-2 and/or Iba1, we performed double immunofluorescence for GFP and Mac-2 or GFP and Iba1. A great majority of GFP-positive cells were immunoreactive for Iba1 (Fig. 3*D–F*). However, double labeling revealed no colocalization between GFP and Mac-2 immunoreactivities in the brains of stroked chimeric mice (Fig. 3*A–C*), thus suggesting that, within the first 72 h after ischemic injury, the cells that preferentially express the Mac-2 marker and proliferate are the resident microglial cells.

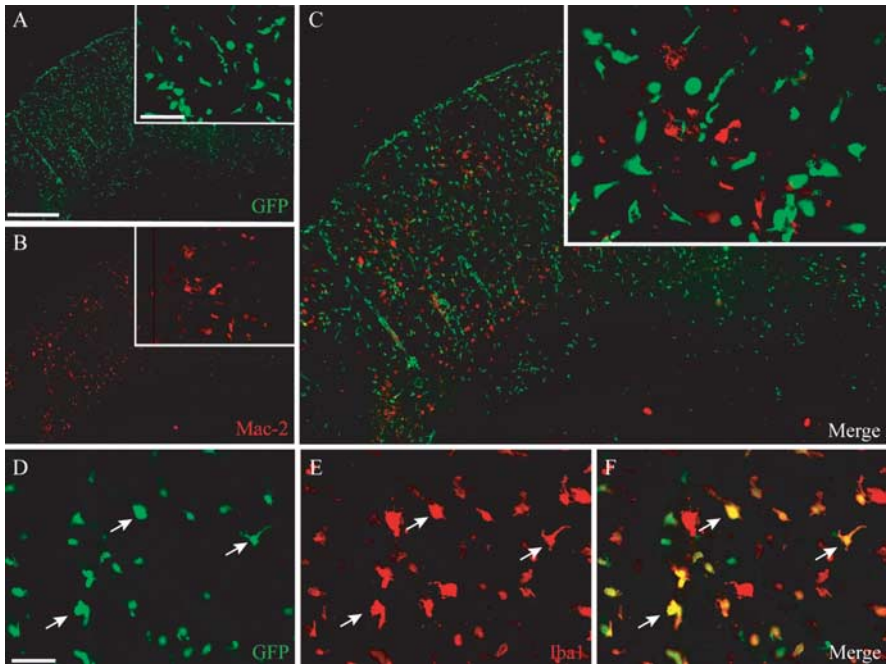

Figure 3. Mac-2$^+$ cells do not colocalize with GFP bone marrow-derived microglia/macrophages in chimeric mice. ***A–C***, No colocalization between Mac-2 and GFP could be found 72 h after transient MCAO in chimeric mice by immunofluorescence. ***D–F***, Majority of infiltrating bone marrow GFP cells colocalize with the marker of microglia/macrophages Iba1 by double immunofluorescence in chimeric mice (white arrows). Scale bars: ***A***, 500 μm; ***A***, inset, ***D***, 50 μm.

Microglial proliferation modulates secretion of proinflammatory cytokines

Previous studies demonstrated that inflammatory mediators such as nuclear factor κB (NF-κB), TNF-α, IL-1β, and IL-6 may influence the extent of neurodegeneration after cerebral ischemia (Dirnagl et al., 1999; Lo et al., 2003). At present, it is not known whether posttraumatic microglial cell proliferation has a role in the regulation of the proinflammatory molecule release. We therefore analyzed temporal pattern of expression and quantified hybridization signals for IκBα, TNF-α, IL-1β, and IL-6. Because NF-κB is implicated in the regulation of several inflammatory pathways, we first analyzed the expression pattern of *in situ* mRNA signals for IκBα (index of NF-κB activation) (Clemens et al., 1997; Laflamme and Rivest, 1999). As shown in Figure 4*F*, no difference in hybridization signals for IκBα was detected 24 h postinjury (WT, 3104 ± 592.9, $n = 4$; CD11b-TK^{mt30}, 2583 ± 684.6, $n = 4$; $p = 0.5862$) (Fig. 4*B*,*C*). However, a twofold increase in IκBα mRNA levels (Fig. 4*F*) was detected in the brain sections of transgenic mice treated with GCV 72 h post-MCAO (WT, 2151 ± 238.1, $n = 4$; CD11b-TK^{mt30}, 4360 ± 169.2, $n = 4$; $p = 0.0003$) (Fig. 4*D*,*E*).

Further, we compared mRNA expression levels for IL-1β, IL-6, and TNF-α using the same time-points, 24 and 72 h after stroke. Normally, transcriptional activation of the genes encoding for the above-mentioned proinflammatory cytokines peaks at 12–24 h after cerebral ischemia (Hill et al., 1999; Offner et al., 2006). As shown in Figure 5, analysis of *in situ* mRNA signals 24 h after MCAO revealed no significant differences in the mRNA levels of IL-1β or TNF-α at this time point (IL-1β: WT, 420.3 ± 58.86, $n = 5$; CD11b-TK^{mt30}, 246.1 ± 40.25, $n = 4$; $p = 0.0545$; TNF-α: WT, 753.4 ± 87.99, $n = 5$; CD11b-TK^{mt30}, 909.8 ± 49.56, $n = 3$; $p = 0.2498$) (Fig. 5*A*,*B*,*E*,*F*). Interestingly, we detected a 1.45-fold decrease in the signal for IL-6 in transgenic brains compared with controls (WT, 1343 ± 101.8, $n = 7$; CD11b-TK^{mt30}, 924.6 ± 76.41, $n = 7$; $p = 0.0065$) (Fig. 5*C*,*D*). However, contrary to a marked decline in mRNA levels of proinflammatory cytokines in the brain section of WT littermates detected 72 h after ischemic injury (Fig. 6), a general increase in proinflammatory signaling occurred in the brains of CD11b-TK^{mt30}-GCV-treated mice. Indeed, we observed a 2.7-fold increase in the number of IL-6 positive cells (WT, 634.7 ± 129.0, $n = 4$; CD11b-TK^{mt30}, 1716 ± 169.8, $n = 5$; $p = 0.0019$) and a 2.15-fold increase in the number of IL-1β positive cells (WT, 200.6 ± 49.35, $n = 4$; CD11b-TK^{mt30}, 431.2 ± 26.96, $n = 4$; $p = 0.0064$), as well as a 2.6-fold increase in the number of cells expressing TNF-α in transgenic mice treated with GCV, compared with controls (WT, 389.0 ± 52.96, $n = 3$; CD11b-TK^{mt30}, 998.8 ± 51.66, $n = 4$; $p = 0.0005$) (Fig. 6*H*). Importantly, the increase in mRNA *in situ* transcripts correlated with an increase in proinflammatory cytokines protein levels. In fact, 72 h after transient MCAO, we observed a significant increase in IL-6 (WT, 18.25 ± 4.589, $n = 4$; CD11b-TK^{mt30}, 70.50 ± 8.742, $n = 4$; $p = 0.0018$) and TNF-α (WT, 52.25 ± 2.898, $n = 4$; CD11b-TK^{mt30}, 78.75 ± 3.326, $n = 4$; $p = 0.0010$) (Fig. 6*G*), and the tendency for IL-1 (WT, 27.50 ± 3.571, $n = 4$; CD11b-TK^{mt30}, 28.50 ± 4.992, $n = 4$; $p = 0.8759$). Note that we detected only few hundred cells that expressed IL-1β mRNA; therefore, the sensitivity of the cytokine array may have not been sufficient to detect significant difference (Fig. 6*G*,*H*). Altogether, these results suggest a marked alteration in the dynamics of the proinflammatory response after

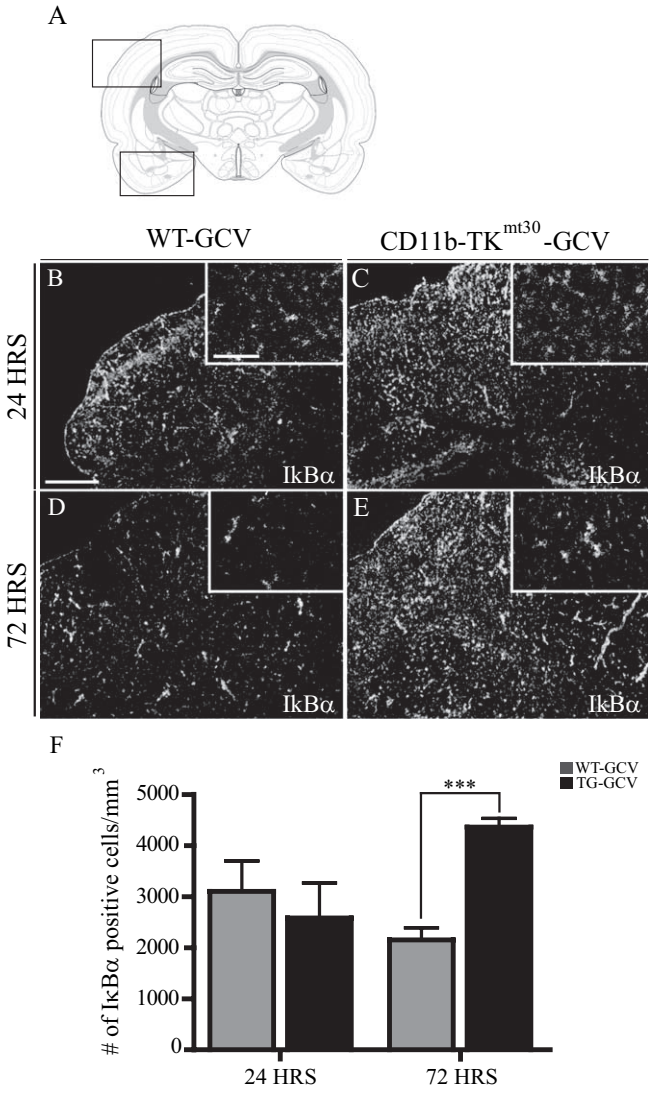

Figure 4. Alteration in the level of IκBα mRNA 72 h after transient MCAO. ***A***, Schematic representation of the brain section used for the *in situ* hybridization experiments. Boxes represent the cortex area magnified in ***B–E*** and in Figures 5 and 6. ***B–E***, Photomicrographs of *in situ* hybridization of IκBα mRNA in WT and transgenic mice treated with GCV 24 h (***B***, ***C***) and 72 h (***D***, ***E***) postinjury. ***F***, Quantification of positive cells in the ipsilateral hemisphere did not reveal any significant changes in transgenic mice compared with the WT mice treated with GCV. Values indicate mean ± SEM ($n = 4$; $p = 0.5862$). At 72 h, an increase in the number of IκBα$^+$ cells in transgenic mice treated with GCV was found compared with the WT-GCV group ($n = 4$; ***$p = 0.0003$). Scale bars: ***B***, 500 μm; ***B***, inset, 50 μm.

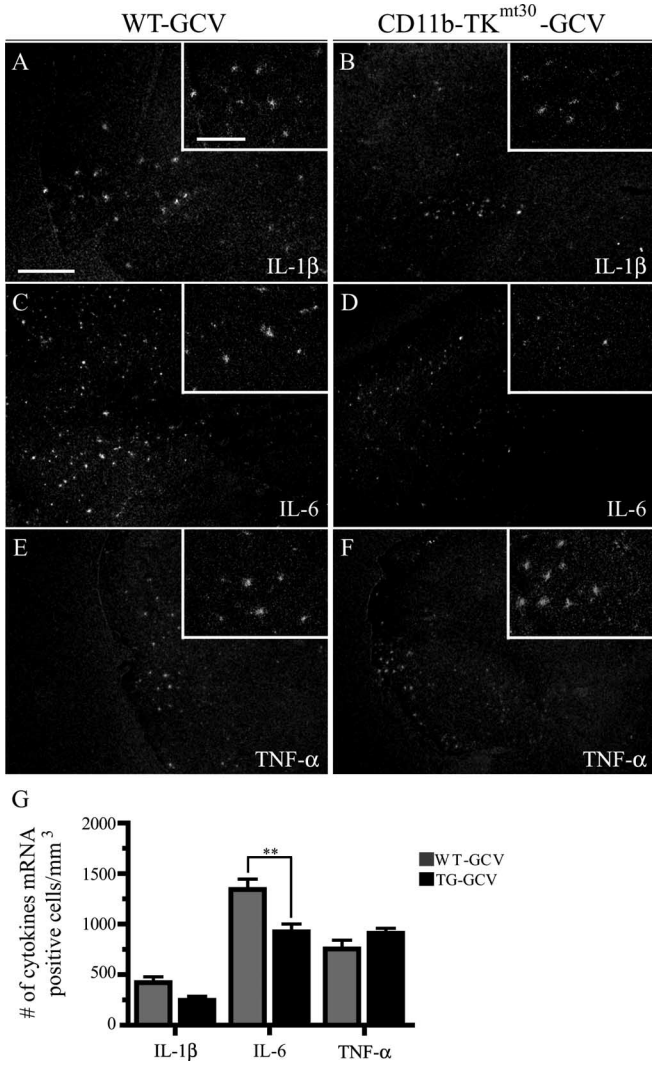

Figure 5. Expression of inflammatory markers 24 h after transient MCAO. ***A–F***, Photomicrographs of *in situ* hybridization for IL-1β (***A***, ***B***), IL-6 (***C***, ***D***), and TNF-α (***E***, ***F***) mRNA in WT and transgenic mice treated with GCV 24 h postinjury. ***G***, No significant change in the number of Il-1 β^+ and TNF-α^+ cells in transgenic mice treated with GCV compared with the WT-GCV treated mice was observed. Data are expressed as mean ± SEM (Il-1 β, $n = 4$, $p = 0.0545$; TNF-α, $n = 3–5$, $p = 0.2498$). An increase was detected in the number of IL-6$^+$ cells in CD11b-TK^{mt30}-GCV compared with the WT-GCV ($n = 7$; **$p = 0.0065$). Scale bars: ***A***, 500 μm; ***A***, inset, 50 μm.

cerebral ischemia in the brains of CD11b-TK^{mt30} mice treated with GCV. Hence, microglial proliferation normally peaking 2–3 d after injury may serve as an important modulator (moderator) of the brain inflammatory response.

Selective ablation of proliferating microglia increases the size of ischemic lesion and number of apoptotic neurons

At present, it is not exactly clear whether or to what extent injury-induced microglial cell proliferation can directly or indirectly affect the survival of adjacent neurons. To address this important question, we first analyzed the size of ischemic lesions in GCV-treated CD11b-TK^{mt-30} mice and controls. As shown in Figure 7, *A* and *B*, the analysis of the TTC staining revealed that the ablation of proliferating microglia after transient MCAO in transgenic mice resulted in a ~13% (WT, 42.98 ± 2.932, $n = 9$; CD11b-TK^{mt30}, 55.44 ± 3.024, $n = 7$; $p = 0.0112$) increase (relative increase of 31%) in the area of infarct 72 h postinjury compared with control WT littermates treated with the nucleotide analog. Importantly, as demonstrated by immunohistochemistry for cleaved caspase-3, the increase in the size of the ischemic lesion was associated with a 2.7-fold increase in the number of apoptotic cells (Fig. 7*C*,*D*). In addition, double labeling for neuronal marker NeuN and cleaved caspase-3 indicated an increase in the number of apoptotic neurons in transgenic animals treated with GCV when compared with controls (Fig. 7*E–J*). Hence, an increase in the infarct area "correlates" with the increase in the number of apoptotic neurons. Note that an increase in caspase-3-positive staining in microglial cells was also detected (Fig. 7*N–P*). Indeed, transgene-derived expression of HSV-1 TK^{mt-30} by microglia sensitizes this cell population to cell death induced by GCV (Black et al., 1996; Gowing et al., 2006). Here, it is important to mention that previous work by Gowing et al. (2006) demon-

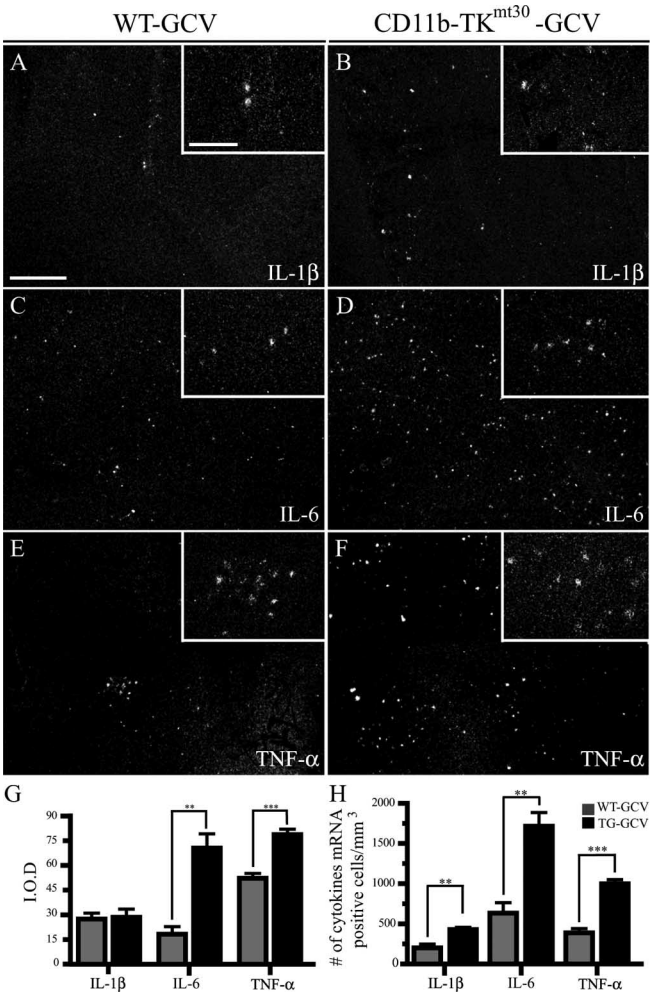

Figure 6. Increase in proinflammatory cytokines 72 h after transient MCAO. ***A–F***, Photomicrographs of *in situ* hybridization for IL-1β (***A***, ***B***), IL-6 (***C***, ***D***), and TNF-α (***E***, ***F***) mRNA in WT and transgenic mice treated with GCV 72 h postinjury. ***G***, Increase in protein levels of proinflammatory cytokines 72 h after transient MCAO. Brain lysates were generated and the presence of key inflammatory cytokine was determined using a cytokines array (RayBiotech). An increase in the protein amount of IL-6 and TNF-α was detected in CD11b-TK^{mt30} mice compared with the WT GCV-treated littermates. No significant change was observed in IL-1β protein expression in transgenic mice treated with ganciclovir compared with the WT GCV-treated group (IL-1β, $n = 4$, $p = 0.8759$; IL-6, $n = 4$, $^{**}p = 0.0018$; TNF-α, $n = 4$, $^{***}p = 0.0010$). ***H***, Significant increases of IL-1β^+, IL-6$^+$, and TNF-α^+ cells were detected in transgenic GCV treated mice compared with the WT-GCV treated mice (IL-1β, $n = 4$, $^{**}p = 0.0064$; IL-6, $n = 5$, $^{**}p = 0.0019$; TNF-α, $n = 4$, $^{***}p = 0.0005$). Data are expressed as mean ± SEM. Scale bars: ***A***, 500 μm; ***A***, inset, 50 μm.

strated no toxic side effects of the short-term GCV treatment or the transgene itself on neuronal population in the CD11b-TK^{mt30} mice. No colocalization of cleaved caspase-3 with the astrocytes marker GFAP was observed in the brain sections of CD11b-TK^{mt30} treated with GCV (data not shown).

Proliferating microglial cells are an important source of IGF-1

Previous findings demonstrated that once activated or in proliferation, microglial cells can produce neurotrophic factors such as BDNF and/or IGF-1 (Miwa et al., 1997; Batchelor et al., 1999; O'Donnell et al., 2002; Nakajima and Kohsaka, 2004). We therefore hypothesized that neurotrophic factors provided by microglial cells may potentially serve as an important source of trophic support for adjacent ischemic neurons. Indeed, immunohistochemical analysis demonstrated an induction of IGF-1 selectively in the area surrounding ischemic lesion (Fig. 8*A–C*) (contralateral side of the brain was devoid of any IGF-1 immunoreactivity) (data not shown). Moreover, a double fluorescent labeling revealed a high level (~100%) of colocalization between IGF-1-positive cells and Mac-2, a marker of activated/proliferating microglia (Fig. 8*A–I*). As further shown in Figure 8*J*, the quantitative analysis of immunofluorescent labeling 72 h after MCAO showed a 1.8-fold decrease in the levels of IGF-1 [control (CTL), 80.13 ± 2.92, $n = 4$; CD11b-TK^{mt30}, 36.54 ± 2.31, $n = 4$; $p < 0.0001$] in the brain sections of transgenic animals treated with GCV compared with the WT littermates treated with GCV.

To obtain the functional proof-of-principle for our hypothesis that proliferating microglia synthesize and secrete trophic factors like IGF-1 and, thus, protect adjacent neurons after injuries, we additionally stimulated ischemia-induced microglial proliferation in WT-littermate mice by injecting M-CSF, an important mitogen for microglial cells (Yoshida et al., 1990; Raivich et al., 1994). Immunohistological analysis revealed a significant increase in the number of proliferating cells (BrdU; CTL, 30,550 ± 350.0; M-CSF, 36 050 ± 150.0; $n = 2$; $p = 0.0048$) associated with a significant increase in the number of Mac-2$^+$ cells (CTL, 10,650 ± 1493; M-CSF, 31,750 ± 13570; $n = 2$; $p = 0.0391$) (Fig. 8*K–M*). Furthermore, the quantitative analysis of immunofluorescent labeling 72 h after MCAO showed a 1.9-fold increase in the levels of IGF-1 in M-CSF-treated mice compared with the saline-injected controls (CTL, 80.13 ± 2,92; M-CSF, 147.9 ± 8.099; $p < 0.0001$) (Fig. 8*J*). As shown in Figure 8*G–I*, a colocalization was observed between Mac-2- and IGF-1-positive cells. These two combined results clearly show that the proliferative response of microglia is important for the production of trophic factors.

Contrary to alterations in the expression levels of IGF-1 in our *in vivo* experiments, 72 h after cerebral ischemia we did not observed detectable changes in the levels of BDNF between our experimental groups. In addition, the double-labeling experiments revealed that 72 h after transient MCAO, BDNF was preferentially expressed in neurons (data not shown).

Discussion

The work presented here provides important *in vivo* evidence of a neuroprotective role of proliferating resident microglial cells in cerebral ischemia. As revealed by Mac-2 staining and the experiments performed on the GFP-chimeric mice, the findings of this study demonstrated that the resident microglia and not the infiltrating bone marrow-derived microglial cells are the first cell population to proliferate after stroke. In addition, by using CD11b-TK^{mt30} and GCV treatment, our results revealed that the selective ablation of proliferating microglial cells after transient MCAO is associated with (1) marked deregulation of postischemic brain inflammatory response, (2) an increase in the size of ischemic lesion and 2.7-fold increase in the number of apoptotic cells, predominantly neurons, and (3) a significant decrease in the levels of neurotrophic factor IGF-1, expressed by Mac-2-positive/proliferating microglia in the areas around ischemic lesion.

Activated microglial cells are the main effectors of the innate immune response in the brain after injuries. Microglial response to brain injury is characterized by a graded stereotypic response comprising a massive expansion (proliferation) of microglial cells that follows the initial stage of microglial activation (Stoll et al., 1998; Raivich et al., 1999b; Ladeby et al., 2005). Microglial proliferation in response to cerebral ischemia is rather a synchronized event and normally peaks a few days (48–72 h) after injury.

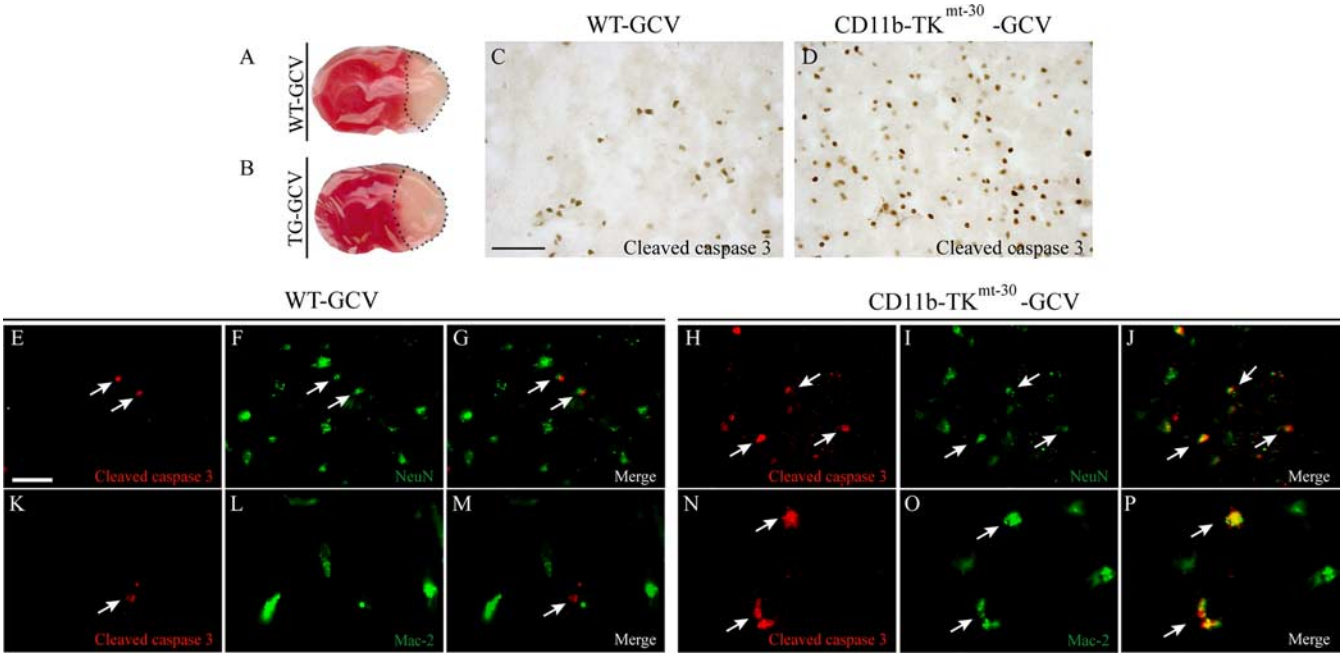

Figure 7. Increase of infarct area and apoptosis in CD11b-TK ^{mt30}-GCV mice 72 h after cerebral ischemia. ***A***, ***B***, Representative brain sections of WT and transgenic brain treated with GCV stained with TTC. The size of ischemic lesion is expressed as a percentage of the control; the equivalent area of contralateral nonischemic hemisphere (100%) was measured 72 h after transient MCAO. A significant increase of ~13% was observed in the size of ischemic lesion in transgenic mice treated with GCV. Each value represents a percentage of the mean value ± SEM ($n = 7–9$; $^*p = 0.05$). ***C***, ***D***, Photomicrographs of immunoreactivities of cleaved caspase-3 in WT and transgenic mice treated with GCV 72 h after transient MCAO. Quantification of labeled cells showed an increase of cleaved caspase-3 $^+$ cells in transgenic mice treated with GCV compared with the WT-GCV. Values indicate mean ± SEM ($n = 4$; $^*p = 0.0422$). ***E–P***, Representative photomicrographs of double immunostaining for cleaved caspase-3 (red) and NeuN (green) (***E–J***) or Mac-2 (green) (***K–P***) in WT and transgenic mice by double immunofluorescence. ***E–J***, Photomicrographs show colocalization for cleaved caspase-3 and NeuN in both groups (white arrows). ***G–J***, In transgenic mice, more NeuN $^+$ cells colocalize with cleaved caspase-3 compared with WT-GCV mice (white arrows) (***H–J***). ***K–M***, In WT-GCV mice, no colocalization was observed for Mac-2 and cleaved caspase-3, but in transgenic mice (***N–P***) some Mac-2 $^+$ cells are cleaved caspase-3 $^+$. Scale bars: ***C***, (in ***E***) ***E–P***, 25 μm.

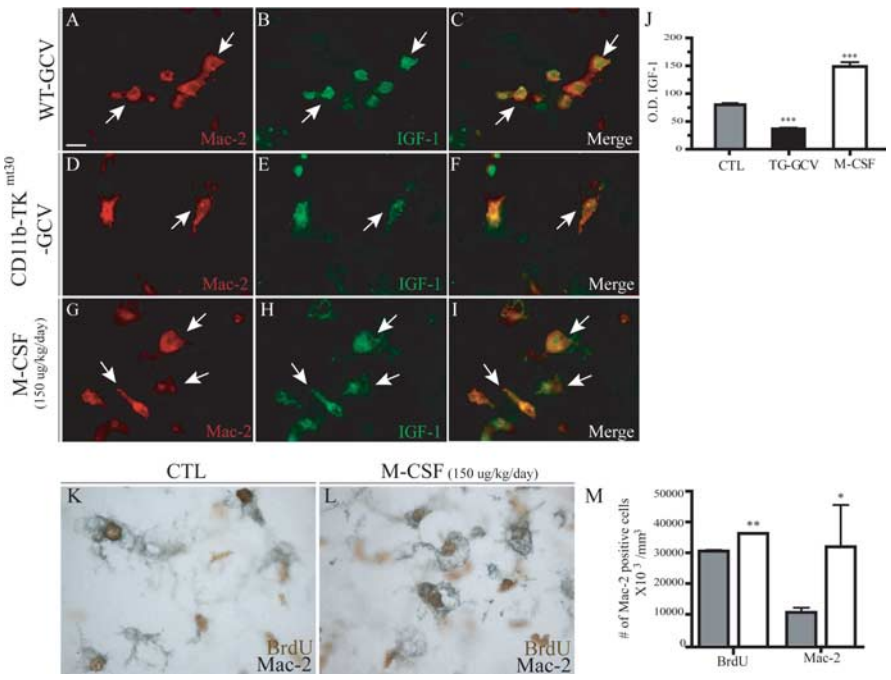

Figure 8. Proliferating microglial cells synthesize and secrete IGF-1. ***A–I***, Double labeling 72 h after transient MCAO reveals colocalization of Mac-2 (red) and IGF-1 (green; white arrows) in WT transgenic mice treated with GCV and in M-CSF-treated mice 72 h postinjury. ***J***, Densitometry quantification reveals a significant (1.8-fold) decrease in IGF-1 expression in the brain section of transgenic mice treated with GCV compared with the WT-GCV and a significant (1.9-fold) increase in IGF-1 levels in the M-CSF-treated group compared with controls. ***K–M***, Double immunohistochemistry for BrdU and Mac-2 reveals an increase in the number of BrdU and Mac-2 positive cells after M-CSF treatment. Data are expressed as mean ± SEM (BrdU, $n = 2$, $^{**}p = 0.0048$; Mac-2, $n = 2$, $^*p = 0.0391$; IGF-1, $n = 2–4$, $^{***}p < 0.0001$). Scale bar, 25 μm.

The possibility that microglial proliferation in response to brain injury may have a distinct biological role has not yet been discussed, mainly because of a lack of adequate experimental models. In the presented work, using a transgenic CD11b-TK $^{mt-30}$ mouse and GCV treatment as a tool, we succeeded in conditionally ablating proliferating microglia without affecting the nondividing cells and/or the process of microglial activation. As revealed by Iba1 and Mac-2 staining, the microglial cells adopted activate morphology, irregular amoeboid shape, and retracted processes, but the numbers were significantly decreased (Fig. 2). Interestingly however, quantitative analysis revealed that in our *in vivo* model system, by injecting GCV we were preferentially targeting/ablating Mac-2-positive cells (~65% reduction in number of Mac-2 cells compared with ~40% loss of Iba-1) (Fig. 2). Moreover, in the series of additional experiments using GFP-chimeric mice (Simard et al., 2006), we identified the Mac-2-positive cells as the activated resident microglial cells. Although the relative cell selectivity of the Mac-2 marker within the first 72 h after ischemic injury remains to be elucidated, our findings clearly suggest that the resident microglial cells, not the infiltrating,

bone marrow-derived inflammatory cells, are the cell population that would first adopt the activated state resulting in early proliferation after cerebral ischemia.

Previous reports indicated a marked increase in proinflammatory cytokine levels peaking 12–24 h after ischemic injury (Hill et al., 1999). Our results obtained from the WT littermates treated with GCV are in agreement with the temporal dynamics of cytokines observed in the previous studies. As expected, an initial increase in the transcripts for TNF-α, IL-1β, and IL-6 detected 24 h after ischemia was followed by an important decline in the mRNA levels of proinflammatory cytokines 72 h after MCAO (Figs. 4, 5). However, contrary to decline in the levels of proinflammatory cytokines observed in control samples, at the same time point, the ablation of proliferating microglial cells resulted in significant increase in the levels of IL1-β, TNF-α, and IL-6, suggesting an important temporal deregulation of proinflammatory cytokine response. Similar patterns of mRNA expression and temporal deregulations were observed in the NF-κB signaling pathway, used as a general marker of inflammatory response in cerebral ischemia (Clemens et al., 1997; Blondeau et al., 2001). Although the exact molecular mechanisms underlying such an alteration in the temporal pattern of expression of proinflammatory responses remains to be elucidated, it is worthy mentioning that in the normal mouse brain, the decline of proinflammatory cytokine response coincides with the peak of postischemic microglial proliferation (48–72 h after stroke). Therefore, it seems that microglial proliferation may play an important role in the regulation/modulation of the proinflammatory responses in cerebral ischemia and possibly other types of acute brain injuries.

Evidence suggests that postischemic inflammation may contribute to brain injury, however, the general hypothesis that inflammatory processes are deleterious and that abrogation of these processes may alleviate brain injury remains untested (del Zoppo et al., 2001; Feuerstein and Wang, 2001). In addition, there is a growing line of evidence suggesting that cellular interaction of activated glial cells and the adjacent affected neurons are much more complex than initially thought (Streit, 2002; Neumann et al., 2006). The presented work may provide an additional step forward in the understanding of the complex microglia-neuron interactions after injuries. The results of our study clearly demonstrated that ablation of proliferating microglial cells exacerbates ischemic injury. Moreover, in our experiments, the significant increase in the size of ischemic lesion was associated with a 2.7-fold increase in the numbers of apoptotic cells, predominantly neurons, suggesting a neuroprotective role of proliferating microglial cells in cerebral ischemia. Our results are in the line with the previous studies performed on the mouse deficient in M-CSF, which is an important mitogen for microglia. In this mouse model (*op/op* mice), a natural frameshift mutation in the coding region of M-CSF resulted in the 70–80% reduction in microglial proliferation (Yoshida et al., 1990; Raivich et al., 1994). Interestingly, the absence of M-CSF and microglial proliferation was associated with enhanced neuronal sensitivity to injuries and increased neuronal cell death after cerebral ischemia (Berezovskaya et al., 1996; Fedoroff et al., 1997). Conversely, addition of M-CSF conferred neuroprotection in the microglial–hippocampal organotypic coculture system (Vincent et al., 2002; Mitrasinovic et al., 2005). At present, the mechanism of M-CSF neuroprotection remains unclear. However, the results of our study suggest that the described neuroprotective effects of M-CSF may be indirect, because of a neuroprotection provided by proliferating microglial cells. Previous *in vivo* and *in vitro* studies demonstrated that activated and proliferating microglial cells may produce neurotrophic molecules such as brain-derived neurotrophic factor (Miwa et al., 1997; Nakajima and Kohsaka, 2004) and/or insulin-like growth factor 1 (O'Donnell et al., 2002). Indeed, the results of our study demonstrated that proliferating microglial cells after cerebral ischemia produce IGF-1 (high levels of colocalization of IGF-1 staining with Mac-2 in two different models), which is consistent with previous findings (O'Donnell et al., 2002). However, in this study, we provided an important additional proof of the biological importance of the endogenous IGF-1 secreted by proliferating microglia. In our *in vivo* model-system, selective ablation of proliferating microglial cells after MCAO was accompanied by a significant (1.8-fold) decrease in the levels of IGF-1 and the significant increase in the numbers of the apoptotic cells, predominantly neurons (Figs. 6, 7). Conversely, injection of microglial mitogen M-CSF after ischemia was associated with a significant increase the number of Mac-2-positive proliferating microglia and 1.9-fold increase in IGF-1 levels. The data presented here provide direct *in vivo* evidence for an antiapoptotic and a neuroprotective role for IGF-1 in ischemic injury (Guan et al., 1993; Beilharz et al., 1998). More importantly, our data suggest a novel functional role for posttraumatic microglial proliferation serving as an endogenous pool of trophic molecules, such as IGF-1, and providing neuroprotection to adjacent injured neurons.

In conclusion, proliferation of activated microglia can be observed in virtually all CNS pathologies, including cerebral ischemia, brain trauma, etc. Based on our results, we propose here a functional role for proliferating microglial cells as modulators of proinflammatory brain response. Moreover, our data revealed a marked neuroprotective potential of proliferating microglial cells serving as an endogenous pool of neurotrophic and antiapoptotic molecules, such as IGF-1. Therefore, selectively targeting (stimulating) microglial proliferation may provide new therapeutic avenues in the treatment of stroke and possibly other neurological disorders.

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