Microglia/Macrophage Polarization Dynamics Reveal Novel Mechanism of Injury Expansion After Focal Cerebral Ischemia

Xiaoming Hu, MD, PhD; Peiying Li, MD, PhD; Yanling Guo, MS; Haiying Wang, PhD; Rehana K. Leak, PhD; Songela Chen; Yanqin Gao, MD; Jun Chen, MD

Background and Purpose—Mononuclear phagocytes are highly plastic cells that assume diverse phenotypes in response to microenvironmental signals. The phenotype-specific roles of microglia/macrophages in ischemic brain injury are poorly understood. A comprehensive characterization of microglia/macrophage polarization after ischemia may advance our knowledge of poststroke damage/recovery.

Methods—Focal transient cerebral ischemia was induced in mice for 60 minutes; animals were euthanized at 1 to 14 days of reperfusion. Reverse-transcriptase polymerase chain reaction and immunohistochemical staining for M1 and M2 markers were performed to characterize phenotypic changes in brain cells, including microglia and infiltrating macrophages. In vitro experiments using a transwell system, a conditioned medium transfer system, or a coculture system allowing cell-to-cell contacts were used to further elucidate the effect of neuronal ischemia on microglia/macrophage polarization and, conversely, the effect of microglia/macrophage phenotype on the fate of ischemic neurons.

Results—Local microglia and newly recruited macrophages assume the M2 phenotype at early stages of ischemic stroke but gradually transformed into the M1 phenotype in peri-infarct regions. In vitro experiments revealed that ischemic neurons prime microglial polarization toward M1 phenotype. M1-polarized microglia or M1-conditioned media exacerbated oxygen glucose deprivation–induced neuronal death. In contrast, maintaining the M2 phenotype of microglia protected neurons against oxygen glucose deprivation.

Conclusions—Our results suggest that microglia/macrophages respond dynamically to ischemic injury, experiencing an early “healthy” M2 phenotype, followed by a transition to a “sick” M1 phenotype. These dual and opposing roles of microglia/macrophages suggest that stroke therapies should be shifted from simply suppressing microglia/macrophage toward adjusting the balance between beneficial and detrimental microglia/macrophage responses. (Stroke. 2012;43:3063-3070.)

Key Words: inflammation ■ macrophage ■ microglia ■ phagocytosis ■ polarization ■ stroke

Microglia/macrophages represent the first line of defense against brain injuries such as stroke. The resident microglia and peripheral macrophages are rapidly mobilized to the site of injury and initiate the release of effector molecules and recruitment of other immune cells.1 The roles of microglia/macrophages in ischemic brain injury, however, are still debated. An increasing number of studies now agree that microglia/macrophages are highly plastic cells that can assume diverse phenotypes and engage different functional programs in response to specific microenvironmental signals.2,3 In particular, in vitro stimulation with lipopolysaccharide and interferon-γ (IFN-γ) promotes the differentiation of “classically activated” M1 microglia/macrophages that typically release destructive proinflammatory mediators.4 In contrast, interleukin (IL)-4 and IL-10 induce an “alternatively activated” M2 phenotype that possesses neuroprotective properties.5,6 The dualistic roles of distinctly polarized macrophage populations have been reported in several central nervous system diseases, including multiple sclerosis7 and spinal cord injury.2 Recently, the concept of microglial M1 and M2 phenotypes also has entered the field of stroke research.8 However, a comprehensive characterization of microglia/macrophage polarization after ischemic brain injury is still missing.

Received April 6, 2012; accepted July 20, 2012.
From the State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences (X.H., P.L., Y.G., Y.Gao, J.C.), Fudan University, Shanghai, China; Department of Neurology and Center of Cerebrovascular Disease Research (X.H., P.L., H.W., S.C., J.C.), University of Pittsburgh, Pittsburgh, PA; Division of Pharmaceutical Sciences (R.K.L.), Mylan School of Pharmacy, Duquesne University, Pittsburgh, PA; Geriatric Research, Educational, and Clinical Center (J.C.), Veterans Affairs Pittsburgh Health Care System, Pittsburgh, PA.
The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.112.659656/-/DC1.

Correspondence to Dr Jun Chen, Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15213 (E-mail chenj2@upmc.edu); or Dr. Xiaoming Hu, Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15213 (E-mail hux2@upmc.edu). © 2012 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.112.659656
In this study, we used a well-established murine model of transient focal cerebral ischemia to analyze the temporal kinetics of microglia/macrophage polarization after stroke. We showed for the first time that local microglia and newly recruited macrophages assume the M2 phenotype at early stages of ischemic stroke but gradually transformed into the M1 phenotype at the sites of injury. In vitro studies revealed that ischemic neurons prime the polarization of microglia toward M1. These M1-polarized microglia exacerbated oxygen glucose deprivation (OGD)-induced neuronal loss. In contrast, maintaining microglia in the M2 phenotype protected neurons against OGD in a cell–cell contact-dependent manner.

**Materials and Methods**

**Murine Model of Transient Focal Ischemia**

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male 10- to 12-week-old C57/BL6 mice (Jackson Laboratory, Bar Harbor, Maine USA) were anesthetized with 1.5% isoflurane in a 30% O2/68.5% N2O mixture under spontaneous breathing conditions. Focal cerebral ischemia was produced by intraluminal occlusion of the left middle cerebral artery (MCA) for 60 minutes as described previously.9 The rectal temperature was controlled by a temperature-regulated heating pad. Regional cerebral blood flow was measured in all stroke animals using laser Doppler flowmetry. Animals that did not show a regional cerebral blood flow reduction to <30% of preischemia baseline levels during MCAO were excluded from further experimentation, as were animals that died during posts ischemic reperfusion. Sham-operated animals underwent the same anesthesia and surgical procedures except MCAO. Animals were randomly assigned to sham and MCAO groups with different reperfusion duration through the use of a lottery-drawing box. All biochemical (polymerase chain reaction [PCR] and analysis) and histological (immunostaining and cell counting) assessments were performed by investigators who were blinded to experimental group assignments. Total of 71 mice (11 sham-operated and 60 ischemic mice) were used in this study, including 7 mice that were excluded from further assessments because of either death after ischemia or failure in ischemic induction.

**Immunohistochemistry and Cell Counting**

Immunohistochemistry was performed on 30-μm free-floating sections. Primary antibodies include goat anti-CD206 (R&D Systems), rat anti-CD11b (Abcam), rabbit anti-Arginase (Santa Cruz Biotechnology), mouse anti-Arginine (Santa Cruz Biotechnology), and rabbit anti-Iba1 (Wako). All images were processed with Image J for counting of automatically recognized cells. The means were calculated from 3 randomly selected microscopic fields in the cortex and striatum of each section, respectively, and 3 consecutive sections were analyzed for each brain. Data are expressed as mean numbers of cells per square millimeter.

**Real-Time PCR**

Total RNA was isolated from sham brain and ischemic brains at using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions; 5 μg was used to synthesize the first strand of cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed on the Opticon 2 Real-Time PCR Detection System (Bio-Rad) using corresponding primers (Table) and SYBR gene PCR Master Mix (Invitrogen). The cycle time values were normalized to GAPDH of the same sample. The expression levels of the mRNAs were then reported as fold changes vs sham control.

**Embryonic Neuronal Culture and Induction of In Vitro Ischemia**

Embryonic cortical neuronal cultures were prepared from 17-day-old Sprague-Dawley rat embryos as previously described.10 To simulate ischemic injury, cultures were submitted to OGD for 60 minutes and then were returned to 95% air, 5% CO2, and normal glucose medium for the time period indicated.10

**Primary Microglia-Enriched Cultures**

Primary microglia-enriched cultures were prepared from the whole brains of 1-day-old pups as described previously.11 After a confluent monolayer of glial cells was obtained (12–14 days after initial seeding), microglia were shaken off, collected, and seeded. For M1 induction, lipopolysaccharide (100 ng/mL) and IFNγ (20 ng/mL) were added to the microglial cultures for 48 hours. For M2 induction, IL-4 (20 ng/mL) was added to the culture for 48 hours.

**Neuron–Microglia (or Microglia-Conditioned media) Cocultures**

Three experimental systems were used: (1) a transwell system allowing contact-independent communication through diffusible factors; (2) a conditioned medium transfer system; and (3) a coculture system allowing cell-to-cell communication via direct microglial–neuronal contacts. To generate neuron–microglia cocultures, 11-day-old neurons cultured in 96-well plate (1×10³/well) were subject to 60 minutes of OGD. Primary microglia (1×10³/well) were then seeded and cultured together with neurons in the presence of lipopolysaccharide plus IFNγ or IL-4 in the culture medium (minimum essential medium containing 10% fetal bovine serum,
10% horse serum, 1 g/L glucose, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 100 μmol/L nonessential amino acids). In some experiments, microglia were seeded in a 96-well transwell (0.4 μmol/L) with lipopolysaccharide plus IFN-γ or IL-4 for 48 hours. Microglia in transwells or microglial-conditioned media (CM) were then added to control or post-OGD neuronal cultures. Neurotoxicity was analyzed 48 hours after coculture using a MAP2 enzyme-linked immunosorbent assay and lactate dehydrogenase (LDH) release.

Quantitative MAP2 Enzyme-Linked Immunosorbent Assay
Neurons were fixed in 96-well plates with 4% paraformaldehyde. After blocking with 10% bovine serum albumin/0.1% Triton X-100 in phosphate-buffered saline for 1 hour, cells were incubated with MAP2 primary antibody (1:1000; Millipore) overnight at 4°C. Alkaline–phosphatase-labeled secondary antibody was then applied (rat antimouse IgG1, 1:1000; Abcam) for 2 hours at room temperature. MAP2 expression was detected using para-nitrophenyl phosphate substrate (Vector Labs). Twenty minutes later, absorbance was read using a SpectraMax microplate reader at 405 nm.

Statistical Analysis
All data are reported as mean±standard error of the mean. Significant differences between means were assessed by analysis of variance and post hoc least significant difference tests for multiple comparisons, unless otherwise indicated. P<0.05 was considered statistically significant. Other methods are available in the online-only Data Supplement.

Results
Reactive Cells in the Ischemic Brain Polarize Toward the M1 Phenotype Over Time
Reactive cells, including the polarized microglia/macrophages and activated astrocytes, are commonly distinguished by their expression of signature genes for surface markers and cytokines/chemokines. Using real-time PCR, we found that the levels of M1-type genes (iNOS, CD11b, CD16, CD32, and CD86) were gradually increased over time from day 3 onward and all but 1 remained elevated for at least 14 days after ischemia (Figure 1A). In contrast, the mRNA expression of all the tested M2 markers, including CD206, Arg1, CCL22, Ym1/2, IL-10, and transforming growth factor-β, was induced beginning 1 to 3 days after MCAO and peaked by 3 to 5 days postinjury. The majority of M2-type genes began to decrease at 7 days after MCAO and returned to preinjury levels by day 14 (Figure 1B).

It is known that some of the M1 and M2 signature genes are expressed not only in microglia/macrophages but also in other central nervous system cells or infiltrating immune cells. The results of real-time PCR therefore reflect the changes of these genes in brain tissues of mixed cell types. To evaluate whether microglia/macrophages were in a particular polarization after MCAO, representative M1-associated or M2-associated marker proteins were analyzed by double
Stroke November 2012

revealed that the M1-like microglia induced by OGD neuronal CM exhibited reduced phagocytosis (Figure 4C) and produced more inflammatory mediators (Figure 4D).

**Effect of M1 and M2 Macrophage on Post-OGD Neuronal Survival**

To elucidate the effect of microglial phenotype on neuronal survival under hypoxic/ischemic conditions, we induced polarized M1 and M2 microglia in vitro using lipopolysaccharide (100 ng/mL) plus IFN-γ (20 ng/mL) or IL-4 (20 ng/mL), respectively, for 48 hours (Supplementary Figure I). These M1 or M2 microglia were then applied in transwells over the non-OGD or post-OGD neuronal cultures (microglia:neuron=1:10). Neuronal survival was ascertained 48 hours later. M1-polarized microglia exacerbated OGD-induced neuronal death, manifested by reduced MAP2 expression (Figure 5A), and increased LDH release (Figure 5B) compared with nonpolarized microglia (M0) and M2-polarized microglia. MAP2 and Hoechst double-staining confirmed the results of the biochemical assays: more condensed/fragmented nuclei were seen in post-OGD neurons cocultured with M1 microglia (Figure 5C). In another set of experiments, CM was collected from M1-polarized, M2-polarized, or nonpolarized microglia and then was applied to OGD or post-OGD neuronal cultures (Figure 5A, 5B). The addition of M1 microglial CM resulted in reduced survival of post-OGD neurons compared with M2 microglial CM. These results suggest that M1 microglia inhibited neuronal survival under pathological conditions via soluble factors. Interestingly, when microglia of different phenotypes were mixed together with neurons in the same well (microglia:neuron=1:10), non-induced microglia promoted the survival of cortical neurons under normal and ischemic/hypoxic conditions. This protective immunofluorescent staining with the microglia/macrophage marker Iba1 on the inner boundary of infarction (Figure 2A). The lack of a specific antibody recognizing specifically either microglia or macrophages precluded the possibility of distinguishing between the local microglia and circulating macrophages that were recruited to the injured brain. Thus, the immunofluorescence for M1 and M2 markers in Iba1+ cells was likely derived from both microglia and macrophages.

Consistent with the real-time PCR results, expression of the M1 marker CD16/32 was low in Iba1+ microglia/macrophage at early stages after MCAO. A significant increase of CD16/32 expression was observed from day 3 onward, which remained elevated until day 14 after ischemia (Figure 2B–D). Very few non-Iba1+ cells expressed CD16/32, indicating that the majority of CD16/32 expression in the penumbral region was associated with microglia/macrophages. In contrast, immunofluorescence for the M2 marker CD206 was increased significantly over control levels on Iba1+ cells at 1 day after MCAO, which remained elevated at 3 to 7 days after MCAO (Figure 3A–3C). This increase was concurrent with the accumulation of microglia/macrophages at the site of injury. Taken together, these data suggest that the microglia/macrophages initially recruited to the site of ischemic injury exhibit mainly the M2 phenotype, but only for a short period of time. At later stages, reactive microglia/macrophages with an M1 phenotype begin to dominate in number at the infarct border.

**Ischemic Neurons Prime Microglial Polarization Toward M1 In Vitro**

To investigate the effect of ischemic neurons on microglial polarization, microglia were treated with CM collected from control neuronal cultures or post-OGD neuronal cultures. Microglial polarization was examined 48 hours later by immunohistochemical staining for CD206 (M2) and inducible nitric oxide synthase (M1). As shown in Figure 4A and 4B, CM from ischemic neurons drove microglial polarization toward M1, whereas CM from control neurons showed no dramatic effect on microglial phenotype. Further studies...
revealed that the M1-like microglia induced by OGD neuronal CM exhibited reduced phagocytosis (Figure 4C) and produced more inflammatory mediators (Figure 4D).

**Effect of M1 and M2 Macrophage on Post-OGD Neuronal Survival**

To elucidate the effect of microglial phenotype on neuronal survival under hypoxic/ischemic conditions, we induced polarized M1 and M2 microglia in vitro using lipopolysaccharide (100 ng/mL) plus IFN-γ (20 ng/mL) or IL-4 (20 ng/mL), respectively, for 48 hours (Supplementary Figure I). These M1 or M2 microglia were then applied in transwells over the non-OGD or post-OGD neuronal cultures (microglia:neuron=1:10). Neuronal survival was ascertained 48 hours later. M1-polarized microglia exacerbated OGD-induced neuronal death, manifested by reduced MAP2 expression (Figure 5A), and increased LDH release (Figure 5B) compared with nonpolarized microglia (M0) and M2-polarized microglia. MAP2 and Hoechst double-staining confirmed the results of the biochemical assays: more condensed/fragmented nuclei were seen in post-OGD neurons cocultured with M1 microglia (Figure 5C). In another set of experiments, CM was collected from M1-polarized, M2-polarized microglia, MAP2 and Hoechst double-staining confirmed the results of the biochemical assays: more condensed/fragmented nuclei were seen in post-OGD neurons cocultured with M1 microglia (Figure 5C). In another set of experiments, CM was collected from M1-polarized, M2-polarized microglia, and then was applied to OGD or post-OGD neuronal cultures (Figure 5A, 5B). The addition of M1 microglial CM resulted in reduced survival of post-OGD neurons compared with M2-polarized CM. These results suggest that M1 microglia inhibited neuronal survival under pathological conditions via soluble factors. Interestingly, when microglia of different phenotypes were mixed together with neurons in the same well (microglia:neuron=1:10), non-induced microglia promoted the survival of cortical neurons under normal and ischemic/hypoxic conditions. This protective effect was further augmented by M2 polarization, suggesting a cell–cell contact-dependent protective mechanism (Figure 5D).

**Discussion**

Recent research in stroke as well as many other neurological diseases points to a dual role of microglia/macrophages in neuronal injury and recovery. In support of a protective effect of microglia/macrophages, it has been reported that selective depletion of proliferative microglia exacerbated ischemic brain injuries and that injections of exogenous microglia into the ischemic brain ameliorated injuries. Microglia/macrophage activation is proposed to benefit the injured brain by removing cell debris and restoring tissue integrity. However, mounting evidence reveals that drastic microglia/macrophage activation leads to secondary expansion of ischemic infarction and deterioration of neurological outcomes. Microglia/macrophages are thought to exacerbate injury by releasing a plethora of neurotoxic substances, impairing poststroke neurogenesis, preventing axon regeneration, and limiting the efficacy of thrombolytic therapy. Therefore, an improved understanding of the dynamic equilibrium between healthy and sick microglia/macrophages is likely to advance our knowledge of poststroke recovery and to be critical for stroke treatment in the future.

In the current study, we demonstrate a differential shift in the M2 to M1 phenotypes in ischemic brain. Soon after an ischemic injury, a majority of microglia/macrophages migrating or infiltrating into the infarct areas initially assume the M2 phenotype, as evidenced by the increased expression of CD206 protein in Iba1+ cells. The present study as well as other reports further indicate that M2 microglia/macrophages are healthier cells with enhanced phagocytic activity and reduced production of inflammatory mediators. Moreover, we demonstrated that M2 microglia/macrophages promote...
the survival of cortical neurons under both normal and ischemic/hypoxic conditions. Such M2 phagocyte- afforded protection seems to be cell–cell contact-dependent, presumably through their ability to clear debris. Therefore, the early recruitment of M2 microglia/macrophages may represent an endogenous effort to clean ischemic tissue and restrict brain damage. Previous studies also suggested that M2 macrophages promote axon growth/sprouting in cultured neurons, suggesting a critical role in tissue recovery and reinnervation. Maintaining the M2 microglia/macrophenotype could benefit the injured brain in many ways. However, as shown in our study, the M2 phagocyte response is transient and phased out within 7 days after injury. In the meantime, M1 microglia/macrophages, which are characterized by reduced phagocytosis and increased secretion of proinflammatory mediators, begin to dominate the injured area. These M1 phagocytes may exacerbate neuronal demise through the release of harmful mediators such as tumor necrosis factor-α and nitric oxide. Moreover, M1 microglia/macrophages have been shown to impair axon regrowth. It is thus conceivable that the M2-to-M1 shift during chronic inflammation after stroke expands neuronal injury and leads to insufficient neuronal recovery. Such an M2-to-M1 shift has been reported in models of spinal cord injury, suggesting that the microglia/macrophenotypic change may be a common pathological mechanism underlying multiple types of central nervous system injuries.

Many questions still remain about M1 and M2 polarization and the phenotypic shift during stroke progression. For example, the exogenous signals that induce the microglia/
Figure 5. Effect of M1 and M2 macrophages on post-oxygen glucose deprivation (OGD) neuronal survival. Microglia cultures were induced toward the M1 or M2 phenotype using lipopolysaccharide (LPS) (100 ng/mL) plus interferon (IFN)γ (20 ng/mL) or interleukin (IL)-4 (20 ng/mL), respectively, for 48 hours. In-transwell microglia or their conditioned media were applied over the non-OGD or post-OGD neuronal cultures for 48 hours. Neuronal survival was quantified by MAP2 enzyme-linked immunosorbent assay (ELISA) (A) and cell death was quantified by LDH release (B). n=6 per group. C, MAP2 and Hoechst staining of OGD neurons cocultured with in-transwell microglia. Images are representative of 3 independent experiments. D, Microglia of different phenotypes were mixed together with non-OGD or post-OGD neurons in the same well for 48 hours. Neuronal survival was quantified by a MAP2 ELISA assay. n=6 per group. *P<0.05, **P<0.01, ***P<0.001.

Macrophage phenotype shift have not yet been identified. Kigerl et al\(^2\) demonstrated that the injured spinal cord microenvironment downregulates the M2 phenotype and upregulates the M1 phenotype. Our in vitro results also illustrate that CM collected from neurons subjected to OGD induce the polarization of microglia toward M1. This suggests that neurons may release soluble factors and/or shed their components to drive the M2–M1 shift. Interestingly, a fully polarized M1 or M2 subpopulation can reverse phenotype and function in response to signals in the microenvironment.\(^21\) Therefore, another question remaining is the identity of the molecular switches inside the microglia/macrophages that control such a dramatic phenotypic change. Recent research on macrophages reveals the importance of IFN response factors in phagocyte phenotype shift. Irf4 and Irf3 were found to encode a key transcription factor that controls M2 macrophage polarization,\(^22,23\) whereas Irf5 encodes a transcription factor that controls M1 polarization.\(^24\) Another study showed that Notch signaling regulates the M1 vs M2 polarization through SOCS3.\(^25\) A CREB-C/EBPβ cascade also was shown to induce M2 macrophage-specific gene expression and promote muscle injury repair.\(^26\) The identification of these critical molecules brings hope to therapies that promote healthy microglia/macrophage responses even under pathological conditions.

Although it is well-accepted that ischemia-induced over-activation of microglia/macrophages amplifies inflammatory neuronal damage and represents a promising target for stroke management, anti-inflammatory agents have failed to improve clinical outcomes in stroke patients so far. Among the factors that might have contributed to the unsuccessful clinical trials, a concern that needs to be emphasized is that a broad suppression of microglia/macrophages may deprive the brain of their normal healthy physiological functions and result in undesired effects. It is worth noting that acute inflammation serves many protective functions, whereas chronic inflammation is more likely to exacerbate injury. In keeping with this classic view of acute vs chronic inflammation, the results of our study demonstrate that microglia/macrophages respond dynamically to ischemic injury, experiencing an early healthy M2 phenotype and then transitioning to a sick M1 phenotype. This dualistic pattern defies a simplistic pigeonholing of microglia/macrophages into a single category and illustrates the sophistication of the endogenous inflammatory response. Taken as a whole, our results suggest that experimental stroke therapies should be shifted from 1-dimensional or blanketed microglia/macrophage suppression toward a more nuanced adjustment of the balance between protective and toxic microglia/macrophage phenotypes.

Sources of Funding
This project was supported by National Institutes of Health grants to J.C. (NS045048, NS36736, NS43802, and NS56118), a grant from the American Heart Association to X.H (10POST4150028), and Chinese Natural Science Foundation grants to Y. Gao (30870794).

Disclosures
None.
References


Microglia/Macrophage Polarization Dynamics Reveal Novel Mechanism of Injury Expansion After Focal Cerebral Ischemia
Xiaoming Hu, Peiying Li, Yanling Guo, Haiying Wang, Rehana K. Leak, Songela Chen, Yanqin Gao and Jun Chen

Stroke. 2012;43:3063-3070; originally published online August 28, 2012; doi: 10.1161/STROKEAHA.112.659656

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/43/11/3063

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2012/08/28/STROKEAHA.112.659656.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia

Xiaoming Hu1,2, MD, Ph.D, Peiying Li1,2, MD, Ph.D, Yanling Guo1, MS, Haiying Wang2, Ph.D, Rehana K Leak3, Ph.D, Songela Chen2, Yanqin Gao1, MD, and Jun Chen1,2,4 MD.

1State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China
2Department of Neurology and Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
3Division of Pharmaceutical Sciences, Mylan School of Pharmacy, Duquesne University, Pittsburgh, PA 15282 USA
4Geriatric Research, Educational and Clinical Center, Veterans Affairs Pittsburgh Health Care System, Pittsburgh, PA 15261, USA

Corresponding authors:
Dr. Jun Chen
Department of Neurology
University of Pittsburgh School of Medicine
Pittsburgh, PA 15213, USA
E-mail: chenj2@upmc.edu
or
Dr. Xiaoming Hu
Department of Neurology
University of Pittsburgh School of Medicine
Pittsburgh, PA 15213, USA
E-mail: hux2@upmc.edu

Cover title: Microglia/macrophage polarization in brain ischemia

Key words: stroke, microglia/macrophage polarization, inflammation, phagocytosis
Figure S1. Microglia cultures were induced toward the M1 or M2 phenotype. Microglia cultures were induced toward the M1 or M2 phenotype using LPS (100 ng/ml) + IFNγ (20 ng/ml) or IL-4 (20 ng/ml), respectively, for 48 hrs. (A) Representative images show CD206, Arg and iNOS stainings. (B) Quantification of TNF-α production from unstimulated, M1 and M2 microglia. n=6 per group. (C-D) Phagocytotic activity of unstimulated, M1, and M2 microglia. The cells were stained with phalloidin to visualize F-actin. (C) Phagocytosis was quantified by counting the number of phagocytosed beads in each cell. n=5-6 per group. (D) Images are representative of three independent experiments. * p<0.01 vs unstimulated and M2 microglia.
Supplementary methods:

**Phagocytosis assay**
Microglia were plated in 96-well plates (5×10⁴ cells/well) and incubated with various treatments for 48h. Nile red fluorescent microspheres (Invitrogen) were solubilized to a concentration of 0.03% solids. Cells were incubated with or without microspheres for 3h. To quench the signal from extracellular or outer plasma membrane-associated microspheres, the medium was removed and the cells were rinsed with 0.25mg/ml trypan blue in PBS for 1min. Cells were lysed using PBST (1% Triton). Intracellular fluorescence was measured using a fluorescence plate reader at 535nm excitation and 575nm emission. For image analysis of phagocytosis, microglia (1×10⁵ cells/well) were plated into 8-well chamber slides (Nunc). Nile red fluorescent microspheres were added as described above. Cells were then rinsed with PBS and fixed in 4% paraformaldehyde. AlexaFluor488 phalloidin (Invitrogen) was added and incubated at room temperature in the dark for 1h. Images were captured with an Olympus confocal microscope.

**Assay for soluble factors in culture media**
Concentrations of TNF-α were measured with a commercial ELISA kit (R&D Systems) according to the manufacturer’s instruction. LDH was measured by a kit from Pointe Scientific. Nitric oxide was measured using a colorimetric reaction with equal volumes of Griess reagent (Invitrogen). Absorbance was read using a SpectraMax microplate reader.