Adoptive Regulatory T-Cell Therapy Preserves Systemic Immune Homeostasis After Cerebral Ischemia

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Background and Purpose—Cerebral ischemia has been shown to result in peripheral inflammatory responses followed by long-lasting immunosuppression. Our recent study demonstrated that intravenous delivery of regulatory T cells (Tregs) markedly protected against transient cerebral ischemia by suppressing neutrophil-derived matrix metalloproteinase 9 production in the periphery. However, the effect of Tregs on systemic inflammatory responses and immune status has not been fully characterized.

Methods—Cerebral ischemia was induced by middle cerebral artery occlusion for 60 minutes in mice or 120 minutes in rats. Tregs were isolated from donor animals by CD4 and CD25 double selection and transferred intravenously to ischemic recipients at 2 hours after middle cerebral artery occlusion. Animals were euthanized on different days after reperfusion. The effects of Tregs on systemic inflammation and immune status were evaluated using flow cytometry, ELISAs, and immunohistochemistry.

Results—Systemic administration of purified Tregs raises functional Tregs in the blood and peripheral organs, including spleen and lymph nodes. These exogenous Tregs remain in the blood and peripheral organs for ≥12 days. Functionally, Treg adoptive transfer markedly inhibits middle cerebral artery occlusion–induced elevation of inflammatory cytokines (interleukin-6 and tumor necrosis factor α) in the blood. Furthermore, Treg treatment corrects long-term lymphopenia and improves cellular immune functions after ischemic brain injury. As a result, Treg-treated animals exhibit decreased bacterial loads in the blood during recovery from cerebral ischemic attack.

Conclusions—Treg treatment did not exacerbate poststroke immunosuppression. On the contrary, Treg-treated animals displayed improved immune status after focal cerebral ischemia. (Stroke. 2013;44:3509-3515.)

Key Words: immunosuppression ■ inflammation ■ stroke ■ T-lymphocytes, regulatory
to accumulate there for >1 month. Increasing numbers of studies suggest that endogenous Tregs influence the development of ischemic brain injury. In our recent study, we reported that intravenous delivery of isolated CD4⁺CD25⁺ Tregs after transient ischemia markedly inhibits cerebral inflammation, reduces brain infarct size, and improves long-term neurological functions. Notably, we demonstrated that Tregs exert neuroprotection from a peripheral location by suppressing neutrophil-derived matrix metalloproteinase 9 and reducing subsequent proteolytic damage of the blood–brain barrier. These findings reveal that Tregs do not necessarily need to enter the brain to modulate central nervous system damage. However, whether and how transferred Tregs affect systemic inflammatory responses and immune status are not well understood.

Using 2 rodent models of focal transient ischemia, we show in this study that intravenous injection of Tregs 2 hours after ischemia significantly reduces peripheral inflammation. We further demonstrate that Treg treatment did not exacerbate poststroke immunosuppression. Remarkably, Treg-treated animals showed robust improvements in immune status after focal cerebral ischemia.

**Methods**

**Rodent Models of Transient Focal Cerebral 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 and Stroke Treatment Academic Industry Roundtable criteria. Male 10- to 12-week-old C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME) 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 (MCAO) for 60 minutes as described previously. This results in moderate brain damage after 3 days, with an n=55.8± mm² infarct size. Rectal temperature was controlled at 37.0±0.5°C during surgery and MCA occlusion (MCAO) using 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 of preischemia baseline levels to <25% during MCAO were excluded from further experimentation. Sham-operated animals underwent the same anesthesia and surgical procedures but were not subjected to MCAO. All animals were randomly assigned to different treatment groups. Finally, all assessments were performed by investigators who were blinded to experimental group assignments.

In the rat model of transient focal cerebral ischemia, transient (120 minutes) cerebral focal ischemia was induced in Sprague–Dawley rats as previously described. This results in moderate brain damage after 3 days, with an n=150.10 x mm² infarct size. Blood was collected 3 days after reperfusion onset.

**Isolation and Adoptive Transfer of CD4⁺CD25⁺ Tregs and Splenocytes**

Single-cell suspensions were prepared from inguinal and axillary lymph nodes and spleens of C57/BL6 mice (8 weeks old). CD4⁺CD25⁺ Treg populations were enriched by negative selection and positive selection with the regulatory T-cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. For the rat study, CD4⁺CD25⁺ Tregs were prepared from Sprague–Dawley rats using the regulatory T-cell isolation kit (R&D system) according to the manufacturer’s instructions. The CD4⁺CD25⁺ Tregs isolated in this manner were >95% enriched, with 82% of the isolated CD25⁺ cells expressing the Treg immunophenotypic marker Foxp3. Recipients received a tail vein injection of 2×10⁶ freshly enriched Tregs or freshly isolated splenocytes in 0.2 mL Dulbecco’s phosphate-buffered saline at 2 hours after reperfusion. For Treg labeling and tracking, Tregs were incubated with 0.5 μmol/L cell tracker orange CMTMR (5-(and-6)-((4-chloromethyl)benzoylamino)tetramethylrhodamine) (Invitrogen) at 37°C for 30 minutes before intravenous injection.

**Cell Preparation for Flow Cytometry**

Spleen, lymph nodes, bone marrow, blood, lung, and brain were collected after MCAO, and single-cell suspensions were prepared for flow cytometric analysis. Briefly, lung and brain were first flushed with PBS and chopped into fine particles in 4 mL of complete RPMI 1640 medium supplemented with 10% FCS. Tissues were then incubated in 10 mL of digestion buffer (2% FBS, 1 mg/mL collagenase II, 0.5 mg/mL of DNase 1 in RPMI 1640 medium) for 1 hour in a 37°C water bath. The suspension was passed through a 70-μm cell strainer, resuspended in 40 mL of complete RPMI 1640, and pelleted at 2000 g for 10 minutes at 4°C. Cells were fractionated on a 30% to 60% percoll gradient (GE Health) at 1000 g for 25 minutes. The mononuclear cells in the interface were washed before staining. Bone marrow was prepared from femur and tibia bones. Peripheral blood was obtained from mice by cardiac puncture, and the red blood cells were lysed by ammonium-chloride-potassium lysis buffer (Sigma-Aldrich). Lymphocytes were isolated from spleens and lymph nodes by mechanical homogenization followed by lysis of red blood cells using ammonium-chloride-potassium lysis buffer. Isolated cells were resuspended at 1×10⁶/mL and stained with CD4 and CD45.1.

**Flow Cytometry**

Cells were stained with anti-mouse CD3, CD4, CD8, B220, NK1.1, Gr-1, CD45.1, and the appropriate isotype controls following the manufacturer’s instructions (eBioscience). For Foxp3 intracellular staining, cells were surface-stained with PB-conjugated anti-CD4 and PE-conjugated anti-CD25 and then permeabilized with the intracellular staining Kit (eBioscience) according to the manufacturer’s protocol. Cells were then stained with allophycocyanin-conjugated anti-Foxp3. Flow cytometric analysis was performed using a fluorescence-activated cell sorter flow cytometer (BD Biosciences).

**Analysis of Ex Vivo Cytokine Production**

Whole blood was diluted 1:5 in RPMI 1640 and incubated at 37°C. For analysis of tumor necrosis factor α (TNF-α) synthesis, samples were stimulated with 100 ng/mL lipopolysaccharide (Sigma-Aldrich) for 4 hours. For analysis of interferon-γ (IFN-γ) and interleukin (IL)-4 production, blood samples were stimulated with 100 μg/mL concanavalin A (Sigma-Aldrich) for 24 hours. For analysis of IL-10 production, blood samples were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin (Sigma-Aldrich) for 5 hours. For analysis of TNF-α, IFN-γ, IL-4, and IL-10 production in spleen cells, 10⁶ cells/mL were stimulated as above. Concentrations of cytokines in culture supernatants were determined using commercially available kits (R&D system).

**Cytokine ELISA**

Blood plasma and cell culture media were collected as described above. Protein concentrations were measured for TNF-α, IL-10, IL-6, IL-4, and IFN-γ (R&D Systems) using commercial ELISA quantification kits according to the manufacturer’s instructions.

**Bacteriological Analysis**

Euthanized mice were washed with 70% ethanol. Blood was collected by cardiac puncture. The lungs were removed after thoracotomy and homogenized in sterile PBS. One hundred microliters of tissue homogenate or blood was serially diluted, plated onto sheep blood agar plates (Hardy Diagnostics), and incubated at 37°C for 24 hours. Bacterial colonies were counted by an observer blinded to experimental group assignment.
Measurement of Infarct Volume
For 2,3,5-triphenyltetrazolium chloride staining, brains were removed and sliced into 7 coronal sections, 1-mm thick each. Sections were immersed in prewarmed 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) in saline for 10 minutes and then fixed in 4% paraformaldehyde. Infarct volume was determined using National Institutes of Health Image J by an observer blinded to experimental group assignment. Infarct volume with correction for brain edema was calculated as the difference between the volume of the contra-lateral hemisphere and the noninfarcted volume of the ipsilateral hemisphere.

Two-Dimensional Laser Speckle Imaging Techniques
Cortical blood flow was monitored using the laser speckle technique. Briefly, a charge-coupled device camera (PeriCam PSI System; Perimed) was positioned above the head, and a laser diode (785 nm) illuminated the intact skull surface to allow penetration of the laser in a diffuse manner through the brain. Speckle contrast, defined as the ratio of the SD of pixel intensity to the mean pixel intensity, was used to measure cortical blood flow because it is derived from the speckle visibility relative to the velocity of the light-scattering particles (blood). This was then converted to correlation intensity, which is inversely and linearly proportional to the scattering particles (blood). This was then converted to correlation rived from the speckle visibility relative to the velocity of the light-scattering particles (blood). This was then converted to correlation intensity, which is inversely and linearly proportional to the scattering particles (blood). This was then converted to correlation intensity, which is inversely and linearly proportional to the mean blood velocity. Laser speckle perfusion images were obtained 10 minutes before MCAO and continued throughout the ischemic period until 5 minutes into the reperfusion. Cortical blood flow was measured again in the same animals at 2 hours after reperfusion. Cortical blood flow changes were recorded over time and expressed as the percentage of pre-MCAO baseline.

Statistical Analysis
Results are presented as means±SEM. The difference in means between 2 groups was assessed by the 2-tailed Student t test. Differences in means between multiple groups were analyzed using 1- or 2-way ANOVA with time or treatment as an independent factor. When the ANOVA showed significant differences, pairwise comparisons between means were performed by post hoc Bonferroni/Dunn tests. In all analyses, P<0.05 was considered statistically significant.

Results
Treg Treatment Elevates the Number of Functional Tregs in the Blood and Spleen After MCAO
To study the peripheral effect of Treg transfer, we first confirmed that adoptive transfer of the therapeutic dose of Tregs (2×10⁶/mouse) at 2 hours after MCAO elevated the number of functional Tregs in the periphery. Flow cytometric analysis demonstrated increases in Treg populations in the blood, spleen, and lymph node of Treg-treated animals compared with splenocyte-injected controls at 1 day after MCAO (Figure 1A and 1B). Similarly, Foxp3 immunostaining revealed increases in Treg populations in the spleens of Treg-treated animals at 1 day after MCAO (Figure 1C). We then examined phorbol 12-myristate 13-acetate–induced (50 ng/mL) and ionomycin-induced (500 ng/mL) IL-10 production in whole blood cultures and splenocyte cultures that were obtained from Treg-treated or splenocyte-treated MCAO animals. ELISAs on the culture media revealed enhanced capacity for IL-10 production in cultures isolated from Treg-treated animals (Figure 1D). These data suggest that Treg transfer significantly elevates the number of functional Tregs and that these viable Tregs produce suppressive cytokines such as IL-10 in the periphery.

Adaptively Transferred Tregs Remain in the Peripheral Blood and Organs for ≥1 Week After Injection
In an attempt to track the adaptively transferred Tregs in recipient mice during the long-term, we used congenic mouse strains bearing allelic variants of CD45. Tregs were isolated from pooled spleens and lymph nodes of CD45.1 mice and then intravenously injected into CD45.2 recipient mice 2 hours after MCAO. Using flow cytometry, we detected transplanted CD45.1+CD4+ Tregs in the spleen, lymph nodes, bone marrow, lung, and blood 1 week after MCAO (Figure 2A). A small number of Tregs had also infiltrated into the brain.

Figure 1. Regulatory T-cell (Treg) treatment elevates the number of functional Tregs in the blood and spleen after middle cerebral artery occlusion (MCAO). A, Flow cytometric comparison of CD25+Foxp3+ Tregs among the CD4+ T-cell populations in blood, spleens, and lymph nodes at 1 day after MCAO and cell transfer. B, Flow cytometric quantification of CD4−CD25+Foxp3+ Treg population in the blood (n=4/group), spleen (n=6–8/group), and lymph nodes (n=4/group) of Treg-treated animals compared with splenocyte-treated or sham-operated animals at 1 day after MCAO. C, Immunohistochemical staining of Foxp3 in the spleen shows an increased number of Tregs in Treg-treated animals at 1 day after stroke. Images are representative of 4 animals per group. D, Interleukin-10 (IL-10) production capacity in ex vivo blood and spleen cultures. Blood and spleen cells isolated from Treg- or splenocyte-treated MCAO animals were cultured for 5 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (600 ng/mL). IL-10 in the medium was quantified by ELISA. n=3 in each group. Data are means±SE. Sp indicates splenocytes. *P<0.05, **P<0.01.
In another series of experiments, we injected cell tracker–labeled Tregs into recipient mice 2 hours after MCAO. Similarly, cell tracker–labeled Tregs were found in multiple peripheral organs and in the ischemic area of the brain at 1 week after MCAO (Figure 2B). Taken together, these data demonstrate that the majority of transferred Tregs remain in the peripheral blood and organs for ≥1 week after injection and may thus influence the systemic immune status of the recipients within this time frame.

Tregs Attenuate Postischemic Peripheral Inflammation

It is known that ischemic stroke induces dynamic and widespread inflammatory responses that involve not only the central, but also the peripheral immune system. Indeed, the concentrations of plasma proinflammatory cytokines IL-6 and TNF-α were increased as early as 1 day after MCAO and remained elevated until ≥7 days after injury. Adoptive therapy with Tregs at 2 hours after MCAO ameliorated these ischemia-induced inflammatory responses at all time points measured (Figure 3A–3B). Plasma concentrations of anti-inflammatory cytokine IL-10 exhibited a slight and transient increase in the Treg-treated group at 1 day after stroke (Figure 3C). We also tested the peripheral effects of Treg transfer in a rat model of stroke. Consistent with results in mice, rats with Treg after treatment exhibited significantly lower levels of IL-6, TNF-α, and IL-1β than splenocyte-treated animals 3 days after ischemia (Figure 3D). Collectively, these data reveal that adoptive transfer of Tregs attenuates postischemic inflammation in peripheral blood.

Tregs Preserve Lymphocyte Populations After Cerebral Ischemia

A long-lasting reduction in the number of lymphocytes has been reported in patients with stroke and is thought to underlie spontaneous infections after stroke. We, therefore, performed differential blood cell counting by flow cytometry to evaluate the effect of Treg transfer on stroke-induced lymphopenia. The number of CD3+CD4+ T helper lymphocytes and CD3+CD8+ cytotoxic T lymphocytes in the blood was significantly reduced at 12 days after MCAO, but this effect was reversed by Treg treatment (Figure 4A). Treg treatment also attenuated loss of blood B220+ B lymphocytes after MCAO. No difference was observed in the number of blood NK1.1+ natural killer cells between Treg-treated and splenocyte-treated groups. A similar trend was observed in spleen T-lymphocyte populations (Figure 4B). These results suggest that Treg treatment corrects the
long-term lymphopenia that is observed after ischemic brain injury.

**Treg Treatment Improves Cellular Immune Functions After MCAO**

Another negative poststroke alteration in the immune system is the impairment of cell-mediated immunity, which is characterized by impaired monocyte function and a shift from Th helper cell (Th) 1 to Th2 cytokine production. We, therefore, performed ex vivo stimulation tests in blood cultures and splenocyte cultures to examine lipopolysaccharide-induced TNF-α production and concanavalin A–induced IFN-γ production as indicators of monocyte and Th cell functions, respectively. As shown in Figure 5A, blood and splenocyte cultures collected from Treg-treated mice at 3 days after MCAO showed enhanced capacity for TNF-α production after lipopolysaccharide stimulation compared with the blood and splenocytes collected from splenocyte-treated animals. The production of IFN-γ (Th1 cytokine) decreased, whereas the production of IL-4 (Th2 cytokine) increased after concanavalin A treatment, resulting in a significantly reduced ratio of IFN-γ/IL-4 in blood cultures collected from MCAO mice. Treg treatment reinstated the blood IFN-γ/IL-4 balance on concanavalin A stimulation (Figure 5B). These results suggest that Treg treatment improves cellular immune functions after MCAO.

**Treg Treatment Reduces the Risk of Spontaneous Infection After MCAO**

We further tested whether Treg treatment affects antimicrobial host defense. Blood and lung homogenates collected 3 days after MCAO demonstrated significant bacterial loads after 24 hours in culture. Cultures of lung homogenates showed no difference between splenocyte- and Treg-treated animals, whereas blood cultures from Treg-treated animals showed decreased bacterial loads compared with splenocyte-treated animals (Figure 5C). Collectively, our results indicate that Treg treatment does not exacerbate poststroke immunosuppression; on the contrary, it improves immune status after MCAO.

**Treg Treatment Reduced Ischemic Brain Injury Without Impairing Reperfusion After MCAO**

Our data showed that Treg treatment dose-dependently reduced the size of infarct after 60 minutes of MCAO in mice (Figure 6C and 6D). A recent study suggests that Treg transfer causes microvascular thrombi, thereby impairing reperfusion to the ischemic brain. To address this concern, we monitored cerebral blood flow at different time points before, during, and after reperfusion and Treg (2×10⁶/mouse) injection using 2-dimensional (2D) laser speckle imaging technique. In contrast to laser Doppler flowmetry, which measures blood flow at a single spot, laser speckle can generate whole-brain images of blood flow. Therefore, laser speckle permits a robust comparison between nonischemic and ischemic hemispheres and can better demonstrate the changes in blood flow in the entire ischemic territory. As shown in Figure 6A and 6B, Treg-treated animals subjected to transient MCAO demonstrated similar extents of reperfusion as in PBS-treated animals.
Regulatory T-cell (Treg) treatment reduces ischemic brain injury without impairing reperfusion after middle cerebral artery occlusion (MCAO). Tregs were injected intravenously 2 hours after reperfusion. Regional cerebral blood flow (CBF) was monitored using 2-dimensional laser speckle imaging techniques. A, Representative images of CBF before MCAO, during MCAO, and at 5 minutes and 24 hours after reperfusion (22 hours after treatment with 2×10⁶ Tregs/mouse). B, Quantification of CBF. Results are expressed as percent change from baseline (pre-MCAO). Treg-treated mice showed similar CBF compared with PBS-treated mice. n=3 to 4 per group. C and D, Effect of various doses of Treg treatment 3 days after MCAO. M=1×10⁶ cells. Data are mean±SE. Sp indicates splenocytes. *P<0.05, **P<0.01.

This unremitting process prevents excessive immune activation while at the same time retaining vigilance against foreign invaders or injury. Recent studies have documented that endogenous Tregs or exogenously transferred Tregs dampen cerebral inflammation and reduce brain damage after ischemic stroke. These findings strongly support a role for Tregs in cerebral immune homeostasis after brain injury. Herein, we extend these findings by showing that Treg transfer after cerebral ischemia also maintains the homeostatic equilibrium of peripheral immune responses, representing an additional mechanism of protection against the damaging sequelae of stroke. We have previously demonstrated that Tregs quickly distribute into different organs, mainly the lymphoid ones, shortly after injection. Within 1 day after adoptive transfer, we are able to detect exogenous Tregs in bone marrow, spleen, lymph nodes, and blood. In the present study, we confirmed that the number of Tregs increase significantly in the spleen and blood at 1 day after Treg transfer. These exogenous Tregs are capable of exerting functional changes, such as an increase in stimulated IL-10 release in blood and spleen cell cultures. We further showed that exogenous Tregs remain in the peripheral organs for ≥1 week after injection. Indeed, previous studies have shown that lymphocytes survive and proliferate in lymphoid organs for ≥21 days after transfer into recipient mice. Adoptively transferred Tregs may, therefore, modulate peripheral immune responses during an extended time frame after injury.

The systemic release of several inflammatory markers after stroke is thought to exert a negative effect on the central nervous system. For example, higher concentrations of plasma proinflammatory markers IL-6, TNF-α, and intercellular adhesion molecule 1 are associated with rapid neurological deterioration and poor functional outcomes in patients with stroke. Our study shows that Treg treatment mitigates postischemic inflammatory cytokines in the periphery and may, therefore, enhance functional recovery after stroke. However, the potent anti-inflammatory effect of Tregs raised the concern that Treg therapy after stroke might further inhibit the already suppressed immune system and result in undesirable side effects, such as an increased risk of infections. Contrary to this expectation, our results indicate that Treg treatment does not exacerbate poststroke immunosuppression. Instead, Treg-treated animals maintained greater lymphocyte populations in the blood and spleen 12 days after MCAO. Furthermore, the immune cells in Treg-treated animals showed improved cell-mediated immunity, with a balanced Th1/Th2 response. This improvement in immune status in Treg-treated stroke mice might be attributed to a direct modulatory effect of Tregs on the immune system, a secondary effect after reduced brain damage, or both. Concomitantly, Treg treatment reduced the risk of spontaneous infection in the blood after MCAO. Taken as a whole, our data suggest that Treg treatment benefits poststroke immune status while simultaneously restricting inflammatory overactivation, in line with a critical role for Tregs in immune homeostasis. Relative to other typical anti-inflammatory treatments that only prevent immune overactivation, Treg treatment seems to strike this difficult balance more effectively.

Despite large positive findings of the protective effects of Tregs in brain, liver, and kidney ischemia/reperfusion models, a recent study raised several concerns on potentially detrimental effects of Tregs. The authors suggest that the accumulation of Tregs in the ischemic brain induces microvascular thrombus formation, impairs reperfusion, and further exacerbates brain damage. However, 2 observations are not consistent with this hypothesis. First, our 2D laser speckle imaging indicates that Treg-treated mice exhibit similar cerebral blood flow recovery as PBS-treated mice. Second, as illustrated by our flow cytometry and cell-tracer studies, greater numbers of injected Tregs accumulate in the liver than migrate into the brain. The relative abundance of Tregs in liver compared with brain is inconsistent with the observation that Tregs induce microvascular thrombi specifically in the ischemic brain but are beneficial to the ischemic liver.

In summary, the present study is the first demonstration that Treg adoptive therapy simultaneously dampens peripheral inflammation and corrects immunosuppression after stroke, in addition to its potent central protective effects. Treg treatment may, therefore, reduce the risk of poststroke infections and hasten stroke recovery. We conclude that further investigations...
of Treg therapy in stroke models are highly warranted, both
to broaden the list of novel Treg molecular targets for rational
drug design and to validate Treg neuroprotection in species
closer to humans.

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**Disclosures**

None.

**References**

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