Adoptive Regulatory T-Cell Therapy Protects Against Cerebral Ischemia

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Objective: Recent evidence suggests that functional deficiency in regulatory T cells (Tregs), an innate immunomodulator, exacerbates brain damage after cerebral ischemia. We therefore evaluated the effect of Treg transfer in rodent models of ischemic stroke and further investigated the mechanism underlying Treg-afforded neuroprotection.

Methods: We examined the therapeutic potential of Tregs and the mechanisms of neuroprotection in vivo in 2 rodent models of ischemic stroke and in vitro in Treg–neutrophil cocultures using a combined approach including cell-specific depletion, gene knockout mice, and bone marrow chimeras.

Results: Systemic administration of purified Tregs at 2, 6, or even 24 hours after middle cerebral artery occlusion resulted in a marked reduction of brain infarct and prolonged improvement of neurological functions lasting out to 4 weeks. Treg-afforded neuroprotection was accompanied by attenuated blood–brain barrier (BBB) disruption during early stages of ischemia, decreased cerebral inflammation, and reduced infiltration of peripheral inflammatory cells into the lesioned brain. Surprisingly, Tregs exerted early neuroprotection without penetrating into the brain parenchyma or inhibiting the activation of residential microglia. Rather, both in vivo and in vitro studies demonstrated that Tregs suppressed peripheral neutrophil-derived matrix metalloproteinase-9 production, thus preventing proteolytic damage of the BBB. In addition to its potent central neuroprotection, Treg treatment was shown to ameliorate poststroke lymphopenia, suggesting a beneficial effect on immune status.

Interpretation: Our study suggests that Treg adoptive therapy is a novel and potent cell-based therapy targeting poststroke inflammatory dysregulation and neurovascular disruption.

Ischemic stroke elicits profound inflammatory response involving both innate and adaptive immunity.1 Innate immune cells such as neutrophils and microglia/macrophages respond promptly to cerebral ischemia and migrate to the injury. Although these cells are indispensable for the clearance of debris and tissue remodeling,2,3 their overactivation releases a large number of cytotoxic molecules. They can also compromise blood–brain barrier (BBB) integrity by producing matrix metalloproteinases (MMPs), allowing the invasion of even more peripheral immune cells.4,5 In addition, cytokines and chemokines produced by activated inflammatory cells further recruit and activate additional immune cells, resulting in a vicious cycle that promotes long-lasting brain damage and worsens neurological deficits.

Members of the adaptive immune system, specifically T lymphocytes, also play a critical role in ischemic brain injury. Most subsets of T cells have been revealed to be detrimental to the ischemic brain.6–8 However, regulatory T cells (Tregs), a specialized subpopulation of T cells, appear to be an endogenous protective mechanism that dampens cerebral inflammation following ischemic stroke.9 In general,
CD4\(^+\)CD25\(^+\) Tregs negatively regulate the immune system and modulate inflammation induced by pathogens and injuries. For example, Tregs suppress effector T cells and other immune cells\(^{10,11}\) through either direct contact with the suppressed cell or release of the immunosuppressive cytokines transforming growth factor (TGF)-\(\beta\) and interleukin (IL)-10.\(^{12}\) Disruptions of Treg function exacerbate inflammation and autoimmunity.\(^{13}\) Tregs increase in the blood several days after the onset of stroke both in patients\(^14\) and in experimental animals.\(^{15}\) This is likely to be an evolutionarily adaptive response that blunts the impact of stroke, because deletion of Tregs worsens both ischemic brain damage and functional outcomes.\(^9\) However, the therapeutic potential of Treg transplantation after ischemic stroke has not been investigated. Even less is known about the precise mechanism through which Tregs protect the brain after stroke.

Using rodent models of focal transient ischemia, we show for the first time that intravenous injections of Tregs even up to 24 hours after ischemia resulted in a marked reduction of brain infarct and a prolonged improvement of neurological functions lasting out to 4 weeks. We further demonstrated that Treg-conferred early neuroprotection is mediated via BBB protection involving an inhibition of peripheral neutrophil-derived MMP9.

**Materials and Methods**

**Ischemia Models**

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Cerebral focal ischemia was induced in mice by intraluminal occlusion of the left middle cerebral artery (MCA) for 60 minutes as described in the Supplementary Methods. Transient (120 minutes) cerebral focal ischemia was induced in Sprague-Dawley rats as described.\(^16\) Experimental procedures were performed following criteria derived from Stroke Therapy Academic Industry Roundtable (STAIR) group guidelines for preclinical evaluation of stroke therapeutics.\(^17\) Cerebral blood flow (CBF) was measured to confirm the vascular occlusion. Animals that did not show a CBF reduction of at least 75% on laser-Doppler flowmetry were excluded (<10% of stroke animals) from further experimentation. Animals that died after ischemia induction were also excluded from experimental groups. Temperature was controlled during the ischemic period. Immediately after surgery, animals were randomly assigned to phosphate-buffered saline (PBS), splenocyte, or Treg treatment groups. Investigators were blinded to treatment groups during cell transfer or vehicle injection and during all outcome assessments.

**Isolation, Labeling, and Adoptive Transfer of Tregs**

Single-cell suspensions were prepared from inguinal and axillary lymph nodes and spleens. CD4\(^+\)CD25\(^+\) Treg populations were enriched by negative selection and positive selection with a regulatory T-cell isolation kit (Miltenyi Biotec, Auburn, CA for mice; R&D Systems, Minneapolis, MN for rats) according to the manufacturer’s instructions. Two mice or 1 rat were needed to obtain 2 \(\times\) 10\(^6\) Tregs. The recipient mouse or rat received a tail vein injection of 2 \(\times\) 10\(^6\) freshly enriched Tregs or freshly isolated splenocytes in 0.2ml PBS at 2, 6, or 24 hours after reperfusion. The 2 \(\times\) 10\(^6\) cell dose was chosen on the basis of our preliminary evaluation of the relationship between Treg dose and therapeutic effect (Supplementary Fig 1A). For Treg labeling and tracking, Tregs were incubated with 0.5 \(\mu\)M cell tracker orange CMTMR (Invitrogen, Carlsbad, CA) at 37°C for 30 minutes before intravenous injection.

**Irradiation and Bone Marrow Transplantation**

To construct bone marrow chimeric mice, 6-week-old recipient male C57/B6 and MMP9\(^{-/-}\) mice underwent lethal gamma irradiation (9.5Gy), followed 6 hours later by intravenous transplantation with bone marrow cells obtained from 6- to 8-week-old syngeneic donors (10\(^7\) cells per recipient). Three groups were studied: (1) wild-type marrow transplanted into wild-type recipients, designated as WT/WT; (2) wild-type marrow transplanted into MMP9\(^{-/-}\) recipients, designated as WT/ MMP9KO; (3) MMP9\(^{-/-}\) marrow transplanted into wild-type recipients, designated as MMP9KO/WT. Six weeks later, blood was obtained in every animal to verify hematopoietic cell reconstitution. Chimeras were subjected to brain ischemia 7 weeks after irradiation.

**Primary Neutrophil Culture and Treatment**

Primary mouse neutrophils from bone marrow and blood were isolated using EasySep Mouse Neutrophil Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer’s instructions. To determine the influence of Tregs on neutrophil-derived MMP9, isolated Tregs were preactivated with CD3/CD28 antibodies (BD Biosciences, Franklin Lakes, NJ)\(^18,19\) for 3 days and then plated into the lower chamber of 96-well Transwell permeable trays (Millipore, Billerica, MA) free of anti-CD3/CD28 stimulation. The enriched neutrophils (1 \(\times\) 10\(^5\) per well) were prestimulated with tumor necrosis factor (TNF)-\(\alpha\) (100ng/ml; Ebioscience, San Diego, CA) for 2 hours for MMP9 induction,\(^20\) and then added to either the lower chamber or the upper chamber of 96-well Transwell trays. The Treg-neutrophil cocultures were maintained at 37°C for another 24 hours. In all conditions, the medium was collected after 24 hours of incubation to quantify the released proteases.

**Statistical Analysis**

Results were presented as mean ± standard error of the mean. The difference in means between 2 groups was assessed by 2-tailed Student t test. Differences in means among multiple groups were analyzed using 1- or 2-way analysis of variance (ANOVA) with time or treatment as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by post hoc Bonferroni/
Dunn tests. In all analyses, $p < 0.05$ was considered statistically significant.

See the Supplementary Material for a full description of all the experimental procedures.

**Results**

**Tregs Reduce Brain Damage after Transient Focal Cerebral Ischemia**

Recent studies that depleted Tregs from the circulation prior to ischemic challenge suggest a beneficial effect of Tregs on late phase ischemic brain injury. However, conflicting findings suggest that Treg depletion fails to affect brain infarct volume. To confirm a direct effect of Tregs on cerebral ischemia and to further evaluate their therapeutic value, we isolated Tregs from donor mice and injected them into recipient mice after MCA occlusion (MCAO; Fig 1). Flow cytometry demonstrated that the isolated CD4$^+$CD25$^+$ Tregs were $>95\%$ enriched, with $82\%$ of them expressing the Treg immunophenotypic marker Foxp3. Transient focal ischemia lasting 60 minutes was induced by MCAO under CBF monitoring before, during, and after MCAO. Animals were randomly assigned to Tregs, splenocyte, or PBS treatment groups. Retrospectively, we found no statistical difference in CBF reduction during and after MCAO among the various experimental groups (see Supplementary Fig 1B). Treg treatment at 2, 6, or 24 hours postischemia significantly attenuated brain infarct and reduced neurological deficits compared to PBS- or splenocyte-treated animals. Mice with splenocyte or PBS treatment developed similar brain infarct volumes as well as cerebral inflammation (Supplementary Fig 2) after MCAO. Splenocyte-treated animals were therefore used in most experiments as controls. The maximal protection was elicited with early Treg transfer 2 hours after MCAO, resulting in approximately 50\% reduction of infarct volume. All subsequent experiments used this optimal post-MCAO administration regimen. Treg treatment did not affect CBF during reperfusion for up to 24 hours as measured by 2-dimensional laser speckle imaging techniques (see Supplementary Fig 1C).

To disentangle the role and interaction of transplanted Tregs with endogenous Tregs, we depleted the endogenous Treg population with a specific CD25 antibody 2 days prior to MCAO (see Fig 1G). Therapeutic numbers of exogenous Tregs protected against early stage ischemic brain injury even in the absence of endogenous Tregs.

We also tested the effect of Treg transfer in a rat model of stroke. Consistent with results in mice, rats with Treg post-treatment developed a significantly smaller infarct than splenocyte- or PBS-treated animals, which was accompanied by a reduced neurological severity score (see Fig 1H).

**Tregs Improve Functional Outcomes and Confer Prolonged Protection after Cerebral Ischemia**

Postischemic sensorimotor dysfunction significantly improved during both the acute and late recovery period after MCAO in Treg-treated mice compared to splenocyte-treated animals, as assessed by the corner test, forelimb placing test, and cylinder test up to 28 days following MCAO (Fig 2A–C). Cresyl violet staining and MAP2 immunostaining revealed profoundly reduced infarct volumes at 7 and 28 days after MCAO, respectively (see Fig 2D, E). Collectively, these results confirmed that Treg treatment actually reduced cerebral tissue loss, rather than simply delaying cell death, and improved long-term neurological function after cerebral ischemia.

**Tregs Attenuate Cerebral Inflammation and Reduce the Early Infiltration of Peripheral Immune Cells into the Ischemic Brain prior to Their Own Central Nervous System Infiltration**

Adoptive transfer of Tregs has been shown to suppress inflammatory responses in several disease models. To investigate the effect of Tregs on postischemic cerebral inflammation, we measured multiple inflammatory markers in the ischemic hemisphere at 24 hours after MCAO. The mRNA levels of IL-6, IL-1$\beta$, IL-17, and TNF-$\alpha$ were substantially increased in splenocyte-treated MCAO mice, and these increases were significantly blunted by Treg treatment (Fig 3A). IL-10 and TGF-$\beta$, 2 main anti-inflammatory mediators released by Tregs, were also upregulated at 24 hours post-MCAO. Unexpectedly, Treg treatment did not change the expression of these 2 cytokines in the brain (Supplementary Fig 3).

Local microglia and infiltrated peripheral immune cells are the major sources of inflammatory cytokines in the injured brain. We then investigated whether Tregs impede the recruitment of peripheral inflammatory cells and/or stabilize resident microglia in the ischemic penumbra (Supplementary Fig 4A), which is a zone of reversible ischemia and salvageable in the first few hours after ischemic stroke onset. Neutrophil, T-cell, and macrophage infiltration was prominent at 1 to 3 days after MCAO in splenocyte-treated mice, but remarkably attenuated in Treg-treated mice (see Fig 3B–E, Supplementary Fig 4B, C). In contrast, an amelioration of microglial activation was not observed until 7 days post-MCAO (see Fig 3B, F, Supplementary Fig 5). These results suggest that the early anti-inflammatory effects of Tregs after ischemia may largely be attributed to their...
FIGURE 1: Adoptive transfer of regulatory T cells (Tregs) confers neuroprotection against focal cerebral ischemia. (A) Scheme for experimental design. Tregs or splenocytes (Sp) were isolated from pooled spleens and lymph nodes (LN) of donors and injected intravenously ($2 \times 10^6$ cells/animal) into recipients at 2, 6, or 24 hours after middle cerebral artery occlusion (MCAO). Time line for parameter measurements is indicated. (B) Representative flow cytometry plots of CD25 and Foxp3 expression on splenocyte, CD4$^+$ T cells after negative selection, and CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ subsets of T cells after double selection. (C–E) Treg-afforded protection in mice at 3 days after 60 minutes of MCAO. (C) Representative 2,3,5-triphenyl tetrazolium chloride–stained coronal sections showed a smaller cerebral infarct in a mouse with adoptively transferred Tregs than in a splenocyte-transferred or phosphate-buffered saline (PBS)-treated mouse. (D) Infarct volumes in mice treated with Tregs at 2 hours ($n = 7$/group), 6 hours ($n = 8–9$/group), or 24 hours ($n = 6–7$/group) after MCAO were significantly reduced. (E) Infarct areas of 7 consecutive coronal sections, 1mm apart, throughout the middle cerebral artery territory in mice that received treatments at 2 hours after MCAO ($n = 7$/group). (F) Treg treatment with 2-hour delay improved neurological deficits in mice over 3 days after MCAO compared to splenocyte or PBS treatment ($n = 7$/group). (G) Exogenous Tregs protected the ischemic brain in the absence of endogenous Tregs. Mice were injected intraperitoneally with either PBS (control) or 300μg of CD25-specific antibody (CD25 mAb) 2 days prior to MCAO. Left: Flow cytometry analysis showing that endogenous Tregs were depleted in anti-CD25 mAb-treated mice. Right: Infarct volume was measured 3 days after MCAO. (H) Treg-afforded protection in rats at 3 days after MCAO. Left: Infarct volume in rats treated with Tregs, splenocytes, or PBS at 2 hours after 120 minutes of MCAO. Right: Treg-treated rats demonstrated reduced neurological severity scores at 1 day and 3 days after MCAO compared to splenocyte- or PBS-treated rats ($n = 5$/group). Data are mean ± standard error. ns = not significant. *$p < 0.05$, **$p < 0.01$. 

ANNALS of Neurology 

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inhibition of peripheral immune cell infiltration rather than to a significant impact on central nervous system (CNS) resident microglia.

In line with a non-CNS targeting mechanism for Treg action, the infiltration of Foxp3⁺ Tregs into brain parenchyma was delayed until 5 days after stroke (see Fig 3H). Using the CD45.1 congenic marker, exogenously transferred Tregs were detected by flow cytometry in the spleen, lymph nodes, bone marrow, lung, liver, and blood, but not in the brain or kidney of MCAO recipients at 1 day post-MCAO (see Fig 3G). These transferred CD45.1⁺CD4⁺ Tregs showed high expression of CD25 and Foxp3 (Supplementary Fig 6C). Similarly, cell tracker-labeled Tregs were found in multiple peripheral organs in the absence of trafficking into the brain parenchyma of MCAO recipients at 1 day after MCAO (see Fig 3H, Supplementary Fig 6A). Taken together, these data verify that the transferred Tregs are located outside the brain while exerting their anti-inflammatory effects by inhibiting peripheral immune cell infiltration into the CNS.

Tregs Do Not Exacerbate Poststroke Immunosuppression

The negative effect of Tregs on some immune responses raises the concern that Treg therapy might further inhibit the already suppressed immune system after stroke.24 We thus performed differential blood cell counting by flow cytometry to evaluate the effect of T reg transfer on stroke-induced immunosuppression. Treg treatment preserved the otherwise significantly reduced blood CD3⁺ T lymphocytes and B220⁺ B lymphocytes following MCAO (Fig 4). Further analysis of the T-cell subpopulation showed a significantly increased number of CD4⁺ T helper cells and CD8⁺ cytotoxic T cells in Treg-treated groups. Tregs had no effect on blood NK1.1⁺ cells. A similar trend was observed in spleen T-lymphocyte populations. Our results indicate that Treg treatment did not exacerbate poststroke immunosuppression.

Tregs Preserve BBB Integrity after Ischemia

The pathology of stroke involves disruption of the BBB and ensuing communication between peripheral immune cells and the brain. We investigated whether Tregs inhibited peripheral immune cell infiltration after cerebral ischemia via BBB protection. The extravasation of 2 tracers, cadaverine Alexa-555 or bovine serum albumin Alexa-555, into the brain parenchyma was prominent at 24 hours after MCAO but reduced by Treg treatment (Fig 5A). Fluorescence quantification revealed significantly lower tracer levels in brain lysates from Treg-treated mice compared to splenocyte-treated...
FIGURE 3: Regulatory T cells (Tregs) attenuate postischemic inflammation and reduce the early infiltration of peripheral immune cells into the brain ahead of their own central nervous system infiltration. (A) Quantitative real-time polymerase chain reaction for mRNA expression of interleukin (IL)-6, IL-1β, IL-17, and tumor necrosis factor (TNF)-α in the ischemic hemispheres from animals with 60 minutes of middle cerebral artery occlusion (MCAO) and 24 hours of reperfusion (n = 6/group). IL-6, IL-1β, IL-17, and TNF-α mRNA were significantly decreased in Treg-treated mice compared to splenocyte (Sp)-treated mice. (B) Representative immunofluorescent staining of myeloperoxidase (MPO), CD3, F4/80, and Iba-1 on brain sections obtained 3 days after MCAO. (C–F) Time courses for the infiltration of MPO+ neutrophilic granulocytes (C), CD3+ T cells (D), and F4/80+ macrophages (E) and the activation of Iba-1+ microglial cells (F) in the ischemic brains of Treg-treated mice compared to splenocyte-treated and sham-operated mice (n = 6/group). (G) Adoptively transferred CD45.1+ CD4+ Tregs were present in the spleen, bone marrow (BM), lymph node (LN), liver, blood, and lung, but not in the brain or kidney at 1 day after MCAO. Plots are representative of 4 animals. (H) Delayed brain infiltration of Tregs after stroke. Cell tracker–labeled Tregs were observed in the brain at 7 days (ii, iv) but not 1 day (i) after MCAO. (iii) Immunohistochemical staining of Foxp3 in brain sections at 5 days after MCAO. Images are representative of sections from 4 animals. Data are mean ± standard error. CTX = cortex; ns = not significant; STR = striatum. *p < 0.05, **p < 0.01.
controls (see Fig 5B–C). Similarly, the intracranial leakage of plasma-derived immunoglobulin G (IgG) was lessened in T reg-treated mice (see Fig 5A, D–F). Importantly, T reg-treated animals showed significantly reduced IgG extravasation (Supplementary Fig 7C) and inflammatory cell infiltration (see Supplementary Fig 7D–F) compared to untreated animals even when the cerebral lesion volumes were matched across groups by titrating MCAO intervals (see Supplementary Fig 7A, B). These data suggest that the BBB protection and anti-inflammatory effects in T reg-treated animals are not simply the consequences of reduced infarct volume.

The integrity of tight junction complexes is associated with paracellular impermeability of the BBB. We assessed the continuity of such complexes using the ZO-1 junctional marker (see Fig 5A). ZO-1 was expressed in a continuous manner in intact animals but was largely disrupted in the infarct areas 24 hours after MCAO. T reg adoptive therapy retained the integrity of ZO-1 expression. The ultrastructure of tight junctions was further observed with transmission electron microscopy (see Fig 5G). Ischemic injury resulted in abnormalities in intercellular tight junctions, manifested by lower electron density and less well-defined basement membranes. T reg treatment after ischemia elicited prominent protection of the ultrastructure of tight junctions and basement membranes. These experiments led to the solid conclusion that the adoptively transferred Tregs ameliorated the BBB disruption in the early phase after stroke.

**Tregs Confer Protection against MCAO by Blunting a Rise in MMP9**

MMP9 rises in the brain and plasma quickly after ischemic stroke and disrupts the BBB. We thus investigated whether T reg treatment could inhibit this increased MMP9 production after MCAO. Gel zymography demonstrated a significant increase in pro-MMP9 and activated MMP9 at 24 hours after ischemic onset in the plasma and, to a lesser extent, in the brain. Remarkably, T reg treatment abolished the ischemia-induced MMP9 elevation almost to sham levels (Fig 6). Quantitative enzyme-linked immunosorbent assay (ELISA) assays further confirmed the inhibitory effect of T regs on plasma MMP9 production after ischemia. The inhibition of central MMP9 expression by Tregs was also confirmed by immunofluorescent staining. At 24 hours after MCAO, MMP9 immunostaining was observed mainly around CD31+ blood vessels in the ischemic zone. Treg treatment reduced the amount of perivascular MMP9. The length of MMP9-positive blood vessels, which was defined by double labeling of MMP9 with CD31, was significantly decreased in Treg-treated brains compared to control MCAO brains. In addition to blood and brain, we also observed the upregulation of MMP9 in the spleen 24 hours after ischemia (Supplementary Fig 8A). Treg treatment dramatically reduced MMP9 staining in the spleen.

To verify a causal connection between MMP9 and Treg-avoided neuroprotection, we subjected MMP9−/− mice to MCAO followed by splenocyte or Treg treatment. Consistent with previous reports, MMP9-deficient mice...
exhibited significant albeit smaller infarct volumes, which was accompanied by reduced IgG extravasation through the BBB (see Fig). Notably, Treg-treated and splenocyte-treated MMP9−/− mice demonstrated a comparable volume of infarct, BBB damage, and neutrophil infiltration (Supplementary Fig 9) early after MCAO.

To further determine whether the beneficial effects of exogenous Tregs are attributable to the MMP9 alteration in peripheral or CNS cells, MCAO was performed in animals that had been subjected to lethal irradiation and bone marrow transplantation. The success of hematopoietic cell reconstitution was confirmed by white blood cell counts (not shown) and measuring MMP9 levels in the blood at 24 hours after MCAO (Table). Tregs showed significant protection in wild-type or MMP9−/− mice that were reconstituted with wild-type
FIGURE 6: Regulatory T cells (Tregs) confer protection against middle cerebral artery occlusion (MCAO) by ameliorating a rise in matrix metallopeptidase 9 (MMP9) production. (A–F) Tregs ameliorated MMP9 production after MCAO. Plasma and brain tissue were obtained at 24 hours after MCAO from splenocyte (Sp)- or Treg-treated mice or sham-operated mice. (A) Representative zymogram comparing brain and plasma MMP9 levels among different treatments. (B, C) Quantified densitometry of MMP9 zymography bands in plasma samples (B, n = 5/group) and in brain lysates (C, n = 5/group). (D) Plasma pro- and active MMP9 levels quantified by enzyme-linked immunosorbent assay. (E) Representative Z-stack confocal image of MMP9 and CD31 double immunostaining. MMP9 immunostaining was observed in the brain parenchyma and around blood vessels in the ischemic zone. The increase in brain MMP9 is accompanied by the prominent leakage of cadaverine Alexa-555 into the brain parenchyma. MMP9 expression in the brain infarct and tracer leakage after MCAO was abolished by Treg treatment. (F) Quantification of the length of MMP9+/CD31+ blood vessels in the brain (n = 4/group). (G–I) Treg-conferred neuroprotection was abolished in MMP9−/− mice. (G) Brain infarcts as measured by MAP2 staining in wild-type and MMP9−/− mice treated with splenocytes or Tregs. (H) Quantification of immunoglobulin G (IgG) leakage determined by positive area of mouse IgG immunohistochemical staining (n = 6/group). (I) The number of infiltrated MPO+ neutrophils at 3 days after MCAO. Brain infarct, IgG leakage, and neutrophil infiltration were significantly decreased in MMP9−/− compared to wild-type mice. Treg treatment failed to confer further protection in MMP9−/− mice. Data are mean ± standard error. CTX = cortex; MPO = myeloperoxidase; ns = not significant; STR = striatum. *p < 0.05, **p < 0.01.
bone marrow (WT/WT and WT/MMP9KO), whereas Tregs had no protective effect in wild-type mice that were reconstituted with MMP9−/− bone marrow (MMP9KO/WT). These results suggest that the Treg-conferred neuroprotection in the acute phase of ischemic brain injury is due to the inhibition of MMP9 expression in bone marrow-derived peripheral hematopoietic cells.

Neutrophil-Derived MMP9 Is a Major Target for Treg Cerebroprotective Action

It is becoming more widely accepted that neutrophils contribute to the MMP9 signatures after brain ischemia.30 Our immunostaining demonstrated that MMP9-loaded neutrophils invaded the brain parenchyma early after MCAO (Fig 7A–C). We also observed numerous MMP9+ neutrophils in the spleen (see Supplementary Fig 8B). Some Gr1+ neutrophils were in close proximity to cell tracker–labeled Tregs in the red pulp area (see Supplementary Fig 6B). Furthermore, the ELISA assay demonstrated a dramatic increase in MMP9 expression in blood neutrophils isolated from MCAO animals (see Fig 7D). Treg treatment significantly reduced MMP9 production in blood neutrophils and inhibited the number of MMP9+ neutrophils in the brain after MCAO. Taken together, these data suggest that adoptively transferred Tregs may exert neuroprotection after cerebral ischemia via the inhibition of neutrophil-derived MMP9.

To confirm the importance of neutrophils in Treg- afforded protection, we used a Gr1-specific antibody to deplete neutrophils 1 day before MCAO (see Fig 7). Consistent with a previous report,31 neutrophil depletion resulted in a significant reduction in plasma MMP9 production, BBB disruption, and infarct volume at 1 day after MCAO. Notably, Treg treatment failed to confer additional protection to neutrophil-depleted animals. These data suggest that neutrophil-derived MMP9 is an essential target for Treg cerebroprotective action.

To further verify the direct effect of Tregs on MMP9 production from neutrophils, we cocultured TNF-α–pretreated neutrophils with or without CD3 and CD28 antibody-primed Tregs (see Fig 7I). MMP9 production was prominent in neutrophils cocultured with T effective cells or without T cells. Tregs markedly inhibited TNF-α–induced MMP9 production from neutrophils when incubated at 1:1 or 1:2 ratios of Tregs:neutrophils; however, they failed to inhibit the production of neutrophil elastase, another important protease in neutrophils, or phorbol 12-myristate 13-acetate–induced superoxide production (Supplementary Fig 10), suggesting a specific inhibitory effect of Tregs on neutrophil MMP9 production. Interestingly, Tregs cultured into the transwells lost their inhibition on MMP9 production from neutrophils, which suggests a mechanism involving direct cell–cell interactions.

Discussion

As a result of numerous failed clinical trials, the STAIR collaborators have established multiple criteria to assess neuroprotective strategies for viability and promise in clinical applications.17 Our results qualify Treg therapy as a promising neuroprotective treatment as defined by STAIR. First, Treg treatment initiated within 2 hours after ischemia exerted potent protection against brain damage in 2 rodent transient focal MCAO models. Second, Tregs were protective out to 1 month after the stroke and improved both short- and long-term neurological functions. Third, Tregs show an extended therapeutic time window of up to at least 24 hours following

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²p < 0.01 versus Sp-treated WT/WT.  
³p < 0.01 versus Sp-treated WT/MMP9KO.  
⁴p < 0.05 versus Sp-treated WT/MMP9KO.  
⁵p < 0.05 versus Sp-treated WT/WT.  
KO = knockout; MMP9 = matrix metallopeptidase 9; Sp = splenocyte; Treg = regulatory T cell; WT = wild type.
FIGURE 7: Neutrophil-derived matrix metalloproteinase 9 (MMP9) is a major target for regulatory T-cell (Treg) cerebroprotective actions. (A–C) Tregs inhibit the infiltration of MMP9-loaded neutrophils into the brain at 3 days after middle cerebral artery occlusion (MCAO). (A) Representative images of MMP9 and myeloperoxidase (MPO) double staining in brain sections. (B) A 3-dimensional confocal image shows MMP9+/MPO+ cells. (C) Quantification of the number of MMP9+/MPO+ cells in the cortex (CTX) and the striatum (STR; n = 4/group). (D) Quantification of blood neutrophil-derived MMP9 at 24 hours after MCAO (n = 4/group). Neutrophils were isolated from the blood of splenocyte (Sp)- or Treg-treated MCAO mice and sham mice at 24 hours after operation. MMP9 was measured in the neutrophil lysate by enzyme-linked immunosorbent assay (ELISA). Neutrophil-derived MMP9 was increased after stroke, and this increase was significantly inhibited by Treg treatment. (E–H) Neutrophil depletion abolished Treg conferred protection. (E) Flow cytometry confirmed neutrophils were depleted in anti-Gr1 monoclonal antibody (Ab; 400 µg/mouse, intraperitoneally)-treated mice. (F) Plasma pro- and active MMP9 levels quantified by ELISA. (G) Quantification of immunoglobulin G (IgG) leakage determined by positive area of mouse IgG immunohistochemical staining (n = 6/group). (H) Infarct volume defined by MAP2 staining (n = 6/group). (I) Tregs inhibited tumor necrosis factor (TNF)-α-induced MMP9 production from cultured neutrophils (n = 6/group). Neutrophils isolated from blood and bone marrow were treated with TNF-α (100 ng/ml) for 2 hours and then cocultured with or without CD3/CD28 antibody-primed Tregs or effective T cells (Teffs) for 24 hours. The release of MMP9 in the culture medium was measured. Tregs, but not Teffs, inhibited the production of MMP9 from TNF-α-challenged neutrophils. Data are mean ± standard error. ns = not significant; PBS = phosphate-buffered saline. *p < 0.05, **p < 0.01.
ischemia. In addition, our ongoing research on long-term effect of Tregs revealed that Treg treatment not only alleviated gray matter injury, but also protected white matter from ischemia (not shown). Recent advances in expanding Tregs ex vivo \(^3\) or in vivo \(^4\) to achieve therapeutic levels further enable their clinical translation.

Consistent with a previous report,\(^9\) we found that Tregs inhibit cerebral inflammation in the brain as early as 1 day after ischemia. The dampened cerebral inflammation was accompanied by a reduced invasion of peripheral immune cells. Although such a generalized inhibitory impact of Tregs on peripheral immune cell trafficking might be explained by their antigen-nonspecific function,\(^9\) the lack of a simultaneous impact on CNS microglia strongly suggests an alternative mechanism involving the preservation of BBB function after ischemia. In line with this notion, Treg-treated MCAO mice showed reduced BBB leakage and preserved tight junction structures. Of note, recent research in amyotrophic lateral sclerosis reported that Tregs may augment M2 microglial polarization and shift the balance of microglia responses from cytotoxicity toward neuroprotection,\(^35\) suggesting that Treg treatment may change microglial properties without affecting their number. Our current results, however, do not support a direct effect of Tregs on microglial properties at 1 day after stroke due to the absence of Treg infiltration and the lack of increase in Treg-derived anti-inflammatory cytokines (IL-10 and TGF-ß) in the brains of Treg-treated animals.

Given our observation that the infiltration of Tregs into the brain was somewhat delayed relative to their early protection against cerebral inflammation, BBB disruption, and brain damage, Tregs must exert early neuroprotection through peripheral means, by either releasing protective mediators into the circulation or targeting other peripheral cells that in turn influence the brain infarct. Liesz and colleagues suggested that IL-10 is a critical mediator utilized by endogenous Tregs to protect the brain, but only at late stages after cerebral ischemia.\(^9\) However, in our study, exogenous Treg treatment did not change IL-10 levels in the brain. Our data show that transfer of Tregs derived from IL-10–deficient mice, either in the presence or absence of endogenous Tregs, still protected against ischemic brain injury at 3 days after MCAO (Supplementary Fig 11). Thus, although IL-10 may be important for the protection elicited by endogenous Tregs against late stages of ischemic injury, it is unlikely to be a direct mediator for exogenous Treg-afforded neuroprotection during early stages of ischemic brain injury. In addition, cerebral levels of TGF-ß, another Treg-derived anti-inflammatory cytokine, were not affected by Treg treatment. All of this evidence disputes a direct protective mediator theory for early Treg action.

In considering the candidates for peripheral cells that are targeted by Tregs, we noted that Tregs inhibited MMP9 production in the blood and the brain as early as 1 day after ischemia. Further in vitro and in vivo studies identified a novel mechanism of Treg action involving potent suppression of MMP9-producing neutrophils. It has been reported in the transient MCAO model of stroke that MMP9 levels went up in the peripheral blood as early as 2 hours after MCAO, maximized at 4 hours, and then gradually subsided but could still be observed until 72 hours. The increase of brain MMP9 is relatively delayed compared to plasma MMP9, beginning at 4 hours, maximising at 24 hours, and remaining at a high level until 72 hours.\(^36\) Neutrophils are major sources of blood MMP9, and their infiltration into the ischemic brain enhances central MMP9 levels by releasing the MMP9 proform.\(^5,37\) Recent data reveal that reperfusion after tPA treatment promotes the degranulation of human neutrophils and release of MMP9.\(^38\) The neutrophil-derived MMP9 has been shown to be important in postischemic BBB breakdown, leukocyte infiltration, and brain damage.\(^5\) The specific inhibition of neutrophil-derived MMP9 by Tregs would thus explain the drastic decrease of MMP9 in the blood and in the brain of Treg-treated animals and the Treg-afforded neuroprotection after cerebral ischemia/reperfusion. In support of a neutrophil–Treg interaction in vivo, infused Tregs were observed in the bone marrow pool, circulating pool, and the marginating pools (spleen, liver, and lung) of neutrophils at 1 day after MCAO. Immunostaining further demonstrated neutrophils in contact with Tregs in the spleen. Furthermore, we showed that neutrophils and MMP9 are critical for Treg-afforded neuroprotection because Tregs lost their early protective effects in peripheral MMP9-deficient or neutrophil-depleted mice. Therefore, although the involvement of other MMP9 secreting cells, such as endothelial cells and macrophages, in the action of Tregs cannot be excluded, our results strongly suggest that neutrophils are a novel direct target for Tregs after ischemic insults.

A poststroke alteration in the systemic immune system is the immunosuppression characterized by loss of leukocytes and impairment of cell-mediated immunity,\(^24\) which predisposes stroke victims to infectious complications. It was important to show that Treg treatment did not exacerbate poststroke immunosuppression from the perspective of future clinical translation of this therapy. Instead, Treg-treated animals showed preserved lymphocyte populations in the blood and spleen after MCAO.
This may be the consequence of less activated immune systems and fewer exhausted peripheral immune cells. It thus seems that Treg treatment benefits poststroke immune status while restricting inflammatory overactivation, in line with its modulatory role in immune homeostasis. However, our study only assessed the short-term effect of Tregs on the number of peripheral immune cells. Further immunologic studies would be necessary to assess the long-term effect of Treg treatment on poststroke immune functions.

In conclusion, we report that Tregs protect the brain against ischemic/reperfusion injury and that this effect is associated with reduced inflammatory responses in the brain. Furthermore, Tregs attenuated BBB disruption following ischemia/reperfusion and subsequent infiltration of peripheral inflammatory cells. We also characterized a neuroprotective mechanism whereby Tregs inhibit neutrophil-derived MMP9 (Fig 8). Our study suggests that Treg adoptive transfer is a novel and potent cell-based therapy specifically targeting poststroke inflammatory dysregulation and neurovascular disruption.

Acknowledgment

This work was supported by the Veterans Health Administration (GRECC pilot grant to J.C.), NIH NINDS grants (NS36736, NS43802, and NS45048 to J.C.), and grants from the American Heart Association (10POST4150028 to X.H., 10SDG2560122 to F.Z.). B.-L.S. was supported by Chinese Natural Science Foundation grants (8107094).

We thank Drs A. Planas and R. K. Leak for detailed comments on the manuscript and helpful discussions; B. Matta, B. Rosborough, Y. Zhu, X. Gao, and G. Li for technical assistance; and S. Giegel for editorial assistance.

Authorship

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Potential Conflicts of Interest
Nothing to report.
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