Distinctive Effects of T Cell Subsets in Neuronal Injury Induced by Cocultured Splenocytes In Vitro and by In Vivo Stroke in Mice

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Background and Purpose—T cells and their subsets modulate ischemic brain injury. We studied the effects of the absence of T cell subsets on brain infarction after in vivo stroke and then used an in vitro coculture system of splenocytes and neurons to further identify the roles of T cell subsets in neuronal death.

Methods—Stroke was induced by middle cerebral artery suture occlusion in mice and infarct sizes were measured 2 days poststroke. Splenocytes were cocultured with neurons, and neuronal survival was measured 3 days later.

Results—A deficiency of both T and B cells (severe combined immunodeficiency) and the paucity of CD4 or CD8 T cells equally resulted in smaller infarct sizes as measured 2 days poststroke. Although a functional deficiency of regulatory T cells had no effect, impaired Th1 immunity reduced infarction and impaired Th2 immunity aggravated brain injury, which may be due to an inhibited and enhanced inflammatory response in mice deficient in Th1 and Th2 immunity, respectively. In the in vitro coculture system, wild-type splenocytes resulted in dose-dependent neuronal death. The neurotoxicity of splenocytes from these immunodeficient mice was consistent with their effects on stroke in vivo, except for the mice with the paucity of CD4 or CD8 T cells, which did not alter the ratio of neuronal death.

Conclusion—T cell subsets play critical roles in brain injury induced by stroke. The detrimental versus beneficial effects of Th1 cells and Th2 cells both in vivo and in vitro reveal differential therapeutic target strategies for stroke treatment.

Key Words: cerebral ischemia ■ stroke ■ T cells ■ Th1 ■ Th2

Inflammation-mediated brain injury is aggravated by both the innate and adaptive immune systems.1 The innate immune response mainly involves macrophages and neutrophils, whereas the adaptive immune response is regulated by T cells and B cells. T cells consist of CD4 and CD8 (cytotoxic T or Tc) T cells. Based on the cytokine profiles and functionality, CD4 T cells are divided into Type 1 helper T cells (Th1), Th2 cells, and regulatory T (Treg) cells.2 Although Th1 cells are proinflammatory, both Th2 cells and Treg cells are anti-inflammatory.3 The adaptive immune system and innate immune cells may crosstalk to regulate brain injury.

The detrimental effects of macrophages and neutrophils in ischemic brain injury have been extensively studied.4–11 Macrophages, which are derived from brain resident microglia and/or circulating monocytes, are activated and recruited a few hours after stroke onset. Macrophages significantly exacerbate brain infarction. Neutrophils are also recruited into the ischemic brain and contribute to brain injury. Nevertheless, only a few groups have studied the roles of T cells and their subsets as well as functional subsets of CD4 T cells in ischemic brain injury. Hurn et al12 showed T cell deficiency robustly reduced infarct size poststroke. Kleinschnitz et al13 and Yalmaz et al14 showed that the absence of CD4 T cells or CD8 T cells resulted in a similar reduction in infarct size as measured 1 day poststroke. In addition, Liesz et al15 showed that Treg cells were responsible for late phase of infarction as measured at 1 week but not for acute infarction measured 1 to 3 days poststroke. Most recently, interleukin-4 deficiency in mice, which results in Th2 impairment,16 led to a larger infarction measured at 2 days poststroke.17 How Th1 deficiency affects brain injury has not been reported, and no studies have compared the deficiency of T cell subsets or functionally distinct CD4 T cell subsets on stroke-induced brain injury in a single experimental setting. We thus investigated these subsets and directly compared their effects on stroke.

Little is known about the protective or detrimental mechanisms of these distinctive T cell and CD4 T cell subsets in brain injury induced by stroke. T cells are recruited into the
ischemic brain as early as 24 hours after stroke.12,14 However, whether physical contact between T cells and neurons causes neuronal death is unknown. To further identify T cell subsets critical to brain injury, we cocultured splenocytes and neurons in a coculture system, allowing direct contact between lymphocytes and neurons. We compared the effects on neuronal death of the splenocytes from mice with the deficiency in total lymphocytes, CD8 T cells, CD4 T cells, or functional distinct CD4 T cells subsets (Th1, Th2, or Treg cells). Moreover, we examined acute brain infarct sizes poststroke in these mice and compared differences between in vitro and in vivo results. Last, we investigated whether Th1 or Th2 deficiency affects brain injury by regulating the activity of innate immune cells such as macrophages and neutrophils in an in vivo experimental setting.

**Materials and Methods**

**Animals**

Mice from Jackson Laboratory (Bar Harbor, ME) were bred at the Stanford animal facility, except for the C57BL/6J wild-type (WT) mice. Immune-deficient animals used included severe combined immunodeficient (SCID) mice with a B6 genetic background, CD8 T cell-deficient mice (B6.129S2-Tap1<sup>tm1Arp/J</sup>), CD4 T cell-impaired mice (B6.129S2-H2<sup>d</sup>Ab1<sup>tm1F1J/J</sup>), Th1-impaired mice (B6.129S2-Mapk9<sup>tm1F1J/J</sup>), Th2-impaired mice (C57BL/6-Iii4<sup>tm1Nnt/J</sup>), and Treg-impaired mice (B6.129X1-Ebi3<sup>tm1Rsb/J</sup>). In addition, we used an enhanced green fluorescent protein mouse (C57BL/6-Tg[CAG-GFP]1310ObLeySo1) for tracking whether splenocytes were washed away in coculture with neurons. All protocols were approved by the Stanford Institutional Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Stroke Model, Neurological Deficit Scores, and Cerebral Blood Flow Measurement**

Male mice, 10 to 12 weeks old (25–30 g), were anesthetized with 2% isoflurane in 70% air and balanced O<sub>2</sub> using a face mask. Middle cerebral artery occlusion was induced by the insertion of a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA) into the cerebral artery occlusion was induced by the insertion of a silicone-coated 6-0 monofilament (Doccol Corp, Redlands, CA) into the middle cerebral artery internal carotid artery for 1 hour followed by reperfusion, as described.15 Rectal temperature was maintained at 37 ± 0.5°C with a heating pad (Harvard Apparatus, Holliston, MA). Heart rate, oxygen saturation, and respiratory rate were monitored continuously (STARR Life Sciences Corp, Allston Park, MA). Animals with no observable deficits immediately after ischemia, those that died within 48 hours, and those with subarachnoid hemorrhage at the time of death were excluded from analysis. Brains were sectioned coronally after 48 hours into 4 slices and stained in 2% 2,3,5-triphenyltetrazolium chloride. Infarct size was analyzed, normalized blindly to the nonischemic hemisphere, and expressed as a percentage and corrected for edema using the National Institutes of Health Image program (Image J 1.37v). Neurological deficit scores were evaluated at 48 hours according to a neurological grading score16 from 0 (no observable neurological deficit) to 4 (unable to walk spontaneously and a depressed level of consciousness). The evaluator was blinded to experimental treatments. In separate animals, regional cerebral blood flow was monitored through a microtip fiberoptic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PerFlux 5000; Perimed AB, Stockholm, Sweden). A small incision was made at the coordinate 1 mm caudal to the bregma and 3.3 mm lateral to the midline in the ischemic hemisphere to expose the skull, and the laser Doppler probe was attached to the exposed skull. Cerebral blood flow was measured 10 minutes before ischemia onset, during (30 minutes after stroke onset) ischemia, and 5 minutes after reperfusion. All data were normalized to the values of cerebral blood flow measured before stroke in WT animals and expressed as relative ratios.

**Coculture of Pure Cortical Neurons or Mixed Neurons and Astrocytes With Splenocytes**

A primary cortical neuronal culture (purity of neurons were >99%) was conducted as described previously.19 Cortex from fetal Swiss Webster mice (Charles Rivers, Wilmington, MA) at 15 days gestation was dissected, collected, and digested in 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA). Cells (4.5 × 10<sup>5</sup>/mL) were plated at 200 μL/well in 96-well plates and incubated at 37°C, 5% CO<sub>2</sub>. After 26 to 30 hours, 60% of the plating medium was replaced by glia conditioned medium with B-27 serum-free supplement. Cyto- sine arabinoside (3 μmol/L) was added to inhibit glial cell proliferation. Cells were continuously incubated without further medium changes until used for coculture with splenocytes at 9 days in vitro.

To generate mixture of neurons and astrocytes primary culture, astrocyte cultures were first prepared as previously described.20 Briefly, cortices freed of meninges were dissected from newborn (Days 1–3) Swiss-Webster mice, minced, and treated with 0.25% trypsin with EDTA (Invitrogen) for 40 minutes at 37°C in a water bath. The cells were resuspended in Dulbecco’s modified Eagle’s medium containing high-glucose, L-glutamine and supplemented with 10% equine serum, 10% fetal bovine serum, and 10 ng/mL epidermal growth factor (Sigma-Aldrich, St Louis, MO) and pipetted to a single-cell suspension. The cell suspension (200 μL/well) was plated in Falcon Primaria 96-well plates (Becton-Dickinson, Franklin Lakes, NJ) at a density of 2 hemispheres/10 mL. On Day 10 to 11, when the astrocytes were 100% confluent, the medium was changed to high-glucose Dulbecco’s modified Eagle’s medium with 10% equine serum. On Day 14, after completely removing the astrocyte growth medium, cortical neurons were dissected and planted on the astrocyte monolayer by 0.9 × 10<sup>5</sup>/well in Dulbecco’s modified Eagle’s medium supplemented with 5% equine serum and 5% fetal bovine serum. Cells were then 1/2 fed by this medium twice per week. On Day 9 of the neuronal-astrocyte culture, cells were cocultured with splenocytes.

To prepare the splenocyte cultures, mouse spleens were chopped into small pieces, homogenized, filtered through a 70-μm strainer twice, and recovered to a 50-μL solution. The solution was centrifuged at 1200 rpm for 5 minutes at room temperature. After removal of the supernatant, 5 mL ACK Lysing buffer (GBCO; Invitrogen) was added to the cell sediment to lyse erythrocytes. After incubation for 5 minutes at room temperature, RPMI1640 was added to stop the lysis reaction and centrifuged at 1200 rpm for 5 minutes at room temperature. The cell pellet was resuspended in 5 mL Dulbecco’s modified Eagle’s medium (Invitrogen) and counted.

Splenocytes were added into pure neuronal cultures or mixed neuron cultures on Day 9 after they were prepared. The coculture system was incubated for another 72 hours. Splenocytes were removed completely by repeated wash and 100 μL Dulbecco’s modified Eagle’s medium + 5% ES was added to each well. Next 10 μL CCK-8 solution was added to each well and incubated at 37°C, 5% CO<sub>2</sub> for 1.5 hours. The absorbance at 450 nm was measured by a microplate reader with reference wavelength at 650 nm.

**Immunofluorescent Staining**

Ischemic or sham-operated mice 48 hours after stroke onset were euthanized with an overdose of isoflurane and perfused with iced phosphate-buffered saline and then embedded in optical cutting temperature tissue frozen medium. The brain was cut into 5-μm sections and fixed with cold acetone. Immunofluorescent staining was carried out under moderate shaking. All washes and incubations were done in 0.1 mol/L phosphate-buffered saline (pH 7.4). Sections were incubated for 1 hour with blocking solution (0.1 mol/L phosphate-buffered saline, 5% equine serum). After washes, sections were incubated overnight at 4°C with rat antimonocline primary antibody for CD68 (diluted 1:200; MCA1957GA; AbD Serotec, Kidlington, Oxford, UK), a marker for reactive macrophages/microglia, or a rabbit antihuman my-
eloperoxidase antibody (MPO, diluted 1:50, #A0398; Dako North America, Inc, Carpinteria, CA). Sections were then rinsed and incubated for 2 hours at room temperature with an Alexa 488-conjugated goat antirabbit (for MPO) or Alexa 488-conjugated goat antirat (for CD68-positive macrophages/microglia, diluted 1:200; Invitrogen) secondary antibodies. The sections were then washed and covered using Vectashield mounting medium with 4’, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). A negative control without primary antibodies was performed in parallel.

The expression of CD68 or MPO was investigated using the optical fractionator method on epifluorescent photomicrographs (Zeiss axiovert inverted scanning microscope; Zeiss). For each animal, 3 sections were chosen for an average value per mouse and the number of immunoreactive cells (for both CD68 and MPO) in the predefined infarct area was counted using Image J software (Image J 1.37v; Wayne Rasband, available through National Institutes of Health). All counts were performed on coded sections to blind the investigator to the treatment group.

Statistics
All results were presented as mean±SEM. Statistical analyses were performed by analysis of variance followed by the Student-Newman-Keuls post hoc test. Tests were considered significant at probability values <0.05.

Table. CBF Was Measured Before Stroke Onset, During Stroke, and After Reperfusion

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Before MCAO</th>
<th>During MCAO</th>
<th>After MCAO</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>1.000±0.038</td>
<td>0.2706±0.0124</td>
<td>0.8580±0.0328</td>
</tr>
<tr>
<td>SCID</td>
<td>1.031±0.036</td>
<td>0.2632±0.0078</td>
<td>0.8830±0.0330</td>
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<tr>
<td>Tap-1 KO (CD8-)</td>
<td>1.019±0.047</td>
<td>0.2642±0.0162</td>
<td>0.8617±0.04082</td>
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<tr>
<td>MHCI KO (CD4-)</td>
<td>1.084±0.039</td>
<td>0.2660±0.0116</td>
<td>0.941±0.0417</td>
</tr>
<tr>
<td>JNK2 KO (Th1-)</td>
<td>1.082±0.0349</td>
<td>0.2642±0.0154</td>
<td>0.8476±0.03626</td>
</tr>
<tr>
<td>IL-4 KO (Th2-)</td>
<td>1.016±0.051</td>
<td>0.2503±0.016</td>
<td>0.8645±0.04335</td>
</tr>
<tr>
<td>Ebi3 KO (Treg-)</td>
<td>1.040±0.035</td>
<td>0.2531±0.0155</td>
<td>0.8941±0.03568</td>
</tr>
</tbody>
</table>

CBF indicates cerebral blood flow; MCAO, middle cerebral artery occlusion; WT, wild-type; SCID, severe combined immunodeficient; KO, knockout; MHC, major histocompatibility complex; IL-4, interleukin-4; Treg, regulatory T cells.

Figure 1. The effects of immune deficiency on infarct size and neurological scores poststroke. Data were divided into Part I (A, C) and Part II (B, D) to focus on comparing T cell subsets and CD4 T cell subsets, respectively. Part I includes the deficiency or paucity of T and B cells, CD4, or CD8 cells. Part II includes the paucity of CD4 T cells or their functional deficiency (Th1, Th2, or Treg). The deficiency for MHCII and Tap-1 that markedly reduces the number of CD4 T cells and CD8 T cells, respectively, is indicated on the figure. A and B. Infarct sizes. Infarct sizes were measured 2 days poststroke. The top panels are representative ischemic brains with TTC staining. The bottom bar graphs represent average infarct sizes. Although Th1 deficiency resulted in smaller infarct volumes than the paucity of CD4 T cells, no significant difference was observed. C and D. Neurological scores. Neurological scores were measured 2 days after stroke. Bar graphs corresponding to infarct sizes compare the effects of T cell and CD4 T cell subset deficiencies on neurological scores. WT indicates wild-type. *, **, *** versus WT, P<0.05, 0.01, and 0.001, respectively. Treg indicates regulatory T cells; MHC, major histocompatibility complex; TTC, 2-3-5-triphenyl tetrazolium chloride.
Results

Distinctive Effects of the Deficiency of T Cell Subsets or CD4 T Cell Subsets in Ischemic Stroke In Vivo

We measured changes in cerebral blood flow during stroke and after reperfusion. Cerebral blood flow was reduced to approximately 27% at the measured cortex and recovered to approximately 87% after reperfusion compared with that before stroke. These values were not significantly different in mice with the deficiency in either T cell subset or functional CD4 T cell subset (Table).

Infarct sizes were measured 2 days after stroke. A deficiency of both T cells and B cells, CD4, or CD8 T cells resulted in similarly reduced infarct sizes (Figure 1A). For CD4 T cell subsets, Th2 deficiency aggravated infarct size and Th1 deficiency inhibited infarct size. The functional deficiency of Treg, however, did not affect infarct size (Figure 1B). Changes in neurological scores after stroke in these animals were consistent with infarct sizes (Figures 1C and 1D).

Th1 Deficiency Inhibited, Whereas Th2 Deficiency Promoted the Inflammatory Response

We examined the effects of Th1 or Th2 deficiency on macrophage and neutrophil activity 2 days after stroke. Immunostaining showed robust protein expression of CD68, the macrophage activity marker, and MPO, the neutrophil activity marker, in the ischemic brains of WT mice (Figure 2A). However, Th1 deficiency inhibited, whereas Th2 deficiency promoted their expression (Figure 2B).

The Neurotoxicity of Splenocytes In Vitro

We used the CCK-8 kit to measure neuronal survival after washing away cocultured splenocytes in vitro. To confirm that splenocytes were completely removed before measuring neuronal survival, we added enhanced green fluorescent protein-positive splenocytes into cultured pure neurons to prove that almost all enhanced green fluorescent protein-positive splenocytes were removed from the coculture after washing (Figure 3C). This ensures that measured cell survival was exclusively derived from the neurons.

We determined the optimal ratio of splenocytes to neurons being able to induce neuronal death. Addition of splenocytes to the culture of pure neurons resulted in a dose-dependent neuronal death (Figure 3A). For the remaining experiments, we selected the ratio of 1:5 (neurons:splenocytes), which resulted in approximately 40% neuronal death. We then added splenocytes to a mixed culture of neurons and astrocytes to test for neuron/astrocyte death using the same ratio of 1:5. In this case, splenocytes did not cause cell death. (Figure 3B).

We further investigated the effects of splenocytes from several immunodeficient animals on cultured neurons. The results show that splenocytes from SCID mice, but not from CD4- or CD8-deficient mice, resulted in less neuronal death compared with splenocytes from WT mice (Figure 4A). Th1-deficient splenocytes caused less neuronal death, whereas Th2-deficient splenocytes significantly aggravated neuronal death. Splenocytes from functional Treg-deficient mice had no effect (Figure 4B).

Figure 2. Th1 deficiency inhibited, while Th2 deficiency promoted inflammation after stroke. A, The top picture shows a representative coronal brain section with cresyl/violet staining, on which the square is drawn to represent the area where pictures of immunostaining were taken and cells were counted. The bottom two panels are representative immunostaining of the macrophage marker, CD68, and neutrophil activity marker, MPO, at 48 hours after stroke in wild type, Th1 and Th2 deficient mice. Scale bar, 20 μm. B, Statistical results of CD68 and MPO positive cell numbers. The immune positive cells were counted and the numbers for wild type mice, Th1 and Th2 deficient mice are portrayed in the bar graphs. *, ** vs WT, P<0.05, 0.01, respectively.
Discussion

To the best of our knowledge our study is the first to systemically examine the effects of splenocytes and several immunodeficiency on neuronal death in an in vitro coculture system, which offers a valuable method to study the effects of lymphocytes on neuronal death. We found that WT splenocytes killed neurons in the pure neuronal culture in a dose-dependent manner. WT splenocytes at a ratio of 1:5 (neuron:lymphocytes) resulted in approximately 40% neuronal death. Nevertheless, WT splenocytes did not cause cell death when cocultured with the mixture of astrocytes and neurons, suggesting that astrocytes may play a protective role.

Figure 3. The establishment of the co-culturing system of splenocytes with pure neurons. A, Splenocytes dose-dependently caused neuronal death in pure neuronal culture. Splenocytes were added to pure neuronal culture according to a series of ratios of neurons to splenocytes, from 1:0.4 to 1:10. Higher concentrations of splenocytes resulted in more neuronal death. Neuronal survival was measured by the CCK-8 kit 3 days after co-culturing, and transformed into a ratio of neuronal death. B, Splenocytes did not cause cell death in mixed neuron and astrocyte culture with the ratio of 1:5 (mixed neurons and astrocytes:splenocytes). Note that survival ratio was not transferred into a ratio of neuronal death in this part of the figure. C, The microscopy study suggests that splenocytes were washed away from the co-culture system before neuronal survival was measured. To confirm that splenocytes were removed before measuring neuronal survival, EGFP positive splenocytes were added to pure neuronal culture. Pictures were taken 3 days later before or after wash. The phase contrast image shows that neurons (arrows) and lymphocytes (arrow heads) co-existed in the culture. The EGFP fluorescent image further shows that EGFP positive lymphocytes existed in the culture before wash. However, after wash with media, almost all EGFP splenocytes were washed away, and only MAP-2 positive neurons were remained. The picture taken from the culture with vehicle treatment without lymphocytes shows higher densities of MAP-2 positive neurons than the one treated with lymphocytes. Scale bar, 20 μm.

Figure 4. The effects of splenocytes on neuronal death in the coculture system. Similarly to Figure 1, the data were divided into Part I (A) and Part II (B). A, Comparison of the paucity of T cell subsets on neuronal death. Splenocytes with total lymphocyte deficiency (T and B cells in SCID mice) and the paucity of CD4 T cells or CD8 T cells in coculture resulted in less neuronal death. B, The effects of the deficiency of individual functional CD4 T cell subsets on neuronal death. The deficient genes and corresponding phenotypes are also labeled. The experimental numbers on the bar graphs represent the total plate well numbers for each cell type, which were repeated 3 times on different days. WT indicates wild-type; SCID, severe combined immunodeficient. **, *** versus WT, P<0.01 and 0.001, respectively.
against lymphocyte-induced neuron death, which is consistent with many previous studies on the neuroprotective effects of astrocytes. Using an in vitro coculture system with pure neurons, we found that splenocytes from SCID mice, but not from the mice with the paucity of CD4 or CD8 T cells, resulted in less neuronal death compared with WT splenocytes. We also found that neuronal death was inhibited by Th1 deficiency, enhanced by Th2 deficiency, and unaltered by functional deficiency of Treg cells.

The in vitro results are largely consistent with the in vivo pathological outcomes, except for the fact that the paucity of CD4 T cells and CD8 T cells in mice resulted in equal reductions in infarct sizes in vivo. The underlying mechanisms for this discrepancy between in vitro and in vivo experiments are unknown. Major factors contributing to the in vitro setting may include different physical interactions between neurons and T cells, various cytokine releases, the lack of ischemia, or the absence of in situ reactions of microglia and other cell types. However, our in vitro data may suggest that the direct physical contact of CD4 T cells or CD8 T cells with neurons is not critical for their detrimental effects. CD4 T cells and CD8 T cells may indirectly affect ischemic brain injury by altering the functions of macrophages, the final direct effectors on neuronal death in vivo. In addition, we found that functional deficiency of Treg cells did not alter neuronal death and acute infarction both in vitro and in vivo; these results are consistent with previous reports.15

We demonstrated the first evidence that Th1 cell deficiency resulted in a robust reduction in infarct size and neuronal death in vivo and in vitro. A previous study showed that the interleukin-4 deficiency in mice resulted in a larger infarction size and neuronal death in vivo and in vitro; these results are consistent with previous reports.15

In conclusion, both in vitro and in vivo studies suggest distinctive effects of T cell subsets as well as CD4 and CD8 T cell subsets in ischemic brain injury. The distinctive effects of the Th1 versus Th2 response in inflammation indicate selective therapeutic targets for stroke treatment.

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Disclosures
None.

References
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