In Vivo Real-Time Multiphoton Imaging of T Lymphocytes in the Mouse Brain After Experimental Stroke

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Background and Purpose—To gain a better understanding of T cell behavior after stroke, we have developed real-time in vivo brain imaging of T cells by multiphoton microscopy after middle cerebral artery occlusion.

Methods—Adult male hCD2-GFP transgenic mice that exhibit green fluorescent protein-labeled T cells underwent permanent left distal middle cerebral artery occlusion by electrocoagulation (n=6) or sham surgery (n=6) and then multiphoton laser imaging 72 hours later.

Results—Extravasated T cell number significantly increased after middle cerebral artery occlusion versus sham. Two T cell subpopulations existed after middle cerebral artery occlusion, possibly driven by 2 T cell subpopulations; 1 had significantly lower and the other significantly higher track velocity and displacement rate than sham.

Conclusions—The different motilities and behaviors of T cells observed using our imaging approach after stroke could reveal important mechanisms of immune surveillance for future therapeutic exploitations. (Stroke. 2011;42:1429-1436.)

Key Words: cerebral infarction ■ cerebral ischemia ■ immune system ■ inflammation

Activation of the immune response is a hallmark of acute stroke and recently T lymphocytes have been revealed as potential therapeutic targets. Mice deficient in lymphocytes have been shown to exhibit reduced infarct volume after transient occlusion of the middle cerebral artery by an intraluminal filament and after permanent occlusion by electrocoagulation (pMCAO). Infarct volume after transient middle cerebral artery occlusion has also reportedly been reduced by the immunosuppressant FTY720, which blocks T cell infiltration into the brain. Despite the deleterious effect of T cells on stroke, subpopulations of T cells may be recruited to the inflamed brain, although this lacks the spatial resolution needed to study individual cells. Epifluorescence video microscopy, an additional in vivo tool, meets the requirements of high resolution but it does not allow real-time imaging in 3-dimensional space. In addition, it has major limitations needing a cranial window that may possibly induce brain injury.

The introduction of multiphoton laser scanning microscopy (MPLSM) represents a major qualitative advancement in immune cell imaging, allowing high-resolution visualization of cells within a 3-dimensional space over time. This technique can image the movements of single cells at depths of hundreds of microns into living tissue and could provide fundamental insight into how T cells and antigen-presenting cells access and behave within the ischemic brain. We have previously used MPLSM to image superfused slices of ischemic brain ex vivo and were able to analyze the detailed processes involved in the immune response, real-time information is required. However, the in vivo imaging approaches developed so far for imaging immune cells after stroke are restricted. MRI has been used for tracking macrophages recruited to the inflamed brain, although this lacks the spatial resolution needed to study individual cells. Epifluorescence video microscopy, an additional in vivo tool, meets the requirements of high resolution but it does not allow real-time imaging in 3-dimensional space. In addition, it has major limitations needing a cranial window that may possibly induce brain injury.

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movements of T cells. However, immune cell mobility and activation are drastically altered in brain slice cultures, where the vascular system is not preserved. MPLSM has recently been used to image T cell behavior in vivo in the lower spinal cord in models of cerebral autoimmune disease. However, considering the difficulties in localizing the ischemic area of interest and that there are far fewer T cells extravasated in stroke compared with autoimmune disease, this in vivo approach has to date never been applied to image T cells after stroke.

We now report real-time in vivo MPLSM imaging and quantitative tracking of endogenous T cell dynamics in the brain after a focal cerebral ischemic insult using transgenic mice whose T cells express green fluorescent protein (GFP). To study T cells in the pMCAO model, it was necessary to image in and near the territory of the middle cerebral artery.
To avoid performing a craniotomy, which has been shown to have pathological effects, we imaged through the thinned skull. The imaging was performed 72 hours after pMCAO, because the results of Jander and Schroeter and our preliminary data suggested that the population of infiltrated T cells was near its peak at this time point.

Materials and Methods

Animals
Adult male CBA/Ca×C57F7BL/10 hCD2-GFP transgenic mice that express GFP under control of the hCD2 promoter (kindly gifted by Dr Dimitris Kioussi, National Institute for Medical Research, London, UK) were maintained on a 12/12-hour light/dark cycle with free access to food and water at the University of Strathclyde, and all the procedures were performed in accordance with local ethical and UK Home Office regulations. GFP is expressed almost exclusively in thymocytes, and virtually all mature thymocytes express it (irrespective of CD4 or CD8 expression); peripheral B cells show approximately 1% of the GFP fluorescence of T cells.

Permanent Middle Cerebral Artery Occlusion
The mice underwent left distal pMCAO by electrocoagulation (n=6) or sham surgery (n=6) under isoflurane anesthesia (induction: 3% in 100% O2; maintenance, 1.5% to 2.0%) with body temperature maintained at 37°C throughout the procedure.

Animal Preparation for MPLSM

Surgical Preparation
Seventy-two hours after middle cerebral artery occlusion, blood was labeled red with either QTracker quantum dots (655 nm, red, Invitrogen; 30 μL mixed with 70 μL phosphate-buffered saline) or rhodamine B isothiocyanate-dextran (RhITC-dextran, 70 kDa, Sigma; 5% in phosphate-buffered saline, 150 μL). Mice were isoflurane-anesthetized with rectal temperature maintained at 36.2°C to 37°C. The left parietal skull was exposed and dried; a point near 2.5 mm lateral, 1 mm posterior to the bregma was marked. A stainless steel plate 0.4 mm thick with a 5-mm hole was glued to the skull with its center over the mark (Figure 1A). Under a dissecting microscope, the skull was thinned using a NSK Vmax Volvere dental drill (Wright Cottrell) with a diamond burr (Diama Labcraft). When the pial vasculature could be clearly seen (Figure 1B), the mouse on its base plate was moved to the imaging microscope and the thinned skull superfused with Ca2+ saline, forming a meniscus to the objective.

Tracking the Coordinates of Recording Sites
To determine the coordinates of each recording site, we took photographs through the dissecting microscope. The sites of imaging...
were identified by their relation to blood vessels and the stereotaxic coordinates (Figure 1C–D). The green autofluorescence of the skull bone was used to determine the underside of the skull, which we defined as \( z=0 \).

**Multiphoton Imaging**

A Ti:sapphire femtosecond laser (Chameleon; Coherent) provided pulsed infrared light through a Radiance 2000 scan head (BioRad) to an upright microscope (Eclipse; Nikon) with a large micrometer-driven stage. A Nikon Fluor 40×, NA 0.80 water immersion objective was used. The maximum beam power at 830 nm was 49.2 mW and at 940 nm 20.5 mW. In naive mice, unintended signs of laser-induced damage such as rolling of cells along vessel walls were not observed in our protocol.

To image GFP-expressing T cells, we used the longest available excitation wavelength, 940 nm. Both of the blood markers used (Qtracker and RhITC-dextran) absorb more strongly at 830 nm than at 940 nm; therefore, in few cases, the vasculature was imaged separately from the T cells.

**Image Acquisition and Processing**

To track extravascular T cells, time series of z-stacks were obtained. Typically, the area imaged was 284 \( \mu \)m \( \times \) 284 \( \mu \)m at 512 \( \times \) 512 pixels and 500 lines per second, the \( z \) step was 2.0 \( \mu \)m, with a total depth of 16 to 20 stacks, and the repetition rate for the stacks was 34 seconds per stack. Four-dimensional reconstructions were analyzed using Velocity 5 software (Improvision).

The location (centroid) of each GFP-positive cell within each 3-dimensional image stack was determined manually by the operator. Only tracks with at least 3 time points were included in the analysis. To quantify T cell behaviors, we calculated, for each cell, its mean velocity as it moved along its track, length covered, displacement (straight line distance from the first position in the track to the last), displacement rate (total displacement during the period of tracking divided by the time it was tracked), and meandering index (displacement/length covered, which provides a measure of a track’s deviation from a straight line). A meandering index of 1 indicates that the track is a perfect straight line; the smaller the value of the meandering index, the greater the meandering of the track.

Images were processed using Adobe Photoshop CS3 (Adobe Systems Europe Ltd). The velocity of cells in the centers of blood vessels was measured by line scans.\(^{22}\)

**Region Compromised by pMCAO**

To check that the point on the skull with coordinates 2.5 mm lateral and 1 mm posterior to bregma was within the damaged area,\(^{23}\) we labeled the brain of 1 pMCAO wild-type mice with Evans blue. A 29-G needle was inserted at these surface coordinates to a depth of 1.5 mm through a craniotomy and 1 \( \mu \)L of Evans blue solution was injected. Twenty-micron coronal brain sections were cut and stained with cresyl violet (Sigma Aldrich) to detect ischemic lesion as previously described.\(^{25}\) In addition, in vivo MPLSM imaging through the skull in an area not too far from the midline but within the lesioned zone. We confirmed that the surface coordinates 2.5 mm lateral, 1.0 mm posterior to bregma met these requirements. Intravenous injection of propidium iodide was used to label dead cells\(^{25}\) and produced little labeling of cells in sham-operated mice, showing that the procedure of thinning the skull caused no cell death or did not compromise the blood–brain barrier (Figure 2A).\(^{26}\) After pMCAO, numerous cells were stained, particularly near the central coordinate point (Figure 2B and Supplemental Figure 1B–C). These results show that in the areas where T cells were imaged after pMCAO, propidium iodide could extravasate from perfused vessels and cross the membranes of many cells. This indicates opening of the blood–brain barrier and, perhaps, debility of the stained cells.\(^{25}\) In addition, in vivo Evans blue staining at the image site (1 \( \mu \)L of Evans blue injected at 1 mm posterior, 2.5 mm lateral from bregma and 1.5 mm ventral to the skull, 72 hours after pMCAO) was colocalized with the autofluorescence produced by the in-

**Immunofluorescence and Confocal Analysis**

Immunofluorescence was performed using previously described protocols\(^{24}\) as described in the Supplemental Methods (http://stroke.ahajournals.org).

**Statistical Analysis**

Results are expressed as mean±SEM of 6 mice per experimental group and nonnormal distributions compared using the 2-tailed Mann-Whitney. One-way analysis of variance and Dunn post hoc test were applied for movement parameters using Prism software (Graph Pad, San Diego, CA). The level of statistical significance was 0.05 per test.

**Results**

As expected, a significant area of lesion was observed at 72 hours after pMCAO (Figure 2). It was our aim to perform in vivo MPLSM imaging through the skull in an area not too far from the midline but within the lesioned zone. We confirmed that the surface coordinates 2.5 mm lateral, 1.0 mm posterior to bregma met these requirements. Intravenous injection of propidium iodide was used to label dead cells\(^{25}\) and produced little labeling of cells in sham-operated mice, showing that the procedure of thinning the skull caused no cell death or did not compromise the blood–brain barrier (Figure 2A).\(^{26}\) After pMCAO, numerous cells were stained, particularly near the central coordinate point (Figure 2B and Supplemental Figure 1B–C). These results show that in the areas where T cells were imaged after pMCAO, propidium iodide could extravasate from perfused vessels and cross the membranes of many cells. This indicates opening of the blood–brain barrier and, perhaps, debility of the stained cells.\(^{25}\) In addition, in vivo Evans blue staining at the image site (1 \( \mu \)L of Evans blue injected at 1 mm posterior, 2.5 mm lateral from bregma and 1.5 mm ventral to the skull, 72 hours after pMCAO) was colocalized with the autofluorescence produced by the in-
No T cells were observed in virtually all areas where the blood was present (Figures 3B, D). The number of extravascular T cells observed in each imaging field were normalized for the volume of the field and averaged for each mouse. The mean T cell density was significantly \((P<0.05)\) in pMCAO mice (5.1±1.2 thousand/mm\(^3\), \(n=6\)) compared with sham-operated mice (sham; 1.9±0.4 thousand/mm\(^3\), \(n=6\)). Note that the true ratio of the T cell populations is greater than the numbers suggest, because the volumes in sham with no injured tissue providing clear confirmation that our imaging site is present inside the compromised area (Figure 2C–D and Supplemental Figure ID–F).

Many areas scanned in sham mice revealed no T cells or rarely more than 1 to 2 extravascular T cells per imaging volume and these were close to the skull (within 20 μm; Figures 3A, C). In contrast, in pMCAO mice, extravascular T cells were observed in virtually all areas where the blood marker was present (Figures 3B, D). The number of extravascular T cells observed in each imaging field were normalized for the volume of the field and averaged for each mouse. The mean T cell density was significantly \((P<0.05)\) higher in pMCAO mice (5.1±1.2 thousand/mm\(^3\), \(n=6\)) compared with sham in all assessed parameters. Single data point and means (bars) are presented. **P<0.01 vs sham; ***P<0.001 vs pMCAO Population 1.

In sham animals, cells moved with an average velocity of 4.78±0.63 μm/min (n=23 T cells) with 39% of them stationary (velocity <2 μm/min\(^2\)). Two main populations were found in pMCAO animals (Figure 5A); 1 population was almost stationary (1.74±0.11 μm/min, n=23 T cells, Population 1) and the other population was quickly moving (8.21±0.10 μm/min, n=25 T cells, Population 2). In addition to exhibiting significantly increased track velocity (Figure 5B), Population 2 showed significantly increased displacement rate (Figure 5C) compared with Population 1 (\(P<0.001\)) and sham (\(P<0.01\)) and a higher meandering index (Figure 5D) compared with Population 1 (\(P<0.001\)) but not sham. Interestingly, Population 1 showed significantly lower \((P<0.01)\) track velocity and displacement rate compared with sham (Figure 5B-C).

With rare exceptions (1 animal in 6 analyzed), the sparse extravascular T cells in sham animals moved slowly and meandered so that their displacement was small (Figures 3A, C; Video 1, Supplemental Material). After pMCAO, most of the numerous T cells (Figures 3B, D) patrolled up and down the perivascular surfaces of blood vessels (Supplemental Figure IIA), sometimes moving from 1 vessel to another (Video 2, Supplemental Material), and few T cells were remote from blood vessels.

T cells, detected in the bloodstream, moved with a similar velocity as erythrocytes (Supplemental Figure IIB–C). After pMCAO, some vessels were poorly perfused, as shown by an absence of fast-moving cells (Supplemental Figure IIC, right panel). Very few T cells were observed on the luminal surface of the vascular endothelium in perfused vessels.

For each mouse, all available T cell tracks were displayed in a single volume (representatives shown in Figure 4, right panels). In the 6 sham-operated mice, the cell movement was generally confined within a radius of <10 μm (Figure 4A), although a few longer displacements were seen (Video 3, Supplemental Material). This behavior is also evident when the tracks were plotted so that their origins coincided (left-hand panels in Figure 4). In contrast, T cells that moved over 10s of microns were observed in all 6 pMCAO mice (representative in Figure 4B), although a subpopulation of almost stationary T cells was also observed.
location in the perivascular space of vessels in contact with components of the neurovascular unit such as astrocytes (glial fibrillary acidic protein-positive) and perivascular macrophages (IB4+/CD11b+; Figure 6).

**Discussion**

Our advances in imaging techniques have allowed us, for the first time to our knowledge, to visualize, define, and quantitatively analyze the movement and behavior of T cells in vivo in real time after stroke. Specifically, we observed a higher number of extravasated T cells in the subarachnoid space after pMCAO than sham and 2 distinct T cell populations in pMCAO mice, 1 that was almost stationary and the second showing higher track velocity and displacement than sham. Infiltrated T cells were observed to patrol along the perivascular surfaces of cerebral vessels.

We have built on technology exploited in infection or autoimmune diseases in which there are several MPLSM studies describing the dynamics of T cells participating in the development of pathology (experimental autoimmune encephalomyelitis, toxoplastic encephalitis, acute viral meningitis). These studies have provided important information on how T cells access the central nervous system and factors that regulate their behavior once in the central nervous system. However, challenges lie ahead for providing insight into neurological diseases such as stroke.

The brain is unique in its development of inflammation because it has no lymphatic system and the blood–brain barrier limits antibody and immune cell entry. Therefore, T cell access is tightly regulated and rare in the normal brain, and despite an increased expression of adhesion molecules during ischemia that facilitates T cell infiltration, there are far fewer T cells in the brain after stroke compared with autoimmune disease. To the best of our knowledge, real-time in vivo T cell movements after stroke have never been successfully visualized and analyzed.

We observed a high number of extravascular T cells 72 hours after pMCAO and only a few T cells after sham. We cannot exclude that the sham procedure (anesthesia, skull thinning) may have induced recruitment of these cells. On the contrary, increased T cell infiltration after stroke is consistent with postmortem immunohistochemistry or flow cytometry studies, although such techniques are limited by the removal, disruption, and processing of tissue at defined time points.

A key observation from our multiphoton microscopy approach is the significant differences in motility, namely track velocity, displacement, and meandering, of extravasated T cells between pMCAO and sham mice. Interestingly, 2 main populations were found in pMCAO mice; both populations showed significantly different dynamics compared with T cells in sham mice. One population showed a mean displacement of 0.25±0.06 μm/min, the other population 3.26±
0.39 µm/min, and sham 1.4±0.4 µm/min. Cordiglieri and colleagues defined in the central nervous system motile cells with displacements >1 µm/min and stationary cells with displacements <1 µm/min; therefore, we consider Population 1 stationary. On the contrary, Population 2 showed track velocity in the range of data already shown in other models of neuroinflammation. The reported differences in track velocities between Population 1 (1.74 µm/min) and Population 2 (8.21 µm/min), in our model, are extremely relevant if compared with T cell velocity in secondary lymphoid organs. We and others have shown that naïve CD4 T cells move at an average velocity 10 µm/min. Both primed and tolerized T cells show slower velocity of approximately 4 to 5 µm/min and cells with velocities <2 µm/min are essentially stationary due to factors like centroid movement. Thus, the observed differences between Populations 1 and 2 and sham in our setting could clearly depend on the different activation status of these cells.

The different motility of the 2 cell populations could reflect the evolution of T cell response from a naïve population to effector/memory cells and/or the behavior of T cells that, after antigen encounter, display an altered profile of adhesion molecules and chemokine receptors. One limitation of our experiments is that GFP expression was not restricted to a specific population of T cells and therefore it is impossible to distinguish which T cell subset was stationary or motile or if this was related to their antigen specificity. Interestingly, recent real-time imaging during recall responses in the lymph node have shown that, in contrast to CD4 T cells, which show slower migration, memory CD8 T cells move more rapidly than naïve cells, suggesting that faster migration could be a defining characteristic of CD8 versus CD4 memory T cells.

Also key to our findings are the paths of these cells once extravasated and we reveal 3 key locations: perivascular, moving from 1 vessel to another, and remote from vessels. The perivascular location of T cells is in line with previous data by Owens who elegantly describes the transmigration of immune cells into the perivascular space and movement of extravasated T cells are likely to be in the perivascular space rather than in the brain parenchyma. We observed T cells patrolling up and down the perivascular surfaces of blood vessels. The object of their survey is unclear, but direct contact of T cells with cellular components of the neurovascular unit has been shown in other inflammatory central nervous system conditions and the perivascular space seems to be a location where immune cells could interact with potential antigen-presenting cells. The immunologic synapse could be formed with astrocytic end feet or perivascular phagocytes, all of which have the capacity to act as antigen-presenting cells. We have attempted to shed further light on these cellular interactions using immunostaining. Although this analysis was performed deeper in the parenchyma compared with the imaged area, we could confirm T cells' location between the endothelium and astrocytic end feet in the perivascular space with a direct contact among astrocytes, macrophages/microglial cells, and T cells. Whether this direct contact is associated with the reported role of the neurovascular unit in stimulating inflammatory responses after stroke is yet to be elucidated, but this would explain the reason for some T cells moving from 1 vessel to another after pMCAO. Lastly, we observed T cells remote from blood vessels in our imaging and again using immunohistochemistry, we observed extravasated T cells quite far from vessels interacting with a microglial cell.

Whether T cells' contribution to cerebral ischemia depends on antigen recognition is still under debate; therefore, a deeper understanding of the cellular interactions between T cells to astrocytes and microglia would help to clarify this issue. By measuring parameters that are likely to influence the character of immune responses (eg, location of T cell activation, location of immune cell interactions, duration and number of interactions, etc) and relating these to the parallel determination of immunologic parameters (eg, cytokine and costimulatory molecules production), a MPLSM strategy could help to analyze these interactions in vivo and hopefully help to elucidate the role of immune cells in stroke.

Conclusions
In conclusion, we have provided previously unavailable information on the dynamics of T lymphocytes recruited by the ischemic brain, which could reveal important mechanisms of immune surveillance after stroke. In addition, our imaging approach represents a major technical advance and an important research tool for the understanding of the complex interactions between the brain and immune cells after ischemia. Combining the development of strategies to deplete specific cell populations/subsets or modify their function and the application of available fluorescent reporters for antigen-presenting cells should allow a dissection of how the behavior of immune cells relates to their function in the ischemic brain. It is hoped that these advances in imaging of immune cells after stroke could provide important information for the development and use of specific therapies for a better management of the immune response in the ischemic brain.

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Disclosures
None.

References


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