Title page

Advances in imaging of new targets for pharmacological intervention in stroke: real time tracking of T cells in the ischaemic brain

Short Running Title: Imaging T cells after focal cerebral ischaemia

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Summary

Background and purpose. T cells may play a role in the evolution of ischaemic damage and repair but the ability to image these cells in the living brain after stroke has been limited. We aim to extend the technique of real time in situ brain imaging of T cells, previously shown in models of immunological diseases, to models of experimental stroke.

Experimental approach. Male C57BL6 mice (6-8 weeks) (n=3) received a total of 2-5 X 10^6 CFSE-labelled lymphocytes from donor C57BL6 mice via i.v. injection by adoptive transfer. Twenty four hours later, recipient mice underwent permanent left distal middle cerebral artery occlusion by electrocoagulation or sham surgery under isoflurane anaesthesia. Female hCD2-GFP transgenic mice that exhibit GFP-labelled T cells underwent MCAO. At 24 or 48 hours post-MCAO, a sagittal brain slice (1500 μm thick) containing cortical branches of the occluded MCA was dissected and used for multiphoton laser-scanning microscopy.

Key results. Our results provide direct observations for the first time of dynamic T cell behaviour in living brain tissue in real time and herein proved the feasibility of multiphoton laser-scanning microscopy for *ex vivo* live imaging of immune response after experimental stroke.

Conclusions and Implications. It is hoped that these advances in imaging of immune cells will provide information that can be harnessed to a therapeutic advantage.

Keywords. Inflammation, leukocytes, brain imaging, middle cerebral artery occlusion, stroke, multiphoton microscopy.

Introduction

Leukocyte infiltration is a major mechanism contributing to the pathogenesis of cerebral ischaemic damage. However, the role of these immune cells in ischaemic damage still needs to be fully understood. Some intriguing proposals that lymphocytes may not be completely harmful for the brain or that manipulation of selected immune cells may result in a therapeutic advantage (Gee et al. 2006; Villoslada et al. 2008) need to be supported by a deep knowledge of the behaviour of these cells during the evolution of ischaemic damage and repair.

Direct observation of dynamic cellular behaviour in living tissues has been facilitated by recent advances in multiphoton laser-scanning microscopy (MPLSM) (Zinselmeyer et al, 2005; Schneider et al. 2006; Maffia et al. 2007; Millington et al. 2007; Cahalan et al.2008). MPLSM provides the ability to illuminate tissues at depth in the absence of significant phototoxicity, allowing analysis of physiological or pathological events over time and has been exploited to study T cells in the brain in experimental autoimmune encephalomyelitis (Flügel et al. 2007; Smorodchenko et al. 2007). We now report, for the first time, real time *in situ* imaging of T cell movement in brain tissue after a focal cerebral ischaemic insult.

Methods

All mice (6-8 weeks) were maintained on a 12/12 h light/dark cycle with free access to food and water at the Biological Procedures Unit, University of Strathclyde, in accordance with local ethical and UK Home Office regulations. Male C57BL6 mice (Harlan-Olac) were sacrificed (n=3) to obtain lymphocytes for adoptive transfer. A total of 2-5 X 10^6 CFSE (Molecular Probe Inc.)-labelled lymphocytes from C57BL6 mice, prepared as previously described (Zinselmeyer et al. 2005; Schneider et al. 2006; Millington et al. 2007), were injected i.v. into age-matched C57BL6 recipients. Twenty four hours later, recipient animals were anaesthetised using 3% isoflurane anaesthesia in 100% O_2 and maintained using 1.5-2%

isoflurane in 100% O_2 as assessed by interdigital reflex. Mice were subjected to permanent left distal middle cerebral artery occlusion (MCAO) by electrocoagulation (Carswell et al. 2005) (males, n=3) or sham surgery (female, n=1). Body temperature was maintained at 37°C throughout procedure.

One female hCD2-GFP transgenic mouse that exhibits GFP-labelled T cells (de Boer et al. 2003) with a very small proportion of GFP-labelled B cells (kindly gifted by Dr. Dimitris Kioussis, NIMR, London, UK) also underwent MCAO.

At 24 or 48 hours post-MCAO, a sagittal brain slice (1500 μm thick) containing cortical branches of the occluded MCA (Fig. 1) was cut, using a Vibratome (Intracel, UK), in oxygenated artificial cerebrospinal fluid (ACSF, containing in mM: NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 2.5, $MgSO_4$ 2, $CaCl_2$ 2 and D-Glucose 10 with 95% O_2/5% CO_2, pH~7.3) (Bushell et al. 2002), glued (Vetbond, 3M) onto a coverslip adhered to the bottom of the imaging chamber and continuously perfused with warmed (35-36°C), gassed (95% O_2 and 5% CO_2) ACSF before and throughout imaging. Imaging was performed using a Radiance 2000MP, MPLSM (Bio-Rad Laboratories Ltd, UK) as described previously (Zinselmeyer et al. 2005; Schneider et al. 2006; Maffia et al. 2007; Millington et al. 2007). The scans were made with 500 lps and between 256 x 256 pixel boxes, and acquired at 20 frames per min. Images were analysed using Volocity software (Improvision, UK).

Results

Initially brain slices were cut in ice-cold (< 4°C) sucrose solution as described previously (Bushell et al. 2002) and imaged at 35-36°C, however, lymphocytes observed in the parenchyma were stationary. This concurs with our own unpublished observations in lymph nodes. Hence, all slices included in the present study were prepared in room temperature ACSF.

Recruitment of labelled lymphocytes in the cerebrovasculature and in the cortex, downstream of occluded MCA in adoptive transfer mice, was observed at 24 hours and 48 hours post- MCAO (Fig. 2A,B respectively). No cells were detectable in the sham-operated mouse (data not shown). Furthermore, since CFSE will label all lymphocyte populations and not just T cells, using an hCD2 GFP transgenic mouse, we imaged, selectively, endogenous T cell movement in the brain at 48 hours post-MCAO (Figure 2C and the accompanying online movie). The average velocity of T cells was 9.4 μm/min although during the imaging period peak velocities approached 32 μm/min compared with average velocities in the region of 12-15 μm/min in lymph nodes previously reported (Zinselmeyer et al. 2005; Miller et al. 2002).

Discussion and conclusion

The presence of T lymphocytes has been previously described by immunohistochemistry at 1-7 days post-MCAO (Li et al. 2005), but their role and behaviour in the parenchyma remains elusive. Our aim was not to quantify T cell recruitment after injury, but rather to provide a feasible protocol for cell detection in the living inflamed brain for future pharmacological targeting. We have proved the feasibility of MPLSM for *in situ* real time imaging of immune response after focal cerebral ischaemia. To the best of our knowledge this is the first demonstration of T cell movement in the brain after cerebral ischaemia and the first application of hCD2-GFP transgenic mice in cerebral ischaemia. Our results have three important implications. Firstly, our visualisation of T cells in the brain implies a dynamic role of T cells, possibly involving direct, physical contact with other cells. Secondly, the use of hCD2-GFP transgenic mice combined with MPLSM represents an important tool for investigating the temporal profile, role and behaviour of endogenous T cells during the evolution of ischaemic damage and repair. Finally, given the growing availability of

transgenic mice expressing fluorescently-labelled cell types (e.g. microglia), our use of adoptive lymphocyte transfer permits future investigations into cellular interactions thereby clarifying lymphocyte activity.

One limitation of the current study is that immune cell mobility and activation could be drastically altered by the slice preparation, therefore the ultimate goal would be the *in vivo* imaging. *In vivo* imaging of immune response in the cortex with multiphoton microscopy has been possible so far only in autoimmune-type disease models, while the dynamic analysis of immune cells behaviour in murine stroke models *in vivo* until now has not been possible due to the technical limitations. A major obstacle is the paucity of immune cells infiltrating the ischaemic area compared with autoimmune disease and another hurdle is the difficulty to implant a cranial window enabling the imaging of the compromised area of interest. On the other hand, *ex vivo* real time imaging bypasses this issue allowing the imaging of the whole cortex, and therefore an easy detection of immune infiltrate. Furthermore, real time imaging of sagittal brain slices could easily allow the visualization of some other brain regions of interest (i.e. striatum, hippocampus) which could be affected by post-ischaemic inflammatory response (Wiart et al. 2007), and which cannot be visualized using conventional *in vivo* multiphoton imaging, due to depth limitation.

For these reasons we strongly believe that our *in situ* real time imaging could represent the first important step towards the in vivo dynamic imaging of immune response after experimental stroke. Our advances in imaging techniques will help define and analyse the cellular interactions, role and behaviour of these immune cells *in situ* after stroke and will help us to understand how intervening in these cellular interactions will affect stroke pathology for future therapeutic exploitations.

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Figure Legends

Figure 1

Representative images of the area of MPLSM imaging (white oval) containing the cortical branches of the occluded MCA (arrow) (A) and of a 1500 μm sagittal slice glued onto a coverslip for MPLSM imaging (B).

Figure 2

Three-dimensional reconstruction of lymphocyte infiltration in the cortex post-MCAO (A+B) At 24 hours post-MCAO fluorescently CFSE-labelled lymphocytes (green) were detectable in a cortical artery and only few cells were infiltrating the parenchyma (A). On the contrary, lymphocyte infiltration in the parenchyma is clearly detectable at 48 hours post-MCAO (B). Each imaged volume consisted of 13 to 21 planes, 2.5 μm apart. **Tracking of T cells in the cortex post-MCAO** (C). At 48 hours post-MCAO endogenous hCD2-GFP T cells were clearly detectable in brain with tracking (yellow lines-red circle) shown in 6 consecutive snapshots representative of 9 minutes of imaging of the same field of view. Bar = 25 μm. The scans were acquired with 500 lps and between 256 x 256 pixel boxes, for a frame rate of 1.95 fps. Images were analyzed using Volocity software (Improvision). The movie of T cells movement in the ischaemic brain tissue is also available as online supplemental data (movie1; view using Quick Time or Real Player).

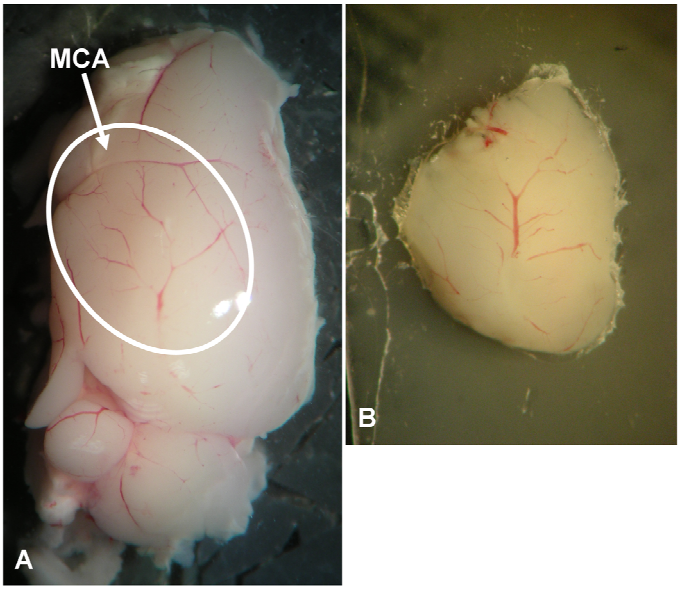

Figure 1

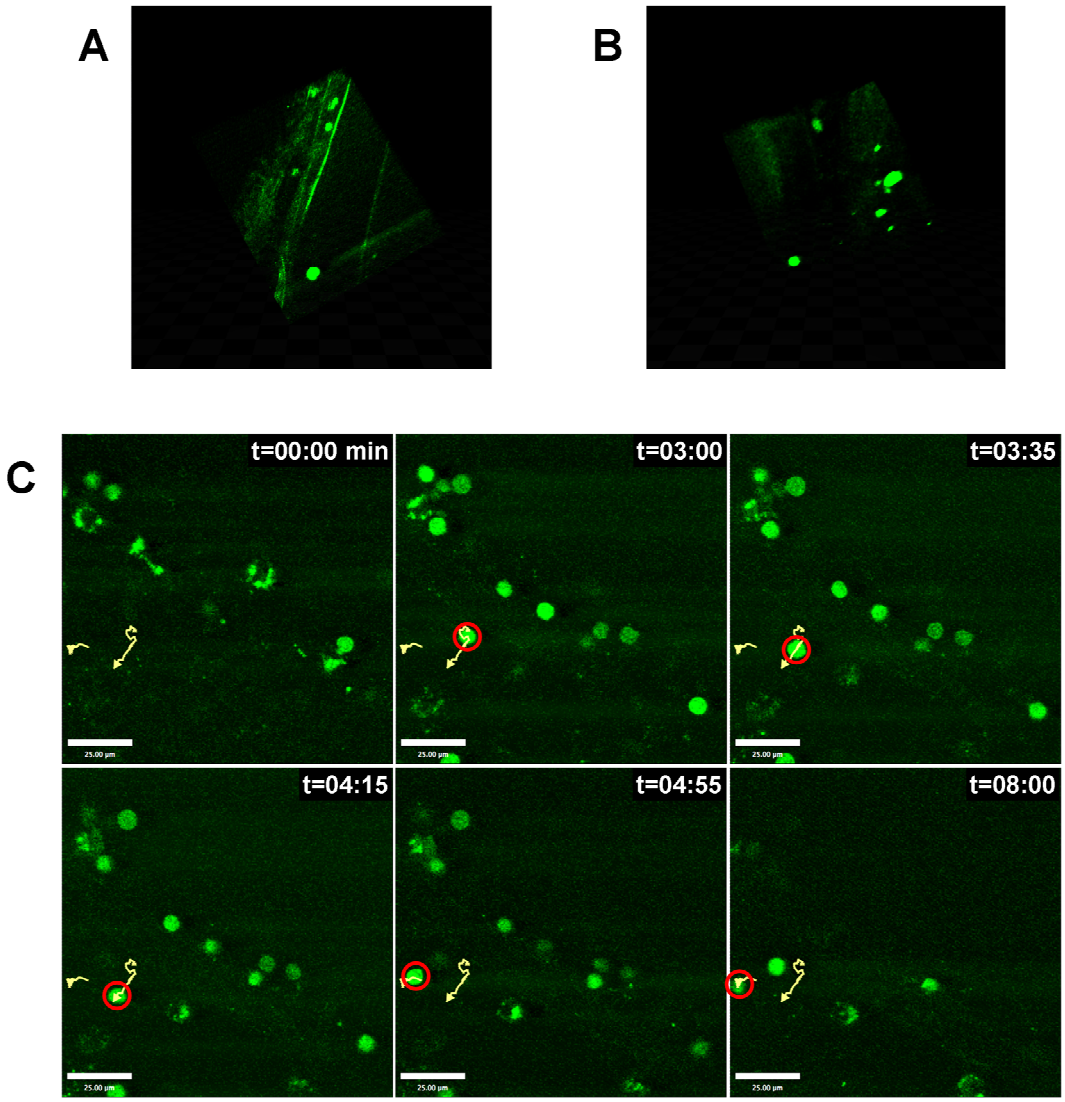

Figure 2

Statement of conflicts of Interest

None.