Understanding Stroke Pathology for Better Treatment

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Bachelor of Biomedical Science Advanced with Honours

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
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Australia
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Abstract

Stroke is a devastating disease that remains the leading cause of mortality and long-term disability worldwide. Despite decades of research and clinical trials, the only pharmacological treatment currently available to acute ischemic stroke patients is recombinant tissue plasminogen activator within 4.5 h of onset. Post-stroke inflammation has been identified as the main form of secondary, delayed injury to the brain following stroke, and effective immunotherapies are therefore urgently needed.

Different branches of the immune system have previously been shown to contribute differently to stroke outcomes. In Chapter 3, we have tested the therapeutic effects of the recombinant cytokine interleukin (IL)-33 on outcome after stroke in mice using 60-min transient middle cerebral artery occlusion (tMCAO). Our data confirmed the damaging effects of Th1-immune responses in the brain following acute stroke and treatment with IL-33 was neuroprotective via Th2-immune switching, through limiting immune cell infiltrations and Th2 polarization. Nevertheless, overall our data show that such Th2 immunotherapies worsen functional outcomes following stroke, possibly due to heightened bacterial infection as a result of diminished resistance via Th1-immune responses. However, combination therapy of IL-33 with broad-spectrum antibiotics was protective both centrally and peripherally. These findings revealed a potentially viable therapeutic pathway for acute ischemic stroke.

In Chapter 4, we examined the effect of another novel anti-inflammatory cytokine, IL-37, in acute stroke. Mice transgenic for human IL-37 were subjected to 60-min tMCAO or photothrombotic stroke. Our data suggest that IL-37, when expressed constitutively in mice, is upregulated in response to acute MCAO-mediated damage, reduces stroke-induced inflammatory profiles, leading to reductions in ischemic brain damage and lung infection,
together with improved clinical outcome. Our study provides direct evidence that IL-37 exerts protection following stroke. However, the protective effects of IL-37 were lost when administered as a recombinant protein to mice following tMCAO. Further studies are still needed in order to identify whether recombinant IL-37 treatment, when optimised, could in fact provide therapeutic benefit in stroke.

A different approach was taken in Chapter 5 to better understand the complex stroke pathology in the MCAO model. We conducted a large-scale retrospective analysis on all control C57Bl/6 mice subjected to either 1 h transient or permanent MCAO performed by various surgeons in our laboratories from 2012-2018. Using bivariable and multivariable analyses, we identified several associations to exist between edema volume and infarct volume or clinical scores in mice with reperfusion therapy. Further, advanced age in these young adult mice (6-40 weeks) was associated with worse functional outcome and greater infection. None of these associations were present in mice subjected to permanent MCAO. We also created several structural equation models to attempt to explain cause-effect relationships between measured parameters.

Collectively, these studies have identified several useful insights for treating ischemic stroke. The work revealed that care should be taken with immunotherapies due to adverse systemic side effects. For the first time, we have demonstrated that reperfusion influences associations among various experimental parameters. Further studies might provide valuable information for the development of treatments and prognoses of ischemic stroke in patients.
Publications during this PhD candidature

Primary Author Publications


Co-authored publications


Conference and symposium abstracts

Oral Presentations

1. Zhang SR, Sobey CG, Drummond GR, Nold CA, Nold MF, Kim HA, Effect of Interleukin-37 on Outcome After Stroke. 1st PAM Student Symposium. La Trobe University, Melbourne, Australia, November 2018. (Won Mike Clarke Award for best presentation)


Poster Presentations


Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals and one submitted publication. The core theme of the thesis is “Understanding Stroke Pathology for Better Treatment”. The ideas, development and writing of the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Pharmacology under the supervision of Professor Christopher Sobey, Dr Hyun Ah Kim and Associate Professor Marcel Nold.

(The inclusion of co-authors reflects the fact that the work came from active collaborations between researchers and acknowledges input into team-based research.)
In the case of Chapters 3 and 4, my contribution to the work involved the following:

<table>
<thead>
<tr>
<th>Publication Title</th>
<th>Chapter 3</th>
<th>Co-author name(s)</th>
<th>Co-author’s contribution</th>
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<tr>
<td>IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke</td>
<td><strong>Status</strong> (published, in press, accepted or returned for revision, submitted)</td>
<td><strong>Nature and % of student contribution</strong></td>
<td><strong>Nature and % of Co-author’s contribution</strong></td>
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<td><strong>Publication Title</strong></td>
<td><strong>Marius Piepke</strong> Performed 16x MCAO surgeries on IL-10&lt;sup&gt;EGFP&lt;/sup&gt; F0xp3&lt;sup&gt;mRFP&lt;/sup&gt; mice, performed flow cytometry on these mice: 3%</td>
<td><strong>No</strong></td>
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<td></td>
<td><strong>Hannah X Chu</strong> Provided technical assistance and guidance on flow cytometry and analyses: &lt;1%</td>
<td><strong>Yes</strong></td>
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<td></td>
<td><strong>Brad R S Broughton</strong> Performed F4/80&lt;sup&gt;3NT&lt;/sup&gt; immunofluorescence staining and associated data analysis: 1%</td>
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<td></td>
<td><strong>Raymond Shim</strong> Performed bacterial plating and analysis: 1%</td>
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<tr>
<td></td>
<td><strong>Connie H Y Wong</strong> Provided technical advice and performed bacterial plating and analysis: 1%</td>
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<td></td>
<td><strong>Seyoung Lee</strong> Performed 4 MCAO surgeries and provided technical advice: &lt;1%</td>
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<td><strong>Megan A Evans</strong> Provided intellectual advice on experimental design: &lt;1%</td>
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<td></td>
<td><strong>Antony Vinh</strong> Provided reagents and intellectual advice on flow cytometry: &lt;1%</td>
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<td><strong>Samy Sakkal</strong> Assisted in cytometric bead array and analysed associated data: 1%</td>
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<td><strong>Thiruma V Arumugam</strong></td>
<td><strong>No</strong></td>
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<td>Provided intellectual advice on experiment design: &lt;1%</td>
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<tr>
<td>Tim Magnus</td>
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<tr>
<td>Provided some resources for flow cytometry and edited the manuscript: &lt;1%</td>
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<tr>
<td>Samuel Huber</td>
<td></td>
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<tr>
<td>Contributed to MCAO surgeries and flow cytometric analysis of some experiments: &lt;1%</td>
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<tr>
<td>Mathias Gelderblom</td>
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<tr>
<td>Assisted in IL-10&lt;sup&gt;GFP&lt;/sup&gt;Foxp3&lt;sup&gt;mRFP&lt;/sup&gt; flow cytometric analysis: 3%</td>
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<td>Grant R Drummond</td>
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<td>Provided input to scientific design and interpretation, edited the manuscript: &lt;1%</td>
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<td>Christopher G Sobey</td>
<td></td>
<td>&lt;1%</td>
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<tr>
<td>Provided funding, designed experiments, interpreted data and assisted with drafting and editing of manuscript: 20%</td>
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<tr>
<td>Hyun Ah Kim</td>
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<tr>
<td>Performed MCAO surgeries, interpreted data and assisted with drafting and editing of the manuscript: 20%</td>
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<td>Publication Title</td>
<td>Status (published, in press, accepted or returned for revision, submitted)</td>
<td>Nature and % of student contribution</td>
<td>Co-author name(s) Nature and % of Co-author’s contribution*</td>
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<td><strong>IL-37 increases in patients after ischemic stroke and protects from inflammatory brain injury, motor impairment and lung infection in mice</strong></td>
<td>Accepted for publication Published on 06 May 2019 Available at: <a href="https://www.nature.com/articles/s41598-019-43364-7">https://www.nature.com/articles/s41598-019-43364-7</a> DOI: 10.1038/s41598-019-43364-7</td>
<td>Performed all animal experiments, analysed and compiled human and animal data, prepared figures, drafted manuscript 65%</td>
<td>Marcel F Nold; Claudia A Nold Provided IL-37tg mouse breeding pairs, intellectual and experimental guidance, assistance with drafting and editing the manuscript: 5% each</td>
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<td>Sung-Chun Tang Provided data on human samples, immunohistochemistry on human brain and performed IL-37 ELISA on human plasma: 5%</td>
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<td>Christine B Bui Provided assistance with IL-37tg mouse colony: &lt;1%</td>
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<td>Thiruma V Arumugam Grant R Drummond Provided advice on experiment design: &lt;1%</td>
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<td>Christopher G Sobey Provided funding, conceived and designed experiments, assisted with drafting and editing of the manuscript: 10%</td>
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<td></td>
<td>Hyun Ah Kim Provided intellectual advice and laboratory assistance on experiments, provided assistance with drafting and editing of the manuscript: 10%</td>
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I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  
Date: 23/04/2019

The undersigned hereby certifies that the above declaration correctly reflects the nature and extent of the student’s and co-authors’ contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:  
Date: 23/04/2019
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## List of Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>3-NT</td>
<td>3-nitrotyrosine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ARRIVE</td>
<td>Animal Research: Reporting of In Vivo Experiments</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BHI</td>
<td>Brain-heart infusion</td>
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<tr>
<td>Breg</td>
<td>Regulatory B cell</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CCA</td>
<td>Common carotid artery</td>
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<td>CCD</td>
<td>Charge0coupled device</td>
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<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CFI</td>
<td>Comparative fit index</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<td>CIA</td>
<td>Cortical infarct area</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Ct</td>
<td>Cycle threshold</td>
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<td>CXCL</td>
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<td>CXC chemokine receptor</td>
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<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
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<tr>
<td>DALY</td>
<td>Disability adjusted life year</td>
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<td>DAMP</td>
<td>Danger associated molecular pattern</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenyindole</td>
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<td>Dendritic cell</td>
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<td>Dimethyl sulfoxide</td>
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<td>ECA</td>
<td>External carotid artery</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>foxP3</td>
<td>Forkhead box P3</td>
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<td>Forward scatter</td>
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<td>Human immunodeficiency virus</td>
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<td>HMGB1</td>
<td>high-mobility group box 1</td>
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<td>HPA</td>
<td>Hypothalamus-pituitary axis</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
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<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>ILC</td>
<td>Innate lymphoid cell</td>
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<tr>
<td>LCA</td>
<td>Left hemisphere cortical area</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCA</td>
<td>Middle cerebral artery</td>
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<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MM</td>
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<td>MPO</td>
<td>Myeloperoxidase</td>
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<td>Mechanical Thrombectomy</td>
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<td>NDMA</td>
<td>N-methyl-D-aspartate</td>
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<td>NF-kB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NGS</td>
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<td>NK</td>
<td>Natural killer cell</td>
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<tr>
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<td>Natural killer T cell</td>
</tr>
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<td>NO</td>
<td>Nitric oxide</td>
</tr>
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<tr>
<td>NOX</td>
<td>NADPH Oxidase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PID</td>
<td>Peri-infarct depolarization</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>Rag</td>
<td>Recombinant activating gene</td>
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<td>RBC</td>
<td>Red blood cell</td>
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<td>RCA</td>
<td>Right hemisphere cortical area</td>
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<td>rCBF</td>
<td>Regional cerebral blood flow</td>
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<tr>
<td>RIA</td>
<td>Right Hemisphere infarct area</td>
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<tr>
<td>RMSEA</td>
<td>Root mean square error of approximation</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rt-PA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SAH</td>
<td>Subarachnoid haemorrhage</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Structural equation modelling</td>
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<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>ST2</td>
<td>suppression of tumorigenicity 2</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLI</td>
<td>Tucker-Lewis index</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>tMCAO</td>
<td>Transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1:
GENERAL INTRODUCTION
1.1 Stroke overview

Stroke is the second leading cause of death after ischemic heart disease and the leading cause of disability around the globe. World Health Organization estimated that almost 6 million deaths are caused by stroke in 2016, contributing to more than 10 % of overall mortality in the world (1-3).

Every nine minutes, someone in Australia will suffer from a stroke. In 2017, there were ~56,000 new or recurring stroke incidents across Australia, and ~12,500 (~22 %) of which did not survive (4). Stroke represents a significant health care and productivity burden to society, with an estimated annual health and non-health financial cost of AUD$5 billion in Australia alone (5-8). Currently there are almost ~480,000 stroke survivors in Australia and it is projected to reach almost 1 million by 2050 (4, 8). Stroke occurs most commonly in older individuals aged 55 or older (9), contributing to the loss of 103,296 disability-adjusted life-years (DALY) in 2011 since 2 out 3 stroke survivors are disabled, requiring assistance or carers to carry out daily living tasks (9). Furthermore, stroke incidence has shown to rise in young or middle-aged adults around the world in recent years. This is particularly concerning in developing countries with low- to middle-income status, where the mean age of stroke incidence and mortality occurs 6-15 years earlier than that of high-income countries (1, 10). Eventually this could lead to even greater socio-economic impact in countries around the world (11-13).

1.1.1 Risk factors

There are a number of risk factors associated with stroke. Non-modifiable risk factors include male sex, older age, genetics and some racial groups (12, 14-16). Modifiable risk factors such as atrial fibrillation, physical inactivity, poor diet, smoking, hypertension, excessive alcohol consumption, diabetes, hyperlipidemia, psychological stress and depression (16-18).
1.1.2 Stroke types

Stroke can be subcategorized into ischemic or hemorrhagic or types depending on underlying pathology. Ischemic stroke accounts for the ~87% of all stroke cases. It occurs when the blood flow to the brain in a cerebral artery is severely interrupted caused by a thrombus or embolus (16, 19, 20). This thesis investigates the therapeutic potential of ischemic stroke and focuses on its pathophysiology. All information provided in the following section is relevant to stroke of ischemic type. Hemorrhagic stroke accounts for the other ~13% of all stroke cases (21, 22). It happens when a weakened blood vessel such as an aneurysm ruptures, causing bleeding into the brain, and subsequent biochemical and physiological disruptions (22). Hemorrhagic stroke is associated with a poor prognosis, with 50% of patients dying within 30 days after initial onset (22, 23).

1.2 Treatments available for stroke

Currently, tissue plasminogen activator (t-PA) is the only approved pharmacological therapy for ischemic stroke (6, 7). However, t-PA administration is limited to stroke patients within 4.5 h of initial onset, thus reducing the eligibility to less than 10% of patients, eventually only 1-2% of patients may benefit from this type of treatment (7). However, a recently completed clinical trial (EXTEND) investigated the possibility of extending t-PA (Alteplase) treatment to up to 9 h following stroke (24). This might provide benefits to significantly more acute stroke patients being admitted to the hospital. In contrast, t-PA treatment after stroke may increase the risk of intracerebral hemorrhage (6).

Endovascular clot retrieval therapies, in particular the mechanical thrombectomy (MTE), have recently been introduced as an alternative or additional approach to stroke treatment. MTE can remove the thrombus from large arteries within minutes, usually with the use of stent retrievers (27). MTE treatment with the second-generation mechanical thrombectomy devices have shown to be safe and able to achieve ~83% recanalization rate in eligible
patients within 6 h of large artery stroke onset, with improved clinical recovery and reduced mortality at 90 days following stroke (28-30), a considerable improvement from the first-generation stent retrievers (21, 31). Furthermore, recent studies have extended the MTE therapeutic window to 16 h in the DEFUSE 3 trial (32), as well as 24 h in the DAWN trial (33) regardless of thrombolytic therapy. Improved functional outcome was observed in both studies (32, 33). However, this treatment is still limited due to the nature of stent retrievers, timing, as well as hospital capabilities. Stroke patients with small artery occlusion, multiple occlusions, or insufficient collateral circulations are not eligible for this treatment. Even after successful MTE, patients with middle cerebral artery occlusion (MCAO) in M1 segments still develop infarctions in the lenticulostriate areas (34). It is worth noting that both t-PA and MTE only aim to restore blood flow in the affected blood vessel following stroke, and they are unable to mitigate the subsequent cellular injury mechanisms that occur later in the brain and periphery. As a result, a better understanding of stroke pathophysiology is essential to develop effective therapies.

1.3 Current progress in stroke trials

Currently there are 223 active clinical trials registered on the database of the U.S. National Library of Medicine (National Institutes of Health) around the world involving ischemic stroke. Two hundred and seven of these studies are investigating potential therapeutic effects of medicinal or mechanical interventions. All these clinical trials can be classified into these 13 categories: post-stroke care and improvement in stroke monitoring devices (n=85), thrombolysis medication therapy (n=39), endovascular clot retrieval (n=17), blood pressure and lipid control in stroke (n=13), targeting biochemical and/or cellular signaling pathways (n=14), anti-coagulation, neuroprotection therapy (n=8), assessment of functional recovery, acupuncture (n=7), stem cell therapy (n=7), body temperature related study (n=6), immunotherapy (n=6), antibiotics or probiotics treatment following stroke (n=2), drug with
unknown mechanisms (n=1), and other various medications or therapies (n=18). It is clear that majority of current clinical trials still focus on reperfusion as well as functional recovery and assessment. Immunomodulation, however, only accounts for less than 3 % of total current trials (n=6), suggesting a great potential exists for further clinical exploration. In fact, experimental targeting of inflammation following acute stroke has gained increased interest in recent years (35-37). Several clinical trials on the efficacy of immunotherapies have also been conducted on stroke patients (38, 39). Unfortunately, like most other clinical trials (40), targeting the activation of microglia (41), infiltration of neutrophils, E-selectin, as well as α4-β1 integrin have all failed to provide any significant benefits in patients following ischemic stroke (39). Furthermore, clinical trials targeting intracellular adhesion molecule 1 (ICAM-1) with the use of enlimomab were terminated pre-maturely due to excessive side effects and higher mortality (42). On the other hand, phase II clinical trials of an interleukin(IL)-1 receptor antagonist (43) or fingolimod (Sphingosine-1-phosphate receptor modulator) regulating the adaptive immune system (44) have displayed promising results. These controversial clinical data suggest that our current understanding of post-stroke inflammation and its pathophysiology are limited (39). Further studies to understand mechanisms of neuroinflammation in acute and delayed stroke are therefore warranted to enable further exploration of potential therapies.

1.4 Pathophysiology of ischemic stroke

1.4.1 General overview

Ischemic stroke triggers a series of complex pathophysiological events that evolve temporally and spatially in the brain, as well as in the periphery (45-47). Within minutes following stroke onset, areas of brain tissue with critical blood flow reduction (typically <20 % of normal blood flow) undergoes permanent necrotic cell death (45, 48-50) as a result of anoxic depolarization, ionic failure, proteolysis, lipolysis, and microtubule disaggregation
(45, 51), forming the irreversible ischemic core. On the other hand, partial blood flow reduction occurs in the area surrounding the ischemic core, called the ‘penumbra’, where collateral blood flow may provide a certain degree of perfusion (typically 20-40 % of normal blood flow), but the tissue is functionally inactive and metabolically unstable under severe stress (49, 50, 52). Over time, cells in the penumbra may eventually undergo death via excitotoxicity, depolarization, inflammation or apoptosis should no treatment or reperfusion be given. Penumbra can comprise a region of brain equal to up to half of the total infarct volume and thus provides an excellent candidate for cell recovery after stroke (49, 50). The following section will describe the series of events that may lead to cell injury following ischemic stroke.

1.4.2. Excitotoxicity

The brain is an organ with a constant high energy demand and little energy reserve. Neurons and astrocytes require an uninterrupted energy substrate supply for proper oxidative phosphorylation and metabolism (53). Excitotoxicity takes place immediately after stroke onset in the ischemic core. It occurs as a result of the toxic accumulation of calcium ions (Ca2+) in the intracellular space caused by excessive release of excitatory amino acids, such as glutamate (50, 54). Soon after the blood flow is interrupted, glucose-dependent adenosine triphosphate (ATP) generation is reduced, which in turn leads to the failure of energy-dependent cellular pumps, causing massive cellular influx of ions and thus energy failure (45, 50), followed by cellular edema and permanent depolarization (45, 50). The influx of Ca2+ ions is essential in stroke pathophysiology as it activates a series of Ca2+-dependent enzymes such as kinases, proteases, lipases and endonucleases that are responsible for the initiation of the injury cascade and eventually apoptosis (50, 55, 56). Furthermore, Ca2+ ions can facilitate the production of reactive oxygen species (ROS) and nitric oxide (NO) via the activation of cyclooxygenase and nitric oxide synthase (NOS) (45, 57). Meanwhile,
impaired reuptake of glutamate causes the release of excessive glutamate into the extracellular space, activating ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. This leads to the further influx of Ca^{2+} ions, propagating a chain of enzymatic activities that eventually cause membrane degradation and cell death by both necrosis and delayed cell apoptosis (45, 50, 58, 59).

Previous studies targeting excitotoxicity with the use of NMDA/AMPA receptor antagonists as a means of neuroprotective therapy were only effective if antagonists were administered before or immediately after stroke, but not without severe side effects (60-63). This was possibly in part due to the narrow therapeutic time window to block excitotoxicity, and the poor selectivity of the NMDA/AMPA receptor antagonists used (60). More recent animal studies suggest that selectively targeting the pro-apoptotic pathway downstream of NMDA receptors with Tat-p53DM or D-JNKI-1 could provide neuroprotection and improve functional outcome within 6 or as much as 12 h after MCAO, respectively (60, 64, 65). The clinical relevance of targeting excitotoxicity should be further explored in future studies.

1.4.3 Peri-infarct depolarization and apoptosis

Peri-infarct depolarization (PID) is a phenomenon that occurs in the penumbra involving repeated depolarizations of neurons in response to the excessive release of glutamate or potassium ions. PID is achieved by the consumption of limited energy provided from the restricted blood flow (45). Over time these cells in the penumbra die and become part of the ischemic core (66-68). PID has been observed in many experimental animal stroke models and causes secondary loss of perfusion in the penumbra area (69). Similarly, in stroke patients, PID and cortical spreading depression following stroke are observed in almost all patients as late as 5 days post-stroke. The duration of PID is found to be associated with poor prognosis (67, 70). Interestingly, preliminary results by Sakowitz et al (71) showed that
the anesthetic ketamine provides acute neuroprotection by inhibiting PID in humans following traumatic brain injury, together with its vasodilatory effect (72). The effects of PID in stroke have not been well elucidated due to the difficulty of its detection in humans (45). Future studies on the exploration and inhibition of PID are needed to potentially identify better neuroprotective therapies.

Excitotoxicity and PID lead to mitochondrial dysfunction and ROS production. While necrosis is the predominant cellular death mechanism following stroke and occurs in the ischemic core, apoptosis is the major form of delayed cell death in the penumbra, where ischemic injury is milder (45, 73-75). Stroke activates two apoptotic pathways in the ischemic penumbra: the intrinsic and extrinsic pathways. In the intrinsic pathway, intracellular Ca\(^{2+}\) overload leads to mitochondrial dysfunction. This then causes the release of apoptotic factors such as cytochrome c, serine protease HrtA2/Omi, and Smac/DIABLO from damaged mitochondria to the cytoplasm (75, 76). Cytochrome c then binds and activates apoptotic protease activating factor 1 (Aparf-1), together with procaspase-9 and dADP/ATP, forming the apoptosome complex (75, 77). The apoptosome then cleaves and activates procaspase-9 to its active form, caspase-9, which in turn activates caspase-3 and initiates the apoptotic cascade (75, 78). Several studies have shown that Aparf-1 can be reduced with Aparf-1-interacing protein in cell culture (79), or lithium or prostaglandin A1 following stroke in rats (80), leading to reduced apoptotic activity. On the contrary, extrinsic pathway of apoptosis involves the activation of cell surface death receptor from tumor necrosis factor receptor superfamily, ultimately forming a death-inducing signaling complex (DISC) (75, 77). The best understood example of this apoptotic pathway involves the engagement of Fas ligand (FasL) to Fas receptor, which then recruits Fas-associated death domain protein (FADD) from the cytoplasmic domain of the receptor. FADD then binds to pro-caspase-8, forming the DISC, which then cleaves and generates activated caspase-8 (75, 77). FasL and Fas have been shown to be upregulated in the rat brain within 48 h after MCAO (81,
Treatment with FasL-neutralizing antibody or rats deficient in FasL display profound protection against cerebral ischemia and markedly smaller brain infarcts (82). Both the intrinsic and extrinsic apoptotic pathways initiate apoptosis through the activation of caspase-3 (75). Further, caspase activation has been observed even within 9 h following stroke and treatment with the caspase inhibitor (z-DEVD-fmk) reduced brain infarct size in mice after cerebral ischemia (83). In the event of irreversible DNA damage after stroke, the tumor suppressor transcription factor, p53, can also induce apoptosis by activating the transcription factor for Fas receptor (75, 84). While our understanding of apoptosis in the context of human stroke is limited, Askalan et al (85) reported that apoptosis is delayed in stroke patients and the expression of caspase-3 can be observed as early as 3 days after stroke onset. With the increasing amount of evidence suggesting the important role of apoptosis, targeting it following stroke appears to be a viable option with wider therapeutic window remains to be further elucidated (85).

1.4.4 Oxidative stress

Whilst normal levels of ROS are important for cellular signaling, and metabolism under physiological conditions, stroke can inactivate antioxidant enzymes and promote the overproduction of ROS through hypoxia, glutamate excitotoxicity or high cytoplasmic Ca²⁺ level (75, 86, 87). The three species of ROS, superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxy radical (OH⁻), may further disrupt cellular structure and signaling (45, 75) also leading to apoptosis. NADPH oxidase (NOX) enzymes have been shown to be a major source of ROS in the central nervous system (CNS) following stroke (88, 89). Numerous studies have reported potentially beneficial outcomes by deletion or inhibition of specific NOX subtypes in cerebral ischemia (90). NO is an essential signaling gas molecule in the regulation of blood flow and inflammation (91, 92) and can be produced by three different enzymatic NOS. During ischemic stroke,
NO production by neuronal NOS (nNOS) increases acutely, followed by sustained NO generation by inducible NOS (iNOS) for 1 week in rats (91). Tissue damage is further exacerbated when O$_2^-$ reacts with NO, leading to the production of highly toxic peroxynitrite (45, 75).

It is important to note that O$_2^-$ production from mitochondria is dramatically higher in the mouse brain following transient MCAO (tMCAO), but not permanent MCAO (pMCAO) (93). Infarct volume was reduced only in the tMCAO model by non-selective NOS inhibitors (94). Interestingly, NO produced by endothelial NOS (eNOS) is neuroprotective through microcirculation improvement and blood brain barrier protection (45). In experimental stroke, mice deficient in eNOS develop larger brain infarcts with smaller penumbral areas (91, 92). Thus, non-selective blockade of NOS enzymes could therefore restrict blood flow following ischemia/reperfusion (92, 94). Future studies on the potential therapeutic benefits of NO modulation in stroke should be explored.
1.4.5 Inflammation

Inflammation is the accumulation and activation of immune cells in the injured or infected tissues. It is initiated during the acute phase of stroke (within minutes to hours) and is likely to become the predominant damaging mechanism within hours and days (59). Inflammation occurs when necrotic and stressed cells release a range of molecules called danger associate molecular patterns (DAMPs) such as heat shock protein, ATP, uridine triphosphate (UTP) and high-mobility group box 1 protein (HMGB1) (94, 96). These DAMPs then activate vascular endothelial cells and local immune cells (e.g. microglia) through the engagement of pattern recognition receptors (PRRs) including toll-like receptor family (TLR1 to 13) and suppression of tumorigenicity 2 (ST2), triggering proinflammatory intracellular signalling cascades and transcription factors, in particular NF-κB, ROS and matrix metalloproteinases (94, 97). This leads to the release of pro-inflammatory cytokines, especially IL-1β, IL-6, IL-17, IL-18 and tumour necrosis factor (TNF)-α, thus initiating local immune responses. Hypoxic environment in the injured area further activates endothelial
cells (98). Animal studies revealed that a series of cell adhesion molecules are upregulated on endothelial cell surface, including P-selectin, E-selectin, intercellular cell adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) (99). They interact with platelets to promote platelet aggregation in the brain microvasculature, forming a thrombus and further blocking cerebral blood supply. Endothelial cells then interact with and recruit circulating leukocytes through the blood-brain barrier (BBB) to injured brain parenchyma, facilitated by adhesion molecules and chemokine gradients, causing a time-dependent and systemic immune activation, cell infiltration and vascular injury (98, 100, 101). Both innate and adaptive immune cells participate in injury mechanisms following stroke (101). Interestingly, inflammation also plays important roles in neuroprotection and tissue repair (101). Due to its wider potential therapeutic window, targeting appropriate inflammation following stroke has attracted attention for potential novel therapies.

1.5 Cells of the innate immune system

1.5.1 Microglia and macrophages

Many cell types are involved in brain infiltration after stroke, and so deeper mechanistic insight into the actions of these cells could help to identify therapeutic targets. Microglia are the brain’s resident macrophages and are responsible for local immune surveillance in the brain (97, 102). Under physiological conditions, microglia constantly survey their environment in a ramified morphology (102). Upon ischemic insult, microglia are the first cell type to become activated, undergoing progressive hyper- or de-ramification, and adopting a phagocytic phenotype in the ischemic hemisphere (97, 102). De-ramified morphology is found in the ischemic core and penumbra and is particularly associated with reperfusion injury such as occurs in the tMCAO model (102). In contrast, in mice subjected to pMCAO, activated microglia are mostly restricted to the penumbra as microglial activation is energy-dependent (103, 104). Microglial activation peaks on days 2-3 and remains present in the
injured hemisphere at a high level potentially for months following stroke (100, 101, 103). Infiltrating macrophages are derived from monocytes originating from the bone marrow and their amoeboid morphology and function resemble an activated microglial cell (97, 101, 105). Macrophages can be distinguished from microglia with their higher expression levels of C-C chemokine receptor type 2 (CCR2), CD45, CD11b, and lower expression of CX3C chemokine receptor 1 (CX3CR1) (101, 105). Following stroke, macrophage infiltration into the brain starts within 24 h and peaks between 7 and 14 days, although at a much lower number than microglia (101, 105).

Microglia/macrophage (MM) activation in response to ischemic brain injury is a complex process and involves their polarization to either pro- (M1-polarised) and anti-inflammatory (M2-polarised) phenotypes (103). Classically activated MMs (M1-like) promote the recruitment of peripheral immune cells and platelets by secreting pro-inflammatory cytokines and chemokines such as IL-1β, IL-12, IL-23, TNF-α, iNOS and major histocompatibility complex (MHC) II (103, 106), leading to the production of even more pro-inflammatory cytokines and chemokines, and thus damaging the integrity of the BBB. This in turn induces more leukocyte infiltration and potential haemorrhagic transformation (common in ischemic stroke patients) in the brain (106). This chain of reactions forms a self-propagating loop, generating more inflammation and eventually enlargement of the brain infarct (96, 98, 100).

On the other hand, alternatively activated MMs (M2-like) take part in tissue repair and wound healing via anti-inflammatory cytokines or growth factors such as IL-4, IL-10, IL-13, TGF-β, platelet-derived growth factor, vascular endothelial growth factor, brain-derived neurotrophic factor (101, 103). Pro- or anti-inflammatory MMs can be activated in response to various local inflammatory stimuli, including the IL-12/IL-4/IL-13 signals from helper T cells and DAMP-TLR interactions (101). Additional polarization phenotypes such as pro-inflammatory Mox or M3 have also been described for mononuclear phagocytes (101), however our knowledge of these cell types remain rather limited. In vitro experiments have revealed that
M1-polarised MMs exacerbate oxygen-glucose deprivation-induced neuronal death, whereas M2-polarised MMs protect neurons against ischemic damage (105). It is worth noting that M2-polarized MMs may provide some degree of functional recovery in mice following stroke, without affecting the infarct volume (107). Targeting M1-polarized MMs towards a M2-polarized phenotype for future therapies may therefore provide limited but useful benefits following acute stroke.

1.5.2 Monocytes

Circulating monocytes are among the first cells to enter the brain through the BBB, and this occurs as early as 3 h post-ischemia (101, 108, 109). They mature and differentiate into macrophages upon entry, and become morphologically indistinguishable from reactive MMs (110). Similar to macrophage activation, monocytes can be broadly categorized into 3 subclasses. Classical pro-inflammatory monocytes express CD14^{high}CD16^{high}CCR2^{high} in humans (or Ly6C^{high}CD45^{high}CCR2^{high} in mice), accounting for the majority of the monocyte subpopulation and responding to injured tissue by releasing pro-inflammatory cytokines to promote further tissue injury in stroke, myocardial infarction and atherosclerosis (111). It has also been found that stroke patients with high levels of monocytes and the chemokine, monocyte-chemoattractant protein (MCP)-1 tend to exhibit worse functional outcome and prognosis (112).

Non-classical anti-inflammatory monocytes do not express CCR2 (CD14^{+}CD16^{high}CCR2^{+} in humans and Ly6C^{low}CD45^{high}CCR2^{+} in mice, respectively) and are responsible for tissue and endothelium repair, lipid removal and anti-inflammatory cytokine release (111). The third monocyte subset – the intermediate monocyte – is less well-defined. These cells express CD14^{high}CD16^{+}CCR2^{+} in humans or Ly6C^{high}CD45^{high}CCR2^{high} in mice and may play important roles in cardiovascular diseases (113) as well as in human immunodeficiency virus-1 (HIV-1) infection (114). In vitro study showed that these intermediate monocytes can
exhibit vastly different inflammatory characteristics when stimulated with pro-, anti- or T helper(Th)2-mediated inflammatory stimuli, suggesting intermediate monocytes might be important in mediating an inflammatory balance following tissue injury (115). In fact, several recent studies have reported that certain Ly6C\textsuperscript{hi} monocytes (pro-inflammatory) enter the brain shortly after stroke in mice, then progressively transform into a macrophage phenotype (F4/80\textsuperscript{+}), while down-regulating expression of the monocyte (Ly6C) cell surface marker, and eventually having the phenotype of an alternatively activated macrophage (M2-polarised macrophage) (108, 116). These data suggest that while some monocyte subsets exacerbate tissue injury, others may mediate tissue repair and dampen inflammation.

1.5.3 Neutrophils

Gelderblom et al (101) found that the infiltration of neutrophils into the brain started 12 h after ischemia-reperfusion injury in C57BL/6 mice, and becomes the predominant infiltrating cell type in the brain by 3 days after stroke. To date, the effect of neutrophils in stroke injury remains controversial. Many previous studies found infiltrating neutrophils exhibit increased activity of myeloperoxidase, neutrophil-extracellular trap formation, histone-3 citrullination and chromatin condensation in various permanent models of stroke in rats (117) and mice (118), contributing to brain infarct development and a worse functional outcome. Indeed, in vivo studies reported that blocking C-X-C motif chemokine receptor 2 (CXCR-2) or very-late-antigen 4 (VLA-4) to prevent the recruitment and transmigration of neutrophils reduces brain infarct development following stroke (119). By contrast, other studies reported that inhibition of neutrophils into the brain, or systemic depletion of neutrophils, does not alter infarct volume in transient models of stroke (117, 120). Furthermore, clinical trials designed to either suppress transmigration (ICAM-1 and VLA-4) or stimulate the activity of neutrophils (granulocyte-colony stimulating factor) have all failed to observe any beneficial or detrimental effects in stroke patients (121). Additionally, depleting neutrophils may also
increase the risk of bacterial infection after stroke and thus worsen clinical outcome (119). It is important to note that neutrophils, similar to mononuclear granulocytes, may also exhibit a pro-inflammatory “N1” and anti-inflammatory “N2” phenotypes. Studies by Cuartero et al (122) showed that rosiglitazone, a PPAR-γ agonist, can polarize neutrophils towards a N2-dominated phenotype expressing Ym-1 and CD206 following MCAO in mice, associated with smaller infarct volumes and less inflammation. Despite these promising data, the effect of N2 neutrophils in stroke, and their relevance in stroke patients and potential therapies remain to be elucidated.

1.5.4 Transition from innate to adaptive immune system
Dendritic cells (DCs) act like a bridge connecting innate immunity with the adaptive immune response. Felder and colleagues found that brain-resident DCs become activated in the ischemic brain by 24 h after ischemia-reperfusion injury, with a significant increase in their expression of co-stimulatory molecules CD80 and CD86 (123). They accumulate in the penumbra and interact with infiltrating T lymphocytes. Interestingly, the infiltration of peripheral DCs into the brain and expression of major histocompatibility complex (MHC) class II does not occur until day 3 (101, 123), suggesting that DCs might play a spatial and especially temporal roles in regulating or maintaining the inflammatory response in ischemic brains. However, further study is needed to clarify the effects of DCs in the context of stroke.

1.6 Cells of the adaptive immune system
1.6.1 T cells
Classically, the adaptive immune response is activated when components of “foreign” antigens are recognized and ingested by antigen presenting cells (APCs) such as DCs and macrophages. These antigens are then processed by APCs and presented by MHC molecules specific to T lymphocytes, along with the co-stimulatory molecule CD80, resulting
in the proliferation and expansion of an antigen-specific T cell population (36). The presence of polarizing cytokines is also required for specific lymphocyte differentiation. Activation of the adaptive immune response is crucial in fighting against infections. However, its activation can potentially be detrimental in various CNS diseases, including stroke, multiple sclerosis and atherosclerosis (124).

T cells can be categorized into two main classes, CD4+ T-helper cells and CD8+ cytotoxic T cells. Infiltration of lymphocytes has been observed in ischemic brains as soon as 3 h after stroke for CD8+ T cells, or at 24 h for CD4+ T cells (101, 109). Although they infiltrate the injured brain tissue in fewer numbers than innate immune cells, these T lymphocytes play a critical role in mediating post-stroke inflammation and resolution both in the brain and in the periphery (125). Hurn et al (126) found that mice with severe combined immunodeficient disease (SCID), which lack the ability to generate T and B lymphocytes, develop smaller infarcts and have better functional outcomes after focal cerebral ischemia as compared to wild-type mice. Furthermore, adoptive transfer of T cells, but not B cells, augmented the clinical deficit and brain injury, suggesting that unlike T cells, B cells may not be critical for stroke damage (127, 128).

CD8+ cytotoxic T cells react to MHC I antigen complexes and can directly kill infected cells by the release of cytotoxins via necrosis or apoptosis. In CNS diseases, CD8+ T cells exert detrimental effects through activating astrocytes, as well as increasing the permeability of the BBB by altering vascular endothelial tight junction proteins through the promotion of VEGF expression (129). Further, some memory CD8+ T cells reside in the CNS in aged individuals. These age-specific resident memory CD8+ T cells contain abundant pro-inflammatory cytokines and could rapidly release inflammatory cytokines in acute CNS disease conditions such as stroke (129). Such an effect would be expected to promote further endovascular damage and worsen brain injury and functional impairment in older patients (130). Suidan et al (129) suggest that using a neuropilin-1 inhibitor to target the
VEGF pathway could prevent the activation of CD8\(^+\) T cells, to provide neuro- and vaso-protection.

On the other hand, CD4\(^+\) T cells respond to MHC II-expressing APCs, and in the presence of a co-stimulating signal and polarizing stimuli, release various cytokines and further activate innate or adaptive immune cells (131). Unlike CD8\(^+\) T cells, not all CD4\(^+\) T cells are detrimental in CNS diseases. Most CD4\(^+\) T cells can be subcategorized into four main subsets, namely Th1, Th2, Th17 and regulatory T (Treg) cells based on their transcriptional factor and cytokine environment (36, 131).

Th1 cells are involved in cell-mediated immunity and phagocytic activity, which are essential for the body’s defence against viral or bacterial infections (132). Activation of Th1 cells by IL-12 releases pro-inflammatory cytokines, including IFN-\(\gamma\), IL-1, TNF-\(\alpha\), IL-6, IL-12, IL-18, chemokine (C-C motif) ligand (CCL) 5, and CCL7 via transcription factors such as T-bet and STAT4 (132-134), hence further activating Th1 immune responses and classical MMs (M1, CD68\(^+\)/CD80\(^+\)). Normal activation of the Th1 immune response is necessary for cell-mediated intracellular pathogen clearance and phagocytic activities (132). Genetic defects in Th1 cellular signalling pathway could lead to a dysfunctional immune response and increased risk of infection or autoimmune diseases (132). However, activation of a Th1 immune response is thought to be associated with “sterile” inflammation in CNS diseases and further contribute to tissue damage in stroke (135), hypertension (136), atherosclerosis and multiple sclerosis (137). Indeed, ischemic brain injury can be aggravated by intracerebroventricular (ICV) injection of the Th1-associated cytokines, IL-1\(\beta\) and TNF-\(\alpha\) (138). Furthermore, MAPK\(^{\text{APK}}\) mice (which lack a Th1 immune response) have been shown to have smaller brain infarcts after stroke, with reduced infiltration of macrophages and neutrophils into the ischemic hemisphere (139).

Th2 cells, on the other hand, are activated by IL-4 or IL-33 (132). They also secrete Th2 cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13, which may act on different receptors to
activate M2 macrophages (CD68+/CD163+) and promote healing (140). The Th2 immune response is responsible for clearance of large pathogens such as helminths (132), but is also responsible for allergic inflammation including asthma and atopic dermatitis (141). Due to the Th1-predominant nature of post-stroke inflammation, the Th2 immune response has been regarded as anti-inflammatory and people with a predominant Th2 immune response (e.g. asthma) exhibit lower risk of myocardial infarction and stroke (142). Similarly, animal studies have revealed that mice deficient in Th2-immune responses exhibit worse functional outcomes when compared to wild-type mice (126). Clinical studies have revealed that circulating Th2 cells and Th2-associated cytokines are linked to a better survival rate after stroke (143), consistent with the concept that a Th2 immune response is protective in stroke settings, whereas a Th1 response can be detrimental, at least acutely.

IL-17 is a pro-inflammatory cytokine mainly produced by Th17 cells (also known as αβ T cells), γδ T cells, as well as several innate immune cell types (144). Under normal conditions, IL-17 is responsible for host defence against many types of pathogens, in particular against bacteria or fungi in the extracellular space, such as Klebsiella pneumoniae and Citrobacter rodentium (144). However, adverse effects of IL-17 have been implicated in autoimmune diseases such as rheumatoid arthritis (145), systemic lupus erythematosus (146), inflammatory bowel disease (147) and psoriasis (148). Elevated Th17 cells and IL-17 levels have also been implicated in patients with stroke (149), hypertension (150) and multiple sclerosis (149). Both Th17 and γδ T cells become activated in response to IL-1β, IL-6, IL-23 and TGF-β through the transcription factor RORγT (136, 151), leading to IL-17 and IL-22 production, neutrophil and macrophage recruitment, and astrocyte activation (127, 128, 144, 152). Interestingly, the primary source of IL-17 following stroke is γδ T cells, rather than Th17 cells (153, 154). This is possibly due to the fact that activation of γδ T cells through the γδ T cell receptor (TCR) does not require antigen-specific priming like the traditional αβ TCR on T cells, thus providing a much more rapid immune response (47). Gelderblom et al
showed that IL-17A neutralising antibody improved functional outcome and reduced brain infarct development by 3 days after tMCAO, potentially suggesting delayed injury mechanisms of IL-17 in stroke.

Regulatory lymphocytes have recently attracted much attention for their beneficial roles in regulating inflammation after stroke. Tregs are usually classified as CD4+CD25+ with nuclear co-expression of forkhead box P3 (FoxP3). Tregs, upon activation, express CD4^{high}CD62^{low}, and play significant immunomodulatory roles in stroke and other inflammatory CNS diseases. Several studies have found that depletion of Tregs prior to stroke leads to infarct volume enlargement, and their reconstitution reverses the brain injury during subsequent stroke (155). Nevertheless, Kleinschnitz et al (156) pointed out that these Tregs may also be responsible for adverse effects by interacting with platelets and causing platelet aggregation in the acute stages of stroke, resulting in augmented ischemic injury. Tregs are also reported to be able to promote the proliferation of neuronal stem cells after stroke via IL-10 and transforming growth factor (TGF)-β (151). Indeed, Tregs are also known to interact with DCs in the periphery to regulate the production of pro-inflammatory cytokines by rendering reactive T cells anergic (157). Adoptive transfer of Tregs up to 24 h after animal MCAO helped maintain immune homeostasis in the periphery following stroke and Treg could reduce the risk of post-stroke infection when compared to controls, which significantly impacts stroke survivors (158, 159). Due to their capacity to limit brain injury in animal studies of stroke, future studies of Tregs on the effect of stroke should be evaluated.

Th9 and follicular helper T (Tfh) cells are the two lesser known subsets of CD4+ T cells (47). Unsurprisingly, their roles in stroke have not yet been extensively studied. The Th9 cell is a recently discovered cell type and is the major cellular source of cytokine IL-9 (160), as well as some IL-21 and IL-10 (161). Th9 cell differentiation occurs in the presence of IL-4 and TGF-β. Functions of IL-9 appears to be multidisciplinary but have been shown to promote allergy (161, 162), exacerbate intestinal inflammation and autoimmune diseases (161), but
reduce tumour activity (160, 162). Furthermore, recent studies have shown that Th9 cells and IL-9 levels are elevated in patients with acute coronary syndrome (163). Neutralization of IL-9 is protective in murine models of atherosclerosis (163) as well as stroke (164). On the other hand, two cytokines produced by Tfh cells, IL-17 and IL-21 are involved in the pro-inflammatory process following stroke (165). It is currently unclear whether Tfh cells contributes to the degree of neuronal damage in stroke. Tfh cells, however, can express CXCR5 and is involved in antigen-specific B cell development in lymphoid organs (151). It is worth noting that classical priming of T cell-mediated immunity requires 4-7 days, but damage caused by these cells in the ischemic brain can be observed within 24 h (127). Thus, these observations suggest that T cells may mediate their injury via several mechanisms (classical and non-classical), at least in the early period following stroke. Indeed, previous reports suggest that T cells accumulating in post-capillary venules of ischemic brain regions are accompanied by aggregated platelets, which may lead to thrombo-inflammation and occlusion of microvasculature (166), although there have also been contradictory findings (127). Further studies are certainly needed to clarify the extensive and complex mechanisms of T cell-mediated injury after stroke.

1.6.2 B cells

In contrast to the reasonably well-defined roles of major T cell subsets in stroke outcome, the effects of B cells in stroke are poorly characterised. Recent work by Schuhmann et al (167) showed that pharmacological depletion or genetic deficiency of B cells does not alter brain infarct volume or functional recovery. Similar results were also observed when mice deficient in recombinant activating gene (Rag\(^{-/-}\)) were transplanted with B cells prior to stroke (167). Yet, recent data also show that intraperitoneal injection of CD19\(^{+}\) B cells can limit brain injury after stroke by releasing the anti-inflammatory cytokine IL-10, and these cells modulate the brain’s immune response from the periphery instead of accumulating in the
brain alone (168). In fact, these IL-10 producing B cells can be classified as regulatory B cells (Bregs) with surface expression of CD19+CD1dhiCD5+ (169). These Bregs represent a subset of powerful anti-inflammatory cells that modulate microglial activity via programmed cell death protein (PD)-1 and release IL-10, thus providing protection to the CNS from ischemic and/or inflammatory damage (168, 169). Akiyoshi et al (168) has suggested that the major source of IL-10 following stroke may actually be Bregs, rather than Tregs, as previously thought. Despite the promising protection by Bregs, some B cells (possibly B2 cells) may cause delayed cognitive impairment by forming a tertiary lymphoid structure with T cells and macrophages, which release autoreactive antibodies that may contribute to impaired cognitive function (170).
Figure 2. Simplified summary of various immune cells and their roles following stroke.

1.7 Novel inflammatory cell types

With a deeper understanding of immunology and its relevance in the context of stroke, more cells are being found to be involved in the post-ischemic environment. Innate lymphoid cells (ILCs), are a group of cells with lymphoid morphology, no RAG-dependent antigen receptors, and no myeloid cell phenotype (171). There are three categories of ILCs and the most studied one is the natural killer (NK) cell, which belongs to the group 1 subclass of ILCs. Group 1 ILCs are characterized by the production of IFN-γ, which can cause necrosis of neuronal cells (171, 172). NK cells infiltrate brain and accumulate in the penumbra within 3 h following stroke, and have been shown to damage the BBB via interferon-inducible
protein-10 (IP-10), and further contribute to the enlargement of the infarct by promoting apoptosis (172). In contrast, group 2 ILCs can be activated by cytokines such as IL-33, which leads to the production of Th2 type cytokines including IL-4, IL-5 and IL-13 to further activate Th2 cells (171, 173). Halim et al (174) reported that activation of ILC-2 is crucial for Th2 activation. These data suggest that group 2 ILCs may be neuroprotective in the brain from inflammation after stroke. Group 3 ILCs include complex subsets with the shared transcription factor, RORγT (173). Similar to Th17 and γδ T cells, group 3 ILCs produce IL-22 and IL-17, leading to neutrophil recruitment and inflammation (173). Based on current knowledge and similarities to IL-17-producing T lymphocytes, it might be predicted that ILC-3 would also be pathogenic in the setting of stroke.

Natural killer T (NKT) cells express both T cell receptors and have in common the NK cell surface marker NK1.1 (175). NKT cells may also participate in post-ischemic brain inflammation. Along with NK cells, they have been observed in the brain after thromboembolic stroke, with an activated phenotype (176). In allergy, they produce large quantities of cytokines such as IL-4 or IL-5 upon activation. Currently, it is still unclear as to how NKT cells may contribute to stroke injury. Wong et al (177) discovered that stroke causes the noradrenaline-mediated arrest of invariant NKT cells in the liver, leading to increased risk of bacterial infection post-stroke.

Inflammation following acute stroke is a complex process that involves both central and peripheral immune activation. It is a double-edged sword as inflammatory mechanisms can augment devastating injuries in the brain, but are also essential to provide subsequent tissue resolution, repair and debris clearance (35, 104, 119, 178). It is a collective effort involving the plethora of immune cells, both “good” and “bad”. Studies investigating a single cell population provide us with concise but limited knowledge on how each immune cell type contributes to ischemic injury. Current concepts in immunotherapy for stroke are therefore focusing on shifting the entire stroke-induced immune profile from pro- to anti-inflammatory,
as appropriate, to provide maximal protection to the brain, as well as the periphery. In order to achieve this, better understanding of immune activation/manipulation mechanisms is required and is the focus of the following section.

1.8 The Th1/Th2 paradigm and possible neuroprotection

As described above, polarization of naïve CD4+ T cells is dependent upon different polarizing cytokines in the surrounding environment. Interestingly, Th1 and Th2 immune responses are often regarded as being mutually exclusive (179). Both Th1 and Th2 immune responses need to act in a coordinated manner to maintain proper immune surveillance and pathogen clearance. This is evident in that pregnancy is linked with a lower ratio of Th1:Th2, which favours the development of maternal tolerance to the fetus (132). An anti-inflammatory shift from a Th1- to a Th2-mediated immune response can be regulated with the reduction of cellular reserve of glutathione in APCs and CD4+ T cells. On the contrary, both Th1 and Th2 immune responses may contribute to pathology under disease conditions (132). For example, the Th1 cytokine IFN-γ is excessively secreted in Crohn’s disease, whereas the Th2 cytokines IL-4 and IL-5 are drastically reduced (132). Treatment with allergen-specific immunotherapy designed to shift from a Th2- to a Th1-dominant phenotype has been successful in treating Th2-mediated allergic reactions (180). Importantly for stroke, most of the damaging mechanisms in CNS diseases (including acute stroke) are associated with the upregulation of Th1 immune responses and further activation of innate immune cells (150). It therefore seems plausible that a Th2-dominant immunity would provide better neuroprotection following many of these CNS conditions, including stroke.

Nevertheless, it is important to note that many CNS diseases involve a complex interplay among almost all branches of immune system including Th17- and Treg-mediated pathways (150). Recent studies identified that the Th17:Treg ratio could play an equally important role as the Th1:Th2 ratio in autoimmune diseases such as multiple sclerosis and Alzheimer’s
It is therefore suggested that immunotherapies following stroke should focus on the crosstalk among Th1, Th2, Th17, Treg, as well as the interaction with innate immune cells. While immunotherapies could provide potential neuroprotection in the acute setting, we should be cautious regarding the long-term effects of these treatments.

1.9 Immunosuppression after stroke

The immune system also participates in long-term systemic immunosuppression after stroke. It is evident that up to 65 % of initial survivors suffer from infections as a result of post-stroke immunosuppression within days after stroke onset (182). Pneumonia (~57 %) and urinary tract infections (~27 %) are particularly common and are associated with worse short and long-term prognoses (182). This may contribute to around 30 % of overall stroke mortality within one year of disease onset (183).

The effect of long-term systemic immunosuppression after stroke has recently attracted much attention. Several studies revealed that post-stroke infections in patients are associated with a greater plasma level of the anti-inflammatory cytokine IL-10, as well as a reduced level of the pro-inflammatory cytokine IFN-γ from day 1 post-stroke (184). This is accompanied by a rapid onset of lymphocyte apoptosis both in the plasma and in various lymphoid organs, leading to a sustained lymphopenia and consequently post-stroke immunodepression in patients, who may eventually develop varying degrees of post-stroke infections. Similarly, lymphopenia and dysfunction of APCs have also been observed in animal models of stroke (185). Post-stroke infection in mice appears to be strain-dependent and influenced by the predominant CD4+ T cell profile in each strain. Th1-prone C57BL/6 mice exhibit mild lung infections following stroke, whereas 129SV or Balb/c mice (Th2-prone) developed serious chest infections, and were susceptible to bacteremia (186). Interestingly, a shift from a Th1 to a Th2 immune response has been observed after stroke in both C57BL/6 mice and also in stroke patients (181).
The precise mechanisms of post-stroke immunosuppression are still unclear. It is suggested that systemic infection might be a result of an over-compensation of the immune system to protect the brain from further ischemic damage against the initial pro-inflammatory insult (142, 187). Several factors, such as neuronal response and gut biology, may collectively contribute to post-stroke immunosuppression. The most investigated concepts are the activation of sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis.

Activation of the SNS after stroke triggers the release of catecholamines (including adrenaline and/or noradrenaline) from the adrenal medulla and the nerve terminal (188). Catecholamines act on immune cells expressing β2-adrenoceptors, such as B cells, macrophages and Th1, but not Th2 cells, to induce apoptosis (189). Eventually the systemic cytokine profile shifts to a Th2-dominant immune response, especially with the release of IL-10 from activated Tregs, DCs and even M2 monocytes. Ultimately this leads to a long-lasting T cell lymphopenia (189). Wong et al (177) also observed that the activation of the sympathetic nervous system impaired the function of hepatic iNKT cells, rendering them unable to fight peripheral infections. The translocation of autologous gut flora through a leaky mucosal membrane damaged during stroke, or from aspiration, may provide a source of the infections (190, 191).

The HPA axis includes the CNS and endocrine system (188). In response to stress, corticotrophin releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus and acts on the adrenal gland, leading to the release of adrenocorticotropic hormone (ACTH). Eventually, ACTH stimulates the release of glucocorticoids, which in turn adversely affect the function of immune cells. Excessive production of pro-inflammatory cytokines like IL-1 and IL6 then act as a negative feedback on the HPA axis and ultimately lead to the suppression of inflammatory mediators (188). Prass and colleagues (181) showed that treatment with either the β-adrenoceptor antagonist, propranolol, or the glucocorticoid antagonist, RU486, could prevent the apoptosis of splenocytes in mice.
following stroke. Interestingly, only propranolol was able to prevent the Th1 to Th2 shift and reduce bacterial infections in both lung and blood, suggesting that the SNS may play a major role in mediating post-stroke infection (181). Nevertheless, many studies have reported that patient serum cortisol levels are elevated at 7 days post-stroke and return to baseline within 3 months (181). Higher cortisol levels are associated with greater dependency following stroke, worse functional outcome, increased bacterial infection as well as higher mortality, although contradictory results have also been reported (181). Future studies are therefore required for a better understanding of the HPA axis and its effects on post-stroke immunosuppression and infection.

1.10 The brain-gut axis

The bidirectional communication between the CNS and the gastrointestinal (GI) tract is often referred to as the brain-gut axis (192). Recently, scientists have discovered the potential significance of the gut immune balance in its contribution to both central and peripheral immune responses in CNS diseases (47). Acute stroke causes the release of DAMPs, cytokines and adhesion molecules from the injury site to the periphery. The vagus nerve and enteric nervous system are then activated. Together, cytokines such as IL-17 and G-CSF are released in the gut, leading to gut dysbiosis, dysmotility and increased permeability (47). As the GI tract consists of >70 % of the body’s total immune system and hosts a wide variety of commensal bacteria (47, 193), more inflammatory cells – especially γδ T cells – migrate from the gut to the brain, contributing to enhanced inflammation and enlarged brain injury (194). Stanley et al (195) suggested that more than 70 % of post-stroke lung infection is the result of gut microbiota disseminating from the small intestine (e.g. enterococcus spp. and Escherichia coli) due to increased gut permeability. Further, bacteria and toxins released from the leaky gut may contribute to a life-threatening sepsis and/or systemic inflammatory response syndrome (193). About half of all stroke patients experience various
GI-related complications both acutely and chronically, including dysphagia (~40 %), constipation (~55.2 %), faecal incontinence (~30 %) and GI bleeding (<10 %) (196). These GI complications are also associated with poorer functional outcome and increased dependency, disability and mortality following stroke (196). Prophylactic antibiotic treatment in animal studies of stroke have provided controversial findings (195). Similarly, no protective effects of antibiotic treatment have been seen in clinical stroke trials (197, 198). Overall, these findings are consistent with the possibility that targeting bacterial dissemination and subsequent organ colonization may provide an approach to reducing post-stroke immunodepression and infection.

1.11 Immunotherapy in stroke

1.11.1 Th2 cytokine therapy on stroke outcome (IL-4 and IL-33)

IL-4 is a classical Th2 cytokine that was first discovered in 1990. IL-4 binds to IL-4Rα, and triggers the heterodimerisation with IL-2Rγ or IL-13Rα1, forming Class I and II IL-4 receptors, respectively, which leads to the activation of intracellular signalling, maturation and differentiation of naïve helper T cells to Th2 cells (199, 200). Many studies have shown that IL-4 plays an important immunosuppressive role in the CNS, such that IL-4 inhibits the activation of astrocytes, induces apoptosis of activated microglia and releases neurotrophic factors to ensure neuronal survival (199). IL-4 has been shown to be protective in many inflammatory, and autoimmune CNS diseases including Alzheimer’s disease, Parkinson’s disease and multiple sclerosis (199, 201). Recently, several studies, including from our lab (see Chapter 3) have found that IL-4 immunotherapy reduces brain injury following stroke via a regulation of anti-inflammatory cytokines such as YM1 and arginase-1, adding to our existing body of knowledge (80). However, long-term clinical outcome of IL-4 immunotherapy remains controversial as IL-4 may promote fibrosis in the lung and increase
the permeability of the intestinal epithelium (202), which could increase bacterial infection in
the periphery following stroke.

IL-33 is a novel cytokine that was first characterized by Schmitz et al in 2005 (203). It belongs
to the IL-1 superfamily and shares a similar structure with other IL-1 pro-inflammatory
cytokines including IL-1α, IL-1β, IL-18, IL-36 and IL-37 (203, 204). IL-33 is highly expressed
in various tissues, including brain, spinal cord, lung, skin, stomach, heart, kidney and spleen
(203). It is usually stored in the nucleus of many cells, such as astrocytes, macrophages,
mast cells and stromal cells, acting as an alarmin and it is released upon mechanical cell
stress, cellular injury or necrosis (205-207).

When released, IL-33 binds to its receptor, ST2 on the surface of Th2 cells, activating
downstream signalling pathways NF-κB and MAP kinases, producing the Th2 cytokines IL-
5 and IL-13 and subsequent Th2 immune responses independent of IL-4 (208). IL-33 can
also activate DCs to stimulate naïve T cells directly into Th2 phenotype (209). IL-33,
additionally, can act as a chemokine to attract Th2 cells to the site of injury (210).
Furthermore, IL-33 has been shown to promote and stimulate the proliferation of Tregs
expressing ST2 in the intestine (204), thus activating M2 macrophages and group 2 ILCs
(171, 204). Together with these mechanisms, IL-33 may contribute to tissue repair and
wound healing, as evident in their contribution in reducing allograft transplant rejection (211).
Due to the unique Th2-promoting profile of IL-33, treatment with IL-33 or inhibition of its
activation can be beneficial in disease settings that are exacerbated by Th1 immune
responses. This includes bacterial or viral infections (208), myocardial infarction (212) and
atherosclerosis (206). Not surprisingly, in diseases that can be aggravated by Th2 immune
responses such as airway hyperactivity, parasite and helminth infections, IL-33 has been
found to exacerbate these conditions (207). Effects of Th2 cytokine on several CNS
diseases are summarised in table 1.
In stroke, post-ischemic brain inflammation is regarded as being Th1-mediated. Guo et al (213) have shown that a genetic polymorphism of IL-33 influences the relative risk of ischemic stroke development (213), suggesting the possible involvement of IL-33 in clinical stroke. Indeed, Yang et al (214) reported that ST2−/− mice displayed markedly increased brain infarct volumes following stroke when compared to wild-type mice. It is worth noting that the risk of systemic infection with IL-33 treatment in stroke is elevated due to the higher Th2 immune profile after treatment and additional interventions may be required to eliminate its augmented bacterial infection. Conversely, IL-33 can also cause the production of a Th1 immune response. NK and NKT cells can produce a large quantity of IFN-γ by directly interacting with IL-33 through surface ST2 (215). Taking that into consideration, the role of IL-33 in post-inflammation needs to be further examined (see Chapter 3).

1.11.2 Interleukin-37

Another relatively novel member of the IL-1 superfamily is IL-37, previously known as IL-1F7. IL-37 was first characterized by Pan and co-workers from in silico research (216). It is structurally similar to other IL-1 ligand members, especially IL-18 (217). Both IL-33 and IL-37 are capable of translocating to the cell nucleus and regulating cellular transcriptional activities, potentially down-regulating pro-inflammatory pathways (218). In humans, IL-37 mRNA levels have been detected in thymus, brain, epithelial cells, uterus and testis constitutively, and its expression can be upregulated in DCs (216, 219). Furthermore, immunohistochemistry staining has shown expression of IL-37 to be present in monocytes (220). Despite been identified earlier than IL-33, our knowledge of IL-37 remains much more limited. A murine homologue of IL-37 is yet to be found.

Recent studies found that the binding of IL-37 to IL-18Rα requires the co-binding of a second IL-1 ligand family, IL-1R8 (221). Unlike IL-18, the binding of IL-37 to IL-18 receptor fails to trigger the release of pro-inflammatory IFN-γ. Instead, it dampens the activity of NF-κB and
other intracellular pro-inflammatory mediators such as JunD and BATF, hence activating STAT3. This causes the proliferation of M2 macrophages (219) and reduction of DCs (222). Expression of IL-37 is also required for the normal activity of Tregs. Silencing the IL-37 gene with siRNA results in an exacerbation of immunoreactivity in human T cell cultures (223). More extensive cell culture studies found that IL-37 helps to reduce pro-inflammatory cytokines in lipopolysaccharide (LPS)-induced inflammation, including IL-1α, IL-1β, IL-6, TNF, M-CSF, GM-CSF and IL-23 in macrophage RAW cell lines (219).

Unlike IL-33, which reduces inflammation by skewing the immune response from Th1 to Th2, IL-37 dampens both Th1 and Th2 immunity (224). This is evident when intranasal administration of IL-37 ameliorates the symptoms of allergic reaction (225), the opposite effect of IL-33. Mice made transgenic for FLAG-tagged human transgene IL-37b show better protection against non-lethal LPS challenge, with significant reductions in IL-1β, IL-6, IL-17 and IFN-γ. In addition, IL-37 protein is not expressed in healthy individuals, and is only activated in inflamed tissues, suggesting that IL-37 reduces inflammation by a negative feedback mechanism (219). Circulating levels of IL-37 in acute stroke patients appear to be elevated acutely following stroke (80), suggesting a potential role of IL-37. Mice transgenic for human IL-37 showed better functional recovery after spinal cord injury, with fewer neutrophils infiltrating into injured sites, and reduction of many pro-inflammatory cytokines including IL-6 and IL-1β (226). Studies of IL-37 on several CNS diseases are summarised in table 1. Currently, no study has yet investigated the potential role of IL-37 in stroke, but the studies described in Chapter 4 have focused on this.
Table 1, Roles of Th2-promoting or immunosuppressive cytokines in cerebrovascular diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cytokines</th>
<th>Role of cytokines/antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic stroke</td>
<td>IL-4</td>
<td>• Intracerebral ventricular injection of IL-4 confers protection in IL-4−/− mice (227)</td>
</tr>
<tr>
<td></td>
<td>IL-33</td>
<td>• IL-4 reduces stroke severity in a rat model of stroke (80)</td>
</tr>
<tr>
<td></td>
<td>IL-37</td>
<td>• IL-33−/− mice displayed augmented stroke pathology (228)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Circulating level of IL-37 is increased in patients with acute stroke (229)</td>
</tr>
<tr>
<td>Multiple sclerosis (MS)</td>
<td>IL-4</td>
<td>• IL-4−/− mice have exacerbated disease severity (230)</td>
</tr>
<tr>
<td></td>
<td>IL-33</td>
<td>• Extracellular vesicle injection containing IL-4 is beneficial in murine model of MS (231)</td>
</tr>
<tr>
<td></td>
<td>IL-37</td>
<td>• IL-33 knockout or deficient mice have worse functional outcome, with greater immune cell activation (232)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Plasma IL-37 is increased in patients with MS, and is correlated with disease severity (233)</td>
</tr>
<tr>
<td>Alzheimer’s disease (AD)</td>
<td>IL-4</td>
<td>• IL-4 gene therapy reduces disease severity in APP/PS1 mice (234)</td>
</tr>
<tr>
<td></td>
<td>IL-33</td>
<td>• IL-33 polymorphism poses altered risks of AD development and severity (235)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• IL-33 treatment reduces disease pathogenesis in APP/PS1 mice (236)</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>IL-4</td>
<td>• IL-4 induces apoptosis and IL-4 depletion protects mice from atherosclerosis (237)</td>
</tr>
<tr>
<td></td>
<td>IL-33</td>
<td>• Treatment with IL-33 reduces the development of atherosclerosis (238)</td>
</tr>
<tr>
<td></td>
<td>IL-37</td>
<td>• Transgenic expression of IL-37 protects mice from atherosclerotic lesions (239)</td>
</tr>
<tr>
<td>Myocardial infarction (MI)</td>
<td>IL-4</td>
<td>• IL-4 is protective in MI by promoting M2 polarization of macrophages (240)</td>
</tr>
<tr>
<td></td>
<td>IL-33</td>
<td>• IL-33 treatment improved disease outcome by inhibiting NF-κB and MARP pathway (212, 241)</td>
</tr>
<tr>
<td></td>
<td>IL-37</td>
<td>• IL-37 level is elevated in patients with acute coronary syndrome; High level of IL-37 is associated with worse clinical outcome (242)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• IL-37 suppresses inflammation and is protective following MI (243)</td>
</tr>
</tbody>
</table>

1.12 Stroke models

A substantial number of pre-clinical studies have been conducted using various animal models of stroke developed in the past five decades in order to investigate stroke pathophysiology and develop new therapeutic strategies (244, 245). The most commonly
used stroke models include intraluminal filament-induced or thromboembolic occlusion of MCA, distal or proximal MCAO, transcranial surgical MCAO, as well as approaches using craniotomy, photothrombosis, endothelin-1, and embolic strokes (244, 245). This section will focus on the two major types of animal models of stroke: the intraluminal filament-induced MCAO and photothrombotic stroke. Ischemia to the brain regions supplied by the MCA is the most common type of ischemic stroke among patients (~70 %) (244). For this, a filament is inserted through the internal carotid artery (ICA) and into the Circle of Willis to occlude the origin of the MCA, and transcranial laser Doppler flowmetry is often implemented to increase confidence that artery occlusion has occurred (244, 245).

1.12.1 Middle cerebral artery occlusion (MCAO)

Intraluminal MCAO would seem to be a reasonable approach to replicate a common form of human stroke in rodent models. This stroke model has been used in >40 % of all experimental stroke studies (245). Whilst it is relatively more invasive than other models such as cortical photothrombosis, it is less so than craniotomy-related models. Intraluminal MCAO in rodents is reported to produce a penumbra and also may result in post-stroke infection – two features that are evident in human strokes (244). Retraction of filament from the ICA lumen can also lead to rapid reperfusion in the MCA region, mimicking effective reperfusion therapy by thrombolysis or clot retraction, and this means of causing reperfusion is relatively reproducible and controllable. Permanent MCAO (i.e. without filament retraction) mimics clinical stroke scenarios in which no reperfusion occurs. Brain infarct development in mice subjected to intraluminal MCAO is time- and strain-dependent. A 30-min or longer occlusion will almost always result in a large infarct volume that takes up a substantial proportion (25-35 %) of the ischemic hemisphere (246). On the other hand, the main criticisms of the MCAO model are that intraluminal MCAO does not cause occlusion by a thromboembolism blood clot, it may potentially induce hyperthermia in mice due to ischemic
injuries induced in hypothalamus (247), and that reperfusion (filament withdrawal) does not resemble clinical thrombolysis therapy (244, 245).

1.12.2 Photothrombotic stroke
Photothrombotic stroke is achieved by the systemic injection of a photoreactive dye such as rose bengal into mice or rats, followed by the irradiation of exposed skull by a cold light source (or laser) to a target region of the cortex (244, 248). Photothrombotic stroke has been used in only ~5% of all experimental stroke studies (245). When the brain tissue is then exposed to light in the presence of the intravascular dye, oxygen-derived free radicals are generated, leading to the local activation of endothelial cells and platelets, and eventually platelet aggregation, vessel obstruction and ischemic brain injury (249). Photothrombotic stroke, when compared to intraluminal MCAO model, has several advantages. The photothrombotic stroke model produces highly reproducible and well-defined ischemic damage in rodents while being less surgically invasive. The localised brain lesion allows the assessment of defects relevant to an injury in that region, such as motor function of the contralateral forelimb following a lesion in the primary motor cortex. Animals subjected to this model of stroke exhibit smaller (less than one-third) brain lesions than those resulting from 60 min MCAO and hence much lower mortality (108, 248). However, it is currently thought that the photothrombotic model of cortical stroke is not well suited for assessment of neuroprotective therapies as little or no penumbra is present (250). In addition, acute vasogenic oedema has been observed in rats following photothrombotic stroke (251).

1.13 Effects of reperfusion on stroke
Many animal studies of stroke utilising the MCAO model involve only a transient period of ischemia prior to filament withdrawal which mimics the scenario of ischemia-reperfusion in stroke patients receiving thrombolysis or clot retraction. As might be expected, reperfusion
generally results in better functional outcomes in animals and patients in comparison to permanent cerebral artery occlusion (252), but this is somewhat offset by a well-established phenomenon of reperfusion-induced brain injury involving a sudden increase in ROS production and inflammation (253). Interestingly, Chu et al (109) reported that despite the lack of restoration of blood flow, a greater number of infiltrating immune cells are present in the brain at 24 h following permanent MCAO compared to mice subjected to transient (1 h) MCAO (109). When there is no or delayed reperfusion, the primary injury mechanism in the brain within the first 3 h is irreversible anoxic-ischemic cell death, with a gradual increase in ROS production (253). In order to better understand stroke pathophysiology, it is important to document and identify the key factors in mediating the differences between the stroke subtypes.

1.14 Pre-clinical to clinical stroke research: Where are we? Are we wasting time and money?

Tissue plasminogen activator and mechanical thrombectomy therapies have provided valuable benefits to a relatively small subset of stroke patients within a limited timeframe. The goals of current stroke research are to solve two major obstacles: 1, to widen the therapeutic window for current thrombolytic or thrombectomy therapies and 2, to develop therapeutic strategies targeting mechanisms of injury including those caused by ischemia-reperfusion (47). A tremendous amount of resources have been invested in pre-clinical stroke research since 1958, with more than 1,200 pharmaceutical compounds being tested (40, 254). Despite promising molecular and cellular targets having been constantly identified from animal experiments, all human clinical trials have failed in “bench-to-bedside” translation. The lack of success could be contributed to by several flaws in pre-clinical and clinical study designs.
Firstly, the quality of design of many pre-clinical and clinical trials is questionable. The majority of pre-clinical experimental studies do not have the ideal sample size estimates, randomisation, or blinding incorporated into the study (61, 255, 256). An analysis from the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) database revealed that pre-clinical animal stroke studies in the past few decades contain an average group sample size of 8, with a statistical power of less than 45 % (255). This suggests that most experimental studies only had a 45 % of chance of succeeding by correctly rejecting the null hypothesis; i.e. many studies are likely to have concluded with a ‘false negative’ finding regarding beneficial effects of a therapeutic intervention in stroke (255), and publication of false negative results of a substantial number of studies has likely resulted. On the other hand, many studies with small sample sizes have tended to report results with low reproducibility and inflated effect sizes, leading to potentially a >40 % probability of a ‘false positive’ outcome (257). Indeed, O’Collins et al (40) reported that 114 clinically tested drugs were not more efficacious than 912 drugs used only in experimental settings. Some drugs should never have entered clinical trials if more stringent drug selection guidelines had been enforced (61). Furthermore, almost all (>98 %) original pre-clinical stroke publications report some form of significant result from a therapeutic intervention, whereas negative results – estimated to be truly present in around ~50 % of total studies – apparently are never published, which reflects a strong bias towards studies with significant outcomes in the stroke academic community (255, 258). Dirnagl (255) pointed out that almost two-thirds of pre-clinical studies failed to report relative cerebral blood flow in the brain following MCAO, thus making it hard to confirm whether a stroke actually took place in each animal (255). Combining all these factors, it is safe to say that many experimental stroke publications still do not produce reliable and clinically relevant results (255).
Secondly, pre-clinical animal models of stroke do not perfectly represent clinical stroke scenarios. As mentioned previously, animal models of stroke, such as MCAO or photothrombosis, can only mimic human stroke to a certain extent and are not able to fully explain the complex nature of human stroke pathophysiology (255). This is particularly relevant when assessing post-stroke ischemia-reperfusion injury mechanisms. Most stroke studies administer neuroprotective agents almost immediately after stroke onset, some proof-of-concept studies even apply therapies prior to stroke surgeries (61). While neuroprotective effects of these drugs might be evident if administered shortly after stroke, they provide no clinical value if they fail to demonstrate similar neuroprotective effects when administered hours or days later (61). This is particularly important as up to 80 % of stroke patients do not get admitted to hospital within 3 h (259). In addition, neuroprotective mechanisms of drugs aim to salvage penumbra from proceeding to permanent injury and infarction (61). Many clinical trials fail to select patients with large penumbral volumes, which could hinder the testability of potentially neuroprotective candidate drugs (61, 256). More importantly, the disconnection between pre-clinical and clinical trials is exacerbated when most animal experiments are performed on young, male mice as opposed to the reality that ~75 % of strokes occur in patients aged 65 or over, regardless of gender and other comorbidities (9). Whilst it is easier to test the effect of drugs in the homogenous environment made possible by a single stroke model, stroke patients usually present with extremely diverse disease origins and complexities, such as different ischemic type or other comorbidities such as hyperglycemia (61, 255). It is therefore important to establish selection criteria for better quality control of clinical outcome when testing a neuroprotective agent.

Thirdly, the outcome measurements in pre-clinical and clinical studies are different. Animal studies usually assess the effects of therapeutic interventions based on infarct development, as well as acute functional recovery. However, long-term functional outcome is usually the
gold-standard for assessing neuroprotective drugs in clinical trials; patient outcomes are tested as late as 90 days following stroke (61, 260). It is promising to see that some recent animal stroke studies have extended the functional assessment timeline to one or even two months following stroke induction (259, 261). Consequently, long-term efficacy of each treatment candidate should be the main focus in animal stroke studies, where the level of brain infarct protection is not the only consideration of effectiveness (61).

1.15 Understanding disease mechanisms for better medical care
Stroke pathophysiology is a complex process involving both damaging and protective mechanisms in the brain and periphery (45). It is thus expected that a cocktail of drugs aimed at dampening time-dependent injury mechanisms, as well as promoting endogenous neuroprotection might provide better outcome than a single drug therapy (61). Targeting inflammation has become increasingly popular in acute stroke therapies, but the multifaceted effects of each intervention need to be carefully investigated, in both animals and humans. Besides, understanding the interrelationships between outcome parameters could lead to the better understanding and prediction of disease outcomes (see Chapter 5) in order to progress the development of personalised medicine. To date, it is still relatively unclear which factors truly determine the extent of ischemia-reperfusion injury following stroke (47). It is thus important for pre-clinical researchers to gain a deeper understanding of the disease pathophysiology in order to be able to identify important treatment targets for better clinical outcomes.
1.16 The aims of the thesis are as follows;

Chapter 3
To test the effect of acute therapy with IL-33 on outcome after stroke

Chapter 4
To assess the effect of human IL-37 expression on outcome after stroke

Chapter 5
To perform retrospective analyses on a large sample of experimental stroke (MCAO) data to explore evidence for associations between key parameters
1.17 References


CHAPTER 2:
GENERAL METHODS
2.1 Ethics approval

All studies were performed in accordance with National Health and Medical Research Council of Australia guidelines for the care and use of animals in research and approved by Monash University Animal Ethics Committee. All studies fully adhere to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (1).

Chapter 3: Approved ethics projects SOBS/2010/10 and MARP/2014/064

Chapter 4: Approved human ethics project by National Taiwan University Hospital Committee of Human Research; Approved animal ethics projects MARP/2014/064 and MARP/2016/038

Chapter 5: no ethics directly applicable

2.2 Animals used in the studies described in this thesis

A total of 741 mice were used for the studies described in Chapters 3 and 4. This consisted of 624 wild-type male (C57BL/6; 6-64 weeks), 12 wild-type female (C57BL/6; 7-23 weeks), 17 male T-bet knockout on C57BL/6 background (8-24 weeks), and 88 male C57BL/6 transgenic mice with human IL-37 gene (IL-37tg, 10-80 weeks). One hundred and two male C57BL/6 mice and 24 IL-37tg mice were excluded from the studies due to: 1, insufficient regional cerebral blood flow profile (rCBF) during stroke surgery and recovery (see below) (N = 40); 2, subarachnoid haemorrhage (SAH) during surgery (N = 15); and 3, other technical difficulties resulting in death or euthanasia prior to scheduled endpoint of study (N = 71).

Mice were purchased through Monash Animal Research Platform and were housed in Monash Animal Research Laboratory (ARL) in a specific pathogen-free environment with 12 h light-dark cycle and temperature maintained at 20-23 °C. Mice were housed either with littermates or individually (after surgery) with ad libitum access to food pellets and water. For Chapter 5, data collected from 716 naïve or vehicle-treated C57BL/6 mice subjected to
middle cerebral artery occlusion (MCAO) between 2012 and 2017 in our laboratory were included in a retrospective analysis. Some of the data from 288 of these mice comprised some of the original work presented in Chapters 3 and 4.

2.3 Animal models of stroke

2.3.1 Middle cerebral artery occlusion (MCAO) model (1 h and 45 min ischemia)

Mice were delivered and housed at ARL for acclimatisation for at least 7 days prior to surgery. Mice were weighed and anaesthetised (i.p.; 27-gauge needle) with a mixture of ketamine (80 mg/kg; Parnell Laboratories, Australia) and xylazine (10 mg/kg; Troy Laboratories, Australia) in 0.9 % saline. Local anaesthetic bupivacaine was also injected (2.5 mg/kg, s.c.) locally to the neck incision area. White petroleum jelly (Vaseline, Unilever, Netherlands) or eye drops (Refresh Night time®, Allergan®, Ireland) were applied onto both eyes to prevent the drying of cornea during surgery. Neck and scalp areas were cleaned with 80 % ethanol and a scalpel blade was used to shave off hair from these regions. A midline incision was made to expose the skull. A laser Doppler probe and its holder (Perimed, Sweden) were attached to the left parietal bone of the skull (roughly 2 mm posterior and 5 mm lateral to the bregma) with superglue (Loctite® 401 Instant Adhesive, Henkel, Germany) and accelerator (Insta-Set®, Bob Smith Industries, USA). Regional CBF in the cortical area supplied by the middle cerebral artery (MCA) was measured and recorded by transcranial laser Doppler Flowmetry (PF5010 LDPM Unit, Perimed, Sweden). Mice were then secured by adhesive tape by their paws onto the surgical platform in a supine position. Body temperature was measured and maintained by a rectal probe connected to a Digitemp temperature controller (Extech Equipment, Australia) linked to a ceramic heat lamp (Exo Terra, Australia) at 37.5 ± 0.5 °C throughout the surgery until consciousness was regained.
Under a dissecting microscope (MZ6, Leica Microsystems, Germany), a midline incision was made on the neck and connective tissue was carefully dissected away with curved fine tip forceps (11274-20, Fine Science Tools, Canada) to expose the right common carotid artery (CCA) bifurcation. A low temperature fine-tipped hot wire cautery (EL11115, Warner & Webster, Australia) was used to cauterise the superior thyroid artery, a branch of the external carotid artery (ECA). The ECA was then ligated by tightly tying two knots with 5-0 silk suture (SM8010, Dynek, Australia) anterior to the bifurcation of the CCA. A cut was then made between the knots using straight spring scissors (15000-08, Fine Science Tools, Canada) to create a stump on the ECA. Connective tissues and vagus nerve were then carefully separated from the adjacent CCA and internal carotid artery (ICA) before placing a silk suture underneath the ICA anterior to the CCA bifurcation to create a bridge. The posterior CCA was then clamped (18055-05, Fine Science Tools, Canada) to stop blood flow, and tension was applied on the bridge to prevent backflow of blood from the ICA. A small nick was made at the ECA stump near the CCA bifurcation, and a 6-0 silicone-coated monofilament (0.21 mm in diameter, 602156, Doccol Corporation, USA) was slowly introduced through the tiny opening, advanced along the CCA before curving into the ICA at the CCA bifurcation to occlude the origin of MCA at its junction with the Circle of Willis. Severe reduction of rCBF (typically >70 %) was confirmed with the transcranial laser Doppler flowmeter. The filament was tied in place with the stump, and the clamp and suture bridge were then removed to allow collateral blood supply to other regions of the brain and limit the ischemic area to that supplied by the MCA. Regional CBF was continuously monitored for signs of spontaneous reperfusion or subarachnoid haemorrhage, which were identified as one or multiple sudden spikes or a gradual decline in rCBF shown by transcranial laser Doppler Flowmetry, respectively. Spontaneous reperfusion was rectified immediately with repositioning of the monofilament to ensure constant MCA occlusion. Mice in which SAH occurred were euthanised and excluded from the cohort. The filament was retracted after
either 45 or 60 min of ischemia to allow reperfusion to the MCA, confirmed by a sharp increase in the rCBF.

Suture and filament were then trimmed with angled spring scissors (15006-09, Fine Science Tools, Canada) before the neck incision was closed with a 6-0 nylon suture (Dynek, Australia), applied with Betadine® (Safoni-Aventis, France) and sprayed with OpSite Spray dressing (Smith & Nephew, UK). rCBF was continuously monitored for a further 30 min until removal of the laser Doppler probe from the skull. The scalp incision was closed with superglue (Loctite® 401 Instant Adhesive, Germany). Each mouse received 1 ml of injectable saline (s.c.) with a 27-gauge needle immediately following reperfusion and was continuously monitored under the heat lamp until consciousness was regained.

A satisfactory rCBF profile was defined as >50 % of rCBF reduction from baseline during ischemia, as well as >50 % of rCBF recovery within the first 10 min of reperfusion. Mice that did not meet these rCBF profile requirements were excluded from further study (described above).

One hundred and twenty-eight mice received sham surgeries, in which they were anaesthetised and the right CCA bifurcation was exposed as for the MCAO surgery, but there was no ligation or clamping of arteries, nor any insertion of a monofilament. Sham-operated mice were randomly chosen from the littermates that would be otherwise subjected to MCAO.

Following recovery from stroke or sham surgery, mice were placed into a new flat bottom square cage with extra sawdust bedding, tissue paper, gel nectar (Able Scientific, Australia), and crushed normal chow in the cage, and normal food and water sitting on the top of the cage. Cages were then placed on heating pads with only half of the cage bottom in direct contact with the heat pad so animals were able to move to warmer or cooler parts of the cage and thus more easily regulate their body temperature. Mice were monitored continuously for the first hour (animal ethics projects SOBS/2010/10 and MARP/2014/064)
or once every hour for the first 3 h (ethics project MARP/2016/038) following surgery and assigned clinical signs severity scores at frequent intervals prior to euthanasia according to a range of parameters, including their activity, appearance, body weight change, body temperature, breathing pattern, coat appearance, excrement, and vocalisation as described in Tables 2.1 and 2.2. For studies approved under animal ethics projects SOBS/2010/10 and MARP/2014/064, frequency of animal monitoring and appropriate actions taken were based on the accumulated clinical signs severity score to ensure the welfare of each mouse. For example, mice were monitored twice daily until scheduled euthanasia if they obtained an accumulated clinical score total of 0-4, closely monitor every 3 hours or euthanise at 5 pm if mice obtained an accumulated clinical score between 5 and 9, mice were immediately euthanised if they obtain an accumulated score of 10 or higher (Table 2.1).

A model of photothrombotic cortical stroke was also performed on some mice in Chapter 4. Details of this stroke procedure are outlined in the Appendix.
Table 2.1 Clinical signs severity scoring chart for experimental stroke in mice for ethics numbers SOBS/2010/10 and MARP/2014/064

<table>
<thead>
<tr>
<th>Clinical Sign*</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Activity</td>
<td>Normal healthy movement</td>
<td>Not moving spontaneously but moves when lifted by tail OR circling</td>
<td>Rolling &lt;2 h prior to scheduled cull time</td>
<td>Rolling &gt;2 h prior to scheduled cull time OR doesn’t move when lifted by tail OR no righting reflex</td>
</tr>
<tr>
<td>B. Appearance</td>
<td>Normal healthy</td>
<td>Hunched</td>
<td>Shaking</td>
<td>Unconscious</td>
</tr>
<tr>
<td>C. Body weight</td>
<td>Normal weight</td>
<td>Acute weight loss &lt;10%</td>
<td></td>
<td>Acute weight loss &gt;10% OR chronic weight loss &gt;15%</td>
</tr>
<tr>
<td>D. Body temperature</td>
<td>Feels warm</td>
<td></td>
<td>Feels cold</td>
<td></td>
</tr>
<tr>
<td>E. Breathing</td>
<td>Normal</td>
<td>Slow or irregular</td>
<td></td>
<td>Gasping OR labored</td>
</tr>
<tr>
<td>F. Coat</td>
<td>Normal</td>
<td>Piloerection</td>
<td></td>
<td>Bleeding OR infected wounds</td>
</tr>
<tr>
<td>G. Feces/urine</td>
<td>Evidence in box</td>
<td>No feces or urine at 9 am on day after surgery</td>
<td>No feces or urine by 9am 2 days after surgery</td>
<td></td>
</tr>
<tr>
<td>H. Vocalization</td>
<td>None</td>
<td>Quiet/low level OR nattering</td>
<td></td>
<td>Loud</td>
</tr>
</tbody>
</table>

* Use monitoring sheet to add scores for each of A-H to calculate total clinical score

Criteria for close monitoring OR immediate euthanasia

<table>
<thead>
<tr>
<th>Total clinical score</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>Monitor at 9:00 am and 5:00 pm</td>
</tr>
<tr>
<td>5-9</td>
<td>Closely monitor at least every 3 hours; euthanize at 5:00 pm</td>
</tr>
<tr>
<td>10 or more</td>
<td>Immediately euthanize</td>
</tr>
</tbody>
</table>

Specific requirements

- Continual monitoring of all mice for at least 1 h after surgery
- Provide crushed/crumbed food to the box
- Provide hydration gel in a dish in the box
- Weigh mice daily
- Monitoring sheet to be kept in the vicinity of the mouse
- Monitor, score and record all mice at 9.00 am and 5.00 pm every day
- If clinical score is 5-9, also monitor, score and record at 12.00 pm and 3.00 pm every day
- If clinical score is 5-9 at 5.00 pm, euthanize (or continue to monitor every 3 h)
- If mouse is cold, provide heat via lamp or mat
- Give 1 ml subcutaneous saline once daily
### Table 2.2 Clinical signs severity scoring chart for experimental stroke in mice for ethics number MARP/2016/038

<table>
<thead>
<tr>
<th>CLINICAL SIGNS</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Activity (circling)</strong></td>
<td>Normal healthy movement</td>
<td>Not moving spontaneously but moves when lifted by tail OR circling within 2 hours prior to scheduled experimental endpoint</td>
<td>Not moving when lifted by tail OR circling at more than 2 hours prior to scheduled experimental endpoint</td>
</tr>
<tr>
<td><strong>B. Activity (rolling)</strong></td>
<td>Normal healthy movement</td>
<td>Rolling within 2 hours prior to scheduled experimental endpoint</td>
<td>Rolling at more than 2 hours prior to scheduled experimental endpoint OR no righting reflex</td>
</tr>
<tr>
<td>C. Appearance</td>
<td>Normal healthy</td>
<td>Hunched</td>
<td>Shaking/Unconscious</td>
</tr>
<tr>
<td><strong>D. Body weight</strong></td>
<td>Normal weight</td>
<td>Acute weight loss &lt;10%</td>
<td>Acute weight loss &gt;10% OR Chronic weight loss &gt;15%</td>
</tr>
<tr>
<td>E. Body temperature</td>
<td>Feels warm</td>
<td>Slow OR Irregular</td>
<td>Gasping OR Labored</td>
</tr>
<tr>
<td><strong>F. Breathing</strong></td>
<td>Normal</td>
<td>Piloerection/ruffled fur</td>
<td>Bleeding OR Infected wounds</td>
</tr>
<tr>
<td><strong>G. Coat</strong></td>
<td>Normal</td>
<td>Evidence in box</td>
<td>No feces or urine by 9am 2 days after surgery</td>
</tr>
<tr>
<td><strong>H. Feces/urine</strong></td>
<td>Evidence in box</td>
<td>No feces or urine at 9 am on day after surgery</td>
<td>No feces or urine at 9 am 2 days after surgery</td>
</tr>
<tr>
<td>I. Vocalization</td>
<td>None</td>
<td>Quiet/low level OR Nattering</td>
<td>Vocalization</td>
</tr>
</tbody>
</table>

### SPECIFIC REQUIREMENTS FOR POST-STROKE MONITORING

- Hourly monitoring of all mice for first 3 hours after surgery
- If signs are present within 2 hours of scheduled experimental endpoint, animal will be monitored every 30 minutes
- Provide crushed/crumbed food to the box
- Provide wet mashed food or gel in a dish in the box
- Weigh mice daily
- Monitoring sheet to be kept in the vicinity of the mouse
- After MCAO surgery, keep mice on heat pad until the end of experiment
- Give 1 mL subcutaneous saline once daily

### CRITERIA FOR CLOSE MONITORING OR IMMEDIATE EUTHANASIA

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score of 0</td>
<td>Monitor daily for the first 5 days and then every 2-3 days</td>
</tr>
<tr>
<td>Any score of 1</td>
<td>Monitor twice daily</td>
</tr>
<tr>
<td>Any score of 2</td>
<td>Immediately euthanise</td>
</tr>
</tbody>
</table>
2.4 Drug preparations

2.4.1 Chapter 3

2.4.1.1 Mouse recombinant interleukin (IL)-4 protein

Recombinant mouse IL-4 carrier free protein was purchased from Biolegend® (118 amino acid, His23-Ser140, > 95% purity; Product code: 574306; USA). Each vial of IL-4 was diluted in 1 % bovine serum albumin (BSA, Sigma-Aldrich, USA) dissolved in injectable saline (m/v) made immediately prior to dilution, to achieve a final concentration of 25 µg/ml. Diluted recombinant IL-4 protein was then aliquoted to sterile, autoclaved Axygen™ MaxyClear Snaplock microtubes (Fisher Scientific, USA) with each containing 5 µg of recombinant IL-4 in 200 µl of 1 % BSA in saline to avoid any freeze-thaw cycle. All microtubes were then stored at -20 °C for further use. For injection, each aliquot containing IL-4, IL-33 or 1% BSA was thawed at room temperature and each animal was injected with 200 µl of aliquot immediately following reperfusion.

2.4.1.2 Mouse recombinant IL-33 protein

Recombinant mouse IL-33 carrier free protein was purchased from Biolegend® (158 amino acid, Ser109-Ile266, >98 % purity; Product code: 580508; USA). Each vial of recombinant IL-33 was diluted in 1 % of BSA (Sigma-Aldrich, USA) dissolved in injectable saline (m/v) made immediately prior to dilution, to achieve a final concentration of 10 µg/ml. Diluted recombinant IL-33 protein was then aliquoted to sterile, autoclaved Axygen™ MaxyClear Snaplock microtubes (Fisher Scientific, USA) with each containing 2 µg or 0.4 µg of recombinant IL-33 in 200 µl of 1 % BSA in saline. All microtubes were then stored at -20 °C until further use. Repeated free-thaw cycles were avoided.
2.4.1.3 Antibiotics

Ampicillin and gentamicin were administered as antibiotic treatments for some mice before and after stroke. Ampicillin and gentamycin were reconstituted and diluted in injectable saline to achieve a final concentration of 1.5 and 40 mg/ml, respectively. Ampicillin and gentamycin were then aliquoted separately and stored at 4 °C until future use. For injection, each mouse was administered subcutaneously a combination of gentamycin (12 mg/kg) and ampicillin (300 mg/kg).

2.4.2 Chapter 4

2.4.2.1 Recombinant human IL-37 protein

Recombinant human IL-37 protein was provided by Prof Marcel Nold and his team from Hudson Institute of Medical Research, Clayton. To maintain protein solubility and stability, recombinant IL-37 protein was stored at -80 °C and diluted in sterile phosphate buffer saline (PBS) solution to 1 % (m/v) on the day of surgery.

2.5 Treatment

2.5.1 Chapter 3

Prior to surgery, individuals in each mouse litter were randomly assigned to vehicle, IL-4 or IL-33 treatment groups equally, where possible. Vehicle (1 % BSA in saline), IL-4 (5 µg) or IL-33 (2 µg or 0.4 µg) were thawed and injected intraperitoneally into the assigned mice with a 27-gauge needle once at 24 h prior to surgery, and again immediately after reperfusion following MCAO. For mice with a scheduled 3-day recovery period, vehicle (1 % BSA) or IL-33 (2 µg) was injected (i.p.) once immediately after reperfusion following MCAO, and again at every 24 h until 2 days following stroke. Among those mice receiving antibiotic therapy, a mixture of ampicillin (100 mg/kg) and gentamycin (4 mg/kg) were injected subcutaneously at least 2 h following the initial vehicle or IL-33 treatment in each group. Antibiotic treatments
were repeated at every 8 h for an additional 2 times. Treatment of each mouse was performed in a blinded fashion.

2.6 Post-stroke functional assessment

A range of assessments were performed on mice to determine their functional deficits immediately before the scheduled euthanasia at either 24 or 72 h following stroke induction. The investigators were blinded to all treatments or mouse genotypes at the time of functional assessment.

2.6.1 Clinical score

Clinical scores were assessed using a modified 6-point scoring system in studies reported in Chapters 3, 4 and 5, as described previously (2, 3). Mice were assigned to a specific clinical score based on the following criteria: 0 – normal motor function; 1 – flexion of torso to one side or swing out of control when lifted by tail; 2 – normal posture at rest, but circling to one side when held by tail on a flat surface; 3 – leaning to one side at rest; 4 – no spontaneous activity and/or rolling to one side on a flat surface; and 5 – death prior to scheduled euthanasia (Chapters 3 and 4 only). In studies reported in Chapter 5, mice with a clinical score of 5 were excluded from the clinical score and reported separately as mortality.

2.6.2 Hanging grip test

Hanging grip tests were performed at the end of some experiments to assess forelimb strength and motor coordination (2, 3). Mice were suspended with both forelimbs gripping a horizontal metal wire (~1 mm in diameter) with a 60 cm span and raised 50 cm above a padded surface. Time taken for mice to fall was recorded for up to 180 s. The procedure was repeated another two times with at least 2 min of rest between each trial. The average
time of the 3 trials was calculated and recorded as hanging wire latency to fall. In studies reported in Chapter 5, mice that achieved a full 180 s of hanging grip time were excluded from the bivariable and multivariable analyses in order to more readily identify true relationships between hanging grip time and various other parameters.

2.6.3 Open field parallel floor test

Spontaneous locomotor activity was assessed in some mice following surgery using an automated behavioural video-tracking and analytic software application (ANY-Maze®, USA). Each mouse was placed in a 20 cm x 20 cm acrylic box with parallel metal rods elevated 10 mm above a metal floor. Metal rods are spaced ~8 mm apart to allow the mouse’s feet to slip through, which is detected by the sensor situated within the metal floor and recorded as foot-slip event. A camera was positioned overhead to record mouse locomotor activity for 300 s, starting 15 s after placing the animal into the box to limit any anxious behaviour due to human handling. A range of parameters were recorded and analysed, including total distance travelled (m), average speed (m/s), total time mobile (s), total mobile episodes, absolute turn angle (°), number of complete rotations (clockwise and anti-clockwise) of the animal’s body, the number and duration of foot-slip s. Total distance travelled (m) and maximum speed (m/s) were reported in these chapters.

Functional assessments specific for photothrombotic stroke are outlined in the Appendix of Chapter 4.

2.7 Evaluation of brain injury

2.7.1 Brain sectioning

Mice were euthanised by isoflurane (Baxter, USA) inhalation overdose at 24 or 72 h following the induction of MCAO or at 7 days following photothrombotic stroke. Mice were decapitated and immediately the brain was gently removed from the skull, snap frozen in
liquid nitrogen and then stored at -80 °C until sectioning. Brains were coronally sectioned with 30 µm and 10 µm in thickness at -17 °C with a cryostat (CM1850, Leica Microsystems, Germany) for infarct determination or immunohistochemistry, respectively. In mice subjected to MCAO, evenly spread coronal sections with 30 µm thickness and separated by 420 µm were collected throughout the brain, and thaw-mounted onto poly-L-lysine coated glass slides (0.1 % in distilled H₂O, Sigma-Aldrich, USA). For brains collected following the photothrombotic model of stroke, evenly spread coronal sections with 30 µm thickness separated by 210 µm were collected spanning the infarct region, and thaw-mounted onto poly-L-lysine coated glass slides. Multiple 10 µm coronal sections (~9-10 sections) were also thaw-mounted onto poly-L-lysine coated glass slides after each 30 µm coronal section collected in 6 regions between bregma 1.18 mm and -1.34 mm. Coronal brain sections 10 µm thick were immediately frozen and stored at -80 °C until future use, whereas 30 µm sections were stored at -20 °C and stained for infarct analysis within 24 h.

2.7.2 Infarct and swelling analysis

To delineate the infarct, 30 µm brain sections were immersed in 0.1 % thionin in acetic acid (Sigma-Aldrich, USA) for 2 min and then washed twice in distilled water to remove excess thionin. Sections were then immersed in 80 % ethanol (v/v), followed by 100 % ethanol (v/v) for 2 min each. Sections were then dried, dipped in xylene, and cover-slipped with DPX-mounting media (LabChem, USA). Stained sections were allowed to dry overnight before images were taken using a charge-coupled device (CCD) camera (Cohu Inc., USA) mounted above a light box (Biotec-Fisher Colour Control, Germany).

Infarct and edema volumes were analysed in a blinded manner, such that the investigator was blinded to treatment and/or genotype of each mouse. Right infarct area (RIA), right hemisphere area (RHA), left hemisphere area (LHA), cortical infarct area (CIA), right cortical area (RCA), and left cortical area (LCA) were measured using ImageJ software (National
Institute of Health, USA) to estimate total infarct volume corrected for edema using the following formula, as described previously (2, 4): Corrected infarct volume (CIV) = [RIA-(RHA – LHA)] x (thickness of section + distance between sections). Total corrected cortical infarct volume is calculated as: CCIV = [CIA-(RCA-LCA)] x (thickness of section + distance between sections). Corrected subcortical infarct volume (CSIV) is the difference between CIV and CCIV. Total edema volume is the total volume difference between the ischemic (right) and ipsilateral (left) hemisphere, calculated using the following formula: [RHA-LHA] x (thickness of section + distance between sections).

2.8 Immunohistochemistry

2.8.1 3,3’-diaminobenzidine (DAB) staining

Dako kits (Dako, Denmark) were used to perform DAB staining on frozen brain sections (10 µm in thickness). Sections were air dried (~10 min) and the borders of sections were circled with a Dako pen (Dako, Denmark). Sections were then fixed in freshly made ice-cold 4 % paraformaldehyde (PFA) for 15 min before washing in 0.01 M of PBS (3 x 5 min). Brain sections were then blocked with peroxidase blocking solution supplied with the Dako kit for 10 min at room temperature. Following washing in PBS (3 x 5 min), sections were blocked with 10 % normal goat serum (NGS; Sigma-Aldrich, USA) diluted in PBS containing 0.2 % Triton-X 100 (Sigma-Aldrich, USA) for 30 min at room temperature. Sections were subsequently incubated in either rabbit anti-CD3 or rabbit anti-myeloperoxidase antibodies at appropriate concentrations (see table 2.3) overnight at 4 °C in a humidified chamber. Sections were washed in PBS (3 x 5 min) the next day, followed by a 2-h incubation with peroxidase-labelled polymer-conjugated goat anti-rabbit IgG immunoglobulin, supplied by the Dako kit at room temperature (see table 2.3). Sections were again washed in PBS (3 x 5 min) and incubated with DAB reagent supplied by the Dako kit for ~7 min in the dark in a fume hood. Brain sections were then washed in distilled water to stop the reaction before
proceeding to hematoxylin nuclear counterstain (Vector Laboratories, USA) for 2 min. Sections were then rinsed with Scott’s Tap Water before being dehydrated in 80 % (v/v), 100 % (v/v) ethanol and xylene for 1 min each, sequentially. Sections then cover-slipped with DPX mounting media (LabChem, USA). Tissue-mounted slides were allowed to dry overnight before being viewed and counted on Olympus light microscope (Olympus BX51, Japan).

2.8.2 Immunofluorescence

Slide-mounted brain sections were airdried and borders of sections were circled with a Dako pen (Dako, Denmark). Sections were then fixed in ice-cold acetone or freshly made 4 % PFA for 15 min before being washed in PBS (3 x 5 min). Antigen-retrieval steps were then carried out with CXCL16 antibody. To perform the antigen retrieval protocol, antigen retrieval buffer (10 mM sodium citrate, 0.05 % Tween 20, pH 6.0) was heated in the microwave until boiling. Sections were then immersed in the hot antigen retrieval buffer for 10-20 min then removed to cool to room temperature. Sections were washed in PBS (3 x 5 min), followed by blocking with 10 % NGS or 2 % BSA for 30 min at room temperature to prevent non-specific binding, where appropriate. Sections were incubated with appropriate primary antibodies (See Table 2.3) at 4 °C overnight in a humidified chamber, then washed in PBS (3 x 5 min) and incubated in appropriate fluorophore-conjugated secondary antibodies (See Table 2.3) for 2 h at room temperature away from light. For direct immunofluorescence staining, same protocol was followed without the incubation of secondary antibody with brain sections. All primary and secondary antibodies were diluted to appropriate concentrations in diluents containing 0.2 % Triton-X in 0.01 M PBS. Alternatively, if the primary antibody was raised in mice, a Mouse-on-Mouse (M.O.M.™) kit (Vector Laboratories. USA) was used and all antibody dilutions were performed in M.O.M.™ diluent made up from the M.O.M.™ kit instructions. To block non-specific binding, mouse Ig Blocking Reagent supplied in the
M.O.M.™ kit was used to incubate sections for 1 h at room temperature. Sections were then washed in PBS (3 x 5 min) and incubated in M.O.M.™ diluent for 5 min at room temperature, followed by the incubation of primary antibodies at 4 °C overnight in a humidified chamber. Sections were then washed with PBS (3 x 5 min), and incubated with M.O.M.™ biotinylated anti-mouse IgG reagent for 10 min. Following a wash with PBS (3 x 5 min), Fluorescein Avidin DCS supplied by the M.O.M.™ kit was applied to sections for 5 min. Finally, all sections were washed with PBS (3 x 5 min), and cover-slipped with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) for nuclear staining. Coverslips were sealed with nail polish for storage. Slides were analysed and photographed using an Olympus Fluorescent Microscope (Olympus BX51, Japan) with appropriate filters (See table 2.3).

DAB or fluorescent positive cells of interest were generally counted throughout the entire hemisphere using a high-power field object lens (20X) paired with a 10X eye piece lens. The contralateral hemisphere was used as a control. In studies reported in Chapter 4, the number of CD45⁺ cells were colocalised with DAPI and counted in three 710 µm x 530 µm fields (200X) in each of the infarcted, cortical and subcortical regions of the ischemic hemisphere, and corresponding mirrored fields in the ipsilateral hemisphere on each section. For GFAP expression analysis, fluorescence intensity was assessed using ImageJ software in each of the peri-infarct regions and the corresponding regions in the contralateral hemisphere spanning 250 µm x 250 µm in each region. Typically, average values of total cell count or fluorescence intensity were analysed in 5-6 whole brain sections from each animal. All analyses of immunohistochemistry slides were conducted by an investigator blinded to the identity of the treatment group.
Table 2.3 List of antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Primary antibodies*</th>
<th>Dilution</th>
<th>Company</th>
<th>Notes</th>
</tr>
</thead>
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<tr>
<td>Rabbit-anti-CD3</td>
<td>1:200</td>
<td>Abcam, USA</td>
<td>DAB staining</td>
</tr>
<tr>
<td>Rabbit-anti-myeloperoxidase</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mouse anti-3-nitrotyrosine</td>
<td>1:50</td>
<td></td>
<td>M.O.M.™ kit</td>
</tr>
<tr>
<td>Mouse anti-CD68</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-GFAP</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-CXCL16</td>
<td>1:800</td>
<td>R &amp; D systems, USA</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488 Rat anti-CD45</td>
<td>1:50</td>
<td>BioLegend, USA</td>
<td>Direct Immunofluorescence Filter: FITC</td>
</tr>
<tr>
<td>Rat anti-F4/80</td>
<td>1:100</td>
<td>Bio-Rad, USA</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-human IL-37</td>
<td>1:500</td>
<td>Invitrogen, USA</td>
<td>Anti-human antibody; M.O.M.™ kit</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Company</th>
<th>Fluorescent filters</th>
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</thead>
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<tr>
<td>Goat-anti-rabbit IgG Alexa Fluor® 594</td>
<td>1:500</td>
<td>Life Technologies, USA</td>
<td>Cy3™</td>
</tr>
<tr>
<td>Goat-anti-rabbit IgG Alexa Fluor® 488</td>
<td>1:200</td>
<td></td>
<td>FITC</td>
</tr>
<tr>
<td>Goat-anti-rat IgG Alexa Fluor® 594</td>
<td>1:500</td>
<td></td>
<td>Cy3™</td>
</tr>
<tr>
<td>Donkey anti-rabbit IgG DyLight® 488</td>
<td>1:800</td>
<td></td>
<td>FITC</td>
</tr>
<tr>
<td>Donkey anti-mouse DyLight® 488</td>
<td>1:800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.O.M.™ biotinylated anti-mouse IgG reagent</td>
<td>1:200</td>
<td>Vector Laboratories, USA</td>
<td>FITC</td>
</tr>
<tr>
<td>Anti-rabbit IgG horse-radish peroxidase conjugate</td>
<td>1:200</td>
<td>Dako, Denmark</td>
<td>Visible light</td>
</tr>
</tbody>
</table>

* All primary antibodies are anti-mouse unless otherwise specified
2.9 Tissue processing for RNA extraction

In some animals, RNA was extracted from the brain, lung and spleen. Mice were euthanised with isoflurane (Baxter, USA) inhalation overdose and each was transcardially perfused with 50 ml of RNase-free PBS (Sigma-Aldrich, USA). Perfusion was performed by inserting a 27 G needle into the left ventricle, with sterile PBS slowly and continuously administered. A small nick was made with a pair of straight spring scissors on the right atrium to allow the escape of dark venous blood into the body cavity. After successful perfusion, lungs were removed from the chest cavity and cleared of connective tissue and blood, before being snap frozen in liquid nitrogen. The spleen was then removed from the abdomen, cleared of connective tissue, cut in half and also snap frozen. Mice were decapitated and the brain was immediately removed from the skull, separated into left and right hemispheres, and snap frozen in liquid nitrogen. All these tissues were then stored at -80 °C until future use.

2.10 RNA extraction

RNA extraction from tissues was performed using the RNeasy Mini Kit (Qiagen, Germany) and TRIzol® reagent (Invitrogen, ThermoFisher Scientific, USA). Specifically, tissues were weighed and added to 1 ml of Trizol® reagent. Tissues were then thoroughly homogenised with a hand-held homogeniser (TMP125-115, Omni Inc., USA). Samples were topped up with additional Trizol® reagent according to measured weights, to achieve a typical final volume of 1 ml of Trizol® reagent for every 100 mg of brain, or every 50 mg of spleen or lungs. In order to dissociate protein-nucleic acid complexes, samples were incubated at room temperature for 5 min, followed by the addition of 20 % volume of chloroform into the mixture. Samples were shaken vigorously for 15 s and incubated again for 2 min at room temperature. Following incubation, samples were centrifuged at 4 °C, 12,000 g for 15 min to allow the separation of RNA from DNA, protein and debris. The upper aqueous phase was then carefully collected from the mixture, while avoiding the interphase and debris. The
aqueous phase was mixed with an equal volume of 70% ethanol (v/v) followed by vortexing. Samples were then transferred to RNeasy Mini spin columns with collection tubes supplied with the RNeasy Mini Kit, and centrifuged at 10,000 g for 30 s. All flow-throughs were discarded. DNase digestion was also performed using the RNase-Free DNase Set (Qiagen, Germany). Each sample was incubated with 80 µl of DNase reagent mixture containing 30 Kunitz units of DNase (in 10 µl of RNase-free water) dissolved in 70 µl of Buffer RDD for 20 min at room temperature. Each sample was washed with 700 µl of buffer RW1 and twice with 500 µl of buffer RPE, with centrifugation at 10,000 g for 30 s to discard the flow-through in between each wash. Subsequently, RNeasy columns were centrifuged at 14,000 g for 1 min to ensure the column membrane was dry. 50 µl of RNase-free water was added to each column membrane, and incubated for 1 min before centrifugation for 1 min at 10,000 g. A NanoDrop 1000 Spectrophotometer (ThermoFisher, USA) was used to measure the light absorbance properties of each RNA sample to determine its purity and concentration. Samples with RNA concentrations lower than 150 ng/µl were discarded. Samples with an RNA concentration greater than 2000 ng/µl were diluted before proceeding to the cDNA conversion. All RNA samples were tested three times with the NanoDrop and an average value was used to determine the RNA concentration. RNA samples with both 260:230 and 260:280 ratios of 2.0 or greater were considered pure and thus acceptable for subsequent cDNA conversion and real-time polymerase chain reaction (rt-PCR) procedures. In the cases of RNA samples having an acceptable yield but low purity, an RNA purification procedure was performed. RNA samples were added to 0.1 volume of 3 M RNase-free sodium acetate (pH 5.2; Thermal Fisher Scientific, USA) followed by vortexing. Samples were then added and mixed thoroughly with 2.2 volumes of ice cold 100% ethanol. Samples were kept at -80 °C for ~1 h before being centrifuged at 14,000 g for 10 min. Supernatant was then decanted, and the remaining traces of pellet washed with 500 µl of 80% ethanol (v/v). Samples were again centrifuged at 14,000 g for 5 min and all supernatant was
removed by carefully inverting the sample on a paper towel to allow it to dry. The remaining pellet was then reconstituted in RNase-free water (typically ~30 µl) for testing of RNA purity and concentration.

2.11 First strand cDNA synthesis
First strand cDNA synthesis was performed using a High Capacity cDNA RT kit (Applied Biosystem, ThermoFisher Scientific, USA). 2 µg of RNA was added to 6.8 µl of master mix supplied by cDNA RT kit, consisting of RT buffer (2 µl), RT random primers (2 µl), dNTP mix (0.8 µl), RNase inhibitor (1 µl), and MultiScribe Reverse Transcriptase (1 µl). Reverse transcription was performed using a thermal cycler (My Cycler, Bio-Rad, USA) using the following protocol: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C before holding at 4 °C. cDNA was then stored at -20 °C for later use.

2.12 Real-time polymerase chain reaction (rt-PCR)
To perform rt-PCR, pre-made first strand cDNA was loaded with Taqman® Universal PCR Master Mix (Applied Biosystems, USA) and pre-designed Taqman® primers to measure the mRNA expression of various genes, including Il6, Chil3 (Ym1), Tnfa, Arg1, Il1b, Il-10, Il33, Il4, Foxp3, Ccl2, Icam1, Il13, Il5, St2, Tgfb1, Vcam1, Ifng, Cxcl16, Tbx21, Gata3, Il23a, Cxcl14, Rorc, Il18, Il12a, Il21, Il22, and human IL-37 (Applied Biosystems, USA). Control genes were selected based on their expression stability in certain tissues (5), for example, Gapdh was selected as the housekeeping gene for brain and lung tissues, whereas for spleen tissue, β-actin was used as the housekeeping gene. All samples were loaded onto a 96-well plate as a triplicate to minimise data variability. rt-PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using the following protocol: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 15 s), annealing and extension (60 °C for 1 min). Fluorescence intensity was measured at the end
of each cycle and a comparative threshold was manually selected (cycle threshold Ct) to quantify gene expression, which was normalised to the housekeeping gene and data were presented as fold-change relative to control mice using the comparative cycle threshold (Ct) method. The formula is shown below:

\[
\text{Fold change} = 2^{-\Delta\Delta C_t}; \text{ in which } \Delta C_t = \text{Ct (mean value of target gene in triplicates) – Ct (mean value of housekeeping gene in triplicates)}; \Delta \Delta C_t = \Delta C_t (target \text{ gene}) – \Delta C_t (average values of the target gene in all control samples)}.
\]

2.13 Bacterial analysis in lungs, liver and mesenteric lymph node

Mice were euthanised by isoflurane inhalation overdose. Dissection of animals for bacterial analyses was carried out under aseptic conditions. All dissection equipment was cleaned and the mouse’s skin was washed with 80% ethanol (v/v). All lobes of the lung, left lateral lobe of liver and mesenteric lymph nodes were isolated, weighed, and immediately transferred to 1 ml of sterile PBS, before being homogenised with a hand-held homogeniser (TMP125-115, Omni Inc., USA). 10 µl of tissue homogenates was transferred and serially diluted up to 4 times by a factor of 10, and each dilution then plated as triplicates onto brain-heart-infusion agar plates supplemented with 5% sheep blood (Monash University, Australia). Plates were inverted and incubated at 37 °C for 18 h. The number of colony forming units (CFUs) were counted and presented as CFUs per mg of tissue using the following formula: CFU/mg = (average CFU counts in the triplicates x dilution factor)/tissue weight in mg.

2.14 Statistical analyses

Parametric values are presented as mean ± standard error of mean (SEM) in Chapters 3 and 4 or mean ± standard deviation (SD) in Chapter 5, as appropriate. Non-parametric values (such as clinical score) are presented as median. Individual data points were
presented on all graphs. For comparison between 2 groups of parametric data, an unpaired t-test was selected for data groups with equal variances, otherwise Welch’s t-test was selected for data analysis. Alternatively, a Mann-Whitney test was used to compare two groups of non-parametric data. For comparison between three or more groups, data were analysed with one-way ANOVA with Bonferroni post hoc tests for parametric values, or Kruskal-Wallis test for non-parametric values. A ROUT test was performed to identify statistical outliers, where indicated. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. USA) in Chapters 3, 4 and part of 5. In Chapter 5, the D’Agostino-Pearson normality test was performed to identify the distribution profiles of each parameter reported. To identify correlations between parameters, bivariable analysis (Spearman rank correlation test) and multivariable analysis (linear or logistic regression) were performed using IBM® SPSS® Statistics Version 24 (IBM, USA) and RStudio Version 1.0.136 (Affero General Public License v3), respectively. Structural equation modelling performed in Chapter 5 was done using IBM®SPSS®Amos 25.0.0 (Build 1338625, IBM, USA). P < 0.05 was considered as statistically significant.
2.15 References


CHAPTER 3:

IL-33 MODULATES INFLAMMATORY BRAIN INJURY BUT EXACERBATES SYSTEMIC IMMUNOSUPPRESSION FOLLOWING ISCHEMIC STROKE
IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke

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Research Article  Cardiology  Inflammation

Stroke triggers a complex inflammatory process in which the balance between pro- and antiinflammatory mediators is critical for the development of the brain infarct. However, systemic changes may also occur in parallel with brain inflammation. Here we demonstrate that administration of recombinant IL-33, a recently described member of the IL-1 superfamily of cytokines, promotes Th2-type effects following focal ischemic stroke, resulting in increased plasma levels of Th2-type cytokines and fewer proinflammatory (3-nitrotyrosine*F4/80*+) microglia/macrophages in the brain. These effects of IL-33 were associated with reduced infarct size, fewer activated microglia and infiltrating cytotoxic (natural killer–like) T cells, and more IL-10–expressing regulatory T cells. Despite these neuroprotective effects, mice treated with IL-33 displayed exacerbated post-stroke lung bacterial infection in association with greater functional deficits and mortality at 24 hours. Supplementary antibiotics (gentamicin and ampicillin) mitigated these systemic effects of IL-33 after stroke. Our findings highlight the complex nature of the inflammatory mechanisms differentially activated in the brain and periphery during the acute phase after ischemic stroke. The data indicate that a Th2-promoting agent can provide neuroprotection without adverse systemic effects when given in combination with antibiotics.

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IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke

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Introduction

Ischemic stroke triggers an extensive inflammatory response in the brain that is thought to be a major mechanism of secondary brain injury. This occurs when cerebral ischemia induces the activation and proliferation of brain-resident microglia and the recruitment of circulating leukocytes, including neutrophils, monocytes, and lymphocytes (1, 2). This acute local inflammatory response is mediated by cytokines and chemokines initially released by ischemic cerebral endothelial cells (3). High levels of proinflammatory cytokines and/or low levels of antiinflammatory cytokines are linked with early worsening and more severe outcomes of stroke (4, 5).

Clinical evidence indicates that elevated numbers of circulating Th2-related cells are associated with a reduced risk of cardiovascular events (6). Unlike Th1 responses, which promote the generation of proinflammatory mediators in the setting of cardiovascular disease, Th2-type responses lead to production of antiinflammatory cytokines, such as IL-4, IL-5, IL-9, IL-13, IL-25, and IL-33 (7, 8). Importantly, the relative predominance of a Th2- versus a Th1-type inflammatory response appears to be crucial for the composition and activity of immune cells entering the brain and consequently for the severity of the stroke.

Stroke triggers a complex inflammatory process in which the balance between pro- and antiinflammatory mediators is critical for the development of the brain infarct. However, systemic changes may also occur in parallel with brain inflammation. Here we demonstrate that administration of recombinant IL-33, a recently described member of the IL-1 superfamily of cytokines, promotes Th2-type effects following focal ischemic stroke, resulting in increased plasma levels of Th2-type cytokines and fewer proinflammatory (3-nitrotyrosine+F4/80+) microglia/macrophages in the brain. These effects of IL-33 were associated with reduced infarct size, fewer activated microglia and infiltrating cytotoxic (natural killer–like) T cells, and more IL-10–expressing regulatory T cells. Despite these neuroprotective effects, mice treated with IL-33 displayed exacerbated post-stroke lung bacterial infection in association with greater functional deficits and mortality at 24 hours. Supplementary antibiotics (gentamicin and ampicillin) mitigated these systemic effects of IL-33 after stroke. Our findings highlight the complex nature of the inflammatory mechanisms differentially activated in the brain and periphery during the acute phase after ischemic stroke. The data indicate that a Th2-promoting agent can provide neuroprotection without adverse systemic effects when given in combination with antibiotics.
eventual outcome after cerebral ischemia (9). Mice lacking IL-4 develop more M1-polarized microglia/macrophages, larger infarcts, and worse functional deficits following cerebral ischemia, whereas administration of recombinant IL-4 abrogates these effects (10) and improves outcome in WT mice (10, 11). Consistent with such an effect, IL-10, which can mediate Th2 cell functions (7), also exerts protective effects whereby its overexpression results in smaller infarcts (12, 13). Conversely, deficiency of IL-10 results in more severe infarcts and functional deficits after stroke (14).

Here, we have tested the hypothesis that pharmacological intervention to modulate the profile of inflammatory mediators using a Th2-promoting cytokine, recombinant IL-33, can limit brain injury and functional impairment during the early phases after stroke. IL-33 is a ligand for suppression of tumorigenicity 2 (ST2), an IL-1 family receptor, and can initiate and amplify Th2-type responses (7, 15). A transmembrane form of ST2 is expressed primarily on Th2 cells to mediate its Th2 effector functions (16). IL-33 can also activate other cell types besides T cells, including mast cells, macrophages, dendritic cells, eosinophils, basophils, NKT cells, and innate-like lymphoid cells, that could contribute to amplification of a Th2-type response (17). In pathological settings, endogenous IL-33 can be released from necrotic cells and degraded by caspases after apoptosis (18).

Protective effects of IL-33 administration have been reported in experimental disease models such as myocardial infarction (19), atherosclerosis (20), inflammatory bowel disease (21), Alzheimer’s disease (22), experimental autoimmune encephalomyelitis (23), and concanavalin A–induced liver damage (24). Further, in tumor-bearing mice, IL-33 suppresses activity of NK cells and dendritic cell maturation, and enhances M2 polarization of macrophages (24). Here, in a mouse model of cerebral ischemia, we have extensively investigated the therapeutic potential of recombinant IL-33 (and in limited studies, IL-4) to enhance Th2-associated inflammatory signaling and thus limit brain injury and clinical impairment. Daily administration of IL-33 (2 μg/d) and IL-4 (5 μg/d) have each been used for therapy in non-stroke experimental studies (25, 26), and so here they were first administered at these doses over 2 days. While these interventions were found to be neuroprotective in association with Th2-related antiinflammatory actions, they also accelerated and/or exacerbated post-stroke Th2-mediated systemic immunosuppression (27–29), leading to lung infection and mortality. These adverse systemic effects were mitigated either by combination therapy with antibiotics or by reducing the dose of IL-33 by 80% (0.4 μg/d for 2 days) and the ischemic period from 60 to 45 minutes. This latter modification also enabled 72-hour follow-up and an examination of the role of IL-10 generation and/or Tregs.

Results
Effects of IL-4 or IL-33 on stroke outcome. Blood flow to the cortical region supplied by the middle cerebral artery (MCA) was reduced by ~80% during MCA occlusion (MCAO), and reperfusion resulted in the prompt return of flow to pre-ischemic levels (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.121560DS1). Compared with vehicle, neither IL-4 nor IL-33 had any effect on blood flow changes (Supplemental Figure 1A). Representative coronal brain sections at 24 hours after MCAO are shown in Figure 1, A–C. Mice treated with IL-4 (5 g daily; total dose, 10 μg) or IL-33 (2 μg daily; total dose, 4 μg) had ~35% smaller infarcts than vehicle-treated mice (Figure 1D), due mainly to smaller cortical infarcts (Figure 1, A–C, and Supplemental Figure 1, D and E).

Vehicle-treated mice subjected to stroke displayed clinical deficits, and reduced hanging grip time and locomotor activity compared with sham-operated mice (Figure 1, E–G). Treatment with IL-33 or IL-4 augmented the clinical deficits and tended to further reduce hanging grip time and locomotor activity after stroke compared with vehicle treatment (Figure 1, E–G, and Supplemental Figure 1, B and C).

Effect of IL-33 on Th1 and Th2 cytokines. The Th1-associated (as opposed to Th2-associated) nature of acute postischemic brain inflammation in C57BL/6 mice was demonstrated by marked increases in expression of the proinflammatory cytokines IL-1 and IL-6 (Figure 2, A and B). By contrast, brain expression of the antiinflammatory Th2 cytokines IL-5 and IL-13 were unaffected by stroke (Figure 2, C and D). Further, administration of IL-33 was confirmed to exert a Th2-promoting effect in vivo by increasing brain expression of the antiinflammatory Th2 cytokines IL-5 in stroke-operated and IL-13 in sham-operated mice (Figure 2, C and D), while profoundly reducing post-stroke expression of IL-1β and IL-6 (Figure 2, A and B). Stroke tended to increase the systemic expression of G-CSF, TNF, and IL-6, but plasma levels of these cytokines were unaffected by IL-33 (Figure 2, E–G). By contrast, treatment with IL-33 increased plasma levels of IL-5 protein by ~400-fold in sham and stroke mice (Figure 2H).
Effect of IL-33 on brain infiltration of immune cells after cerebral ischemia. At 24 hours, there were 7-fold more leukocytes present in the ischemic hemisphere of vehicle-treated mice than in sham controls (Figure 3A). IL-33 treatment had no overall effect on the total number of leukocytes present in the brain at 24 hours (Figure 3A). However, among the ~15,000 leukocytes (CD45hi), ~20% were neutrophils, and treatment with IL-33 tended to further increase this number (Figure 3B). Total monocytes present in the brain were increased by ~10-fold after stroke in vehicle-treated mice, and this increase was attenuated by IL-33 treatment (Figure 3C and Supplemental Figure 2A). While IL-33 had no effect on the stroke-induced increase in CD4+ T cells (Figure 3D), the treatment substantially reduced the numbers of CD8+ T cells, CD4-CD8- double-negative (DN) T cells, NK cells, and NKT cells infiltrating the brain after stroke (Figure 3, E–H, and Supplemental Figure 2, B and C). The effect of IL-33 on NKT cells (Figure 3H) was largely confined to the CD4+ NKT cell population (Figure 3, I and J, and Supplemental Figure 2D). CXCL16 is a chemoattractant for activated CD8+ T cells and NKT cells, and indeed immunohistochemical staining revealed that this effect of IL-33 was associated with fewer CXCL16+ cells present after stroke (Figure 3K).

Effect of IL-33 on microglial/macrophage activation and polarization after cerebral ischemia. Flow cytometry indicated a trend toward a 50% increase in microglia (CD45+CD11b+F4/80+; P > 0.05) present at 24 hours...
in the ischemic hemisphere of vehicle-treated mice, whereas no such trend was present in IL-33–treated mice after stroke (Figure 4A). Moreover, immunohistochemistry revealed that stroke increased the number of activated microglia/macrophages and that this increase was partly attenuated by IL-33 treatment (Figure 4B, \( P = 0.07 \); Supplemental Figure 3A; \( P = 0.07 \)). Both flow cytometry (Figure 4C) and immunohistochemistry (Figure 4D) indicated that there was a ~5-fold increase in macrophages in the brain following stroke. Furthermore, IL-33 tended to reduce macrophage content (Figure 4, C and D), and there were ~50% fewer proinflammatory (3-nitrotyrosine\(^+\) [3-NT\(^+\) F4/80\(^+\)]) microglia/macrophages present in the ischemic brains of mice treated with IL-33 versus vehicle (Figure 4E and Supplemental Figure 3B).

Figure 2. Pro- and antiinflammatory cytokine expression in the brain and plasma after stroke. Expression of (A) \( \text{Il-1}\beta \), (B) \( \text{Il-6} \), (C) \( \text{Il-5} \), and (D) \( \text{Il-13} \), in the ischemic hemisphere at 24 hours after stroke, quantified using qPCR. Circulating levels of (E) G-CSF, (F) TNF, (G) IL-6, and (H) IL-5 at 24 hours after stroke. Data are presented as mean ± SEM. *\( P < 0.05 \), 1-way ANOVA with Bonferroni post hoc test. Brain qPCR, \( n = 5–12 \); plasma cytokine: \( n = 2–12 \) (\( n = 2 \) for sham IL-33 only). Veh, Vehicle.
Effect of IL-33 on splenocytes and circulating immune cells after cerebral ischemia. Treatment with IL-33 increased spleen weight by ~25% compared with vehicle controls in both sham- and stroke-operated mice (Supplemental Figure 4A, both \( P < 0.05 \)), as has been reported (15). Spleen weight was reduced by 10%–15% at 24 hours after stroke in mice treated with either vehicle or IL-33, and this reduction was statistically significant in the latter treatment group (Supplemental Figure 4A). At 24 hours after stroke in both vehicle- and IL-33–treated mice, there were ~40% fewer splenocytes than in sham-operated mice, and this reduction was statistically significant with IL-33 treatment (Supplemental Figure 4B). This trend toward a reduction in total splenocytes at 24 hours after stroke was notable among CD4+ T cell and NK cell subsets (Supplemental Figure 4, C and D), but not in NKT cells, macrophages, or monocytes (Supplemental Figure 4, E–G). Treatment with IL-33 generally had no effect on numbers of any splenocytes examined, except for a 2- to 4-fold increase in macrophages and monocytes after stroke (Supplemental Figure 4, B–G).
Consistent with the peripheral effects of stroke to reduce splenocyte numbers, the number of circulating leukocytes also tended to be reduced (by 30%-40%) at 24 hours in both vehicle- and IL-33–treated mice, compared with sham mice (Supplemental Figure 5A). There were no statistically significant effects of stroke and/or IL-33 detected among the different cell populations studied, although circulating CD4+ T cells were typically ~40% fewer after stroke in each group (Supplemental Figure 5, B–F).

**Effect of IL-33 on post-stroke mortality, functional deficits, and lung infection.** Despite the clear effects of the Th2-promoting IL-33 treatment to reduce infarct size in association with reduced Th1-type inflammation in the brain and periphery among survivors (Figure 1, A–D), we were surprised that IL-33 exacerbated clinical score and reduced hanging grip time in comparison to vehicle treatment (Figure 1, E–G). This exacerbation of post-stroke morbidity in mice administered IL-33 was accompanied by a tendency toward a greater post-stroke mortality rate at 24 hours (Figure 5A).

Post-stroke immunodepression and infection is an emerging concept in the understanding of stroke outcomes, and it is associated with a shift in the environment of the immune system from a Th1-driven (proinflammatory) one in the initial stages toward a Th2-skewed (antiinflammatory) setting during the subacute period after stroke (30). We thus investigated the effect of stroke on lung infection at 24 hours in our model and the potential impact of IL-33 treatment. There was virtually no evidence of bacterial infection in lung homogenates from any sham-operated mice treated with either vehicle or IL-33 (Figure 5B). However, consistent with the known consequences of post-stroke immunodepression (31), lung infection was commonly detected in vehicle-treated mice after stroke (Figure 5B). Furthermore, the bacterial load in the lungs was increased by a further 5-fold in mice subjected to stroke and treated with IL-33 (Figure 5B). We then demonstrated that post-stroke lung infection could be prevented if IL-33–treated mice were supplemented with antibiotics (ampicillin and gentamicin; Figure 5B). This combination therapy also reduced mortality and clinical and functional deficits to levels similar to (but not less than) those observed in vehicle-treated mice subjected to stroke (Figure 5, A, C, and D). Importantly, we confirmed that IL-33 therapy in combination with antibiotics reduced infarct size when assessed at

**Figure 4. Microglial cells and macrophages in the brain after stroke.** Total number of microglial cells quantified using (A) flow cytometry and (B) immunohistochemical staining of CD68+ cells in the ischemic hemisphere at 24 hours after stroke. Total number of macrophages and/or microglia quantified using (C) flow cytometry and (D) immunohistochemical staining of F4/80+ cells in the ischemic hemisphere at 24 hours after stroke. (E) The number of F4/80+ cells that coexpress 3-nitrotyrosine (3-NT) in the ischemic hemisphere is also shown. Data are presented as mean ± SEM. *P < 0.05, 1-way ANOVA with Bonferroni’s post hoc test. Flow cytometry, n = 8–13; CD68, n = 8–10; F4/80 and 3-NT, n = 5–7. S, sham; C, contralateral; I, ischemic; M/M, microglia/macrophages.
either 24 or 72 hours after stroke and when treatment was initiated after cerebral ischemia (2 μg IL-33 daily; total dose, 6 μg in 72-hour cohort; Figure 6, A–F).

We similarly demonstrated in T-bet–/– mice, which lack Th1-type cytokines, that an enhanced Th2 immune response caused by IL-33 following stroke (in the absence of Th1 immunity) is detrimental, and resulted in >70% (13 of 18) mortality (see also Supplemental Figure 6A). Consistent with our data in C57BL/6 mice (Figure 1, A–E), IL-33 administration resulted in neuroprotection of T-bet–/– survivors despite the worsened clinical deficit (Supplemental Figure 6, A and B).

**Effect of IL-33 on IL-10 expression by infiltrating immune cells.** Despite its overall neuroprotective effects (e.g., Figure 1, A–D), the exacerbation of post-stroke morbidity and mortality by IL-33 without antibiotics (Figure 1, E–G, and Figure 5, A–D) limited our ability to explore the underlying mechanisms of neuroprotection beyond the acute phase of stroke. Therefore, in subsequent experiments we administered a 5-fold-lower daily dose of IL-33 (0.4 μg) to separate cohorts of mice, and examined potential target populations of cells infiltrating the ischemic brain over 3 days and responding to IL-33. For these studies, we utilized IL-10/Foxp-3 double reporter mice in order to identify cells expressing the antiinflammatory cytokine IL-10 and/or the Treg marker FoxP3. We also induced a milder level of cerebral ischemia by using 45 minutes rather than 60 minutes of MCAO. Thus, in these experiments, there was no mortality over 3 days in IL-33–treated mice (vs. 2 deaths in vehicle-treated mice; Figure 7A) and no effect of IL-33 on clinical deficit (Figure 7B). In a separate cohort of mice, we confirmed that this lower dose of IL-33 did not exacerbate the systemic effects of stroke (i.e., lung infection, latency to fall in hanging wire test, clinical score) while tending to exert a level of neuroprotection similar to that of the higher dose (Supplemental Figure 7).

We considered Tregs to be a candidate cell type for mediating neuroprotective effects of IL-33 after stroke. Indeed, IL-33 treatment resulted in a >3-fold increase in the number of infiltrating Tregs in the brain.
at 3 days after stroke (Figure 7C), and the number of Tregs expressing IL-10 was 4-fold greater than in vehicle-treated mice (Figure 7D). IL-33 treatment also doubled the number of infiltrated Tregs expressing the IL-33 receptor ST2 as compared with control mice (Figure 7E).

Similar to the effect of high-dose IL-33 at 24 hours, low-dose IL-33 treatment had no effect on the total number of leukocytes (including CD4+ T cells) present in the brain at 3 days after stroke (our unpublished observations). Interestingly, low-dose IL-33 had no effect on the numbers of NK cells (Figure 7F) or NKT cells (our unpublished observations), but resulted in a 4-fold increase in IL-10–expressing NK cells in the brain at 72 hours (Figure 7G).

Discussion

There is growing evidence that a Th2-type inflammatory response can exert neuroprotective effects following cerebral ischemia via limiting inflammation-driven infarct progression. We recently found marked differences in the brain immune cell composition between Th1- and Th2-prone mouse strains following cerebral ischemia together with a milder stroke outcome in the latter strain (9). Here we have extended this finding by exploring the therapeutic potential of a recombinant Th2-type cytokine administered to provide neuroprotection in ischemic stroke.

There are several important findings of this study. First, we show that brief (daily for 2–3 days) systemic administration of the Th2-promoting cytokines IL-33 and IL-4 reduces acute brain injury after cerebral ischemia-reperfusion. Second, despite limiting infarct development, there was no effect on total immune cell infiltration, suggesting that IL-33 may have limited Th1-mediated injury by modulating the inflammatory environment, including the entry or activation state(s) of certain cell subsets. This included a reduced number of activated microglia; fewer monocytes, 3-NT"F4/80" proinflammatory microglia/macrophages, CD8+ T cells, CD4+ NKT cells, DN T cells, and NK cells; but more neutrophils and IL-10–expressing Tregs. Third, the DN T cells were increased after stroke in vehicle-treated mice, but not in IL-33–treated animals. We found that a subset of these DN T cells were present in much lower numbers than DN NKT cells, suggesting the IL-33–sensitive increase in DN T cells was instead mostly due to mucosa-associated invariant T cells and/or γδ T cells (32–34), which are also responsible for release of proinflammatory cytokines in disease settings (35, 36). Fourth, despite its neuroprotective effects, administration of IL-33 in the acute phase of stroke can also promote the acceleration of a
systemic switch from a Th1- to a Th2-type inflammatory response that is associated with post-stroke immunosuppression, exacerbated bacterial infection in the lungs, and increased morbidity and mortality. Fifth, our detection of changes in immune cell numbers (due to stroke and/or IL-33) in the brain but not the blood at 24 hours suggests that the neuroprotection by IL-33 was related directly to its effects in the brain rather than any peripheral actions. Sixth, administration of antibiotics in combination with IL-33 prevents the increased post-stroke morbidity and mortality caused by the Th2-promoting cytokine while preserving its neuroprotective effects.

We investigated whether administering a novel cytokine, IL-33, can impact the outcome after ischemic stroke by modulating the post-stroke immune response in an antiinflammatory manner. Th2-promoting effects of IL-33 were confirmed by (i) increased expression of IL-5 and IL-13; (ii) attenuation of the stroke-induced increases in proinflammatory cytokines IL-1β and IL-6; and (iii) fewer 3-NT=F4/80+ cells, consistent with fewer M1-polarized proinflammatory macrophages (37) in the post-stroke brain. Indeed, systemic administration of either IL-33 or the classical Th2 cytokine IL-4 markedly reduced the volume of cerebral infarction developing by 24 and 72 hours after ischemia-reperfusion.

Figure 7. Effects of low-dose IL-33 on functional outcomes and leukocyte subsets at 72 hours. Survival (A) and clinical score (B) at 72 hours after stroke. Flow cytometric quantification of (C) FoxP3+CD4+ T cells, (D) IL-10+FoxP3+CD4+ T cells, (E) percentage of ST2-expressing FoxP3+CD4+ T cells, (F) NK cells, and (G) IL-10+ NK cells in the ischemic hemisphere of IL-10eGFPFoxp3mRFP mice at 72 hours after stroke. Data are presented as mean ± SEM. *P < 0.05; Student’s unpaired t test. Flow cytometric analysis, n = 4–6. Veh, vehicle.
Tregs are established to modulate post-stroke inflammatory brain injury, possibly via an IL-10 signaling pathway (38). Our flow cytometric data demonstrate that IL-33 increased the numbers of Foxp3+CD4+ Tregs, including IL-10–expressing Tregs in the brain, and that a higher percentage of these Tregs expressed the IL-33 receptor protein ST2 following IL-33 treatment. While a role of Tregs in mediating IL-33–induced neuroprotection has been questioned (39), other studies have asserted that Tregs are an important target of IL-33. For example, administration of IL-33 ameliorated experimental colitis by promoting a Foxp3+ Treg response, whereby the protective effect was abolished in the absence of Tregs (40). Furthermore, mice lacking ST2 exhibited Treg dysfunction consistent with an involvement of IL-33/ST2 signaling in Treg development and function (41). Further, IL-33 stimulated IL-2 secretion by dendritic cells to drive ST2 expression and expansion of CD4+Foxp3+ Tregs (42). Indeed, ST2+ Tregs represent an important subset of activated Foxp3+ cells (~10% of CD3+CD4+CD25+ cells in thymus and spleen) (42). Finally, a recent study by Yang et al. demonstrated that neuroprotection by IL-33 in vitro is mediated by ST2-expressing microglia (43). Taken together, our data support the likely role of Tregs in IL-33–mediated neuroprotection after stroke.

We investigated another mechanism by which IL-33 could suppress the recruitment of lymphoid cells into the ischemic brain. The transmembrane chemokine CXCL16 and its receptor, CXCR6, are expressed in neurons, microglia, and astrocytes — cells that release soluble CXCL16 in response to proinflammatory stimuli (44). Soluble CXCL16 acts as a chemoattractant for activated CD8+ T cells, NKT cells, and Th1-polarized T cells that express CXCR6 (45, 46). We found immunohistochemical evidence for a marked increase in the expression of CXCL16 in the ischemic brain, which was attenuated by IL-33. These data may indicate that reduced CXCL16 expression contributed to the reduced chemoattraction by IL-33 of damaging lymphoid cells, such as CD8+ T cells and NKT cells, into the ischemic brain.

This study also addressed the importance of elevated Th2-related immune function on the severity of post-stroke infection. Stroke-associated pneumonia, secondary to the suppression of systemic immune cell function, is now understood to contribute to morbidity and mortality after ischemic stroke (27, 31). For example, stroke leads to leukopenia and splenic atrophy with fewer splenocytes, but more CD4+Foxp3+ Tregs not only in the spleen (47), but also in the ischemic hemisphere (2). Experimental and clinical data suggest that the degree of post-stroke immunosuppression is proportional to the severity of ischemic brain injury (48). Thus, we encountered adverse side effects of IL-33 administration after stroke manifested as increased 24-hour mortality, more severe bacterial infection of the lungs, and worsened functional impairment in survivors despite a 35%–50% smaller infarct volume than in vehicle-treated controls. We administered a dose of IL-33 (2 mg/kg per day for 2–3 days) that has been used safely for many weeks in other disease models (25). However, it seems likely that in our model of severe cerebral ischemia (60 minutes MCAO), the adverse systemic effects of IL-33 were a consequence of its Th2-promoting immunosuppression to increase the susceptibility to bacterial infection, because these effects were abrogated by antibiotics. Subsequent studies to examine the role of IL-10 and/or Tregs over 72 hours avoided such effects by instead reducing the dose of IL-33 by 80% in combination with a milder stroke (45-minute MCAO).

In summary, we have demonstrated that short-term administration of a Th2-type cytokine, IL-33, exerts neuroprotection after cerebral ischemia, which involves an augmented Th2:Th1 cytokine profile, fewer proinflammatory and cytotoxic subsets of immune cells, and more IL-10–expressing Tregs infiltrating the injured brain. Further, our findings suggest that administration of such Th2-promoting therapies in severe cases of acute stroke is likely to be safer in combination with antibiotics to limit the risk of exacerbating post-stroke immunosuppression and bacterial infections.

**Methods**

**Animals.** This study fully adheres to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (49). All animal experiments were conducted in accordance with National Health and Medical Research Council of Australia guidelines for the care and use of animals in research and were approved by the Monash University Animal Ethics Committee. Mice had free access to water and food pellets before and after surgery.

A total of 456 male C57BL/6 mice provided by the Monash Animal Research Platform aged 8–12 weeks were studied. Mice were excluded from the study if they (i) died during the surgical procedure (n = 18), (ii) experienced subarachnoid hemorrhage (n = 16), or (iii) were euthanized due to <65% reduction in regional cerebral blood flow (rCBF) during MCAO or <50% recovery of rCBF within 10 minutes of
reperfusion (n = 23). To study subsets of Tregs, we used male double reporter IL-10−eGFPFoxp3mRFP mice that were backcrossed on a C57BL/6 background for >10 generations and produced by our group (50), aged 12–16 weeks (n = 8). To study the role of Th1 immunity on the enhanced Th2 response subsequent to IL-33 therapy, we used male T-bet−/− (T-bet−) mice backcrossed on a C57BL/6 background for >10 generations (obtained from Monash Animal Research Platform), aged 8–12 weeks, which lack the T-box transcription factor Thx21 (n = 28); thus, CD4+ T cells from these mice do not produce the Th1-type cytokine IFN but secrete elevated levels of Th2-type cytokines in response to inflammatory stimuli.

*Treatments.* Recombinant mouse IL-4 (5 μg; 574306, BioLegend) or IL-33 (2 μg; 580508, BioLegend) was injected intraperitoneally 24 hours before and immediately after reperfusion into C57BL/6 and T-bet−/− mice. Control mice were injected with the vehicle, 1% BSA. To determine the effect of preventing systemic bacterial infection, some animals received a combination of ampicillin (100 mg/kg) and gentamicin (4 mg/kg) subcutaneously. For experiments with a 24-hour end point, administration of antibiotics occurred 24 hours before, 1 hour after, and 8 hours after stroke. In experiments in which there was a 72-hour end point, animals received daily IL-33 injections starting immediately after reperfusion, and antibiotics were administered twice a day starting 1 hour after stroke. A separate cohort of animals (IL-10−eGFPFoxp3mRFP mice) received 0.4 μg IL-33 intraperitoneally 24 hours before and immediately after reperfusion. Mice were randomized into different treatment groups and experiments were conducted in a blinded fashion.

**Transient focal cerebral ischemia.** Focal cerebral ischemia was induced by transient intraluminal filament–induced occlusion of the right MCA, as described previously (51, 52). Mice were anesthetized with ketamine-xylazine (80 and 10 mg/kg, respectively; intraperitoneally). Rectal temperature was monitored and maintained at 37.5°C ± 0.5°C using an electronic temperature controller (Testronics) linked to a heat lamp throughout the procedure and until animals regained consciousness. Briefly, the right proximal common carotid artery was clamped, and a 6-0 nylon monofilament with silicone-coated tip (Doccol Corp.) was inserted and gently advanced into the distal internal carotid artery, 11–12 mm distal to the carotid bifurcation, occluding the MCA at the junction of the circle of Willis. Severe (typically ~80%) reduction in rCBF was confirmed using transcranial laser Doppler flowmetry (Perimed) in the area of cerebral cortex supplied by the MCA. The filament was then tied in place, and the clamp was removed. After 1 hour of cerebral ischemia, the monofilament was retracted to allow reperfusion for 23 or 71 hours. The low-dose IL-33 group and IL-10−eGFPFoxp3mRFP mice were given a milder stroke (45 minutes of occlusion) to reduce acute mortality and enable 3 days of post-stroke survival. Reperfusion was confirmed by an immediate increase in rCBF, which reached the preischemic level within 5 minutes. The wound was then closed, and the animal was allowed to recover. Regional CBF was recorded for 30 minutes of reperfusion. Sham-operated mice were anesthetized, and the right carotid bifurcation was exposed and dissected free from surrounding connective tissue, but no filament was inserted. All animals were administered 1 ml sterile saline via a subcutaneous injection for rehydration after surgery. Gel nectar (Able Scientific) was placed inside the cage, and access to chow food and water was provided. All animals’ boxes were placed on heat pads after surgery until euthanasia.

**Clinical score assessment.** At the end of the experiment (24 or 72 hours after induction of stroke/sham surgery), clinical assessment was performed by an observer blinded to experimental groups using a 6-point scoring system (51, 52): 0, normal motor function; 1, flexion of torso and contralateral forelimb when mouse is lifted by the tail; 2, circling when mouse held by the tail on a flat surface; 3, leaning to one side at rest; 4, no spontaneous motor activity; 5, death within the 23- or 71-hour reperfusion period. A hanging wire test was also performed in which mice were suspended from a wire 30 cm high for up to 180 seconds, and the average time of 3 trials with 5-minute rest periods in between was recorded. Locomotor activity was assessed using the ANY-maze video tracking system, in which mice were placed in a 20 × 20–cm acrylic box with a floor made of steel rods spaced 8 mm apart and raised 10 mm above a base steel plate. Automated software detects and records exploratory parameters such as distance traveled during a 10-minute period.

**Cerebral infarct and edema volumes.** Mice were euthanized at 24 or 72 hours by isoflurane overdose, followed by decapitation. The brains were immediately removed and snap frozen with liquid nitrogen. Coronal sections (30 μm) separated by ~420 μm were stained with thionine (0.1%) to delineate the infarct. Images of the sections were captured with a CCD camera mounted above a light box. Infarct volume was quantified as described previously (52, 53) using image analysis software (NIH ImageJ) and corrected for brain edema, estimated using the following formula: corrected infarct volume = [left hemisphere area – (right hemisphere area × 2)] / 2.


deleted
area – right hemisphere infarct area) × (thickness of section + distance between sections)] (54, 55). Edema-corrected infarct volumes of individual brain sections were added to give a 3D approximation of the total infarct volume. Total, cortical, and subcortical infarct volumes were quantified individually.

**Quantitative PCR.** Mice were euthanized at 24 hours by isoflurane overdose and intracardially perfused with RNase-free PBS. The brain was removed from the skull, and after removal of the cerebellum and olfactory bulb, the right (ischemic) hemisphere was snap-frozen in liquid nitrogen. RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse transcribed into cDNA using an RT² First Strand Kit (QIAGEN). Levels of RNA expression of selected genes, including Il-1β, Il-5, Il-6, and Il-13, were measured using predesigned TaqMan gene expression assays. Gene expression data were normalized to the housekeeping gene GAPDH (Applied Bioscience) with the ΔΔCt method (56). Results were presented as fold change relative to vehicle-treated sham-operated mice.

**Plasma cytokines.** Mice were euthanized at 24 hours by isoflurane overdose, and blood was collected via cardiac puncture. Plasma samples were analyzed for the cytokines G-CSF, TNF, IL-6 and IL-5 using BD Cytometric Bead Array Cell Signaling (CBA) Flex Set System (BD Biosciences). Flex Set standard, capture bead master mix, and PE detection reagents were prepared as described in the Cell Signaling Master Buffer Kit (BD Biosciences). Samples were analyzed by FACSDiva Version 6.1.1 using forward scatter–A (FSC-A), FSC-W, side scatter–A(SSC-A), SSC-W, PE-A, APC-A, and APC-Cy7-A plots as described in the BD CBA Flex Set Templates for Flow Cytometers manual (BD Biosciences).

**Flow cytometry.** Mice were euthanized at 24 or 72 hours by isoflurane overdose, followed by blood collection by cardiac puncture, and the whole mouse was then intracardially perfused with PBS, and brain and spleen were collected. Leukocytes were purified from blood using red blood cell lysis buffer (155 mmol/l NH₄Cl, 10 mmol/l KHCO₃, and 3 mmol/l EDTA). Spleens were mechanically dissociated and passed through 70-μm nylon cell strainers (BD Falcon) to obtain a single-cell suspension. Cells were then lysed with red blood cell lysis buffer and washed with PBS containing 1% BSA. The brain was removed from the skull and, after removal of the cerebellum and olfactory bulb, was separated into left (contralateral) and right (ischemic) hemispheres. Each hemisphere was dissociated mechanically in digestion buffer containing collagenase type XI (125 U/ml), hyaluronidase (60 U/ml), and collagenase type 1-S (450 U/ml) in Ca²⁺/Mg²⁺-supplemented PBS (MilliporeSigma) and incubated at 37°C for 45 minutes with gentle agitation (550 rpm). The mixture was then passed through 70-μm nylon cell strainers to obtain a single-cell suspension. After washing with PBS (350 g, 10 minutes at 4°C), the cell pellet was resuspended in 3 ml 30% Percoll (GE Healthcare), underlaid with 70% Percoll, and centrifuged for 20 minutes at 1,400 g at room temperature without the use of a brake. Cells at the interphase of 2 density gradients were collected and washed with PBS containing 1% BSA (350 g, 10 minutes at 4°C) for staining.

All cells were incubated with appropriate antibodies (listed in Tables 1 and 2) at 4°C in darkness for 20 minutes (Table 1) or 30 minutes (Table 2). After staining, cells were analyzed by LSRII flow cytometer (BD Biosciences; Table 1) or LSR Fortessa (BD Biosciences; Table 2), and FlowJo software (Tree Star Inc.). Countbright counting beads (Invitrogen) were included to define the absolute number of cells in the samples.

Gating strategy (Table 1): Forward and side scatters were used to identify single cells. Dead cells were excluded with Live/Dead markers (near infrared or aqua). Cells were gated for CD45hi and CD45med populations as described previously (2). Microglial cells were identified as CD45med-CD11b+F4/80+. CD45hi cell populations were then divided into myeloid and subdivided into monocytes (CD45hiCD11b+Ly6C⁺), macrophages (CD45hiCD11b+F4/80⁺), neutrophils (CD45hiCD11b+Ly6C⁻Ly6G⁺), and lymphoid cells, which included T cells (CD45hiCD3⁺), B cells (CD45hiCD19⁺), NK cells (CD45hiNK1.1⁺CD3⁻), and NKT cells (CD45hiNK.1⁺CD3⁺). Two panels of antibodies were included for the study, one of which was employed with each animal. Panel 1 enabled the quantification of microglia and leukocytes with myeloid origin. This included macrophages (F4/80⁺), neutrophils (Ly6G⁺), and monocytes (Ly6C⁺), which were further subdivided into Ly6Ch and Ly6Cmed monocytes. Panel 2 enabled the identification and quantification of lymphoid cells consisting of B cells (CD19⁺), NK cells (NK1.1⁺CD3⁺), NKT cells (NK1.1⁺CD3⁺), and T cells (CD3⁺). T cells were further subdivided into CD4⁺ T cells, CD8⁺ T cells, CD4⁺CD8⁺ T cells, and CD4⁺CD8⁻ DN T cells. CD25⁺ Tregs were also identified from CD4⁺ T cell populations. Fluorescence Minus One controls were also included in both panels to define positive populations for CD11b, Ly6C, F4/80, CD3, CD19, and CD25.
Gating strategy (Table 2): Forward and side scatters were used to identify single cells. Cells were gated for CD45+ and then divided into T cells (CD45+CD3+) and B cells (CD45+CD20+). CD45+CD20− cells were subdivided into T cells (CD3+), NK cells (NK1.1+CD3−), and NKT cells (NK1.1+CD3+). T cells were then further subdivided into CD4+ T cells and CD8+ T cells. CD4+ T cells were gated on IL-10 versus FoxP3 (quadrangle gates) and then on CD25 versus ST2 in all 4 populations. B cells, NK cells, and CD8+ T cells were also gated on IL-10 versus ST2 (quadrangle gates).

**Immunofluorescence.** Brain sections were immunofluorescently labeled for CXCL16 (membrane-bound CXCL16) or CD68 (activated microglia/macrophages [M/M cells]), or double-labeled for 3-NT (peroxynitrite marker) and F4/80 (M/M cell marker), to identify proinflammatory M/Ms (57).

Frozen brains were sectioned (10 μm) and thaw-mounted onto poly-L-lysine–coated slides. Multiple serial coronal brain sections that spanned the infarct region were taken for analysis. Sections were fixed in 4% paraformaldehyde for 15 minutes, washed in 0.01 M PBS (3 × 10 minutes), and blocked with 2% BSA (for CXCL16) or 10% goat serum and a Mouse on Mouse Ig blocking reagent (Vector Laboratories) for 1 hour. Sections were then incubated with a polyclonal goat anti-CXCL16 antibody (1:800, AF503, R&D Systems), a mouse-CD68 [ED1] antibody (1:100, ab31630, Abcam), or a mouse 3-NT antibody (1:50, ab61392, Abcam), and a rat anti-F4/80 antibody (1:100, 123147, BioLegend) overnight in a humidified chamber. The following day, sections were washed in 0.01 M PBS (3 × 10 minutes) and...
incubated in a chicken anti-goat Alexa Fluor 488 (1:200, A-21467, Invitrogen) secondary antibody or a goat anti-rat Alexa Fluor 594 (1:500, A-11007, Invitrogen) secondary antibody for 2 hours (3-NT+ and F4/80-labeled sections only). Brain sections were then washed in 0.01 M PBS (3 × 10 minutes) and incubated in biotinylated anti-mouse IgG reagent (Vector Laboratories) for 10 minutes. After washing in 0.01 M PBS (3 × 10 minutes), fluorescein avidin DCS (Vector Laboratories) was applied onto the sections for 5 minutes. Sections were washed in 0.01 M PBS (3 × 10 minutes), coverslipped, and examined using an Olympus fluorescence microscope. Numbers of CXCL16+ cells, CD68+ cells, 3-NT+ cells, and F4/80+ cells in the right (ischemic) hemisphere were counted for each brain section by researchers blinded to treatment groups. For colocalization studies, data were presented as percentage of 3-NT+ cells of total F4/80+ cells. All appropriate secondary antibody controls were performed to ensure that there was no nonspecific binding.

Bacteriological analysis. Animals were euthanized at 24 hours by inhalation of isoflurane and sprayed with 80% ethanol to maintain sterility. Blood was collected by cardiac puncture, followed by thoracotomy. The lungs, liver, and spleen were removed and homogenized in 1 ml sterile PBS using an Omni Tissue Master 125 Homogenizer (Omni International). To determine CFU, 10 μl tissue homogenate or blood was serially diluted and plated onto brain heart infusion (BHI) agar plates supplemented with 5% defibrinated horse blood (Australian Ethical Biologicals). Plates were incubated at 37°C for 18 hours, and bacterial colonies were counted.

Statistics. Values are presented as mean ± SEM. Results of the hanging wire test, infarct volume, quantitative PCR (qPCR), plasma cytokine value, flow cytometry, immunohistochemistry, and bacteriological analysis were analyzed using 1-way ANOVA with Bonferroni's post hoc test with selected multiple comparisons or 2-tailed Student’s unpaired t test, as appropriate. The clinical score was expressed as the median result per group and was analyzed using a Kruskal-Wallis test with Dunn’s post hoc test. A P value less than 0.05 was considered statistically significant. Statistical analyses were carried out using GraphPad Prism (GraphPad Software).

Study approval. All animal studies and procedures were approved by the Monash University Animal Ethics Committee and fully complied with National Health and Medical Research Council of Australia guidelines for the care and use of animals in research.

Author contributions
SRZ, MG, CGS, and HAK designed the study. SRZ, MP, RS, CHYW, BRSB, SS, MG, HAK, HXC, and SL conducted various experiments, and acquired and analyzed data. AV, TM, SH, GRD, MAE, and TVA provided important reagents and valuable feedback on the manuscript. SRZ, CGS, and HAK wrote and edited the manuscript.

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Supplemental Figure 1. Regional cerebral blood flow, infarct volumes and functional outcome. (A) Regional cerebral blood flow during 1 h middle cerebral artery occlusion and 30 min reperfusion, (B) hanging wire latency to fall (C) clinical score, (D) cortical and (E) subcortical infarct volumes, and (F) hemispheric swelling at 24 h after stroke. Data are presented as mean±SEM for (A, B, and D-F) and as median scores for (C). *P<0.05 compared with vehicle; Student’s unpaired t-test for (B), Mann-Whitney test for (C), and one-way ANOVA with Bonferroni post hoc for (D-F). Blood flow: n=18-30; hanging wire: n=15-29; clinical score: n=17-34; infarct volumes: n=12-15. Veh, Vehicle.
Supplemental Figure 2. Representative flow cytometry panels of leukocytes in the brain after stroke. Gating strategies for (A) monocytes, (B) T cells, (C) NK and NKT cells and (D) CD4+CD8+ NKT cells at 24 h after stroke in mice treated with vehicle (Veh) or IL-33.
Supplemental Figure 3. Representative images of microglia and macrophages in the brain after stroke using immunohistochemistry. (A) Immunohistochemical staining of CD68+ cells in the ipsilateral hemisphere of sham-operated mice, or stroke-operated mice treated with vehicle or IL-33. (B) Double immunohistochemical staining of F4/80 and 3-nitrotyrosine (3-NT) in the ischemic hemisphere of mice treated with vehicle or IL-33 at 24 h after stroke. Veh, Vehicle. Scale bar = 50 μm.
Supplemental Figure 4. Leukocytes in the spleen after stroke.

Normalised spleen weight is shown in (A). Flow cytometric quantification of (B) total leukocytes, (C) CD4⁺ T cells, (D) NK cells, (E) NKT cells, (F) macrophages, and (G) monocytes in the spleen at 24 h after stroke. Data are presented as mean±SEM. *P<0.05; One-way ANOVA with Bonferroni post hoc test. Spleen weight: n=7-25; total leukocytes: n=11-19; subsets: n=3-8. Veh, Vehicle.
Supplemental Figure 5. Leukocytes in the circulation after stroke. Flow cytometric quantification of (A) total leukocytes, (B) monocytes, (C) CD4+ T cells, (D) CD4+CD25+ T cells (E) NK cells and (F) NKT cells per ml of blood at 24 h after stroke. Data are presented as mean±SEM. One-way ANOVA with Bonferroni post hoc test. Total leukocytes: n=11-19; subsets: n=3-8. Veh, Vehicle.
Supplemental Figure 6. Functional outcome and infarct volume in T-bet⁻/⁻ mice. (A) Clinical score and (B) total infarct volume at 24 h after stroke. Data are presented with median scores in (A) and as mean±SEM in (B). *P<0.05; Mann-Whitney test for (A) and Student’s unpaired t-test for (B). Clinical score: n=11-18; infarct volume: n=5-10. Veh, Vehicle.
Supplemental Figure 7. Infarct volume, post-stroke infection and functional outcome in mice with 45 min of MCA occlusion. (A) Total infarct volume, (B) bacterial load in lung, (C) hanging wire latency to fall, and (D) clinical score at 24 h after stroke. Data are presented as mean±SEM for (A-C) and as median scores in (D). Student’s unpaired t-test for (A-C) and Mann-Whitney test for (D); n=7-8. Veh, Vehicle; IL-33 (Low), Low dose IL-33.
CHAPTER 4:

IL-37 INCREASES IN PATIENTS AFTER ISCHEMIC STROKE AND PROTECTS FROM INFLAMMATORY BRAIN INJURY, MOTOR IMPAIRMENT AND LUNG INFECTION IN MICE
IL-37 increases in patients after ischemic stroke and protects from inflammatory brain injury, motor impairment and lung infection in mice

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Acceptance letter

From: "scientificreports@nature.com" <scientificreports@nature.com>
Reply-To: "scientificreports@nature.com" <scientificreports@nature.com>
Date: Tuesday, 23 April 2019 at 8:37 pm
To: Chris Sobey <C.Sobey@latrobe.edu.au>
Subject: Scientific Reports: Decision letter for SREP-19-02925A

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Abstract

Post-stroke inflammation may contribute to secondary brain injury and systemic immunosuppression. Interleukin (IL)-37 is an immunosuppressive cytokine belonging to the IL-1 superfamily with no mouse homologue yet identified, the effects of which have not been studied in stroke. Here we report: 1) the effect of ischemic stroke on circulating IL-37 in humans; and 2) the effect of IL-37 on stroke outcome measures in mice transgenic for human IL-37 (IL-37tg). We found that in the first 3 days after ischemic stroke in 55 patients, the plasma abundance of IL-37 was ~2-fold higher than in 24 controls. In IL-37tg mice, cerebral ischemia-reperfusion resulted in marked increases in plasma IL-37 (~9-fold) and brain IL-37 mRNA (~7,000-fold) at 24 h compared with sham-operated IL-37tg mice. Further, compared with wild-type (WT) mice subjected to cerebral ischemia-reperfusion, IL-37tg mice exhibited less severe locomotor deficit, smaller cerebral infarcts and reduced bacterial lung infection. In the ischemic hemisphere, there were 60% fewer pro-inflammatory microglia-macrophages and up to 4-fold higher expression of anti-inflammatory markers in IL-37tg compared to WT mice. Our data show that IL-37 expression is increased following ischemic stroke in humans and IL-37tg mice, and may exert protective effects by modulating post-stroke inflammation in the brain and periphery.
**Introduction**

Stroke is a leading cause of death and disability, with more than 800,000 cases occurring annually in the USA alone.\(^1\) Ischemic stroke accounts for 87% of stroke cases, and is caused by the occlusion of a cerebral artery by a thrombus or embolus, resulting in neuronal death in the directly affected brain region.\(^1\) Currently, the only available treatments are recombinant tissue plasminogen activator (rt-PA) within 4.5 h of stroke onset and/or mechanical clot retrieval within 8 h in the presence of either rt-PA or anticoagulants. Due to these time constraints, only ~15% of ischemic stroke patients can receive any such treatment.\(^2-4\)

In order to identify novel therapeutic targets for development of additional stroke therapies with wider application, we will need to first gain a better understanding of mechanisms and mediators that may influence stroke outcome.

Post-stroke inflammation has been well documented in animal models, and is believed to contribute to secondary brain injury.\(^5,6\) Inflammation is initiated by the activation of resident microglia, followed by increases in cell adhesion molecules and chemokines, leading to the infiltration of immune cells through the blood-brain barrier.\(^5,6\) Both innate and adaptive immune responses contribute to the post-ischemic inflammation. Several types of inflammatory cells enter the brain parenchyma and may release pro-inflammatory mediators, such as cytokines and reactive oxygen species, and thus promote further tissue damage and infarct enlargement.\(^7-9\) Some infiltrating immune cells, such as T regulatory cells and M2-polarized microglia-macrophages, can instead exhibit anti-inflammatory characteristics in the post-stroke brain.\(^9-11\)

Inflammation after stroke may also be associated with altered systemic immune function, lymphopenia and splenic atrophy,\(^12,13\) and can render individuals susceptible to infection.\(^14,15\) Indeed, ~30% of fatalities in stroke patients are associated with infections, especially in the form of pneumonia.\(^16,17\) Thus, an understanding of the mechanisms of post-ischemic inflammation is crucial to identify potential therapies.
Here, we have investigated a relatively newly identified cytokine named IL-37 (previously known as IL-1F7) belonging to the IL-1 superfamily, and its regulation and action in stroke. IL-37 is currently the only IL-1 family cytokine of which a murine homologue has not yet been identified. In humans and also mice, IL-37 binds to IL-18Rα, forming a complex with IL-1R8 (SIGIRR) and may act either intracellularly or extracellularly on a variety of tissues including brain where the IL-37a isoform is thought to be preferentially expressed. While IL-37b – the isoform expressed by these mice – is the most studied isoform, a total of five IL-37 isoforms (a-e) exist and are distributed differentially in various organs and tissues in humans. Unlike most other IL-1 family members, IL-37 is anti-inflammatory and its mRNA is inducible in several innate and adaptive immune cell types such as monocytes, neutrophils, natural killer cells, as well as plasma cells. Effects of IL-37 have been investigated in endotoxic shock, inflammatory bowel disease, myocardial infarction, spinal cord injury, multiple sclerosis and liver ischemia. While a recent pilot study reported IL-37 to be elevated in serum of 5 ischemic stroke patients, there is no information on regulation or effect of IL-37 in the setting of stroke. Thus, we have investigated: 1) the abundance of IL-37 in the blood of control and ischemic stroke patients, and in post-mortem brain sections; and 2) the effect of IL-37 on stroke outcome measures in mice transgenic for human IL-37 (IL-37tg). The mRNA encoding IL-37 contains an instability sequence such that, even under the control of the constitutively active CMV promoter in these IL-37tg mice, an inflammatory stimulus (e.g. as in the ischemic brain) is required for mRNA upregulation and thus protein production.
Results

**IL-37 expression is elevated in brain and plasma after acute ischemic stroke**

To investigate the regulation of IL-37 in the setting of stroke, we explored IL-37 protein and mRNA in humans after stroke and in mice in a model of this disease. In patients with acute ischemic stroke, the plasma abundance of IL-37 was approximately double that of control patients 3 days after the event \((P=0.038; \text{Fig. 1a})\), but there was no correlation with two indicators of stroke severity – the National Institute of Health Stroke Scale (NIHSS, \(\rho=-0.14, P=0.304\)) or Glasgow Coma Scale scores \((\rho=0.10, P=0.488)\). Post-stroke plasma IL-37 abundance on day 3 was similar to that recorded within 2 days of stroke onset \((170\pm245 \text{ pg/ml})\). Similarly, in IL-37tg mice subjected to ischemic stroke, plasma IL-37 was ~8-fold higher than in sham-operated IL-37tg mice 24 h after surgery \((P<0.001; \text{Fig. 1b})\; \text{note that WT mice do not express IL-37}\). As in human patients after stroke, plasma IL-37 was not correlated with post-stroke clinical scores in mice \((\rho=0.07, P=0.733)\).

Immunohistochemical analysis of post-mortem human brain sections obtained from a person who died after ischemic stroke indicated IL-37-expressing cells accumulating within the infarct but not in the non-ischemic hemisphere \((\text{Fig. 2a,b})\). We were unable to perform immunohistochemical analysis of IL-37 in mouse brain due to cross-reactivity of the antibody in WT tissue \((\text{not shown})\). However, brain expression of IL-37 mRNA was profoundly increased in IL-37tg mice specifically in the ischemic hemisphere after stroke \((\sim7,000\text{-fold}; P=0.005; \text{Fig. 2c})\). These IL-37tg mice also had a lower median clinical score than concurrently studied WT counterparts subjected to stroke on the same day \((\text{Fig. 2d})\). Furthermore, there was a strong trend towards a negative correlation between brain expression of IL-37 mRNA and clinical score in mice after stroke \((\rho=-0.50, P=0.085)\), whereas this was not the case for plasma IL-37 protein \((\rho=-0.07, P=0.833)\).
**Effect of IL-37 on stroke outcomes**

Following insertion of the monofilament to occlude the middle cerebral artery (MCA) and induce cerebral ischemia, blood flow to the region of cortex supplied by the MCA was reduced by ~75% in both WT and IL-37tg mice. Rapid, near-complete reperfusion was achieved in all mice following monofilament withdrawal, and the presence of IL-37 had no effect on changes in regional cerebral blood flow (rCBF) (Supplementary Fig. S1). No anatomical differences were observed between WT and IL-37tg mice upon examination of gross anatomy of the cerebral vasculature, including the Circle of Willis and its branches (not shown). We observed no difference in post-stroke mortality at 24 h, with deaths in 16.3% (7/43) of WT and 15.9% (7/44) of IL-37tg mice.

Open field testing indicated no difference in motor function between WT and IL-37tg mice at 24 h after sham surgery ($P=0.176$ and $P=0.230$; Figs. 3a and c, respectively). By contrast, virtually all mice subjected to stroke exhibited deficits in motor function (Figs. 3b and d). We observed that these functional deficits were significantly less severe in IL-37tg than in WT mice following stroke; for example, IL-37tg mice travelled 2.5 times further ($P=0.036$) and achieved a 2-fold higher maximum speed ($P=0.019$) than WT mice after stroke (Figs. 3b and d). Cerebral infarct volume was ~30% smaller in IL-37tg versus WT mice ($P=0.034$; Figs. 4a-c), particularly in the subcortex (Supplementary Fig. S1).

**Regulation of anti-inflammatory mediators in mouse brain after stroke.**

We examined mRNA levels of several inflammation-related genes known to be associated with stroke in the brain. As seen for IL-37 (Fig. 2a), several other anti-inflammatory markers, namely *Foxp3* ($P=0.012$), *Ym1* ($P=0.006$), *Il-10* ($P=0.018$), *Il-13* ($P=0.002$) and *Tgfβ* ($P=0.008$) were up to 3-fold increased in the ischemic hemisphere following stroke in IL-37tg compared to WT mice (Figs. 5a-e).
**Effect of IL-37 on brain infiltration of immune cells following stroke**

Immunohistochemistry revealed that there were similar numbers of CD45+ leukocytes, neutrophils (MPO+), T cells (CD3+), astrocytes (GFAP+) (Supplementary Fig. S2) and microglia-macrophages (Fig. 6a) in the ischemic hemisphere of WT compared with IL-37tg mice after stroke. However, using immunohistochemistry we observed that the activation/polarization states of microglia-macrophages in the ischemic hemisphere were markedly different between IL-37tg and WT mice: IL-37tg mice exhibited 55 % fewer pro-inflammatory cells (F4/80+3-NT+; \( P=0.004 \); Fig. 6b) and 73 % more alternatively activated cells (F4/80+3-NT-; \( P=0.010 \); Fig. 6c) than WT mice, which reflected a ratio strongly polarized towards an ‘alternative activation’ pattern (\( P<0.001 \); Fig. 6d).

**Effect of IL-37 on post-stroke lung infection and anti-inflammatory cytokines**

Targeting post-stroke brain inflammation with anti-inflammatory therapy may be neuroprotective, but may entail detrimental systemic consequences, such as increased lung infection.\(^{15}\) However, lungs of IL-37tg mice exhibited 73 % fewer bacterial colony forming units than WT mice after stroke (\( P=0.005 \); Fig. 7a). This was associated with 2.7-fold greater lung expression of Il-10 mRNA (\( P=0.006 \); Fig. 7b), whereas in contrast to brain (i.e. Fig. 2c), IL-37 mRNA in the lungs tended to be reduced by stroke (\( P=0.075 \); Fig. 7c).
Discussion

There are five major findings of this study. First, ischemic stroke resulted in a marked increase in plasma IL-37 in both humans and IL-37tg mice. Second, this was associated with augmented abundance of IL-37 in the ischemic brain tissue of one stroke patient and in mice. Third, compared with WT mice subjected to cerebral ischemia-reperfusion, IL-37tg mice exhibited less severe locomotor deficit and smaller cerebral infarcts. Fourth, the neuroprotection evident in IL-37tg mice involved fewer pro-inflammatory microglia-macrophages and higher expression of anti-inflammatory cytokines in the ischemic brain hemisphere. Fifth, post-stroke bacterial lung infection was profoundly reduced in IL-37tg compared to WT mice.

It is known that activation of certain anti-inflammatory signalling pathways can promote neuroprotection following stroke, but may not necessarily avoid adverse systemic effects. For example, previously we found that systemic administration of IL-33, a Th2-promoting cytokine, is neuroprotective, but these beneficial effects were offset by accelerated systemic immunosuppression and augmented bacterial infections. In contrast to IL-33, IL-37 is inducible by a broad range of pro-inflammatory mediators, including cytokines such as those generated in the brain following ischemic stroke. As a powerful suppressor of innate and adaptive immunity, it then reduces non-specific, T helper (Th) 1, Th2 and Th17 immune responses via negative feedback inhibition. Here we assessed the effect of ischemic stroke in humans on the regulation of IL-37 in blood and the post-mortem brain. Furthermore, we utilised transgenic mice for human IL-37 to similarly assess the effect of stroke on IL-37 regulation in that species, and to investigate the effect of IL-37 on brain injury, motor impairment and bacterial infection following stroke. Unlike humans, these IL-37tg mice express IL-37b driven by the CMV promotor. Expression of this splice variant is therefore ubiquitous in these mice, including in the brain cells or in immune cells infiltrating
the brain, thus allowing us to investigate the effects of IL-37 in various disease settings including stroke.

Our data show that plasma IL-37 was increased following stroke in both humans and IL-37tg mice, and that brain (but not lung) abundance of IL-37 was also augmented, consistent with its production being driven by pro-inflammatory signalling.\textsuperscript{19,41,42} We noted that a lower clinical score in mice was more strongly related to expression of \textit{IL-37} mRNA in the brain than with circulating IL-37 protein, consistent with the concept that IL-37 generated locally in the brain is likely to be the cause of neurological protection. Likely cellular sources of plasma IL-37 are peripheral blood mononuclear cells, including monocytes-macrophages and dendritic cells,\textsuperscript{43} and brain expression of IL-37 may occur in astrocytes, microglia and infiltrating macrophages.\textsuperscript{28,35} We were unable to perform IL-37 co-localisation study with these immune cells due to the cross-reactivity of human IL-37 antibody on mouse tissues. Indeed, it was noteworthy that whereas transgenic expression of IL-37 had no effect on total numbers of immune cells infiltrating the post-ischemic mouse brain, there was a marked reversal in the ratio of pro-inflammatory (3-NT\textsuperscript{+}; indicative of peroxynitrite-induced oxidative damage in pro-inflammatory microglia-macrophages) to anti-inflammatory (3-NT\textsuperscript{-}) microglia-macrophages (F4/80\textsuperscript{+} cells)\textsuperscript{10,13} in IL-37tg compared with WT mice. These findings, which were associated with higher expression of anti-inflammatory markers in the ischemic brain, are consistent with reports that IL-37 reduces the pro-inflammatory effects of immune cells, especially macrophages.\textsuperscript{19,44,45}

MCA occlusion-reperfusion in WT and IL-37tg mice produced a similar rCBF profile and thus equivalent severity of ischemia, but it resulted in a milder degree of functional impairment and a smaller infarct volume in IL-37tg mice. These protective effects of IL-37 are analogous to the greater residual locomotor function displayed by IL-37tg mice after spinal cord injury.\textsuperscript{28} Here, the amelioration of locomotor deficit and brain injury were associated with less bacterial infection of the lungs after stroke at 24 h following stroke, a timepoint at which we
find post-stroke lung infection in mice to be maximal. Previous studies\textsuperscript{15,46} have shown that post-ischemic functional outcome (i.e. clinical score and locomotor activity) after stroke does not strictly correlate with cerebral infarct volume; rather, other factors such as lung infection can contribute significantly to post-stroke morbidity.\textsuperscript{14} It is therefore likely that the multifactorial benefits of IL-37, including neuroprotection and reduction of lung infection, produced the improvement of functional outcome in combination. It has been suggested that the post-stroke lung infections are the result of bacteria translocating from a leaky intestinal epithelium.\textsuperscript{47,48} Since IL-37 has been demonstrated to ameliorate intestinal inflammation and protect barrier functions,\textsuperscript{26,40} it is tempting to speculate that such protection of the intestine may contribute to the rescue of IL-37tg mice from bacterial infection in the lungs.

There are some limitations of this study. Firstly, WT and IL-37tg mice were housed separately as controls. Future studies that include co-housing prior to study to synchronise microbiomes could assess the role of the gut microbiome environment in our findings. Secondly, here we investigated the effect of IL-37 on post-stroke outcome at 24 h only, and it will be important to clarify if the IL-37-dependent protective effects are sustained over a longer period. Thirdly, as this study was restricted to young to middle aged adult male mice, future studies should evaluate the effects of IL-37 in females and also in aged mice of both sexes.

In summary, this study has provided the first data assessing the effect of ischemic stroke on the regulation of IL-37 in humans and IL-37tg mice, and of the impact of IL-37 on stroke outcome measures. Our findings reveal that IL-37 is augmented in the brain and plasma following ischemic stroke, and exerts protection by modulating post-stroke inflammation in the brain and periphery. IL-37 may therefore have potential as a novel therapeutic approach in stroke, and future work to explore the therapeutic potential of post-stroke administration of recombinant IL-37 is therefore warranted.
Material and Methods

Patients
The study was approved by the National Taiwan University Hospital Committee of Human Research and conducted in accordance with the Helsinki Declaration of 1975 (and as revised in 1983). Written informed consent was obtained from the patients or from the next of kin of patients with decreased consciousness. The study included 55 ischemic stroke patients admitted within 24 h of symptom onset. Head MRI or repeated CT examination was performed at admission and at 24 h after symptom onset to confirm the diagnosis of acute ischemic stroke. Patients were excluded if they had received rt-PA or had active infection, autoimmune disease or were under steroid therapy. The study also included 24 control subjects matched for sex and age who were free of cerebrovascular disease for >12 months. Venous blood was obtained from controls or from patients on day 3 after stroke onset. Clinical information including stroke presentation, risk factors, co-morbidities, blood pressure, plasma lipid data (see below) were collected at the time of admission from all study subjects. The severity of stroke was assessed by the National Institutes of Health Stroke Scale (NIHSS) and the Glasgow Coma Score (GCS). Patient characteristics are shown in Table 1.

Human blood and brain tissue
Venous blood samples (n=79) were collected from control patients (n=24) or ischemic stroke patients on day 3 after stroke onset (n=55). Controls included here were stable patients of the clinic who had an ischemic stroke >2 years previously. Blood was immediately placed into EDTA-treated anticoagulation tubes and centrifuged at 300 xg for 15 min at 4 °C to obtain plasma, which was removed with a Pasteur pipette and immediately frozen at -80 °C for future analysis. The plasma levels of IL-37 were determined using a commercially available ELISA kit (see below).
A sample of human brain tissue was obtained from an anonymized autopsy patient in National Taiwan University Hospital with approval from the National Taiwan University Hospital ethics committee. The patient was a 38-year-old female who had acute myocarditis. Right hemispheric infarct developed during the admission with mortality outcome. The brain tissue was collected within 48 h of death and specimens were fixed in 4 % buffered formalin for at least 3 weeks before paraffin embedding, then sections were stained according to standard immunohistochemistry procedures described below using primary antibodies against IL-37 (Clone: 37D12; Thermal Fisher Scientific, USA) and the Dako REAL™ EnVision™ Detection System (Dako, Denmark). Briefly, brain sections were deparaffinized by heating at 60 °C for 30 min followed by xylene application. Sections were then rehydrated by passing through a series of decreasing concentrations of ethanol (100%, 90%, 70% and 50%) for 5 min each step. Sections were then washed in 0.1 M of PBS. Endogenous peroxidase was quenched with 3% hydrogen peroxidase for 10 min. Sections were then blocked with IL-37 antibody (1:100) at 4 °C overnight. Sections were washed the following day, and stained with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV). Dako REAL™ DAB+ Chromogen was applied to sections after washing. Sections were dehydrated with ethanol and xylene prior to being mounted with DPX mountant (VWR International, USA).
**Animals**

A total of 147 male mice (average age = 17.6 weeks) were used in this study. Homozygous mice transgenic for human IL-37 (IL-37tg) were generated as described previously. Our IL-37tg mouse colony was bred from the original colony, which was backcrossed onto C57BL/6 mice for >10 generations. To generate the mice, fertilised eggs from C57BL/6 mice were injected with a pIRES IL-37b expressing plasmid and genotypes were identified at the age of 3-4 weeks. Animals studies have been reported in full compliance with the ARRIVE guidelines. Both C57Bl/6 (WT: n=71) and IL-37tg mice (n=76) were bred at Monash Animal Research Platform, and were housed in separated neighbouring boxes in Monash Animal Research Laboratory prior to experimentation. Of these, 38 were excluded from the study because of: (1) death during the surgical procedure (WT, n=9; IL-37tg, n=3); (2) <50 % reduction in rCBF relative to the pre-occlusion level during cerebral artery occlusion (WT, n=7; IL-37tg, n=15); or (3) subarachnoid hemorrhage due to filament insertion (WT, n=1; IL-37tg, n=3). Mice were housed in a specific pathogen-free environment with free access to food and water. All experiments were approved by Monash University Animal Ethics Committee (Project MARP/2014/064 and MARP/2016/038) and performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, National Health and Medical Research Council of Australia. Animal characteristics are shown in Table 2.

**Transient focal cerebral ischemia in mice**

Mice were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively, i.p.). Rectal temperature was maintained at 37 ± 0.5 °C by a heat lamp throughout the surgery until the animal regained consciousness. Both sham and stroke surgical procedures have been described previously. Ischemia was achieved by inserting a 6-0 silicone-coated nylon monofilament (Doccol, USA) into the distal internal carotid artery and
advancing it to occlude the origin of the MCA. Reperfusion was achieved by retraction of the monofilament after 1 h of occlusion. Successful ischemia-reperfusion was confirmed with transcranial laser-Doppler flowmetry (Perimed, Sweden) in each animal, and was defined as >50 % reduction of rCBF during ischemia and >50 % of recovery of rCBF towards baseline within 10 min of reperfusion. rCBF was recorded for a further 30 min after reperfusion. Sham-operated mice were anesthetized and the right carotid bifurcation was exposed without insertion of a filament. Wounds were then closed, and mice were allowed to recover in a clean cage placed on a heating pad for 23 h. All mice were injected s.c. with 1 ml of sterile saline during their recovery.

**Assessment of motor activity and clinical score in mice**

At 24 h after sham or MCA occlusion surgery, mice were subjected for 5 min to a modified open field with parallel rod floor test for which they were placed in an acrylic box (20 x 20 cm) with raised parallel metal rods across the floor, and locomotor data were automatically collected and analysed by the device software (ANY-maze, Stoelting, USA). Some mice (N = 12 for WT, N = 13 for IL-37tg) were assessed according to a five-point ‘clinical scoring’ system, 0: normal motor function; 1: flexion of torso and contralateral forelimb when lifted by tail; 2: circling to one side when held by tail on a flat surface, but normal posture at rest; 3: leaning to one side at rest; 4: absence of spontaneous activity. Clinical scoring was performed by an investigator blinded as to the genotype and surgical protocol.

**Cerebral infarct and edema volume in mice**

Following motor assessment, mice were killed by isoflurane inhalation overdose and decapitation. Brains were removed immediately and snap frozen in liquid nitrogen. Coronal brain sections (30 µm) separated by ~420 µm were cut and thaw-mounted onto glass slides coated with poly-L-lysine. Sections were then stained with thionin (0.1 %) to delineate the
infarct area. Total, cortical, subcortical infarct and edema volumes were estimated as described previously.\textsuperscript{15,46}

**Immunohistochemistry**

Immunohistochemical analysis was performed in brain sections for several cell types, including pan leukocytes (CD45\textsuperscript{+}), microglia-macrophages (F4/80\textsuperscript{+} that were positive or negative for 3-nitrotyrosine; 3-NT), T lymphocytes (CD3\textsuperscript{+}), neutrophils (myeloperoxidase, MPO\textsuperscript{+}) and astrocytes (GFAP\textsuperscript{+}). Frozen coronal brain sections (10 µm) were thaw mounted onto poly-L-lysine coated glass slides. A 3,3\textquoteright-diaminobenzidine (DAB) kit (Dako, Denmark) was used to detect CD3\textsuperscript{+} T cells or MPO\textsuperscript{+} neutrophils. Sections were air dried before fixing in ice-cold 4 % paraformaldehyde for 15 min. Sections were then blocked with peroxidase for 10 min followed by washing in 0.01 M phosphate-buffered saline (PBS). Sections were then blocked in 10 % normal goat serum for 30 min and incubated with primary antibodies overnight at room temperature (rabbit-anti-CD3, 1:200; Catalogue: ab215212, or rabbit-anti-MPO, 1:100; Catalogue: ab188211, Abcam, UK). They were washed the next day, followed by staining with peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulins for 2 h. DAB was applied onto sections after washing. All sections were then counterstained with 25 % hematoxylin for 2 min and dehydrated with ethanol and xylene prior to being mounted with DPX mountant (VWR International, USA). Immunofluorescent staining was also performed on frozen brain sections to detect CD45\textsuperscript{+} leukocytes (Alexa Fluor 488 rat-anti-mouse CD45; Clone: 30-F11, Biolegend, USA) or GFAP\textsuperscript{+} astrocytes (rabbit anti-GFAP, 1:500; Catalogue: ab7260, Abcam, UK). For both, sections were stained with primary antibody (1:50 or 1:500, respectively) overnight at 4 °C. Following a wash the next day, GFAP-stained sections were incubated with secondary antibody (Alexa Fluor 594 goat anti-rabbit, 1:200; Catalogue: A-11034, Invitrogen, USA) for 2 h at room temperature prior to counterstaining with DAPI. Double immunohistochemical
staining with 3-NT (1:50 Catalogue: ab61392, Abcam, UK) and F4/80 (1:100; Clone: Cl:A3-1, Bio-Rad, USA) was also performed to detect pro-inflammatory (i.e. ‘M1-polarized’) macrophages, as described previously.10,13,15 Sections were washed in 0.01 M PBS, coverslipped, and examined using an Olympus fluorescence microscope. Numbers of CD3+, MPO+, F4/80+ or GFAP+ cells in the left (contralateral) and right (ischemic) hemispheres were counted in 6 brain sections per mouse by a researcher blinded to group identity. CD45+ cells colocalised with DAPI were counted at 200x (in a 710 µm x 530 µm field) in each of the ischemic and contralateral hemispheres of 3 sections per mouse. For colocalization studies, data were presented as percentage of 3-NT+ cells of total F4/80+ cells. For analysis of GFAP expression, fluorescence intensity was analysed using ImageJ software (NIH, USA) in two 250 µm x 250 µm areas within the peri-infarct or corresponding contralateral regions, and normalised to the WT contralateral hemisphere. All appropriate secondary antibody controls were performed in the absence of corresponding primary antibodies to ensure that there was no nonspecific binding.

**Bacterial analysis in mouse lungs**

Mice were killed by isoflurane inhalation overdose. Using sterile technique, lungs were removed and homogenised in 1 ml of sterile PBS. Ten µl of tissue homogenate was serially diluted 10-fold. Homogenates and their diluents were then pipetted onto brain heart infusion agar plates supplemented with 5 % horse blood, and plates were incubated at 37 °C for 18 h after which bacterial colony forming units were counted.

**Quantitative real-time PCR**

Some mice (n=44) were killed by isoflurane inhalation overdose and perfused with sterile PBS (50 ml) via the left ventricle prior to decapitation and brain removal. Hemispheres were separated and immediately frozen in liquid nitrogen. Total RNA was extracted with TRIzol
reagent (Thermofisher Scientific, USA) and RNeasy Mini Kit (Qiagen, Germany) followed by cDNA conversion with Quantitect Reverse Transcription kit (Qiagen). Quantitative RT-PCR was performed with TaqMan Gene expression primers (Applied Biosystems, USA) including Il-1b, Tnf-a, Il-23a, Il-12a, Foxp3, Chil3, Il-10, Il-13, Tgfb, and IL-37 using Bio-Rad CFX96TM real-time PCR machine (Bio-Rad, USA). Data were expressed as fold change (2^{\Delta\Delta CT}) relative to WT sham-operated mice, with the exception of IL-37 in stroke-operated mice, which was normalized to IL-37tg sham-operated mice.

**ELISA for IL-37 in human and mouse plasma**

The level of circulating IL-37 in patients and mice was determined using a human IL-37 ELISA kit (Adipogen, Switzerland). Measurements were performed in duplicate and the results were averaged. Samples with obvious hemolysis, which was visually detected as a pink to red tinge, were not used for measurements. 100 µl of neat plasma was placed into wells coated with anti-human IL-37 antibody in duplicate. Plates were then sealed, incubated overnight at 4 °C and washed, followed by addition of Detection Antibody (Adipogen). Plates were washed again and incubated at 37 °C for 1 h. Horse radish peroxidase-conjugated anti-rabbit IgG was added, the plate was washed again and 3,3’,5,5’-tetramethylbenzidine substrate solution (Adipogen) was added to each well for 10 min in the dark to allow for colour development. Stop solution was then added and absorption was read at 450 nm by a CLARIOstar microplate reader (BMG Labtech, Germany). The standard curve was interpolated in a quadratic equation.

**Statistical analysis**

Data are generally presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed with GraphPad Prism 7.1 (GraphPad Software Inc, USA). Results were analyzed by two-tailed one-way analysis of variance (ANOVA) with Bonferroni post-
hoc test, unpaired Student’s t-test or Welch’s t-test, as appropriate. In some cases, pairs of parameters collected from cohorts of patients or mice were assessed for correlation using Spearman’s rank correlation test. A ROUT test was conducted to exclude statistical outliers. The study was performed in a blinded manner wherever possible. As each animal was assigned to a code on the animal card for MCAO surgery, investigators were not strictly blinded to the genotype of animal during the surgery itself or during the clinical scoring (0-4) immediately prior to euthanasia. However, the post-stroke mobility was analysed using automated software, and therefore was unbiased. Furthermore, all subsequent analyses of tissues such as infarct volume, gene expression, immunohistochemistry and lung infection were performed in a blinded manner. \( P<0.05 \) was considered statistically significant.
Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia (NHMRC) [Project Grants APP1064686, APP1085323]; and NHMRC Senior Research Fellowships (GRD and CGS).
Author contributions

SRZ, MFN, CGS and HAK designed the study; SRZ and SCT conducted various experiments, acquired and analysed data. MFN, CBB, CAN, TVA and GRD provided important reagents and intellectual feedback on the manuscript. SRZ, MFN, CGS and HAK wrote and edited the manuscript. All authors reviewed the manuscript.
Competing Interests

The authors declare no competing interests.
Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
### Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Control (N = 24)</th>
<th>Acute stroke (N = 55)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± SD)</td>
<td>72 ± 8</td>
<td>68 ± 14</td>
<td>0.247</td>
</tr>
<tr>
<td>Males – number (%)</td>
<td>19 (79)</td>
<td>36 (65)</td>
<td>0.292</td>
</tr>
<tr>
<td>NIHSS at admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no./total no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>0/55 (0)</td>
<td></td>
</tr>
<tr>
<td>1-4</td>
<td></td>
<td>10/55 (18)</td>
<td></td>
</tr>
<tr>
<td>5-15</td>
<td></td>
<td>19/55 (35)</td>
<td></td>
</tr>
<tr>
<td>16-20</td>
<td></td>
<td>10/55 (18)</td>
<td></td>
</tr>
<tr>
<td>21-42</td>
<td></td>
<td>16/55 (29)</td>
<td></td>
</tr>
<tr>
<td>Glasgow Coma Scale at admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no./total no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-8</td>
<td></td>
<td>5/50 (10)</td>
<td></td>
</tr>
<tr>
<td>9-12</td>
<td></td>
<td>18/50 (36)</td>
<td></td>
</tr>
<tr>
<td>13-15</td>
<td></td>
<td>27/50 (54)</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td></td>
<td>154 ± 29</td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg; mean ± SD) at admission</td>
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</tr>
<tr>
<td>Blood pressure classification*</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>no./total no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>4/52 (8)</td>
<td></td>
</tr>
<tr>
<td>Elevated</td>
<td></td>
<td>4/52 (8)</td>
<td></td>
</tr>
<tr>
<td>Hypertension stage 1</td>
<td></td>
<td>6/52 (11)</td>
<td></td>
</tr>
<tr>
<td>Hypertension stage 2</td>
<td></td>
<td>38/52 (73)</td>
<td></td>
</tr>
<tr>
<td>Smoking – no./total no. (%)</td>
<td>4/14 (29)</td>
<td>23/54 (43)</td>
<td>0.379</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>8/24 (33)</td>
<td>25/54 (37)</td>
<td>0.748</td>
</tr>
<tr>
<td>High plasma triglyceride (&gt;150 mg/dl)</td>
<td>11/24 (46)</td>
<td>18/54 (33)</td>
<td>0.803</td>
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<tr>
<td>High plasma total cholesterol (&gt;200 mg/dl)</td>
<td>6/24 (25)</td>
<td>18/54 (33)</td>
<td>0.239</td>
</tr>
</tbody>
</table>

*Normal: SBP <120 mmHg and DBP <80 mmHg; Elevated: SBP 120-129 mmHg and DBP <80 mmHg; Hypertension stage 1: SBP 130-139 mmHg or DBP 80-89 mmHg; Hypertension stage 2: SBP >140 mmHg or DBP >90 mmHg.
Table 2. Animal characteristics.

<table>
<thead>
<tr>
<th>Animal Characteristics</th>
<th>WT (N = 54)</th>
<th>IL-37tg (N = 53)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>17.9 ± 0.7</td>
<td>17.6 ± 0.8</td>
<td>0.559</td>
</tr>
<tr>
<td>Pre-surgical weight (g)</td>
<td>33.6 ± 0.5</td>
<td>31.5 ± 0.6</td>
<td>0.012</td>
</tr>
<tr>
<td>Post-surgical weight (g)</td>
<td>31.5 ± 0.6</td>
<td>28.9 ± 0.5</td>
<td>0.003</td>
</tr>
<tr>
<td>Reduction in rCBF during MCAO (%)</td>
<td>73.5 ± 1.6</td>
<td>74.3 ± 1.5</td>
<td>0.723</td>
</tr>
<tr>
<td>Level of reperfusion relative to baseline (%)</td>
<td>96.7 ± 4.4</td>
<td>88.8 ± 5.2</td>
<td>0.256</td>
</tr>
</tbody>
</table>

rCBF, relative cerebral blood flow; MCAO, middle cerebral artery occlusion. Data are mean ± SD.
References


Figure 1. Ischemic stroke increases IL-37 in the plasma level of humans and mice. (a) Circulating level IL-37 protein is shown for control subjects (n=24) and patients 3 days after an ischemic stroke (n=55). (b) IL-37 protein abundance is shown for IL-37tg mice at 24 h after either sham surgery (n=8) or ischemic stroke (n=23). Data are presented as mean ± S.E.M.; *P<0.05, Welch’s t-test.
Figure 2. IL-37 is increased in the brain after stroke in a human and in IL-37tg mice, and is associated with a lower median clinical score in mice. Photomicrographs showing IL-37 immunoreactivity is (a) absent in a brain section from the contralateral hemisphere and (b) present (arrows) in the ischemic hemisphere of a stroke patient. Scale bar = 100 μm. (c) Expression of IL-37 mRNA in brains of IL-37tg mice 24 h after sham surgery (n=8) or in the ischemic hemisphere after stroke (n=13). The mean of the sham group is set as 1.0. **P<0.01; Welch's t-test. (d) Clinical score for wild-type (WT; n=12) and IL-37tg mice (n=13) at 24 h after stroke (P=0.747; Mann-Whitney test). Data in (c) and (d) are presented as mean ± S.E.M. or median, respectively.
Figure 3. Post-stroke functional deficit is less in IL-37tg mice. Measures of post-stroke mobility, namely total distance travelled in 5 min (a,b) and maximum speed attained (c,d), were obtained 24 h after sham surgery (a,c; n=8 for each group) or after stroke surgery (b,d; n=37 for IL-37tg and n=33 for WT). Data are shown as mean ± S.E.M; *P<0.05, unpaired Student's t-test.
Figure 4. Infarct volume is reduced in IL-37tg mice after stroke. Coronal brain sections are shown from (a) a WT and (b) an IL-37tg mouse 24 h post-stroke with the infarct perimeter delineated in yellow. Images are representative of n=24 mice per group. (c) Total infarct volume is shown as mean ± S.E.M; *P<0.05, Welch’s t-test.
Figure 5. IL-37 increases anti-inflammatory cytokines in the brain after stroke. (a) Foxp3, (b) Ym1, (c) Il-10, (d) Il-13 and (e) Tgfb were quantified by qPCR in the ischemic hemisphere of WT and IL-37 tg mice (n=8-13) 24 h after stroke. Data are presented as mean ± S.E.M.; *P<0.05 and **P<0.01, one-way ANOVA with Bonferroni post-hoc test.
Figure 6. IL-37 reduces pro-inflammatory, and increases anti-inflammatory microglia-macrophages in the brain after stroke. (a) Immunohistochemical quantification of total microglia-macrophages (F4/80+) in the ischemic hemisphere 24 h after stroke. Also shown are numbers of (b) pro-inflammatory (3-nitrotyrosine[3-NT]-positive) and (c) anti-inflammatory (3-NT-negative) F4/80+ cells, and (d) the ratio of F4/80+3-NT+ to F4/80+3-NT- microglia-macrophages in WT and IL-37tg mice. All data are n=6 and are presented as mean ± S.E.M.; *P<0.05, unpaired Student’s t-test.
Figure 7. Post-stroke lung infection is reduced in IL-37tg mice. (a) Bacterial load in the lung of WT (n=23) and IL-37tg (n=20) mice, and expression of (b) IL-37 and (c) IL-10 (n=6-7) mRNA 24 h after stroke are shown. CFU, colony forming unit. Data are presented mean ± S.E.M.; *P<0.05 and **P<0.01, unpaired Student’s t-test.
**Supplementary Data**

**IL-37 increases in patients after ischemic stroke and protects from inflammatory brain injury, motor impairment and lung infection in mice**

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Running Head: Interleukin 37 in stroke

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\* Contributed equally
Supplementary Figure S1: Regional cerebral blood flow (rCBF), infarct and edema volumes after stroke.

(a) Blood flow

(b) Cortical infarct

(c) Subcortical infarct

(d) Edema

Supplementary Figure S1. Regional cerebral blood flow (rCBF), infarct and edema volumes after stroke. (a) rCBF during middle cerebral artery occlusion and reperfusion (n=20 in each of WT and IL-37tg mice). (b) Cortical, (c) subcortical and (d) hemispheric edema volumes in WT (n=26) and IL-37tg (n=28) mice at 24 h after stroke. Data are presented as mean ± S.E.M.; *P<0.05, Welch’s t-test for (b-d).
Supplementary Figure S2: Immunohistochemical quantification of immune cells and astrocytes in the ischemic hemisphere of WT mice after stroke.

Supplementary Figure S2. Immunohistochemical quantification of immune cells and astrocytes in the ischemic hemisphere of WT (n=7-9) and IL-37tg (n=7-9) mice after stroke. Numbers of (a) CD45+ leukocytes, (b) MPO+ neutrophils, (c) CD3+ T cells and (d) GFAP astrocytes at 24 h after stroke. Data are presented as mean ± S.E.M.; unpaired Student’s t-test.
CHAPTER 5:
RETROSPECTIVE ANALYSIS OF 5 YEARS OF LABORATORY DATA TO IDENTIFY INTERRELATIONSHIPS BETWEEN OUTCOME MEASURES IN EXPERIMENTAL STROKE
Retrospective analysis of 5 years of laboratory data to identify interrelationships between outcome measures in experimental stroke

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Abstract

Pre-clinical stroke studies model the pathophysiology of clinical stroke where a range of parameters are measured to assess the severity of outcome. However, post-stroke pathology is complex and variable, and associations between parameters are difficult to identify. We performed a retrospective large-scale analysis on 716 control C57BL/6 mice subjected to either transient (1 h) middle cerebral artery occlusion (tMCAO) or permanent MCAO (pMCAO). Structural equation modelling (SEM) was used to construct models to identify cause-effect relationships among numerous variables recorded at 24, 48, or 72 h post-stroke. Our collective data demonstrate that following tMCAO, infarct volume is fully developed within 24 h, whereas brain edema continues to evolve. Permanent MCAO resulted in larger infarct (70 %) and edema volumes (>2-fold) than tMCAO. Eighty-five percent of lung infections resolved within 48 h of tMCAO, accompanied by improved functional outcome. There was greater leukocyte infiltration in brains of mice receiving pMCAO, and more severe leukopenia. Multivariable analyses revealed that edema was positively correlated with infarct volume (Beta 0.778) and clinical score (Beta 0.365) in tMCAO, but not pMCAO. Age (during young adult hood; i.e. 6-40 weeks old) was correlated positively with lung infection (Beta 0.540), and negatively with mobility (Beta -0.322) following tMCAO. However, after pMCAO, age did not appear to affect infarct volume, functional outcomes or lung infection. Bivariable analysis (Spearman’s rank correlation) showed clinical score to be negatively correlated with circulating leukocytes (ρ=-0.286), but positively correlated with brain leukocytes (ρ=0.332) following tMCAO, but not pMCAO. This large-scale analysis of animal experiments has thus provided insight into relationships between variables not available when studies are analysed in isolation.
Introduction

Stroke is a leading cause of mortality and disability worldwide. Ischemic stroke accounts for ~87% of all stroke cases (1) and contributes to 7 million incidents each year globally. Despite the fact that mortality rate has gradually declined in the past two decades due to improved medical care and dedicated stroke units in many countries, more patients require advanced medical treatment and care as a result of stroke-induced disabilities, with an estimated annual cost of US$40.1 billion in 2014 and projected to be ~US$129 billion in 2035 in the USA alone (1, 2).

Ischemia of the brain region supplied by the middle cerebral artery is the most common type of stroke in humans. Animal models provide a platform to study stroke pathology with the potential for reproducible pathophysiological effects (3). Middle cerebral artery occlusion (MCAO) by insertion of an intraluminal filament method is the most widely used model in rodents (3-6) in which the development of infarct core and penumbra, post-stroke inflammation, and immunosuppression have been well characterised (7-10). Furthermore, transient or permanent placement of the intraluminal filament allows the study of ischemia-reperfusion injury or prolonged ischemic injury without reperfusion, respectively.

Stroke may lead to a series of pathological mechanisms that augment the initial ischemic damage, including excitotoxicity, peri-infarct depolarisation, inflammation, apoptosis, systemic immunosuppression and infection (9, 11, 12). Potential therapeutic effects of more than 1,200 compounds that target various cellular pathways have been investigated in experimental models of stroke since the first clinical study in 1958 (13). However, to date, only the thrombolytic drug (recombinant tissue plasminogen activator, rt-PA) and mechanical thrombectomy have been approved for clinical use (14, 15). Thus, many clinical trials have failed to validate the most effective candidates reported from animal research (14), emphasising that there remains a significant translational gap between preclinical and clinical stroke research (7).
Currently, preclinical stroke research mostly focuses on the therapeutic effect of a single or multiple drug(s) administered post-stroke, with – to our knowledge – little or no work seeking to better understand the complex interrelationships between various pathophysiological outcome measures (e.g. infarct volume vs edema volume). Moreover, understanding various interactions between post-stroke outcome parameters is not practical in small study designs. Hence, we have collected data from all successfully completed MCAO surgeries in C57BL/6 mice performed in our lab since 2012 and compiled information on various routinely-collected parameters into a central database in order to conduct this retrospective meta-analysis. We have restricted this study to data from the ‘control’ mice in a variety of published (9, 16-32) and unpublished studies that were either untreated (i.e. ‘naïve’) or treated with the vehicle that was relevant to the specific study. The current work aims to identify any potential association(s) between post-stroke parameters that may contribute to stroke outcome, including the effects of reperfusion.
Methods

Animals

A total of 716 MCAO surgeries were performed on C57BL/6 mice sourced from Monash Animal Research Platform, Asutralian Research Council, or Walter and Eliza Hall Institute between January 2012 and November 2017 at Monash University Animal Research Laboratories or Department of Pharmacology, Clayton, Australia. Animal selection criteria and animal count for each category is shown in a flow chart in Figure 1. All untreated (n=372) or vehicle-treated mice (n=344) that recovered following the MCAO surgery (see below) are included in this study. Each of these mice were studied as a control for comparison with others receiving a specific treatment(s) as part of numerous separate studies performed in the lab during this period. Only the ‘control’ mice from these studies are included in this analysis. Mice were of either sex and 6-40 weeks of age. Here, a successful MCAO surgery is defined as >50 % reduction in regional cerebral blood flow (rCBF) during ischemia relative to the pre-ischemic level as detected by trans-cranial laser Doppler flowmetry, and – in mice subjected to transient MCAO (tMCAO; n=520) – this was followed by restoration of rCBF by >50 % (relative to the pre-ischemic level) within the first 10 min of reperfusion. Other mice (n=196) were subjected to permanent occlusion of the MCA (pMCAO) whereby the filament was not withdrawn to initiate reperfusion. Mice were excluded from the original study and hence this meta-analysis if they: (i) failed to achieve this rCBF profile; (ii) died during the surgical procedure, or (iii) experienced subarachnoid haemorrhage. Animals were housed, and surgeries were performed in a specific-pathogen-free environment. After these exclusions were applied, the vehicle treatments administered to the 716 remaining mice in this analysis were as follows: untreated (n=372); 200 µl of 1 % bovine serum albumin, BSA i.p. [n=128]; 200 µl of dimethylsulfoxide i.p. [n=79]; 200 µl of injectable saline i.p. [n=62]; 200 µl of Hank’s Balanced Salt Solution i.p. [n=49]; 200 µl of antibody isotype control i.p. [n=13]; or ≤200 µl of injectable water s.c. [n=13]. All animal experiments were approved by the
Monash University Animal Ethics Committee and were conducted in accordance with the National Health and Medical Research Council of Australia guidelines for the care and use of animals in research. Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were fully adhered to in each study (33).

**Middle cerebral artery occlusion (MCAO)**

Each MCAO surgery (total = 716) in this study was performed by one of five different surgeons from our laboratory (Hyun Ah Kim: n=244; Hannah X Chu: n=200; Seyoung Lee: n=125; Megan A Evans: n=103; Shenpeng R Zhang: n=44). All surgeons followed the same MCAO protocol to ensure reproducibility of the model of tMCAO or pMCAO, as described previously (9, 31, 34). Mice were anaesthetised with ketamine-xylazine (80 and 10 mg/kg, respectively; i.p.). Focal cerebral ischemia was achieved by inserting an intraluminal filament into the cerebral circulation in order to fully occlude the origin of the right MCA, as follows. A mid-line incision was made, and the right common carotid artery was exposed and then clamped. A 6-0 nylon monofilament with a silicone-coated tip (Doccol Co., USA) was gently inserted into the distal carotid artery, occluding the MCA at the junction with the Circle of Willis. A reduction (typically ~70 %) in rCBF was confirmed and recorded using transcranial laser-Doppler flowmetry (Perimed, Sweden) overlying the cortical region supplied by the MCA. The monofilament was secured in place and the clamp was removed. For tMCAO, the filament was retracted after 1 h of ischemia to allow reperfusion for either 23, 47 or 71 h. Reperfusion (typically restoration of rCBF to >80 % of the pre-ischemic level) to the cerebral cortex was confirmed using laser-Doppler flowmetry. For pMCAO, the monofilament was left in place for 24 or 48 h until euthanasia. A temperature controller (Testronics, Australia) connected to a heat lamp was used to continuously monitor and maintain body temperature at 37.5 ± 0.5 °C measured with a rectal probe throughout the procedure until the animal regained consciousness. All mice received 1 ml of injectable
saline (s.c.) for rehydration immediately after surgery. Mice were then housed in individual cages placed on a heatpad for the duration of recovery period until scheduled euthanasia, with free access to gel nectar (Able Scientific, Australia), food and water.

**Behavioural assessments**

Behavioural/motor function outcomes were assessed at the end of the experiment (24, 48 or 72 h post-stroke), as described previously (35, 36). A clinical score (0-5) was assigned to each mouse using a modified 6-point scoring system: 0 for normal motor function; 1 if flexion of torso and contralateral forelimb occurred when lifted by the tail; 2 if circling when held by the tail on a flat surface; 3 if leaning to one side at rest; 4 for no spontaneous activity; 5 for death after recovery from surgery but prior to scheduled euthanasia.

A hanging wire test was performed in 481 mice by suspending the mouse on a wire, elevated by 30 cm from a bench surface, for up to 180 s and the latency to fall (s) was recorded. Mice were tested 3 times with 5-min breaks in between. The average latency to fall during the 3 trials was included in this study. To avoid potential statistical bias in the bivariable analyses, hanging wire data were excluded from bivariable analyses (see below) for each mouse that hung onto the wire for the full 180 s in any of the three trials (n=30; total included n=451).

Additionally, locomotor activity was assessed in some mice (n=314) to evaluate post-stroke mobility. Mice were placed in a 20 cm x 20 cm acrylic box with a camera above to record the animal’s movement for 5 min. Automated video tracking software (ANY-maze; Stoelting Co., USA) was used to measure the total distance travelled within the 5-min period.

**Infarct volume analysis**

Mice were euthanised at 24, 48 or 72 h post-stroke by isoflurane overdose. Following decapitation, brains were removed and immediately snap frozen using liquid nitrogen. Infarct area was measured using thionin (0.1 %)-stained coronal sections (30 µm) separated by
420 µm, as described previously (9, 31). Images were captured using a CCD camera and infarct volume was analysed using Image J software (NIH, USA) and corrected for edema volume, using the following formula, as described previously (31, 37): corrected infarct volume = [left hemisphere area – (right hemisphere area – right hemisphere infarct area) x (thickness of section + distance between sections)]. Corrected infarct volumes were added to give a three-dimensional total infarct volume. Similarly, total edema volume was calculated from the sum of differences between the areas of right and left hemispheres throughout the coronal brain sections. Additionally, infarct and edema volumes of each animal were normalised by the left hemisphere area at Bregma -1.06 mm, to account for any variation in brain size. Furthermore, injury was assessed specifically in the insular region of the brain including the agranular, dysgranular, and granular insular cortices (Bregma 2.46 mm to -1.22 mm). Brain infarct in the insular cortex was scored based on a 3-point scale: 0 = absence of infarct; 1 = partial infarction; and 2 = complete infarction.

**Lung infection measurement**

Bacterial load in the lungs was quantified in 196 mice at 24 (n=116), 48 (n=8) or 72 h (n=12) after tMCAO or 24 h after pMCAO (n=60). Lungs were removed and homogenized in 1 ml of sterile phosphate buffered saline (PBS) with a tissue homogenizer (Omni International, USA). Ten µl of the tissue homogenate was serially diluted and plated onto brain heart infusion agar supplemented with 5 % defibrinated horse blood (Australian Ethical Biologicals, Australia). Following incubation at 37 °C for 18 h, bacterial colonies were counted and the colony forming units (CFU) per mg of lung tissue were calculated.

**Flow cytometric analysis**

Immediately after euthanasia (at 24, 48 or 72 h by isoflurane overdose), blood was collected via cardiac puncture, followed by intracardiac perfusion with PBS. Tissue collection and
preparation of single-cell suspensions were described previously (9). Various gating strategies were established in multiple studies to identify the total leukocyte population and subsets in the blood, spleen and/or brain (9, 20, 31, 34).

Briefly, circulating leukocytes were purified with red blood cell lysis buffer and resuspended in 1 % BSA in PBS. Spleen was mechanically dissociated and passed through a 70 µm nylon cell strainer (BD Falcon, Australia) to obtain a single-cell suspension. Splenic leukocytes were purified with red blood cell lysis buffer and resuspended in 1 % BSA in PBS. Brain was divided into hemispheres and mechanically dissociated in digestion buffer containing collagenase type XI, hyaluronidase, and collagenase type I-S (125 U/ml, 60 U/ml, and 450 U/ml, respectively, in PBS supplemented with Ca$^{2+}$ and Mg$^{2+}$) at 37 °C for 45 min. Brain homogenates were passed through a 70 µm nylon cell strainer to obtain a single-cell suspension. Cells were then separated by the density gradient using 30 % and 70 % Percoll (GE Healthcare), leukocytes were isolated from the interphase and resuspended in 1 % BSA in PBS.

For consistency, flow cytometric data are presented as cells per ml for blood cells, or as the total number of leukocytes per brain hemisphere or spleen. Animals (n=131) for which there are data on the number of each of: total leukocytes, monocyte/macrophages, microglia, neutrophils, T and B lymphocytes, are included in this study.

**Statistical analysis**

Group data are presented as scatter plots indicating mean ± standard deviation (SD) except for the non-parametric clinical score and insular infarct data, in which the median is indicated. Basic statistical analysis of group data was performed using GraphPad Prism 7.1 (GraphPad Software Inc, USA). Results were analysed by unpaired t-test, Welch’s t-test, or one-way analysis of variance (ANOVA) with Bonferroni post-hoc test, as appropriate. A
ROUT test was performed to exclude statistical outliers on infarct and edema analyses only. P<0.05 was considered statistically significant.

The D'Agostino-Pearson normality test indicated that none of the recorded variables followed a normal distribution. Furthermore, as there are some non-parametric variables present in the data set (i.e. clinical score and insular cortex infarct severity), Spearman’s rank correlation coefficient test was used on the entire data set to identify potential relationships between pairs of variables using IBM® SPSS® Statistics Version 24 (IBM, USA), prior to multivariable analyses (see below). Data are presented with a Spearman’s rank correlation coefficient (ρ) should a monotonic relationship exist. Only variables with P≤0.1 in the bivariable analysis (see Suppl. data) were selected for multivariable analyses, as outlined below.

Multivariable analyses (using RStudio Version 1.0.136; Affero General Public License v3) assessed the relationship of an outcome variable (e.g. infarct volume, edema volume, lung infection, distance travelled or circulating leukocytes) with other independent variables, where possible. For this, linear or logistic regression was used to assess the association of each these major outcome variables of interest against other covariates, for parametric or non-parametric data, respectively. Covariates such as age, spleen weight and hanging wire time were also included in the model. P<0.05 was considered to be statistically significant. Figures were generated and presented if P<0.05 in appropriate correlation tests. Data are presented with standardised beta coefficients, Beta, as a measurement of correlational strength, along with 95% confidence interval (CI) and P value. Due to low sample sizes, only bivariable analyses were performed on most data from flow cytometric studies. A brief description of the data selection process for bivariable and multivariable analyses is shown in a flow chart in Figure 2.
**Structural equation modelling**

Potential models from structural equation modelling (SEM) were developed to identify cause-effect relationship(s) between various parameters measured following ischemic stroke. SEM models were developed from assumed knowledge based on experience, as well as trial and error. Four SEM models were constructed using IBM®SPSS®Amos 25.0.0 (Build 1338625, IBM, USA) and fitted with 435 observations from tMCAO animal data, and one SEM model was fitted with 196 observations from pMCAO animal data. Paths were drawn between variables to indicate the potential relationships. In all SEM models, the model estimator was set to the maximum likelihood with estimated means and intercepts to compensate for incomplete observations. Data are presented as standardized regression weights following the path analysis and P<0.05 was considered to be statistically significant for each relationship. Goodness of fit was measured using three methods: SEM models with comparative fit index (CFI) >0.9; Tucker-Lewis Index (TLI) >0.9; and the Root Mean Square Error of Approximation (RMSEA) <0.05 considered satisfactory fit.
Results

Animal characteristics

Animal characteristics and variables recorded are shown in Table 1 and in many cases also presented in graphical form for completeness (Figs 1-2). Five hundred and twenty out of 716 mice included in this study received tMCAO surgery. Of these, 435, 33 and 52 mice were euthanised at 24, 48, and 72 h after ischemia, respectively. The remaining 196 mice received pMCAO surgery, 187 mice were euthanised at 24 h, and 9 female mice were euthanised at 48 h. Whilst all mice that received tMCAO were males, there were 138 male (70.4 %) and 58 female (29.6 %) mice that received pMCAO. Data on some experimental parameters were unavailable for some mice as the data was either not recorded at the time of procedure or is missing.

Temporal pathophysiological profile in mice following tMCAO

Despite a considerable degree of variability in infarct volume, the data indicate that the extent of stroke-induced brain infarction is likely to be fully established by 24 h after tMCAO (Fig 3A). In contrast, edema volume appears to continue to increase for at least 72 h after tMCAO (Fig 3B). Splenic atrophy accompanied these effects of tMCAO, whereby spleen weight was reduced by 20 % at 72 h (Fig 3C). The mean level of bacterial infection in the lungs was highest at the 24 h timepoint following tMCAO and was typically minimal or absent at 48 and 72 h (Fig 3D). Latency to fall in the hanging wire test was also quite variable at 24 h after tMCAO, but was statistically greater by 72 h (Fig 4A). Clinical score was also statistically lower by 72 h after tMCAO (Fig 4B), at which time there was a tendency for increased mobility (Fig 4C) as compared to data at 24 h. Consistently, most deaths occurred within the first 24 h after tMCAO (Fig 4D).
Effect of reperfusion at 1 h after MCAO

Permanent MCAO resulted in ~70 % larger brain infarcts and >2-fold larger edema volume than tMCAO at 24 h (Fig 3A and B). It was notable that infarction was present in the insular cortex of virtually all mice (54/55; 98.2 %) subjected to pMCAO but only ~20 % (45/228; 19.7 %) at 24 h following tMCAO (Table 1). Furthermore, prolonged occlusion of the MCA (i.e. pMCAO) led to a greater functional deficit at 24 h than tMCAO as indicated by a higher clinical score (Fig 4B) and higher mortality (Fig 4D) but a similar level of lung infection (Fig 3D).

We compared the immune cell profiles in the brains, blood and/or spleens of mice subjected to either tMCAO or pMCAO at 24 h after stroke (Table 2). The number of leukocytes infiltrating the brain was 2.2-fold higher in mice subjected to pMCAO than in those that received tMCAO (Table 2). This was associated with a greater number of neutrophils (3.8-fold higher) and B cells (2.8-fold higher). By contrast, there were ~40 % fewer resident microglia, and a trend for fewer (by 30-40 %) infiltrating macrophages and T cells in the brains of mice at 24 h after pMCAO compared with tMCAO (Table 2). There was profound leukopenia at 24 h in mice subjected to pMCAO, with ~70 % fewer circulating cells of both myeloid and lymphoid lineages than in mice at 24 h after tMCAO (Table 2). On the other hand, splenic leukocyte number tended to be ~2-fold higher at 24 h in mice subjected to pMCAO than in those after tMCAO, and this increase was particularly due to 3-4-fold higher numbers of T and B lymphocytes (Table 2).

Effect of sex on stroke outcome after pMCAO

Animal characteristics are shown in Table 3 comparing male and female mice subjected to pMCAO at 24 h. Female mice had ~23 % lower pre-surgical body weight than male mice of matched age. Furthermore, female mice could hang on a wire >4-fold longer than male mice, but there was no sex difference between other measures of functional outcome (e.g. clinical
score and mobility) and female mice tended to have 40% lower levels of lung infection (Table 3). Interestingly, while female mice exhibited ~20% smaller infarcts, edema volume was 50% greater than in male mice (Table 3). Infarction was present in the insular cortex of ~96% of female but only 61% of male mice (Table 3).

**Multivariable analyses**

Multivariable regression modelling confirmed that body weight was associated with age (Beta 0.561, 95% CI 0.073 to 20.267) and spleen weight (Beta 0.157, 95% CI 0.015 to 0.054) in all mice subjected to either tMCAO or pMCAO (Fig 5).

In mice subjected to tMCAO, infarct volume was positively correlated with edema volume (Beta 0.778, 95% CI 0.79 to 1.10, P<0.0001, Fig 6A, Table 4) but this was not the case in mice subjected to pMCAO (Fig 6B, Table 5). Infarct volume was positively correlated with clinical score in mice subjected to tMCAO (Beta 0.343, 95% CI 0.00 to 0.04, P=0.043, Fig 6E, Table 12), and likewise in pMCAO (Beta 0.321, 95% CI -0.879 to 10.263, P=0.021, Fig 6F, Table 5). On the other hand, edema volume was positively associated with clinical score in mice following tMCAO (Beta 0.365, 95% CI 2.478 to 8.512, P<0.0001, Fig 6C, Table 6) but not pMCAO (Fig 6D).

Remarkably, there was a strong positive correlation between lung infection and age in mice subjected to tMCAO (Beta 0.616, 95% CI 613.47 to 1,080.23, P<0.0001, Fig 7A, Table 8), but no correlation between lung infection and spleen weight (Beta -0.080, 95% CI -645.93 to 272.22, P=0.420, Table 8). However, these relationships were absent in mice subjected to pMCAO (Fig 7B). As might be expected, the level of infection was negatively associated with the latency to fall in the hanging wire test in mice subjected to pMCAO (Beta -0.539, 95% CI -241.27 to -3.73, P=0.044, Fig 7D, Table 9). However, this was not the case after tMCAO after multivariable analysis (Fig 7C, Table 8).
As a variable that reflects clinical outcome, distance travelled in 5 min may be used to assess the functional recovery. We found that distance travelled was negatively correlated with age (Beta -0.322, 95% CI -0.005 to -0.001, P=0.016, Fig 8A, Table 10) and positively correlated with spleen weight (Beta 0.327, 95% CI 0.002 to 0.007, P=0.003, Fig 8C, Table 10) in mice subjected to tMCAO. Similarly, distance travelled was positively correlated with spleen weight in mice subjected to pMCAO (Beta 0.385, 95% CI 0.025 to 0.131, P=0.005, Fig 8D, Table 11). Clinical score, another measure of functional outcome, was also negatively associated with spleen weight (Beta -0.343, 95% CI -0.010 to -0.011, P<0.001) and positively associated with infarct volume (Beta 0.343, 95% CI 6.49 x 10^{-4} to 0.037, P=0.043, Fig 6E) in mice subjected to tMCAO (Table 12).

**Immune cell profile**

Multivariable analysis revealed that circulating leukocytes were positively associated with spleen weight in mice subjected to pMCAO (Beta 0.630, 95% CI 88.554 to 1727.006, P=0.034, Fig 9B, Table 13), but not in tMCAO. Bivariable analyses were performed on selected flow cytometric data due to generally small samples. In mice subjected to tMCAO, bivariable analyses found that certain subsets of circulating leukocytes were associated with stroke outcome. Whilst circulating leukocytes were not correlated with any clinical outcome in mice subjected to either tMCAO or pMCAO (Fig 9C-E), circulating T cells were positively correlated with clinical score ($\rho$=0.364, P=0.011, n=48, Fig 10A) and negatively correlated with distance travelled ($\rho$=-0.521, P=0.005, n=27, Fig 10E). Moreover, consistent with an adverse effect of circulating T cells in mice following tMCAO, our data suggest that circulating T cells were negatively correlated with hanging grip time following pMCAO ($\rho$=-0.841, P=0.036, n=6, Fig 10D). On the other hand, post-stroke clinical outcomes do not correlate with other circulating leukocytes ($\rho$=-0.078, P=0.581, n=52 in tMCAO and $\rho$=-0.182, P=0.517, n=15 in pMCAO)
Flow cytometric analysis in mice subjected to tMCAO showed that clinical score was positively correlated with the number of brain-infiltrating leukocytes ($\rho=0.364$, $P=0.011$, $n=48$, Fig 11A), T lymphocytes ($\rho=0.485$, $P=0.001$, $n=42$, Fig 11C) and with a trend in B lymphocytes ($\rho=0.426$, $P=0.078$, $n=18$, Fig 11H) at 24 h following stroke. Brain-infiltrating macrophages, however, were positively correlated with post-stroke mobility (i.e. distance travelled; $\rho=0.615$, $P=0.033$, $n=12$, Fig 11G). By contrast, following pMCAO, although no correlation was observed between total brain leukocytes and clinical score (Fig 11B), a strong positive relationship was present between brain-infiltrating T lymphocytes and clinical score ($\rho=0.556$, $P=0.020$, $n=17$, Fig 11D). Infiltrating neutrophils, on the other hand, were strongly negatively correlated with clinical score in mice subjected to pMCAO ($\rho=-0.557$, $P=0.006$, $n=23$, Fig 11F), but not tMCAO ($\rho=0.182$, $P=0.273$, $n=38$, Fig 11E). Interestingly, splenic macrophages and neutrophils were negatively associated with brain infarct volume (macrophages: $\rho=-0.651$, $P=0.022$, $n=12$, Fig 12A and neutrophils: $\rho=-0.578$, $P=0.049$, N=12, Fig 12B). Likewise, these splenic myeloid cells were negatively correlated with clinical score (macrophages: $\rho=-0.372$, $P=0.047$, $n=29$, Fig 12C and neutrophils: $\rho=-0.326$, $P=0.090$, $n=28$, Fig 12D). Due to the small sample size, we were unable to identify whether these effects were also present in mice subjected to pMCAO. While it was expected that spleen weight was positively correlated with the number of spleen leukocytes ($\rho=0.374$, $P=0.017$, $n=40$, Fig 12E), spleen leukocyte count was not correlated with post-stroke mobility in mice subjected to tMCAO.

**Structural Equation Modelling (SEM)**

Four SEM models were constructed using data collected from the tMCAO group and 1 SEM model was constructed from the pMCAO data. Out of 435 animals from the tMCAO group, 27 sample moments (sample size for each SEM model) were included in SEM models 1 and 2, and 20 sample moments were included in SEM models 3 and 4. Spleen weight is
presented as spleen weight (in mg) standardized to pre-surgical body weight (in g) in all 5 SEM models labelled as we found that spleen:body weight ratio provided more consistent results in SEM analyses.

**Model 1 for tMCAO metadata**

The first SEM from tMCAO mice is described in Fig 13 with 5 hypothesised relationships. We found that infarct volume positively predicts edema volume (standardized coefficient = 0.593), which can also be predicted by the age of the animal (standardized coefficient = 0.145). Furthermore, age is a predictor of low spleen:body weight ratio (standardized coefficient = -0.185), as well as reduced post-stroke mobility (standardized coefficient = -0.139). Lung infection was also positively related to the age of the animal (standardized coefficient = 0.561). This model seems to be a good fit for the tMCAO metadata and all relationships from this model are statistically significant (CFI=0.950, TLI=0.894, and RMSEA=0.043).

**Model 2 for tMCAO metadata**

Similar to Model 1, Model 2 (Fig 14) shows that a larger infarct volume is predictive of a larger edema volume (standardized coefficient = 0.592). While it is hypothesized that both edema volume and age have negative effects on post-stroke mobility (distance travelled), only the effect of edema volume is statistically significant (edema volume: standardized coefficient = -0.205, P=0.007; and age: standardized coefficient = -0.128, P=0.055). Moreover, lung infection and low spleen:body weight ratio are again predicted by age (standardized coefficient = 0.561 and -0.185, respectively). This model seems to be a good fit for the tMCAO metadata and 4 out of 5 relationships are statistically significant (CFI=0.956, TLI=0.908, and RMSEA=0.039).
Model 3 for tMCAO metadata

Model 3 (Fig 15) describes 4 hypothesized relationships. It shows that infarct volume positively predicts edema volume (standardized coefficient = 0.592), while post-stroke mobility (distance travelled) is positively related to spleen:body weight ratio, and negatively related to edema volume (standardized coefficient = 0.225 and -0.219, respectively). Furthermore, a high spleen:body weight ratio is predictive of a low level of lung infection (standardized coefficient = -0.281). This model appears to be a good fit to the tMCAO metadata and all 4 relationships from this model are statistically significant (CFI=0.995, TLI=0.987, and RMSEA=0.015).

Model 4 for tMCAO metadata

Model 4 (Fig 16) explains 4 hypothesized relationships. Consistent with the previous 3 models, edema volume is positively predicted by infarct volume (standardized coefficient = 0.592). A high spleen:body weight ratio is predictive of a low level of lung infection (standardized coefficient = -0.278). Additionally, a large edema volume is predictive of low post-stroke mobility (standardized coefficient = -0.218). However, the hypothesized relationship between lung infection and short distance did not reach statistical significance (p = 0.052). The model appears to be a good fit to the tMCAO metadata and 3 out of 4 relationships from this model are statistically significant (CFI=1.000, TLI=1.000, and RMSEA=0.014).

Model 1 for pMCAO metadata

Four hypothesized relationships are described in this model (Fig 17). The higher spleen:body weight ratio is predictive of a lower clinical score (standardized coefficient = -0.212). The hypothesized relationship between edema volume and clinical score is not statistically significant (standardized coefficient = -0.223, P=0.079). Furthermore, clinical
score has a positive effect on the level of lung infection (standardized coefficient = 0.287), which also is predictive of a worse stroke outcome (shorter hanging wire time; standardized coefficient = -0.383). This model appears to partially explain the pMCAO metadata as 3 out of 4 relationships are statistically significant. However, more sample sizes may be required to achieve a good fit (CFI=0.8, TLI=-1.299, and RMSEA=0.163).
Discussion

There are several important findings from this study. First, brain infarct fully develops by 24 h post-stroke and is associated with the degree of swelling following 1 h of tMCAO in C57BL/6 mice. Second, the extent of lung infection in mice peaks at 24 h post-stroke, and reduces thereafter with an improved clinical score and grip strength at 72 h. Third, reperfusion has a profound impact on stroke outcome and pathophysiology. For example, edema volume (i.e. brain swelling) is associated with infarct volume and clinical score in mice subjected to tMCAO, but not in mice subjected to pMCAO. Fourth, even for young adult mice aged 2-7 months, age is remarkably associated with the extent of post-stroke lung infection, and mobility after tMCAO, but not pMCAO. Fifth, a greater number of splenic and brain-infiltrating leukocytes is associated with a worse clinical outcome. Further, whilst a greater number of myeloid cells in the brain is associated with milder stroke outcomes, a greater number of lymphocytes in the blood and brain is associated with more severe outcomes.

Consistent with previous findings in mice (9, 16-32, 38, 39), we have shown that 60 min of severe cerebral ischemia (i.e. tMCAO) followed by reperfusion results in an established brain infarct in mice within 24 h, accompanied by infiltration of various immune cells. Permanent MCAO resulted in a larger infarct at 24 h than tMCAO indicating that the infarct size is dependent on the length and severity of ischemia. In this model, the infarct typically develops from the striatum and expands into the cortex as the inflammatory response spreads (3, 40). Previous studies using 90 min of MCAO in both rats and mice have indicated that infarct growth occurs mostly within the first 12 h of ischemia, independent of reperfusion (38, 41, 42), consistent with our metadata. By contrast, infarct expansion appears to be slower in non-human primates and humans, in which there is a delayed progression of infarct development for 7-10 days (43, 44).
A lower clinical score was observed at 24 h if reperfusion occurred, but other functional parameters including hanging grip time and distance travelled, as well as the level of lung infection, appeared to be independent of reperfusion. It has been suggested that acute functional assessment in animal models following stroke induction could be affected by various factors other than brain infarct, such as level of anesthesia, stroke recovery or bacterial infection (9, 44, 45). Here, for hanging group test, we have excluded animals with hanging grip results with a full 180 s in any of the trials they performed. In doing so, we removed the assessment cap set in the experiment and we were able to minimise the potential bias that may cause the correlation scale to be “off the chart”. Interestingly, the present data indicate that lung infection in our mouse model largely resolves after 24 h, consistent with a strong Th1-dominant immune profile of C57BL/6 mice to assist in the clearance of bacteria (46, 47).

Our data showed that female mice have ~20 % smaller brain infarcts than male mice at 24 h after pMCAO in young adults, with moderately less functional impairment. Indeed, pre-clinical studies have reported that estrogen exerts a beneficial effect on stroke outcome in both male and female mice (21, 36, 48, 49) via suppression of pro-inflammatory responses in the brain and periphery (50-52). However, after menopause, stroke is more common in patients aged 55 or older (53), and tends to be most severe and frequent in elderly women typically aged over 80 (54, 55). Despite a better functional outcome overall, we show that female mice have more brain swelling following stroke than male mice, which is consistent with clinical findings (56). A larger degree of cerebral edema will lead to greater intracranial pressure (57), which may explain the greater mortality rate in female mice as compared to male mice from our study.

We investigated the interrelationships between multiple variables using a multivariable analysis. Stroke commonly occurs in the aged population (53), however most pre-clinical stroke studies are conducted in young adult animals. Similarly, our present analysis was
restricted to young adult mice aged 6-30 weeks (equivalent to approx. 18-35 years in humans) (58). We found that whereas infarct development was independent of age, increased age within this period of young adulthood was associated with more severe lung infection and poorer mobility at 24 h following ischemia-reperfusion injury, but not following pMCAO. While it is known that elderly patients have an increased risk of post-stroke lung and urinary tract infections (59-62), to our knowledge our data are the first to identify an age-dependent effect of stroke on infection incidence within the period of relatively young adulthood. It has been well documented that post-stroke infection occurs in more than 30% of acute stroke patients and contributes to worse functional recovery and increased mortality (59, 63-67).

Brain infarction is accompanied by varying degrees of cerebral edema. Here, a strong positive correlation between edema and infarct volume was identified in mice subjected to tMCAO. Furthermore, edema volume was associated with a worse clinical score, suggesting that brain swelling may be a principal/major indicator of post-stroke morbidity and mortality in mice subjected to ischemia-reperfusion injury. On the other hand, infarct volume was a stronger indicator of functional outcome in mice following both tMCAO and pMCAO. Those stroke patients with greater edema volumes predictably express higher acute NIHSS scores and worse 3-month stroke outcomes (68-70), consistent with the metadata from our animal studies. Thrombolytic therapy in stroke patients is often associated with intracerebral hemorrhage and/or cerebral edema (71). This is possibly due to the disruption of blood-brain barrier due to either a direct effect of rt-PA or to reperfusion injury (68, 72). While both infarct and edema volumes are valid therapeutic targets in animal models of stroke, our data here indicate that reperfusion contributes differently to the relationships between infarct volume, edema volume and post-stroke functional outcome.

Following pMCAO, lung infection was negatively correlated with hanging wire time. However, this relationship was absent following tMCAO when adjusted for age in the
multivariable analysis. Age, on the other hand, was associated with the extent of lung infection and mobility following tMCAO. Indeed, elderly patients have a higher incidence of pneumonia and worse functional outcomes (60, 73). Several factors could potentially affect bacterial clearance in aged individuals such as impaired pathogen clearance in the mucosal layers, reduced chest wall mobility or lung compliance, and immune dysregulation, especially following stroke (74). It is worth noting that stroke in patients is likely to involve more complicated and heterogeneous processes than in animal models of stroke. In addition to advanced age, risk factors for developing pneumonia in stroke patients also include aphasia, motor dysfunction and cognitive impairment (60). Our data suggest that reperfusion may also contribute to lung infections especially in older (although young to middle-aged adult) animals. Studies in stroke patients do not generally report whether such infections are related to whether or not thrombolytic therapies were administered. Our findings suggest that reperfusion therapy in stroke patients with increasing age potentially increases the risk of infection, at least in young adults. However, further study is needed to clarify this issue in older individuals.

We investigated effects on the peripheral immune system – in the spleen and circulation – following stroke. After stroke, there is a dynamic change in spleen weight associated with apoptosis of splenic leukocytes (9) and this can persist for up to 7 days (75-77). Our multivariable analyses indicate that a lower post-stroke spleen weight is associated with a worse mobility and fewer circulating leukocytes following either tMCAO or pMCAO. This possibly is consistent with catecholamine-mediated splenic atrophy leading to greater immunosuppression in the periphery, rendering the animal more susceptible to post-stroke infection (78, 79). We used the spleen weight as a rudimentary indication of the spleen leukocyte count in the multivariable analysis as we had a large sample size, and indeed it was reflective of the total number of live leukocytes it contained. Following stroke, splenocytes are released into the circulation and enter the ischemic brain within 2 d, further
contributing to the ischemia-induced damage. Consistent with pre-clinical studies, patients with smaller spleens are reported to have worse functional recoveries and higher NIHSS scores (80). We did not have a sufficiently large sample size to assess the effect of the spleen changes after pMCAO.

Our data are consistent with the concept that higher myeloid cell counts in the brain and spleen result in a protective phenotype in mice, whereas more T or B lymphocytes in the circulation, spleen and brain predict a worse outcome. Roles of innate immune cells in cardiovascular diseases have been long debated. Higher expression of M2-polarised microglia/macrophages, Ly6C\textsuperscript{hi} monocytes, and N2-polarised neutrophils are reportedly associated with milder outcomes following stroke (9, 20, 34, 81-83). Our data suggest that a higher number of circulating T lymphocytes, especially in the acute phase, could serve as an excellent biomarker for poor short-term stroke recovery regardless of whether reperfusion occurs. Our bivariable analysis between clinical outcome and flow cytometry profile suggests that reperfusion therapy-induced brain salvage and functional protection following acute stroke in mice may be partly due to the resulting fewer leukocytes translocating to the brain from periphery, as well as lower T and B cell counts in the circulation, at least acutely.

We have used structural equation modelling (SEM models) to identify and construct complex models of the interrelationships between observed (independent) and latent (outcome) variables. Interestingly, with this approach we show for the first time that age (within a relatively early period of adulthood) exerts a significant impact on several important parameters following tMCAO. Our data indicate that the extent of brain swelling following tMCAO is largely dependent on age and infarct volume. Furthermore, we found that the extent of lung infection is not a direct effector of post-stroke immobility following tMCAO, but may play a stronger role following pMCAO, consistent with the concept that reperfusion therapy can also alleviate systemic pathology after stroke independently of its direct effect to limit infarct size.
There are several limitations of this study. Although the overall sample size is relatively large for a pre-clinical study, only some of the parameters were assessed in any individual mice and so our analysis is limited by the smaller sample size of certain parameters, which reduced the sensitivity and/or specificity of the data. In mice subjected to tMCAO, we found that increasing age during early to mid-adulthood can impact on post-stroke edema volume, lung infection and mobility. Inclusion of data from much older mice (e.g. >12 months) will provide a more complete assessment of the role of age in outcome measures after stroke, a clinical event that is most common in advanced age (84, 85). Additionally, estrous cycle stage in these female mice and potential sex-dependent effects in the tMCAO model warrant investigation. Furthermore, while it is intriguing that greater brain injury and clinical deficits are associated with fewer myeloid cells and more lymphocytes in the circulation, spleen or brain, it will be important to identify the relevant cell subsets.

In summary, this study has used a meta-analysis approach on a body of data collected in our laboratory over a 5-year period to identify interrelationships between pathophysiological parameters in mice following MCAO surgery. We have identified that 24 h after MCAO surgery, brain edema is closely correlated with infarct size after ischemia-reperfusion but not if reperfusion does not occur. Overall, our data suggest that reperfusion in the tMCAO model following stroke could lead to quite different pathophysiological scenarios from that occurring after pMCAO. Interestingly, increasing age – even within relatively young adulthood (e.g. 6-25 weeks) – was associated with more severe lung infection, and lower post-stroke mobility following ischemic-reperfusion, underlying mechanisms of which require study. By contrast, no such effects of age were detected after pMCAO (i.e. when there was no reperfusion). We found evidence that higher levels of myeloid cells in the brain and spleen are associated with reduced neurological injury following stroke, whereas poorer functional outcomes are associated with higher T and/or B cell counts in the brain and circulation after either tMCAO or pMCAO, suggesting a potentially useful biomarker. Further studies are
warranted to better understand stroke pathophysiology so that new biomarkers and therapeutic targets can be identified.
References


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</tr>
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<td>Age (days) mean ± SD [N]</td>
<td>73 ± 28 [433]</td>
<td>68 ± 13 [33]</td>
</tr>
<tr>
<td>Number of males (%)</td>
<td>435 (100)</td>
<td>33 (100)</td>
</tr>
<tr>
<td>Pre-surgical weight (g) mean ± SD [N]</td>
<td>28.3 ± 3.5 [366]</td>
<td>26.4 ± 2.8 [23]</td>
</tr>
<tr>
<td>Hanging wire time (s) mean ± SD [N]</td>
<td>36.5 ± 45.5 [318]</td>
<td>38.8 ± 26.7 [13]</td>
</tr>
<tr>
<td>Distance (m) mean ± SD [N]</td>
<td>1.5 ± 2.4 [214]</td>
<td>2.6 ± 3.5 [6]</td>
</tr>
<tr>
<td>Clinical score (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51/399 (12.8)</td>
<td>3/31 (9.7)</td>
</tr>
<tr>
<td>1</td>
<td>62/399 (15.5)</td>
<td>8/31 (25.8)</td>
</tr>
<tr>
<td>2</td>
<td>49/399 (12.3)</td>
<td>2/31 (6.5)</td>
</tr>
<tr>
<td>3</td>
<td>128/399 (32.1)</td>
<td>9/31 (29.0)</td>
</tr>
<tr>
<td>4</td>
<td>75/399 (18.8)</td>
<td>3/31 (9.7)</td>
</tr>
<tr>
<td>5</td>
<td>34/399 (8.5)</td>
<td>6/31 (19.3)</td>
</tr>
<tr>
<td>Infarct volume (mm³) mean ± SD [N]</td>
<td>38.7 ± 24.8 [245]</td>
<td>38.8 ± 28.6 [14]</td>
</tr>
<tr>
<td>Edema volume (mm³) mean ± SD [N]</td>
<td>26.7 ± 20.2 [245]</td>
<td>41.4 ± 46.6 [14]</td>
</tr>
<tr>
<td>Insular infarct (%)</td>
<td>Absent</td>
<td>183/228 (80.3)</td>
</tr>
<tr>
<td>Partial</td>
<td>23/228 (10.1)</td>
<td>1/14 (7.2)</td>
</tr>
<tr>
<td>Full</td>
<td>22/228 (9.6)</td>
<td>3/14 (21.5)</td>
</tr>
<tr>
<td>Spleen weight (mg) mean ± SD [N]</td>
<td>66.7 ± 19.6 [216]</td>
<td>59.0 ± 12.4 [13]</td>
</tr>
</tbody>
</table>

[N]: N number
Table 2. Flow cytometric characteristics of mice that received tMCAO and pMCAO.

<table>
<thead>
<tr>
<th>Cell counts</th>
<th>Transient MCAO</th>
<th>Permanent MCAO</th>
<th>P value tMCAO vs pMCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental endpoint (h)</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Brain cell count (cells) mean ± SD [N]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>8,957 ± 7,011 [60]</td>
<td>10,393 ± 7,161 [12]</td>
<td>--</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1,138 ± 1,168 [44]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2,118 ± 1,939 [43]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>1,819 ± 4,963 [50]</td>
<td>2,225 ± 738.8 [6]</td>
<td>--</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>767.4 ± 594.8 [29]</td>
<td>872.7 ± 382.5 [6]</td>
<td>--</td>
</tr>
<tr>
<td>Microglia</td>
<td>10,054 ± 7,713 [36]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Blood cell count (x10³ cells/ml) mean ± SD [N]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>22.08 ± 16.73 [41]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>271.36 ± 176.81 [46]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Spleen cell count (x10⁶ cells/spleen) mean ± SD [N]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.32 ± 0.55 [37]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.51 ± 4.01 [36]</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>1.96 ± 1.78 [43]</td>
<td>3.18 ± 1.28 [6]</td>
<td>--</td>
</tr>
</tbody>
</table>

Statistical tests were performed between tMCAO and pMCAO data at 24 h following stroke. [N]: N number; Welch’s t-test or unpaired t-test where appropriate; *P<0.05**P<0.01, ***P<0.001, ****P<0.0001.
Table 3. Animal characteristics of mice that received pMCAO based on sex.

<table>
<thead>
<tr>
<th>Animal Characteristics</th>
<th>Permanent MCAO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Animal Count</td>
<td>138</td>
<td>58</td>
</tr>
<tr>
<td>Age (days)</td>
<td>63 ± 11</td>
<td>64 ± 10</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[137]</td>
<td>[58]</td>
</tr>
<tr>
<td>Pre-surgical weight (g)</td>
<td>27.3 ± 2.4</td>
<td>21 ± 1.9</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[107]</td>
<td>[58]</td>
</tr>
<tr>
<td>Hanging wire (s)</td>
<td>27.0 ± 42.5</td>
<td>116.7 ± 66.0</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[97]</td>
<td>[11]</td>
</tr>
<tr>
<td>Distance (m)</td>
<td>2.2 ± 4.7</td>
<td>2.0 ± 2.6</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[16]</td>
<td>[41]</td>
</tr>
<tr>
<td>Clinical score Median</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>[N]</td>
<td>[125]</td>
<td>[58]</td>
</tr>
<tr>
<td>Mortality rate</td>
<td>10.4%</td>
<td>25.9%</td>
</tr>
<tr>
<td>Infarct volume (mm³)</td>
<td>73.5 ± 26.8</td>
<td>58.6 ± 13.6</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[31]</td>
<td>[24]</td>
</tr>
<tr>
<td>Edema volume (mm³)</td>
<td>44.4 ± 24.3</td>
<td>66.3 ± 23.8</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[31]</td>
<td>[24]</td>
</tr>
<tr>
<td>Insular infarct Median score (%)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Absent = 0</td>
<td>1/31 (3.2)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>Partial = 1</td>
<td>11/31 (35.5)</td>
<td>1/24 (4.2)</td>
</tr>
<tr>
<td>Full = 2</td>
<td>19/31 (61.3)</td>
<td>23/24 (95.8)</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>72.5 ± 19.0</td>
<td>65.1 ± 16.5</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[40]</td>
<td>[43]</td>
</tr>
<tr>
<td>Lung infection (CFU/mg)</td>
<td>12,197 ± 11,482</td>
<td>7,659 ± 11,265</td>
</tr>
<tr>
<td>mean ± SD [N]</td>
<td>[16]</td>
<td>[44]</td>
</tr>
</tbody>
</table>

Unpaired t-test was performed for all parameters except clinical score and insular infarct, which were analysed with Mann-Whitney test. [N]: N number; **P<0.01, ***P<0.001, ****P<0.0001.
Table 4. Multivariable analysis for infarct volume in mice 24 h after tMCAO

<table>
<thead>
<tr>
<th>Infarct volume tMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edema volume</td>
<td>0.992</td>
<td>0.072</td>
<td>0.816</td>
<td>0.85 – 1.14</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>Lung infection</td>
<td>-0.868 x 10^{-5}</td>
<td>4.142 x 10^{-5}</td>
<td>-0.012</td>
<td>-9.09 – 7.35 (x10^{-5})</td>
<td>0.834</td>
</tr>
<tr>
<td>Distance travelled</td>
<td>0.189</td>
<td>0.568</td>
<td>0.020</td>
<td>-1.32 – 0.94</td>
<td>0.740</td>
</tr>
</tbody>
</table>

Residual standard error: 14.47; Degree of freedom: 98; ****P<0.0001

Table 5. Multivariable analysis for infarct volume in mice 24 h after pMCAO

<table>
<thead>
<tr>
<th>Infarct volume pMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edema volume</td>
<td>-0.022</td>
<td>0.115</td>
<td>-0.025</td>
<td>-0.25 – 0.21</td>
<td>0.851</td>
</tr>
<tr>
<td>Clinical score</td>
<td>5.571</td>
<td>2.336</td>
<td>0.321</td>
<td>0.88 – 10.26</td>
<td>0.021*</td>
</tr>
</tbody>
</table>

Residual standard error: 21.78; Degree of freedom: 50; *P<0.05

Table 6. Multivariable analysis for edema volume in mice 24 h after tMCAO

<table>
<thead>
<tr>
<th>Edema volume tMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung infection</td>
<td>-2.683 x 10^{-5}</td>
<td>5.446 x 10^{-5}</td>
<td>-0.047</td>
<td>-13.49 – 8.12 (x10^{-5})</td>
<td>0.623</td>
</tr>
<tr>
<td>Distance travelled</td>
<td>-0.747</td>
<td>-0.787</td>
<td>-0.097</td>
<td>-2.31 – 0.82</td>
<td>0.345</td>
</tr>
<tr>
<td>Clinical score</td>
<td>5.495</td>
<td>1.520</td>
<td>0.365</td>
<td>2.48 – 8.51</td>
<td>&lt;0.000****</td>
</tr>
</tbody>
</table>

Residual standard error: 19.23; Degree of freedom: 97; ****P<0.0001

Table 7. Multivariable analysis for edema volume in mice 24 h after pMCAO

<table>
<thead>
<tr>
<th>Edema volume pMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen weight</td>
<td>-0.497</td>
<td>0.362</td>
<td>-0.319</td>
<td>-1.25 – 0.27</td>
<td>0.190</td>
</tr>
<tr>
<td>Hanging wire</td>
<td>0.156</td>
<td>0.150</td>
<td>0.242</td>
<td>-0.16 – 0.47</td>
<td>0.315</td>
</tr>
</tbody>
</table>

Residual standard error: 29.15; Degree of freedom: 16;

Table 8. Multivariable analysis for lung infection in mice 24 h after tMCAO

<table>
<thead>
<tr>
<th>Lung infection tMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>SE</td>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>869.800</td>
<td>142.200</td>
<td>0.616</td>
<td>613.47 – 1,080.23</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>-186.900</td>
<td>230.400</td>
<td>-0.080</td>
<td>-645.93 – 272.22</td>
<td>0.420</td>
</tr>
<tr>
<td>Hanging wire</td>
<td>850.000</td>
<td>1,867.300</td>
<td>-0.047</td>
<td>-2,870.67 – 4,570.68</td>
<td>0.650</td>
</tr>
</tbody>
</table>

Residual standard error: 32,270; Degree of freedom: 74; ****P<0.0001
### Table 9. Multivariable analysis for lung infection in mice 24 h after pMCAO

<table>
<thead>
<tr>
<th>Lung infection pMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance travelled</td>
<td>-340.300</td>
<td>-0.122</td>
<td>-1,799.55 – 1,118.94</td>
<td>0.621</td>
<td>NA</td>
</tr>
<tr>
<td>Hanging wire</td>
<td>-122.500</td>
<td>-0.539</td>
<td>-241.27 – -3.73</td>
<td>0.044*</td>
<td>7D</td>
</tr>
</tbody>
</table>

Residual standard error: 9.747; Degree of freedom: 12; *P<0.05

### Table 10. Multivariable analysis for distance in mice 24 h after tMCAO

<table>
<thead>
<tr>
<th>Distance travelled tMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.026</td>
<td>-0.322</td>
<td>-0.01 – -0.00</td>
<td>0.016*</td>
<td>8A</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>0.042</td>
<td>0.327</td>
<td>0.00 – 0.01</td>
<td>0.003**</td>
<td>8C</td>
</tr>
<tr>
<td>Lung infection</td>
<td>3.285 x 10^-6</td>
<td>7.217 x 10^-6</td>
<td>0.059</td>
<td>-1.11 – 1.77 (x10^-5)</td>
<td>0.650</td>
</tr>
</tbody>
</table>

Residual standard error: 2.006; Degree of freedom: 74; *P<0.05, **P<0.01

### Table 11. Multivariable analysis for distance in mice 24 h after pMCAO

<table>
<thead>
<tr>
<th>Distance travelled pMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen weight</td>
<td>0.078</td>
<td>0.385</td>
<td>0.03 – 0.13</td>
<td>0.005**</td>
<td>8D</td>
</tr>
<tr>
<td>Lung infection</td>
<td>7.867 x 10^-6</td>
<td>3.758 x 10^-5</td>
<td>0.027</td>
<td>-6.75 – 8.32 (x10^-5)</td>
<td>0.835</td>
</tr>
</tbody>
</table>

Residual standard error: 3.09; Degree of freedom: 54; **P<0.01

### Table 12. Multivariable analysis for clinical score in mice 24 h after tMCAO

<table>
<thead>
<tr>
<th>Clinical score tMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct volume</td>
<td>0.019</td>
<td>0.343</td>
<td>0.00 – 0.04</td>
<td>0.043*</td>
<td>6E</td>
</tr>
<tr>
<td>Edema volume</td>
<td>9.138 x 10^-3</td>
<td>0.140</td>
<td>-0.01 – -0.03</td>
<td>0.402</td>
<td>6C</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>0.026</td>
<td>-0.343</td>
<td>-0.01 – -0.01</td>
<td>&lt;0.001***</td>
<td>NA</td>
</tr>
<tr>
<td>Lung infection</td>
<td>1.311 x 10^-6</td>
<td>3.360 x 10^-6</td>
<td>0.039</td>
<td>-5.39 – 8.01 (x10^-6)</td>
<td>0.698</td>
</tr>
</tbody>
</table>

Residual standard error: 1.159; Degree of freedom: 70; *P<0.05, ***P<0.001

### Table 13. Multivariable analysis for circulating leukocytes in mice 24 h after pMCAO

<table>
<thead>
<tr>
<th>Blood leukocytes pMCAO</th>
<th>Unstandardized Coefficient</th>
<th>Standardized Coefficient</th>
<th>95% CI</th>
<th>P value</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>883.2</td>
<td>0.330</td>
<td>-638.83 – 2,405.18</td>
<td>0.212</td>
<td>NA</td>
</tr>
<tr>
<td>Spleen weight</td>
<td>907.8</td>
<td>0.630</td>
<td>88.55 – 1,727.01</td>
<td>0.034*</td>
<td>9B</td>
</tr>
</tbody>
</table>

Residual standard error: 17,790; Degree of freedom: 7; *P<0.05
Figure 1. Animal selection criteria and animal count in each category based on the types of stroke induced and the timing of euthanasia. MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow; SAH, subarachnoid haemorrhage.
Mouse entry with recorded parameters

Select mice with 23 h of stroke recovery, separate based on stroke type (tMCAO or MCAO)*

Bivariant analyses using SPSS on selected parameters

D’Agostino-Pearson normality test

Obtain Spearman correlation coefficient (ρ), and P-value for each pair of analyses

P ≤ 0.1 for any pair

No

Select all relevant, independent variables for each outcome variable

linear or logistic regression for multivariable analyses

Report Beta coefficient, 95% CI and P-values in table

Correlation identified; Generate graphs using results from bivariant analyses, report ρ, P-value and N for each pair of parameters

And

Compare using the same parameters between tMCAO and pMCAO group, generate graphs

* Correlation tests between age and pre-surgical bodyweight or spleen weight are inclusive of all data regardless of stroke type or duration

Figure 2. Data selection process for bivariant and multivariable analyses. tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion.
Figure 3. Temporal pathophysiological profile in mice after tMCAO or pMCAO. (A) Infarct volume, (B) edema volume, (C) spleen weight and (D) lung infection at 24, 48 or 72 h after stroke. Data are presented as mean±SD. One-way ANOVA with Bonferroni post-hoc test. ****P<0.0001; (A, B) n=14-245, (C) n=13-216, (D) n=8-115. tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion.
Figure 4. Functional outcomes in mice after tMCAO or pMCAO. (A) Latency to fall in the hanging wire test, (B) clinical score, (C) distance travelled in the open field test, and (D) mortality rate between 0-24 h, 24-48 h or 48-72 h after stroke. Data are presented as mean±SD in A and C, and as median in B. One-way ANOVA with Bonferroni post-hoc test for A and C; Kruskal-Wallis test for B. **P<0.01, ***P<0.001, ****P<0.0001; (A) n=13-318; (B) n=25-365; (C) n=6-214; (D) n=27-405. tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion.
Figure 5. Correlation between age and pre-surgical body weight or post-stroke spleen weight in mice after tMCAO or pMCAO. (A) Body weight is correlated with age (ρ=0.539, P < 0.0001, n=599). (B) Age is weakly associated with spleen weight (ρ=0.127, P=0.015, n=362). All mice subjected to tMCAO received 1 h of ischemia. tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s rank correlation rank. *P<0.05.
Figure 6. Correlation between edema volume and infarct volume or clinical score in mice after tMCAO or pMCAO. (A) There is a strong correlation between edema and infarct volumes in mice following tMCAO (\( \rho = 0.609, \ P < 0.000, \ n = 245, \) Table 4). (B) No correlation is present between edema and infarct volumes after pMCAO (\( \rho = -0.131, \ P = 0.341, \ n = 55, \) Table 5). (C) Edema volume is correlated with clinical score following tMCAO (\( \rho = 0.218, \ P = 0.001, \ n = 245, \) Table 6), but (D) not following pMCAO (\( \rho = -0.182, \ P = 0.192, \ n = 53 \)). (E) Infarct volume is correlated with clinical following tMCAO (\( \rho = 0.316, \ P < 0.000, \ n = 245, \) Table 12), as well as following (F) pMCAO (\( \rho = 0.275, \ P = 0.047, \ n = 53 \), Table 5). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *\( P < 0.05 \).
Figure 7. Correlation between lung infection and age or hanging grip time in mice after tMCAO or pMCAO. (A) There is a positive correlation between lung infection and age in mice following tMCAO ($\rho=0.256$, $P=0.006$, $n=116$, Table 8), but (B) not following pMCAO ($\rho=0.033$, $P=0.801$, $n=60$). (C) Lung infection is not correlated with the latency to fall on the hanging wire test following tMCAO ($\rho=-0.183$, $P=0.107$, $n=79$, Table 8), (D) but is negatively associated following pMCAO ($\rho=-0.557$, $P=0.031$, $n=15$, Table 9). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *$P < 0.05$. 
Figure 8. Correlation between distance travelled and age or spleen weight in mice after tMCAO or pMCAO. (A) There is a negative correlation between distance travelled and age in mice following tMCAO ($\rho=-0.210$, $P=0.002$, $n=214$, Table 10). (B) No correlation is present between distance travelled and age following pMCAO ($\rho=-0.003$, $P=0.980$, $n=57$). (C) Distance travelled is positively correlated with spleen weight following tMCAO ($\rho=0.218$, $P=0.017$, $n=151$, Table 10), (D) as well as following pMCAO ($\rho=0.360$, $P=0.006$, $n=57$, Table 11). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *$P < 0.05$. 

\[ tMCAO \]

A  Distance Travelled vs Age  \[ \rho = -0.210^* \]

B  Distance Travelled vs Age  \[ \rho = -0.003 \]

C  Distance Travelled vs Spleen Weight  \[ \rho = 0.218^* \]

D  Distance Travelled vs Spleen Weight  \[ \rho = 0.360^* \]
Figure 9. Correlation between circulating leukocytes and spleen weight or functional outcome in mice after tMCAO or pMCAO. (A) There is no correlation between circulating leukocytes and spleen weight in mice following tMCAO ($\rho=-0.042$, $P=0.778$, n=47), but a strong positive correlation following pMCAO ($\rho=0.758$, $P=0.011$, n=15). (C) Circulating leukocytes are not correlated with clinical score following tMCAO ($\rho=-0.022$, $P=0.876$, n=52), as well as pMCAO ($\rho=-0.182$, $P=0.5172$, n=15). (E) Similarly, no correlation is present between circulating leukocytes and mobility (distance travelled, $\rho=0.154$, $P=0.409$, n=31). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *$P < 0.05$. 
Figure 10. Correlation between circulating T cells and functional outcomes in mice after tMCAO or pMCAO. (A) There is a positive correlation between circulating T cells and clinical score in mice following tMCAO (ρ=0.364, P=0.011, n=48), but (B) no such correlation is present in mice following pMCAO (ρ=0.617, P=0.192, n=6). (C) There is no correlation between circulating T cells and hanging grip time in mice following tMCAO (ρ =-0.028, P=0.856, n=44), but (D) there is a strong negative correlation following pMCAO (ρ=-0.841, P=0.036, n=6). (E) There is a strong negative correlation between circulating T cells and post-stroke mobility (distance travelled, ρ=-0.521, P=0.005, n=27). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *P < 0.05.
Figure 11. Correlation between various brain-infiltrating leukocytes (total), T cells, neutrophils, macrophages or B cells and functional outcome in mice after tMCAO or pMCAO. (A) There is a positive correlation between infiltrating leukocytes and clinical score in mice following tMCAO ($\rho=0.364$, $P=0.011$, $n=48$), but (B) not following pMCAO ($\rho=-0.180$, $P=0.333$, $n=31$). Brain infiltrating T cells are positively correlated with clinical score following both (C) tMCAO ($\rho=0.485$, $P=0.001$, $n=42$) and (D) pMCAO ($\rho=0.556$, $P=0.020$, $n=17$). (E) Brain infiltrating neutrophils are not correlated with clinical score in mice following tMCAO ($\rho=0.182$, $P=0.273$, $n=38$) but (F) a strong negative correlation was found following pMCAO ($\rho=-0.557$, $P=0.006$, $n=23$). (G) Brain infiltrating macrophages are strongly correlated with distance travelled following tMCAO ($\rho=0.615$, $P=0.033$, $n=12$). (H) Brain infiltrating B cells are not correlated with clinical score following tMCAO ($\rho=0.426$, $P=0.078$, $n=18$). tMCAO, transient middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion. Spearman’s correlation rank. *$P < 0.05$. 


Figure 12. Correlation between splenic macrophages, neutrophils or leukocytes and infarct volume, functional outcomes or spleen weight in mice after tMCAO. Both macrophages and neutrophils in the spleen are negatively correlated with infarct volume (A, $\rho=-0.651$, $P=0.022$, $n=12$; and B, $\rho=-0.578$, $P=0.049$, $n=12$) and clinical score (C, $\rho=-0.372$, $P=0.047$, $n=29$; and D, $\rho=-0.326$, $P=0.090$, $n=28$) following tMCAO. (E) Spleen leukocytes are positively correlated with spleen weight following tMCAO ($\rho=0.374$, $P=0.017$, $n=40$). tMCAO, transient middle cerebral artery occlusion. Spearman’s correlation rank. *$P < 0.05$
Figure 13. Model 1: Graphical representation of the structural equation modelling of pathophysiological profile in mice following transient middle cerebral artery occlusion. Paths are labelled with standardised regression coefficients. Green arrows indicate positive coefficients, whereas red arrows indicate negative coefficients. A high coefficient is visualised with a darker shade. Circles represent unobserved variances estimated in the SEM model, which are not directly measured but inferred from other observed variables. All paths presented are statistically significant (P<0.05). Standardised path coefficient and covariance estimates are labelled on each path as numeric values. BW, body weight.
Figure 14. Model 2: Graphical representation of the structural equation modelling of pathophysiological profile in mice following transient middle cerebral artery occlusion. Paths are labelled with standardised regression coefficients. Green arrows indicate positive coefficients, whereas red arrows indicate negative coefficients. A high coefficient is visualised with a darker shade. Circles represent unobserved variances estimated in the SEM model, which are not directly measured but inferred from other observed variables. Statistically non-significant path is presented in grey (P>0.05). Standardised path coefficient and covariance estimates are labelled on each path as numeric values. BW, body weight.
Figure 15. Model 3: Graphical representation of the structural equation modelling of pathophysiological profile in mice following transient middle cerebral artery occlusion. Paths are labelled with standardised regression coefficients. Green arrows indicate positive coefficients, whereas red arrows indicate negative coefficients. A high coefficient is visualised with a darker shade. Circles represent unobserved variances estimated in the SEM model, which are not directly measured but inferred from other observed variables. All paths presented are statistically significant (P<0.05). Standardised path coefficient and covariance estimates are labelled on each path as numeric values. BW, body weight.
Figure 16. Model 4: Graphical representation of the structural equation modelling of pathophysiological profile in mice following transient middle cerebral artery occlusion. Paths are labelled with standardised regression coefficients. Green arrows indicate positive coefficients, whereas red arrows indicate negative coefficients. A high coefficient is visualised with a darker shade. Circles represent unobserved variances estimated in the SEM model, which are not directly measured but inferred from other observed variables. Statistically non-significant path is presented in grey (P>0.05). Standardised path coefficient and covariance estimates are labelled on each path as numeric values. BW, body weight.
Figure 17. Model 1: Graphical representation of the structural equation modelling of pathophysiological profile in mice following permanent middle cerebral artery occlusion. Paths are labelled with standardised regression coefficients. Green arrows indicate positive coefficients, whereas red arrows indicate negative coefficients. A higher coefficient is visualised with a darker shade. Circles represent unobserved variances estimated in the SEM model, which are not directly measured but inferred from other observed variables. Statistically non-significant path is presented in grey (P>0.05). Standardised path coefficient and covariance estimates are labelled on each path as numeric values. BW, body weight.
CHAPTER 6:
GENERAL DISCUSSION
6.1 Summary of thesis

This thesis has examined various effects of immunotherapies following ischemic stroke and gained a deeper understanding of stroke pathophysiology in mice. Using the mouse MCAO model of ischemic stroke, the findings have underlined the importance of Th1-type inflammation in the contribution to acute stroke pathology, and revealed that the ensuing brain injury may be mitigated by systemic treatment with Th2-promoting cytokines, IL-4 and IL-33 (Chapter 3), or by transgenic expression of the human anti-inflammatory cytokine, IL-37 (Chapter 4). Nevertheless, the findings raise caution regarding the use of Th2-associated immunotherapies (IL-4 and IL33) in that they may augment the risk of post-stroke bacterial lung infection, which may ultimately exacerbate clinical outcome and even lead to higher post-stroke mortality. The data suggest that treatment with antibiotics, when used in combination with Th2-promoting cytokines, can lessen such detrimental effects. On the other hand, endogenous expression of the anti-inflammatory gene, IL-37, provided clear protection against adverse effects of ischemic stroke both in the brain and in the periphery. Finally, a large-scale retrospective analysis of data (Chapter 5) from all naïve and vehicle-treated MCAO-operated C57BL/6 mice in our laboratory since 2012 has revealed some complex and fascinating interrelationships among measured parameters following stroke. Our data suggest that many factors might be taken into consideration for predicting whether the benefit of reperfusion will outweigh the potential risks. This chapter will briefly discuss the above findings as a whole, current clinical implications of post-stroke immunotherapies and prognosis, limitations and questions that are yet to be answered regarding post-stroke pathology.

6.2 Targeting inflammation with IL-33 and IL-37 in stroke

The studies presented in Chapter 3 demonstrated protective effects of acute Th2-promoting cytokine therapies in the brain of mice following stroke. Using flow cytometry and real-time
PCR, our data confirmed previous findings (1-4) that acute tMCAO is associated with a massive influx of leukocytes into the ischemic hemisphere within 24 h, along with abundant local production of pro-inflammatory cytokines, leading to inflammation-mediated mechanisms of damage to the ischemic brain (5). Indeed, our data suggest that acute treatment with IL-33 exerts a dose-dependent protective effect against ischemic brain damage, by skewing local and systemic inflammatory profiles to a Th2-phenotype, which are shown to be protective in various CNS diseases (6). Furthermore, the endogenous transgenic expression of the human anti-inflammatory cytokine IL-37 was found to result in a similar level of brain protection following tMCAO. Despite the fundamental differences between the two cytokines regarding their molecular and cellular mechanisms of action (7, 8), neither IL-33 nor IL-37 altered the infiltration profiles of immune cells entering the brain after stroke. Instead, both cytokines appeared to exert anti-inflammatory switching effects on cellular activity, i.e. by reducing the pro-inflammatory activity of microglia/macrophages and increasing local mRNA expression of anti-inflammatory markers such as IL-10 and foxP3. Our initial data therefore suggest that targeting post-stroke inflammation with IL-33 or IL-37 may provide some previously undiscovered benefits following stroke pathology, at least in the brain.
6.3 The disadvantages of IL-33 immunotherapy and its clinical relevance

Anti-inflammatory cytokine therapy following stroke may provide useful neuroprotective benefits to prevent secondary ischemic damage. However, our study described in Chapter 3 was the first to provide some important insights into the drawbacks of such treatment in CNS diseases. We postulated that the worsened clinical outcomes and mortality occurring as a result of IL-33 treatment were due to increased bacterial lung infections. In an apparent effort to protect the brain from further inflammatory damage, after a stroke the body induces a series of complex immunomodulatory mechanisms to limit the effects of overactivation of local immune responses, and this involves a systemic shift from a Th1 to a Th2-dominant phenotype in both stroke patients and in animal models (9, 10). A Th2 immune response thus promotes tissue healing and regeneration, but can also lead to long-term immunosuppression that is seen in many stroke patients (11, 12). It is plausible that IL-4 and IL-33, as Th2-promoting cytokines, can accelerate this shifting process, but exacerbating acute lung infections after stroke. Consistent with such an effect in stroke, similar detrimental effects of IL-33 have been reported in other disease conditions such as Dengue viral infection (13) and respiratory viral infections (14).

Our data suggested that post-stroke functional deficits exacerbated by IL-33 treatment could be mitigated by combination therapy with antibiotics to eliminate the development of lung infections. It is worth noting that the post-stroke clinical outcomes and survival rates in mice receiving combination therapy (recombinant IL-33 and antibiotics) were not necessarily better than in those mice that only received vehicle and/or antibiotics following stroke, similar to many clinical trials with prophylactic antibiotic treatment (15, 16). Since clinical translation of therapies for stroke must focus mainly on improving functional outcomes, as opposed to quantitatively reducing the extent of brain injury (17), the translational potential of IL-33, even in combination with antibiotics, may be too low to justify pursuing further.
6.4 The undiscovered potential and disadvantages of IL-37 in stroke

Unlike administration of recombinant IL-33, endogenous expression of human IL-37 gene in mice exhibited markedly lower lung infection and consequently better post-stroke mobility than wild type C57BL/6 mice. A possible explanation for this is that endogenous IL-37 usually first becomes activated at the site of injury and in relevant immune cells, as evident in stroke (Chapter 4) and other diseases (18), whereas the peripheral administration of high-dose anti-inflammatory cytokines may rapidly lead to systemic changes of inflammatory profile, which may then trigger unfavourable outcomes such as post-stroke immunosuppression. Indeed, IL-37 is reported to be upregulated and released into the circulation in acute autoimmune inflammatory conditions but not in sufficient levels to fully offset the effects of pro-inflammatory mediators also released (18-22). The expression of IL-37 only becomes upregulated in the presence of inflammatory stimuli, and its post-stroke level may therefore correlate with stroke severity, similar to previously reported studies in myocardial infarction (20), arthritis (22) and multiple sclerosis (19). Overall, these features may make IL-37 a potentially excellent biomarker in predicting disease severity and prognosis.

Despite the promising effects of IL-37 in acute stroke that were revealed here in IL-37tg mice, we did not observe any therapeutic benefits using human recombinant IL-37 (rIL-37; 40 µg/kg) in a separate cohort of wild type mice subjected to tMCAO (n= 10 per group; See Appendix Fig 7.7). This may have been due to poor stability of systemically administered rIL-37. The dose was selected based on previous evidence (23, 24), but we are yet to determine the stability and optimal dose for rIL-37 in mice following stroke so that this direction for stroke therapy might be further explored. Furthermore, the long-term, systemic effects of rIL-37 treatment on peripheral immune system remain to be determined.

Additional preliminary studies suggested that IL-37 did not provide neuroprotection in middle-aged IL-37tg mice subjected to photothrombotic stroke (see Appendix 7.2).
Compared with the MCAO model, the model of photothrombosis-induced cortical stroke induces much milder brain injury with a low-grade, delayed inflammatory response in the brain (25, 26). Photothrombotic stroke in rodents is particularly useful for studies of long-term functional recovery from stroke due to its less severe nature. Peripheral effects of photothrombotic stroke are usually negligible and seldomly studied (26, 27). Thus, the activity of IL-37 in our transgenic mice might be expected to remain low, and hence it is perhaps not surprising that any protective or beneficial effects of IL-37 were minimal or absent.

### 6.5 Retrospective analysis of interrelationships between outcome measures in experimental stroke

In Chapter 5, a large-scale retrospective analysis was conducted to examine some common parameters that were routinely assessed during mouse MCAO experiments in our laboratory. MCAO is the most widely used animal model for mimicking human stroke pathology and for investigate potential drug efficacy in stroke (28). In our efforts to better understand MCAO disease pathophysiology and how it might reflect post-stroke conditions in humans, we made several novel findings regarding the interrelationships among such parameters, which may in future serve to better understand stroke in animals and humans. For example, we found that the age of the animal plays a much more prominent role on many post-stroke functional parameters than previously thought, especially in tMCAO even within cohorts of young or middle-aged adults aged up to 40 weeks, equivalent to ~40 human years (29). Historically it has been common that mostly young adult mice, and in most cases males, are used for experimental stroke studies, due mainly to economic reasons as well as the desire to limit sex hormone-related variability (30, 31). However, it is now well recognised that efficacy of a treatment in pre-clinical stroke studies of young adult males does not necessarily translate to similar efficacy in older animals and/or females, let alone in human
stroke patients (30). Indeed, ~75 % of strokes occur in older patients aged 55 or more (32). Our data strongly suggest that future pre-clinical stroke studies should involve aged or at least middle-aged animals where possible.

Chapter 5 studies also revealed some strong effects of reperfusion on stroke outcomes. Whilst reperfusion therapies have shown to provide some benefits in mice 24 h after stroke, associated risks such as lung infections and potential haemorrhage should not be ignored. Our data suggest that careful prognostic evaluation may be needed to assess whether reperfusion therapy following stroke could provide sound clinical benefits in mice with certain characteristics, such as those with advanced age. This also raises the strong possibility that all pre-clinical neuroprotective agents should be tested in experimental strokes both with and without reperfusion to determine their optimal effectiveness before eventual extension into clinical studies.

Numerous biomarkers have been investigated over the past few decades in order to be able to better predict stroke severity and prognosis from shortly after time of onset (33, 34). Here, we found evidence to suggest that the total numbers of either circulating or brain infiltrating T lymphocytes are reflective of poorer clinical outcome within 24 h of stroke, regardless of whether reperfusion has occurred. Our findings could be included in the growing body of literature of biomarkers for stroke development and prognosis. Future studies on the specific subtypes of T cells (and probably other immune cells) should also be performed to assess whether suppression of these T cell subtypes, for example, could improve stroke outcomes both in the short and long term.

6.6 Limitations and future directions

It is apparent that various mechanisms collectively contribute to stroke pathophysiology and outcomes, such as infarct development, infection and functional recovery. While targeting inflammation has been shown here to be beneficial in proof-of-concept animal stroke
models, immunotherapies alone might not be sufficient to produce the best possible outcome following stroke. Immune responses could perhaps be ideally targeted in a relevant temporal and spatial manner, i.e. in acute and/or long-term settings, as well as in the brain and/or periphery. Like IL-33, a seemingly effective neuroprotective agent might inadvertently produce severe delayed toxic effects following stroke that may be less easily identified in current common study designs (17). It will be vital that the long-term effects of novel stroke interventions are first examined in pre-clinical settings. It seems plausible that clinical trials must eventually explore whether targeting of multiple ischemic mechanisms based on their temporal and spatial importance can provide superior outcomes. Indeed, a cocktail of drugs or interventions targeting not only the occlusive blood clot, but also subsequent mechanisms of inflammation, infection and apoptosis may provide better treatment strategies for stroke recoveries (35).

Protocols examined in Chapters 3 and 4 typically involved administration or expression of the therapy within 1 h of stroke onset, which is well within the 4.5 h thrombolytic timeframe in humans (28). We do not know whether similar benefits of IL-33 or IL-37 might be observed beyond the current therapeutic window, which is the case for up to 90 % of stroke patients who present for medical attention too late to be eligible for thrombolytic therapy (36). Future work with such immunomodulatory agents should thus utilise both transient and permanent stroke animal models, and they should also be assessed when administered after the 4.5 h timeframe.

In these three chapters, all data were collected in mice with a C57BL/6 background, which are known to have a Th1-dominant phenotype (37), and are thus more prone to ischemic damage than other common laboratory mouse strains such as ICR, BALB/C and CBA (38). Similarly, in humans, patients with distinct immune or genetic profiles are known to exhibit different risks or recovery outcomes in stroke (39, 40). It will therefore be important for a potential neuroprotective agent to be tested on various mouse strains to ascertain the
conditions in which most benefit might be achieved, so that the treatment might be tailored to an ideal clinical population. To effectively achieve this, a deep understanding of all post-stroke parameters and their interrelationships is required, as well as biomarkers identified to be able to quickly match individual patients to established genetic and immune profiles to guide the most suitable therapeutic approaches.

6.7 Conclusion

This thesis has utilised mouse models to identify benefits and risks associated with Th2-type cytokine therapy on outcomes after ischemic stroke. Targeting inflammation in stroke appears to be able to provide a promising therapeutic pathway that requires further exploration before effective translation into the clinic is appropriate. Clearly, there may be risks associated with side-effects of individual cytokines, and so systemic consequences of such therapies, such as lung infection, should be closely monitored especially when systemic administration is employed. Finally, this thesis has also identified several important interrelationships between various disparate parameters underlying stroke pathology. It is suggested that the mechanistic differences between transient and permanent MCAO in their resulting profiles of outcome measures should be carefully evaluated to guide better treatment and care of stroke patients.
6.8 References


38. Yang G, Kitagawa K, Matsushita K, Mabuchi T, Yagita Y, Yanagihara T, et al. C57BL/6 strain is most susceptible to cerebral ischemia following bilateral common carotid


CHAPTER 7:
APPENDICES
This section includes some methodologies and results that are specific to single chapters but not described elsewhere in this thesis.

7.1 Chapter 3

7.1.1 Additional information for flow cytometry on innate lymphoid cells

Preliminary experiments were performed to determine the level of innate lymphoid cells (ILC) in the brains and lungs of some mice following stroke. Protocols of ILC extractions and quantifications are described below.

Mice were euthanised by isoflurane (Baxter, USA) inhalation overdose at 24 h following the induction of MCAO and transcardially perfused with 50 ml of PBS. Perfusion was performed by inserting a 27 G needle into the left ventricle, and a small nick was made with a pair of straight spring scissors on the right atrium to remove blood. After successful perfusion, all lobes of the lung were removed from the chest cavity, cleared of connective tissues and adjacent organs. Brains were gently removed from the skull following decapitation and separated into left and right hemispheres using a scalpel blade. Lungs and brain hemispheres were processed using Lung Dissociation Kit and Adult Brain Dissociation Kit, respectively (Miltenyi Biotec, Germany) to obtain single-cell suspensions.

Each lung tissue was placed in a gentleMACS C Tube (Miltenyi Biotec, Germany) containing 1x Buffer S (2.4ml), Enzyme D (100 µl) and Enzyme A (15 µl) supplied in the Lung Dissociation Kit, before being loaded upside down onto the sleeve of the gentleMACS Dissociator (Miltenyi Biotec, Germany). gentleMACS program m_lung_01 was then run on the Dissociator before incubation at 37 °C for 30 min whilst being continuously rotated. C tubes containing lung tissues were then placed back onto the gentleMACS Dissociator to run the program m_lung_02. Following termination of protocol, C Tubes were then briefly centrifuged for tissue collection. Tissue samples were resuspended with 1x Buffer S and then transferred through a 70-µm MACS SmartStrainer (Miltenyi Biotec, Germany) into 15 ml falcon tubes to obtain single-cell suspensions. Cell strainers were then washed with 1x
Buffer S to ensure all remaining tissues were collected. Single-cell suspensions were then centrifuged at 300xg for 10 min and supernatants were removed. Cells were then resuspended with 2 ml of FACS buffer (2% of foetal calf serum (ThermoFisher Scientific, USA) and 2 mM of ethylenediaminetetraacetic acid (EDTA; Merck USA) in sterile 1x PBS) and placed on ice in preparation for ILC staining.

Brain tissues were mechanically dissociated with a scalpel blade on a petri dish and then placed into a C Tube containing 1950 µl of enzyme mix 1 (50 µl of Enzyme P in 1900 µl Buffer Z) and 30 µl of enzyme mix 2 (10 µl of Enzyme A in Buffer Y), all supplied in an Adult Brain Dissociation Kit, before being loaded upside down onto the sleeve of the gentleMACS Dissociator with heaters. The gentleMACS program 37C_ABDK_01 was then run. Following brief centrifugation to collect all tissue samples at the bottom of the tube, brain samples were resuspended with Dulbecco’s phosphate-buffered saline containing calcium, magnesium, glucose and pyruvate (D-PBS; ThermoFisher Scientific, USA) and then passed through a 70-µm MACS SmartStrainer (Miltenyi Biotec, Germany) into 15 ml falcon tubes to obtain single-cell suspensions. Cell strainers were then washed with D-PBS to ensure all remaining tissues were collected. Brain cell suspensions were then centrifuged at 300xg for 10 min and supernatant were then removed. To perform debris removal, cells from each brain hemisphere were resuspended in 4 ml of ice-cold D-PBS mixture containing 900 µl of Debris Removal Solution (supplied by Adult Brain Dissociation Kit). Tissues samples were mixed well and then carefully overlayed with 4 ml of D-PBS in a 15 ml tube. Samples were then centrifuged at 3,000xg for 10 min at 4 °C with full acceleration and brake. Three phases were formed following centrifugation, and the top two phases were carefully aspirated and discarded. The remaining cells were gently resuspended and topped up to 15 ml with D-PBS, followed by centrifugation at 1,000xg for 10 min at 4 °C and aspiration of supernatant. Finally, red blood cells were removed from the cell suspension using Red Blood Cell Removal Solution supplied in the Adult Brain Dissociation Kit. In detail, cell pellets were
resuspended in 1 ml of 1x ice-cold Red Blood Cell Removal Solution and incubated at 4 °C for 10 min. 10 ml of ice-cold PB buffer (PBS with 0.5% Bovine serum albumin, pH 7.2) were then added before the centrifugation at 300xg at 4 °C for 10 min. Following complete aspiration, single brain cell suspensions were resuspended with 2 ml of FACS buffer and placed on ice in preparation for ILC staining.

For ILC staining, a list of antibodies used and dilution factors are described in table 7.1. Samples were washed with 1 ml of 1x PBS and centrifuged at 300xg for 5 min at room temperature, followed by aspiration. Viability staining (200 µl, Zombie Aqua, 1:200) was then performed on each sample for 15 min at room temperature in the dark. Cells were then washed with 2 ml of FACS buffer and centrifuged at 300xg at 4 °C for 5 min, followed by aspiration of supernatant. Samples were then incubated with Fc-receptor blocker (CD16/CD32; 1:50; BD Pharmingen, USA) at 4 °C for 15 min in the dark. Cell surface staining was then performed using appropriate antibodies diluted in FACS buffer listed in table 7.1 at 4 °C for 30 min in the dark. Following a wash with FACS buffer and centrifugation at 300xg for 5 min at 4 °C, supernatant was aspirated, and cell pellets were resuspended in 300 µl of Foxp3 Fixation/Permeabilization working solution (BioLegend, USA) at 4 °C for 30 min in the dark. Cells were then washed with 2 ml of Foxp3 Fixation/Permeabilization buffer before being centrifuged at 300xg for 5 min at 4 °C. Cells were decanted and intracellular staining was performed using antibodies diluted with Fixation/Permeabilization buffer with appropriate concentrations (as listed in table 7.1) at 4 °C for 30 min in the dark. Cells were then washed in 2 ml of 1x Fixation/Permeabilization buffer followed by the centrifugation at 300xg for 5 min at 4 °C. Fluorescence minus one (FMO) was included as a negative control for each antibody listed in table 7.1. Cells were then decanted and resuspended in 200 µl of FACS buffer, and analysed by a Fortessa flow cytometer (BD Biosciences, USA) and FlowJo Software (Tree Star Inc, USA).
7.1.2 Gating Strategies

All single cells were gated through side scatter (SSC) and forward scatter to remove debris. Dead cells (zombie aqua™+) were excluded from the analysis. Live cells were then gated for CD45, a general leukocyte marker, before being subdivided to identify the 3 subtypes of ILCs. All ILCs were gated and express CD45⁺CD4⁻TCRβ⁻CD11b⁻CD11c⁻TER-119⁻B220⁻CD3 epsilon⁻GR-1⁻. Type 1 ILCs were then identified as being T-bet\textsuperscript{high}. Type 2 ILCs were identified as GATA3⁺KLRG1⁺, and type 3 ILCs were identified as RoRγT⁺NkP46⁺ (Figure 7.1)
<table>
<thead>
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<th>Dilution</th>
<th>Target cells</th>
<th>Host/Isotype</th>
<th>Supplier</th>
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<td>Mouse IgG2a, κ</td>
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<td>PerCP/Cy5.5</td>
<td>1/500</td>
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<td>T cells &amp; NK cells</td>
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**Cell surface**

**Intracellular**

RORγT PE 1/200 Immature CD4+CD8+ thymocytes Mouse IgG2a, κ BD Biosciences
Tbet PE-CF594 1/25 Th1 cells Mouse IgG1, κ BD Biosciences
GATA3 BV421 1/25 Th2 cells Mouse IgG2b, κ BioLegend

Table 7.1. Antibodies used in flow cytometry experiments to determine innate lymphocyte cells
Figure 7.1 Gating strategies for innate lymphoid cells types 1-3.
7.1.3 Results

The number of ILCs in brains and lungs are presented as percentage of total live cells and shown in figures 7.1 and 7.2.

![Type 1 ILC in brain](image)

![Type 2 ILC in brain](image)

![Type 3 ILC in brain](image)

Figure 7.1 Innate lymphoid cell (ILC) counts in the brain following stroke. Flow cytometric quantification of (A) type 1 ILCs, (B) type 2 ILCs, and (C) type 3 ILCs in each hemisphere at 24 h after stroke. N = 4-7; S, sham; C, contralateral; I, ischemic; Veh, vehicle.
Figure 7.2 Innate lymphoid cell (ILC) counts in the lung following stroke. Flow cytometric quantification of (A) type 1 ILCs, (B) type 2 ILCs, and (C) type 3 ILCs in lung at 24 h after stroke. N = 3-8; Veh, vehicle.
Figure 7.3 Gating strategy for brain panel 1
Figure 7.4 Gating strategy for spleen, blood panel 1
Figure 7.5 Gating strategy for panel 2
7.2 Chapter 4

7.2.1 Photothrombotic stroke

Focal stroke targeting primary motor cortex (M1) was induced by photothrombosis in some male mice (11-18 months old). Under isoflurane anaesthesia (2-3% isoflurane in O₂; Baxter, USA), mice were placed in a stereotaxic apparatus (Stoelting, UK)) with the skull exposed. Under isoflurane anaesthesia (2-3% isoflurane in O₂; Baxter, USA), mice were placed in a stereotaxic apparatus (Stoelting, UK)) with the skull exposed. A cold light source (KL1500 LCD, Zeiss, Germany) attached to a 40x objective lens producing an illumination with 2 mm in diameter was securely placed directly above the left primary motor cortex, 1.5 mm lateral from the bregma. 200 μl of Rose Bengal (1% m/v in normal saline; Sigma, USA) was administered intraperitoneally and allowed to circulate for 5 min, followed by the illumination using the cold-light source for 15 min through the intact skull. The head wound was then closed with tissue adhesive (Vetbond™, 3M, USA). Sham-operated mice were injected with Rose Bengal but not subjected to light illumination. Body temperature was monitored rectally and maintained at 37.0 ± 0.5 °C with a heat pad throughout the surgical procedure until the animal regained consciousness. Mice were monitored twice daily for the first 3 days, and then at least once daily until functional testing and euthanasia on day 7.

7.2.2 Behavioural assessment for mice subjected to photothrombotic stroke

Two different functional assessments described below were performed on day 7 following photothrombotic stroke in order to capture subtle functional and motor deficits induced by this milder model of ischemic brain injury. Both functional assessments were performed on mice once immediately prior to stroke induction to establish the baseline readings. Functional assessment was evaluated by investigators blinded to the genotype or treatment of the animals.
7.2.2.1 Cylinder test – spontaneous forelimb task

The cylinder test assesses forelimb asymmetry following unilateral stroke (1, 2). Mice were placed in a glass cylinder (19 cm x 13 cm, H x D), and the duration that one or both forelimbs spent on the cylinder wall supporting when rearing was recorded for 5 min using a camera located directly above the cylinder. Video recording of each cylinder test was played at 20% of real time speed to quantitatively determine forelimb preference. Total time spent on the left, or the right, or both paws was recorded. The asymmetry index was then calculated using the following formula: - percentage of time ipsilateral use : percentage of time contralateral use.

7.2.2.2 Grid walking test

The grid walking test was also performed in mice following photothrombotic stroke in order to measure the degree of limb impairment (1, 2). Animals were placed in a box measuring 45 cm x 25 cm x 20 cm (L x W x H) and a metal grid with 11 mm x 11 mm openings above the floor. A camera was placed at floor level to record “foot-slips”, where any limb slipped through the metal grid, over 5 min. Videos were analysed at half speed and the number of foot-slips on each limb was counted. The total number of front or rear foot-slips by the right paws were divided by the total number of slips by the left paws to establish the asymmetry index.
Figure 7.6 Infarct and functional outcomes in mice at 7 days post photothrombotic stroke. (A) Total infarct volume. (B) Cylinder test for assessment of forepaw asymmetry. Grid walking testing for asymmetric deficits in (C) forepaws and (D) hind paws. N = 3-4 for sham, n = 9-10 per group for stroke; WT, wild type
Figure 7.7 Brain infarct, functional outcome and lung bacterial infection in mice treated with recombinant IL-37, 24 h following transient 60 min MCAO. (A) Total infarct volume, (B) clinical score, (C) hanging wire latency to fall. Parallel floor testing indicating (D) total distance and (E) number of foot slips per metre travelled within 5 min. (F) Bacterial infection in lungs at 24 h following stroke. Veh, Vehicle; rIL-37, recombinant IL-37; Student’s unpaired t-test or one-way ANOVA with Bonferroni post-hoc test, as appropriate.
7.2.3 References


### 7.4 Chapter 5

**Table 7.2. Correlation table for mice at 24 h after 60 min tMCAO**

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## Part 4 of 16

### Transient MCAO

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| 41| Clinical score           | Rho                      | 0.140 | 0.182 | 0.485**
|   | Sig. (2-tailed)          |                          | 0.397 | 0.273 | 0.001 |
| 43| N                        |                          | 39  | 38  | 42  |
| 44| Post-surgical bodyweight (g) | Rho | -0.429 | 0.470 | -0.310 |
|   | Sig. (2-tailed)          |                          | 0.188 | 0.171 | 0.243 |
| 46| N                        |                          | 11  | 10  | 16  |
| 47| Blood leukocytes (cells/ml) | Rho                    | -0.049 | 0.195 | 0.084 |
|   | Sig. (2-tailed)          |                          | 0.813 | 0.349 | 0.654 |
| 49| N                        |                          | 26  | 25  | 31  |

*P<0.05, **P<0.01 (two tailed)
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*P<0.05, **P<0.01 (two tailed)
Table 7.3. Correlation table for mice at 24 h after permanent MCAO

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*P<0.05, **P<0.01 (Two tailed); Variables with P<0.1 are labeled in yellow, Black or blue texts represent cells situated above or below the center dividing line, respectively, as indicated by green cells.
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*P<0.05, **P<0.01 (Two tailed)
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| 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 2 | Age (days) | Rho | 0.046 | 0.055 | 0.119 |
| 3 |   | Sig. (2-tailed) | 0.653 | 0.464 | 0.327 |
| 4 |   | N | 99 | 182 | 70 |
| 5 | Pre-surgical bodyweight (g) | Rho | -0.143 | 0.119 | 0.896** |
| 6 |   | Sig. (2-tailed) | 0.188 | 0.129 | 0.000 |
| 7 |   | N | 86 | 163 | 68 |
| 8 | Sex | Rho | 0.324** | -0.024 | -0.802** |
| 9 |   | Sig. (2-tailed) | 0.001 | 0.750 | 0.000 |
| 10 |   | N | 99 | 183 | 70 |
| 11 | Insular infarct | Rho | 0.511* | -0.305* | -0.462** |
| 12 |   | Sig. (2-tailed) | 0.011 | 0.026 | 0.003 |
| 13 |   | N | 24 | 53 | 40 |
| 14 | Infarct volume (mm$^3$) | Rho | -0.321 | 0.275* | 0.020 |
| 15 |   | Sig. (2-tailed) | 0.126 | 0.047 | 0.904 |
| 16 |   | N | 24 | 53 | 40 |
| 17 | Edema volume (mm$^3$) | Rho | 0.458* | -0.182 | 0.039 |
| 18 |   | Sig. (2-tailed) | 0.024 | 0.192 | 0.809 |
| 19 |   | N | 24 | 53 | 40 |
| 20 | Brain size (mm$^2$) | Rho | -0.221 | 0.008 | -0.060 |
| 21 |   | Sig. (2-tailed) | 0.299 | 0.956 | 0.712 |
| 22 |   | N | 24 | 53 | 40 |
| 23 | Normalized infarct volume | Rho | -0.314 | 0.259 | 0.013 |
| 24 |   | Sig. (2-tailed) | 0.135 | 0.061 | 0.935 |
| 25 |   | N | 24 | 53 | 40 |
| 26 | Normalized edema volume | Rho | 0.442* | -0.191 | 0.047 |
| 27 |   | Sig. (2-tailed) | 0.030 | 0.171 | 0.774 |
| 28 |   | N | 24 | 53 | 40 |
| 29 | Post-surgical spleen weight (mg) | Rho | -0.418** | 0.019 | 0.281* |
| 30 |   | Sig. (2-tailed) | 0.009 | 0.862 | 0.031 |
| 31 |   | N | 38 | 83 | 59 |
| 32 | Spleen weight : pre-surgical bodyweight ratio | Rho | -0.372* | -0.090 | -0.245 |
| 33 |   | Sig. (2-tailed) | 0.024 | 0.428 | 0.064 |
| 34 |   | N | 37 | 80 | 58 |
| 35 | Lung infection (CFU/mg) | Rho | -0.557* | 0.169 | 0.208 |
| 36 |   | Sig. (2-tailed) | 0.031 | 0.197 | 0.111 |
| 37 |   | N | 15 | 60 | 60 |
| 38 | Distanced travelled (m) | Rho | 0.308 | -0.518** | -0.212 |
| 39 |   | Sig. (2-tailed) | 0.264 | 0.000 | 0.114 |
| 40 |   | N | 15 | 57 | 57 |
| 41 | Hanging grip time (s) | Rho | 1 | -0.498** | -0.277 |
| 42 |   | Sig. (2-tailed) | 0.000 | 0.180 |
| 43 |   | N | 99 | 97 | 25 |
| 44 | Clinical score | Rho | -0.498** | 1 | 0.192 |
| 45 |   | Sig. (2-tailed) | 0.000 | 0.112 |
| 46 |   | N | 97 | 183 | 70 |
| 47 | Post-surgical bodyweight (g) | Rho | -0.277 | 0.192 | 1 |
| 48 |   | Sig. (2-tailed) | 0.180 | 0.112 |

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### Permanent MCAO

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7.6 Co-authored publications published during PhD candidature


Full-length Article

Treatment with an interleukin-1 receptor antagonist mitigates neuroinflammation and brain damage after polytrauma

Mujun Sun a, Rhys D. Brady a,b, David K. Wright c,d,f, Hyun Ah Kim b, Shenpeng R. Zhang b,e, Christopher G. Sobe y b, Maddison R. Johnstone b, Terence J. O’Brien a,f, Bridgette D. Semple a,f, Stuart J. McDonald b,1, Sandy R. Shultz a,f,1,⇑

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Keywords:
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Fracture
Multitrauma
IL-1β
Neuroinflammation
MRI

A B S T R A C T

Traumatic brain injury (TBI) and long bone fracture are common in polytrauma. This injury combination in mice results in elevated levels of the pro-inflammatory cytokine interleukin-1β (IL-1β) and exacerbated neuropathology when compared to isolated-TBI. Here we examined the effect of treatment with an IL-1 receptor antagonist (IL-1ra) in mice given a TBI and a concomitant tibial fracture (i.e., polytrauma). Adult male C57BL/6 mice were given sham-injuries or polytrauma and treated with saline-vehicle or IL-1ra (100 mg/kg). Treatments were subcutaneously injected at 1, 6, and 24 h, and then once daily for one week post-injury. 7–8 mice/group were euthanized at 48 h post-injury. 12–16 mice/group underwent behavioral testing at 12 weeks post-injury and MRI at 14 weeks post-injury before being euthanized at 16 weeks post-injury. At 48 h post-injury, markers for activated microglia and astrocytes, as well as neutrophils and edema, were decreased in polytrauma mice treated with IL-1ra compared to polytrauma mice treated with vehicle. At 14 weeks post-injury, MRI analysis demonstrated that IL-1ra treatment after polytrauma reduced volumetric loss in the injured cortex and mitigated track-weighted MRI markers for axonal injury. As IL-1ra (Anakinra) is approved for human use, it may represent a promising therapy in polytrauma cases involving TBI and fracture.

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1. Introduction

Polytrauma involves significant injury to at least two body regions and is a common consequence of motor vehicle accidents, slips and falls, industrial accidents, and war (McDonald et al., 2016). The combination of traumatic brain injury (TBI) plus extracranial injury (ECI) is a common form of polytrauma, and cases involving significant TBI result in the highest risk of mortality amongst polytrauma patients (Pape et al., 2010; McDonald et al., 2016).

TBI is a leading cause of death and disability worldwide (Blennow et al., 2012). TBI involves a heterogeneous, complex and evolving pathophysiology, including primary and secondary injury mechanisms (Blennow et al., 2012). Primary injury involves direct tissue damage by mechanical forces at the moment of impact, and may include blood-brain barrier (BBB) disruption, necrosis and axonal shearing (Blennow et al., 2012). The primary injury also initiates secondary injury processes including neuroinflammation, cerebral edema, oxidative stress, apoptosis and further BBB breakdown (Blennow et al., 2012). Secondary injury processes can occur within minutes to hours after TBI, and may persist into chronic stages and contribute to neurodegeneration (Blennow et al., 2012; Shultz et al., 2015b).

BBB disruption in TBI provides unprecedented access for peripheral factors to enter the brain, and the migration of peripheral factors into the injured brain can impact neuropathology (Hang et al., 2004; Utagawa et al., 2008). Because ECI (e.g., long bone fracture) results in robust biological responses (e.g., systemic inflammation), the potential for peripheral involvement in the...
pathobiology of TBI may be substantially greater in polytrauma patients than for those with isolated-TBI (McDonald et al., 2016). For example, there is some evidence that a concomitant ECI can negatively impact functional outcomes in TBI patients (van Leeuwen et al., 2012; Leitgeb et al., 2013; Lingsma et al., 2013). Furthermore, our group recently reported that mice given a concomitant TBI and tibial fracture had increased neuroinflammation, BBB disruption, edema, brain damage, and behavioral deficits compared to mice given an isolated-TBI. Notably, these changes occurred in the presence of increased interleukin-1β (IL-1β) levels in the brain (Shultz et al., 2015a).

IL-1β is a key pro-inflammatory cytokine both centrally and peripherally (Allan et al., 2005; Webster et al., 2017). IL-1β production is highly upregulated post-TBI, stimulates astrocytes, microglia and neurons, and modulates neuroinflammation (Ciallella et al., 2002; Allan et al., 2005; Semple et al., 2010). IL-1β is also implicated in other injury mechanisms occurring in TBI, such as edema, neuronal hyperexcitability and cell death (Allan et al., 2005; Lazovic et al., 2005). The type 1 IL-1 receptor (IL-1R) mediates the biological functions of IL-1β, and the endogenous IL-1 receptor antagonist (IL-1ra) competes with IL-1β for IL-1R binding (Allan et al., 2005). Previous rodent studies demonstrate improved outcomes from experimental brain injury after treatment with recombinant IL-1ra, in mice over-expressing IL-1ra, and in mice lacking the IL-1R (Sanderson et al., 1999; Tehranian et al., 2002; Lazovic et al., 2005). Because our previous findings implicate IL-1β as an important pathophysiological factor involved in the interactive effects of TBI and ECI, here we assessed the therapeutic effects of a clinically approved IL-1ra, Anakinra, in a polytrauma model featuring closed-skull TBI and concomitant tibial fracture.

2. Materials and methods

2.1. Mice

C57BL/6 male mice (n = 96) were obtained from the Australia Animal Research Centre, and housed individually under a 12-h light/dark cycle with access to food and water ad libitum. Mice were 12 weeks of age at the time of injury. All procedures were comply with the ARRIVE guidelines, were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee, and were within the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes by the Australian National Health and Medical Research Council.

2.2. Experimental groups

Mice were randomly assigned to one of four experimental groups: sham-injuries + vehicle treatment (SHAM+VEH); sham-injuries + IL-1ra treatment (SHAM+IL-1ra); TBI + fracture + vehicle treatment (POLY+VEH); and TBI + fracture + IL-1ra treatment (POLY+IL-1ra). Mice that died immediately after polytrauma (n = 9, 15.8% mortality rate) were excluded from the study. A subset of mice were euthanized at 48 h post-injury, and the remaining mice were euthanized at 16 weeks post-injury after behavioral testing and in vivo MRI. The number of mice/group for the 48-h recovery experiments was: SHAM+VEH, n = 8; SHAM+IL-1ra, n = 7; POLY+VEH, n = 8; POLY+IL-1ra, n = 8. The number of mice/group for the long-recovery experiments was: SHAM+VEH, n = 12; SHAM+IL-1ra, n = 12; POLY+VEH, n = 16; POLY+IL-1ra, n = 16.

2.3. Polytrauma

The polytrauma model featuring a concomitant closed-skull TBI and tibial fracture was performed as previously described (Flierl et al., 2009; Shultz et al., 2015a; Brady et al., 2016). Each mouse was placed in an anesthesia induction chamber containing 4% isoflurane for 2 min. Once anesthetized, mice were placed in a nose cone that maintained the anesthetic (2% isoflurane). All mice received 0.05 mg/kg of buprenorphine analgesic subcutaneously. To induce a tibial fracture, an incision was made inferior of the right knee of the mouse. An entry point into the medullary canal of the tibia was made using a 26 G needle, and an intramedullary rod (000 stainless steel insect pin, 0.25 mm diameter) was inserted inside the medullary canal. A fracture was generated in the tibial midshaft, and visualised via X-ray (DEXCO DX-3000L; DEXCOWIN, Korea) to confirm a transverse, non-comminuted fracture. The initial intramedullary rod was removed and a new rod (00 stainless steel insect pin, 0.3 mm diameter) was inserted that remained in situ for the remainder of the study. Sham-injury for the fracture procedure consisted of the same procedures, except no fracture was inflicted. After a total surgery/anesthesia duration of 10 min (i.e., immediately following the tibial fracture), a weight-drop device was used to induce a closed-skull TBI. The weight-drop device consisted of a guided- and weighted-rod with a blunt silicone-covered impact tip (3 mm diameter). The mouse was removed from the nose cone and an incision was made along the midline of the scalp to reveal the skull surface. The mouse was then stabilized on the weight-drop device platform, the weighted-rod was released from a distance of 2.5 cm, and the impact tip made contact between the sagittal and coronal suture of the right hemisphere (i.e., directly above the primary somatosensory cortex; 1.5 mm posterior, and 1.5 mm lateral relative to Bregma; Franklin & Paxinos, 2007). The rod was retracted immediately after the impact occurred, and the scalp incision was sutured. The sham TBI procedure was identical to that described for the TBI procedure, except the weighted-rod was not released. Duration of apnea, loss of consciousness (i.e., hind-limb withdrawal to toe pinch), and self-righting reflex were recorded as indicators of acute injury severity (Table 1) (Shultz et al., 2014, 2015b).

2.4. IL-1ra treatment

Mice were injected subcutaneously with recombinant human IL-1ra (100 mg/kg, Anakinra, Kineret<sup>®</sup>, Swedish Orphan Biovitrum, Sweden) or saline-vehicle at 1, 6, and 24 h post-injury, and then once daily for one week post-injury. This dose and treatment length is similar to previous experimental brain injury studies that have found neuroprotective effects with IL-1ra treatment (Sanderson et al., 1999; Greenhalgh et al., 2010). Furthermore, the initial clinical trial with Anakinra in TBI patients (Helmy et al., 2014) used the same dose and administration method, and a similar treatment length (i.e., 5 days). Mice that were euthanized at 48 h post-injury received their last treatment 2 h before euthanization.

2.5. Quantitative real-time PCR (RT-qPCR)

At either 48 h or 16 weeks post-injury (i.e., after behavioral testing and MRI), mice (6-8/group/recovery time) were briefly anesthetized with isoflurane and decapitated. The ipsilateral somatosensory cortex directly under the impact site was rapidly dissected, frozen in liquid nitrogen, and stored at ~80 °C for RT-qPCR. Frozen tissue was homogenised with PureZOL<sup>®</sup> RNA isolation reagent, and total RNA were extracted using the Auralum<sup>®</sup> Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, USA) (McDonald et al., 2011). 600 ng of RNA for each sample was then reverse transcribed to cDNA. Primers were made commercially by GeneWorks Pty Ltd (Adelaide, SA, Australia). The primer sequences for the genes of interest are summarized in Table 2, and included markers for microglia/macrophages and reactive
Table 1
Acute injury measures. The POLY groups had significantly longer apnea, unconsciousness (i.e., hindlimb withdrawal reflex to toe pinch), and self-righting reflex times (seconds ± SEM) than the SHAM groups. There were no statistically significant differences between the two POLY groups. * = POLY groups different than SHAM groups, p < 0.01.

<table>
<thead>
<tr>
<th></th>
<th>SHAM + VEH</th>
<th>SHAM + IL-1ra</th>
<th>POLY + VEH</th>
<th>POLY + IL-1ra</th>
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<tr>
<td>Apnea (sec)</td>
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<td>22.21 ± 2.66</td>
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<td>Hindlimb (sec)</td>
<td>30.95 ± 3.92</td>
<td>31.74 ± 2.75</td>
<td>156.13 ± 14.04</td>
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<td>Self-righting (sec)</td>
<td>41.80 ± 3.71</td>
<td>42.90 ± 3.23</td>
<td>225.54 ± 24.46</td>
<td>224.13 ± 24.50*</td>
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</table>

Table 2
RT-qPCR markers. Genes of interest included the pan-macrophage marker ionized calcium binding adaptor molecule 1 (Iba1); the pro-inflammatory microglia/macrophage (i.e., M1) markers CD16, CD32, CCL2, CCL4, CCL7, CCL12, and IL-1β; the anti-inflammatory microglia/macrophage (i.e., M2) markers IL-4 Rα, CD36, dual specificity protein phosphatase 1 (DUSP1), and transforming growth factor-β1 (TGF-β1); and the reactive astrocyte markers glial fibrillary acidic protein (GFAP), lipocalin-2 (Lcn2), and vimentin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence 5’–3’</th>
<th>Reverse primer sequence 5’–3’</th>
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<tr>
<td><strong>Microglia/Macrophage Markers</strong></td>
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<tr>
<td>Iba1</td>
<td>GAGATTCGACGGAGAAGAAA</td>
<td>TGGGATCATCAGGAGATTTG</td>
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<td><strong>Pro-inflammatory</strong></td>
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<td>CD32</td>
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<td>CCL4</td>
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<td>CCL12</td>
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<td>Vimentin</td>
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<td>CAGCTCGGAGACTGTCTTA</td>
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astrocytes (Ekmark-Lewén et al., 2010; Jang et al., 2013; Morganti et al., 2016; Sandhir et al., 2008; Wang et al., 2013). qPCR was run using the iCycler iQ Multi-Colour RT-PCR detection system (using SsoFast™ EvaGreen®, Bio-Rad, Hercules, USA). To establish specificity of DNA products, melt-curve analysis was performed. Cycle threshold (Ct) values were collected for analysis, and data was normalised to internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward, 5’–CATACCAGGAAATGAGCTTG–3’; reverse, 5’–CATACAGGAAAATGAGCTTG–3’). Relative quantification of genes of interest mRNA expression was determined using the 2−ΔΔCt method (McDonald et al., 2011).

2.6. Immunohistochemistry

To identify neutrophils in the injured cortex, myeloperoxidase (MPO)-positive cells were counted in the same mice described for RT-qPCR (5–6 mice/group/recovery time; Brair et al., 2011). Frozen brain samples were sectioned into serial coronal brain sections (10 μm), thaw-mounted onto poly-L-lysine coated slides, and fixed in 4% paraformaldehyde for 15 min. Sections were then washed in 0.01 M PBS (3 × 5 min) and blocked with peroxidase (Dako, CA, USA) for 10 min. Sections were then washed in 0.01 M PBS (3 × 5 min) and then blocked with 10% goat serum for 30 min. Sections were then incubated with a polyclonal goat anti-MPO antibody (1:100, Abcam, Cambridge, UK) overnight in a humidified chamber at 4°C. The following day, sections were washed in 0.01 M PBS (3 × 5 min) and incubated in peroxidase-labelled polymer conjugated goat anti-rabbit immunoglobulin (Dako, CA, USA) for 2 h. Brain sections were then washed in 0.01 M PBS (3 × 5 min) and incubated with diaminobenzidine solution (Dako, CA, USA) for 5 min. After washing in distilled water, sections were dehydrated in the order of 70% ethanol, 100% ethanol and xylene. Sections were cover slipped using DPX mounting media and MPO-positive cells were counted using an Olympus microscope, and normalized to the area of tissue analyzed.

2.7. Cerebral edema analysis

Brain water content was assessed as an indicator of cerebral edema in the same mice that were used for RT-qPCR (6–8/group; Flierl et al., 2009). The cortex immediately adjacent (i.e., anterior) to the impact site, as well as the equivalent cortex from the contralateral hemisphere, were rapidly dissected, immediately weighed (wet weight), and then dried at 100 °C for 24 h. The tissue was again weighed (dry weight), and the following formula was used to determine brain water content: water content (%) = (wet weight − dry weight)/wet weight.

2.8. Behavioral testing

Mice underwent behavioral testing beginning at 12-weeks post-injury. With the exception of the rotarod, all tests were recorded by an overhead camera and objectively analyzed by Ethovision tracking software (EthoVision®, Noldus, USA). Testing was conducted by an investigator blinded to experimental conditions. Open field was used to assess locomotor and anxiety-like behavior (Shultz et al., 2012, 2014). The open field was a circular arena (100 cm diameter) shielded by 30 cm high walls. A circular area (66 cm diameter) in the arena was defined as the middle field. The mouse was released in the center and allowed to explore for 5 min. Distance travelled, time in the middle field, and middle field entries were calculated.
Anxiety-like behavior was assessed using the elevated-plus maze (San Diego Instruments, USA) (Shultz et al., 2012). The maze consisted of 2 opposing opened arms and 2 opposing closed arms (30 cm × 6 cm) shaped like a “plus”. The closed arms were shielded by walls (15 cm high), while the opened arms were not. Each mouse was placed in the center of the maze facing an open arm and allowed to explore for 5 min. The proportion of time spent in the open arms and total distance travelled were calculated.

Motor function was assessed by the rotarod (Harvard Apparatus, Holliston, USA) (Shultz et al., 2014). The apparatus consisted of a rotating barrel (3 cm diameter) divided by walls into four equal lanes (5 cm width). Three trials were performed each day for two consecutive days. For each trial, the mouse was placed on the rotating barrel with a start speed of 0.0027 g, and the speed accelerated to 0.27 g over a 5-min period (i.e. 4 to 40 r.p.m.). The duration of time on the rotarod was recorded.

The water maze was used to assess spatial cognition (Shultz et al., 2011; Minter et al., 2016). The apparatus consisted of a circular pool (1.63 m diameter) filled with opaque water (21–23 °C). An escape platform was hidden 0.5 cm below the water surface in a quadrant of the pool and this location was maintained throughout testing. Visible cues were fixed around the perimeter of pool. Mice completed 4 trials/day for 5 days with a maximum trial duration of 60 s. Each trial started as the mouse was released at one of four randomized points, and ended when the mouse located the platform, or 60 s had elapsed in which case the experimenter would guide the mouse to the platform. Search time required for each trial was calculated.

The three-chamber task was used to assess social interest (Nadler et al., 2004; Semple et al., 2017). The apparatus (39 cm × 42 cm) consisted of three chambers, and the test mouse was first given 10 min to habituate in the empty apparatus. Immediately following habituation, a 10 min test involved the test mouse having a choice between spending time in the central (neutral) chamber or two adjacent chambers, one containing a wire cup-enclosed stimulus mouse (age, strain and sex-matched) and the other containing an empty cup. A mouse exhibiting normal sociability favors proximity of the stimulus mouse in preference over the empty cup.

2.9. MRI acquisition

Following behavioral testing (i.e., 14 weeks post-injury), mice underwent in vivo MRI scanning under isoflurane anesthetic (2%) (Shultz et al., 2015a). MRI was performed using a 4.7 Tesla Bruker Avance III scanner fitted with a BGA1252 actively shielded gradient set and cryogenically cooled surface coil (Bruker Biospec, Ettlingen, Germany). A three-plane localization sequence followed by multi-slice scout images in coronal, axial and sagittal planes were completed to establish the correct position of the mouse brain. A T2-weighted image was obtained in the axial plane using a 2D rapid acquisition with relaxation enhancement (RARE) sequence with the following imaging parameters: repetition time (TR) = 12,000 ms; effective echo time (TEeff) = 40 ms; RARE factor = 8; field of view (FOV) = 19.2 × 19.2 mm²; matrix size = 160 × 160; spatial resolution = 120 × 120 μm²; number of slices = 64; slice thickness = 120 μm; and number of excitations = 4. A diffusion-weighted image was also acquired in the axial plane using an echo planar imaging sequence with the following imaging parameters: repetition time (TR) = 7,500 ms; TE = 37 ms; shots = 2; FOV = 24.0 × 16.0 mm²; matrix size = 96 × 64; spatial resolution = 250 × 250 μm²; number of slices = 30; and slice thickness = 250 μm. Diffusion weighting was performed with diffusion duration = 6 ms, diffusion gradient separation = 15 ms and b-value = 3,000 s/mm² in 126 non-collinear directions with 4 non-diffusion (b0) images.

2.10. MRI analyses

For volumetric analysis, regions of interest (ROI) including cortex, hippocampus and corpus callosum were outlined in each hemisphere on 15 consecutive coronal T2-weighted images using ITK-SNAP software (www.itksnap.org) by an experimenter who was blinded to experimental conditions (Shultz et al., 2014, 2015a). The first image outlined contained the most anterior portion of the hippocampus (i.e., approximately -9 mm from Bregma), with the consequent images posterior to this. Therefore, the ROIs encompassed the impact site of the weight-drop. Volumes were calculated using MATLAB (Mathworks, Natick, USA).

Diffusion-weighted imaging processing was performed using MRtrix (www.mrtrix.org) and DTI-TK (www.dti-tk.sourceforge.net) (Wright et al., 2016a,b). Spatial intensity inhomogeneity was corrected by estimating the bias field across the mean b0 image and images were normalized for white matter signal intensity. A study template image was constructed using DTI-TK and subject-to-template diffeomorphisms calculated for each mouse. The fibre orientation distribution (FOD) was estimated for each mouse brain using multi-tissue constrained spherical deconvolution with group average response function (Tournier et al., 2004). Whole brain tractograms of two million streamlines were generated using the iFOD2 tractography algorithm and warped into template space using the subject-to-template diffeomorphisms (Tournier et al., 2010). Three track-weighted images were generated: track density image (TDI), mean curvature, and the average pathlength map (APM). TDI, mean curvature, and APM were then analyzed using track-based spatial statistics (TBSS) (Smith et al., 2006). Fractional anisotropy (FA), trace (TR), radial diffusivity (RD) and axial diffusivity (AD) images were also calculated and warped into template space using the subject-to-template diffeomorphisms for analysis with TBSS. Permutation testing was performed using randomise, with 5,000 permutations fully corrected for family-wise error and with threshold-free cluster enhancement (Winkler et al., 2014).

2.11. Fracture biomechanics

The effect of IL-1ra treatment on the biomechanical properties of healing tibial fractures was assessed using a three-point bending test in the POLY groups at 16 weeks post-injury (Brady et al., 2014). The tibias were dissected, the intramedullary rod was removed, and the tibia was immersed in silicone oil, and stored at −20 °C. Samples were mounted on the three-point bending apparatus with the fulcrum directly overlying the fracture site. Each sample was loaded at a constant rate of 1.67 mm/s, with load and deflection data recorded continuously using transducers connected to an x–y plotter by preamplifiers. There were no significant findings on bone fracture site biomechanical properties including peak force to failure and stiffness (data not shown).

2.12. Statistical analysis

All outcomes, with the exception of TBSS testing (see “MRI analyses” section), were analyzed with SPSS 24.0 software (IBM Corp, Armonk, USA). Biomechanical fracture properties were analyzed using Welch’s t-test. Water maze, rotarod, and three-chamber social task was analyzed by mixed-design analysis of variance (ANOVA), with injury and treatment as between-subjects factors and day or chamber as within-subjects factor. All other outcomes were analyzed by two-way ANOVA, with injury and treatment as between-subjects factors. Bonferroni post hoc comparisons were carried out when appropriate. Statistical significance was set as p ≤ 0.05.
3. Results

3.1. Effect of IL-1ra treatment on markers for microglia/macrophages after polytrauma

There was a significant treatment-injury interaction on gene expression of the pan-macrophage marker Iba1 (F(1,26) = 6.012, p < 0.05) in the injured cortex at 48 h post-injury. Post hoc analyses indicated that the POLY+VEH mice had increased mRNA levels of Iba1 in the ipsilateral cortex compared to all other groups (p < 0.01, Fig. 1A), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There were also significant main effects for injury (F(1,26) = 27.373, p < 0.001) and treatment (F(1,26) = 8.740, p < 0.01) at 48 h post-injury.

There were significant treatment-injury interactions on gene expression of the pro-inflammatory (i.e., M1) microglial/macrophage markers CD16 (F(1,26) = 6.463, p < 0.05, Fig. 1B) and CD32 (F(1,26) = 4.997, p < 0.05, Fig. 1C), in the injured cortex at 48 h post-injury. Post hoc analyses indicated that the POLY+VEH mice had increased mRNA levels of CD16 and CD32 compared to all other groups (p < 0.01), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. At 48 h post-injury, there was also a significant main effect for injury on gene expression of the pro-inflammatory microglial/macrophage markers CCL2 (F(1,26) = 9.066, p < 0.01; Fig. 1D), CCL4 (F(1,26) = 11.412, p < 0.01; Fig. 1E), CCL12 (F(1,26) = 8.281, p < 0.01; Fig. 1F), CD16 (F(1,26) = 26.978, p < 0.001), and CD32 (F(1,26) = 18.579, p < 0.001). There was also a significant main effect for treatment on gene expression of the pro-inflammatory microglia/macrophage markers IL-1β (F(1,26) = 4.774, p < 0.05; Fig. 1H), CD16 (F(1,26) = 7.762, p < 0.05), and CD32 (F(1,26) = 6.534, p < 0.05) in the injured cortex at 48 h post-injury.

There was a significant treatment-injury interaction on gene expression of the anti-inflammatory (i.e., M2) microglial/macrophage marker IL-4 Rα (F(1,26) = 5.589, p < 0.05, Fig. 2A). Post hoc analyses indicated that the POLY+VEH mice had increased mRNA levels of IL-4 Rα compared to all other groups (p < 0.01), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There were also significant main effects for injury (F(1,26) = 8.398, p < 0.01) and treatment (F(1,26) = 4.462, p < 0.05) on IL-4 Rα gene expression levels at 48 h post-injury. There was also a significant main effect for injury on gene expression of the anti-inflammatory microglial/macrophage markers CCL2 (F(1,26) = 7.857, p < 0.01; Fig. 2B), DUSP1 (F(1,26) = 5.574, p < 0.05; Fig. 2C), and TGF-β1 (F(1,26) = 16.310, p < 0.001; Fig. 2D) in the injured cortex at 48 h post-injury. There were no significant findings at 16 weeks post-injury.

3.2. Effect of IL-1ra treatment on markers for astrocyte activation after polytrauma

There was a significant treatment-injury interaction on gene expression of the activated astrocyte marker Lcn2 (F(1,26) = 4.397, p < 0.05) in the injured cortex at 48 h post-injury. Post hoc analyses indicated that the POLY+VEH mice had significantly increased mRNA levels of Lcn2 in the ipsilateral cortex compared to all other groups (p < 0.01, Fig. 3A), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There were also significant main effects for injury (F(1,26) = 10.090, p < 0.01) and treatment (F(1,26) = 4.834, p < 0.05) on Lcn2 at 48 h post-injury.

A significant main effect for injury on mRNA levels of GFAP (F(1,26) = 16.149, p < 0.001, Fig. 3B) and vimentin (F(1,26) = 10.108, p < 0.01, Fig. 3C) in the injured cortex was also found at 48 h post-injury, with POLY mice having increased GFAP and vimentin. There were no significant findings at 16 weeks post-injury.

3.3. IL-1ra treatment reduced neutrophils in the injured cortex after polytrauma

There was a significant treatment-injury interaction on the number of MPO-positive neutrophils/mm² (F(1,18) = 4.473, p < 0.05) in the injured cortex at 48 h post-injury. Post hoc analyses indicated that the POLY+VEH mice had significantly increased MPO-positive neutrophils/mm² compared to all other groups (p < 0.01, Fig. 4E), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There were no significant findings at 16 weeks post-injury.

3.4. IL-1ra treatment reduced cerebral edema after polytrauma

There was a significant treatment-injury interaction (F(1,27) = 5.593, p < 0.05) on the measure of brain water content in the ipsilateral cortex at 48 h post-injury. Post hoc analyses indicated that the POLY+VEH mice had significantly increased brain water content in the injured cortex compared to all other groups (p < 0.01, Fig. 5A), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There were also significant main effects for injury (F(1,27) = 13.989, p < 0.01) and treatment (F(1,27) = 5.747, p < 0.05) on brain water content at 48 h post-injury. There were no significant findings at 16 weeks post-injury.

3.5. There was no effect of injury or treatment on behavioral measures

There were no statistically significant findings between the experimental groups on any of the behavioral measures (Fig. 6). However, all mice spent longer on the rotarod as testing progressed (F(1,52) = 59.226, p < 0.001, Fig. 6B), preferred the stimulus mouse chamber over the empty chamber during the three-chamber social task (F(1,52) = 87.729, p < 0.001, Fig. 6C), and had shorter search latencies in the water maze as testing progressed (F(2,208) = 44.590, p < 0.001, Fig. 6D).

3.6. IL-1ra treatment reduced brain atrophy after polytrauma

MRI was used to assess ROI volumes at 14 weeks post-injury (Fig. 7A). There was a significant treatment-injury interaction (F(1,52) = 9.545, p < 0.01) on ipsilateral cortex volume. Post hoc analyses indicated that the POLY+VEH group had significantly reduced volume of ipsilateral cortex compared to all other groups (p < 0.05, Fig. 7B), whereas the POLY+IL-1ra mice did not significantly differ from SHAM groups. There was also a significant main effect for injury (F(1,52) = 17.226, p < 0.001) on ipsilateral cortex volume.

There was also a significant treatment-injury interaction (F(1,52) = 4.811, p < 0.05) on ipsilateral hippocampus volume. Post hoc analyses indicated that POLY+VEH mice had significantly reduced volume of ipsilateral hippocampus compared to SHAM+VEH (p < 0.01, Fig. 7D), whereas the POLY+IL-1ra mice did not significantly differ from SHAM+VEH. There was also a significant main effect for injury (F(1,52) = 6.384, p < 0.05) on ipsilateral hippocampus volume.

A significant main effect for injury was also found on ipsilateral (F(1,52) = 19.269, p < 0.001, Fig. 7F) and contralateral (F(1,52) = 4.739, p < 0.05, Fig. 7G) corpus callosum volumes, with POLY mice having significantly less volume compared to SHAM mice.

3.7. IL-1ra treatment mitigated white matter damage after polytrauma

TBSS analyses of track-weighted imaging and DTI measures assessed markers of white matter integrity at 14 weeks post-injury. When compared to POLY+VEH mice, POLY+IL-1ra mice had significantly increased APM in bilateral corpus callosum, fimbria, and fornix.
IL-1ra treatment reduced gene expression of pro-inflammatory microglia/macrophage markers after polytrauma. At 48 h post-injury, POLY + VEH mice had significantly increased mRNA levels of the pan-macrophage marker Iba1 (A), as well as the pro-inflammatory microglial/macrophage markers CD16 (B) and CD32 (C), in the ipsilateral cortex compared to all other groups. The POLY mice also had significantly increased mRNA levels of the pro-inflammatory microglial/macrophage markers CCL2 (D), CCL4 (E), CCL7 (F), and CCL12 (G) in the ipsilateral cortex at 48 h post-injury compared to SHAM mice. The IL-1ra treated mice had significantly decreased mRNA levels of the pro-inflammatory marker IL-1β (H) in the ipsilateral cortex at 48 h post-injury compared to VEH-treated mice. No statistically significant findings were found at 16 weeks post-injury.*** = different than all other groups, * = different than SHAM, p < 0.05. # = different than VEH-treated, p < 0.05. Results are relative to SHAM + VEH for each time-point. Error bars indicate SEM.

Fig. 1. IL-1ra treatment reduced gene expression of pro-inflammatory microglia/macrophage markers after polytrauma. At 48 h post-injury, POLY + VEH mice had significantly increased mRNA levels of the pan-macrophage marker Iba1 (A), as well as the pro-inflammatory microglial/macrophage markers CD16 (B) and CD32 (C), in the ipsilateral cortex compared to all other groups. The POLY mice also had significantly increased mRNA levels of the pro-inflammatory microglial/macrophage markers CCL2 (D), CCL4 (E), CCL7 (F), and CCL12 (G) in the ipsilateral cortex at 48 h post-injury compared to SHAM mice. The IL-1ra treated mice had significantly decreased mRNA levels of the pro-inflammatory marker IL-1β (H) in the ipsilateral cortex at 48 h post-injury compared to VEH-treated mice. No statistically significant findings were found at 16 weeks post-injury.*** = different than all other groups, * = different than SHAM, p < 0.05. # = different than VEH-treated, p < 0.05. Results are relative to SHAM + VEH for each time-point. Error bars indicate SEM.
bria, internal capsule, optic track and cerebral peduncle, and ipsilateral external capsule (Fig. 8A); significantly increased curvature in ipsilateral corpus callosum, internal capsule, external capsule, optic track and cerebral peduncle (Fig. 8B); and significantly increased TDI in ipsilateral internal capsule and cerebral peduncle, and contralateral optic track (Fig. 8C). These findings suggested increased white matter integrity in the POLY+IL-1ra mice.

4. Discussion

TBI and fracture are common components of polytrauma (Grosswasser et al., 1990), and clinical studies have reported that ~40% of TBI patients experienced ECI (Siegel et al., 1991; Leitgeb et al., 2013). We recently demonstrated that this injury combination in mice results in elevated IL-1β levels and exacerbated neu-
Ropathology when compared to isolated-TBI (Shultz et al., 2015a). Therefore, here we examined the effect of treatment with the IL-1ra, Anakinra, in mice given a TBI and tibial fracture. We found that POLY+IL-1ra mice had reduced expression of markers associated with pro- (i.e., M1) and anti-inflammatory (i.e., M2) microglia/macrophages, as well as astrogliosis at 48 h post-injury compared to POLY+VEH mice. There was also a significant reduction in the number of MPO-positive neutrophils and cerebral edema in the injured cortex of POLY+IL-1ra mice compared to POLY+VEH mice at 48 h after polytrauma. At 14 weeks post-injury, in vivo MRI indicated that IL-1ra treatment after polytrauma significantly reduced volumetric loss in the injured cortex and mitigated track-weighted MRI markers for axonal injury. We also tested the biomechanical properties of the healing tibial fractures and found that they were unaffected by IL-1ra at 16 weeks post-injury. Together these findings suggest that IL-1β/IL-1R signalling may be an important modulator of neuropathology in polytrauma involving TBI and fracture, and that treatment with IL-1ra may be a safe and effective treatment to improve neurological outcomes in individuals who suffer this injury combination.

4.1. IL-1β/IL-1R signalling in the neuropathology of polytrauma

IL-1β is a major pro-inflammatory cytokine that can be produced by neurons, microglia, astrocytes, endothelial cells, and peripheral leukocytes, is rapidly and highly upregulated after both TBI and fracture, and can signal/activate a number of cells (e.g., microglia, astrocytes, leukocytes) via the IL-1R to promote neuroinflammation (Allan et al., 2005; Webster et al., 2017). We have previously shown that brain levels of IL-1β after a TBI are exacerbated by a concomitant fracture, and that this is associated with elevated neuroinflammation and worsened outcomes relative to an isolated-TBI. Neuroinflammation is a common secondary injury mechanism in TBI that has been associated with other pathophysiological mechanisms (e.g., edema, BBB breakdown, and apoptosis).
and chronic neurological consequences including neurodegenerative disease (Kumar and Loane 2012; Hellewell et al., 2016). Neutrophil infiltration, microglia/macrophage activation, and astrogliosis are all hallmarks of the neuroinflammatory response after TBI, and each can produce molecules that further perpetuate neuroinflammation and contribute to neurotoxic effects (Kumar
and Loane, 2012). Consistent with our previous findings, here we found evidence of a robust neuroinflammatory response after polytrauma, as indicated by an increased number of neutrophils, and elevated gene expression of markers for microglia and astrocyte activation at 48 h post-injury. Supporting a central role for IL-1β in this process, we found that treatment with IL-1ra after polytrauma significantly attenuated many of these effects.

A particularly interesting finding relates to the markers used to assess microglia activation/phenotype. Here we found that polytrauma increased the gene expression of markers that have previously been associated with M1 (i.e., pro-inflammatory) and M2 (i.e., anti-inflammatory) microglia/macrophage activation (Ekmark-Lewén et al., 2010; Jang et al., 2013; Sandhir et al., 2008; Wang et al., 2013), and that IL-1ra treatment after poly-

Fig. 7. Track-weighted imaging analyses. TBSS analyses of the track-weighted imaging measures APM (A), mean curvature (B), and TDI (C) were completed to assess markers of white matter integrity at 14 weeks post-injury. When compared to POLY + VEH mice, POLY + IL-1ra mice had significantly increased APM in bilateral corpus callosum, fimbria, internal capsule, optic tract and cerebral peduncle, and ipsilateral external capsule (A, red-yellow voxels = $p < 0.05$); significantly increased curvature in ipsilateral corpus callosum, internal capsule, external capsule, optic tract and cerebral peduncle (B, blue voxels = $p < 0.05$); and significantly increased TDI in ipsilateral internal capsule and cerebral peduncle, and contralateral optic track (C, green voxels = $p < 0.05$). These findings suggested increased white matter integrity in the POLY + IL-1ra mice.

Fig. 8. Behavioral testing. At 12 weeks post-injury there were no statistically significant differences between any of the experimental groups found on the time in the middle area of the open field (A), the time spent on the rotarod (B), the time spent in the stimulus mouse chamber (black) relative to the empty chamber (white) in the three-chamber social task (C), the search time required to locate the platform during water maze testing across consecutive testing days (D). For B and D: Black line = SHAM; Blue line = POLY; Solid line = VEH; Dotted line = IL-1ra. Error bars indicate SEM.
trauma mitigated this effect. Although this finding appears somewhat counterintuitive (i.e., polytrauma increased anti-inflammatory markers and an anti-inflammatory IL-1ra treatment decreased anti-inflammatory markers), it is important to note that M1 and M2 polarization are concurrent following TBI; that there are temporal complexities involved in this process; that the classification of microglia as M1/pro-inflammatory versus M2/anti-inflammatory is now recognized to be oversimplified and has shifted to a spectrum-like profile; and that future research is still required to characterize and understand the various roles of different inflammatory factors throughout the inflammatory process (Morganti et al., 2016; Ransohoff, 2016).

Cerebral edema is another common acute event after TBI that contributes to neuropathology and poor outcomes, and has been linked to IL-1R-mediated neuroinflammation (Lazovic et al., 2005). Consistent with our previous studies, here we found that polytrauma induced significant cerebral edema at 48 h post-injury. While the precise mechanisms linking IL-1β and edema formation remain to be elucidated, our finding that IL-1ra treatment significantly reduced edema after polytrauma aligns with other studies reporting that the modulation of IL-1R signalling can attenuate cerebral edema after brain injury (Yang et al., 1997; Masada et al., 2001; Lazovic et al., 2005).

In vivo MRI is a clinically relevant method that allows for the assessment of brain injury. Brain atrophy is common after TBI, including mild to moderate TBI (MacKenzie et al., 2002). At 14 weeks post-injury an ROI-based analysis of structural T2-weighted MRI revealed that the POLY+VEH group had less ipsilateral cortex volume compared to all other groups, and less ipsilateral hippocampus volume relative to the SHAM+VEH group. Importantly, the volumes of the ipsilateral cortex and hippocampus of POLY+IL-1ra mice did not differ from sham-injured mice. Because axonal injury is common in TBI, we further assessed this with diffusion MRI markers. Track-weighted imaging analysis is a relatively new diffusion MRI that provides a number of measures (Wright et al., 2016b, 2017). APM represents the average length of tracks passing through each voxel, curvature map represents the average curvature of all tracks passing a voxel, and TDI represents the density of tracks passing through each voxel. Decreases in these measures, as seen in POLY+VEH mice, are indicators of axonal injury. In contrast, POLY+IL-1ra mice had increased track-weighted measure values in white matters areas such as corpus callosum, fimbria, internal capsule, external capsule, optic tract, and cerebral peduncle, when compared to POLY+VEH mice, suggesting that IL-1ra treatment mitigated axonal injury after polytrauma.

As neuroinflammation and edema are important contributors to neuropathology after TBI, it is possible that the attenuation of these mechanisms by IL-1ra treatment contributed to the improved long-term neuropathological outcomes in the POLY+IL-1ra mice in this study. However, it is also possible that IL-1ra exerted protection via other pathways related to IL-1β/IL-1 signalling, such as cell death, BBB breakdown, and neuronal hyperexcitability (Allan et al., 2005), and future studies should explore this. While IL-1ra mitigated much of the inflammation after polytrauma, there was still some evidence for increased gene expression of markers for activated microglia and astrocyte in the POLY+IL-1ra group, and this might have contributed to loss of corpus callosum volume in these mice. Alternatively atrophy of the corpus callosum in the POLY+IL-1ra mice might be accounted for by irreversible damage at the moment of impact, or other secondary injury mechanisms involved in TBI.

There are some limitations that should be considered when interpreting the findings. The acute post-mortem studies involved a single recovery time (i.e., 48 h post-injury). 48 h was chosen in an attempt to efficiently assess a number of different inflammatory/pathological processes at a single acute post-injury time. However, because there are temporal complexities with these processes it is likely that our study did not capture each of the peak activation periods (Webster et al., 2017), and future post-mortem studies should include additional post-injury times. Another limitation is that some of the RT-qPCR markers associated with different microglia/macrophage phenotypes and reactive astrocytes are non-specific. For example, although Lcn2 is expressed by astrocytes and is a mediator of astrogliosis (Jang et al., 2013), it is also expressed by endothelial cells and neutrophils under inflammatory conditions (Jin et al., 2014). Therefore, considering that neutrophils were also found to be affected by injury and treatment in a manner similar to Lcn2, it is possible that both neutrophils and astrocytes contributed to the Lcn2 changes observed. Therefore, conclusions made regarding Lcn2 and other non-specific markers must be made with caution, and future studies are required to determine the exact cellular sources of these markers as well as IL-1β. The current study also found no differences between the groups at 12 weeks post-injury on behavioral measures. This may be due to a number of factors including the sensitivity of the behavioral measures, the recovery time tested, and/or the relatively mild nature of the TBI administered in this polytrauma model (Shultz et al., 2011; Wright et al., 2016b). More specifically, the behavioral assessment we used may have lacked sensitivity to detect subtler functional deficits, or failed to measure an outcome that was affected by the injury (e.g., post-traumatic epilepsy; Diamond et al., 2015; Liu et al., 2016). Furthermore, the neurological consequences observed in the POLY+VEH mice were mild in comparison to those produced by more severe models (e.g., fluid percussion injury or controlled cortical impact; Shultz et al., 2015b; Semple et al., 2017), thus IL-1ra treatment may have functional benefits had a more severe model with clear behavioral deficits been utilized. In regards to the timing of behavioral testing, previous studies have demonstrated that neuroinflammation and associated degeneration can occur in the chronic post-TBI period (Ramlackhansingh et al., 2011). Therefore, it is possible functional deficits may have eventually manifested in the POLY+VEH mice had a longer recovery period been tested in this study. Furthermore, the IL-1ra treatment may have increased the rate of recovery had acute or sub-acute time-points been tested. Taken together, future studies could administer a more severe TBI, incorporate behavioral outcomes with increased sensitivity, or test at different post-injury time-points to further evaluate the benefit of IL-1ra on functional outcomes after polytrauma and better characterize recovery in this model.

4.2. The clinical application of IL-1ra treatment?

Anakinra is a commercially-available, FDA-approved human recombinant IL-1ra, that has progressed to a phase II, open label, randomized-control clinical trials in severe TBI (Helmy et al., 2014, 2016). In this trial, 20 severe TBI patients (i.e., GCS < 8) were recruited in the first 24 h after injury, monitored for 6 h, and then randomized 1:1 to receive either Anakinra treatment or control. Anakinra (100 mg) was delivered via subcutaneous injection, with four further doses given at 24 h intervals. Control patients were monitored and sampled in an identical manner but received no drug. Results from this study indicated that Anakinra is safe and has good brain penetrance. Specifically, the mean concentrations of IL-1ra in treated versus controls 6 h after treatment onset was 243 ng/mL vs 67 pg/mL in plasma and 123.6 pg/mL vs 27.6 pg/mL in brain microdialysate (Helmy et al., 2016). Although our current study was limited in that no blood was collected from the mice, and brain tissue was not analysed for IL-1ra levels, previous rodent brain injury studies have consistently reported good IL-1ra brain penetrance using doses similar to those in our current study (Greenhalgh et al., 2010). The lack of blood collection also pre-
vented us from examining the impact of the treatment on circulating inflammatory markers that may have contributed to the neurological changes observed.

The initial clinical trial has also found that IL-1Ra treatment can modify neuroinflammation in TBI patients (Helmy et al., 2014, 2016). Of particular interest, in the first 48 h after injury the TBI patients treated with Anakinra had increased levels of cytokines (e.g., MCP-1, GM-CSF, IL-1) typically associated with an M1 microglia/pro-inflammatory response, whereas the control TBI patients had increased levels of cytokines (e.g., IL-4, IL-10, MDC) typically associated with an M2 microglia/anti-inflammatory response. Similar to our study, the supposed anti-inflammatory IL-1Ra treatment had a somewhat counterintuitive effect – in this case the treated patients had increased levels of M1 markers. Consistent with our earlier discussion related to M1/M2 classification, the authors of the clinical trial emphasized that a simple pro-versus anti-inflammatory categorization is no longer valid and that inflammatory factors can play a range of roles after injury, both toxic and protective.

Still, the clinical trial findings are somewhat inconsistent with the findings from the current study, as the POLY+IL-1Ra mice had decreased levels of M1 markers 48 h post-injury. There are a number of reasons that might account for this. The clinical trial initiated the Anakinra treatment > 6 h post-injury versus 1 h post-injury in the current study. Considering the temporal complexities related to the expression of different inflammatory factors, that the same inflammatory factor may have a different function/effect at different times, and inter-species temporal differences in regards to inflammation, the timing of the treatment and analysis may have contributed to the contrasting M1 findings. The clinical trial also involved patients at the most severe end of the TBI spectrum, with predominantly diffuse injury patterns, and the microdialysis catheter was intentionally placed away from any focal injuries. Conversely, our study involved a relatively mild and focal injury pattern, and the analysis involved tissue directly beneath the impact site. Therefore, it is possible that the injury pattern (e.g., diffuse versus focal) and brain region examined may affect inflammatory outcomes. The clinical trial also had a highly heterogeneous TBI population relative to our preclinical study. For example, age and biological sex can affect inflammatory factors, and the clinical trial involved male and female participants that ranged from 18 to 61 years of age, whereas our study included only young adult males. Furthermore, the clinical trial did not differentiate between TBI with or without extracranial injury, whereas the current study was only done in mice given polytrauma.

Related to this, whether IL-1Ra treatment may be more suitable in polytrauma versus isolated TBI is an important question. Unfortunately, because the current study failed to incorporate isolated TBI groups, we were unable to compare the effects of IL-1Ra treatment in these two settings. However, the findings from our previous paper and/or the current study do indicate that polytrauma results in exacerbated IL-1β and related pathology compared to an isolated TBI, and that IL-1Ra treatment mitigates the effects of polytrauma. Based on these findings, it is possible that TBI patients presenting with polytrauma may have an increased likelihood of experiencing IL-1R-mediated pathophysiology, and that this process may be heightened or prolonged, relative to an isolated TBI. Considering the heterogeneous nature of TBI, polytrauma patients may represent a readily identifiable subgroup of TBI patients that are likely to benefit from IL-1Ra treatment. This does not imply that IL-1Ra is a less appropriate treatment for isolated TBI that involves IL-1R-mediated pathophysiology, nor are we aware of any evidence that indicates that the two scenarios warrant a different IL-1Ra treatment strategy. These are important questions that should be examined in future studies.

Finally, it is important to discuss the therapeutic window of IL-1Ra treatment after TBI. Because IL-1β is rapidly upregulated after TBI, it is likely that the optimal treatment window is in the acute period of TBI, which may limit this treatment strategy to the emergency setting. However, our previous study with this polytrauma model found that IL-1β levels were increased for at least 1 month post-injury (Shultz et al., 2015ab), and other studies have reported chronic neuroinflammation in TBI patients (Ramlachhansingh et al., 2011). Therefore, it is possible that delayed treatment with IL-1Ra may be beneficial in some circumstances, although future studies are required to examine this.

4.3. Conclusions

IL-1Ra treatment after polytrauma reduced inflammation and edema at 48 h post-injury, and mitigated damage to the injured cortex and white matter at 14 weeks post-injury. Anakinra is a commercially-available, FDA-approved human recombinant IL-1ra, that has progressed to phase II clinical trials in TBI patients. However, there have been many translational failures in TBI drug discovery, which may be in part due to the heterogeneity and temporal complexities of TBI pathophysiology. For example, not all TBI patients will experience a robust inflammatory response, and the therapeutic window to target IL-1β may be short-lived. Therefore, IL-1Ra may only be appropriate for TBI patients that have a strong, exacerbated, or prolonged neuroinflammatory response that actively involves IL-1β. Such a response may be related to genetics, TBI mechanics/severity, or the presence of an ECI. Although future studies are required to examine the influence of ECs on TBI pathophysiology and functional recovery, and how IL-1Ra modifies these outcomes, in patients, such endeavours are warranted considering our findings here, the high incidence of concomitant TBI + ECI, and the lack of effective intervention.

Conflict of interest

The authors declare no conflict of interest.

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References


MouseMove: an open source program for semi-automated analysis of movement and cognitive testing in rodents

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The Open Field (OF) test is one of the most commonly used assays for assessing exploratory behaviour and generalised locomotor activity in rodents. Nevertheless, the vast majority of researchers still rely upon costly commercial systems for recording and analysing OF test results. Consequently, our aim was to design a freely available program for analysing the OF test and to provide an accompanying protocol that was minimally invasive, rapid, unbiased, without the need for specialised equipment or training. Similar to commercial systems, we show that our software—called MouseMove—accurately quantifies numerous parameters of movement including travel distance, speed, turning and curvature. To assess its utility, we used MouseMove to quantify unilateral locomotor deficits in mice following the filament-induced middle cerebral artery occlusion model of acute ischemic stroke. MouseMove can also monitor movement within defined regions-of-interest and is therefore suitable for analysing the Novel Object Recognition test and other field-related cognitive tests. To the best of our knowledge, MouseMove is the first open source software capable of providing qualitative and quantitative information on mouse locomotion in a semi-automated and high-throughput fashion, and hence MouseMove represents a sound alternative to commercial movement analysis systems.

The Open Field (OF) test is amongst the most commonly used assays for monitoring exploratory behaviour and locomotor activity in laboratory animals1. The OF test relies on the principle that rodents and other laboratory animals will innately explore novel surroundings. Ideally, the OF should be devoid of visual landmarks, olfactory cues and be situated in a quiet dimly lit room; so as to encourage exploration and ambulation, to minimise learning/memory triggers and to reduce stress on the laboratory animal during testing. It is because of these simple underlying principles and low animal handling requirements that the OF test represents a relatively quick, reproducible and robust assay. Accordingly, the OF test has been used to assess neurological effects across a wide array of experimental paradigms, including acquired brain injury2, psychostimulant administration3, stress/anxiety induction4, aging5, gender5, circadian cycling6, differing genophenotypic backgrounds7 and environmental factors8.

Despite its widespread popularity, the vast majority of research groups rely upon commercial systems for recording and digitally analysing the OF test. These commercial system use an overhead video camera or a laser-gridded arena, and quantify movement parameters using proprietary software. Examples of commercial systems include Ethovision9, Noldus10, ANY-maze11, and AcqKnowledge12. Each of these systems operate in an automated fashion, requiring no user input and therefore also suffer from the same challenges as commercial systems mentioned above.

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current commercial systems capable of performing OF testing include the ANY-maze (Stoelting Co; IL, USA), EthoVision® XT (Noldus; Wageningen, The Netherlands), TopScan (Clever Sys Inc.; VA, USA), and Opto-Varimex (Columbus Instruments; OH, USA). While these commercial systems are excellent in their recording and analytical capability, they are relatively expensive, offer little methodological transparency or flexibility and often restrict OF testing to laboratories with the financial means to establish specialised behavioural suites.

Numerous open source programs have been released as alternatives to the commercial OF systems9–13. All but one of these prior programs restricts its analyses to two main aspects of movement: travel distance and time spent within a defined region-of-interest (ROI). The most recent freeware, EthoWatcher, represents a significant improvement in that it allows users to analyse a wider array of rodent movement parameters such as grooming and rearing behaviours13. This being said, many other routine aspects of movement, including stationary fraction, object speed, laterality differences or ROI time, in combination with capacity for batch analysis, are currently not addressed by existing programs. As a result, commercial systems still offer vastly superior performance and throughput for OF test analysis.

Here we describe MouseMove—an open source program that offers semi-automated and high-throughput analysis of a wide array of movement parameters, including distance travelled, mean speed, speed variance, stationary fraction and laterality (i.e. left/right turn, turning offset and curvature). Using a model of acquired brain injury (the transient middle cerebral artery occlusion [MCAo] model of experimental stroke), which is well known to cause altered locomotion14,15, we show that MouseMove can discern both quantitative and qualitative differences in movement across an OF. Importantly, using a mechanical calibration system, we demonstrate that MouseMove tracks changes in distance, speed and laterality with >96% accuracy. MouseMove also has a region-of-interest function which allows quantitative analysis of cognitive tests such as the Novel Object Recognition (NOR) test. Taken together, MouseMove represents a sound and freely available alternative to commercial platforms for analysing arena-related assays such as the OF test and the NOR test.

Results

Design of an OF, automated image processing and movement analysis using MouseMove. An OF arena was first assembled to test MouseMove. As shown in Fig. 1, the OF had a circular white melamine floor, black plastic walls, was surrounded by a white polyester curtain and had a webcam positioned above its centre. For each OF test, a mouse is placed in the centre of the arena and video footage captured.
('experiment video,' Fig. 2a; refer to Methods for details). Directly after testing, a ~10 second video of the empty arena is captured ('background video,' Fig. 2b). The open source program, ImageJ, is then initialised and the 'experiment' and 'background' videos are uploaded (Fig. 2c) using our ImageJ macro Preprocessing.ijm (Supplementary File 1). The macro automatically performs the following stepwise processes: Step 1—thresholding of the raw videos to create binary videos. Step 2—\(x, y\) alignment between the experiment and background videos (Fig. 2d,e). Step 3—subtraction of the aligned background video from the experiment video (Fig. 2f). Step 4—object recognition and trajectory generation using the published MTrack2 plugin\(^{16}\). The macro generates a saveable image of the mouse's cumulative trajectories (Fig. 2g) and a text file stipulating the \(x, y\) coordinates of the mouse over time. Next, MouseMove's graphical user interface (GUI) is initialised. Pertinent parameters (video frame rate, range of frames to
be analysed, spatial scale of the video) and the location of the macro-generated text file are entered using MouseMove.exe (Supplementary File 2). A detailed analysis of the movement patterns is then automatically performed, whereby MouseMove measures the fractional time spent stationary, distance travelled, speed mean/standard error of the mean (s.e.m.) and various indices of laterality (number of left turns, number of right turns, ratio of left:right turns (LRratio), turn offset and curvature radius). By default, the analytical results of MouseMove are depicted in both a visual/graphical form (Fig. 2h) and as a saveable text file (Fig. 2i).

Mechanical calibration of MouseMove. To calibrate MouseMove we took video footage of a mouse-shaped object affixed to a perspex arm being driven by a speed-controlled rotor (Fig. 3a). We tested MouseMove’s tracking capabilities across a range of defined speeds and curvatures by varying the position of the mouse-shaped object along the perspex arm (Fig. 3b). Via this approach, we found that MouseMove quantified movement parameters such as distance travelled, speed and curvature radius with >96% accuracy (Fig. 3c). Importantly, the calibration settings encompass the range of movement typically exhibited by adult mice (as determined by batch analysis of >150 mice; data not shown).

As an additional means with which to assess accuracy, we attempted to correlate the findings of MouseMove with that of EthoWatcher software for the speed-controlled rotor videos (Fig. 3) and for the videos of sham- or MCAo-operated mice (Figs 4 and 5). Unfortunately, this comparison could not be made due to inconsistencies in the object tracking function of EthoWatcher (data not shown).

MouseMove analysis can be used to quantify reduced locomotor activity in mice after experimental stroke. To demonstrate the utility of MouseMove in an experimental setting, we used it to analyse the OF behaviour of mice that had undergone the MCAo model of acute ischaemic stroke, which is well known to produce focal unilateral cerebral infarction and impaired locomotor activity in
rodents. MouseMove highlighted both qualitative (Fig. 4a and Supplementary Video 1) and quantitative (Fig. 4b–d) differences in locomotor activity between sham-operated mice and MCAo-operated mice. In particular, MouseMove resolved a ~3-fold reduction in mean speed (data not shown), a ~3-fold reduction in total distance travelled (Fig. 4c), and a ~70-fold increase in sedentary behaviour (stop time fraction; Fig. 4d) in MCAo-operated mice, relative to sham-operated mice.

MouseMove vector analysis can be used to quantify laterality deficits in mice after experimental stroke. The unilateral brain damage characteristic of the MCAo model not only reduces general locomotor activity, but also produces laterality deficits such as circling. Analysis of the OF behaviour using MouseMove clearly quantified this altered laterality, with MCAo-operated mice exhibiting a marked increase in turning bias (Fig. 5a,b) and tighter circling (Fig. 5c,d), relative to sham-operated mice. Thus, MouseMove provides a detailed quantification of altered OF behaviours following experimental stroke in mice.

MouseMove’s region-of-interest (ROI) analysis can be used to quantify episodic recognition memory. The OF test can also be used to measure anxiety whereby increased fractional time spent close to the walls indicates increased anxiety. Alternatively, apparatus (e.g. objects, cues, stimuli, mazes) can be placed into the OF to allow cognitive tests to be performed. In both these instances, movement needs to be analysed within defined sub-regions of the OF. Accordingly, we added a ROI function (see ROI tab in MouseMove.exe) to expand the utility of MouseMove and allow the quantification of certain cognitive assays.

To use the ROI function, videos are captured and handled in the same fashion as for the OF test. Once the video parameters and input files have been loaded into MouseMove’s GUI, one needs to click on the ‘ROI’ tab of the GUI and stipulate the centre and radius (in pixels) of each ROI. Up to 4 circular ROIs

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**Figure 4. Analysis of locomotor activity in mice after experimental stroke using MouseMove.** (a) Cumulative trajectories of a representative MCAo-operated mouse and a sham-operated mouse over 100 s intervals in the OF. (b) Plot of distance travelled over time for a representative sham- and MCAo-operated mouse in the OF. Numerical annotations indicate the mean speed for these same sham- and MCAo-operated mice over the 400 s period. (c,d) ‘Distance travelled’ (c) and ‘Fractional time spent stationary’ (d) for sham-operated (n = 6) and MCAo-operated (n = 7) mice over a 400 s period in the OF. Dot points represent the mean value for individual mice. Line and error bars represent the cohort mean ± s.e.m. **p < 0.01 and *p < 0.05 as determined by two-sided unpaired t-test without (c) or with Welch’s correction (d).**
can be measured simultaneously. The fractional time spent within each ROI (i.e. ‘ROI fractions’) is then automatically measured and the results added to the saveable text file (Fig. 2i).

To exemplify this ROI functionality, we modified the OF to perform the NOR test (see Methods for details). The NOR test is widely used to assess episodic memory \(^{18}\). It involves habituating a mouse in an OF containing two identical objects, then replacing one of the identical objects with a novel object (Fig. 6a,b). The time spent exploring the novel object, relative to the familiar object, is a gauge of episodic recognition memory \(^{18}\). As shown in Fig. 6c–e, the increased tendency of mice to explore the novel object was easily detectable using the ROI function of Move. Based on these findings, Move should not only enable semi-automated quantitative OF testing, but also allow digital analysis of cognitive tests such as the NOR.

**Discussion**

The OF test was first described by Hall and Ballachey in 1932 \(^{19}\). Today, the OF test is the canonical assay for comparative assessment of changes in locomotor activity. Here we demonstrate Move to be an alternative, publicly available means of analysing the OF test in a semi-automated fashion. Move has two downloadable components: an ImageJ macro and a separate program with a custom-built GUI. Move utilises the pre-existing plugin, MTrack2 \(^{16}\), to robustly track object movement. The \(x,y\) coordinates output file of the MTrack2 plugin is then fed into Move’s GUI for quantitation of movement parameters including distance travelled, speed mean/S.E.M, fractional time spent stationary and indices of laterality (number of left turns, number of right turns, ratio of left:right turns \([LRatio]\), turning offset and curvature). Lastly, Move provides a visual overview of the cumulative tracking and graphical overview of distance travelled and speed distribution (Fig. 2h).

There are several important caveats with Move when compared to the commercial OF analytical systems. For instance, whereas commercial systems offer an integrated package with an OF, movement recorder and analytic software, Move users will be required to build their own OF, mount a video camera, install the ImageJ program and download our pre-processing macro and Move.exe file. Building your own video-monitored OF, however, is straightforward and can be as simple as placing a ~0.4m high circular wall on an appropriately coloured floor with an overhead webcam (Fig. 1). In addition, whilst Move measures many of the same locomotion parameters as the ANY-MAZE and
other commercial systems, it does not analyse higher-order events such as rearing or defecation and is incapable of distinguishing the rodent’s head from its tail.

In spite of these limitations, MouseMove offers numerous advantages to researchers who do not have access to a commercial OF analytical system. First, the script of MouseMove is publicly available and can therefore be customised. Once an OF is built, we show here that the MouseMove-based protocol can be used to provide accurate OF data in a semi-automated fashion, without the need for advanced equipment or specialist training. Second, the time taken for MouseMove to process and analyse video footage is relatively short compared to the time needed to perform these tasks manually (typically 10 min versus 3 h per OF test, respectively). MouseMove has undergone extensive in-house testing and (from March 2014-April 2015) has been used to quantify the locomotion of >150 mice (using high-throughput batch analysis; data not shown) across two different OFs in different research facilities. Third, we show that the ROI function expands the utility of MouseMove and allows analysis of OF-related cognitive tests such as the NOR test. Finally, while the studies described here have utilised MouseMove to analyse altered OF behaviours following experimental stroke in mice, this program could equally be suitable for the measurement of altered locomotion in other rodent models of brain injury/stimulation.

Figure 6. Analysis of novel object recognition using MouseMove’s region-of-interest function. (a,b) Video still of a representative mouse during familiarization (a) and test (b) sessions of the Novel Object Recognition (NOR) test. (c,d) Cumulative trajectories of the same mouse during familiarization (c) and test (d) sessions in 200 s intervals. The blue overlaid circles indicate that each ROI was centred on the stimuli (i.e. the lego tower and flask) and sized to be 30 pixel lengths in radius (i.e. a ~25 cm diameter circle). (e) Relative time spent exploring the 2nd object (i.e. the lego tower in the test session or flask #2 in the familiarization session) versus the 1st object (i.e. flask #1 in both the test and familiarization sessions). Dot points represent the mean value for individual mice (total of n = 7 mice). Lines connect the values for the same mouse across both NOR sessions. **p < 0.01 as determined by two-sided paired t-test.
In conclusion, MouseMove is an open source, semi-automated and customisable means of performing the OF test. Accordingly, it should broaden usage of, not only the well characterised and popular OF test, but also other cognitive assays such as the NOR test.

**Methods**

**Materials.** Webcams used were the QuickCam E3560 or a HD Webcam C615 (Logitech; Lausanne, Switzerland). Videos were converted using Weeny Free Video Converter 2 version 2.1 (www.weeny-soft.com). The video pre-processing utilised ImageJ 1.50a (National Institutes of Health, USA) and the MTrack2 plugin\(^{16}\) (designed by Nico Stuurman, Vale laboratory, University of California, CA, USA).

**Experimental animals.** All animal procedures and methods were performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and the Use of Animals for Experimental Purposes in Australia. All animal procedures and methods for this study were approved by the institutional Animal Ethics Committee (AEC), either AMREP AEC or Monash Animal Research Platform AEC. Experiments used adult male C57BL/6 mice (aged 8–12 weeks). Mice were maintained under a 12-hour light/dark cycle with ad libitum access to food and water.

**Open Field (OF) Test.** The OF test was performed according to the guidelines of Gould and colleagues\(^1\). In brief, OF testing was done in a quiet (~60 decibel) and dimly lit (~27 lux) room. Two OFs were designed to test MouseMove. The first OF (Fig. 1d) was used for the MCAo cohort (Figs 4 and 5) and featured a white melamine circular floor (0.79 m in diameter, Fig. 1a) and a black corrugated plastic wall (0.4 m in height, Fig. 1c). To minimize spatial cues, the OF was surrounded by a white opaque polyester curtain suspended from a bicycle wheel which, in turn, was suspended on top of semi-rigid fibreglass tent poles (Fig. 1b,c). A HD C615 webcam was fixed to the hub of the wheel ~1 m above the OF and connected via USB cable to a computer. The second OF (Fig. 1e) was used for mechanical calibration (Fig. 3) and for NOR testing (Fig. 6) and featured a white melamine circular floor (0.9 m in diameter, Fig. 1a), a black corrugated plastic wall (0.4 m in height, Fig. 1c) and was surrounded by a white opaque polyester curtain suspended from a rail affixed to the wall. A QuickCam E3560 webcam was fixed to the rail ~1.5 m above the centre of the OF and connected via USB cable to a computer (Fig. 1b,c). The OF was wiped with 70% (v/v) ethanol before each test to minimise olfactory cues. For testing, a mouse was placed in the centre of the OF and its movement recorded for 15 min. The mouse was then removed from the OF and a ~10 s video of the empty arena was captured. Note, we recommend that future users build an OF with similar dimensions and mount their video camera at a similar position as that shown in Fig. 1; this is because, whilst our software offers good flexibility in terms of arena size/camera placement, extreme departure (e.g. >2-fold) from our OF dimensions will require a compensatory change to be made to the Preprocessing.ijm and MouseMove.exe settings.

**Middle Cerebral Artery occlusion (MCAo) model of ischaemic stroke.** The MCAo model was performed as described previously\(^{20}\). Mice underwent sham surgery or 1 h occlusion of the middle cerebral artery followed by 23 h of reperfusion and then OF testing.

**Novel Object Recognition (NOR) test.** The NOR test was performed according to the guidelines of Leger et al.\(^{19}\) with minor modifications. In brief, four different laminated A4-sized pictures were equally spaced around the walls of the OF to facilitate spatial orientation (Fig. 6a). Two identical 225 ml tissue culture flasks (160 mm high, 90 mm wide, 38 mm deep) were filled with sucrose, parafilm-sealed then placed at opposite ends of the OF (~15 cm from the wall; Fig. 6a). A mouse was then allowed to explore the OF for 15 min whilst being video recorded (referred to as the ‘familiarisation’ session). 20 h later, one of the tissue culture flasks was replaced with a tower of lego pieces (tower was 170 mm high, 63 mm wide, 63 mm deep) and the same mouse was allowed to explore the OF for 15 min whilst being video recorded (referred to as the ‘test’ session). Note, after both familiarization and test sessions a ~10 s video of the empty arena was also captured.

**Video capturing, conversion and pre-processing.** Videos are acquired at a spatial resolution of 640 × 480 pixels per frame (1–4 mm per pixel depending upon OF configuration) and a temporal resolution of 25 frames per second. Videos are then converted into MJPEG-compressed .avi files at full spatial and temporal resolution using Weeny Free Video Converter 2. The pre-processing macro (Preprocessing.ijm downloadable as Supplementary File 1) is then launched and the folder containing the converted .avi files is selected. Our pre-processing macro then spatially bines the videos into 320 × 480 before performing object segmentation/tracking whereby the ‘background video’ is subtracted from the ‘experiment video’. The subtracted video is then thresholded using the ImageJ’s ‘Minimum’ algorithm. To perform anti-aliasing, we applied ImageJ’s ‘erode’ and ‘dilate’ filters to the thresholded image. In our experience, anti-aliasing reduces artefacts (e.g. faeces) and is necessary for robust object segmentation/tracking. Object segmentation/tracking is then performed by feeding the anti-aliased video into the MTrack2 plugin. The output file generated by the MTrack2 plugin is then fed into MouseMove’s GUI for quantitation of movement parameters. Note, we recommend that future users capture/convert video footage with...
the same temporal and spatial resolution, because significant departure (e.g. >2-fold) from these video parameters will require a compensatory change to be made to the Preprocessing.jim.

**MouseMove GUI design and algorithms.** LabVIEW 12.0 Development System (National instruments, TX, USA) was used to build MouseMove.exe. MouseMove contains a 'file import function' which allows reading of the trajectory data file generated by our ImageJ macro and a 'file export function' for saving of the analysis results into a comma separated values text file. By default, MouseMove downsamples the tracking coordinates by a factor of 10 (i.e. effectively adjusting the tracking to 2.5 frames per second). The downsampled tracking coordinates are then used for analysis. To simplify analyses, it is assumed that mice only move in the forward direction (supported by our batch analysis of >150 mice where ~90% of movement occurs in the forward direction; data not shown).

For trajectory analyses: Let \( n \) be the total number of data points for the pre-processed trajectory. At \( i \)-th point \((t_i, x_i, y_i)\) from the pre-processed data, the distance increment is defined by Equation #1:

\[
\Delta d_i = \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}.
\]

If \( \Delta d_i = 0 \), 'stop' status was assigned to this time point. Stop time fraction was then determined by normalizing 'stop' time points over the \( n \). The first \((x_{i}, y_{i})\) and second \((x_{i+1}, y_{i+1})\) time derivatives of the displacement at each point \((x_i, y_i)\) were calculated using the 2\(^{nd}\) order central discrete differentiation method with a window of three consecutive coordinates. For example, for \( i = 0, 1, 2, \ldots, n-1 \),

\[
x_i = \frac{x_{i+1} - x_{i-1}}{t_{i+1} - t_{i-1}}
\]

\( x_{i-1} \) is the first element in initial condition, and \( x_n \) is the first element in final condition. The instantaneous speed is given by Equation #2:

\[
v_i = \sqrt{x_i^2 + y_i^2}.
\]

For region-of-interest analyses: The 'ROI fraction' calculated by MouseMove calculates the fractional time spent within each ROI. In other words, a 'ROI1_fraction' of 0.3 indicates that the tracked object spent 30% of its time within ROI 1. Note, each ROI is circular in shape and is defined by its centre and radius (coordinates which are manually entered into MouseMove's ROI GUI).

For laterality analyses: A sliding window of 3 frames is used for all laterality measures. Within each sliding window, the vector across frames #2–3 is expressed relative to the vector across frames #1–2. The directionality of the mouse movement at \( i \)-th point is determined by the angle of the vector \((x_i, y_i)\) in Equation #3: \( \theta_i = \arctan2(y_i, x_i) \). Thus, Equation #4: \( \Delta \theta_i = \theta_i - \theta_{i-1} \) tells which direction the mouse will move in. Assuming a forward moving mouse cannot instantaneously turn over 90 degree, the decision rule of the directionality is: forward left \((0 < \Delta \theta_i < \pi/2)\), Fig. 5a, left), forward right \((- \pi/2 < \Delta \theta_i < 0)\), Fig. 5a, right), forward straight \((\Delta \theta_i = 0)\) and backward \((|\Delta \theta_i| \geq \pi/2)\). For each trajectory, the ratio of the left- against right-oriented time fractions (LRatio) was calculated (Fig. 5b, left y-axis) and \(|1 - LRatio|\) is regarded as a further indication of movement laterality (Fig. 5b, right y-axis). To quantify curvature radius at each point of the trajectory (Fig. 5c,d) we used Equation #5:

\[
\rho_i = \frac{\left(k_i^2 + \frac{1}{y_i^2}ight)^{3/2}}{\left|k_i y_i - \frac{1}{y_i} x_i\right|}.
\]

**Instruction for operating MouseMove**

**Prerequisites:**

(1) A computer with a Microsoft Windows operating system.

(2) ImageJ 1.50a (Fiji) ; can be downloaded from http://fiji.sc/Downloads

(3) Preprocessing.jim can be downloaded via the link for Supplementary file 1.

(4) MouseMove.exe can be installed after downloading via the link for Supplementary file 2. Users may be prompted to install LabVIEW if not already installed on their computer.

**Step-wise instructions:**

(1) Create a new folder which contains at least one experiment video file and one background video file (.avi file format with a spatial resolution of 640 × 480 and a temporal resolution of 25 frames per second). The same name for corresponding experiment and background videos should be used, but with the suffix 'empty' added to the background video file name. Note, multiple videos can also be placed in the same folder for batch analysis, however, the names of the corresponding experiment/background videos must be paired (e.g. experiment #1 filename = ‘MCAo mouse a.avi’, background #1 filename = ‘MCAo mouse a empty.avi’, experiment #2 filename = ‘MCAo mouse b.avi’ and background #1 filename = ‘MCAo mouse b empty.avi’).

(2) Open the macro Preprocessing.jim in ImageJ.

(3) Click ‘Run’ and the interface will prompt you to identify where the video-containing folder is located. The macro will preprocess all videos in the folder and save the output trajectory data as ‘TkResults …txt’ files. Note: because of ImageJ’s memory restrictions, videos are segmented into 5000 frame lengths, and therefore the output files are named by combining the original video name with the frame range (e.g. ‘TkResults_MCAo mouse a_1-5000.txt’).
(4) Open the MouseMove.exe and click the yellow ‘folder open’ button to import one TkResults file. MouseMove will then automatically recognise and stitch together the other segments from the same experiment. After this, MouseMove automatically displays its analysis results.

(5) To analyse different frame range, recalibrate OF spatial and temporal parameters, downsample the video and stipulate the centre and radius of an ROI (in the ROI tab) simply change these parameters in MouseMove’s GUI and click ‘Update’.

(6) To start a new analysis of other preprocessed data files, simply click the yellow ‘open folder’ icon and import a new file-of-interest. The software will automatically update the results.

(7) Click the ‘Save’ button to save the analysis result as a comma separated values text file (.csv) within the original video-containing folder.

Note, example ‘background’ and ‘experiment’ video files can be downloaded as Supplementary files 3 and 4, respectively, and used to test the proper functioning of Preprocessing.jim macro and MouseMove.exe.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism® 6.04 (GraphPad Software, Inc.). Results are expressed as mean ± s.e.m. For each cohort, the number of independent experiments and the statistical test employed is indicated in the respective legend. p < 0.05 was considered to be statistically significant.

References


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Author Contributions

Additional Information
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**Supplementary Material**


**List of additional supplementary files**

**Supplementary Video 1 | OF testing of sham-operated and MCAo-operated mice.**
Video compares footage of a sham-operated and a MCAo-operated mouse before and after processing with *Preprocessing.ijm.*

**Supplementary File 1 | Preprocessing.ijm macro (requires unzipping).**

**Supplementary File 2 | MouseMove.exe installer (requires unzipping).**

**Supplementary File 3 | Example .avi ‘background’ video for testing MouseMove.**

**Supplementary File 4 | Example .mp4 ‘experiment’ video for testing MouseMove.** Note, one needs to convert this file into .avi format before using it to test *MouseMove.*