Mechanistic insights to infections after stroke for better targeted therapeutics

Raymond Shim

Bachelor of Science (Honours)

A thesis submitted for the degree of Doctor of Philosophy at

Monash University in 2020

Department of Medicine

Monash Health, Clayton

Copyright notice

Abstract

Stroke is a devastating disease that is a major cause of death and disability around the world and is a leading contributor to mortality in Australia. Complications that are traditionally associated with stroke include neurological and functional impairments. However, an understated complication in patients with stroke suffer is infection. Infections occur in up to two-thirds of stroke survivors, with bacterial lung infection (pneumonia) being a leading cause of death. Consequently, preventing and reducing the occurrence of post-stroke infection in patients is of utmost priority in stroke research. Studies show that patients with more severe strokes are especially more prone to developing pneumonia. The high incidence of pneumonia in patients with severe stroke was initially believed to be due to impairments in swallowing function (dysphagia), allowing for direct entry of pathogens into the lung from oral and gastric contents. Attempts to reduce aspiration and dysphagia using nasogastric tubing and mechanical ventilators do not reduce the incidence of post-stroke infection and are even considered to be risk factors. Furthermore, trails exploring the efficacy of preventive antibiotic therapy in reducing post-stroke infection have failed. Therefore, exploring the mechanisms to infection after stroke is paramount for the discovery of new treatments and therapeutic strategies. Evidence now suggests that the risk to infection is contributed by a suppression of antibacterial immunity that occurs following stroke. A dysfunction of the sympathetic nervous system (SNS) has been shown to be a main mediator of post-stroke immunosuppression via activation of the β-adrenergic receptors (β-AR). In particular, previous work from our laboratory provides evidence that β-AR activation on the hepatic invariant natural killer T (iNKT) cell is a primary driver of immunosuppression and infection after experimental stroke. However, while it is understood that increased stroke severity increases the risk to infection, the relationship between cerebral infarct size on immunosuppression requires further elucidation.

The overarching goal of this thesis is to expand our understanding of the way increased stroke severity impacts on host immunity and on the mechanisms to immunosuppression. This project aims

to 1) determine whether infarct size and location promotes immunosuppression and lung infection after stroke, 2) explore the contribution of the SNS and the β_2 adrenergic receptor on post-stroke infection, and 3) test the efficacy of specific iNKT cell activators, α-galactosylceramide (α-GalCer) and two structural analogues, in boosting immunity as potential therapeutic option to reduce post-stroke infection. This project utilises a versatile mouse model of ischemic stroke known as the middle cerebral artery occlusion (MCAO) in order to control the severity of stroke. The main findings of this thesis show that cerebral infarct size increases the risk to lung infection by severely impairing pulmonary immunity. In contrast, the location of cerebral infarct does not influence the susceptibility to post-stroke infection. We also show that SNS dysregulation and β_2-adrenergic receptor activation contributes post-stroke infection, but other factors have a greater role, especially following severe stroke. Finally, we demonstrate that therapeutic use of α-GalCer provides protection against post-stroke lung infection.

Publications during enrolment

Thesis-related publications:

1. **Shim, R.**, Wen, S.W., Wanrooy, B.J., Rank, M., Thirugnanachandran, T., Ho, H., Sepehrizadeh, T., de Veer, M., Srikanth, V.K., Ma, H., Phan, T.G., Sobey, C.G. and Wong, C.H.Y. (2019) Stroke severity, and not infarct location, increases the risk to infection. *Translational Stroke Research*. doi:10.1007/s12975-019-00738-3
2. **Shim, R.** and Wong, C.H.Y. (2018) Complex interplay of multiple biological systems that contribute to post-stroke infections. *Brain, Behaviour, and Immunity* (70:10-20). doi:10.1016/j.bbi.2018.03.019

Other publications throughout candidature:

1. Wen, S.W., **Shim, R**., Ho, L., Wanrooy, B.J., Srikhanta, Y.N., Prame Kumar, K., Nicholls, A.J., Shen, S., Sepehrizadeh, T., de Veer, M., Srikanth, V.K., Ma, H., Phan, T.G., Lyras, D. and Wong, C.H.Y. (2019) Advanced age promotes colonic dysfunction and gut-derived lung infection after stroke. *Aging Cell*. doi:10.1111/acel.12980
2. Gan, P.Y., Chan, A., Ooi, J.D., Dick, J., Nagai, K., O'Sullivan, K.M., Oudin, V., **Shim, R.**, Kitching, A.R. and Holdsworth, S.R. (2019) Biologicals targeting T helper cell subset differentiating cytokines are effective in the treatment of murine anti-myeloperoxidase glomerulonephritis. *Kidney International* (96:1121-1133)
3. Zhang, S.R., Piepke, M., Chu, H.X., Broughton, B.R.S., **Shim, R.**, Wong, C.H.Y., Lee, S., Evans, M.A., Vinh, A., Sakkal, S., Arumugam, T.V., Magnus, T., Huber, S., Gelderblom, M., Drummond, G.R., Sobey, C.G. and Kim, H.A. (2018) IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke. *JCI Insight*. doi:10.1172/jci.insight.121560

4. Gan, P.Y., Fujita, T., Ooi, J.D., Alikhan, M.A., Dick, J., **Shim, R.**, Odobasic, D., O'Sullivan, K.M., Kitching, A.R. and Holdsworth, S.R. (2017) Pathogenic role for γδ T cells in autoimmune anti-myeloperoxidase glomerulonephritis. *The Journal of Immunology* (199 [9]:3042-3050)

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 two original papers published in peer reviewed journals. The core theme of the thesis is infection after stroke as a result of suppressed immunity. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Medicine at Monash Health, Clayton under the supervision of Dr. Connie Wong, Dr. Shu Wen Wen and Prof. Christopher Sobey.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research

In the case of chapters 1 and 3, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision, submitted)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	Complex interplay of multiple biological systems that contribute to post-stroke infection	Published	90%. Concept and writing manuscript	*Connie Wong*, input into manuscript 10%	No
3	Stroke severity, and not cerebral infarct location, increases the risk to infection	Published	70%. Concept, performing experiments, data collection, data analysis, writing manuscript	*Shu Wen Wen*, 5% Performing experiments and manuscript input *Brooke Wanrooy*, 2% Performing experiments *Michelle Rank*, 3% Performing experiments *Tharani Thirugananachandran*, 2% Performing experiments *Luke Ho*, 2% Data analysis *Tara Sepehrizadeh*, 2% Performing experiments and data collection *Michael de Veer*, 1% Performing experiments *Velandai Srikanth*, 1% Data analysis *Henry Ma*, 1% Data collection *Thanh Phan*, 2% Data collection and manuscript input *Christopher Sobey*, 4% Manuscript input	No

				Connie Wong, 5% Concept, Performing experiments and manuscript input	

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Raymond Shim

Student signature: **Date:**

I hereby certify 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 name: Dr. Connie Wong

Main Supervisor signature: **Date:**

Acknowledgements

The work throughout these few years would not have come to fruition without the tremendous help and support from friends, peers, and colleagues that I have met along the way.

First and foremost, my supervisors, Connie, Shu, and Chris. No student could have asked for a better supervisory team. Connie, your guidance and mentorship over the past 7 years is unmatched. You have a knack of being able to calm me down and make everything seem simple. The generosity you have shown to me and others is truly amazing, and I strive to become even half the supervisor you are. Word cannot express how much you have helped me both in my work and in my personal life. I would not be here without you. Shuey, thank you for all your help and advice over these years. Since you joined, you light up the lab with your presence and make everything a joy (even facs). And also, thanks for letting me come around to your office to annoy you after I went to annoy Connie. To Chris, your experience and intellect in stroke and research in general helped elevate my own research and widen my ways of thinking. I will have to come around to pick your brain more often in the future.

To the rest of the Wong Lab, Sj, Alyce, Kathryn and Brooke, thanks for being a great crew to work with. I will treasure the time we spend in and (mostly) out of the lab. Trips down to Clayton Rd for food and snacks is definitely a highlight of these few years. From being in this lab from the start and seeing the how it's turned out has made me feel extremely proud to be a member of the Wong Lab – another testament to the incredible guidance that Connie has given all of us. The way we mesh and work together is unrivalled by any other lab.

For my friends and colleagues at the CID, Louisa, Megan, and Poh, spending time at work by not doing work has helped make my PhD journey much smoother. I will always treasure the times we spend doing crosswords or getting coffee. To the rest of CID and members of SCS reception, your

presence has made working in the CID an extremely enjoyable experience. I am lucky to have been given so many opportunities at CID and SCS.

The work in this thesis would not have been possible without the extraordinary help from many collaborators. I would like to thank Dr. Michelle Rank for her assistance with the PT stroke model and general chats when I come to visit your lab, and Prof. Thanh Phan for your eccentric passion for research to ultimately improve the well-being of patients with stroke and elevating the research from aim 1 with your inputs. Further thanks to my panel members, Dr. Suzie Miller, Dr. Sarah Jones, and Dr. Poh-Yi Gan for your constant encouragement and constructive criticism during my milestones. And finally thank you to the core facilities at MMCAF, Monash Histology, and MBI for your assistance.

Finally, thank you to my family and friends for their support. Thank you to Korey, Nat, Court, and Olivia for the fun times and food. Gigantic thanks to Dr. Angela Nguyen – I actually don't think I could have submitted on time without you. Thank you for staying up late with me and keeping me awake while I write my thesis. Thank you for calming me down during times of (extreme) stress and making the mountain that is the thesis more manageable. But most of all, thank you for your company and the much-needed distractions from thesis writing in the last few months. You can now stop rubbing the fact that you're a doctor in my face. These are the best friends anyone could ever asked for.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.

Contents

Copyright notice I

Abstract II

Publications during enrolment IV

Thesis including published works declaration VI

Acknowledgements IX

List of figures XVI

List of abbreviations XVIII

Chapter One 1

Overview 2

Published literature review 3

The autonomic nervous system 14

The sympathetic nervous system and immunity 15

SNS implication on lymphoid organs 16

SNS effect on immune cells 18

Implication of the SNS and the β-ARs in diseases 20

Issues with studies exploring Adrb2 function in immunity 21

The β-ARs in post-stroke infection stroke 22

The invariant natural killer T cell 23

Activation and mechanisms of iNKT cell polarisation 25

Stroke, SNS activation and iNKT cells 28

Current project and statement of thesis aims 29

Chapter Two 34

Mice 35

Mouse model of ischemic stroke – the middle cerebral artery occlusion (MCAO) 35

Preclinical MRI 36

Neurological assessment 37

Brain infarct quantification 37

Bacteriological analysis 37

Enumeration of circulating leukocytes 38

Flow cytometry 38

Serum cytokine analysis 40

Statistical analysis 40

Chapter Three 42

Supplemental material 58

Chapter Four 64

Introduction 65

Methods 69

Mice 69

β-blocking treatments....69
Plasma collection and circulating catecholamine analysis....69
List of other techniques used but previously described:....70
Results....71
Plasma DA and NA increase at 3 h following severe stroke....71
Non-selective β-adrenergic receptor blocking does not reduce post-stroke infection following severe stroke....72
Inhibiting Adrb2 reduces infection following moderate but not severe stroke....72
Deficiency of Adrb2 does not alter post-stroke infection....74
Discussion....75
Figures....82
Chapter Five....102
Introduction....103
Methods....107
Mice....107
Treatments....107
Serum cytokine and alanine aminotransferase (ALT)....108
Culturing bacteria and testing antibacterial immunity induced by α-GalCer analogues....108
List of other techniques used as described in chapter 2:....108
Results....110

Role of pulmonary iNKT cells in the post-stroke lung 110

Deficiency of iNKT cells prevents the loss of pulmonary lymphocytes 110

Mice deficient in iNKT cells are not protected from post-stroke lung infection 111

Treatment of α-GalCer reduces post-stroke infection 112

α-GalCer induces pulmonary and systemic production of IFN-γ 113

The protection against lung infection conferred by α-GalCer is not seen in iNKT cell deficient mice 114

Analogues of α-GalCer induce rapid antibacterial immunity 115

Analogues of α-GalCer do not reduce post-stroke infection 115

Infection challenge after stroke 117

Discussion 118

Differential roles of lung and liver iNKT cells 118

Evaluation of DB06-9 and SKC8-27 121

Roadblocks to translation of glycolipid-based therapies 124

Figures 127

Chapter Six 144

Shedding light on the mechanisms to post-stroke infection 145

Complexity of mechanisms behind post-stroke infection and immunosuppression 147

Strategies to reduce infection in patients with stroke 151

Limitations and future directions 153

Concluding remarks 157

References 161

Appendix 189

Appendix 1 – Animal monitoring sheet 190

Appendix 2 – Clinical signs severity scoring chart 191

Appendix 3 – Antibodies used for flow cytometry 192

Appendix 4 – Flow cytometry gating strategies 194

List of figures

Figure 1.1 Structure of α-galactosylceramide (α-GalCer) and mechanism of invariant natural killer T (iNKT) cell activation. 31

Figure 1.2 Molecular structure of α-GalCer glycolipids, OCH and C-glycoside. 32

Figure 1.3 Proposed mechanism of glycolipid loading onto CD1d by antigen presenting cells (APC) for inducing T-helper 1 (Th1) or T-helper 2 (Th2) iNKT cell responses 33

Figure 4.1 Generation and metabolism of catecholamines by sympathetic nerve endings 83

Figure 4.2 Plasma 3,4-dihydroxyphenylalanine (DOPA) within the first 3 h after stroke are unchanged. 85

Figure 4.3 Plasma dopamine (DA) is elevated at 3 h after severe stroke. 87

Figure 4.4 Changes to plasma dihydroxyphenylacetic acid (DOPAC) within the first 3 h after stroke. 89

Figure 4.5 Plasma noradrenaline (NA) is elevated at 3 h following severe stroke. 91

Figure 4.6 Plasma adrenaline (AD) is unchanged within the first 3 h after stroke. 93

Figure 4.7 Plasma dihydroxyphenylglycol (DHPG) is unchanged within the first 3 h after stroke. 95

Figure 4.8 Non-selective inhibition of β-adrenergic receptors with PPL does not improve post-stroke infection after severe experimental stroke. 96

Figure 4.9 Specific Adrb2 antagonism does not prevent infection after severe stroke. 97

Figure 4.10 Specific Adrb2 antagonism does not prevent infection after moderate stroke. 98

Figure 4.11 Specific Adrb2 antagonism reduces infection after moderate stroke in comparison to severe stroke. 99

Figure 4.12 Deficiency of Adrb2 does not protect from post-stroke infection. 100

Figure 5.1 The molecular structures of α-GalCer, DB06-9 and SKC8-27. 127

Figure 5.2 Pulmonary iNKT cells remain unchanged after stroke. 128

Figure 5.3 iNKT cell deficiency preserves CD4+ T cell populations in the lung after stroke. 130

Figure 5.4 iNKT cell deficient mice are neither protected or more prone to post-stroke lung infection. 131

Figure 5.5 Treatment with α-GalCer reduces post-stroke lung infection. 133

Figure 5.6 α-GalCer treatment enhances systemic IFN-γ production and lowers lung IL-10 134

Figure 5.7 α-GalCer treatment does not protect iNKT cell deficient mice from bacterial lung infection. 135

Figure 5.8 The structural analogues of α-GalCer, DB06-9 and SKC8-27, enhance antibacterial immunity. 137

Figure 5.9 Analogue treatment after stroke does not reduce post-stroke lung infection. 139

Figure 5.10 Glycolipid treatment after stroke does not alter long-term recovery of cerebral oedema. 141

Figure 5.11 Treatment with α-GalCer could not reduce artificial infection at 7 h following stroke. 142

Figure 6.1 Hypothesised temporal profile of mechanisms to post-stroke infection. 159

List of abbreviations

30 min tMCAO: 30 minute transient MCAO

60 min tMCAO: 60 minute transient MCAO

6-OHDA: 6-hydroxydopamine

Adrb1: β1 Adrenergic receptor

Adrb2: β2 Adrenergic receptor

Adrb3: β3 Adrenergic receptor

ALT: Alanine-aminotransferase

ANS: Autonomic nervous system

APC: Antigen presenting cell

BCL-6: B cell lymphoma 6 protein

BHI: Brain heart infusion

C: Carbon

cAMP: Cyclic adenosine monophosphate

CCL: Chemokine C-C motif ligand

CCR-: C-C chemokine receptor

CFU: Colony forming units

CNS: Central nervous system

CXCL-: C-X-C motif chemokine ligand

CXCR: C-X-C chemokine receptor

DA: Dopamine

DAMPS: Danger associated molecular patterns

DBH: Dopamine-β-hydroxylase

DHPG: Dihydroxyphenylglycol

DMSO: Dimethyl sulfoxide

DOPA: 3,4-dihydroxyphenylalanine

DOPAC: dihydroxyphenylacetic acid

E4BP4: E4 promoter-binding protein 4

EAE: Experimental autoimmune encephalomyelitis

HLA-DR: Human leukocyte antigen-DR

HMGB1: High-mobility group box 1

HPA axis: Hypothalamic-pituitary-adrenal axis

HSC: Haematopoietic stem cells

IFN-γ: Interferon γ

Ig: Immunoglobulin

IL: Interleukin

iNKT: Invariant natural killer T cell

LAAAD: L-aromatic-amino acid decarboxylase

LB broth: Lauria-Bertani broth

LPS: Lipopolysaccharide

MAO: Monamine oxidase

MCAO: Middle-cerebral artery occlusion

MHC: Major histocompatability complex

NA: Noradrenaline

NGT: Nasogastric tubing

NK cell: Natual killer cell

NKT cell: Natural killer T cell

PAMPS: Pathogen associated molecular patterns

PAT: Preventive antibiotic therapy

PBMCs: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCT: Procalcitonin

PET: Positron emission tomography

PKA: Protein kinase A

PLZF: Promyelocytic leukemia zinc finger protein

pMCAO: Permanent middle cerebral artery occlusion

PNMT: Phenylethanolamine N-methyltransferase

PPL: Propranolol

PRV: Psudorabies virus

PSNS: Parasympathetic nervous system

PT: Photothrombotic

RAGE: Receptors for advanced glycation end products

RBC: Red blood cell

RORγt: Retinoid-related orphan receptor γt

RT: Room temperature

SEM: Standard error of the mean

SNS: Sympathetic nervous system

SPF: Specific pathogen free

TBI: Traumatic brain injury

TCR: T cell receptor

Th1: T-helper 1

Th2: T-helper 2

tMCAO: Transient middle-cerebral artery occlusion

TNF-α: Tumour necrosis factor α

TYR: Tyrosine

WT: Wildtype

α-GalCer: α-galactosylceramide

β-AR: β Adrenergic receptor

Chapter One

Literature review

Overview

This chapter will begin by including a published review of the literature that describes stroke and frequent complications associated with this disease. In particular, this review highlight that a major contributor to complications following stroke is bacterial infection and outlines the diverse biological pathways that contribute to the development of post-stroke infection. This review was published in the journal *Brain, Behaviour, and Immunity* (2018).

In exploring the potential mechanisms of post-stroke infection in this thesis, I have specifically expanded on the role of the sympathetic nervous system (SNS) and further discuss the pathways to SNS-controlled immunity. Moreover, research in our laboratory has previously descried the contribution of invariant natural killer T (iNKT) cells in mediating stroke-induced immunosuppression. As such, I will provide the background related to this research and examine the capacity of targeting iNKT cells using therapeutic interventions to combat post-stroke infection

Published literature review

Brain, Behavior, and Immunity 70 (2018) 10–20

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Review Article

Complex interplay of multiple biological systems that contribute to post-stroke infections

Raymond Shim, Connie H.Y. Wong *

Centre for Inflammatory Diseases, Department of Medicine, Monash Medical Centre, Monash University, Clayton, Victoria, Australia

ARTICLE INFO

Article history:
Received 11 December 2017
Received in revised form 14 March 2018
Accepted 14 March 2018
Available online 21 March 2018

Keywords:
Stroke
Immunosuppression
Bacterial infection
Therapeutics

ABSTRACT

Stroke is a leading contributor of death and disability around the world. Despite its recognised debilitating neurological deficits, a devastating clinical complication of surviving stroke patients that needs more attention is infection. Up to half of the patients develop infections after stroke, and a high proportion of them will die as a direct consequence. Major clinical trials that examined preventive antibiotic therapy in stroke patients have demonstrated this method of prevention is not effective as it does not reduce incidence of post-stroke pneumonia or improve patient outcome. Additionally, retrospective studies evaluating the use of β-blockers for the modulation of the sympathetic nervous system to prevent post-stroke infections have given mixed results. Therefore, there is an urgent need for more effective therapeutic options that target the underlying mechanisms of post-stroke infections. The understanding that infections are largely attributable to the "stroke-induced systemic immunosuppression" phenomenon has begun to emerge, and thus, exploring the pathways that trigger post-stroke immunosuppression is expected to reveal potential new therapeutics. As such, we will outline the impacts that stroke has on several biological systems in this review, and discuss how these contribute to host susceptibility to infection after stroke. Furthermore, the emerging role of the gut and its microbiota has recently come to surface and intensifies the complex pathways to post-stroke infection. Finally, we identify potential avenues to combat infection that target the pathways of stroke-induced systemic immunosuppression to ultimately improve stroke patient outcome.

Contents

1. Clinical relevance of post-stroke infection . . . 11
2. Clinical trials to prevent infections after stroke . . . 11
   2.1. Antibiotics . . . 11
   2.2. Beta-blockers . . . 12
3. The impact of stroke on biological pathways . . . 12
   3.1. Stroke and neurological complications . . . 12
   3.2. Immune changes after stroke . . . 13
      3.2.1. Local immune changes . . . 13
      3.2.2. Systemic immune changes after stroke . . . 13
      3.2.3. The relationship of stroke severity and infarct location on immunosuppression . . . 14
      3.2.4. Immune exhaustion . . . 14
   3.3. Sympathetic activation in post-stroke infection . . . 15
   3.4. Gut microbiome as a source of post-stroke infection . . . 15
4. Potential therapeutics to post-stroke infections . . . 17

* Corresponding author at: Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, VIC 3168, Australia.
E-mail address: connie.wong@monash.edu (C.H.Y. Wong).

https://doi.org/10.1016/j.bbi.2018.03.019
0889-1591/

5. Concluding remarks . . . 17
Disclosure of potential conflicts of interest . . . 18
Funding . . . 18
References . . . 18

1. Clinical relevance of post-stroke infection

Stroke is a highly prevalent and debilitating neurovascular disease that continues to be a leading cause of death and disability around the world. In 2015, strokes accounted for almost 12% of all deaths globally and contributed to the second highest loss of disability-adjusted life years (DALYs) (Feigin, 2016; Feigin et al., 2017). With better healthcare over the past decades, mortality rate from strokes has decreased, however the disease incidence continues to increase, and it is predicted to rise by 36% by 2030 (Strong et al., 2007). As a consequence, stroke creates a hefty financial burden on the health care system of society (Mozaffarian et al., 2015).

Stroke occurs when there is a blockage (ischemic) or rupture (haemorrhagic) of a cerebral blood vessel and impairs blood flow into the brain. Deprivation of blood and nutrients to areas of the brain during stroke can result in severe neurological deficits and patients suffer a range of functional impairments (Kimura et al., 2004). However, a less well known, but a highly prevalent clinical complication that stroke patients suffer is infection, with pneumonia and urinary tract infections (UTIs) being most common (Langhorne et al., 2000; Vernino et al., 2003). Infections have been reported to occur in up to two-thirds of stroke survivors, with patients that have more severe strokes at greater risk (Hug et al., 2009; Langhorne et al., 2000). Some studies have suggested that infection may be a marker/symptom of the extent of cerebral damage (Vargas et al., 2006). However, there has been more evidence to suggest that post-stroke infection (namely pneumonia) is independently associated with poor patient outcome and may even contribute to mortality in up to 30% of stroke patients (Aslanyan et al., 2004; Heuschmann et al., 2004; Hilker et al., 2003; Kwan and Hand, 2007; Vermeij et al., 2009). Furthermore, stroke patients with pneumonia may be at higher risk of recurring stroke (Erdur et al., 2015), potentially due to mechanisms such as alterations in immunohematological mechanisms, pro-inflammatory cytokine activation, platelet activation, and endothelial dysfunction (Emsley and Hopkins, 2008). The current standard treatment for stroke patients diagnosed with infection is prompt administration of antibiotics, however caution needs to be taken regarding this therapeutic approach due to potentially harmful adverse effects, and an alarming rise in antibiotic-resistant strains of bacteria (Yan et al., 2015). As such, there is an urgent unmet clinical need for more effective and targeted treatments to reduce infections after stroke. Therefore, this review will describe the current and emerging treatments to post-stroke infection, outline the biological mechanisms underlying the increased susceptibility to infections in stroke patients (focussing on the post-acute phase), and highlight some potential targeted therapeutics.

2. Clinical trials to prevent infections after stroke

2.1. Antibiotics

Antibiotics are antibacterial agents that target various essential bacterial components, processes, and functions to either inhibit the growth or directly kill the microbe. While antibiotics are heavily relied upon to treat infectious diseases, antibiotics have not improved over the last 20 years and there is a growing clinical concern of antibiotic-resistant strains of bacteria (Gould, 2016). Despite this, research has been conducted to assess the efficacy of preventive antibiotic therapy (PAT) to prevent infection, its associated complications and improve stroke patient outcome. Experimental models provide proof-of-concept by showing that PAT could improve survival rates in post-stroke animals by preventing infections and reducing neurological deficits (Hetze et al., 2013; Meisel et al., 2004). Indeed, these studies gave justification for the evaluation of PAT in randomized clinical trials, although the beneficial outcomes of PAT were not observed in stroke patients in preliminary trials (Chamorro et al., 2005; Harms et al., 2008). Consistent with these studies, trials within the last 5 years, including the Preventive Antibiotics in Stroke Study (PASS) (Westendorp et al., 2015) and STROKE-INF (Kalra et al., 2015) trials, did not show an improvement in patient outcome and suggest that PAT is not better than standard treatment. Recent meta-analyses also provide further overwhelming evidence that reaffirms the ineffectiveness of PAT in improving stroke patient outcome (van de Beek et al., 2009; Vermeij et al., 2018; Zheng et al., 2017). Importantly, while PAT could reduce the incidence of overall infection, most studies did not show a reduced incidence of pneumonia in patients that underwent PAT (Kalra et al., 2015; van de Beek et al., 2009; Vermeij et al., 2018; Westendorp et al., 2015), which may suggest that pneumonia is a greater contributor to worsened stroke patient outcome. Thus, while antibiotics are effective for therapeutic treatment of infection, their prophylactic benefits are not observed and cannot be recommended to clinically prevent infections in stroke patients.

The most recent published trial for PAT was the STRAWINSKI trial which used circulating procalcitonin (PCT) levels in patient blood to predict the onset of stroke-associated pneumonia and treat prophylactically with antibiotics (Ulm et al., 2017). Patients with a PCT level of greater than 0.05 ng/ml were considered likely to get infection and were treated with antibiotics, while the control group was given standard care. The types of antibiotics used in the trial were inconsistent between treating physicians and there was only a 65% compliance of protocol. As such, there was no difference in infection rates between PCT-guided group and the control group between 0 and 7 days after stroke and no improvement of stroke outcome was observed as assessed by modified Rankin Scale. Based on this study and others, PAT is not recommended as it was not better than standard treatment (Kalra et al., 2015; Ulm et al., 2017; Westendorp et al., 2015).

The use of PAT is furthermore discouraged as it comes with the growing concern of antibiotic resistance. A range of antibiotics were tested against bacteria in the sputum samples of stroke patients with pneumonia. Culturable bacteria commonly associated with pneumonia were tested for, including *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Surprisingly, there was a 100% resistance rate in *S. aureus* against penicillin, erythromycin, oxacillin and ciprofloxacin, 100% resistance rate in *E. coli* against ceftriaxone and ticarcillin, and 100% resistance rate in *P. aeruginosa* against ceftriaxone (Yan et al., 2015). Indeed, while this study was conducted in a single hospital and is not representative of hospitals around the world, this study may explain the failure of antibiotic treatment in patients with post-stroke pneumonia.

Antibiotics may also come with the cost of adverse effects. One example is in the Early Systemic Prophylaxis of Infection After

Stroke study (ESPIAS) where the fluoroquinolone, levofloxacin, was chosen as the antibiotic for the PAT group (Chamorro et al., 2005). Compared to the placebo group, not only was levofloxacin prophylaxis unable to reduce infection rates within 7 days post-stroke, it was also associated with worsened stroke outcome at 90 days post-stroke (Chamorro et al., 2005). Although, worsened stroke outcome was not observed by 11 days post-stroke in the Preventive Antibacterial Therapy in Acute Ischemic Stroke (PANTHERIS) trial, which used the fourth-generation fluoroquinolone, moxifloxacin (Harms et al., 2008). This may suggest that adverse effects of fluoroquinolones occur in the post-acute phase of stroke. Complementary animal studies using the fluoroquinolone, enrofloxacin, revealed no differences in behavioral and neurological impairment within 1 week post-stroke in the enrofloxacin-treated group (Zierath et al., 2015). However, by 1 month post-stroke, enrofloxacin-treated animals began displaying worsened neurological impairment (Zierath et al., 2015). These detrimental effects on stroke outcome may be due to the γ-aminobutyric acid (GABA) inhibitory and/or the excitatory properties of fluoroquinolones (Chamorro et al., 2005; Sarro and Sarro, 2001), and thus their use to treat patients with stroke or other neurological disorders must be cautioned. Further evidence arguing against the use of preventive antibiotics for stroke-associated infection have demonstrated antibiotic therapies disrupt the natural microbiota of the gastrointestinal tract, resulting in complications such as increases in gut barrier permeability (Bischoff et al., 2014) and risk of inflammatory bowel disease (Shaw et al., 2011). To investigate the impact of antibiotic pre-treatment on stroke outcome, one study used a quintuple broad-spectrum antibiotic cocktail to deplete the gut microbiota, and then stopped antibiotic treatment 72 h prior to stroke surgery in mice (Winek et al., 2016b). From five days following stroke, clinical symptoms of colitis were observed in microbiota-depleted animals – as evident by weight loss, severe diarrhoea, and intestinal epithelia damage – and resulted in greater mortality rates compared to post-stroke animals with specific-pathogen-free (SPF) microbiota. By recolonising microbiota-depleted animals with SPF microbiota, post-stroke outcomes were improved and demonstrates the deleterious effects of preventive antibiotic therapy on stroke outcome. While this is an extreme scenario that does not exactly reflect what is usually achieved by the normal antibiotic regimens used in patients, this study does highlight the importance of keeping a normal gut microbiota to maintain stroke patient health and importantly suggests that future research should explore antibiotic-independent therapies to treat post-stroke infection.

2.2. Beta-blockers

With the challenges of antibiotics, new approaches are urgently needed to reduce stroke-associated infection. Stroke has been recently identified to induce robust changes to immunity, ultimately resulting in immunosuppression and leaving the host at greater risk to infection (Shim and Wong, 2016). One mechanism in which post-stroke immunosuppression is believed to occur is through activation of the sympathetic nervous system (SNS) (Schulze et al., 2014). Effector molecules of the SNS can act upon the adrenergic receptors on immune cells to induce immunosuppressive effects and thus studies exploring the potential of adrenergic receptor blockers to reduce stroke-associated infection have been conducted (Maier et al., 2015; Sykora et al., 2015). Several experimental studies have used β-blockers to inhibit downstream SNS activation to reduce symptoms of post-stroke immunosuppression and decrease infection (Prass et al., 2003; Wong et al., 2011). This prompted retrospective studies into β-blocker use and its association to infections after stroke. Promising results were found in several studies, where β-blocker use (either pre-stroke or on-stroke use) was associated with reduced incidences of infection and improved mortality rates (Dziedzic et al., 2007; Sykora et al., 2015). However, other studies found no improvements in infection incidence or stroke patient outcome with β-blocker use (Barer et al., 1988; Maier et al., 2015). Additional retrospective studies did not find an association of β-blocker use with reduced infection rates. In fact, in the PASS study, β-blocker use was found as a risk factor for infections as patients were twice as likely to get infections and mortality and unfavourable outcomes were worse off after 3 months (Westendorp et al., 2016). It is unclear whether selective or non-selective β-blockers were used by stroke patients in the PASS study, however future studies should differentiate types of β-blockers used. Indeed, non-selective β-blocker use within 3 days following admission was associated with increased infection rates, while selective β-blocker use was not associated with infection (Starr et al., 2017). These studies give merit for a well-designed randomised clinical trial to provide a definitive answer to whether β-blockers can be used to prevent or treat post-stroke infection.

A common flaw which come with antibiotics and β-blockers that contributes to their failure is their lack of specificity to the underlying mechanisms of post-stroke infection. Changes to the body following stroke disrupts multiple biological processes that may ultimately leave patients more susceptible to infections and thus discovery of a single "cure-all" treatment is improbable. It is likely that treatments may require multiple of highly specific therapeutics that can be used in combination and target the precise mechanisms of post-stroke susceptibility to infection. Therefore, understanding the impact of stroke on the complex interplay of the biological pathways that result in infections is required to identify therapeutic targets.

3. The impact of stroke on biological pathways

3.1. Stroke and neurological complications

Pneumonia is a devastating complication as it worsens stroke patient outcomes (Vermeij et al., 2009). It is well understood that the neurological complications that occur in stroke patients can result in dysphagia (difficulty in swallowing) and aspiration (entry of material into the respiratory tract), leaving patients at greater risk of pneumonia (Hoffmann et al., 2017; Teramoto, 2009). In fact, stroke patients with dysphagia that aspirate are 11-times more likely to develop pneumonia (Martino et al., 2005). The pathophysiology of dysphagia and aspiration is multi-factorial and includes factors such as substance P levels, damage to cortical and subcortical regions of the brain, decreased level of consciousness, mechanical ventilation, gastric tube feeding, and patient immobility (Cohen et al., 2016; Teramoto, 2009). Until recently, dysphagia and aspiration were believed to be the main contributors to infections after stroke. However, experimental and clinical studies suggest that dysphagia and aspiration alone cannot explain the high incidence of post-stroke pneumonia (Hoffmann et al., 2017; Teramoto, 2009). Experimentally, to cause pneumonia and bacteraemia to a similar degree, sham-operated control animals required an inoculation of 1000-fold more *Streptococcus pneumoniae* compared to post-stroke animals (Prass et al., 2006). This notion is also supported in clinical settings, whereby healthy patients that aspirate to a similar extent to stroke patients do not develop pneumonia (Harms et al., 2008). Furthermore, aspiration only occurs in half of the stroke patients that develop pneumonia (Riquelme et al., 1996). Therefore, additional factors that contribute to the susceptibility to infection in stroke patients have begun to emerge, such as stroke-induced impairment to immunity.

3.2. Immune changes after stroke

3.2.1. Local immune changes

The hypoxic environment in the brain during a stroke restricts the delivery of nutrients, disrupts ATP synthesis to cause cell apoptosis and necrosis at the ischemic core, whereby dying cells release danger associated molecular patterns (DAMPs) (Liesz et al., 2015a). DAMPs can activate nearby microglial cells through pattern recognition receptors (PRRs) to induce downstream inflammatory pathways such as activation of NF-κB and the mitogen-activated protein (MAP) kinase pathways (Yilmaz and Granger, 2010). Activated microglia secrete pro-inflammatory molecules to initiate inflammation at the site of cerebral vascular occlusion. Shortly upon resolution of the blockage and reperfusion of the ischemic core, re-oxygenation of affected areas results in reactive oxygen species (ROS) production by mitochondrial mediated mechanisms and adds to the highly inflammatory environment (Abramov et al., 2007). While this inflammatory environment is crucial for initiation of wound healing and clearance of cell debris, excessive recruitment of peripheral immune cells is detrimental to the brain. In fact, the infiltration of peripheral immune cells promotes tissue swelling and increases the intracranial pressure in the brain within the skull, which has been shown to increase fatalities by 80% in stroke patients with malignant MCA infarction (Vahedi et al., 2007). In addition, various immune cells have roles in infarct development, which have been previously described (reviewed in (Shim and Wong, 2016)). It is conceivable that cerebral inflammation that occurs within hours after stroke onset promotes secondary damage and further collateral brain injury within the confines of the skull. An emerging dogma that is gaining momentum suggests that to limit the damage to the brain, there is a subsequent systemic immune shift from a pro-inflammatory to an anti-inflammatory dominant state. This shift in systemic immunity arises after a localised brain injury induced by stroke is thought to occur as a neuroprotective mechanism that attempts to dampen the highly inflammatory environment in the brain after a stroke (Prass et al., 2003; Römer et al., 2015). Despite this, it is becoming clear this immune shift renders the host unable to fight off pathogens and becomes susceptible to infections.

3.2.2. Systemic immune changes after stroke

Over the past two decades, there have been experimental and clinical evidence to show large changes in systemic immunity. Within the first 4–6 h after stroke, systemic inflammation has been reported with increased levels of interleukin- (IL-) 6, interferon- (IFN-) γ, and CXCL-1 in the plasma of post-stroke animals (Chapman et al., 2009; Offner et al., 2006a). However, the initial systemic inflammation is short-lasting and is followed by severe immunosuppression. This is particularly apparent from the dramatic changes in major lymphoid organs that are critical for the development of innate and adaptive immune responses after stroke. There have been suggestions that splenic contraction may be partially due to mobilisation of macrophages and monocytes into the bloodstream which may contribute to cerebral inflammation and infarct development (Ajmo et al., 2008; Pennypacker and Offner, 2015) Although, macroscopic shrinking of the spleen and thymus observed in post-stroke mice can be largely attributed to apoptosis of splenic and thymic T, B and NK cells within 12 h post-stroke (Prass et al., 2003). This was seen to extend to up to 96 h post-stroke, where a 90% decrease in splenocyte numbers was seen in post-stroke animals (Offner et al., 2006b). Atrophy of the spleen post-stroke resulted in a decrease T cell mitogenic factors, as well as increased numbers and activation of splenic regulatory T cells (Tregs) that secret the anti-inflammatory cytokine IL-10 (Offner et al., 2006a,b). As a result, T cells are unable to proliferate or secrete the required pro-inflammatory and bactericidal cytokines, and hence increases the host susceptibility to infections after stroke. Consistent with these animal studies, ultrasound findings in a clinical setting showed around 40% of stroke patients have reduced splenic volume (Vahidy et al., 2016). Furthermore, stroke patients who later developed infections had reduced splenic volume, decreased numbers of circulating B cell and effector T cells, but with increased Treg cell numbers and levels of IL-10 compared to healthy hospital controls (Jiang et al., 2017).

Stroke-induced immunosuppression is evident in a range of immune cell types. Innate immune cells are the first line of defence against invading pathogens. Despite increased systemic granulocyte numbers after stroke, infections still readily occur (Laban et al., 2015; Westendorp et al., 2011). This suggests a functional impairment and inability to target pathogens by innate immune cells, such as neutrophils and monocytes. Neutrophils are the first responders to sites of injury and infection, where they secrete soluble inflammatory and anti-microbial factors (Mantovani et al., 2011). Neutrophils are also known to catch pathogens by release of neutrophil extracellular traps (NETs), and phagocytose, and kill internalised pathogens by oxidative burst (Brinkmann et al., 2004). In patients with ischemic stroke, the efficiency of NETosis was found to be impaired at 1 day following stroke but returned to baseline levels at day 5 upon *in vitro* stimulation (Ruhnau et al., 2014). Additionally, oxidative burst in neutrophils were drastically decreased shortly after stroke and up to 7 days compared to healthy controls, whereas there was no difference in neutrophil migration (Ruhnau et al., 2014). Similarly, in a small cohort of haemorrhagic stroke patients, a 40% reduction in neutrophil oxidative burst for up to 10 days was observed following stroke (Seki et al., 2010). Future studies to rescue neutrophil bactericidal function may be warranted although care should be taken as impairment of neutrophil function may be a mechanism to prevent recurring stroke. Not only were neutrophils the most abundant immune cell in thrombi of ischemic stroke patients, some neutrophils were also seen to be initiating NETosis in histological analysis (Laridan et al., 2017). Thrombolytic therapies with tissue plasminogen activator (t-PA) in combination with DNAse to account for presence of neutrophils and NETs in clots may present as a more effective treatment regime than t-PA alone in reducing thrombus weight (Laridan et al., 2017).

Macrophages are derived from circulating monocytes, and similar to neutrophils, can phagocytose pathogens and kill through oxidative burst (Sica and Mantovani, 2012). Macrophages influence immunity through secretion of a range of cytokines and are also critical in bridging innate and adaptive immunity, whereby presentation of their major histocompatibility complexes (MHC) (or human leukocyte antigen [HLA]) along with other costimulatory molecules prime T cells to fight infections (Sica and Mantovani, 2012). Pioneer observations show reduced HLA-DR expression and TNF-α production by monocytes, despite increased monocyte numbers (Harms et al., 2008; Urra et al., 2009). When comparing circulating monocytes from stroke patients with or without infection, monocytes taken from stroke patients with infections had reduced efficacy in oxidative burst activity and thus emphasises the role of monocytes/macrophages in post-stroke infections (Ruhnau et al., 2014). Experimentally, downregulation of MHC class II was seen in macrophages of post-stroke rats. In fact, the reduced expression of MHC class II was observed in alveolar macrophages at 3 days post-stroke and may offer an explanation to susceptibility to post-stroke bacterial pneumonia (Deng et al., 2016). 'Endotoxin tolerance' has recently been shown in monocytes post-stroke, whereby these cells were unable to produce tumour necrosis factor- (TNF-) α and IFN-γ to initiate a bactericidal immunity upon lipopolysaccharide (LPS) stimulus (Deng et al., 2016; Haeusler et al., 2008; Hernández-Jiménez et al., 2017). Interestingly, a role of t-PA to reduce inflammatory pathways has been

described, such that LPS-induced expression of TNF-α, IL-1b, IL-6 and CCL3 by bone marrow derived macrophages was suppressed by either inactive or active forms of t-PA (Mantuano et al., 2017). In addition, HLA-DR expression was lower in LPS-stimulated monocytes from stroke patients compared to healthy volunteers (Hernández-Jiménez et al., 2017). However, it is currently unknown whether MHC expression on other professional antigen presenting cells (APCs) are affected.

While there is evidence to confirm the contribution of stroke-induced innate immunosuppression to the susceptibility to infection, immunosuppression can persist for months and years following ischemia (Theodorou et al., 2008; Zhang et al., 2014). In fact, throughout a 10-year period, over 17% of stroke patients were readmitted due to infection (Rohweder et al., 2017). Stroke triggers a complex and multi-phasic immune response, with prolonged modulation of the host innate and adaptive immunity (Theodorou et al., 2008; Zhang et al., 2014). The most profound stroke-induced effect that occurs is the drastic loss of circulating, splenic, and thymic T cells (Offner et al., 2006b; Prass et al., 2003; Ruhnau et al., 2016; Wong et al., 2017). Along with this, T cell immunity shifts from a pro-inflammatory T-helper 1 (Th1) type to an anti-inflammatory T-helper 2 (Th2) type immune response (Prass et al., 2003). A Th2 immunity is characterised by increased levels of immunosuppressive cytokines such as IL-4 and IL-10, whereas a Th1 immunity is characterised by the dominance of inflammatory cytokines like IFN-γ and TNF-α. Regulation of the Th1/Th2 immune balance is critical in health and disease, where a Th1-skewed response is commonly preferred to combat infections, while a Th2-skewed immunity is generally favoured to alleviate inflammation and inflammatory diseases. We and others have described a systemic lowering of the Th1/Th2 ratio that occurs resulting from increased production of IL-4 and IL-10 in stroke patients (Prass et al., 2003; Theodorou et al., 2008; Wong et al., 2017). Clinically, it was demonstrated that this Th2 dominant immunity persisted for over 34 months following stroke onset (Theodorou et al., 2008). Furthermore, work from our lab recently reported that IL-10 levels were significantly higher in stroke patients that developed infections (Wong et al., 2017), which is validated by earlier observations from the ESPIAS trial (Chamorro et al., 2006). In addition to lowered numbers of T cells and a Th2-skewed response, reduced MHC class II expression and ineffective costimulation for T cell activation is also evident after stroke (Hug et al., 2011). Taken together, there is a growing body of evidence indicating impaired T cell immunity after stroke.

B cells have a role in production of antibodies, as well as phagocytosis and antigen presentation (Mauri and Bosma, 2012). Marginal zone B cells have roles in rapidly (within 1–3 days) producing immunoglobulin M (IgM) in response to bacterial infection. Despite their importance in host defence and bacterial clearance, little is known about suppression or dysfunction of B cell activity following stroke. Recently, it was revealed that there was a loss of splenic B cells via apoptosis, reduced antigen capture, and a decrease in circulating IgM levels that lead to bacterial lung infections from 1 day following stroke onset in experimental mice. These findings were paralleled in stroke patients whereby a significant reduction in circulating IgM levels occurred within 3 h following stroke compared to hospital controls, and stroke patients that acquired infection had even less IgM than stroke patients without infections (McCulloch et al., 2017). In a separate study, it was found that serum IgG levels in stroke patients was reduced, and this reduction was even more prominent in patients with stroke-associated infection (Liesz et al., 2015b). As deficiencies in immunoglobulin levels is a risk-factor for infections (Mawhorter and Yamani, 2008), preventing the loss of immunoglobulin-producing B cells may prevent stroke-associated infections, although the underlying mechanisms in immunoglobulin loss are poorly understood.

3.2.3. The relationship of stroke severity and infarct location on immunosuppression

The extent of post-stroke immunosuppression may also be dependent on factors such as severity of stroke and the location of cerebral infarct. During the post-acute stage of ischemic stroke, extensive infarct result in reduced spleen size and splenic cellularity due to impaired regulation of the sympathetic and parasympathetic nervous systems (Mracsko et al., 2014). Additionally, larger cerebral infarcts or increased stroke severity was associated with increased IL-10 production, a decrease in Th1/Th2 ratio (Wong et al., 2017) and, impaired monocyte function and lymphocytopenia (Hug et al., 2009). In fact, in one study, extent of brain injury was a predictor for post-stroke pneumonia (Hug et al., 2009). Overall, it is understood that larger infarct size predisposes to greater susceptibility to infection and worse outcomes for stroke patients (Vargas et al., 2006). However, the relationship between infarct size and location with immunosuppression to result in post-stroke infection is less clear (Minnerup et al., 2010). Lesions localising within the superior and middle temporal gyri were recently reported to be associated with immunosuppression (characterised by lymphopenia and loss of HLA-DR expression on monocytes) and increased incidence of pneumonia after stroke (Urra et al., 2017). Other regions of the brain, such as the insular cortex, has garnered previous attention as damages to this brain region has been associated with increased plasma catecholamine levels (Walter et al., 2013). Furthermore, infarct involvement of the insular cortex has been associated with neutrophil and regulatory T cell alterations, and an increased risk of post-stroke infection (Walter et al., 2013). Further studies are required to elucidate the relationship between infarct location and infection.

3.2.4. Immune exhaustion

While there is overwhelming evidence supporting post-stroke immune impairment in both innate and adaptive immunity, the precise mechanisms contributing to stroke-induced immunosuppression require further elucidation. One suggestion for stroke-induced immunosuppression is the induction of an immediate (within hours) systemic pro-inflammatory response following stroke to exhaust the immune response which leaves the host susceptible to infection (Offner et al., 2006a). A recent study identified high-mobility group box 1 (HMGB1) protein in its role in exhausting immunity and proposed a model of post-stroke immunosuppression (Liesz et al., 2015a). During the early phases of stroke, release of HMGB1 from dying neurons escapes into the circulation where it can act on receptors for advanced glycation end products (RAGEs) on monocytes in the bone marrow and spleen. The activation of splenic monocytes results in the secretion of pro-inflammatory cytokines and induces the 'sickness behavior' seen shortly after stroke. Furthermore, HMGB-1 stimulation of RAGE on bone marrow-derived monocytes promotes their expansion and release into the circulation during the subacute phase of stroke. These bone marrow-derived monocytes are functionally similar to myeloid derived suppressor cells (MDSCs) in their ability to suppress T cell immunity through arginase-1 (Arg-1) secretion (Liesz et al., 2015a). The findings of this study demonstrating HMGB-1-mediated expansion of bone marrow-derived monocytes may explain the increased circulating monocyte numbers in the blood from 1 day following stroke in patients (Vogelgesang et al., 2008), and the subsequent loss of T cells via apoptosis mediated by Arg-1 (Sippel et al., 2015). Nonetheless, the researchers of this study also recognise the role of catecholamines in influencing post-stroke immunity independently to the HMGB-1/RAGE pathway (Liesz et al., 2015a).

3.3. Sympathetic activation in post-stroke infection

The emerging concept of "brain-controlled immunity" offers an additional explanation for post-stroke immunosuppression. Catecholamines, effector molecules of the SNS, are described to shift immunity to a Th2 dominant response following stroke (Prass et al., 2003). Indeed, circulating catecholamine levels are elevated 1–3 days following stroke (Akıl et al., 2015), suggesting that the effect of catecholamines on peripheral immune cells may be robust and have potentially long-lasting consequences. Notably, noradrenaline, produced by nerve terminals during stress, acts upon adrenergic receptors that are widely expressed on immune cells (Anne et al., 2007). In fact, most of immunosuppressive effects can be attributed to β-adrenergic receptor activation. For example, catecholamines mediate the inhibition of CTLA-4 expression on T cells (Vogelgesang et al., 2010), induces apoptosis of lymphocytes (Prass et al., 2003), reduces IFN-γ production, promotes alternatively activated (M2) macrophages (Grailer et al., 2014) and reduces oxidative burst activity in phagocytes (Ruhnau et al., 2014). Additionally, the activation of β-adrenergic receptors by catecholamines leads to downregulation of NF-κB which prevents pro-inflammatory cytokine gene transcription (TNF-a, IL-6, IL-1b) whilst promoting anti-inflammatory cytokine production (IL-10) (Stolk et al., 2016).

Many studies have shown that the immunosuppressive effects of catecholamines can be attenuated by using β-adrenergic receptor antagonists. The administration of propranolol in post-stroke mice sustained the Th1 response (Prass et al., 2003), prevented the loss of marginal zone B cells and circulating IgM (McCulloch et al., 2017). Additionally, blockade of β-adrenergic signalling preserved post-stroke spleen sizes, and retained natural killer (NK) cell bactericidal functions (Liu et al., 2017). These are all contributing factors that resulted in the reduction of infection and mortality after stroke. However, while β-adrenergic receptors are expressed widely on immune cells, it is unknown whether the effects of catecholamines act directly on immune cells or whether specific types of immune cell are responsible for inducing downstream immunosuppression following β-adrenergic receptor activation. Previous findings from our laboratory support the latter. We showed that the invariant natural killer T (iNKT) cell was able to respond to distant brain injury within 4 hrs following stroke to initiate an immune response toward a Th2 bias through the production of IL-4 and IL-10 (Wong et al., 2011). Indeed, sympathetic activation of iNKT cells was responsible for the altered host immune defence as administration of propranolol could ameliorate iNKT cell behavioral and functional impairment, systemic immunosuppression and infection rates (Vogelgesang et al., 2010). Therefore, this suggests that activation of the iNKT cell with noradrenaline promotes the downstream immunosuppression that occurs following stroke. The findings of catecholamine-mediated immunosuppression prompted clinical studies into β-blocker therapy to prevent infections following stroke. However, as discussed, retrospective studies found unfavourable outcomes were associated with β-blocker use and suggests that catecholamines do not solely explain post-stroke immunosuppression. To support this, some experimental studies found that SNS inhibitors somewhat prevented the loss of NF-κB (Zuo et al., 2016), partially rescued MHC class II levels and LPS-induced TNF-α and IFN-γ expression in macrophages (Deng et al., 2016; Wang et al., 2016).

Dysregulation of the hypothalamic-pituitaryadrenal (HPA) axis and subsequent glucocorticoid production has also been implicated in stroke-induced immunosuppression but to a lesser extent than catecholamines (Mracsko et al., 2014; Prass et al., 2003). It is likely that the HPA axis and SNS activation work in unison following stroke to contribute to immunosuppression. Combination treatment to block the action of β-adrenergic receptor and glucocorticoid receptor in post-stroke mice were superior in preventing splenic atrophy and NK cell death compared to single treatment to inhibit either β-adrenergic receptor or glucocorticoid receptor (Liu et al., 2017). Whether glucocorticoid blockers are associated with improved outcomes in stroke patients requires further elucidation.

3.4. Gut microbiome as a source of post-stroke infection

There has been an interest in the importance of the gut and its microbiota in neurological diseases (Bansal et al., 2009; Houlden et al., 2016). Subtle hints that have risen throughout the literature in the past decade point to the involvement of the gut microbiota in post-stroke infections. One of the earliest studies found that at least 95% of culturable bacteria from the blood and lungs of post-stroke mice were made up of *E. coli* – a common gut microbe (Prass et al., 2003). In some stroke patients however, causative bacteria that contribute to pneumonia could not be detected in sputum samples (Vargas et al., 2006; Walter et al., 2007), potentially suggesting the presence of anaerobic bacteria that require extremely specific growth conditions (Westendorp et al., 2011). SNS-induced dysfunction of gastrointestinal tract motility was later identified to occur following stroke (Schaller et al., 2006), while others showed impairments in intestinal permeability in different forms of brain injury (Bansal et al., 2009). Additionally, it was recently revealed that changes in microbial diversity occurred in stroke and transient ischemic attack patients, whereby a depletion of beneficial bacteria, belonging to *Bacteroides*, *Prevotella*, and *Faecalibacterium* genera, was detected in faecal samples (Yin et al., 2015). More recent evidence from our laboratory and others have demonstrated the importance of the gut microbiota in post-stroke infections (Crapser et al., 2016; Stanley et al., 2016). We described the dissemination of gut bacteria into the periphery following stroke (Stanley et al., 2016). In a small cohort of infected post-stroke patients, we found over 70% of culturable bacteria from the blood, sputum and urine were bacteria normally part of the intestinal microbiota, including *E. coli*, *Enterococcus* spp., and *Morganella morganii*. Complementary to this, post-stroke SPF mice had increased bacterial loads in the lung, liver and spleen – a result not seen in post-stroke germ-free (GF) mice, suggesting bacterial infection originated from the host's own microbiota. Further bacteriological analysis in the peripheral organs of post-stroke mice revealed a significant increase of bacterial communities in the lungs, while there was a reduction of that in the ileum and colon. Moreover, SourceTracker predicted that around 60% of bacterial communities in the lungs of post-stroke mice originated from the liver and small intestine (Stanley et al., 2016). Whether microbes of the gut enter the periphery through a hematogenous route is still under dispute. We and others provide proof of concept as positive blood cultures were detected in post-stroke animals (Prass et al., 2003; Wong et al., 2011). To support the notion that pathogens may disseminate through the bloodstream after stroke, positive blood cultures have been reported in up to 10% of stroke patients with infection (Dettenkofer et al., 2001; Hilker et al., 2003; Vargas et al., 2006). There may be several reasons that can account for the low rate of positive blood cultures in stroke patients including 1) timing of blood collection, 2) presence of microorganisms that cannot be cultured with traditional culturing techniques, 3) microorganisms disseminate through a non-hematogenous route (e.g. lymphatics), and 4) causative organism may originate from an external source (e.g. gastric tubes). Nonetheless, the findings from these studies provide evidence that the causative agents of post-stroke bacterial pneumonia may be contributed by the host's own gut microbiota (Stanley et al., 2016).

For gut microbes to disseminate into the periphery, there must be a dysfunction in gut barrier integrity (Balzan et al., 2007). This has been shown in patients with brain injury where a common complication is gastrointestinal dysfunction (Pilitsis and Rengachary, 2001), characterised by the disruption of gut epithelial cell tight junctions (Hang et al., 2003). In an experimental model, we have also shown that gut barrier integrity is impaired following stroke, as evident by reduced expression of the tight junction protein Zonula occludens-1 (ZO-1) (Stanley et al., 2016). In fact, the ileum was most susceptible to post-stroke gut permeability which transiently peaked at 3 h after stroke onset. We proposed this increased permeability in the gut barrier allowed for intestinal bacteria to translocate and disseminate from the gut into the periphery. We confirmed this by inoculation of mice with *E. coli* or *Enterococcus fecalis* following stroke and subsequently detecting these bacteria only in peripheral tissue including the blood and lungs of post-stroke mice. Furthermore, we demonstrated the involvement of the SNS in inducing gut permeability post-stroke as β-adrenergic receptor blockade inhibited gut barrier permeability and significantly reduced bacterial loads in peripheral organs (Doyle and Buckwalter, 2017). Therefore, β-adrenergic receptor activation after stroke triggers robust intestinal barrier breakdown and systematic immunosuppression enabling commensal bacteria in the gut to disseminate into the periphery and allow fatal infections to take place. We have outlined the interaction between the brain, gut and immune system after stroke schematically in Fig. 1.

Clearly, the activation of the SNS can influence the gut and its microbiome, but the interaction of the gut microbiota and the brain is a two-way street. The composition of the gut microbiota has been highlighted to impact stroke outcomes. Although stroke induces dysbiosis of the gut microbiota (Houlden et al., 2016; Yamashiro et al., 2017; Yin et al., 2015), changes in gut microbiota

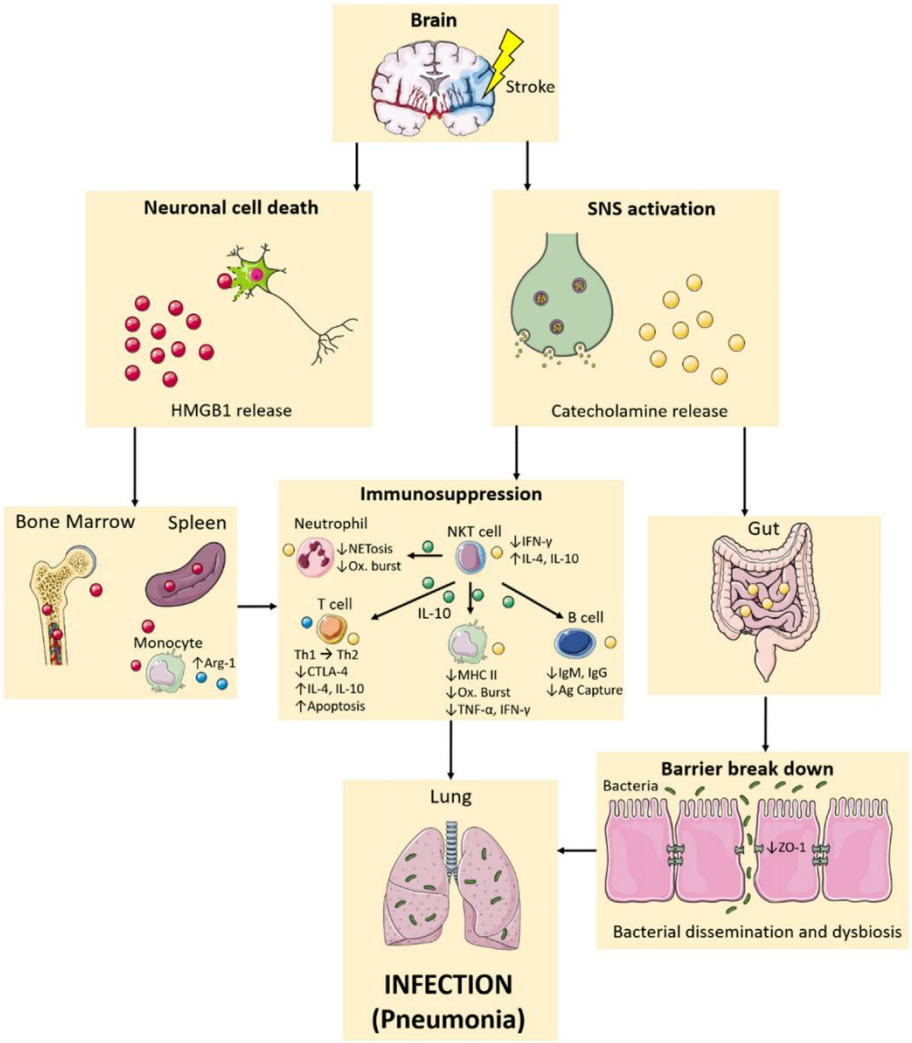


Fig. 1. The mechanisms of immunosuppression and infection after stroke. During stroke, impaired blood flow into the brain induces neuronal cell death. Consequently, dying cells release high mobility group box 1 (HMGB1) protein, which can escape into the periphery and act upon splenic and bone marrow-derived monocytes. HMGB1-activated monocytes secrete arginase-1 (Arg-1) to suppress T cells and induce apoptosis. In contrast, hyper-activation of the sympathetic nervous system (SNS) following stroke promotes catecholamine release. Catecholamines may act directly on various immune cells, or upon the natural killer T (NKT) cell to induce downstream immunosuppressive effects at a systemic level. Furthermore, SNS activation may also impact the gut, where there a reduction in Zonula occludens-1 (ZO-1) expression allows for gut barrier breakdown. As a result, the suppressed immunity is unable to fight bacteria that disseminate into the periphery and therefore stroke patients suffer from infectious complications, such as pneumonia. Images adapted from Servier Medical Art (Creative Commons).

composition has been subsequently shown to exacerbate post-stroke neuro-inflammation, potentially through the migration of IL-17- and IFN-γ secreting intestinal T cells into the brain (Singh et al., 2016). Furthermore, disrupting the natural composition of the gut flora with a combination of antibiotics before stroke onset worsened the mortality rates in post-stroke mice at days 3 and 7. This worsened outcome could be reduced by restoring microbiota-depleted mice with conventional microbiota of SPF mice (Winek et al., 2016a). These data further dissuade the use of preventive antibiotic treatments to treat post-stroke infections. In contrast, pre-stroke treatment with amoxicillin to induce gut dysbiosis could reduce brain infarct volume at 3 days following stroke (Benakis et al., 2016). In this study, it was found that the neuroprotection was due to an expansion of intestinal regulatory T cells and reduction of IL-17-producing T cells (Benakis et al., 2016). The recent discoveries of the multi-directional communication between the brain, immune system, gut and its microbiome highlight the complexity of the interplay between biological systems in stroke. Despite this intricacy, these exciting new findings and pathways allow for the unlocking of novel therapeutic potential in targeting the microbiota to treat post-stroke complication.

4. Potential therapeutics to post-stroke infections

As immunosuppression is a major risk factor for infections, the use of immuno-modulants to prevent the shift to a Th2 phenotype (or induce the shift to a Th1 phenotype) may be plausible to prevent post-stroke infection. As discussed above, the release of HMGB-1 from dying cells of the brain could leak into the periphery and act upon RAGE receptors on splenic and bone marrow immune cells to induce immune exhaustion following stroke (Liesz et al., 2015a). . The RAGE receptor is a member of the immunoglobulin superfamily that can be bound to the surface of cells or found in a soluble form (Fukami et al., 2014). Soluble form of RAGE (sRAGE) can sequester RAGE ligands and acts as a competitive inhibitor to membrane bound RAGE. This has been used in models of atherosclerosis to reduce inflammation (Ha et al., 2013), and thus may also have similar uses in stroke. In fact, sRAGE treatment in post-stroke mice was shown to reduce plasma IL-6 levels and partially attenuate spleen atrophy (Liesz et al., 2015a). As RAGE has more than one ligand (e.g. β-sheet fibrillin and advanced glycation end products), more specific sequestration of HMGB-1 using monoclonal antibodies may be preferential as treatment and has been shown to reduce systemic inflammation following stroke (Liesz et al., 2015a). Therefore, more studies are required to explore the potential of targeting the HMGB-1/RAGE pathway to reduce post-stroke systemic inflammation and immunosuppression to improve patient outcomes.

Evidently, the mechanisms to post-stroke immunosuppression and multifactorial. We showed that the Th1 to Th2 shift in stroke can be initiated though the activation of the β-adrenergic receptors on iNKT cells. This may suggest that β-blockers may be useful to ultimately prevent infections after stroke, however a number of prospective studies did not find an association of β-blocker use with reduced infection (Maier et al., 2015; Westendorp et al., 2016). Therefore, therapeutic strategies aimed at targeting iNKT cells to reverse post-stroke immunosuppression may serve as a possible therapeutic avenue. The iNKT cell response can be specifically modified using α-galactosylceramide (α-GalCer). Professional APCs present α-GalCer via CD1d to activate iNKT cells through their T cell receptors (Kawano et al., 1997). In addition to the specificity of α-GalCer in activating only iNKT cells, iNKT cells activated in this manner robustly produce both IFN-γ and IL-4 (Im et al., 2009). Indeed, administering α-GalCer therapeutically in post-stroke mice significantly reduced bacterial load in peripheral organs (Wong et al., 2011). For this therapeutic option to be translated into the clinic, we must first confirm the involvement of iNKT cells in stroke patients. In a small cohort of stroke patients, a robust and sustained activation of iNKT cells was observed in stroke patients from admission and up to 90 days following stroke onset. Additionally, iNKT cell activation was correlated with increased systemic IL-10, and a decreased Th1/Th2 ratio to demonstrate an anti-inflammatory shift in immunity following stroke (Wong et al., 2017). As such, modulation of iNKT cell responses has become an even more attractive method to reduce post-stroke infection. However, it should be noted that care should be taken when designing immuno-stimulatory therapeutics to combat infection as dampening inflammation may be a defence mechanism to reduce brain damage and prevent recurring stroke. Future studies will be required to ensure brain infarcts are not exacerbated with the use of immuno-stimulants.

The emerging role of the gut microbiota in stroke-associated infection is relatively new and extremely complex (Stanley et al., 2016), but targeting the post-stroke gut barrier breakdown offers another avenue to prevent infectious complications in stroke patients. In our study, blocking SNS activation with propranolol could prevent gut permeability, thus β-adrenergic receptor antagonists may be developed as a potential therapeutic. However, as β-adrenergic receptors are expressed on a wide range of cells throughout the body, this treatment will have a broad spectrum of effects. Additionally, the use of β-blockers to prevent post-stroke infections has not been associated with better outcomes in stroke patients and suggests exploring more targeted treatments should be the focus (Westendorp et al., 2016). Addressing gut leakiness by targeting tight junction regulation may provide more specific therapeutic options but further elucidation of these mechanisms is clearly required. The zonulin pathway of gut barrier impairment has been implicated in inflammatory diseases and autoimmunity (Fasano, 2011), but it is currently unknown whether this is implicated in stroke. Furthermore, while we provide evidence of potential paracellular bacterial transmigration via decreased ZO-1 expression, we cannot exclude the possibility of transcellular mechanisms of bacterial dissemination following stroke (Wen and Wong, 2017). It is currently unknown how the sympathetic overdrive after stroke alters gut physiology, yet it is feasible that stroke alters gut permeability by induction of intestinal epithelial or enteric neuronal cell death. Further studies will need to identify more precise mechanisms in bacterial translocation after stroke.

5. Concluding remarks

Considerable progress has been made in identifying mechanisms to develop potential therapeutics that specifically target individual pathways of post-stroke infection. These pathways include a) immune exhaustion, triggered by HMGB-1/RAGE pathway, b) immunosuppression, triggered by a shift of Th2 immunity systematically and by iNKT cells in a SNS-dependent manner, and c) dissemination of gut bacteria, due to impaired gut barrier integrity. Preventing immunosuppression through immunomodulation may be a viable option, however future studies must ensure that these do not exacerbate brain damage after stroke. Repairing gut barrier integrity may also be feasible but precise mechanisms underlying gut barrier breakdown and bacterial dissemination need to be elucidated. Further research may also consider using combination therapy to tackle the multifactorial processes that work in concert to ultimately place stroke patients susceptible to infection. However, we note that the mechanisms of post-stroke infections are not limited to the discussed pathways and thus further studies are required to elucidate other channels of

intervention. A better understanding of the precise molecular events leading up to the impaired host antibacterial defence after stroke will take us one very significant step closer to developing powerful and targeted treatment regimens for stroke patients, ultimately improving patient outcomes.

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Funding

This work was supported by National Heart Foundation (NHF, Australia; 100863) and National Health and Medical Research Council (NHMRC, Australia; APP1104036). The financial supports have no role in conducting the research and/or preparation of the article.

References

Abramov, A.Y., Scorziello, A., Duchen, M.R., 2007. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J. Neurosci. 27, 1129–1138.
Ajmo, C.T., Vernon, D.O., Collier, L., Hall, A.A., Garbuzova-Davis, S., Willing, A., Pennypacker, K.R., 2008. The spleen contributes to stroke-induced neurodegeneration. J. Neurosci. Res. 86, 2227–2234.
Akıl, E., Tamam, Y., Akıl, M.A., Kaplan, I., Bilik, M.Z., Acar, A., Tamam, B., 2015. Identifying autonomic nervous system dysfunction in acute cerebrovascular attack by assessments of heart rate variability and catecholamine levels. J. Neurosci. Rural Practice 6, 145.
Anne, M., Juha, K., Makikallio, T., Mikko, T., Olli, V., Kyosti, S., Heikki, H., Vilho, M., 2007. Neurohormonal activation in ischemic stroke: effects of acute phase disturbances on long-term mortality. Curr. Neurovasc. Res. 4, 170–175.
Aslanyan, S., Weir, C., Diener, H.C., Kaste, M., Lees, K.R., 2004. Pneumonia and urinary tract infection after acute ischaemic stroke: a tertiary analysis of the GAIN International trial. Eur. J. Neurol. 11, 49–53.
Balzan, S., de Almeida Quadros, C., De Cleva, R., Zilberstein, B., Cecconello, I., 2007. Bacterial translocation: overview of mechanisms and clinical impact. J. Gastroenterol. Hepatol. 22, 464–471.
Bansal, V., Costantini, T., Kroll, L., Peterson, C., Loomis, W., Eliceiri, B., Baird, A., Wolf, P., Coimbra, R., 2009. Traumatic brain injury and intestinal dysfunction: uncovering the neuro-enteric axis. J. Neurotrauma 26, 1353–1359.
Barer, D., Cruickshank, J., Ebrahim, S., Mitchell, J., 1988. Low dose β blockade in acute stroke ("BEST" trial): an evaluation. British Med. J. (Clin. Res. ed.) 296, 737.
Benakis, C., Brea, D., Caballero, S., Faraco, G., Moore, J., Murphy, M., Sita, G., Racchumi, G., Ling, L., Pamer, E.G., 2016. Commensal microbiota affects ischemic stroke outcome by regulating intestinal [gamma][delta] T cells. Nat. Med. 22, 516–523.
Bischoff, S.C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., Tilg, H., Watson, A., Wells, J.M., 2014. Intestinal permeability–a new target for disease prevention and therapy. BMC Gastroenterol. 14, 189.
Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. Science 303, 1532–1535.
Chamorro, A., Amaro, S., Vargas, M., Obach, V., Cervera, A., Torres, F., Planas, A., 2006. Interleukin 10, monocytes and increased risk of early infection in ischaemic stroke. J. Neurol. Neurosurg. Psychiatry 77, 1279–1281.
Chamorro, A., Horcajada, J., Obach, V., Vargas, M., Revilla, M., Torres, F., Cervera, A., Planas, A., Mensa, J., 2005. The early systemic prophylaxis of infection after stroke study a randomized clinical trial. Stroke 36, 1495–1500.
Chapman, K.Z., Dale, V.Q., Dênes, Á., Bennett, G., Rothwell, N.J., Allan, S.M., McColl, B. W., 2009. A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke. J. Cereb. Blood Flow Metab. 29, 1764–1768.
Cohen, D.L., Roffe, C., Beavan, J., Blackett, B., Fairfield, C.A., Hamdy, S., Havard, D., McFarlane, M., McLauglin, C., Randall, M., 2016. Post-stroke dysphagia: a review and design considerations for future trials. Int. J. Stroke 11, 399–411.
Crapser, J., Ritzel, R., Verma, R., Venna, V.R., Liu, F., Chauhan, A., Koellhoffer, E., Patel, A., Ricker, A., Maas, K., 2016. Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice. Aging (Albany NY) 8, 1049.
Deng, Q.-W., Yang, H., Yan, F.-L., Wang, H., Xing, F.-L., Zuo, L., Zhang, H.-Q., 2016. Blocking sympathetic nervous system reverses partially stroke-induced immunosuppression but does not aggravate functional outcome after experimental stroke in rats. Neurochem. Res. 41, 1877–1886.
Dettenkofer, M., Ebner, W., Els, T., Babikir, R., Lücking, C., Pelz, K., Rüden, H., Daschner, F., 2001. Surveillance of nosocomial infections in a neurology intensive care unit. J. Neurol. 248, 959–964.
Doyle, K.P., Buckwalter, M.S., 2017. Does B lymphocyte-mediated autoimmunity contribute to post-stroke dementia? Brain, Behav, Immun. 64, 1–8.
Dziedzic, T., Slowik, A., Pera, J., Szczudlik, A., 2007. Beta-blockers reduce the risk of early death in ischemic stroke. J. Neurological Sci. 252, 53–56.
Emsley, H.C., Hopkins, S.J., 2008. Acute ischaemic stroke and infection: recent and emerging concepts. Lancet Neurol. 7, 341–353.
Erdur, H., Scheitz, J.F., Ebinger, M., Rocco, A., Grittner, U., Meisel, A., Rothwell, P.M., Endres, M., Nolte, C.H., 2015. In-hospital stroke recurrence and stroke after transient ischemic attack: frequency and risk factors. Stroke 46, 1031–1037.
Fasano, A., 2011. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. Physiological Rev. 91, 151–175.
Feigin, V., 2016. Global, regional, and national disability-adjusted life years (Dalys) for 315 diseases and injuries and healthy life expectancy (Hale), 1990–2015: a systematic analysis for the global burden of disease study 2015. Lancet 388, 1603–1658.
Feigin, V.L., Norrving, B., Mensah, G.A., 2017. Global burden of stroke. Circulation Res. 120, 439–448.
Fukami, K., Yamagishi, S.-I., Okuda, S., 2014. Role of AGEs-RAGE system in cardiovascular disease. Curr. Pharmaceutical Des. 20, 2395–2402.
Gould, K., 2016. Antibiotics: from prehistory to the present day. J. Antimicrob. Chemother. 71, 572–575.
Grailer, J.J., Haggadone, M.D., Sarma, J.V., Zetoune, F.S., Ward, P.A., 2014. Induction of M2 regulatory macrophages through the β2-adrenergic receptor with protection during endotoxemia and acute lung injury. J. Innate Immun. 6, 607–618.
Ha, C.H., Kim, S., Chung, J., An, S.H., Park, S., Choi, D., Kwon, K., 2013. Inhibitory effect of soluble RAGE in disturbed flow-induced atherogenesis. Int. J. Mol. Med. 32, 373–380.
Haeusler, K.G., Schmidt, W.U., Föhring, F., Meisel, C., Helms, T., Jungehulsing, G.J., Nolte, C.H., Schmolke, K., Wegner, B., Meisel, A., 2008. Cellular immunodepression preceding infectious complications after acute ischemic stroke in humans. Cerebrovascular Dis. 25, 50–58.
Hang, C.-H., Shi, J.-X., Li, J.-S., Wu, W., Yin, H.-X., 2003. Alterations of intestinal mucosa structure and barrier function following traumatic brain injury in rats. World J. Gastroenterol.: WJG 9, 2776.
Harms, H., Prass, K., Meisel, C., Klehmet, J., Rogge, W., Drenckhahn, C., Göhler, J., Bereswill, S., Göbel, U., Wernecke, K.D., 2008. Preventive antibacterial therapy in acute ischemic stroke: a randomized controlled trial. PLoS One 3, e2158.
Hernández-Jiménez, E., Gutierrez-Fernández, M., Cubillos-Zapata, C., Otero-Ortega, L., Rodríguez-Frutos, B., Toledano, V., Martínez-Sánchez, P., Fuentes, B., Varela-Serrano, A., Avendaño-Ortiz, J., 2017. Circulating monocytes exhibit an endotoxin tolerance status after acute ischemic stroke: mitochondrial DNA as a putative explanation for poststroke infections. J. Immunol. 198, 2038–2046.
Hetze, S., Engel, O., Römer, C., Mueller, S., Dirnagl, U., Meisel, C., Meisel, A., 2013. Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach. J. Cereb. Blood Flow Metab. 33, 846–854.
Heuschmann, P.U., Kolominsky-Rabas, P.L., Misselwitz, B., Hermanek, P., Leffmann, C., Janzen, R., Rother, J., Buecker-Nott, H.-J., Berger, K., 2004. Predictors of in-hospital mortality and attributable risks of death after ischemic stroke: the German Stroke Registers Study Group. Arch. Internal Med. 164, 1761–1768.
Hilker, R., Poetter, C., Findeisen, N., Sobesky, J., Jacobs, A., Neveling, M., Heiss, W.-D., 2003. Nosocomial pneumonia after acute stroke: implications for neurological intensive care medicine. Stroke 34, 975–981.
Hoffmann, S., Harms, H., Ulm, L., Nabavi, D.G., Mackert, B.-M., Schmehl, I., Jungehulsing, G.J., Montaner, J., Bustamante, A., Hermans, M., 2017. Stroke-induced immunodepression and dysphagia independently predict stroke-associated pneumonia–The PREDICT study. J. Cereb. Blood Flow Metab. 37, 3671–3682.
Houlden, A., Goldrick, M., Brough, D., Vizi, E.S., Lénárt, N., Martinecz, B., Roberts, I., Denes, A., 2016. Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production. Brain, Behav., Immun. 57, 10–20.
Hug, A., Dalpke, A., Wieczorek, N., Giese, T., Lorenz, A., Auffarth, G., Liesz, A., Veltkamp, R., 2009. Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection. Stroke 40, 3226–3232.
Hug, A., Liesz, A., Muerle, B., Zhou, W., Ehrenheim, J., Lorenz, A., Dalpke, A., Veltkamp, R., 2011. Reduced efficacy of circulating costimulatory cells after focal cerebral ischemia. Stroke 42, 3580–3586.
Im, J.S., Arora, P., Bricard, G., Molano, A., Venkataswamy, M.M., Baine, I., Jerud, E.S., Goldberg, M.F., Baena, A., Karl, O., 2009. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. Immunity 30, 888–898.
Jiang, C., Kong, W., Wang, Y., Ziai, W., Yang, Q., Zuo, F., Li, F., Xu, H., Li, Q., Yang, J., 2017. Changes in the cellular immune system and circulating inflammatory markers of stroke patients. Oncotarget 8, 3553–3567.
Kalra, L., Irshad, S., Hodsoll, J., Simpson, M., Gulliford, M., Smithard, D., Patel, A., Rebollo-Mesa, I., Investigators, S.-I., 2015. Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial. Lancet 386, 1835–1844.
Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. Science 278, 1626–1629.

Kimura, K., Minematsu, K., Kazui, S., Yamaguchi, T., 2004. Mortality and cause of death after hospital discharge in 10,981 patients with ischemic stroke and transient ischemic attack. Cerebrovascular Dis. 19, 171–178.
Kwan, J., Hand, P., 2007. Infection after acute stroke is associated with poor short-term outcome. Acta Neurologica Scandinavica 115, 331–338.
Laban, K.G., Rinkel, G.J., Vergouwen, M.D., 2015. Nosocomial infections after aneurysmal subarachnoid hemorrhage: time course and causative pathogens. Int. J. Stroke.
Langhorne, P., Stott, D., Robertson, L., MacDonald, J., Jones, L., McAlpine, C., Dick, F., Taylor, G., Murray, G., 2000. Medical complications after stroke a multicenter study. Stroke 31, 1223–1229.
Laridan, E., Denorme, F., Desender, L., François, O., Andersson, T., Deckmyn, H., Vanhoorelbeke, K., De Meyer, S.F., 2017. Neutrophil extracellular traps in ischemic stroke thrombi. Ann. Neurol.
Liesz, A., Dalpke, A., Mracsko, E., Antoine, D.J., Roth, S., Zhou, W., Yang, H., Na, S.-Y., Akhisaroglu, M., Fleming, T., 2015a. DAMP signaling is a key pathway inducing immune modulation after brain injury. J. Neurosci. 35, 583–598.
Liesz, A., Roth, S., Zorn, M., Sun, L., Hofmann, K., Veltkamp, R., 2015b. Acquired immunoglobulin G deficiency in stroke patients and experimental brain ischemia. Exp. Neurol. 271, 46–52.
Liu, Q., Jin, W.-N., Liu, Y., Shi, K., Sun, H., Zhang, F., Zhang, C., Gonzales, R.J., Sheth, K. N., La Cava, A., 2017. Brain ischemia suppresses immunity in the periphery and brain via different neurogenic innervations. Immunity 46, 474–487.
Maier, I.L., Karch, A., Mikolajczyk, R., Bähr, M., Liman, J., 2015. Effect of Beta-blocker therapy on the risk of infections and death after acute stroke-a historical cohort study. PLoS One 10, e0116836.
Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. 11, 519–531.
Mantuano, E., Azmoon, P., Brifault, C., Banki, M.A., Gilder, A.S., Campana, W.M., Gonias, S.L., 2017. Tissue-type plasminogen activator regulates macrophage activation and innate immunity. Blood. blood-2017-2004-780205.
Martino, R., Foley, N., Bhogal, S., Diamant, N., Speechley, M., Teasell, R., 2005. Dysphagia after stroke. Stroke 36, 2756–2763.
Mauri, C., Bosma, A., 2012. Immune regulatory function of B cells. Ann. Rev. Immunol. 30, 221–241.
Mawhorter, S., Yamani, M.H., 2008. Hypogammaglobulinemia and infection risk in solid organ transplant recipients. Curr. Opin. Organ Transpl. 13, 581–585.
McCulloch, L., Smith, C.J., McColl, B.W., 2017. Adrenergic-mediated loss of splenic marginal zone B cells contributes to infection susceptibility after stroke. Nat. Commun. 8.
Meisel, C., Prass, K., Braun, J., Victorov, I., Wolf, T., Megow, D., Halle, E., Volk, H.-D., Dirnagl, U., Meisel, A., 2004. Preventive antibacterial treatment improves the general medical and neurological outcome in a mouse model of stroke. Stroke 35, 2–6.
Minnerup, J., Wersching, H., Brokinkel, B., Dziewas, R., Heuschmann, P.U., Nabavi, D. G., Ringelstein, E.B., Schäbitz, W.-R., Ritter, M.A., 2010. The impact of lesion location and lesion size on poststroke infection frequency. J. Neurol. Neurosurg. Psychiatry 81, 198–202.
Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., de Ferranti, S., Despres, J.-P., Fullerton, H.J., Howard, V.J., 2015. Heart disease and stroke statistics-2015 update: a report from the american heart association. Circulation 131, e29.
Mracsko, E., Liesz, A., Karcher, S., Zorn, M., Bari, F., Veltkamp, R., 2014. Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke. Brain, Behav., Immun. 41, 200–209.
Offner, H., Subramanian, S., Parker, S.M., Afentoulis, M.E., Vandenbark, A.A., Hurn, P. D., 2006a. Experimental stroke induces massive, rapid activation of the peripheral immune system. J. Cereb. Blood Flow Metab. 26, 654–665.
Offner, H., Subramanian, S., Parker, S.M., Wang, C., Afentoulis, M.E., Lewis, A., Vandenbark, A.A., Hurn, P.D., 2006b. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J. Immunol. 176, 6523–6531.
Pennypacker, K.R., Offner, H., 2015. The role of the spleen in ischemic stroke. J. Cereb. Blood Flow Metab. 35, 186–187.
Pilitsis, J.G., Rengachary, S.S., 2001. Complications of head injury. Neurological Res. 23, 227–236.
Prass, K., Braun, J.S., Dirnagl, U., Meisel, C., Meisel, A., 2006. Stroke propagates bacterial aspiration to pneumonia in a model of cerebral ischemia. Stroke 37, 2607–2612.
Prass, K., Meisel, C., Höflich, C., Braun, J., Halle, E., Wolf, T., Ruscher, K., Victorov, I.V., Priller, J., Dirnagl, U., 2003. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1–like immunostimulation. J. Exp. Med. 198, 725–736.
Riquelme, R., Torres, A., El-Ebiary, M., de la Bellacasa, J.P., Estruch, R., Mensa, J., Fernández-Solá, J., Hernández, C., Rodriguez-Roisin, R., 1996. Community-acquired pneumonia in the elderly: a multivariate analysis of risk and prognostic factors. Am. J. Respiratory Crit. Care Med. 154, 1450–1455.
Rohweder, G., Salvesen, Ø., Ellekjær, H., Indredavik, B., 2017. Hospital readmission within 10 years post stroke: frequency, type and timing. BMC Neurol. 17, 116.
Römer, C., Engel, O., Winek, K., Hochmeister, S., Zhang, T., Royl, G., Klehmet, J., Dirnagl, U., Meisel, C., Meisel, A., 2015. Blocking stroke-induced immunodeficiency increases CNS antigen-specific autoreactivity but does not worsen functional outcome after experimental stroke. J. Neurosci. 35, 7777–7794.
J. Ruhnau J. Schulze B. von Sarnowski M. Heinrich S. Langner C. Pötschke A. Wilden C. Kessler B.M. Bröker A. Vogelgesang 2016 Reduced numbers and impaired function of regulatory T cells in peripheral blood of ischemic stroke patients Mediators Inflamm. 2016.
Ruhnau, J., Schulze, K., Gaida, B., Langner, S., Kessler, C., Bröker, B., Dressel, A., Vogelgesang, A., 2014. Stroke alters respiratory burst in neutrophils and monocytes. Stroke 45, 794–800.
Sarro, A., Sarro, G., 2001. Adverse reactions to fluoroquinolones. An overview on mechanistic aspects. Curr. Med. Chem. 8, 371–384.
Schaller, B.J., Graf, R., Jacobs, A.H., 2006. Pathophysiological changes of the gastrointestinal tract in ischemic stroke. Am. J. Gastroenterol. 101, 1655.
Schulze, J., Vogelgesang, A., Dressel, A., 2014. Catecholamines, steroids and immune alterations in ischemic stroke and other acute diseases. Aging Dis. 5, 327.
Seki, Y., Sahara, Y., Itoh, E., Kawamura, T., 2010. Suppressed neutrophil respiratory burst in patients with haemorrhagic stroke. J. Clin. Neurosci. 17, 187–190.
Shaw, S.Y., Blanchard, J.F., Bernstein, C.N., 2011. Association between the use of antibiotics and new diagnoses of Crohn's disease and ulcerative colitis. Am. J. Gastroenterol. 106, 2133–2142.
Shim, R., Wong, C.H., 2016. Ischemia, immunosuppression and infection—tackling the predicaments of post-stroke complications. Int. J. Mol. Sci. 17, 64.
Sica, A., Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. J. Clin. Invest. 122, 787–795.
Singh, V., Roth, S., Llovera, G., Sadler, R., Garzetti, D., Stecher, B., Dichgans, M., Liesz, A., 2016. Microbiota dysbiosis controls the neuroinflammatory response after stroke. J. Neurosci. 36, 7428–7440.
Sippel, T.R., Shimizu, T., Strnad, F., Traystman, R.J., Herson, P.S., Waziri, A., 2015. Arginase I release from activated neutrophils induces peripheral immunosuppression in a murine model of stroke. J. Cerebral Blood Flow Metabolism.
Stanley, D., Mason, L.J., Mackin, K.E., Srikhanta, Y.N., Lyras, D., Prakash, M.D., Nurgali, K., Venegas, A., Hill, M.D., Moore, R.J., 2016. Translocation and dissemination of commensal bacteria in post-stroke infection. Nat. Med. 22, 1277–1284.
Starr, J.B., Tirschwell, D.L., Becker, K.J., 2017. Increased infections with β-blocker use in ischemic stroke, a β2-receptor mediated process? Neurological Sci. 38, 967–974.
Stolk, R.F., van der Poll, T., Angus, D.C., van der Hoeven, J.G., Pickkers, P., Kox, M., 2016. Potentially inadvertent immunomodulation: norepinephrine use in sepsis. Am. J. Respir. Crit. Care Med. 194, 550–558.
Strong, K., Mathers, C., Bonita, R., 2007. Preventing stroke: saving lives around the world. Lancet Neurol. 6, 182–187.
Sykora, M., Siarnik, P., Diedler, J., Lees, K., Alexandrov, A., Bath, P., Bluhmki, E., Bornstein, N., Claesson, L., Davis, S., 2015. β-Blockers, pneumonia, and outcome after ischemic stroke evidence from virtual international stroke trials archive. Stroke 46, 1269–1274.
Teramoto, S., 2009. Novel preventive and therapeutic strategy for post-stroke pneumonia.
Theodorou, G., Marousi, S., Ellul, J., Mougiou, A., Theodori, E., Mouzaki, A., Karakantza, M., 2008. T helper 1 (Th1)/Th2 cytokine expression shift of peripheral blood CD4+ and CD8+ T cells in patients at the post-acute phase of stroke. Clin. Exp. Immunol. 152, 456–463.
Ulm, L., Hoffmann, S., Nabavi, D., Hermans, M., Mackert, B.-M., Hamilton, F., Schmehl, I., Jungehuelsing, G.-J., Montaner, J., Bustamante, A., 2017. The randomized Controlled STraWinSKi Trial: procalcitonin-Guided antibiotic Therapy after Stroke. Front. Neurol. 8.
Urra, X., Cervera, A., Obach, V., Climent, N., Planas, A.M., Chamorro, Á., 2009. Monocytes are major players in the prognosis and risk of infection after acute stroke. Stroke 40, 1262–1268.
Urra, X., Laredo, C., Zhao, Y., Amaro, S., Rudilosso, S., Renú, A., Prats-Galino, A., Planas, A.M., Oleaga, L., Chamorro, Á., 2017. Neuroanatomical correlates of stroke-associated infection and stroke-induced immunodepression. Brain, Behav., Immun. 60, 142–150.
Vahedi, K., Hofmeijer, J., Juettler, E., Vicaut, E., George, B., Algra, A., Amelink, G.J., Schmiedeck, P., Schwab, S., Rothwell, P.M., 2007. Early decompressive surgery in malignant infarction of the middle cerebral artery: a pooled analysis of three randomised controlled trials. Lancet Neurol. 6, 215–222.
Vahidy, F.S., Parsha, K.N., Rahbar, M.H., Lee, M., Bui, T.-T., Nguyen, C., Barreto, A.D., Bambhroliya, A.B., Sahota, P., Yang, B., 2016. Acute splenic responses in patients with ischemic stroke and intracerebral hemorrhage. J. Cereb. Blood Flow Metab. 36, 1012–1021.
van de Beek, D., Wijdicks, E.F., Vermeij, F.H., de Haan, R.J., Prins, J.M., Spanjaard, L., Dippel, D.W., Nederkoorn, P.J., 2009. Preventive antibiotics for infections in acute stroke: a systematic review and meta-analysis. Arch. Neurol. 66, 1076–1081.
Vargas, M., Horcajada, J.P., Obach, V., Revilla, M., Cervera, Á., Torres, F., Planas, A.M., Mensa, J., Chamorro, Á., 2006. Clinical consequences of infection in patients with acute stroke is it prime time for further antibiotic trials? Stroke 37, 461–465.
Vermeij, F.H., Op Reimer, W.J.S., De Man, P., Van Oostenbrugge, R.J., Franke, C.L., De Jong, G., De Kort, P.L., Dippel, D.W., 2009. Stroke-associated infection is an independent risk factor for poor outcome after acute ischemic stroke: data from the Netherlands Stroke Survey. Cerebrovascular Dis. 27, 465–471.
Vermeij, J.D., Westendorp, W.F., Dippel, D.W., van de Beek, D., Nederkoorn, P.J., 2018. Antibiotic therapy for preventing infections in people with acute stroke. Cochrane Database Syst. Rev. 1. CD008530.

Vernino, S., Brown, R.D., Sejvar, J.J., Sicks, J.D., Petty, G.W., O'Fallon, W.M., 2003. Cause-specific mortality after first cerebral infarction a population-based study. Stroke 34, 1828–1832.
Vogelgesang, A., Grunwald, U., Langner, S., Jack, R., Bröker, B.M., Kessler, C., Dressel, A., 2008. Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. Stroke 39, 237–241.
Vogelgesang, A., May, V.E., Grunwald, U., Bakkeboe, M., Langner, S., Wallaschofski, H., Kessler, C., Bröker, B.M., Dressel, A., 2010. Functional status of peripheral blood T-cells in ischemic stroke patients. PLoS One 5, e8718.
Walter, U., Knoblich, R., Steinhagen, V., Donat, M., Benecke, R., Kloth, A., 2007. Predictors of pneumonia in acute stroke patients admitted to a neurological intensive care unit. J. Neurol. 254, 1323–1329.
Walter, U., Kolbaske, S., Patejdl, R., Steinhagen, V., Abu-Mugheisib, M., Grossmann, A., Zingler, C., Benecke, R., 2013. Insular stroke is associated with acute sympathetic hyperactivation and immunodepression. Eur. J. Neurol. 20, 153–159.
Wang, H., Yan, F.-L., Cunningham, M., Deng, Q.-W., Zuo, L., Xing, F.-L., Shi, L.-H., Hu, S.-S., Huang, Y., 2016. Potential specific immunological indicators for stroke associated infection are partly modulated by sympathetic pathway activation. Oncotarget 7, 52404.
Wen, S.W., Wong, C.H., 2017. An unexplored brain-gut microbiota axis in stroke. Gut Microbes, 1–6.
Westendorp, W.F., Nederkoorn, P.J., Vermeij, J.-D., Dijkgraaf, M.G., van de Beek, D., 2011. Post-stroke infection: a systematic review and meta-analysis. BMC Neurol. 11, 110.
Westendorp, W.F., Vermeij, J.-D., Brouwer, M.C., Roos, Y., Nederkoorn, P.J., van de Beek, D., Investigators, P., 2016. Pre-stroke use of beta-blockers does not lower post-stroke infection rate: an exploratory analysis of the preventive antibiotics in stroke study. Cerebrovascular Dis. 42, 506–511.
Westendorp, W.F., Vermeij, J.-D., Zock, E., Hooijenga, I.J., Kruyt, N.D., Bosboom, H.J., Kwa, V.I., Weisfelt, M., Remmers, M.J., ten Houten, R., 2015. The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial. Lancet.
Winek, K., Dirnagl, U., Meisel, A., 2016a. The gut microbiome as therapeutic target in central nervous system diseases: implications for stroke. Neurotherapeutics 13, 762–774.
Winek, K., Engel, O., Koduah, P., Heimesaat, M.M., Fischer, A., Bereswill, S., Dames, C., Kershaw, O., Gruber, A.D., Curato, C., 2016b. Depletion of cultivatable gut microbiota by broad-spectrum antibiotic pretreatment worsens outcome after murine stroke. Stroke 47, 1354–1363.
Wong, C.H., Jenne, C.N., Lee, W.-Y., Léger, C., Kubes, P., 2011. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. Science 334, 101–105.
Wong, C.H., Jenne, C.N., Tam, P.P., Léger, C., Venegas, A., Ryckborst, K., Hill, M.D., Kubes, P., 2017. Prolonged activation of invariant natural killer T cells and TH2-skewed immunity in stroke patients. Front. Neurol. 8.
Yamashiro, K., Tanaka, R., Urabe, T., Ueno, Y., Yamashiro, Y., Nomoto, K., Takahashi, T., Tsuji, H., Asahara, T., Hattori, N., 2017. Gut dysbiosis is associated with metabolism and systemic inflammation in patients with ischemic stroke. PLoS One 12, e0171521.
Yan, L., Qing, Y., Xingyi, J., Hongbo, Q., 2015. Etiologic diagnosis and clinical treatment of multiple drug-resistant bacteria infection in elderly patients with stroke-associated pneumonia after neurosurgery. Cell Biochem. Biophys. 71, 731–734.
Yilmaz, G., Granger, D.N., 2010. Leukocyte recruitment and ischemic brain injury. Neuromol. Med. 12, 193–204.
Yin, J., Liao, S.X., He, Y., Wang, S., Xia, G.H., Liu, F.T., Zhu, J.J., You, C., Chen, Q., Zhou, L., 2015. Dysbiosis of gut microbiota with reduced trimethylamine-N-oxide level in patients with large-artery atherosclerotic stroke or transient ischemic attack. J. Am. Heart Assoc. 4, e002699.
Zhang, S., He, W.-B., Chen, N.-H., 2014. Causes of death among persons who survive an acute ischemic stroke. Curr. Neurol. Neurosci. Rep. 14, 1–11.
Zheng, F., von Spreckelsen, N., Zhang, X., Stavrinou, P., Timmer, M., Dohmen, C., Goldbrunner, R., Cao, F., Zhang, Q., Ran, Q., 2017. Should preventive antibiotics be used in patients with acute stroke? A systematic review and meta-analysis of randomized controlled trials. PLoS One 12, e0186607.
Zierath, D., Kunze, A., Fecteau, L., Becker, K., 2015. Effect of antibiotic class on stroke outcome. Stroke 46, 2287–2292.
Zuo, L., Shi, L., Yan, F., 2016. The reciprocal interaction of sympathetic nervous system and cAMP-PKA-NF-kB pathway in immune suppression after experimental stroke. Neurosci. Lett. 627, 205–210.

Although the roles of SNS and iNKT cells in regulating stroke-induced immunosuppression and development of post-stroke infections were outlined in the published review, it was not sufficient for the purpose of this thesis. Therefore, these concepts will be explored more in-depth below.

The autonomic nervous system

The autonomic nervous system (ANS) is a branch of the peripheral nervous system that regulates the involuntary or subconscious bodily processes such as heart rate, blood pressure, respiration, and digestion [2]. Autonomic nerves detect physiological changes and utilizes subdivisions of the ANS to induce responses that alter or regulate homeostasis. There are two major subdivisions of the ANS, known as the parasympathetic nervous system (PSNS) and the SNS, and they have opposing roles in regulating bodily functions. The PSNS primarily uses the neurotransmitter acetylcholine released from the vagus nerve endings to return the body to a resting or relaxed state [2]. Therefore, the PSNS is commonly referred to mediate the "rest and digest" functions such as lowering heart rate, reduce cardiac contractility and enhance digestion [3]. On the other hand, stress stimuli activate the SNS which employs neurotransmitters, known as catecholamines, which are released from sympathetic nerve terminals. Bodily responses to the SNS and catecholamines mediate the "fight or flight" response to increase cardiac output and blood pressure, and slow down gastrointestinal processes [4]. Major stressors that activate the SNS are external stimuli that can be visually or physically perceived. However, internal stimulus, which include inflammation, tissue injury or infection, can also initiate this stress response [3]. It is these internal stimuli that play an important role in health and disease and implicates the SNS in the regulation of immunity in a bidirectional manner following stroke and other diseases.

The sympathetic nervous system and immunity

In response to internal stress, sensory neurons in the periphery detect the abundance of various stimuli, including cytokines, such as interleukin (IL) -1, -6, and tumour necrosis factor alpha (TNF-α), and other antigens, such as danger associated molecular patterns (DAMPS) and pathogen associated molecular patters (PAMPS), and send signals to the brain [5]. The brain responds by activating sympathetic neurons through the spinal cord to release catecholamines as the signalling relay that induces the physiological impact of the SNS throughout the body. Noradrenaline (NA) is the major catecholamine released by sympathetic nerves and nerve terminals. NA acts upon the adrenergic receptors which are G-protein coupled receptors that are expressed on many cell types throughout the periphery and nervous system [4]. They can be classified into 3 main groups, the α_1- , α_2- and β receptors, each of which can be further subdivided into three subgroups [4]. Activation of the adrenergic receptors induce a multitude of physiological responses that depend on the organ or cell type in which it is expressed. Activation of the α_1 receptors induce the release of Ca^{2+} and primarily results in physiological responses such as smooth muscle contraction [6]. The α_2 receptors are predominantly expressed on presynaptic neurons where they are considered inhibitory as their activation by NA inhibits further release of NA [6]. The β-adrenergic receptors (β-AR) are known for their roles in varying cardiac output due to their abundant expression in the heart [7]. Activation of β_1-adrenergic receptor (Adrb1) in cardiac muscle allows entry of Ca^{2+} and thus increases arterial contractility and cardiac output [4, 8], while β_2- adrenergic receptor (Adrb2) are commonly located on smooth muscle cells in the airways to induce relaxation [4]. Finally, the β_3 adrenergic receptor (Adrb3) is mainly expressed by adipose cells where they mediate lipolysis and thermogenesis upon activation [9].

SNS implication on lymphoid organs

The nervous system and immune system have been traditionally viewed as mutually exclusive biological processes. However, studies dating back in the 80's began alluding to the notion that these systems are more intertwined than initially believed. Studies were able to utilise electron microscopy to visualise sympathetic nerve terminals within the white pulp of the spleen and demonstrated these nerve terminals were in close proximity with immune cells such as T cells, B cells and dendritic cells [10, 11]. We now know that sympathetic neurons innervate various lymphoid organs, including primary lymphoid organs (bone marrow and thymus) [12-14], as well as secondary lymphoid organs (lymph nodes) [15-17] and therefore have direct access to major immune sites to regulate immune cell function.

Primary lymphoid organs host the development of T and B lymphocytes, innate immune cells of the myeloid lineage (including neutrophils and macrophages). Tracing the location of sympathetic neurons can be performed by injecting the pseudorabies virus (PRV) into lymphoid tissues [18]. Infecting PRVs enter sympathetic nerves from the synaptic terminal and travel into the cell body for replication and generation into virus particles that are released and infect connected neurons. The PRV can then be labelled to visualise sympathetic neural pathing within the organ. This technique revealed sympathetic nerves line blood vessels throughout the bone marrow [19, 20]. Furthermore, these sympathetic nerves in the bone marrow are within proximity of a subset of quiescent haematopoietic stem cells (HSC) [21, 22]. Due to the high innervation of the bone marrow, these studies suggest that neurotransmitters, such as NA, have direct access to the bone marrow stroma where haematopoiesis occurs. As such, it is unsurprising to find that SNS signalling can regulate development of immune cells. In cases of prolonged SNS activation induced by long-term

stress in mice and human participants, transcription and translation of genes associated with myelopoiesis is promoted, and an associated increase output of monocyte and granulocytes out of the bone marrow [23]. Additionally, SNS activation results in the excessive mobilisation of immune cells from the bone marrow to the periphery by regulating the chemokine gradient of C-X-C motif chemokine ligand (CXCL-) -12 [24, 25]. In a healthy state, the CXCL12 chemokine gradient is tightly regulated to control egress of bone marrow derived immune cells. However, upon SNS signalling or changes in circadian rhythm, β-AR activation reduces the expression of CXL12, thus resulting in excess release of progenitor cells into the periphery [26]. Importantly, these hematopoietic effects of stress were inhibited by pharmacological blockage of the β-ARs or chemical ablation of neurons expressing adrenergic receptors to suggest the involvement of SNS signalling in haematopoiesis [13, 23, 26].

Similarly, PRV tracing technology have also been applied in the spleen. To demonstrate neural innervation of the spleen, earlier studies injected PRV into the spleen of rats and were able to detect PRV labelling within the spinal cord and brain at 60 h and 110 h post-infection [27]. Sympathetic nerves fibres that innervate the spleen have been observed along the splenic artery and vein, and branch into T cell-rich zones in the rat [10]. However, these studies utilised histological imaging techniques that only enable partial visualisation of the adrenergic network within the spleen. More recently, distribution of the splenic adrenergic network has been revealed by advanced three-dimensional imaging. Using ImmuView, the sympathetic architecture was observed to spread throughout the parenchyma of the murine spleen in a branch-like manner [15]. In regard to the functional role of the SNS in the spleen, this study demonstrated that NA-dependent activation of Adrb2 on immune cells impeded bacterial clearance in the spleen [15]. Importantly, this alludes to the ability of NA to directly modulate immune cell function.

SNS effect on immune cells

While α adrenergic receptors have a higher affinity for NA compared to the β-ARs its role on immune cell function have not been well characterised. This is due to little to no expression of these receptors on immune cells. Studies that attempted to identify the presence of α adrenergic receptors on immune cells have also been controversial. The majority of studies report the absence of α_1 adrenergic receptors on peripheral blood mononuclear cells (PBMCs) in a healthy state, including T cells, B cells, NK cells and monocytes [28-30]. However, when the immune response is activated, α adrenergic receptors in immune cells can be upregulated to enhance immunity. For example, activation of the α_1 adrenergic receptor on macrophages can induce TNF-α secretion in mice in response to caecal ligation and puncture [31]. In contrast, the β-ARs are consistently reported to be constitutively expressed on immune cells at a basal state with Adrb2 being most highly expressed [5, 32]. For this reason, the modulation of immune responses by the SNS and noradrenaline is thought to be mediated by the Adrb2 and therefore will be further discussed.

The activation of Adrb2 on immune cells is shown to be associated with immunosuppression. Of the innate immune cells, neutrophils are among the first immune cells to migrate to sites of injury and infection, where they mediate antibacterial effects via respiratory burst and release of antibacterial mediators [33]. Studies show that neutrophil respiratory burst can be inhibited by Adrb2 agonists [34, 35]. Furthermore, *in vivo* superfusion of NA over mouse cremaster muscle tissue inhibits the ability of neutrophils to transmigrate toward bacterial-derived chemoattractant [36]. These effects are likely mediated by Adrb2 signalling on neutrophils due to the significantly higher relative expression of Adrb2 compared to the other α and β-ARs [36].

For monocytes and macrophages, Adrb2 agonism also drive immunosuppressive effects. Monocyte-derived macrophages express toll-like receptors that detect the presence of bacterial PAMPS, such as lipopolysaccharide (LPS), and induce TNF-α production in response. With the addition of NA following *in vitro* stimulation with LPS, human monocyte-derived macrophages increased production of the immunosuppressive cytokine IL-10 while TNF-α production is suppressed [37, 38]. These effects were suggested to be mediated by the Adrb2 as macrophages from mice deficient of Adrb2 were unable to produce IL-10 despite the addition of NA [37].

The effects of Adrb2 activation have also been characterised on cells of the adaptive immune system. Activation of Adrb2 can enhance the suppressive function of regulatory T cells [39], induce IL-10 secretion, and reduce the trafficking of lymphocytes through peripheral tissues and secondary lymphoid organs [40]. The retention of mature T cells in secondary lymphoid organs after Adrb2 signalling was found to be mediated by enhancing T cell expression of C-C chemokine receptor (CCR-) -7 and C-X-C chemokine receptor (CXCR-) -4 [40-42]. Lymphocyte activation via Adrb2 signalling also reduces secretion of cytokines required for proliferation, such as IL-2 and interferon γ (IFN-γ), and enhances regulatory T cell function though mechanisms dependent on cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)-dependent phosphorylation [39, 43]. Furthermore, Adrb2 signalling has been shown to inhibit or disrupt NF-κB gene transcription in T cells and monocytes to prevent production of proinflammatory cytokines and molecules [44, 45]. Therefore, due to the innervation of sympathetic nerves throughout major immune organs and the role of Adrb2 activation in general immunosuppression, activation of the SNS has been implicated in the regulation of immune functions during many disease states.

Implication of the SNS and the β-ARs in diseases

Pioneering studies implicated the role of SNS and immunity in various stress scenarios. Exercise-induced stress was shown to promote the proliferation of suppressive T cells and reduce the expression of IL-2 on lymphocytes in healthy volunteers [46]. These effects were suggested to be induced by Adrb2 activation as treatment with the global β-blocker, propranolol, could prevent these alterations, while treatment with the β_3-receptor antagonist, metoprolol, had no effect [46]. However, chronic stress and SNS dysregulation can enhance the progression of various immune-mediated diseases. A meta-analysis found that stress-related psychological factors were associated with increased incidence of cancer and poor survival rates [47]. Indeed, due to the immunosuppressive functions of the SNS, immunity against tumours become impaired. In addition, NK cells, which are known to target tumour cells expressing low amounts of major histocompatibility complex, were reported to have decreased tumour cytotoxic activity during high stress states [48, 49]. Furthermore, the accumulation of myeloid-derived suppressor cells can facilitate the development of cancer by suppressing anti-tumour immunity in response to SNS signalling [50]. The resulting metastasis of cancers are likely to be mediated by the β-ARs as experimental and clinical studies demonstrate delayed disease progression with β-blocker use [51-55]. Furthermore, β-AR activation on lymphocytes may be critical in inducing suppression of tumour immunity as mice deficient in mature lymphocytes had delayed stress-induced tumour growth [56].

Sympathetic activation can also negatively impact on the clearance of infections [49]. In bacterial infection with *Listeria monocytogenes*, ablating sympathetic nerves or inhibiting NA uptake in mice improved bacteria clearance in the spleen and liver [57]. This was later confirmed to be due to β-AR activation as preventing β-AR signalling effectively reduced bacterial burden by enhancing IFN-γ and TNF-α production by splenocytes [58, 59]. Similar

findings were reported in models of *Klebsiella pneumoniae* infection, where sympathetic nerve ablation or inhibiting NA uptake (desipramine) improved survival in mice and reduced bacterial load via monocyte production of IL-6 [60].

Importantly, β-AR signalling may also be critical in regulating or balancing inflammatory immune responses. Adrb2 deficient mice had enhanced systemic TNF-a production and reduced IL-10 levels compared to wildtype controls following LPS-induced sepsis. Subsequently, mice with Adrb2 deficiency succumbed to endotoxemia even with sub-lethal doses of LPS, possibly due to overactivation of proinflammatory pathways [37]. The regulation of β-AR signalling is also evident in scenarios of chronic inflammation and autoimmunity. Adrb2 deficient mice display more severe symptoms of colitis compared to wildtype mice [37]. In experimental autoimmune encephalomyelitis (EAE) that is commonly used for modelling multiple sclerosis, chemical sympathectomy or depletion of NA worsens disease severity [61, 62]. Indeed, stimulating the β-ARs using agonists suppressed relapsing EAE in Lewis rats [63, 64] and improved clinical scores of EAE in mice [62]. Overall, these studies demonstrate the critical role SNS and β-AR activation in the regulation or development of various disease states.

Issues with studies exploring Adrb2 function in immunity

A majority of studies that examine the role of NA on immune responses exclusively rely on the use of pharmacological molecules that inhibit (e.g. propranolol), stimulate (e.g. isoproterenol), or ablate (i.e. 6-hydroxydopamine; 6-OHDA) sympathetic nerves. This is due to the limitations of generating a strain of Adrb2 deficient mice on a background that is more relevant to human immunity [65]. Currently, the Adrb2 deficient mouse is commercially available on an FVB/N background which allows for better success in generating transgenic

mice in comparison to the C57BL/6J strain of mice [66]. However, it is advantageous to have knockout strains of mice with the C57BL/6J background due to a closer modelling of human immunity and disease. The first studies that generated the Adrb2 deficient mouse strain on the FVB/N background found little changes in various parameters of adaptive immunity, including lymphoid organ cell numbers and T cell-dependent antibody responses in naïve conditions in comparison to mice with the Adrb2 receptor [67, 68]. The lack of observable immune changes in Adrb2 deficient mouse were reasoned to be due to compensatory mechanisms as a result of the inherent lack of the Adrb2 receptor in this mouse strain, which were independent of increased expression of other adrenergic receptors. The authors suggest that adoptive transfer of Adrb2 deficient leukocytes into a wildtype mouse may demonstrate the direct effect of Adrb2 deficiency on immunity [65]. However, Adrb2 knockout mouse strains used in these studies and also in more recent studies were bred on an FVB/N background [67-70], making adoptive transfers into other strains of mice not possible [65, 68]. Therefore, creating an Adrb2 deficient mouse on a common background, such as C57Bl/6, will enable further investigation into Adrb2 in various immunological diseases.

The β-ARs in post-stroke infection stroke

In the published literature review included in this chapter, we described the changes to immunity that occur as a result of β-adrenergic signalling after stroke (see section 3.3 in review on page 8). These studies rely on global inhibition of the β-ARs with propranolol, or ablation of noradrenergic neurons using 6-OHDA to show these effects. However, while these studies were pivotal in identifying the sympathetic activation as a mechanism to susceptibility to infection after stroke, little is known about whether the Adrb2 is solely responsible for these changes. One study examined the effects of selective and non-selective β-blockers on infection

rates in patients with stroke [71]. Interestingly, non-selective β-blocker use was associated with increased infections while this association was not seen in patients receiving selective β-blockers. Therefore, this study suggests that each β-AR may have differential roles in post-stroke immune suppression and emphasises the importance of teasing out the role of the Adrb2 in post-stroke infection.

Whether the SNS acts on a multitude of immune cells, or only on specific subtypes to induce immunosuppressive effects after stroke is unclear. In a previous study, we identified the invariant natural killer T (iNKT) cell as a cell type that is rapidly activated after stroke via β-adrenergic signalling and induces systemic immunosuppression following stroke [72]. As such, iNKT cells may be a primary mediator of post-stroke immunosuppression and understanding the role and functions of iNKT cells may reveal potential avenues to reduce post-stroke infection.

The invariant natural killer T cell

The natural killer T (NKT) cell is a subset of non-classical lymphocytes that share the properties of NK cells and T cells. In humans and mice, they are predominantly found in the liver where they make up approximately 5% and 40% of hepatic lymphocytes in humans and mice, respectively [73, 74]. Their frequencies in the periphery also vary, where they make up between 0.1-3% of total leukocytes in the blood, bone marrow and spleen [73, 75]. There are two main subsets of NKT cells that are categorised based on the specificities of their T cell receptor (TCR). The iNKT cells (or type 1 NKT cells) are the most common type of NKT cells, making up around 80% of all NKT cells in mice and humans, while the remainder is made up of variant NKT cells (type II NKT cells) [75]. Concentrating on the iNKT cells, it is defined by the specificity of their T cell receptor, which is made up of a highly conserved α chain,

encoded by Vα24 in humans (or Vα14 in mice) and the Jα18 gene segments. The β chain is also highly restricted and encoded by the Vβ8.2, Vβ7 and Vβ2 in mice, and Vβ11 in humans [76]. Unlike conventional T cells that recognise peptides presented by major histocompatibility complexes (MHC), iNKT cells recognise glycolipids presented by antigen presenting cells on the non-classical MHC-like complex CD1d. Like the iNKT cell TCR, CD1d is also highly conserved across many animal species, suggesting that iNKT cells play an essential role in immunity. In fact, iNKT cells has been implicated in many immunological diseases, including allergy [77, 78], autoimmunity [79, 80], cancers [81, 82], and infectious diseases [83, 84].

The iNKT cells can be considered as a master regulator of immunity. They are most well-known for secreting high quantities of cytokines including IL-1β, -2, -3, -5, -10, -13, -17, -21, and TNF-α [76]. Importantly, iNKT cells can also produce IFN-γ and IL-4 – cytokines that enhance T-helper cell responses. T cells are major cells of the adaptive immune system (along with B cell) that can be separated into two major classes, T-helper cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells). The T-helper cells can be further classified into the T-helper 1 (Th1) cells or T-helper 2 (Th2) that are defined by the cytokines in which they are activated by and the cytokines they secrete [85]. Upon activation with IL-12, naïve T cells differentiate into Th1 cells and produce the effector cytokines IFN-γ and IL-2. This, in turn, recruit phagocytes, such as macrophages, and enhances phagocytosis and digestion of bacterially infected cells [86]. As such, the Th1 response is also known as cell mediated immunity and is critical for the defence against bacterial infection. However, Th1 responses are associated with autoimmune diseases such as rheumatoid arthritis [87]. In contrast, IL-4 induces differentiation of T cells into Th2 cells to further produce IL-4, -10 and -13 . These cytokines promote B cell release of immunoglobulin E (IgE), which promotes mast cell and eosinophil degranulation. The Th2 response is also known as the humoral immune response [88]. It is vital for tissue repair but it has also been implicated in diseases such as asthma and

allergy [88]. Due to the ability of the iNKT cell to produce both IFN-γ and IL-4, the iNKT cells can facilitate activation of both Th1 and Th2 immunity, respectively [89]. As such, iNKT cells become an attractive candidate for targeted therapeutic for various immunological diseases. For example, stimulating iNKT cells to enable the Th1 response via IFN-γ can enhance immunity for fighting cancers and infections, while inducing a Th2 response via IL-4 may assist in alleviating some autoimmune diseases.

Activation and mechanisms of iNKT cell polarisation

The most well-known activator of iNKT cells is α-galactosylceramide (α-GalCer), an α-linked glycosphingolipid, comprised of a sugar ring that is α-linked to an 18-carbon (C) sphingosine base chain with a 26-C fatty acyl chain [90] (**Figure 1.1A**). The original name for α-GalCer is KRN7000 due to its discovery in 1993 from an Okinawan marine sponge (*Agelas mauritianus*) by Kirin Brewery Company during a screen for compounds that induce anti-tumour and immunostimulatory responses [91]. It was found to have strong anti-tumour activity which were initially thought to be mediated by natural killer (NK) cells by presentation on antigen presenting cells (APCs) [92]. Later, α-GalCer was identified to specifically activate NKT cells that possess the TCR encoded by Vα14 [90, 93], which is now recognised to be the iNKT cell. α-GalCer is readily loaded to create a complex with CD1d on APCs (in both humans and mice) and the TCR of the iNKT cell [94]. Activation of iNKT cells in this manner induces rapid and robust production of both IFN-γ and IL-4 (**Figure 1.1B**) [94]. Recently, the development of structural analogues of α-GalCer has been a large focus as alterations to α-GalCer structure can change iNKT cell responses.

At the molecular level, the 26C and 18C carbon chains respectively bind to regions of the CD1d molecule known as the A' channel and F' channel [95, 96]. As such, altering the

structure of α-GalCer can affect the overall binding of α-GalCer to CD1d and TCR. In early studies, it was believed that structural modifications in the sugar group, glycosidic bond or lengths and saturation of fatty acid chains of α-GalCer could modify iNKT cell responses by altering binding kinetics of the CD1d-glycolipid-TCR complex, as well as varying the stability and concentration of the CD1d-glycolipid complex [97-99]. Two analogues of α-GalCer that have been extensively used to explore the mechanisms of iNKT cell polarisation. These are C-glycoside and OCH, which induce a Th1- and Th2-skewing response, respectively (**Figure 1.2**) [100]. Previous reports show that analogues such as OCH that have truncated sphingosine chain, reduced IFN-γ expression while maintaining IL-4 expression by iNKT cells following *in vitro* stimulation – factors associated with a Th2 response [79, 80, 101]. By assessing the binding kinetics, this study suggested that modulation of iNKT cell by analogues with truncated sphingosine chains reduced length of TCR stimulation compared to α-GalCer. This would trigger short-lasting calcium influxes, leading to translocation of transcription factors to promote *il-4* gene transcription. Conversely, sustained stimulation of the iNKT cell TCR by the CD1d-α-GalCer complex would induce prolonged calcium influxes for translocation of transcription factors to produce both IL-4 and IFN-γ [101]. However, another study showed that the Th1 biasing compound, C-glycoside, was unstable when bound to CD1d and provided less TCR stimulation compared to OCH to suggest that the strength of binding between TCR-glycolipid-CD1d was not an explanation of iNKT cell polarisation [100]. Therefore, the duration of receptor engagement may play a role in iNKT cell cytokine responses by altering binding kinetics of the CD1d-α-GalCer-TCR complex, however this is inconclusive at the present time.

More recently, the method of glycolipid loading to CD1d and the association of lipid rafts with the CD1d-glycolipid complex is a proposed mechanism in polarising iNKT cell responses. In this model, rapid and direct surface loading of glycolipids onto CD1d induces a

Th2 biased immunity, whereas α-GalCer is intracellularly loaded onto CD1d before being presented (**Figure 1.3**) [94, 102]. In contrast, analogues that bias a Th1 response also required intracellular loading of glycolipid onto CD1d and presentation on the cell membrane as well as an association with cholesterol-rich lipid rafts [102-104]. Furthermore, emerging evidence suggests that modulations of iNKT cell responses may be dependent on the glycolipid-presenting cell. Glycolipid presentation by CD1d on regulatory B cells induced iNKT cell-mediated suppression of Th1 immunity to protect against experimental rheumatoid arthritis, while this protection was not seen in mice deficient in CD1d-expressing B cells [105]. Clearly, understanding the mechanisms of iNKT cell polarisation unlocks the potential of creating immune-polarising compounds for treatment in a wide range of diseases.

Research exploring the potential of α-GalCer and targeting the iNKT cell in diseases began in the cancer field. As mentioned above, α-GalCer was initially shown to induce potent anti-tumour immunity in the B16 melanoma cancer model [93]. This protection was dependent on α-GalCer presentation by CD1d-expressing cells to iNKT cells to enhance direct tumour cell killing via degranulation [93]. More recently, α-GalCer has been tested in models of bacterial infection [106]. During *Streptococcus pneumoniae* infection, a common microbe that causes pneumonia, 90% of iNKT cell-deficient mice succumbed to pulmonary infection compared to only a 25% mortality rate in wildtype mice [107]. Furthermore, activation of iNKT cell with α-GalCer could effectively clear urinary infection with *Escherichia coli*, that was mediated by systemic production of IFN-γ, TNF-α and IL-10 [108]. Importantly, these studies demonstrate the importance of iNKT cells in antibacterial immune defence.

Stroke, SNS activation and iNKT cells

The role of the iNKT cell in post-stroke infection has been seldom explored. In a previous study, we showed that hepatic iNKT cells are in a prime position to detect and respond to distant brain damage in a murine model of ischemic stroke [72]. Early activation of hepatic iNKT cells, characterised by a "pirouetting" phenotype and increased CD69 expression (T cell activation marker), induced systemic immunosuppression via IL-10 production and lead to bacterial infection after stroke. Importantly, we validated our experimental findings in the clinical settings, where circulating iNKT cells were shown to be activated and increased levels of plasma IL-10 was detected in patients with stroke upon admission into hospital [109]. In mice, post-stroke activation of iNKT cells was mediated by β-adrenergic activation as therapeutic treatment with propranolol could prevent iNKT cell-mediated immunosuppression (further described in section 3.3 of the published literature review in this chapter on page 8) [72]. Furthermore, we showed that activating iNKT cells with α-GalCer within a therapeutic timeframe following cerebral ischemia promoted hepatic iNKT cells to produce IFN-γ, leading to reduced bacterial infection after stroke. Importantly, the protection from post-stroke infection conferred by propranolol or α-GalCer was not seen in iNKT cell deficient mice to demonstrate that iNKT cells are responsible for the modulation of post-stroke immunity and are a viable therapeutic target to combat post-stroke infection. However, these studies were performed on mice with a BALB/c background, which are more susceptible to infectious complications compared to mice on a C57BL/6 background [110]. This is due to BALB/c mice having a Th2-dominant immunity, while C57BL/6 mice have a Th1-dominant immunity that is considered to be more reflective of immunity in humans [111, 112]. Furthermore, the risk of exacerbating long-term cerebral injury after stroke with immunostimulatory therapies such as

α-GalCer was not assessed. Therefore, determining whether α-GalCer treatment affects the infarct volume of post-stroke mice is also yet to be determined.

While α-GalCer was effective in reducing post-stroke infection in mice, side effects of α-GalCer, such as liver injury [113], has prevented its translation into the clinic. Examining analogues of α-GalCer may potentially avoid these detrimental side effects. Additionally, finding analogues that preferentially drive a Th1 response may be beneficial to prevent further post-stroke immunosuppression, lessen infection and therefore improve stroke outcomes. However, because collateral brain injury following stroke is driven by inflammatory responses [114], shifting immunity toward an inflammatory Th1 response could potentially exacerbate neurological deficits and increase the possibility of recurring strokes. Conversely, analogues of α-GalCer that skew for a Th2 immunity may improve neurological outcomes in exchange for greater immunosuppression and susceptibility to infection after stroke. As such, finding α-GalCer analogues that induces a sufficient iNKT cell response to prevent post-stroke infection, yet does not exacerbate brain injury, would be optimal to improve the overall outcome of patients with stroke.

Current project and statement of thesis aims

In this chapter, some areas that require further elucidation have been identified and will be addressed in the subsequent chapters. Firstly, while infections, such as pneumonia, are common and most fatal complications after stroke, the association of cerebral infarct volume on post-stroke infection remain unclear. Additionally, infarctions of certain brain regions may also impact on the susceptibility to post-stroke infection. As such, Aim 1 of this thesis explores the effect of various severities of stroke on post-stroke immunity and infection, and will

elucidate the relationship between infarction of various brain regions on post-stroke lung infection in both experimental and clinical settings.

Secondly, Aim 2 will outline how SNS activation can contribute to post-stroke infection by inducing systemic immunosuppression. Using pharmacological activators and inhibitors of adrenergic receptors, post-stroke activation of the β-ARs on immune cells by catecholamines has been identified as a major pathway in which immunosuppression occurs. However, it is unclear whether the Adrb2 receptor is specifically responsible for systemic post-stroke immune alterations. Therefore, Aim 2 of my PhD thesis will explore the role of Adrb2 in post-stroke immunosuppression through the use of specific Adrb2 inhibitors and *Adrb2* knockout mice.

Finally, our group has previously identified that adrenergic activation of the hepatic iNKT cells is a major contributor to post-stroke immunosuppression. However, as the most fatal and common type of infection occurs in the lung, Aim 3 of this PhD thesis will further delineate the role of pulmonary iNKT cells in post-stroke lung immunity. Furthermore, immunomodulatory capabilities of iNKT cells will be harnessed by therapeutically treating post-stroke animals with α-GalCer and two synthetic structural analogues to demonstrate the potential of activating iNKT cells to reduce post-stroke infection.

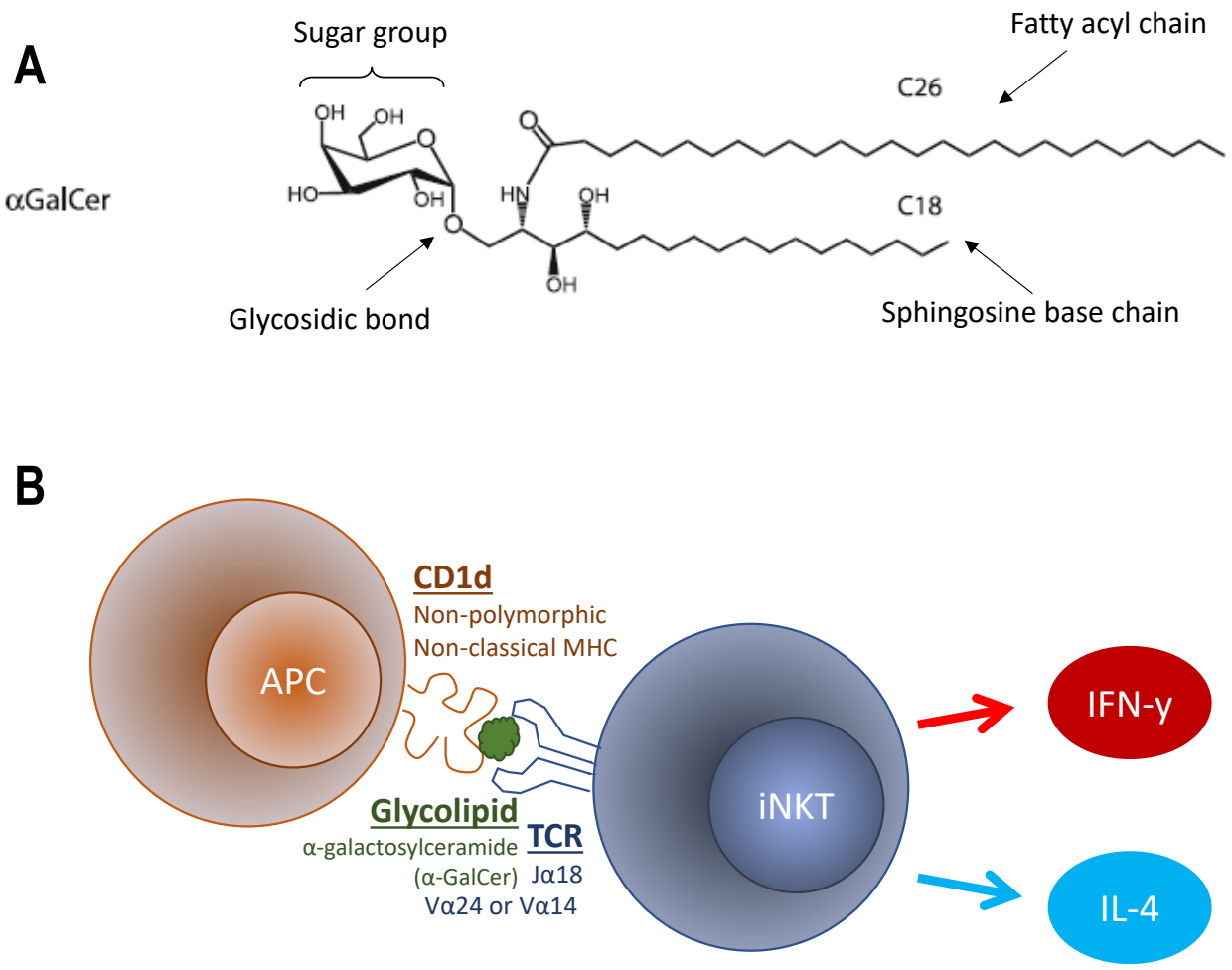


Figure 1.1 Structure of α-galactosylceramide (α-GalCer) and mechanism of invariant natural killer T (iNKT) cell activation.

α-GalCer is composed of a sugar ring group that is glycosidically bound to an 18-carbon (C18) fatty sphingosine base chain and a 26-carbon (C26) fatty acyl chain (A). Figure adapted from [100]. The glycolipid, α-GalCer, is the most well-studied activator of iNKT cells. It is rapidly loaded onto the non-classical MHC molecule known as CD1d that is expressed by antigen presenting cells (APC). The APCs present α-GalCer to the TCR of the iNKT cell, which is encoded by the Vα24 (humans) or Vα14 (mice) and Jα18 (both humans and mice) gene segments. Activation of iNKT cells in this manner induces robust production of both IFN-γ and IL-4 (B).

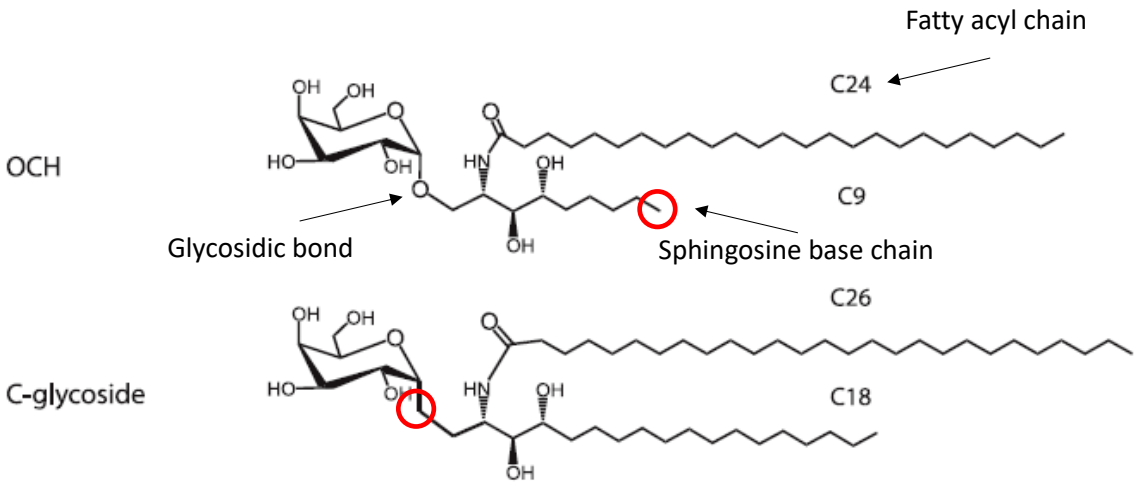


Figure 1.2 Molecular structure of α-GalCer glycolipids, OCH and C-glycoside.

Modulations to the structure of α-GalCer can alter the cytokine output by iNKT cells following activation. The analogue, OCH, differs from α-GalCer by a truncated sphingosine base chain that is 9-carbon long. Similarly, the analogue, C-glycoside, differs by the absence of the glycosidic bond. Red circles denote differences in analogue structure compared to α-GalCer. Figure adapted from [100].

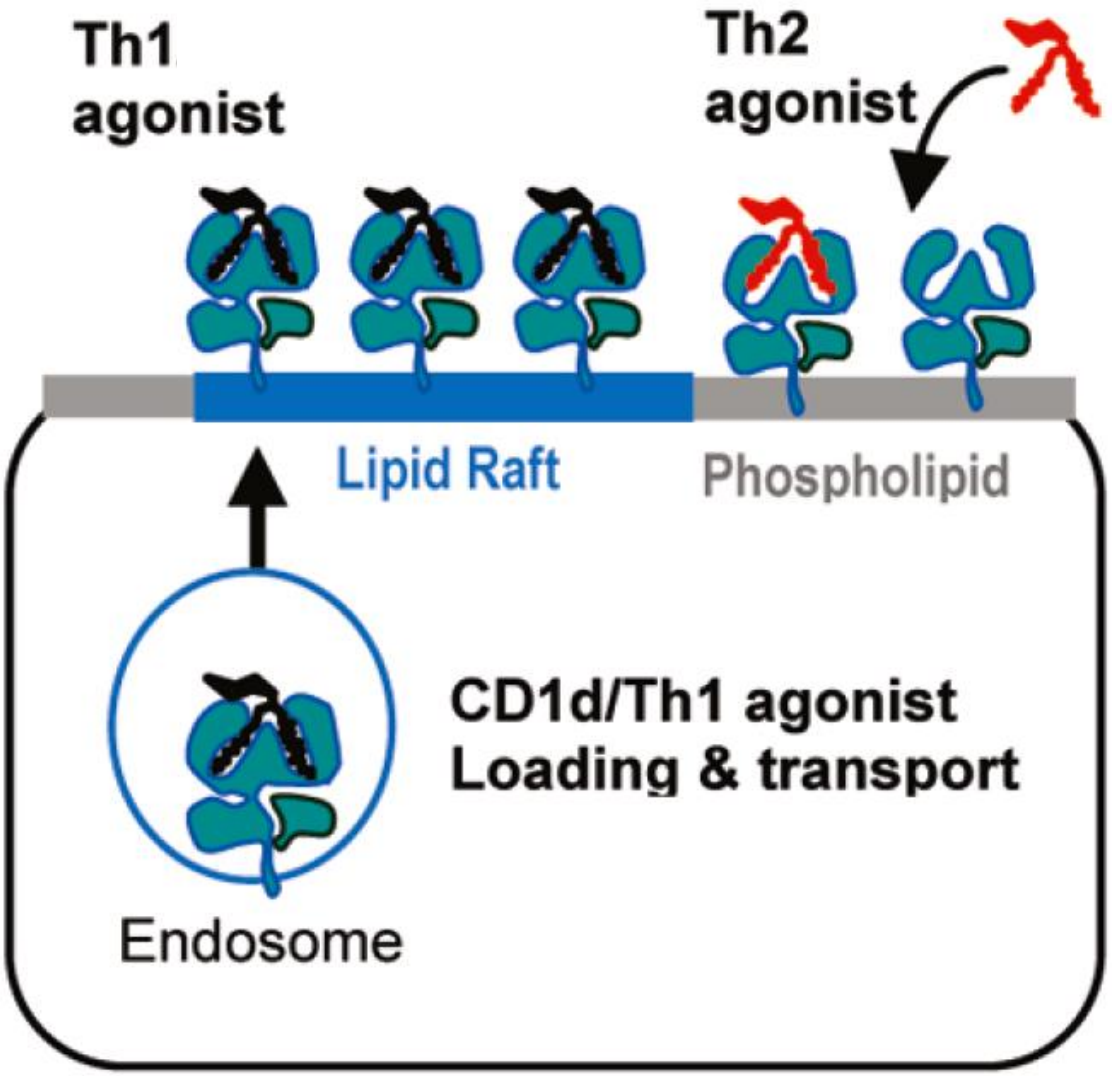


Figure 1.3 Proposed mechanism of glycolipid loading onto CD1d by antigen presenting cells (APC) for inducing T-helper 1 (Th1) or T-helper 2 (Th2) iNKT cell responses

Glycolipids that induce Th1 responses are taken up by APCs and are loaded onto CD1d within endosomes. The glycolipid-CD1d complex is then transported to the surface of the APC and the glycolipid is presented on CD1d with cholesterol-rich lipid domain (left). In contrast, glycolipids that induce Th2 responses are freely loaded onto CD1d on the cell surface for presentation to iNKT cells (right). Figure adapted from [102].

Chapter Two

General materials and methods

Mice

Adult male C57Bl/6 mice of 7-12 weeks were obtained from Monash Animal Research Platform (MARP, Clayton, VIC, Australia) and housed at Monash Medical Centre Animal Facility (MMCAF) in specific pathogen-free (SPF) conditions. Mice were acclimatised for a minimum of 7 days before the beginning of any experimental procedure. All procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCB/2016/10, Monash Medical Centre, Clayton, Victoria, Australia). All animals had access to water and food *ad libitum*, maintained in temperature-controlled rooms under a 12-hr light-dark cycle and allowed to acclimatise for at least 7 days before experimentation.

Mouse model of ischemic stroke – the middle cerebral artery occlusion (MCAO)

The mouse model of ischemic stroke was performed as previously described [115]. Mice were anaesthetised by intraperitoneal injection of ketamine (Claris) at 150 mg/kg and xylazine (Ilium) at 10 mg/kg. Once anaesthetised, an incision at the top of the head was made to expose the skull, and a laser Doppler holder (PeriFlux System 5000, Stroke model kit 407, Perimed, Järfälla-Stockholm, Sweden) was temporarily glued to the right side of the skull to monitor blood flow in the right hemisphere of the brain. The fur at the site of the neck incision was removed and the area was sterilised with 80% ethanol. A 1-2 cm incision was made along the midline of the throat to expose the trachea. The right common carotid artery, external carotid artery, and internal carotid artery were dissected away from any connective tissue. A vessel clip was used on the right common carotid artery, and a slit was made in the external carotid artery. A silicone-coated monofilament with a diameter of 0.21-0.23mm (Doccol Corporation) was immediately inserted into the external carotid artery and advanced into the internal carotid artery to occlude blood flow into the MCA. A successful occlusion of blood flow to the brain was determined by a ~70% drop in Doppler reading. The external carotid artery was then tied

off and the vessel clip removed. In this study, we utilised both reperfused and non-reperfused models of MCAO. In the reperfused model, the monofilament was retracted to allow for reperfusion of blood flow into the MCA following either 30 or 60 min of occlusion. Conversely, in the non-reperfused model, the monofilament was not retracted, and MCA was occluded for the duration of the experimental time point. The incision at the neck was then sutured, Doppler probe removed, and the head incision closed. As a control, potential surgical stress that is unavoidable to induce MCAO in mice was modelled in a sham surgery, whereby similar surgical procedures were performed without the insertion of the monofilament. After surgery, all mice were allowed to recover overnight on a heat pad and monitored and weighed hourly for up to 8 h post-surgery. Mice were then monitored daily for up to 4 days where they were humanely euthanised at each experimental timepoint. See **Appendix 1 and 2** for monitoring sheet and clinical severity scoring charts, respectively.

Preclinical MRI

An Agilent 9.4T small animal MRI scanner was used to obtain T2-weighted scans for brain oedema volume quantification during longitudinal studies. Animals were continuously anaesthetised using 1% isoflurane prior to and for the duration of the scan. The Fast Spin Echo T2-weighted imaging parameters were: slice thickness = 0.3 mm; repetition time = 4000 ms; echo time = 42.528 ms; echo train length = 4; averages = 6; flip angle = 90°; FOV= 20x20 mm and matrix size= 128x128. MRI scans were used to determine brain oedema volume for each animal using ImageJ (NIH, Bethesda, MD, USA). MRI was performed by Dr. Tara Sepehrizadeh and Gang Zheng from the Monash Biomedical Imaging facility.

Neurological assessment

At 8 h and every 24 h following surgery until the experimental endpoint, neurological assessment was performed using a six-point scoring system [116]: 0 = normal motor function; 1 = flexion of torso and contralateral forelimb when lifted by tail; 2 = circulating when mouse is held by tail on flat surface; 3 = leaning to one side at rest; 4 = no spontaneous motor activity; 5 = death.

Brain infarct quantification

At experimental endpoints, mice were anaesthetised and humanely euthanised via cervical dislocation. The head of the mouse was cut off and skin peeled back to expose the skull. The skull at the top of the head was removed and the brain was carefully dissected out of the skull. Brains were slowly frozen over liquid nitrogen. Coronal sections at a thickness of 30 μm were collected at every ~420 μm. For infarct staining, sections were submerged in 0.1% thionin (Sigma) for 2 mins, 70% ethanol for 2 mins, and 100% ethanol for 2 mins, with washing performed with deionised water between each step. Sections were dried and submerged in Histosol before mounting coverslips using DPX Mountant (Thermo Fisher). Images were captured using a Canon PowerShot SX730 HS mounted above a light box. Infarct volume was measured using ImageJ (NIH, Bethesda, MD, USA) and corrected for brain oedema using the formula: corrected infarct volume = [left hemisphere area – (right hemisphere area – right hemisphere infarct area) × (thickness of section + distance between sections)] [116].

Bacteriological analysis

At experimental endpoints, mice were humanely euthanised and sterilised with 80% ethanol. The skin at the chest was cut away and tools were resterilised with 80% ethanol before dissecting the lung out of the chest cavity. Lungs were weighed, placed in sterile phosphate

buffered saline (PBS – 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and homogenised under sterile conditions. For determination of colony forming units (CFUs), 10 µl of tissue homogenate was serially diluted and plated in triplicates onto brain heart infusion (BHI) agar plates, supplemented with 5% sheep blood as supplied by the Department of Microbiology at Monash University. Plates were incubated at 37°C in aerobic conditions and bacterial colonies quantified after 24 h. Data is expressed as CFU/g of tissue using the equation: (triplicate average × $10^{\text{dilution factor}}$)/tissue weight.

Enumeration of circulating leukocytes

At experimental endpoints, mice were anaesthetised by isoflurane inhalation and whole blood collected via cardiac puncture using a 21G needle. 10 µl of whole blood was added to an Eppendorf tube containing 190 µl of crystal violet cell counting solution (0.05% crystal violet w/v). Circulating leukocytes were enumerated on a haemocytometer using the equation: averaging the cell count in the outer four quadrants × dilution factor × 10^4. Cell counts were expressed as number of leukocytes/ml of blood.

Flow cytometry

To examine the immune cell composition of circulating and lung leukocytes, flow cytometric analyses were performed on single-cell suspensions. At experimental endpoints, mice were anaesthetised, and blood was taken via cardiac puncture and immediately placed into tubes containing EDTA (Sarsdtet). Circulating leukocytes were pelleted (centrifuging for 5 mins at 491 × *g* at 4ºC) and washed with FACS buffer (PBS with 2% foetal calf serum, 4 mM EDTA). The cell pellet then underwent red blood cell (RBC) lysis by resuspending in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 3 min at room temperature (RT). Cells were washed with FACS buffer and cell lysis repeated if required to

obtain a single-cell suspension for antibody staining. For the lung, all lobes were placed on ice and finely minced using scissors. Lungs were placed in digestion buffer containing 3 ml of 0.5 mg/mL of collagenase D (Sigma-Aldrich) in full media (RPMI supplemented with 10% foetal calf serum, 1% Pen/Strep, 4 mM L-Glut) and digested for 40 min at 37ºC with gentle agitation. To stop the digestion, 8 ml of cold PBS was added to the lung homogenate and samples placed on ice. Samples were then passed through a 70 µm filter, washed with FACS buffer and pelleted for RBC lysis by resuspending in 3 ml of ACK lysis buffer for 3 min at RT with inversion. To stop RBC lysis, 8 ml of PBS was added, and cells further pelleted and washed with FACS buffer for filtration through a 40 µm filter. Single-cell suspensions were added to a 96-well plate, pelleted and resuspended in fluorescent antibody cocktails containing Fc receptor blocker (2.4G2, BD Biosciences) with either: a) PE-conjugated anti-CD45 (30-F11, eBioscience), FITC-conjugated anti-CD11b (M1/70, BioLegend), APC-Cy7-conjugated anti-Ly6C (AL-21, BD Pharmingen), BV510-conjugated anti-Ly6G (1A8, BioLegend), PE-Cy7-conjugated anti-F4/80 (BM8, eBioscience); or b) PE-conjugated anti-CD45 (F11, eBioscience), APC-conjugated anti-CD3e (145-2C11, BD Biosciences), PE-Cy7-conjugated anti-CD4 (RM4-5, eBiosciences), FITC-conjugated anti-CD8 (53-6.7, BD Biosciences), e450-conjugated anti-B220 (RA3-6B2, eBiosciences). Cell viability was determined using 7-Aminoactinomycin D (7AAD; BioLegend).

For analysis of iNKT cells, the single-cell suspensions were pelleted and resuspended in a fluorescent antibody cocktail containing: Fc receptor blocker (2.4G2, BD Biosciences), APC eFluor780-conjugated anti-CD45 (30-F11, Invitrogen), APC-conjugated anti-TCRβ (H57-597, BD Pharmingen), PE-conjugated PBS-56-loaded CD1d-tetramer (NIH tetramer facility, Atlanta, GH), FITC-conjugated anti-CD69 (H1.2F3, BD Pharmingen). Cells were then permeabilised using the Cytofix/Cytoperm Plus kit (BD Biosciences) as per manufacturer's

protocol and intracellular cytokines were stained with PE Cy7-conjugated anti-IFN-γ (XMG1.2, eBioscience) and PerCP-Cy5.5-conjugated anti-IL-10 (JES5-16e3, eBioscience).

Cells were run along with Counting Beads (BD Biosciences) and quantified on a BD LSRFortessa (BD Biosciences), where data was analysed using FlowJo (v10.0.7, Tree Star Inc.). To differentiate immune cell populations, all live leukocytes were firstly defined as viability dye negative (7AAD or Live/Dead dye) and $CD45^+$, and further subtyped into neutrophils ($CD11b^+/Ly6G^+$), monocytes ($CD11b^+/Ly6C^+$) T-helper cells ($CD3^+/CD4^+$), cytotoxic T cells ($CD3^+/CD8^+$), B cells ($B220^+$) and iNKT cells ($TCR\beta^+/CD1d\text{-}tet^+$). See **Appendix 3 and 4** for list of antibodies and gating strategies, respectively.

Serum cytokine analysis

To obtain sera, mice were euthanised and whole blood was taken via cardiac puncture. Blood was allowed to clot for 25 min at RT and centrifuged at 2.4 × *g* for 5 min for sera collection. For lungs, the left lobe was homogenised in 700 µl of cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma). Lung homogenates were centrifuged at 6000 × *g* for 10 min at 4ºC. Sera and lung supernatant were collected and stored at -80ºC until analysis. A mouse IFN-γ (AN-18) and IL-10 OptEIA ELISA set (BD Biosciences) were used as per manufacturer's protocol to quantify cytokine levels in the serum and lung.

Statistical analysis

Data were analysed into GraphPad Prism 8. When comparing normally distributed data between two groups, an unpaired *t*-test or Mann-Whitney *u*-test was used following exclusion of significant outliers using Grubbs' test. For comparisons of more than two groups, a One-way ANOVA with Sidak's multiple comparisons test was used for normally distributed data,

while a Kruskal-Wallis test with Dunn's multiple comparisons test was used for non-parametric data. All graphs are presented as mean ± standard error of the mean (SEM). Comparisons were considered statistically significant if the *p-value (p)*<0.05.

Chapter Three

The impact of infarct size and location on post-stroke infection

Translational Stroke Research
https://doi.org/10.1007/s12975-019-00738-3

ORIGINAL ARTICLE

Stroke Severity, and Not Cerebral Infarct Location, Increases the Risk of Infection

Raymond Shim[1] · Shu Wen Wen[1] · Brooke J. Wanrooy[1] · Michelle Rank[2,3] · Tharani Thirugnanachandran[4] · Luke Ho[1,5] · Tara Sepehrizadeh[6] · Michael de Veer[6] · Velandai K. Srikanth[5] · Henry Ma[4] · Thanh G. Phan[4] · Christopher G. Sobey[7] · Connie H. Y. Wong[1]

Received: 22 June 2019 / Revised: 9 September 2019 / Accepted: 10 September 2019

Abstract

Infection is a leading cause of death in patients with stroke; however, the impact of cerebral infarct size or location on infectious outcome is unclear. To examine the effect of infarct size on post-stroke infection, we utilised the intraluminal middle-cerebral artery occlusion (MCAO) mouse model of ischemic stroke and adjusted the duration of arterial occlusion. At 1 day following stroke onset, the proportion of mice with infection was significantly greater in mice that had larger infarct sizes. Additionally, the presence of lung infection in these mice with severe strokes extended past 2 days, suggestive of long-term immune impairment. At the acute phase, our data demonstrated an inverse relationship between infarct volume and the number of circulating leukocytes, indicating the elevated risk of infection in more severe stroke is associated with reduced cellularity in peripheral blood, owing predominately to markedly decreased lymphocyte numbers. In addition, the stroke-induced reduction of lymphocyte-to-neutrophil ratio was also evident in the lung of all post-stroke animals. To investigate the effect of infarct location on post-stroke infection, we additionally performed a photothrombotic (PT) model of stroke and using an innovative systematic approach of analysis, we found the location of cerebral infarct does not impact on the susceptibility of post-stroke infection, confirming the greater role of infarct volume over infarct location in the susceptibility to infection. Our experimental findings were validated in a clinical setting and reinforced that stroke severity, and not infarct location, influences the risk of infection after stroke.

Keywords Stroke · Infection · Infarct volume · Infarct location

Introduction

Ischemic stroke is a debilitating cerebrovascular disease that is a leading contributor to death and disability worldwide. While stroke is traditionally associated with disability and devastating neurological deficits, a major cause of poor outcome and death for patients with stroke is infection [1]. Infections are reported in up to 64% of patients and can develop within

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12975-019-00738-3) contains supplementary material, which is available to authorized users.

✉ Connie H. Y. Wong
connie.wong@monash.edu

[1] Centre for Inflammatory Diseases, Department of Medicine at Monash Health, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, VIC 3168, Australia

[2] Department of Anatomy and Neuroscience, School of Biomedical Sciences, The University of Melbourne, Parkville, Victoria, Australia

[3] School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria, Australia

[4] Stroke and Ageing Research Group, Department of Medicine at Monash Health, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, Victoria, Australia

[5] Department of Medicine (Academic Unit), Peninsula Clinical School, Central Clinical School, Monash University, Frankston, Victoria, Australia

[6] Monash Biomedical Imaging, Monash University, Clayton, Victoria, Australia

[7] Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Victoria, Australia

Published online: 10 November 2019

3 days of stroke onset [2]. Bacterial pneumonia is the most common fatal infectious complication in stroke and contributes significantly to extended hospital stay, worsening of neurological outcome, development of further complications, and death [3]. As such, recent research has focused on strategies to reduce the occurrence of post-stroke infection in order to improve patient outcome. Most notably, recent clinical trials tested the efficacy of preventive antibiotic therapy (PAT) to reduce the occurrence of post-stroke infection [4–6]. Unfortunately, findings showed that PAT could not reduce the incidence of post-stroke pneumonia nor provide additional benefits over standard treatment [5–9]. In light of the heterogeneity of stroke amongst patients, it is not surprising that the therapeutic strategy of a "one-size-fits-all" treatment would fail. Therefore, a greater understanding of the effect of different stroke phenotypes on host defence and the associated life-threatening sequelae of infection is crucial for facilitating the development of better targeted treatments.

The elevated risk of infection in patients with stroke suggests that immune functions that normally facilitate bacterial clearance are weakened. Supporting this notion, findings from clinical and experimental studies demonstrated that stroke can reshape systemic immune responses. Within hours to days of stroke onset, there is evidence for profound loss of circulating lymphocytes (lymphocytopenia), reduced spleen cellularity, and impaired immune cell functions [10–12]. Recent studies have proposed that the degree of immune impairment and susceptibility to infection is linked to the severity of stroke. Indeed, stroke patients with larger cerebral infarcts display increased circulating levels of interleukin-10 (IL-10), reduction in Th1/Th2 ratio [12], impairment of monocyte and lymphocyte function [13], and reduced spleen cellularity [14]. These clinical observations support the notion that the extent of brain injury induced by stroke can potentially be used to predict infection risk [15–17]. Experimental studies with animal models have been utilised to delineate the mechanisms underlying how larger brain infarction promotes greater immune impairment. However, the majority of these studies employ a single model of stroke, possibly overlooking the effect of cerebral reperfusion and varying stroke severity on peripheral immunity and post-stroke infection.

Another factor that may impact the susceptibility to post-stroke infection is the area of the brain in which cerebral ischemia occurs. Lesions involving the superior and middle temporal gyri, orbitofrontal cortex, or the superior longitudinal fasciculus have been associated with infection development in a clinical setting [17]. In addition, infarction of the insular cortex has been reported to associate with prolonged dysphagia [18]—a well-known risk factor for post-stroke pneumonia [19–21]. Clinical studies have observed an impaired systemic immune function, increased susceptibility to infection, and worse functional outcome in patients with strokes involving the insular cortex [22–24]. However, the relationship may be confounded as the insular cortex is a common area of infarction in the middle cerebral artery (MCA) territory [25]. Indeed, a recent study suggests that the detrimental outcomes of insular strokes were instead due to larger infarct volumes [26]. This was supported by other studies that found no associations of insular strokes with infection or immune alterations after correcting for infarct volume [27, 28]. In light of this, the relationship between stroke infarct size, localisation and post-stroke infection clearly remains controversial and requires further study.

In this study, we aimed to investigate the role of cerebral lesion size following reperfused or non-reperfused ischemic stroke on the extent and frequency of post-stroke infection over time. In addition, we assessed systemic immune parameters, including leukocyte counts and composition in the blood, lung, and spleen. Furthermore, to assess the contribution of infarct localisation on infection, independent of infarct size, we utilised an innovative systematic approach to explore the effect of cerebral infarction involving 12 brain regions on post-stroke infection. Finally, we conducted a retrospective clinical study whereby the findings are consistent with our experimental study, demonstrating that cerebral lesion size is an important determinant for infection, and not stroke infarct location.

Methods

Mice

Adult male C57Bl/6 mice of 7–12 weeks were obtained from Monash Animal Research Platform (MARP, Clayton, VIC, Australia) and housed at Monash Medical Centre Animal Facility (MMCAF) in specific pathogen-free (SPF) conditions. All procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCB/2016/10, Monash Medical Centre, Clayton, Victoria, Australia).

For photothrombotic (PT) stroke experiments, 7–8-week-old male C57Bl/6J mice were sourced from Animal Resources Centre (ARC, Canning Vale, Western Australia, Australia) and housed under SPF conditions at RMIT University Animal Facility (RAF, Bundoora, Victoria, Australia). Procedures for PT strokes were approved by the RMIT Animal Ethics Committee (AEC1613, RMIT University, Bundoora, Victoria, Australia).

All animals had access to water and food ad libitum, maintained in temperature-controlled rooms under a 12-h light-dark cycle and allowed to acclimatise for at least 7 days before experimentation.

MCAO

The mouse model of ischemic stroke was performed as previously described [29]. Briefly, mice were anaesthetised by

intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Once anaesthetised, an incision at the top of the head was made to expose the skull, and a laser Doppler holder (PeriFlux System 5000, Stroke model kit 407, Perimed, Järfälla-Stockholm, Sweden) was glued to the right side of the skull to monitor blood flow in the right hemisphere of the brain. The fur at the site of the neck incision was removed and the area was sterilised with ethanol. A 1–2-cm incision was made along the midline of the throat to expose the trachea. The right common carotid artery, external carotid artery, and internal carotid artery were dissected away from any connective tissue. A vessel clip was used on the right common carotid artery, and a slit was made in the external carotid artery. A silicone-coated monofilament with a diameter of 0.21–0.23 mm (Doccol Corporation) was immediately inserted into the external carotid artery and advanced into the internal carotid artery to occlude blood flow into the MCA. A successful occlusion was determined by a ~ 70% drop in Doppler reading. The external carotid artery was tied off and the vessel clip removed. In this study, we utilised both reperfused and non-reperfused models of middle cerebral artery occlusion (MCAO). In the reperfused model, the monofilament was retracted to allow for reperfusion of blood flow into the MCA following either 30 or 60 min of occlusion. Conversely, in the non-reperfused model, the monofilament was not retracted, and MCA was occluded for the duration of the experimental time point. The incision at the neck was then sutured, Doppler probe removed, and the head incision closed. As a control, potential surgical stress was mimicked in a sham surgery, whereby similar surgical procedures were performed without the insertion of the monofilament. After surgery, all mice were allowed to recover overnight on a heat pad. Mice were then monitored daily for up to 4 days where they were humanely euthanised at each experimental timepoint.

Photothrombotic Vascular Occlusion

Photothrombotic vascular occlusion creates an ischemic infarction/stroke with clear and well-delimited borders that are highly reproducible. All photothrombotic stroke procedures were performed under aseptic conditions at the RMIT University Animal Facility. Mice were anesthetised with inhaled isoflurane (5% induction and 1.5–2% maintenance in medical oxygen at 0.2 ml/min flow rate) and placed onto a heating pad to maintain body temperature at 37 °C, as measured by a rectal thermal probe (Stoelting Rodent Warmer X2, Stoelting Co., Wood Dale, IL, USA). Once anesthetised, fur on the scalp was removed with electric clippers, the mouse was positioned in a digital stereotaxic frame (Stoelting Co., Wood Dale, IL, USA), and the skin over the scalp cleaned with 70% ethanol. Using a scalpel, an incision was made in the skin overlying the desired infarct area and the skull exposed. Infarct coordinates, as determined using the Mouse Brain Atlas [30] were marked on the skull with the aid of the digital stereotactic frame. Mice were then injected with Rose Bengal i.p. (Merck KGaA, Darmstadt, Germany) and after 3.5 min the area of the skull overlying various cortices was illuminated with a 565 nm LED using a 1500-μm-diameter fibre optic cable (ThorLabs, Newton, NJ, USA) for 15 or 20 min.

To produce an infarct targeting the sensorimotor cortex, mice were injected with 80 mg/kg i.p. of Rose Bengal, and the skull over the sensorimotor cortex representation (2.2 mm right of bregma) was illuminated for 15 min. Due to the anatomical position of the insular cortex, the thickness of the skull greatly obstructs penetration of light into this area of the brain. Therefore, to produce an infarct targeting the insular cortex, mice were injected with a dosage of 120 mg/kg i.p. of Rose Bengal and the skull over the insular cortex representation (2.8 mm depth from Bregma on the right lateral surface of the skull) was illuminated for 20 min. In order to access the insular cortex region with the fibre optic cable, mice were positioned with the head tilted to the left. All Rose Bengal doses and illumination times were optimised for the desired cortical lesion size and location in preliminary experiments. After surgery, all mice were allowed to recover overnight on a heat pad and post-surgical analgesia was provided by buprenorphine (0.05 mg/kg s.c. every 12 h for 24 h).

Preclinical MRI

An Agilent 9.4 T small animal magnetic resonance imaging (MRI) scanner was used to obtain T2-weighted scans for brain oedema volume quantification during longitudinal studies. Animals were continuously anaesthetised using 1% isoflurane prior to and for the duration of the scan. The Fast Spin Echo T2-weighted imaging parameters were as follows: slice thickness = 0.3 mm; repetition time = 4000 ms; echo time = 42.528 ms; echo train length = 4; averages = 6; flip angle = 90°; FOV = 20 × 20 mm and matrix size = 128 × 128. MRI scans were used to determine brain oedema volume for each animal using ImageJ (NIH, Bethesda, MD, USA).

Neurological Assessment

At 8 h and every 24 h following surgery until the experimental endpoint, neurological assessment was performed using a six-point scoring system [31]: 0 = normal motor function; 1 = flexion of torso and contralateral forelimb when lifted by tail; 2 = circulating when mouse is held by tail on flat surface; 3 = leaning to one side at rest; 4 = no spontaneous motor activity; 5 = death.

Infarct Quantification

At experimental endpoints, brains were dissected and slowly frozen over liquid nitrogen. Coronal sections at a thickness of

30 μm were taken at every ~420 μm. Sections were stained with 0.1% thionin (Sigma) to visualise brain infarct. Images were captured using a Canon PowerShot SX730 HS mounted above a light box. Infarct volume was measured using ImageJ (NIH, Bethesda, MD, USA) and corrected for brain oedema using the formula: corrected infarct volume = [left hemisphere area − (right hemisphere area − right hemisphere infarct area) × (thickness of section + distance between sections)] [31].

Bacteriological Analysis

At experimental endpoints, the lung was dissected and weighed. Tissues were placed in sterile PBS and homogenised under sterile conditions. For determination of colony forming units (CFUs), 10 μL of tissue homogenate was serially diluted and plated onto brain heart infusion (BHI) agar plates, supplemented with 5% sheep blood. Plates were incubated at 37 °C and bacterial colonies quantified after 24 h.

Circulating Leukocytes Analyses

At experimental endpoints, mice were anaesthetised and whole blood collected via cardiac puncture. Quantification of circulating white blood cells (WBCs) was performed using a crystal violet cell counting solution (0.05% crystal violet *w/v*).

Flow Cytometry

To examine the immune cell composition of circulating and lung leukocytes, flow cytometric analyses were performed on single-cell suspensions. Blood was taken via cardiac puncture and immediately placed into tubes containing EDTA. Circulating leukocytes were washed with FACS buffer, underwent RBC lysis, and washed again to obtain a single-cell suspension for staining. For the lung, a single-cell suspension was achieved by mincing the tissue, digestion in 0.5 mg/mL of collagenase D (Sigma-Aldrich), RBC lysis and finally followed by filtration through a 40 μm filter. Single-cell suspensions were pelleted and resuspended in fluorescent antibody cocktails containing Fc receptor blocker with either: (a) PE-conjugated anti-CD45 (30-F11, eBioscience), FITC-conjugated anti-CD11b (M1/70, BioLegend), APC-Cy7-conjugated anti-Ly6C (AL-21, BD Pharmingen), BV510-conjugated anti-Ly6G (1A8, BioLegend), PE-Cy7-conjugated anti-F4/80 (BM8, eBioscience); or b) PE-conjugated anti-CD45 (F11, eBioscience), APC-conjugated anti-CD3e (145-2C11, BD Biosciences), PE-Cy7-conjugated anti-CD4 (RM4-5, eBiosciences), FITC-conjugated anti-CD8 (53-6.7, BD Biosciences), e450-conjugated anti-B220 (RA3-6B2, eBiosciences). Cell viability was determined using 7-aminoactinomycin D (BioLegend). Cells were run along with Counting Beads (BD Biosciences) and quantified on a BD LSRFortessa (BD Biosciences), where data was analysed using FlowJo (v10.0.7, Tree Star Inc.). For cell populations, all leukocytes were defined as $CD45^+$, where they were further separated into neutrophils ($CD11b^+/Ly6G^+$), monocytes ($CD11b^+/Ly6C^+$) T-helper cells ($CD3^+/CD4^+$), cytotoxic T cells ($CD3^+/CD8^+$), and B cells ($B220^+$). The lymphocyte-neutrophil ratio was calculated by dividing the sum of T and B cells by the number of neutrophils.

Mouse Cerebral Infarct Location Analysis

A database of all experimental animals in this study was created. Of all the experimental animals, data from mice with an experimental endpoint of 1 day following stroke were combined for further retrospective analysis. Mice that underwent preclinical MRI scans were excluded as these animals were not culled until day 4. Any mice with data missing or unavailable were also excluded. Four double-blinded investigators examined thionin-stained brain sections from all included mice to determine infarct involvement within various brain regions. Twelve brain regions were examined: caudoputamen, insular cortex, somatomotor area, somatosensory area, piriform area, gustatory areas, visceral areas, striatum ventral region, hypothalamus, thalamus, midbrain, and hippocampal region (as defined by the Allen Brain Atlas—accessible from http://mouse.brain-map.org/static/atlas). For each data set, investigators recorded the number of sections in which infarct occurred within each specified brain region.

Patient Sampling

A retrospective cohort design was used, where all information was obtained from scanned medical and electronic records available at the time of data collection. All patients primarily admitted for an acute stroke to Monash Medical Centre between 16 January 2015 and 10 February 2016 were enrolled, and the data presented here are a subset of that used in a previously published article [32]. Patients with haemorrhagic stroke, transient ischemic attacks, or those who developed a stroke as a complication of hospitalisation and thus had a separate reason for admission, were excluded. Included patients were followed-up for development of infections until the day of discharge from the acute ward or death, depending on which came first. If death occurred in the sub-acute setting (rehabilitation unit) within the same admission, it was also recorded. All data extraction was performed by a sole, blinded investigator (L.H.) who was not involved in infection assessment. The severity of stroke was determined in most cases using the National Institute of Health Stroke Scale (NIHSS) within 24 h of admission. If that was not documented, a retrospective NIHSS was obtained on the documented signs and symptoms on admission. Computed tomography (CT) brain scans at stroke onset were used to differentiate between

ischemic stroke, taking care not to misclassify those with haemorrhagic transformation within an ischemic stroke.

Patient Data Evaluation and Analysis

All infections that developed while patients were in the acute stroke unit were recorded. Infections were defined with reference to the Centre of Disease Control (CDC) criteria for infections, which required certain clinical, pathological, radiological and microbiological evidence.

All relevant evidence relating to a potential infection were recorded, systematically reviewed by a senior physician (V.K.S.) with reference to the CDC/NHSN criteria and classified as "Definite", "Probable" or "Unlikely". Definite infections were diagnosed in those with sufficient information to meet the CDC/NHSN criteria. Probable infections were diagnosed in those with insufficient information to meet the CDC/NHSN criteria but were regarded as a likely infection by the physician. Unlikely infections were diagnosed in those with sufficient information to not meet the criteria.

Furthermore, data were collected on the use of antibiotics, and invasive procedures such as intravenous cannula (IVC), indwelling catheter (IDC), nasogastric tube (NGT) and endotracheal tube (ETT). The use of pre-admission medications that may influence immune function and infection risk including antibiotics, immunosuppressive medications and beta-blockers were obtained through pharmacy records. The presence or history of comorbidities that may predispose to infection risk were also recorded.

Statistics

Experimental data were input into GraphPad Prism 7 and analysed using one-way ANOVAs with Sidak's multiple comparisons test to compare the means of multiple groups, except for non-parametric data where a Kruskal-Wallis test with Dunn's multiple comparisons test was used. For comparison of data between two groups, an unpaired t-test or Mann-Whitney test was used depending on the distribution of data. Difference in infection rate between groups was analysed with a χ^2 test. Comparisons were considered significant if the p value was < 0.05.

To analyse the contribution of each independent variable to the presence of infection in our mouse data set, we used the multivariate adaptive regression spline (MARS) algorithm (a non-parametric regression analysis) from the *earth* package, R [33]. This method can be used to capture non-linear relationships between each predictor variable and the outcome variable in a data-driven manner. The analysis assesses each data point for each predictor as a knot and fits a linear regression model to either side of a hinge point to achieve the smallest error.

For patient data, a multivariable logistic regression analysis was performed by a blinded investigator (V.K.S) and used to determine the association between stroke and the presence of infection after adjusting for confounding factors of age, sex, stroke severity, the use of medications affecting infection risk (specifically immunosuppressive including corticosteroids, immunological therapies, disease-modifying anti-rheumatic drugs, chemotherapy), the presence of any comorbid conditions affecting infection risk (such as diabetes mellitus, chronic lung disease, chronic liver disease, active malignancy), the use of indwelling urinary catheter, the presence of NGT feeding and cerebral infarct location defined as cortical versus non-cortical.

Results

Infection Is more Prevalent after a Severe Stroke

To investigate the effect of stroke severity on infection, we used the MCAO model of ischemic stroke and adjusted the duration of occlusion with the aim of achieving varying severities, and examined the effect of reperfusion. Our models of MCAO were: 30 min transient MCAO (tMCAO) = 30 min of arterial occlusion with reperfusion; 60 min tMCAO = 60 min of arterial occlusion with reperfusion; permanent MCAO (pMCAO) = occlusion of MCA throughout the experimental period. The volume of cerebral infarct lesion, amount of cerebral oedema, neurological deficit and lung infection were therefore all assessed at 24 h following stroke onset (Fig. 1). Histological analysis of thionin-stained brain sections (Fig. 1a) revealed an increase of infarct volume with increasing duration of MCA occlusion (Fig. 1b). This result is consistent with the more sensitive imaging modality of in vivo MRI, where oedema volume, assessed in a blinded manner, was significantly increased with occlusion time (Fig. 1c). The successful induction of increasing stroke severities was also confirmed through neurological scoring where mice showed enhanced neurological impairment with increasing duration of arterial occlusion (Fig. 1d). From the parameters examined, we thus established mild (30 min tMCAO), moderate (60 min tMCAO), and severe (pMCAO) models of experimental stroke.

Bacterial pneumonia is a frequent and often fatal complication after stroke, both in experimental and clinical settings [34]. However, the roles of cerebral reperfusion and stroke severity on post-stroke infection remain unclear. As such, we next examined the contribution of stroke severity and duration of occlusion on the prevalence of post-stroke infections. Bacterial cultures of lung tissue revealed an increasing presence of culturable bacteria following prolonged occlusion of MCA, with mice that underwent pMCAO having the most dramatic increase compared to the sham surgical control (Fig. 1e). Interestingly, there appears to be no difference in

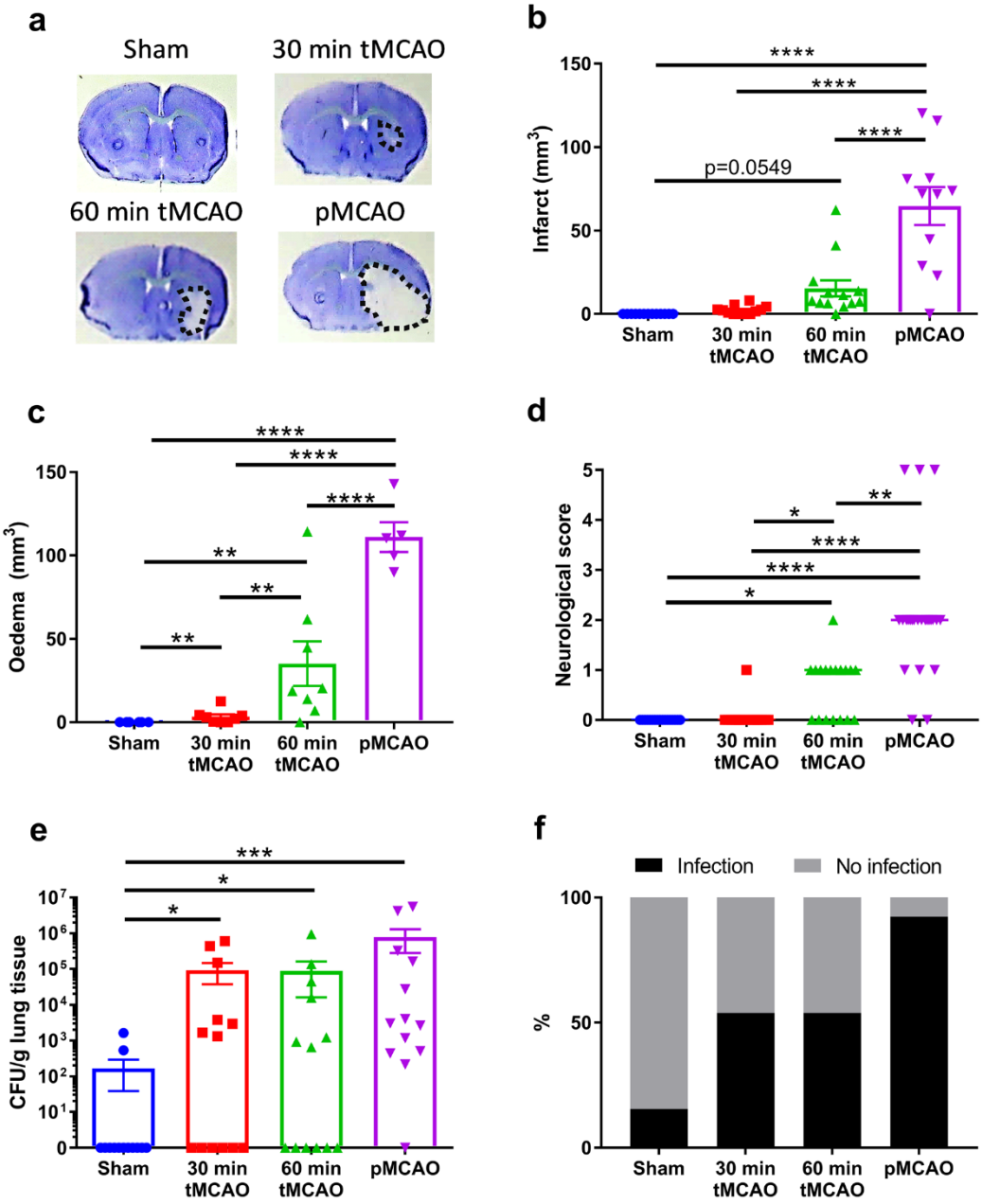


Fig. 1 Infection is more frequent at 1 day following severe stroke. Stroke (30 min tMCAO, 60 min tMCAO, or pMCAO) or sham surgery was performed in independent mice and culled 1 day after surgery. Upon euthanisation, brains were sectioned and stained with 0.1% thionin, where infarct region is denoted in white and outlined with a black dotted line (**a**). Infarct volume was calculated using ImageJ (**b**). Similarly, a separate cohort of mice underwent MRI scans at 1 day after surgery to determine oedema volumes (**c**). Prior to euthanasia, neurological score was recorded, where 0 = normal motor function; 1 = flexion of torso and contralateral forelimb when lifted by tail; 2 = circling when mouse is held by tail on flat surface; 3 = leaning to one side at rest; 4 = no spontaneous motor activity; 5 = death within 24 h (**d**). Lungs were collected for bacteriological analysis, where bacterial load was measured (e) and infection rate was also recorded (**f**). Graphs are presented as mean ± SEM. Neurological score data is presented as median. $n \geq 10$ mice per group or $n \geq 4$ for MRI study, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, one-way ANOVA normally distributed data or Kruskal-Wallis test for bacteriological analysis

the amount of culturable lung bacteria between post-stroke animals. However, when comparing lung infection rate after stroke, the proportion of mice that acquired lung infection significantly increased with stroke severity (χ^2 test for trend =

13.93, $p = 0.0002$) (Fig. 1f). Taken together, with our established models of stroke severity, our findings demonstrate that stroke severity has a substantial impact on the host susceptibility to lung infection.

Infection Peaks at 1 Day After Experimental Stroke

To assess the effect of stroke severity on infection risk over time, we conducted a longitudinal study of up to 4 days following the onset of mild, moderate, or severe stroke. We monitored the development and resolution of oedema in the brain via in vivo MRI in the same animals over the experimental period (Fig. 2a). Over the course of 4 days, mice that underwent 30 min or 60 min tMCAO surgery had oedema peaking at 1 day following stroke onset that returned to baseline by day 4 (Fig. 2b). Consistent with this, the infarct volume was not larger at day 2 or 4 after these models of tMCAO (Fig. 2c). The recovery of oedema volume toward baseline corresponded with neurological scores, where mice displayed normal motor function by 4 days following stroke onset (Fig. 2d). However, mice that were subjected to pMCAO displayed larger brain oedema (Fig. 2b) and infarct (Fig. 2c) volumes after 1 day and remained significantly higher than transient or sham stroke at day 4, with neurological deficit evident throughout the experimental period (Fig. 2d). To assess longer term impact of stroke on lung infection, we collected the lungs and performed bacteriological analyses via culturing at days 2 or 4 following surgery. By 2 days after either 30 min or 60 min tMCAO, lung infection diminished to baseline levels (Fig. 2e). Conversely, lung infection was still evident in mice that underwent pMCAO at day 2, and this returned to baseline at 4 days following stroke onset (Fig. 2e). These findings indicate that the peak of lung infection occurs at 1 day following experimental stroke and requires a longer period of time to resolve following a severe, non-reperfused stroke.

Non-reperfused Cerebral Ischemia Results in Severe and Prolonged Immune Alterations

We next explored the effect of stroke on peripheral immunity by examining differences in leukocyte composition in the blood and lung, and in spleen weight within 4 days following stroke onset. Consistent with previous reports, stroke onset decreased total circulating leukocyte numbers (Fig. 3a). In fact, circulating leukocyte number was inversely correlated with infarct volume ($p = 0.0085$, $r^2 = 0.1412$) (Fig. 3b). This finding suggests that a more pronounced immune alteration occurs with a greater severity of stroke. Temporally, circulating leukocyte levels remained lower than sham levels throughout the experimental period in mice that underwent pMCAO (Fig. 3c). The spleen followed a similar trend whereby spleen weights were less than sham level at 1 day following all models of stroke. At day 2 post-surgery, spleens in mice that underwent 30 or 60 min tMCAO were equivalent to sham weight, while spleen weights remained low throughout the 4-day experimental period in mice that underwent pMCAO (Fig. 3d). As infection in the lung was most prominent at 1 day following stroke, we further analysed differences in immune cell populations in the blood and lungs at this timepoint. The lymphocyte-neutrophil ratio in blood has previously been used as a predictor for community-acquired bacterial pneumonia [35, 36] and more recently in post-stroke pneumonia in humans [37]. In our experimental study, we used flow cytometry to assess the immune cell composition in the blood as well as at the site of infection (lung) of post-stroke mice at 1 day following surgery and we calculated the lymphocyte-neutrophil ratio. Compared with sham mice, there was a markedly lower lymphocyte-neutrophil ratio in the blood and lung after all severities of stroke, which was most pronounced in mice subjected to pMCAO (Fig. 3 e and f). This reduced lymphocyte-neutrophil ratio in the blood and lung was primarily due to the loss of lymphocytes, including B cells and $CD4^+$ T cells and $CD8^+$ T cells, while neutrophil numbers remain unchanged (Supp. Fig. 1 and Supp. Fig. 2). These data demonstrate that stroke induces a drastic change in peripheral immune cell populations, with severe strokes producing longer lasting changes.

The Location of Cerebral Infarction Does Not Impact on Post-Stroke Infection

Whether the specific location of a cerebral infarct promotes greater susceptibility to infection remains controversial as brain lesions in the MCA territory can affect many common areas. As such, we sought to determine if damage of different cerebral cortices would induce systemic immune changes that alter host susceptibility to post-stroke lung infection. Using the PT stroke model, we accurately targeted the insular or sensorimotor cortex as confirmed through thionin staining (Fig. 4a). The dose of Rose Bengal and the duration of light exposure to induce cerebral infarct within the sensorimotor or insular cortex were adjusted to create comparable infarction in order to directly investigate the effect of infarct location without the influence of infarct size. Indeed, cerebral infarction generated with this model in the insular or sensorimotor cortex displayed no significant differences in infarct size (Fig. 4b). Moreover, our findings demonstrated that spleen weight (Fig. 4c) and circulating leukocyte counts (Fig. 4d) at 1 day following PT stroke onset were comparable in mice that underwent cerebral injury in the insular or sensorimotor cortex. Additionally, there was no difference in the degree or rate of infection following stroke within the insular or sensorimotor cortex (Fig. 4e). These data suggest that the localisation of a brain infarct induced by PT stroke, specifically when comparing the insular and sensorimotor cortex, does not change host susceptibility to post-stroke infection.

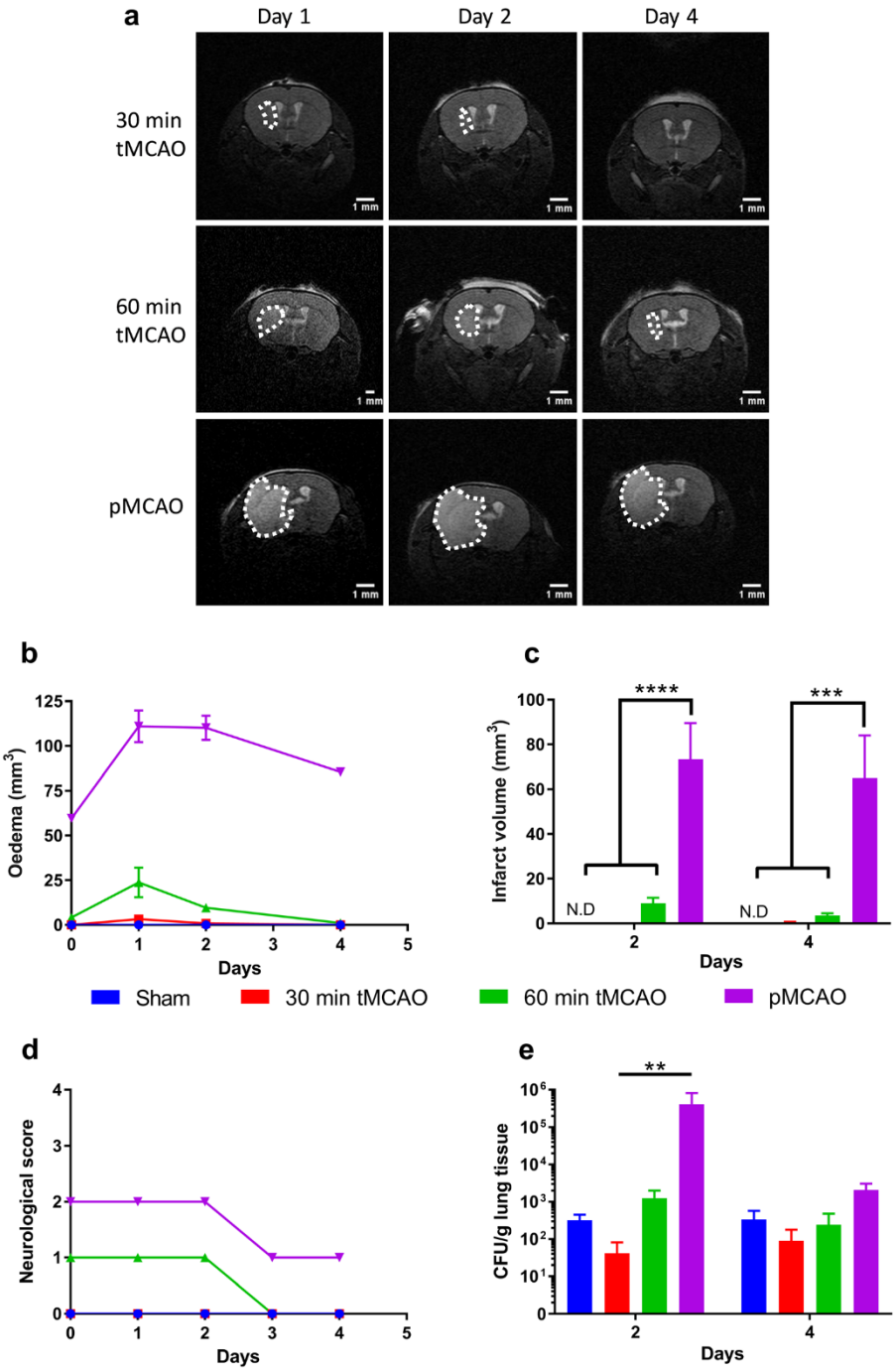


Fig. 2 Infection peaks at 1 day following severe stroke and resolution of infection is extended. Mice underwent either a 30 min tMCAO, 60 min tMCAO, or pMCAO and were monitored daily for up to 4 days following stroke. At 1, 2, and 4 days following surgery, mice were anaesthetised and underwent MRI scans (**a**) to longitudinally monitor oedema volumes over the experimental timepoint (**b**). Similarly, separate cohorts of mice were euthanised at 2 and 4 days after surgery in order to quantify infarct volume (**c**). Neurological scores were recorded daily until the end of the experimental endpoint (d). Lungs were also taken to quantify bacterial load (**e**). Data are presented as mean ± SEM. Neurological score data is presented as median. $n \geq 6$ per group or $n \geq 4$ for MRI study. $^{**}p < 0.01$ and $^{****}p < 0.0001$ using a one-way ANOVA for normally distributed data, or Kruskal-Wallis test for bacteriological analysis

Infarct Volume Is a Greater Determinant of Post-Stroke Infection

Furthermore, we performed a systematic analysis of our experimental data to assess whether cerebral infarct volume or infarct location influenced the risk of acquiring lung infection. Only mice with experimental endpoints at 1-day post-stroke (at the peak of infection) were included in our analysis. Mice that underwent MRI scanning, flow cytometric analysis, or had data sets with missing or unavailable data were excluded. The final study cohort used for retrospective analysis included 61 MCAO or PT stroke

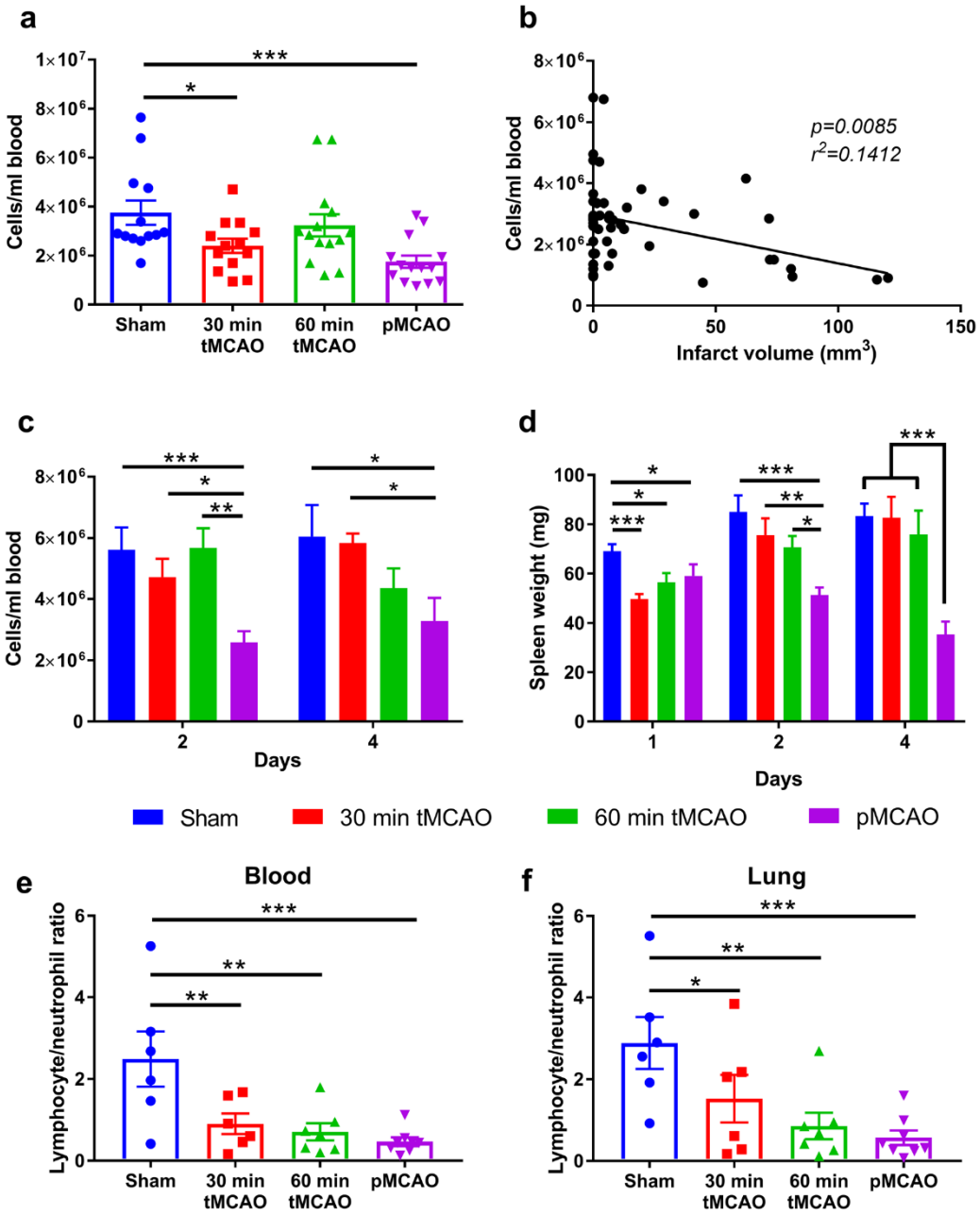


Fig. 3 Immunosuppression comes with increasing stroke severity. At 1 day following MCAO, mice were anaesthetised, and blood was collected via cardiac puncture. Circulating leukocyte counts were enumerated (**a**) and could be inversely correlated with infarct volume (**b**). In separate cohorts of mice, circulating leukocyte numbers were measured at 2- and 4-days following surgery (**c**). Furthermore, spleen weights at day 1, 2 and 4 were also measured (**d**). At 1 day following surgery, flow cytometric analyses of the blood (**e**) and lung (**f**) was performed and a lymphocyte/neutrophil cell ratio was determined. Data are represented as mean ± SEM, where $n \geq 13$ per group for raw leukocyte counts and $n \geq 6$ for flow cytometry data, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA

mice. In a double-blinded manner, 12 brain regions were annotated for assessment of infarct involvement: caudoputamen, insular cortex, somatomotor area, somatosensory area, piriform area, gustatory areas, visceral area, striatum ventral region, hypothalamus, thalamus, midbrain and hippocampal region. A MARS model was used for analysis of the relationship between predictors and infection. Our data indicate that the only key predictor for

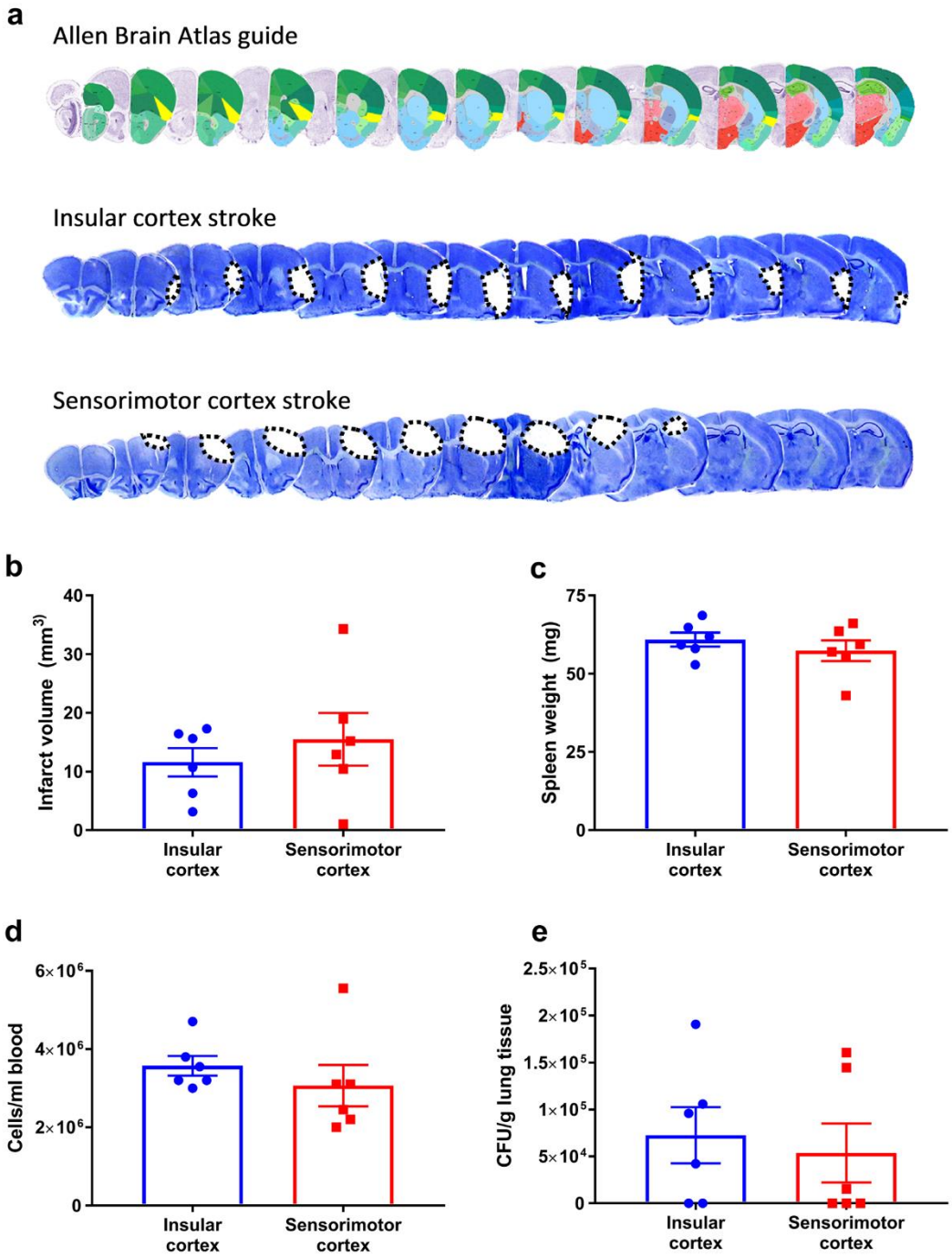


Fig. 4 Infarct localisation in the insular cortex does not worsen infection. Comparison of the insular cortex (denoted in yellow), as defined by the Allen Brain Atlas (found at http://mouse.brain-map.org/static/atlas), against thionin-stained brain sections with infarct achieved using the photothrombotic (PT) stroke model (denoted in white and outlined with dotted lines) (**a**). Infarct volumes were measured between insular stroke and motor cortex stroke (**b**). Additionally, spleen weight (**c**), circulating leukocyte count (**d**), and lung bacteria (**e**) were measured. Data are represented as mean ± SEM, where $n \geq 6$ per group

early infection was infarct volume, with larger lesions increasing the risk of acquiring lung infection at 1 day following stroke. The odds ratio for infarct volume at a hinge below 14.95 was 1.005 (95% CI 1.002–1.059, $p =$

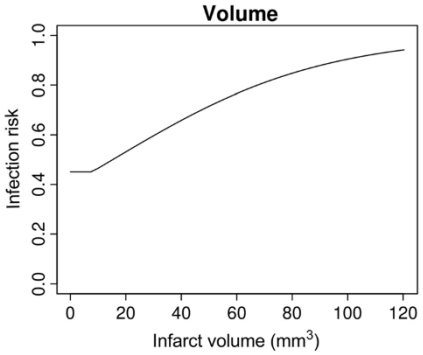


Fig. 5 Relationship between volume of infarct and the development of infection

0.03) (Fig. 5). The MARS analysis also confirmed that there was no significant relationship between different infarct locations to the risk to infection. In contrast to some previous studies, the involvement of the insular was not an important determinant of lung infection. These findings support the risk of post-stroke infection to be more dependent on the extent of cerebral ischemia than the location of cerebral infarct.

Stroke Severity Is a Clinical Risk Factor for Infection

Of the total 633 patient records obtained, 201 did not meet the selection criteria (including patients with haemorrhagic stroke) and were therefore excluded from the study. This left a study cohort of 432 patients with ischemic stroke. Patient characteristics are presented in Table 1. The mean age of the study cohort was 71.45 years (standard deviation [SD] 14.9), with 44% being female (*N* 190). Among the study sample, cerebral infarction in the cortical region was most common and the proportion of patients with infarcts in the left hemisphere was similar to that of the right hemisphere. Overall, 44 patients acquired infection, accounting for an overall incidence of 10%. The most common types of infection were UTI (*N* 18, 40.9%) and pneumonia (*N* 17, 38.6%). Patients with multiple infections (*N* 4, 9.09%) and other infections (eye/ear/nose/throat, gastrointestinal, other respiratory infections excluding pneumonia, skin/soft tissue, and unknown) were much less prevalent (*N* 1, 2.27%).

Using a multivariable logistic regression, shown in Table 2, greater age (OR 1.05, 95% CI 1.01–1.08), greater stroke severity (OR 1.07, 95% CI 1.02–1.12), and use of indwelling catheter (OR 7.42, 95% CI 3.14–17.53) were independently associated with the risk of infection (all $p < 0.01$). Sex, comorbidities, use of immunosuppressants, use of pre-stroke antibiotics, use of β-blockers, and the use of NGT were not independently associated with a risk of infection. Furthermore, our analysis of cerebral infarct location between cortical and non-cortical was not independently associated with a risk of infection (OR 1.04, 95% CI 0.46–2.35).

Table 1 Descriptive statistics of patients with ischemic stroke

	Ischemic stroke ($n = 432$)
Age (mean/SD)	71.45 (14.9)
Female sex	190 (44.0%)
Days in acute ward (median)	4 (2.5–7)
Location of stroke:	
- Cortical	230 (53.2%)
- Subcortical	48 (11.1%)
- Cerebellar	18 (4.2%)
- Brainstem	15 (3.5%)
- Multiple	42 (9.7%)
- Unknown	79 (18.3%)
Hemisphere of stroke	
- Left	182 (50.8%)
- Right	155 (43.3%)
- Both	21 (5.9%)
Severity/NIHSS (mean/SD)	7.77 (7.5)
Death	42 (9.7%)
Prevalence of infection	43 (10.0%)
Day of onset of infection (mean/SD)	3.07 (2.8)
Site of infection	
- Eye/ENT	1 (2.32%)
- Gastrointestinal	1 (2.32%)
- Lung (except pneumonia)	1 (2.32%)
- Pneumonia	17 (39.5%)
- Skin/soft tissue	1 (2.32%)
- Urinary tract	18 (41.9%)
- Unknown	1 (2.32%)
- Multiple	4 (9.30%)
Antibiotics prescribed	55 (12.8%)
IV line inserted	427 (98.8%)
IDC inserted	55 (12.7%)
NGT inserted	27 (6.3%)
ETT inserted	12 (2.8%)
Comorbid conditions	
- Diabetes	105 (24.3%)
- Chronic lung disease	42 (9.7%)
- Chronic liver disease	5 (1.2%)
- Malignancy	51 (11.8%)
- HIV	0 (0.00%)
- Autoimmune disease	8 (1.9%)
Medications	
- Beta-blockers	134 (31.0%)
- Immunosuppressants	29 (6.7%)
- Antibiotics (pre-stroke)	16 (3.7%)

ENT ear, nose and throat, *ETT* endotracheal tube, *HIV* human immunodeficiency virus, *IDC* indwelling urethral catheter, *IV* intravenous, *NGT* nasogastric tube, *SD* standard deviation

Table 2 Multivariable regression of predictors of infection after acute ischemic stroke

	Adjusted OR (95% CI)	*P* value
Age	1.05 (1.01–1.08)	0.005*
Sex	0.77 (0.35–1.65)	0.494
NIHSS	1.07 (1.02–1.12)	0.008*
Comorbidities	1.21 (0.56–2.63)	0.624
Immunosuppressants	2.30 (0.70–7.61)	0.177
Pre-stroke antibiotics	1.93 (0.40–9.34)	0.414
Beta-blockers	1.32 (0.61–2.87)	0.476
Use of NGT	2.16 (0.73–6.44)	0.165
Use of IDC	7.42 (3.14–17.53)	0.000*
Location	1.04 (0.46–2.35)	0.923

Location defined as cortical versus non-cortical

IDC indwelling urethral catheter, *NGT* nasogastric tube, *NHISS* National Institutes of Health Stroke Scale, *OR* odds ratio

Discussion

Infection is a major clinical problem for patients with stroke [3, 34]. However, our understanding of post-stroke infection is limited largely due to the heterogeneity of stroke pathology and severity amongst patients. Additionally, it is currently unclear to what degree the impact of infarct localisation in certain brain regions has on the extent of post-stroke immune alterations and infection development. In the present study, we investigated the relationship between cerebral infarct size and location on systemic immunity and spontaneous lung infection after stroke in mouse models. Our findings suggest that stroke severity, and not the location of cerebral infarct, is associated with a greater susceptibility to infection in both our experimental and clinical studies. We suggest that this increased susceptibility to infection in mice with larger cerebral infarcts is associated with a greater systemic change in immune cell populations, as demonstrated by a profound decrease of the lymphocyte-neutrophil ratio in the blood and lungs of post-stroke animals.

The intraluminal MCAO technique is a well-established preclinical model of stroke [38]. Furthermore, in this model, the duration of arterial occlusion can be easily modified with the retraction of the monofilament, thus allowing for the investigation of reperfused and non-reperfused cerebral ischemic injury. As such, to examine the impact of stroke severity on infection, we adjusted the duration of occlusion to achieve cerebral infarction of varying sizes (30 or 60 min tMCAO), as well as to model patients who did not have reperfusion (pMCAO). This allowed for direct comparison between each severity of stroke and the impact on host immunity and development of infection. While our bacteriological analysis of the lung showed no difference in the degree of infection between the various MCAO models, we demonstrate that the rate of infection is greater in mice that underwent pMCAO (and thus developed a larger infarct volume), the most severe form of experimental stroke. Specifically, we show that more than 90% of mice subjected to pMCAO acquired lung infection within 1 day following stroke onset. This contrasts with around a 50% infection rate in mice subjected to either 30 or 60 min of tMCAO. A prior study focusing on the tMCAO model demonstrated bacterial growth from lung homogenates of all mice that underwent a 90 min MCAO model, compared to half of that in mice at 5 days following 30 min MCAO [39]. In comparison, our data demonstrates that lung infection peaked at day 1, and the resolution of infection was delayed only in mice subjected to the most severe stroke. Nevertheless, our experimental finding indicating that stroke severity predicts the host susceptibility to infection is consistent with our clinical study, and others, where infarct volume and stroke severity (defined by NIHSS) are strong predictors of post-stroke infection [16, 27, 32].

Conceivably, there is a physiological response to protect the host from immune-mediated tissue damage that is offset by the downregulation of immune responses after stroke with the side effect of rendering patients susceptible to infection. Characteristics of post-stroke immunosuppression include profound loss of circulating lymphocytes and atrophy of the spleen [14, 39]; however, limited studies have explored this relationship with stroke severity over an extended time period. Our study suggests that alterations in immunity are dependent on the extent of cerebral ischemia, as we have shown a significant negative correlation between infarct volume and circulating leukocyte numbers at the peak of lung infection. Additionally, our results show a reduction in the lymphocyte-neutrophil ratio in the circulation and lung across all severities of stroke, though this was most pronounced following extensive cerebral infarct induced by pMCAO. Indeed, a decrease of the lymphocyte-neutrophil ratio in the blood has been recently shown to be a predictor of post-stroke pneumonia [37] and is clinically used to predict infection and pneumonia [35–37]. However, to the best of our knowledge, we are the first to show the decrease in the lymphocyte-neutrophil ratio in the lung (site of infection), which may explain the high rate of lung infection following stroke in both experimental models and in the clinic. Furthermore, while lymphocytes and the adaptive immune response could be expected to control infection 5–7 days after stroke, the reduced numbers of these cells do not fully explain the observed increase in infection 24 h following stroke onset. In contrast, cells of the innate immune system, such as neutrophils, serve as the first line of defence and consist of mechanisms that function to promptly respond and protect the host from infection. Indeed, we have previously reported dysfunction of neutrophil activation and chemotaxis in post-stroke mice [40]. Moreover, similar deficiencies were found in circulating blood neutrophils from stroke patients [41], potentially suggesting the risk of post-stroke infection is due to reduced neutrophil function rather than number.

In addition, we show that mice subjected to pMCAO continued to have reduced levels of circulating leukocytes over a 4-day period, while leukocytes in mice with mild and moderate infarct sizes induced by tMCAO recovered to baseline levels by 2 days following stroke. The loss of overall circulating leukocytes may be explained by previous studies that reported a profound loss of lymphocytes following extensive infarcts over a 7-day timeframe [39]. Prolonged alteration of systemic immunity in mice that underwent severe stroke, represented by persistent low circulating leukocyte counts and splenic atrophy, possibly contributes to a delayed clearance of bacteria in the lung. Indeed, in tMCAO models of stroke, the resolution of lung infection was accompanied by the return of leukocyte counts and spleen weight back to sham levels. These findings may also suggest that patients with severe stroke remain immunosuppressed and are more prone to secondary infection. Clinically, immunosuppression has been reported to persist for months and years following stroke [42]. In fact, over 17% of stroke patients were readmitted due to infection throughout a 10 year period [43]. Whether the long-term susceptibility to infection was due to patients having more severe strokes was not delineated in these studies. Our study suggests that patients with greater stroke severity may present with prolonged immune impairment, thus it is feasible that they would require additional management to control potential infectious complications. Indeed, this may be the ideal patient cohort in which antibiotic prophylaxis may be beneficial. However, results from recent clinical trials have suggested against the use of antibiotic prophylaxis as it does not improve outcome in post-stroke patients. Patients with severe acute ischemic stroke that were prophylactically treated with antibiotics had increased risk of infection during hospitalisation and did not show improvements in mortality within the short-term [44]. Furthermore, procalcitonin-guided antibiotic treatment in patients with stroke also did not improve the rate of pneumonia, mortality or patient functional outcome [7]. As such, with the apparent changes to immunity that occurs following stroke, a possible therapeutic alternative to antibiotics is immunomodulatory treatments that will induce an anti-bacterial host immunity to effectively limit post-stroke infection but at the same time does not exacerbate inflammatory processes in the brain.

In our analysis of our patient cohort, age was a predictor of post-stroke infection. It is well established that advanced age is linked to worsened stroke severity and poorer functional outcome [45–47]. These may be due to age-related factors that impede recovery after stroke, such as cell senescence, low-grade systemic inflammation and decline in immune function [46, 48, 49]. The increased risk to post-stroke infection in older patients may come as a result of decreased immune function; however, the underlying mechanisms are unclear. Recent work from our laboratory demonstrated a breakdown in gut barrier integrity, allowing for the dissemination and translocation of gut-derived bacteria into the periphery, including the lung [50]. We have also recently showed that advanced age contributes to intestinal inflammation that exacerbates gut permeability and structural breakdown of the colonic barriers even after mild stroke injury [51]. The increasing focus on the effect of ageing in age-related disease will potentially open alternative avenues of therapeutic intervention to reduce infection in patients with stroke, especially for older patients who are most at risk.

We next attempted to determine whether the location of cerebral infarction influences host susceptibility to infection following stroke, and so we adopted the PT stroke model to accurately target the sensorimotor and insular cortex. This method allowed us to induce cerebral damage in two different areas of the brain with comparable infarct volumes. Our findings indicate PT-induced damage to the sensorimotor or insular cortex resulted in comparable changes in overall circulating leukocyte numbers, nor the degree or rate of infection. Thus, our data suggest that infarct location does not contribute additional risk to post-stroke infection. While previous clinical reports described patients with infarcts involving the right insular cortex having worse long-term functional outcomes [23], and associated with pneumonia [22, 52, 53], this may be due to larger lesion volumes compared to patients with non-insular strokes [54]. The worsened outcome observed in patients with infarct localising to the insular cortex may be explained by the vascular anatomy of the brain. Infarction of the insular cortex requires total occlusion of the M1 and M2 segments of the MCA, resulting in lack of cerebral blood flow to the rest of the MCA territory and thus larger infarct size [54, 55]. Indeed, after adjusting for infarct volume, the associations with death following insular stroke were negated [26, 28]. In addition, a number of studies show no association between infarction of particular brain regions and infection after adjusting for covariates such as infarct size [16, 27]. Our study echoes these findings, but due to the retrospective nature of our clinical data, we could only perform rudimentary analysis of cerebral infarct location by distinguishing cortical versus non-cortical regions and post-stroke infection in our patient cohort. Nevertheless, we address the relationship between cerebral infarct location and infection risk by taking a more systematic and clinically relevant approach with our experimental data. Specifically, we retrospectively examined twelve brain regions and conducted MARS analysis to identify whether infarct involvement in these brain regions could be associated with infection in our animal model. Our data confirmed that only infarct volume was associated with the risk of post-stroke infection.

In summary, we demonstrate that a larger infarct volume after stroke predisposes the host to greater susceptibility to infection. Our data suggest that this is due to a more prolonged and pronounced alteration in the cellular composition of systemic immunity. Furthermore, the location of cerebral infarct does not provide additional host susceptibility to post-stroke infection as indicated by studies targeting specific brain regions, and by the

retrospective comparison of cerebral infarct location and infection that we show here. Therefore, our study highlights that infarct volume is of greater importance than infarct location in the risk of post-stroke immune alterations and infection.

Acknowledgements The authors acknowledge the facilities and scientific and technical assistance of the National Imaging Facility, a National Collaborative Research Infrastructure Strategy (NCRIS) capability, at the Monash Biomedical Imaging, Monash University. The authors acknowledge the facilities and technical assistance of Monash Histology Platform, at the Department of Anatomy and Developmental Biology, Monash University.

Funding Information This work is supported by the National Heart Foundation (NHF, Australia; 100,863), CSL Centenary Fellowship and the National Health and Medical Research Council (NHMRC, Australia: APP1104036). The financial supports have no role in conducting the research and/or preparation of the article.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Heuschmann PU, Kolominsky-Rabas PL, Misselwitz B, Hermanek P, Leffmann C, Janzen R, et al. Predictors of in-hospital mortality and attributable risks of death after ischemic stroke: the German Stroke Registers Study Group. Arch Intern Med. 2004;164(16):1761–8.
2. Langhorne P, Stott D, Robertson L, MacDonald J, Jones L, McAlpine C, et al. Medical complications after stroke a multicenter study. Stroke. 2000;31(6):1223–9.
3. Vermeij FH, op Reimer WJS, De Man P, Van Oostenbrugge RJ, Franke CL, De Jong G, et al. Stroke-associated infection is an independent risk factor for poor outcome after acute ischemic stroke: data from the Netherlands Stroke Survey. Cerebrovasc Dis. 2009;27(5):465–71.
4. Hetze S, Engel O, Römer C, Mueller S, Dirnagl U, Meisel C, et al. Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach. J Cereb Blood Flow Metab. 2013;33(6):846–54.
5. Kalra L, Irshad S, Hodsoll J, Simpson M, Gulliford M, Smithard D, et al. Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial. Lancet. 2015;386(10006):1835–44.
6. Westendorp WF, Vermeij J-D, Zock E, Hooijenga IJ, Kruyt ND, Bosboom HJ, et al. The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial. Lancet. 2015;385:1519–26.
7. Ulm L, Hoffmann S, Nabavi D, Hermans M, Mackert B-M, Hamilton F, et al. The randomized controlled STraWinSKi trial: procalcitonin-guided antibiotic therapy after stroke. Front Neurol. 2017;8.
8. van de Beek D, Wijdicks EF, Vermeij FH, de Haan RJ, Prins JM, Spanjaard L, et al. Preventive antibiotics for infections in acute stroke: a systematic review and meta-analysis. Arch Neurol. 2009;66(9):1076–81.
9. Vermeij JD, Westendorp WF, Dippel DW, van de Beek D, Nederkoorn PJ. Antibiotic therapy for preventing infections in people with acute stroke. Cochrane Database Syst Rev. 2018;1:CD008530. https://doi.org/10.1002/14651858.CD008530.pub3.
10. Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, et al. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J Immunol. 2006;176(11):6523–31.
11. Prass K, Meisel C, Höflich C, Braun J, Halle E, Wolf T, et al. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1–like immunostimulation. J Exp Med. 2003;198(5):725–36.
12. Wong CH, Jenne CN, Tam PP, Léger C, Venegas A, Ryckborst K, et al. Prolonged activation of invariant natural killer T cells and TH2-skewed immunity in stroke patients. Front Neurol. 2017;8.
13. Hug A, Liesz A, Muerle B, Zhou W, Ehrenheim J, Lorenz A, et al. Reduced efficacy of circulating costimulatory cells after focal cerebral ischemia. Stroke. 2011;42(12):3580–6.
14. Mracsko E, Liesz A, Karcher S, Zorn M, Bari F, Veltkamp R. Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke. Brain Behav Immun. 2014;41:200–9.
15. Haeusler KG, Schmidt WU, Föhring F, Meisel C, Helms T, Jungehulsing GJ, et al. Cellular immunodepression preceding infectious complications after acute ischemic stroke in humans. Cerebrovasc Dis. 2008;25(1–2):50–8.
16. Hug A, Dalpke A, Wieczorek N, Giese T, Lorenz A, Auffarth G, et al. Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection. Stroke. 2009;40(10):3226–32.
17. Urra X, Laredo C, Zhao Y, Amaro S, Rudilosso S, Renú A, et al. Neuroanatomical correlates of stroke-associated infection and stroke-induced immunodepression. Brain Behav Immun. 2017;60:142–50.
18. Broadley S, Croser D, Cottrell J, Creevy M, Teo E, Yiu D, et al. Predictors of prolonged dysphagia following acute stroke. J Clin Neurosci. 2003;10(3):300–5.
19. Hoffmann S, Harms H, Ulm L, Nabavi DG, Mackert B-M, Schmehl I, et al. Stroke-induced immunodepression and dysphagia independently predict stroke-associated pneumonia–the PREDICT study. J Cereb Blood Flow Metab. 2017;37(12):3671–82.
20. Nakajoh K, Nakagawa T, Sekizawa K, Matsui T, Arai H, Sasaki H. Relation between incidence of pneumonia and protective reflexes in post-stroke patients with oral or tube feeding. J Intern Med. 2000;247(1):39–42.
21. Steinhagen V, Grossmann A, Benecke R, Walter U. Swallowing disturbance pattern relates to brain lesion location in acute stroke patients. Stroke. 2009;40(5):1903–6.
22. Harms H, Reimnitz P, Bohner G, Werich T, Klingebiel R, Meisel C, et al. Influence of stroke localization on autonomic activation, immunodepression, and post-stroke infection. Cerebrovasc Dis. 2011;32(6):552–60.
23. Sposato LA, Cohen G, Wardlaw JM, Sandercock P, Lindley RI, Hachinski V, et al. Effect of right insular involvement on death and functional outcome after acute ischemic stroke in the IST-3 trial (Third International Stroke Trial). Stroke. 2016;47(12):2959–65.
24. Walter U, Knoblich R, Steinhagen V, Donat M, Benecke R, Kloth A. Predictors of pneumonia in acute stroke patients admitted to a neurological intensive care unit. J Neurol. 2007;254(10):1323–9.
25. Phan TG, Donnan GA, Wright PM, Reutens DC. A digital map of middle cerebral artery infarcts associated with middle cerebral artery trunk and branch occlusion. Stroke. 2005;36(5):986–91.
26. Laredo C, Zhao Y, Rudilosso S, Renú A, Pariente JC, Chamorro Á, et al. Prognostic significance of infarct size and location: the case of insular stroke. Sci Rep. 2018;8(1):9498.

27. Minnerup J, Wersching H, Brokinkel B, Dziewas R, Heuschmann PU, Nabavi DG, et al. The impact of lesion location and lesion size on poststroke infection frequency. J Neurol Neurosurg Psychiatry. 2010;81(2):198–202.
28. Borsody M, Gargano JW, Reeves M, Jacobs B. Infarction involving the insula and risk of mortality after stroke. Cerebrovasc Dis. 2009;27(6):564–71.
29. Connolly ES, Winfree CJ, Stern DM, Stern DM, Solomon RA, Pinsky DJ. Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. Neurosurgery. 1996;38(3):523–32.
30. Paxinos G, Franklin KB. Paxinos and Franklin's the mouse brain in stereotaxic coordinates: Academic Press; 2019.
31. Zhang SR, Piepke M, Chu HX, Broughton BR, Shim R, Wong CH, et al. IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke. JCI Insight. 2018;3(18).
32. Phan TG, Kooblal T, Matley C, Singhal S, Clissold B, Ly J, et al. Stroke severity versus dysphagia screen as driver for post-stroke pneumonia. Front Neurol. 2019;10.
33. Friedman JH. Multivariate adaptive regression splines. Ann Stat. 1991;19(1):1–67.
34. Chamorro Á, Urra X, Planas AM. Infection after acute ischemic stroke a manifestation of brain-induced immunodepression. Stroke. 2007;38(3):1097–103.
35. Curbelo J, Bueno SL, Galván-Román JM, Ortega-Gómez M, Rajas O, Fernández-Jiménez G, et al. Inflammation biomarkers in blood as mortality predictors in community-acquired pneumonia admitted patients: importance of comparison with neutrophil count percentage or neutrophil-lymphocyte ratio. PLoS One. 2017;12(3):e0173947.
36. de Jager CP, Wever PC, Gemen EF, Kusters R, van Gageldonk-Lafeber AB, van der Poll T, et al. The neutrophil-lymphocyte count ratio in patients with community-acquired pneumonia. PLoS One. 2012;7(10):e46561.
37. Nam K-W, Kim TJ, Lee JS, Kwon H-M, Lee Y-S, Ko S-B, et al. High neutrophil-to-lymphocyte ratio predicts stroke-associated pneumonia. Stroke. 2018;49(8):1886–92.
38. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989;20(1):84–91.
39. Liesz A, Hagmann S, Zschoche C, Adamek J, Zhou W, Sun L, et al. The spectrum of systemic immune alterations after murine focal ischemia: immunodepression versus immunomodulation. Stroke. 2009;40(8):2849–58.
40. Nicholls AJ, Wen SW, Hall P, Hickey MJ, Wong CH. Activation of the sympathetic nervous system modulates neutrophil function. J Leukoc Biol. 2018;103(2):295–309.
41. Ruhnau J, Schulze K, Gaida B, Langner S, Kessler C, Bröker B, et al. Stroke alters respiratory burst in neutrophils and monocytes. Stroke. 2014;45(3):794–800.
42. Theodorou G, Marousi S, Ellul J, Mougiou A, Theodori E, Mouzaki A, et al. T helper 1 (Th1)/Th2 cytokine expression shift of peripheral blood CD4+ and CD8+ T cells in patients at the post-acute phase of stroke. Clin Exp Immunol. 2008;152(3):456–63.
43. Rohweder G, Salvesen Ø, Ellekjær H, Indredavik B. Hospital readmission within 10 years post stroke: frequency, type and timing. BMC Neurol. 2017;17(1):116.
44. Tziomalos K, Ntaios G, Miyakis S, Papanas N, Xanthis A, Agapakis D, et al. Prophylactic antibiotic treatment in severe acute ischemic stroke: the Antimicrobial chemopRrophylaxis for Ischemic STrokEIn MaceDonIa–Thrace Study (ARISTEIDIS). Intern Emerg Med. 2016;11(7):953–8.
45. Manwani B, Liu F, Xu Y, Persky R, Li J, McCullough LD. Functional recovery in aging mice after experimental stroke. Brain Behav Immun. 2011;25(8):1689–700.
46. Ritzel RM, Lai Y-J, Crapser JD, Patel AR, Schrecengost A, Grenier JM, et al. Aging alters the immunological response to ischemic stroke. Acta Neuropathol. 2018;136(1):89–110.
47. Yager JY, Wright S, Armstrong EA, Jahraus CM, Saucier DM. The influence of aging on recovery following ischemic brain damage. Behav Brain Res. 2006;173(2):171–80.
48. Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, et al. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immun Ageing. 2005;2(1):8.
49. Wen SW, Wong CH. Aging-and vascular-related pathologies. Microcirculation. 2019;26(2):e12463.
50. Stanley D, Mason LJ, Mackin KE, Srikhanta YN, Lyras D, Prakash MD, et al. Translocation and dissemination of commensal bacteria in post-stroke infection. Nat Med. 2016;22(11):1277–84.
51. Wen SW, Shim R, Ho L, Wanrooy BJ, Srikhanta YN, Prame Kumar K, et al. Advanced age promotes colonic dysfunction and gut-derived lung infection after stroke. Aging Cell. 2019:e12980.
52. Kemmling A, Lev MH, Payabvash S, Betensky RA, Qian J, Masrur S, et al. Hospital acquired pneumonia is linked to right hemispheric peri-insular stroke. PLoS One. 2013;8(8):e71141.
53. Walter U, Kolbaske S, Patejdl R, Steinhagen V, Abu-Mugheisib M, Grossmann A, et al. Insular stroke is associated with acute sympathetic hyperactivation and immunodepression. Eur J Neurol. 2013;20(1):153–9.
54. Kodumuri N, Sebastian R, Davis C, Posner J, Kim EH, Tippett DC, et al. The association of insular stroke with lesion volume. Neuroimage Clin. 2016;11:41–5.
55. Türe U, Yaşargil MG, Al-Mefty O, Yaşargil DC. Arteries of the insula. J Neurosurg. 2000;92(4):676–87.

Supplemental material

Stroke severity, and not cerebral infarct location, increases the risk of infection

Raymond Shim[1], Shu Wen Wen[1], Brooke J. Wanrooy[1], Michelle Rank[2,3], Tharani Thirugnanachandran[4], Luke Ho[1,5], Tara Sepehrizadeh[6], Michael de Veer[6], Velandai K. Srikanth[5], Henry Ma[4], Thanh G. Phan[4], Christopher G. Sobey[7], Connie H. Y. Wong[1*]

[1]Centre for Inflammatory Diseases, Department of Medicine at Monash Health, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, Victoria, Australia.

[2]Department of Anatomy and Neuroscience, School of Biomedical Sciences, The University of Melbourne, Parkville, Victoria, Australia

[3]School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria, Australia.

[4]Stroke and Ageing Research Group, Department of Medicine at Monash Health, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, Victoria, Australia

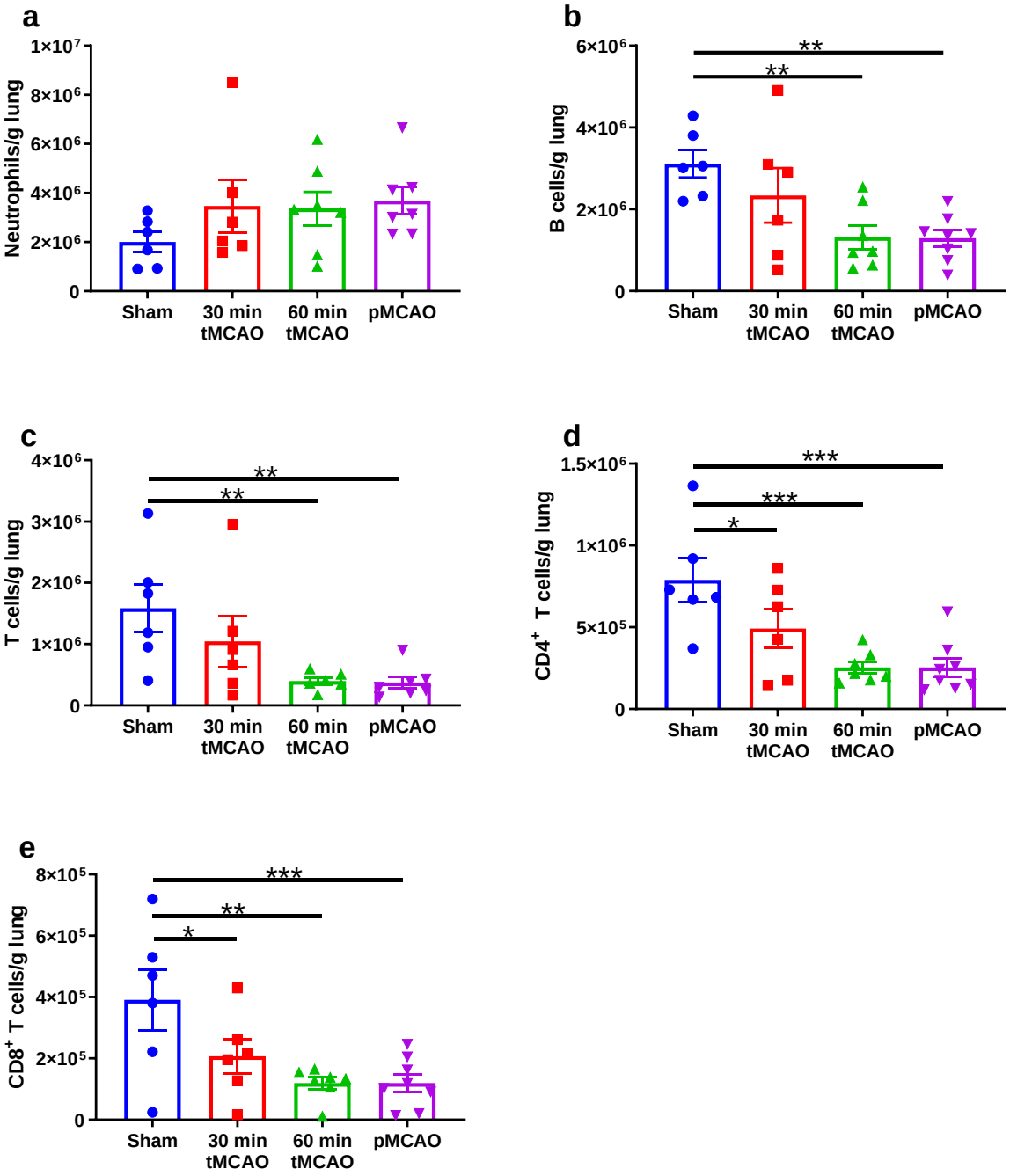
[5]Department of Medicine (Academic Unit), Peninsula Clinical School, Central Clinical School, Monash University, Frankston, Victoria, Australia.

[6]Monash Biomedical Imaging, Monash University, Clayton, Victoria, Australia.

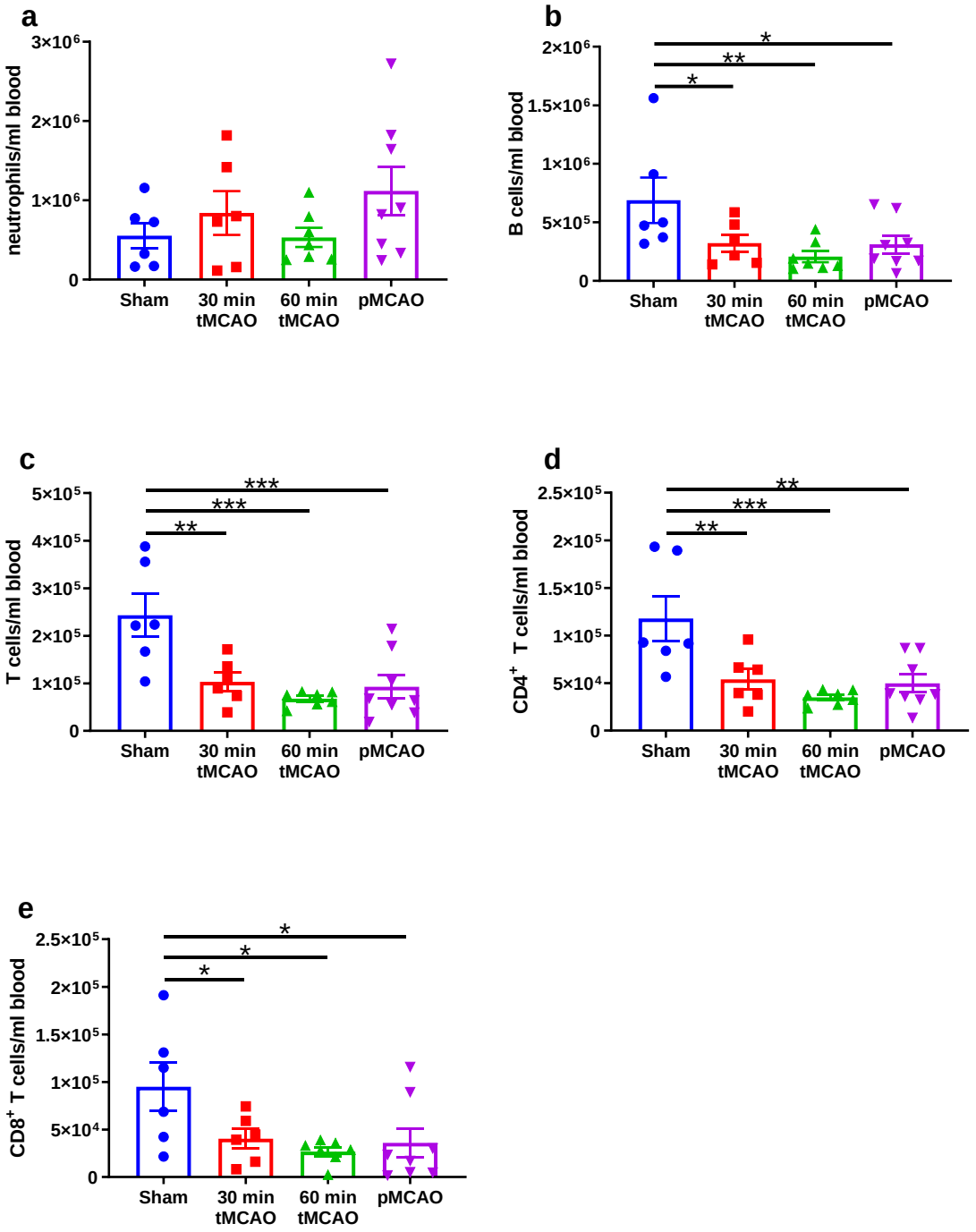
[7]Department of Physiology, Anatomy and Microbiology, School of Life Sciences, La Trobe University, Bundoora, Victoria, Australia.

*Address for correspondence: Connie H. Y. Wong, PhD., Centre for Inflammatory Diseases, Department of Medicine at Monash Health, School of Clinical Sciences, Monash Medical Centre, Monash University, Clayton, VIC 3168; Email: connie.wong@monash.edu

Supplementary figures



Supp. fig. 1 Flow cytometric analyses of neutrophil and lymphocyte numbers in the lung of post-stroke mice. Mice underwent 30 min tMCAO, 60 min tMCAO, pMCAO or sham surgery. At 1 day following surgery, flow cytometric analyses of the lung was performed where neutrophil (a), B cell (b), T cell (c) numbers were quantified. T cells were further separated into $CD4^+$ (d) and $CD8^+$ (e) T cells. Data are represented as mean ± SEM, where $n \geq 6$ per group, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA.


a
neutrophils/ml blood
3×10⁶
2×10⁶
1×10⁶
0
Sham
30 min tMCAO
60 min tMCAO
pMCAO
b
B cells/ml blood
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
*
**
*
Sham
30 min tMCAO
60 min tMCAO
pMCAO
c
T cells/ml blood
5×10⁵
4×10⁵
3×10⁵
2×10⁵
1×10⁵
0
***
***
**
Sham
30 min tMCAO
60 min tMCAO
pMCAO
d
CD4⁺ T cells/ml blood
2.5×10⁵
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
0
**
***
**
Sham
30 min tMCAO
60 min tMCAO
pMCAO
e
CD8⁺ T cells/ml blood
2.5×10⁵
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
0
*
*
*
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Supp. fig. 2 Flow cytometric analyses of neutrophil and lymphocyte numbers in the blood of post-stroke mice. Mice underwent 30 min tMCAO, 60 min tMCAO, pMCAO or sham surgery. At 1 day following surgery, blood was taken via cardiac puncture and flow cytometric analysis was performed where neutrophil (a), B cell (b), T cell (c) numbers were quantified. T cells were further separated into $CD4^+$ (d) and $CD8^+$ (e) T cells. Data are represented as mean ± SEM, where $n \geq 6$ per group, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA.

Chapter Four

The role of the sympathetic nervous system in post-stroke infection

Introduction

The high prevalence of post-stroke infection indicates impaired antibacterial immunity. Indeed, we showed that the loss of circulating leukocytes and decreased spleen size are key characteristics of post-stroke immune impairment in Chapter 3. Additionally, we demonstrated that the loss of circulating leukocytes is dependent on stroke severity and leads to a greater risk to developing infection [117]. However, the mechanisms underlying these observations remain unclear. Based on existing evidence, we hypothesise that post-stroke immunosuppression is driven by a dysfunction of the sympathetic nervous system (SNS). The SNS mediates bodily processes in response to stress via the innervation of tissues and organs throughout the body. When activated, sympathetic nerves around the body secrete neurotransmitters known as catecholamines that act as effector molecules. Noradrenaline (NA) is the catecholamine that is predominantly released by sympathetic postganglionic fibres in the periphery [4]. After stroke, symptoms of impaired cardiac function, heart rate variability, baroreflex sensitivity, and blood pressure changes provide strong evidence of altered SNS activity [118, 119]. Furthermore, elevated circulating NA has been reported within the first 7 days after stroke in patients [120-122]. Increased presence of NA precursors and metabolites in the plasma may also suggest sympathetic overflow [1]. Therefore, understanding the generation and metabolism of NA may reveal the dominant pathways for NA generation and may allow for opportunities to investigate the association between SNS dysfunction and post-stroke infection.

In the peripheral sympathetic nerve endings, synthesis of NA begins with uptake of tyrosine from the bloodstream, sourced from the breakdown of dietary protein. Tyrosine is converted into 3,4-dihydroxyphenylalanine (DOPA) and then dopamine (DA) via tyrosine hydroxylase and L-aromatic-amino acid decarboxylase, respectively [1]. The fate of DA diverges to be converted into dihydroxyphenylacetic acid (DOPAC) or transported into vesicles

for conversion to NA by dopamine-β-hydroxylase (DBH). From here, NA is then transported out of the cell where it can act upon adrenergic receptors on non-neuronal cells to have downstream receptor-mediated effects. Alternatively, and more commonly, NA re-enter neuronal cells via the cell membrane noradrenaline transporter, recycled and re-transported into vesicles for further excretion, or is metabolised into dihydroxyphenylglycol (DHPG) by monamine oxidase (MAO) and then secreted into the bloodstream [1]. A summary of the synthesis and metabolism of NA is depicted in **Figure 4.1**. Excess NA or the metabolites, DOPA, DOPAC and DHPG, found in the circulation is indicative of sympathetic activity. In the setting of stroke, patients were found to have an increased levels of plasma NA [120, 122], suggestive of SNS hyperactivity. Excessive levels of circulating NA may have further cardiac and immunological implications in various organs in the periphery (as outlined in Chapter 1).

Once in the periphery, NA can act on the adrenergic receptors: α_1, α_2, β_1, β_2 and β_3. Of the five main adrenergic receptors, adrenergic receptor β_2 (Adrb2) is the most widely expressed on immune cells [4]. Findings from various preclinical studies suggest activation of Adrb2 on immune cells leads to immunosuppressive effects. Specifically, Adrb2 activation on immune cells has been shown to inhibit respiratory burst in neutrophils [34], interleukin (IL-)-8 production by monocytes [123] and modulate regulatory T cell apoptosis [124]. The activation of β-adrenergic receptors on immune cells in the setting of experimental stroke was also shown to contribute significantly to stroke-induced immunosuppression, including apoptosis of lymphocytes [125], reduced IFN-γ production, induction of alternatively activated (M2) macrophages and down regulation of other pro-inflammatory cytokines [126]. The administration of non-selective β-adrenergic receptor inhibitors (β-blockers) such a propranolol (PPL) has also been demonstrated to reduce the immunosuppressive effects after experimental stroke. For example, post-stroke mice administered PPL showed a sustained Th1 response [125], no loss of marginal zone B cells and circulating IgM levels [127], preserved

splenic atrophy [125, 128] and retained natural killer (NK) antibacterial function [129], and prevented the immunosuppressive functions of the iNKT cell [72]. As a result, use of β-blockers has proven beneficial in reducing infection after experimental stroke.

Unfortunately, the translation of β-blockers for preventing infection in patients with stroke yields mixed findings. While some retrospective clinical studies found that β-blocker use was associated with reduced rates of infection and improved mortality rates [130, 131], a larger portion of literature showed β-blockers did not improve the incidence of post-stroke infection or patient outcomes [132, 133]. In fact, some studies report a positive association between the use of β-blockers and the incidence of infection and risk of mortality in patients with stroke [134]. This discrepancy between clinical studies may be due to the lack of clarity as to whether selective or non-selective β-blockers were tested. This may be of importance as it was recently reported that non-selective β-blockers was associated with increased infections, while selective β-blockers had no effect [71].

With these conflicting reports, the relationship between SNS dysregulation and systemic NA release on β-adrenergic receptor activation and post-stroke infection requires further elucidation. We hypothesise a mechanism underlying post-stroke immunosuppression is the hyperactivation of the SNS, resulting in increased NA release in the periphery to allow for systemic activation of β-adrenergic receptors on immune cells. We propose the interaction between NA with its receptor contributes to functional suppression in host antibacterial immunity and increases susceptibility to infection after stroke. Furthermore, due to the immunosuppressive role of the Adrb2 in other disease models, we suggest that the classic hallmarks of impaired immunity after stroke is primarily driven by this receptor. Therefore, in this chapter, we measure SNS activity by quantifying plasma catecholamines in acute timepoints following stroke. Additionally, we determine the degree in which the β-ARs and specifically the Adrb2 are responsible for post-stroke infection.

Methods

Mice

Wildtype (WT) male mice (C57Bl/6J) mice were used as described in Chapter 2. For experiments using knockout mice, male *Adrb2*$^{-/-}$ (JAX laboratories) mice were bred on a C57Bl/6J background for 10 generations. The genotype of *Adrb2*$^{-/-}$ mice was confirmed by the genotyping facilities from Transnetyx. All procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCB/2016/10, Monash Medical Centre, Clayton, Victoria, Australia). Animals were randomly assigned to the experimental groups to prevent selection bias. All animals had access to water and food *ad libitum*, maintained in temperature-controlled rooms under a 12 h light-dark cycle and allowed to acclimatise for at least 7 days before experimentation.

β-blocking treatments

For experiments using PPL (Sigma-Aldrich), mice were treated with 30 mg/kg at 0 h, 4 h, and 8 h after surgery via intraperitoneal. injection. For experiments using ICI-118551 (Sigma-Aldrich), mice were treated with 1 mg/kg at 0 h, 4 h and 8 h after surgery via i.p. injection. The dosage for PPL and ICI-118551 were obtained from previous publications [72, 135]. Saline injections in separate mice were used as a control group for comparison.

Plasma collection and circulating catecholamine analysis

In naïve mice or following 15 min, 30 min, 60 min and 3 h after stroke or sham surgery, whole blood was taken from mice via cardiac puncture and immediately transferred into tubes containing 20 µl of 250 mM egtazic acid (EGTA) and 100 mM glutathione. Samples were centrifuged for 10 min at 220 × *g* at 4ºC for plasma collection and stored at -80ºC until

catecholamine quantification. Plasma catecholamines were extracted and quantified as previously described [136, 137]. Briefly, plasma was placed in 0.5 ml of 0.4 M perchloric acid containing 0.01% EDTA with 3,4-dihydroxybenzylamine. Samples were centrifuged and supernatant was collected. Catecholamines were extracted from the supernatant using alumina adsorption, separated by high performance liquid chromatography, and quantified by colorimetric detection.

List of other techniques used but previously described:

- 30 min, 60 min tMCAO and pMCAO (pg 37)
- Brain infarct quantification (pg 37)
- Bacteriological analysis (pg 37)
- Enumeration of circulating leukocytes (pg 38)
- Statistical analysis (pg 40)

Results

Plasma DA and NA increase at 3 h following severe stroke

For this study, we utilised the three experimental models developed in Chapter 3 to model stroke severity: mild (30 min transient MCAO [tMCAO]); moderate (60 min tMCAO); and severe (permanent MCAO [pMCAO]). To investigate the effect of stroke on the SNS, we firstly quantified the levels of circulating NA, as well as other catecholamines involved in NA generation and metabolism at 15 min, 30 min, 60 min and 3 h following induction of the 3 models of stroke. The catecholamines measured were DOPA (**Figure 4.2**), DA (**Figure 4.3**), DOPAC (**Figure 4.4**), NA (**Figure 4.5**), AD (**Figure 4.6**), and DHPG (**Figure 4.7**).

In the first 3 h following stroke, plasma DOPA remain unchanged compared to sham and naïve animals (**Figure 4.2A-E**). Similarly, stroke induction did not significantly alter DA concentrations in plasma at 15 min, 30 min and 60 min (**Figure 4.3A-D**) in comparison to sham mice. In contrast, a drastic increase in plasma DA was observed at 3 h following pMCAO compared to mild and moderate stroke groups, as well as to sham mice (**Figure 4.3E**). This significant elevation of DA at 3 h post-pMCAO may impact on the production of its downstream metabolites, DOPAC and NA. While levels of plasma DOPAC remain unchanged after stroke compared to sham at all timepoints (**Figure 4.4B-D**), a change in NA concentration was observed. Specifically, NA levels was only elevated at 3 h in mice that underwent pMCAO in comparison to mice that underwent sham and, 30 min and 60 min tMCAO surgery (**Figure 4.5B-E**). This data suggests that DA is primarily converted in NA and not DOPAC in mice that underwent pMCAO. Despite the rise of circulating NA after 3 h post-pMCAO, the levels of its metabolites, AD (**Figure 4.6B-E**) and DHPG (**Figure 4.7B-E**) in the plasma were not changed compared to sham treatment.

Non-selective β-adrenergic receptor blocking does not reduce post-stroke infection following severe stroke

Circulating NA only represents approximately 5-10% of total NA released from nerve endings [138]. Additionally, the peak of NA release may occur before 15 min or after 3 h post-stroke, which was not assessed. Nonetheless, with the hypothesis that NA induces immunosuppression and susceptibility to infection via β-adrenergic receptor activation after stroke, we examined the effectiveness of inhibiting β-adrenergic receptors non-selectively with PPL in reducing post-stroke lung infection. We also examined the impact of PPL treatment on hallmarks of immune suppression in mice that underwent pMCAO. In mice that underwent pMCAO and treated with saline, spontaneous lung infection was evident at 1 day following surgery (**Figure 4.8A**). However, unlike previous reports that showed PPL was effective in reducing post-stroke infection in mild to moderate models of stroke [72, 125], post-stroke treatment with PPL could not reduce the frequency or degree of infection following the more severe pMCAO (**Figure 4.8A**). Post-stroke immunosuppression has been previously characterised by a loss of circulating leukocytes and spleen cellularity [117, 125, 139]. While circulating leukocytes (**Figure 4.8B**) and spleen weight (**Figure 4.8C**) decreased in mice that underwent pMCAO with saline treatment compared to sham mice treated with saline, PPL treatment could not rescue or improve these characteristics of immunosuppression. These findings suggest that blockade of β-adrenergic receptors with non-selective inhibitors cannot reduce post-stroke infection in a severe model of ischemic stroke.

Inhibiting Adrb2 reduces infection following moderate but not severe stroke

The leading hypothesis by which post-stroke immunosuppression and infection occurs is through activation of β-adrenergic receptors on immune cells. To test the role of the Adrb2

in post-stroke infection, we treated post-stroke mice with the selective Adrb2 antagonist, ICI-118551. While saline-treated stroke mice presented with higher bacterial lung infection compared to sham mice as expected, ICI-118551 treatment could not reduce the levels of bacteria presented in the lung following pMCAO (**Figure 4.9A**). Similarly, mice that underwent pMCAO regardless of ICI-118551 or saline treatment, had reduced circulating leukocytes compared to sham (**Figure 4.9B**). Interestingly, ICI-118551 was effective at preventing a loss in spleen weight initiated by stroke when compared to sham (**Figure 4.9C**). Overall, this suggests specific pharmacological blockade of Adrb2 immediately following stroke onset cannot reduce infection following severe stroke.

The administration of β-blockers has previously been shown to reduce infection following moderate experimental stroke [72, 125]. Therefore, we next tested whether the efficacy of Adrb2 blockers is dependent on stroke severity by administering ICI-118551 to mice that underwent a moderate stroke (60 min tMCAO). In contrast to previous reports, while there was prominent lung infection in saline-treated mice that underwent 60 min tMCAO compared to sham, ICI-118551 treatment could not reduce infection (**Figure 4.10A**). Furthermore, there were no changes to circulating leukocyte numbers (**Figure 4.10B**) or spleen sizes (**Figure 4.10C**) between treatment groups. Due to our finding that β-blockers (PPL) do not reduce post-stroke infection following severe stroke (**Figure 4.8**), we next determined whether the effectiveness of ICI-118551 was impacted by stroke severity. When we compared ICI-118551-treated mice that underwent pMCAO to ICI-118551-treated mice that underwent 60 min MCAO, lung infection was significantly reduced (**Figure 4.11A**). ICI-118551 treatment could also prevent the loss of circulating leukocytes after 60 min tMCAO, compared to the pMCAO counterpart (**Figure 4.11B**). No changes to spleen sizes were detected between 60 min tMCAO surgery mice versus pMCAO surgery mice that were treated with ICI-118551 (**Figure 4.11C**).

Deficiency of Adrb2 does not alter post-stroke infection

To further interrogate the role of Adrb2 on post-stroke infection, we utilised Adrb2 deficient mice and performed 60 min tMCAO. Similar to previous findings, spontaneous lung infection was detected in WT mice that underwent 60 min tMCAO compared to sham (**Figure 4.12A**). Interestingly, this was not accompanied by the hallmark signs of post-stroke immunosuppression as there were no changes to circulating leukocyte numbers (**Figure 4.12B**) or spleen weight (**Figure 4.12C**) between sham-operated and post-stroke WT mice. Similarly, mice with Adrb2 deficiency had similar levels of lung infection following 60 min tMCAO in comparison to their stroke WT counterpart (**Figure 4.12A**). Between Adrb2 deficient mice that underwent sham or 60 min tMCAO, there were no changes to circulating leukocyte levels (**Figure 4.12B**). Additionally, there were also no statistically significant differences in circulating leukocyte count between WT and Adrb2 deficient mice that underwent 60 min tMCAO (**Figure 4.12B**). Spleen sizes decreased in Adrb2 deficient mice that underwent 60 min tMCAO in comparison to its sham counterpart, however this was not different in comparison to WT mice that underwent 60 min tMCAO (**Figure 4.12C**). Overall, these findings suggest that the inherent lack of Adrb2 does not alter the susceptibility to post-stroke infection.

Discussion

Infection after stroke has traditionally been associated with precipitants such as aspiration, indwelling catheters, immobility, or other health comorbidities [140]. Although these factors may play a role, more recent evidence suggests stroke results in impairment of the immune system that can additionally contribute to post-stroke infection. Indeed, the loss of leukocytes in the blood and macroscopic shrinking of the spleen has been shown by us and others as an indicator of post-stroke immunosuppression in both clinical and experimental stroke settings [117, 125, 139, 141]. There is also evidence of impaired antibacterial immune cell function after stroke, including reduced expression of major histocompatibility complex (MHC) class II and interferon-γ (IFN-γ) release by macrophages from animals and humans with stroke [142, 143]. In addition, there are reports of reduced NK cell killing function [129], and loss of marginal zone B cells and circulating IgM in post-stroke mice [127]. While post-stroke immunosuppression is apparent, the mechanisms in which this occurs is unclear. A better understanding of the underlying mechanisms will help reveal new therapeutic targets aimed at reversing this impairment to reduce the rate of infection after stroke.

Findings from a number of clinical and preclinical studies indicate SNS is an important modulator of immune responses after stroke. Given that plasma NA and other catecholamines are elevated in patients with stroke [122, 127, 144], the notion of NA-mediated post-stroke immunosuppression is a possibility. In fact, elevated plasma NA was associated with reduced MHC expression and greater susceptibility to post-stroke infection in patients [145]. While, NA is known to be an agonist of all adrenergic receptors (and is in fact a more potent agonist of the α adrenergic receptors), the Adrb2 is predominantly expressed by immune cells and activation of the Adrb2 has been better associated with immunosuppression than the α adrenergic receptors [4]. In experimental studies, many have attempted to show the

involvement of the Adrb2 using β-receptor antagonists (PPL) or ablation of NA-producing sympathetic neurons (6-hydroxydopamine [6-OHDA]) [72, 125, 127, 129]. Administration of β-receptor agonists or ablating sympathetic neurons have proven effective in reversing some of the immunosuppressed phenotype (such as reduced spleen cellularity and loss of circulating leukocytes) seen following stroke to suggest the role of the SNS [72, 125, 128, 141]. Despite this, efficacy of using β-blocker use to reduce post-stroke infection in patient cohorts has been controversial [71, 130, 132-134]. The discrepancy in findings may be due to the lack of stratifying patients to stroke severity. Therefore, in this chapter, we have designed experiments to examine i) the level of circulating NA and its metabolites following various models of stroke, ii) the effectiveness of adrenergic receptor inhibitors in reducing infection after either a moderate or severe model of stroke, and iii) the role of the Adrb2 in immunosuppression and risk to infection after stroke.

In comparison to mice that underwent sham surgery, there were no changes to the plasma catecholamines DOPA, DOPAC, ADR, and DHPG within the first 3 h after the various models of ischemic stroke. Only at the 3 h timepoint were elevations in plasma DA and NA detected following severe stroke when compared to mild and moderate severities of stroke. There may be a multitude of reasons as to why a more pronounced elevation in plasma catecholamines was not seen in our experiments. Firstly, 3 h post-stroke may be too acute for SNS dysregulation to be detected in the circulation. Indeed, one study showed plasma NA was elevated at 5 h following experimental stroke [146], while others only show an elevation after the first day after stroke in patients [121, 144]. This suggests that catecholamine release is a dynamic process that is continuously regulated and that the increase in catecholamines may only be detected after 3 h following cerebral ischemia. Secondly, plasma NA may not be a good indicator of SNS activation. The concentration of NA in the plasma is dependent on the rate of release and the rate of removal from the plasma whereby reduced removal of NA from

the plasma can increase detection of plasma NA without a change in SNS activity [1]. The contrary may also occur, where despite an increase in SNS activity in certain organs, an increase in NA uptake by various cells in respective organs would lead to no change in plasma NA. Furthermore, plasma NA only represents up to 10% of all NA produced by sympathetic nerve endings and therefore is not fully representative of SNS activity [1, 138, 147]. Lastly, while measuring plasma NA is relatively simple, the half-life of NA is short (approx. 2 min) and the sensitivity and reproducibility of NA detection assays are low [147-149]. Therefore, caution is required for the interpretation and implications of these findings as the relationship between plasma NA on SNS activity is complex [1].

New methods of measuring SNS activity are required, especially to measure changes to SNS activity in various organs due to the deep innervation of sympathetic neurons in major immune sites. A recent study established a protocol for imaging catecholamine uptake by cardiomyocytes in healthy volunteers [150]. This study used two radioactively tagged analogues of catecholamines that were safe for administration into humans and were detectable using positron emission tomography (PET) scanning. Future experiments that delineate the effect of stroke on the SNS may also consider measuring the levels of enzymes involved in NA generation and metabolism to provide further supporting evidence of potential SNS dysregulation. Measuring the changes to NA uptake or enzymes involved in NA biosynthesis at sites of infection (i.e. lung) may be of importance to indicate whether SNS hyperactivation correlate with immune impairment in these organs.

To overcome the shortcomings of measuring plasma NA, we next tested the efficacy of β-blockers in reducing post-stroke infection. The use of PPL, a non-selective β-blocker, in experimental models of stroke has previously proven to reduce post-stroke infection [72, 125]. However, the majority of studies explored this in a mild or moderate severity of stroke (equivalent to the 30 or 60 min tMCAO models used in this study). Here, we showed that PPL

was unable to reduce post-stroke infection in a severe model of stroke, suggesting that β-adrenergic receptor activation is not the primary driver of SNS-mediated immunosuppression and that alternative mechanisms are in play. More importantly, the non-reperfused nature of pMCAO is more reflective of the clinical settings whereby less than 15% of patients with stroke receive thrombolytic or thrombectomy interventions [151].

Past studies indicate that the susceptibility to post-stroke infection is also contributed by immune exhaustion, whereby overstimulation of immunity in the few hours after stroke leads to exhaustion of immune cells by 24 h after stroke [152]. Immune exhaustion following stroke is initiated by release of high-mobility group box 1 (HMGB1) from dying neurons in the ischemic core of the brain. HMGB1 can then escape into the periphery to act on receptors for advanced glycation end products (RAGEs) on monocytes in the bone marrow and spleen. This activation of monocytes promotes a strong production of proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, IL-1β, and IL-6, and induces a "sickness behaviour" phenotype in post-stroke mice. Additionally, HMGB1 promotes proliferation of immunosuppressive bone marrow-derived monocytes that reduces T cell immunity [152].

Additional mechanisms of post-stroke infection is the breakdown of intestinal barriers that allow for the dissemination of gut-derived bacteria to peripheral tissues (such as the lung) [153]. The loss of epithelial tight junction proteins in the colon may be mediated by increased levels of colonic TNF-α, especially in aged post-stroke animals [154]. We suggest that in the case of a mild-moderate stroke, β-adrenergic receptor activation by catecholamines is primarily responsible for post-stroke immunosuppression, however, as severity of stroke worsens, other mechanisms of post-stroke infection come into play to cause infection. This notion is supported by the results of our experiments into our experiments examining the effectiveness of the selective Adrb2 antagonist, ICI-118551, in reducing post-stroke infection. We showed that Adrb2 antagonism could reduce post-stroke infection after a model of moderate stroke, but not

after a model of severe stroke. Therefore, a complex interplay of multiple biological systems is likely to be at play in the event of a severe stroke, and thus blockade or manipulation of multiple pathways may be needed to reverse post-stroke immunosuppression.

We next aimed to further elucidate the role of the Adrb2 in post-stroke infection using Adrb2 deficient mice. Previous studies exploring Adrb2 deficiency utilised mice in mixed genetic backgrounds. We overcame this by backcrossing the strain to C57Bl/6J mice for over 10 generations. In our study, Adrb2 deficiency did not alter the rate of infection and therefore suggests that the Adrb2 is not single-handedly involved in the susceptibility to post-stroke infection. In a prospective study, the use of non-selective β-blockers versus the use of adrenergic receptor β_1 (Adrb1) within the first 3 days following admission on post-stroke infection was explored [71]. This study reported that the use of non-selective β-blockers was associated with increased infections, while use of Adrb1 antagonists did not have an association with infection. As such, authors suggest that the increased association of infection with non-selective β-blocker use was mediated by blocking Adrb2 [71], although our present findings do not support this. However, in our study, data involving the Adrb2 deficient mice should be interpreted with caution. The inherent lack of Adrb2 signalling pathway in these mice may have an impact on findings. Furthermore, previous reports of overexpression of β_1- and β_3-adrenergic receptors in brown adipose tissue in Adrb2 KO mice [155] are suggestive of compensatory mechanisms by other adrenergic receptors in the absence of Adrb2.

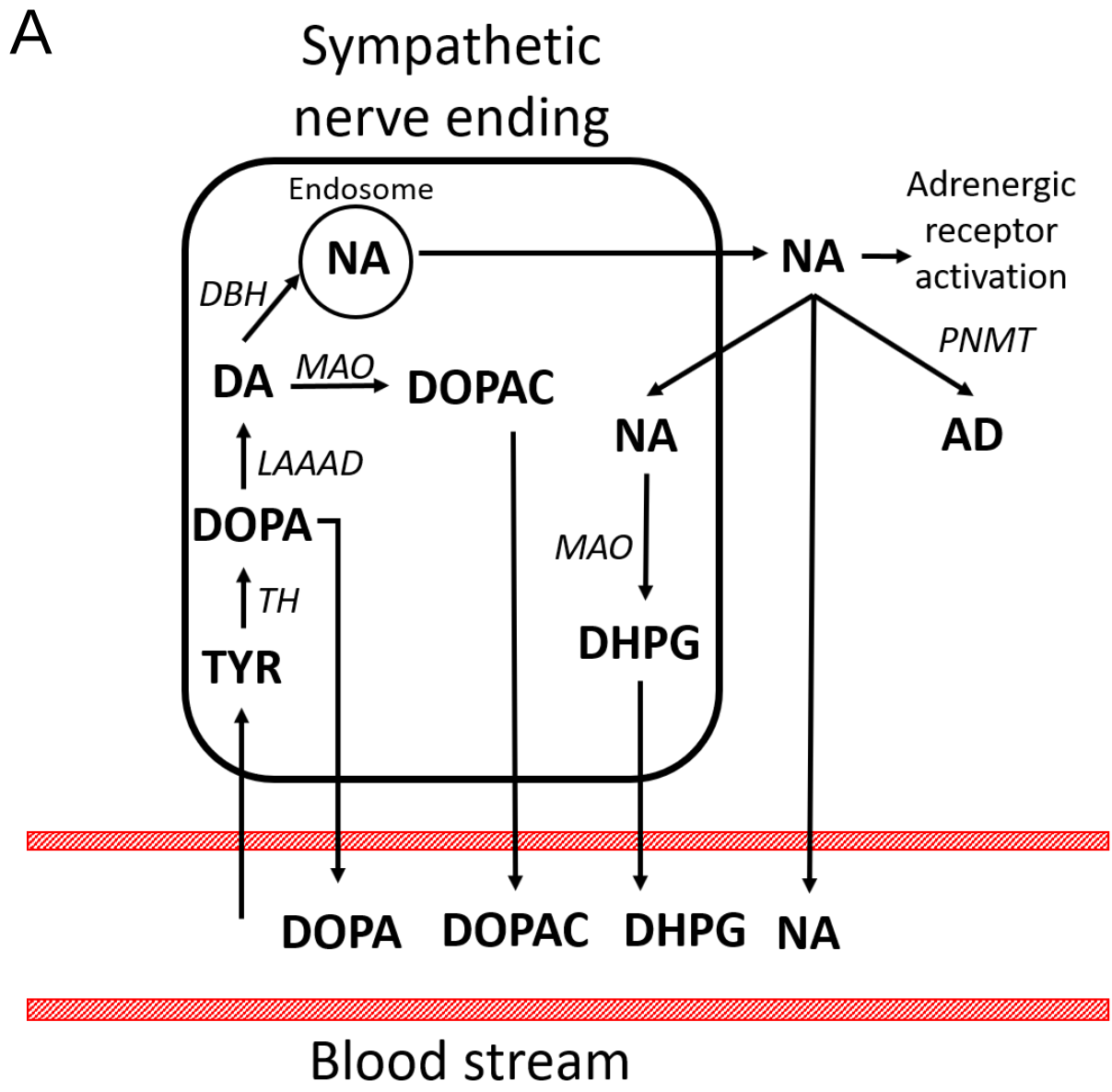
To better understand the impact of stroke on Adrb2 signalling and immunosuppression, ICI-118551 was administered during and following stroke. ICI-118551 treatment has been previously reported to partially reduce post-stroke immunosuppression by sustaining lymphocyte production of IFN-γ [139]. Importantly, the remainder of the immunosuppressive symptoms could be explained by activation of the hypothalamic-pituitary-adrenal (HPA) axis in that study [139]. The HPA axis works in tandem with the SNS in response to stress to

produce glucocorticoids in response to production of adrenocorticotropic hormone. Glucocorticoid blockade after experimental stroke could prevent the loss of T lymphocytes in the blood and preserve spleen size [139]. Furthermore, a combination treatment of β-receptor antagonists and glucocorticoid receptor antagonist was superior in preventing splenic atrophy and NK cell death compared to β-receptor antagonist or glucocorticoid receptor antagonist alone. Therefore, this highlights the role for the HPA axis in post-stroke infection and is likely that HPA axis and SNS activation both collaborate to contribute to post-stroke immunosuppression.

The role of NA and activation of the Adrb2 still requires further study. The experimental approach we took was blockade of adrenergic receptors, while the supplementation of SNS metabolites, such as NA, has yet to be explored in the context of infection after stroke. The administration of NA would require osmotic pumps to allow for small and sustained doses of NA throughout the experimental timepoints. Surgical insertion of osmotic pumps containing NA following mild stroke surgery could be used to model SNS dysregulation that typically would be seen only following a severe stroke. Then, examining the bacterial load in the lung of stroke mice treated with saline against stroke mice treated with NA may answer whether NA mediates post-stroke infection. Similar experiments could be performed using selective β-receptor agonists, such as isoprenaline, to explore the role of the β-receptors in post-stroke immunosuppression. Additionally, imaging of catecholamine uptake in various organs after stroke may also be achievable using PET and radioactively labelled catecholamines to allow for analysis in experimental and clinical stroke [150]. The uptake of catecholamines by immune cells in the lung would be of particular interest as this may offer a reason as to why lung infection is the most common type of infection in post-stroke animals as well as in patients with stroke. Overall, the mechanisms to post-stroke infection are clearly complex, multifaceted and may influence one another. Therefore, there is value in

simultaneously exploring these processes (which may include immune exhaustion, gut barrier breakdown, and autonomic dysfunction) for a better understanding of the mechanisms to post-stroke infection.

Figures


A
Sympathetic nerve ending
Endosome
NA
DBH
DA
MAO
DOPAC
LAAAD
DOPA
TH
TYR
NA
Adrenergic receptor activation
PNMT
NA
AD
MAO
DHPG
DOPA
DOPAC
DHPG
NA
Blood stream

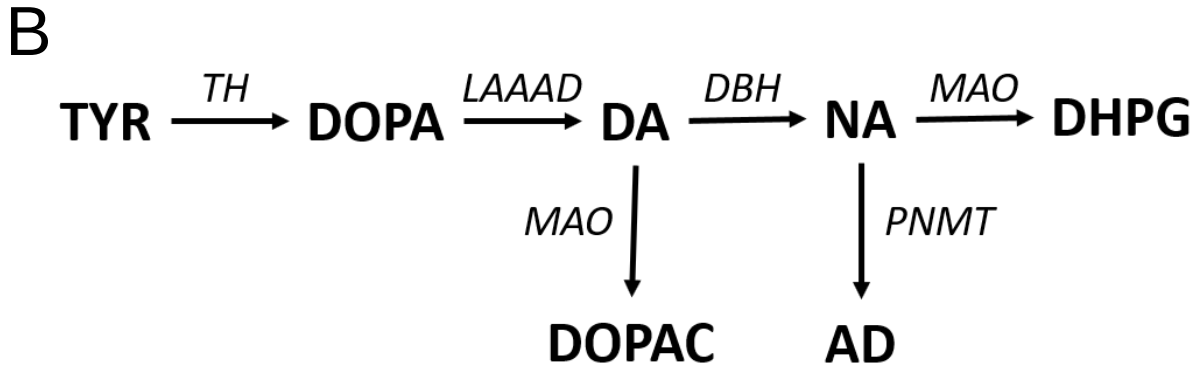
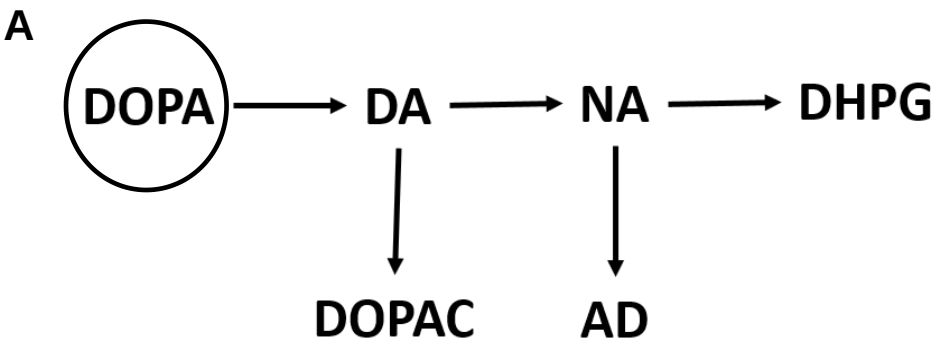

B
TYR
TH
DOPA
LAAAD
DA
DBH
NA
MAO
DHPG
MAO
PNMT
DOPAC
AD

Figure 4.1 Generation and metabolism of catecholamines by sympathetic nerve endings

Circulating tyrosine (TYR) enters the sympathetic nerve ending and is converted into 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH). The enzyme L-aromatic-amino acid decarboxylase (LAAAD) then converts DOPA into dopamine (DA), while excess DOPA are release into the bloodstream. Next, DA is either convert by monamine oxidase (MAO) into dihydroxyphenylacetic acid (DOPAC) to be released into the bloodstream or transported into endosomes for conversion by dopamine-β-hydroxylase (DBH) into noradrenaline (NA) and transported out of the cell. The release out of sympathetic neurons allows NA to either interact with adrenergic receptors on other cell types, escape into the periphery, convert into adrenaline (AD) by phenylethanolamine N-methyltransferase (PNMT), or re-enter the neuronal cell. Here, NA converts into dihydroxyphenylglycol (DHPG) by MAO for release into the bloodstream (A). A simplified depiction of NA generation and metabolism is also depicted (B). Figure adapted from [1].


A
DOPA
DA
NA
DHPG
DOPAC
AD

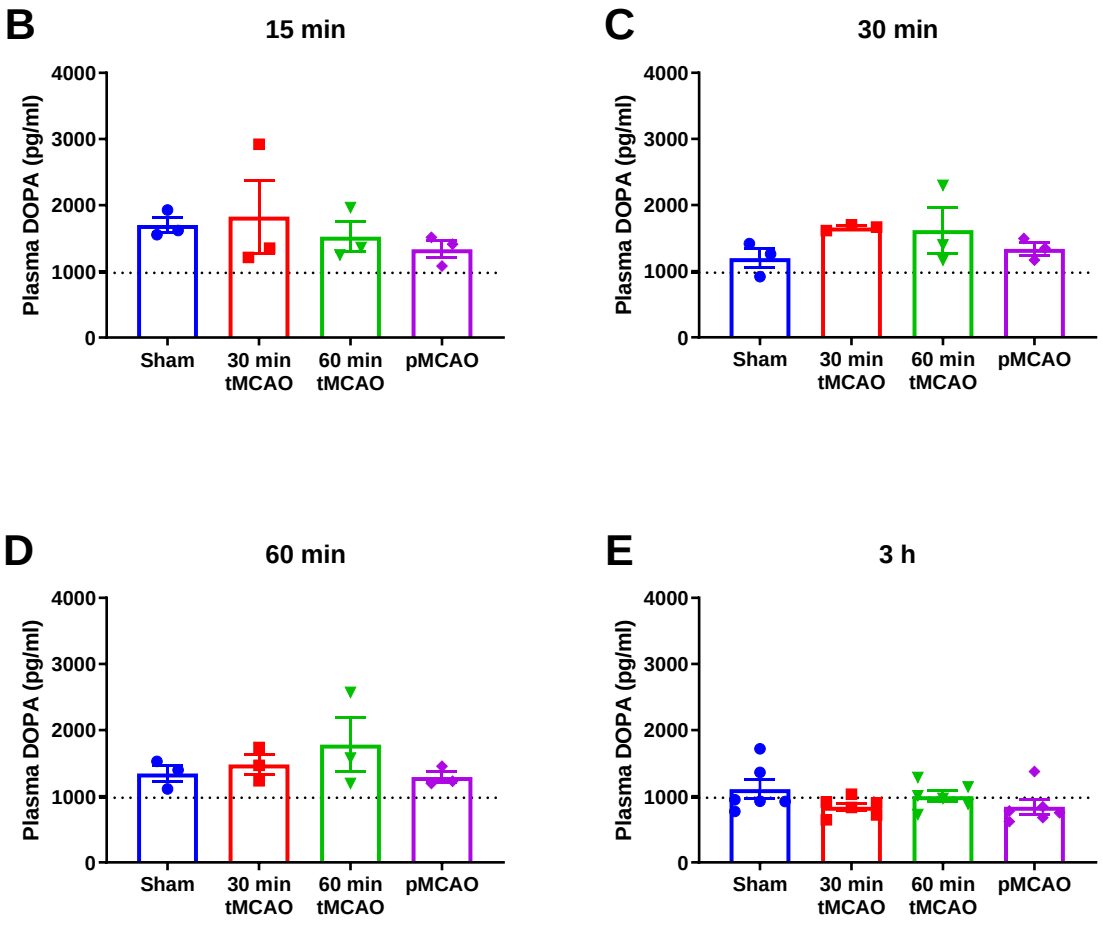
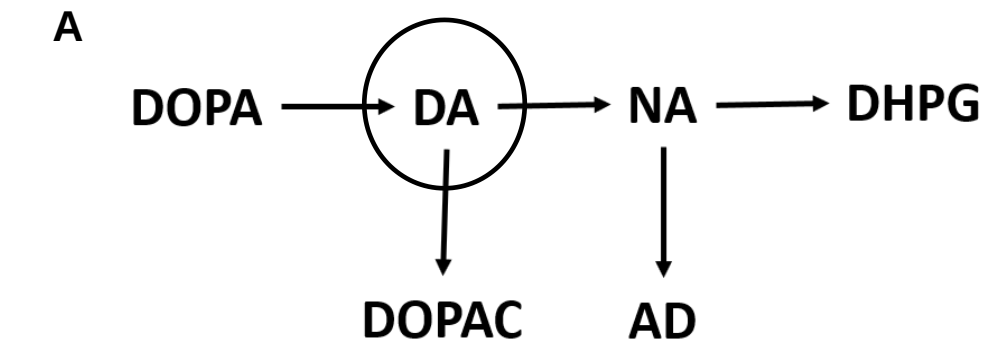

B
15 min
C
30 min
D
60 min
E
3 h
Plasma DOPA (pg/ml)
4000
3000
2000
1000
0
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Figure 4.2 Plasma 3,4-dihydroxyphenylalanine (DOPA) within the first 3 h after stroke are unchanged.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was stored at -80^{o}C until analysis of catecholamines via HPLC. Following SNS activation, DOPA is the first catecholamine that is converted from circulating tyrosine in the nerve endings (A). Plasma DOPA was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, one-way ANOVA with multiple comparisons. Dotted lines denote levels of plasma DOPA in naïve mice. Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.


A
DOPA
DA
NA
DHPG
DOPAC
AD

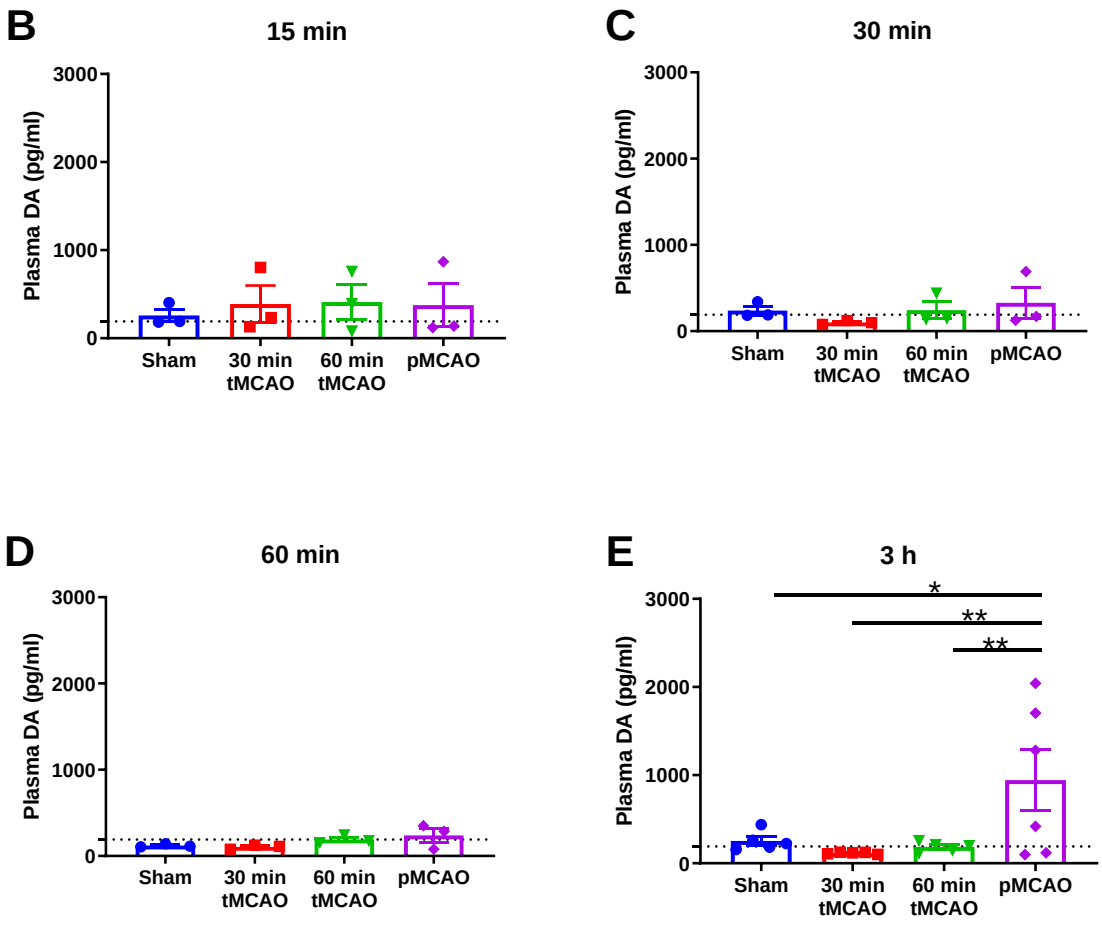

B
15 min
C
30 min
D
60 min
E
3 h
Plasma DA (pg/ml)
3000
2000
1000
0
Sham
30 min tMCAO
60 min tMCAO
pMCAO
*
**
**

Figure 4.3 Plasma dopamine (DA) is elevated at 3 h after severe stroke.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was collected from whole blood and stored at -80^{o}C until analysis of catecholamines via HPLC. DOPA is converted into DA in the catecholamine synthesis pathway (A). Plasma DA was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, *p<0.05, **p<0.01, one-way ANOVA with multiple comparisons. Dotted lines denote levels of plasma DA in naïve mice. Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.

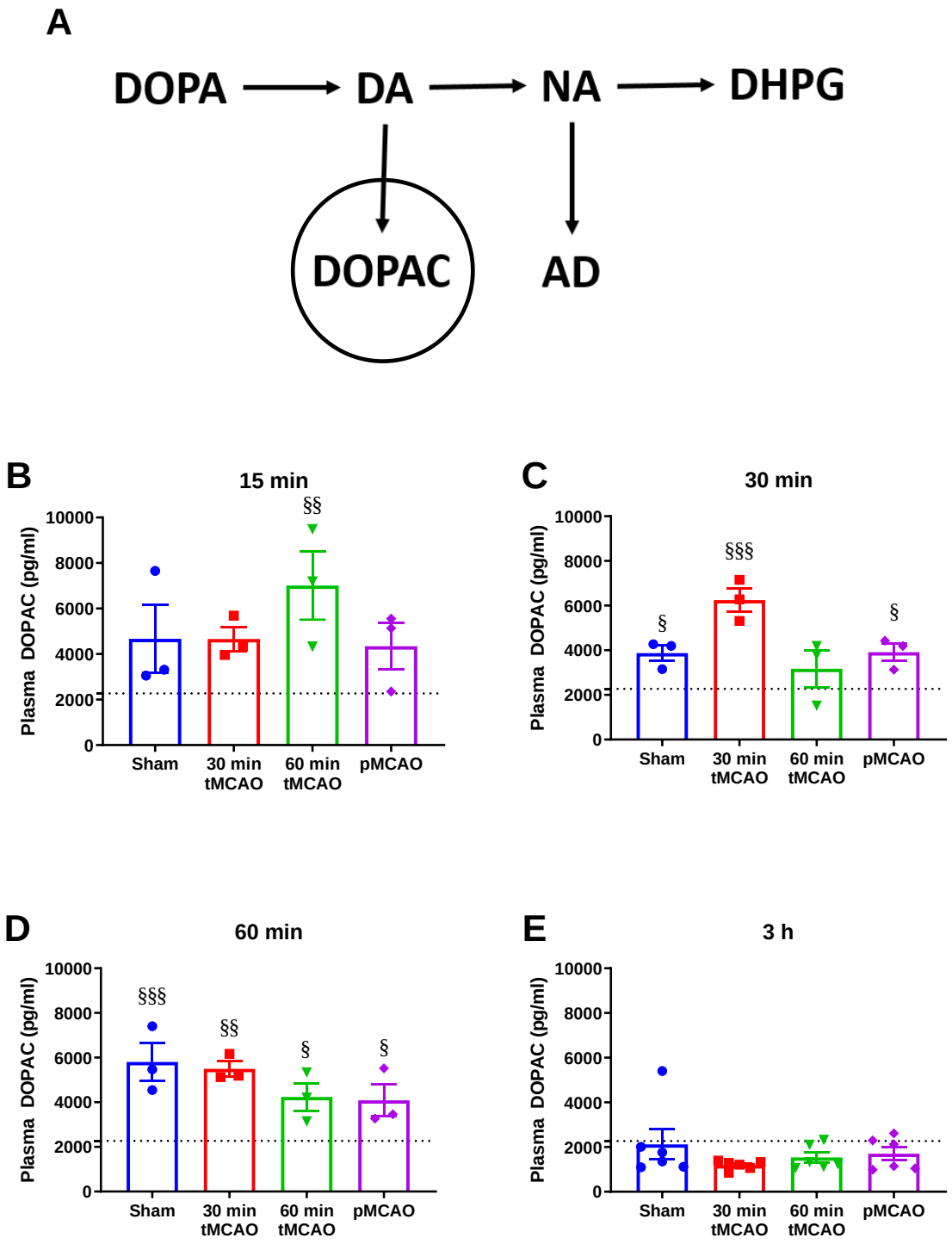

A
DOPA
DA
NA
DHPG
DOPAC
AD
B
15 min
C
30 min
D
60 min
E
3 h
Plasma DOPAC (pg/ml)
10000
8000
6000
4000
2000
0
§§
§§§
§
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Figure 4.4 Changes to plasma dihydroxyphenylacetic acid (DOPAC) within the first 3 h after stroke.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was collected from whole blood and stored at -80^{o}C until analysis of catecholamines via HPLC. In the catecholamine synthesis pathway, DA can be metabolised into DOPAC or noradrenaline (NA) (A). Plasma DOPAC was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, one-way ANOVA with multiple comparisons, where $^{\S}p<0.05$, $^{\S\S}p<0.01$ and $^{\S\S}p<0.001$ in comparison to plasma DOPAC in naïve mice (denoted by dotted lines). Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.

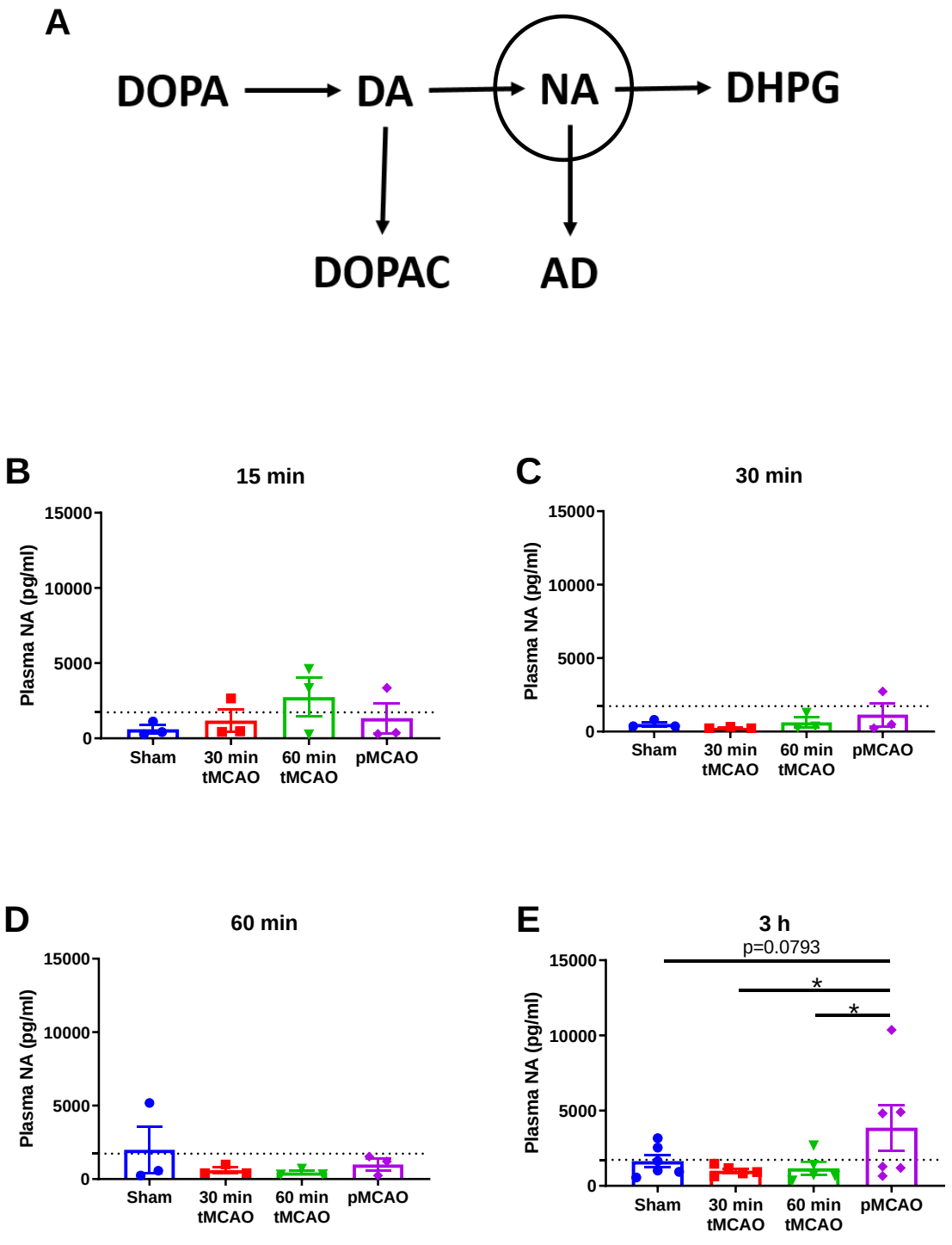

A
DOPA
DA
NA
DHPG
DOPAC
AD
B
15 min
C
30 min
D
60 min
E
3 h
p=0.0793
*
*
Plasma NA (pg/ml)
15000
10000
5000
0
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Figure 4.5 Plasma noradrenaline (NA) is elevated at 3 h following severe stroke.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was collected from whole blood and stored at -80^{o}C until analysis of catecholamines via HPLC. In the catecholamine synthesis pathway, DA is predominantly converted into NA (A). Plasma NA was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, one-way ANOVA with multiple comparisons. Dotted lines denote levels of plasma NA in naïve mice. Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.

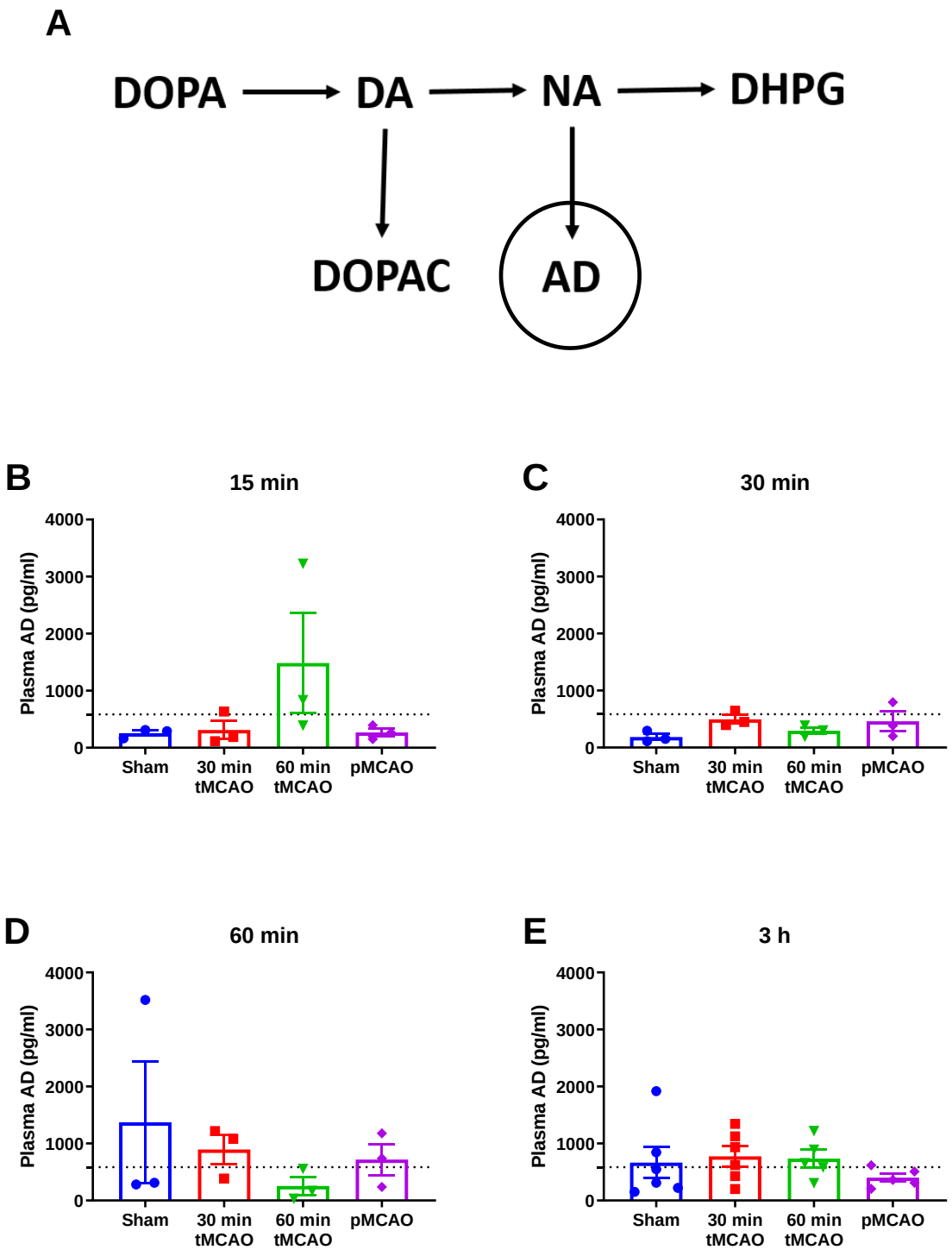

A
DOPA
DA
NA
DHPG
DOPAC
AD
B
15 min
C
30 min
D
60 min
E
3 h
Plasma AD (pg/ml)
4000
3000
2000
1000
0
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Figure 4.6 Plasma adrenaline (AD) is unchanged within the first 3 h after stroke.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was collected from whole blood and stored at -80^{o}C until analysis of catecholamines via HPLC. In the catecholamine synthesis pathway, NA is can be converted into AD (A). Plasma AD was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, one-way ANOVA with multiple comparisons. Dotted lines denote levels of plasma AD in naïve mice. Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.

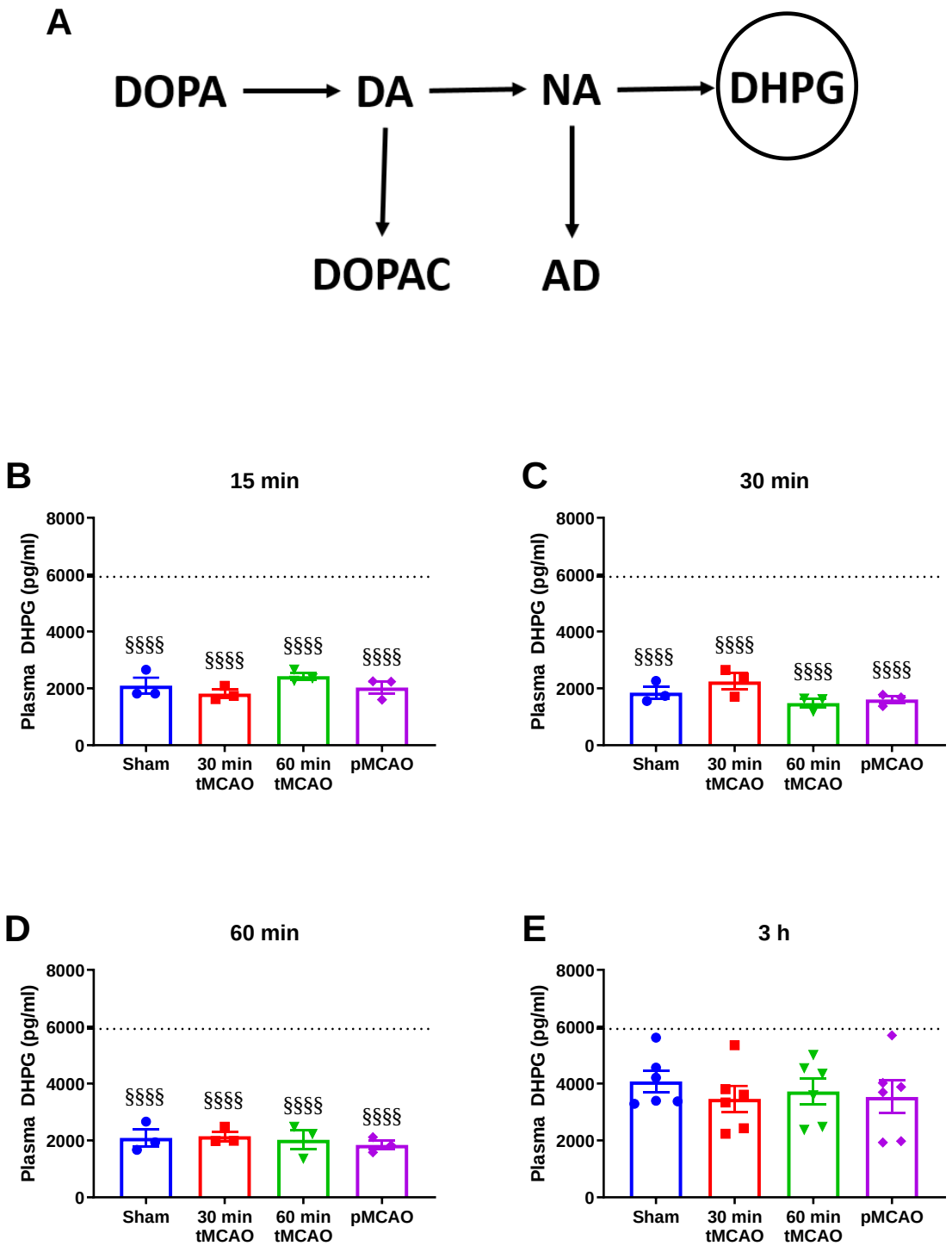

A
DOPA
DA
NA
DHPG
DOPAC
AD
B
15 min
C
30 min
D
60 min
E
3 h
Plasma DHPG (pg/ml)
8000
6000
4000
2000
0
§§§§
Sham
30 min tMCAO
60 min tMCAO
pMCAO

Figure 4.7 Plasma dihydroxyphenylglycol (DHPG) is unchanged within the first 3 h after stroke.

Whole blood from post-stroke mice were added to tubes containing EGTA and centrifuged for plasma collection. Plasma was collected from whole blood and stored at -80ºC until analysis of catecholamines via HPLC. In the catecholamine synthesis pathway, NA is can be converted into DHPG (A). Plasma DHPG was measured at 15 min (B), 30 min (C), 60 min (D), and 3 h (E) post-surgery. Data are represented as mean ± SEM, n=3 mice per group in 15 min, 30 min and 60 min timepoints and n=6 for 3 h timepoints, one-way ANOVA with multiple comparisons where $^{\S}p<0.05$, $^{\S\S}p<0.01$ and $^{\S\S}p<0.001$ in comparison to plasma DHPG in naïve mice (denoted by dotted lines). Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; NA, noradrenaline; AD, adrenaline; DHPG, dihydroxyphenylglycol.

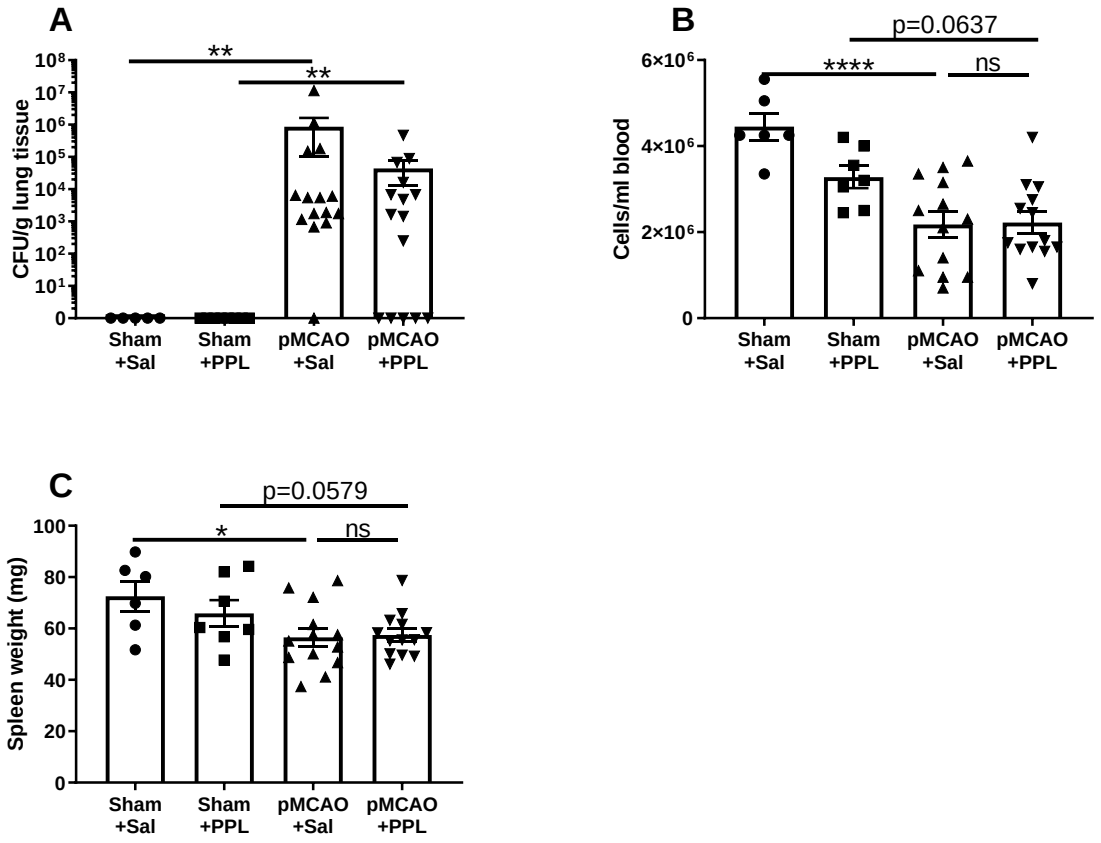


Figure 4.8 Non-selective inhibition of β-adrenergic receptors with PPL does not improve post-stroke infection after severe experimental stroke.

Mice that underwent pMCAO or sham surgery were treated with saline or PPL (30mg/kg) at 0 h, 4 h and 8 h following surgery. At 24 h after surgery, mice were humanely euthanised, where lungs were collected for bacterial analysis (A), circulating leukocyte count was enumerated in whole blood (B), and spleen weights recorded (C). Data are presented as mean ± SEM, where n≥6 and *p<0.05, **p>0.01, ****p>0.0001, one-way ANOVA for normally distributed data or Kruskal-Wallis test for bacteriological analysis.

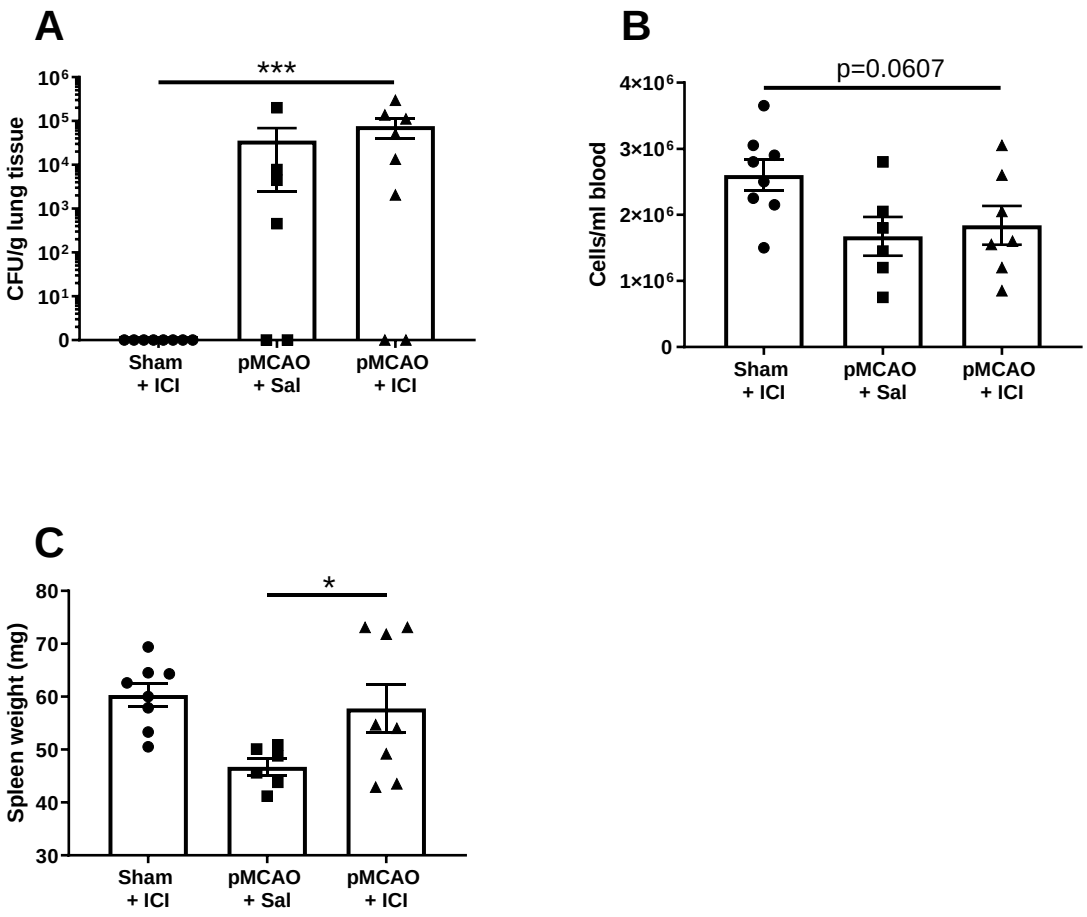


Figure 4.9 Specific Adrb2 antagonism does not prevent infection after severe stroke.

Mice underwent pMCAO or sham surgery and were treated with ICI-118551 (ICI) (1mg/kg) or saline at 0 h, 4 h, and 8 h after surgery. At 24 h after surgery, mice were humanely euthanised, where lungs were collected for bacterial analysis (A), circulating leukocyte count was enumerated in whole blood (B), and spleen weights recorded (C). Data are presented as mean ± SEM, where n≥6 where *p<0.05, ***p>0.001 using a one-way ANOVA for normally distributed data or Kruskal-Wallis test for bacteriological analysis.

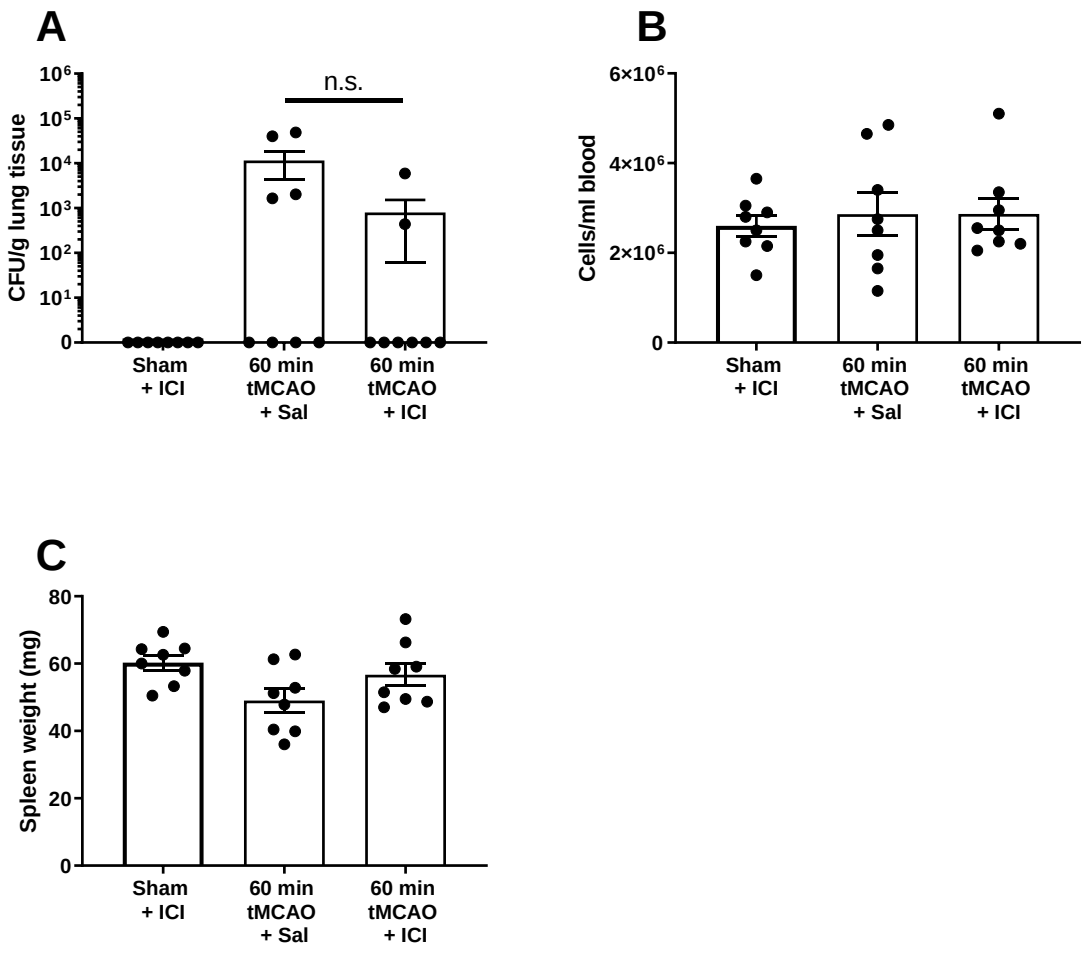


Figure 4.10 Specific Adrb2 antagonism does not prevent infection after moderate stroke.

Mice underwent 60 min tMCAO or sham surgery and were treated with ICI-118551 (ICI) (1mg/kg) or saline at 0 h, 4 h, and 8 h after surgery. At 24 h after surgery, mice were humanely euthanised, where lungs were collected for bacterial analysis (A), circulating leukocyte count was enumerated in whole blood (B), and spleen weights recorded (C). Data are presented as mean ± SEM, where n≥6, one-way ANOVA for normally distributed data or Kruskal-Wallis test for bacteriological analysis.

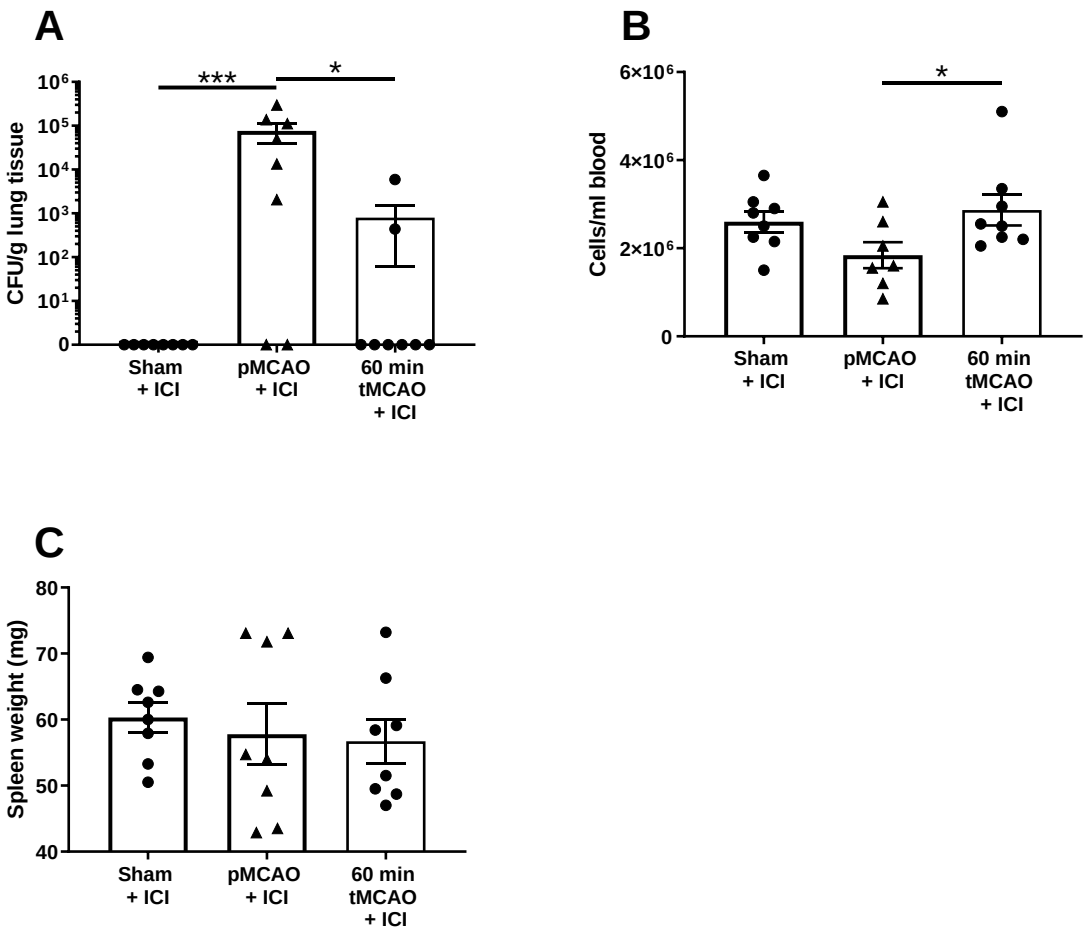


Figure 4.11 Specific Adrb2 antagonism reduces infection after moderate stroke in comparison to severe stroke.

Mice underwent stroke (pMCAO or 60 min tMCAO) or sham surgery and were treated with ICI-118551 (ICI) (1mg/kg) at 0 h, 4 h, and 8 h after surgery. At 24 h after surgery, mice were humanely euthanised, where lungs were collected for bacterial analysis (A), circulating leukocyte count was enumerated in whole blood (B), and spleen weights recorded (C). Data are presented as mean ± SEM, where n≥6 and *p<0.05, ***p>0.001 using one-way ANOVA for normally distributed data or Kruskal-Wallis test for bacteriological analysis. Data presented here are were obtained from Figures 4.9 and 4.10.

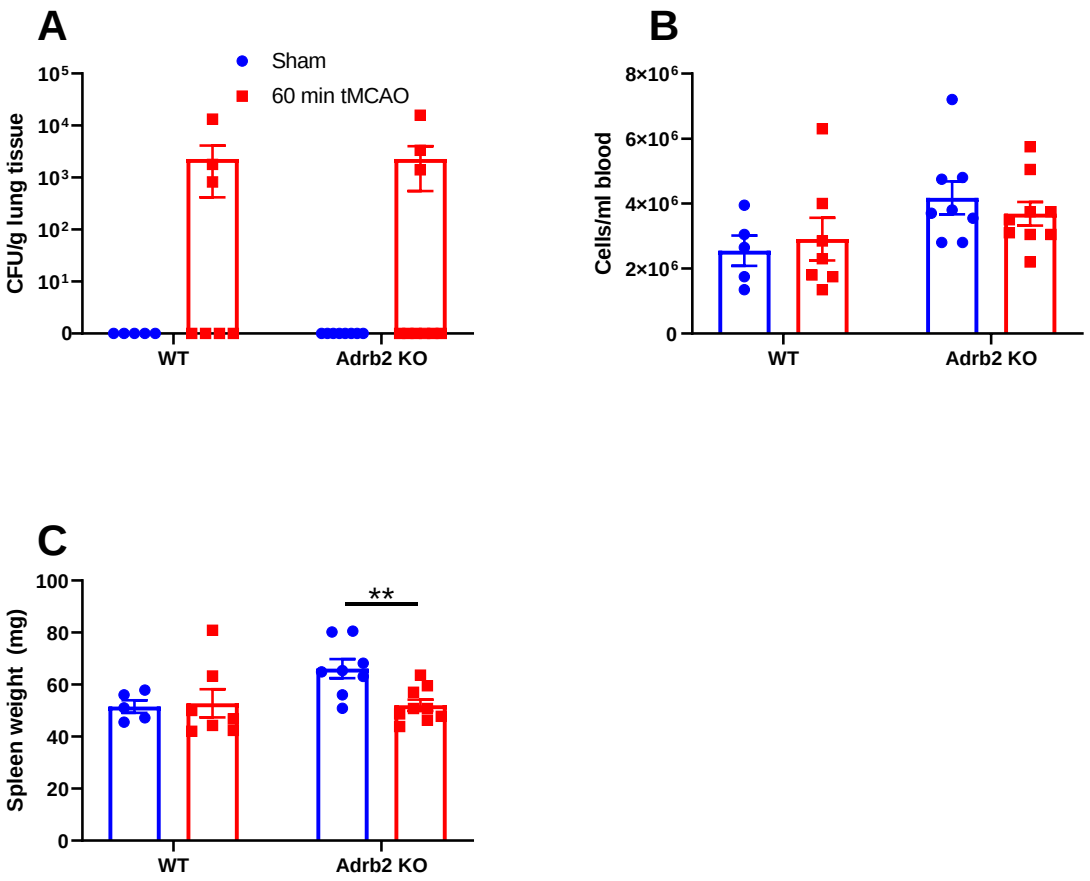


Figure 4.12 Deficiency of Adrb2 does not protect from post-stroke infection.

Wildtype (WT) or Adrb2 deficient (Adrb2 KO) mice underwent 60 min tMCAO or sham surgery. At 24 h following surgery, mice were humanely euthanised, where lungs were collected for bacteriological analysis (A), circulating leukocytes enumerated (B), and spleen weights recorded (C). Data are presented as mean ± SEM, where n≥5 for experiments using Adrb2 KO mice where n≥5, **p>0.01, one-way ANOVA for normally distributed data or Kruskal-Wallis test for bacteriological analysis.

Chapter Five

Targeting iNKT cells for treatment of post-stroke infection

Introduction

Currently, the gold-standard treatment for post-stroke infection is the administration of antibiotics. However, various clinical studies have shown that therapeutic use of antibiotics cannot improve patient outcome [156]. This initiated interest in preventive antibiotic therapy (PAT) as an alternative solution to prevent infections after stroke. Early experimental studies showed that PAT reduced the development of infections and fever, and significantly reduced mortality in post-stroke animals [157, 158]. However, as described in Chapter 1, these beneficial effects of PAT did not translate in clinical trials because of two major issues: 1) PAT did not reduce the incidence of post-stroke pneumonia, and 2) PAT did not improve patient outcome [140, 159-163]. Therefore, with the failure of PAT along with the looming threat of antibiotic resistance, alternative therapeutics to reduce post-stroke infection are urgently required.

Research in the past decade has focused on elucidating the underlying mechanisms of post-stroke infection in order to identify opportunities for therapeutic intervention. Causative mechanisms to post-stroke infection has been traditionally associated with direct entering of pathogens to sites of infection (i.e. lung) via means such as aspiration and use of gastric tubing [164-166]. It is now understood that reduced immunity after stroke increases the risk of infection, although few studies have integrated the underlying mechanisms. It is likely the mechanisms are dependent on a multitude of dynamic processes that create difficulties in the discovery of targeted treatments and therapeutics to prevent or reduce post-stroke infection [167, 168]. Nevertheless, we have previously identified that invariant natural killer T (iNKT) cells in the liver mediates systemic post-stroke immunosuppression, resulting in greater susceptibility to infection [72]. Therefore, therapeutic targeting of the iNKT cell may present as a viable option to reverse post-stroke infection.

The iNKT cell (or type 1 NKT cell) is an innate-like T cell, characterised by the expression of CD3 and a T cell receptor (TCR), which consists of a highly conserved α chain encoded by the *Jα18* gene segment [76]. Unlike conventional T cells, iNKT cells recognise glycolipid antigens presented on the non-polymorphic MHC-like complex, CD1d. Due to the highly conserved nature of CD1d and the iNKT cell TCR across many animal species, iNKT cells have been suggested to play an essential role in immunity [76]. Additionally, owing to their ability to produce a vast array of immunomodulatory molecules, iNKT cells are capable of activating or suppressing immunity and thus function as a master-regulator of immunity [73].

The type of immunity induced by iNKT cells depend on their mode of activation. The most well studied activator of iNKT cells is α-galactosylceramide (α-GalCer), which is presented by CD1d on antigen presenting cells to the TCR of iNKT cells [90, 100]. Activation of the iNKT cell via α-GalCer stimulates iNKT cells to produce copious amounts of interferon (IFN-) γ and interleukin- (IL-) 4 [80, 100]. However, IFN-γ and IL-4 have conflicting roles in immunity. In the current understanding of T-helper (Th) immunology, IFN-γ facilitates a Th1 response, while simultaneously inhibiting the Th2 response. Conversely, IL-4 induces a Th2 response, while inhibiting a Th1 response [87, 88]. Therefore, due to the contradictory effects of IFN-γ and IL-4 in immunity, there has been a large focus in controlling the type of immunity elicited by the iNKT cell. Skewing iNKT cell responses to specifically induce either IFN-γ or IL-4 production can be achieved by altering the structure of α-GalCer [97, 99, 100, 104]. Indeed, the capacity of structural analogues of α-GalCer to polarise iNKT cell responses toward either a Th1 or Th2 response could have benefits in treating a wide range of diseases. Furthermore, as α-GalCer treatment has shown to induce liver toxicity in mice [113], modified compounds or analogues derived from α-GalCer may reduce this and therefore should be considered.

The iNKT cells are predominantly found in the liver, where they comprise of approximately 40% of hepatic lymphocytes in mice and up to 5% in humans [73, 75]. As such, the function of iNKT cells in models of inflammation, infection and injury have been most widely explored in the liver [169, 170]. Indeed, previous work in our laboratory demonstrated that hepatic iNKT cells can remotely respond to distant brain injury within the first 4 h following an experimental model of ischemic stroke [72]. This resulted in increased intracellular IL-10 production by hepatic iNKT cells and spontaneous lung infection within 24 h post-stroke. These findings were reflected in the clinic as we could detect the activation of circulating iNKT cells, which correlated with increased plasma IL-10 in a stroke severity-dependent manner. Furthermore, this increase in IL-10 was exacerbated in patients with post-stroke infection, suggesting the iNKT cells may mediate post-stroke immunosuppression via IL-10 [109]. Furthermore, therapeutic administration of α-GalCer induced the production of systemic IFN-γ and a significant reduction of bacterial load within the lung after experimental stroke [72] and demonstrates the potential of harnessing the iNKT cell in reducing post-stroke infection.

While immune suppression following stroke can be mediated by hepatic iNKT cells, it is unclear whether the same phenotype extends to iNKT cells residing in other tissue compartments. Of note, the immunological profile of lung iNKT cells is of interest due to the high frequency of fatal bacterial pneumonia in patients with stroke. In a healthy state, most pulmonary iNKT cells in mice reside in the vasculature where they patrol for foreign antigen, while a small population reside in the extravascular space [171]. Upon pulmonary infection with *Streptococcus pneumoniae*, production and release of IFN-γ by pulmonary iNKT cells were critical for neutrophil-mediated clearance of bacteria in the lungs and survival of mice [172]. This was dependent on CD1d presentation of glycolipids from *S. pneumoniae* and group B *Streptococcus* to activate iNKT cells to produce antibacterial IFN-γ and IL-17, which

promoted host protection [173]. Furthermore, the CD1d-dependent recruitment of neutrophils into the extravascular space in-turn promotes migration of intravascular iNKT cells into the alveoli via chemokine (C-C motif) ligand 17 (CCL17), which was required for bacterial clearance [171]. Due to the high frequency of lung infection following stroke, there is a requirement to explore the changes in iNKT cell number and activation status in the lung.

Evidently, the role of pulmonary iNKT cells is important in lung immunity and defence against bacterial infection. Therefore, in this chapter, we sought to elucidate the role of the pulmonary iNKT cells in post-stroke infection to test the hypothesis that activation of pulmonary iNKT cells in this manner is immunosuppressive, thus impairs antibacterial immunity in the lung, and leads to increased susceptibility to infection. In addition, we examined the capacity to activate iNKT cells using α-GalCer as a therapeutic to reduce post-stroke infection. Finally, we examined the efficacy of two α-GalCer analogues to enhance immunity to protect from systemic infection and infection challenged after stroke, with the hope of finding an effective alternative to α-GalCer with minimal adverse effects.

Methods

Mice

Wildtype (WT) male mice (C57Bl/6J) mice were used as described in chapter 2. For experiments using iNKT cell deficient mice, male *Ja18*$^{-/-}$ (JAX laboratories) mice were bred on a C57Bl/6J background for 10 generations. The genotype was confirmed by the genotyping facilities from Transnetyx. All procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCB/2016/10, Monash Medical Centre, Clayton, Victoria, Australia). Animals were randomly assigned to experimental groups to prevent selection bias. All animals had access to water and food *ad libitum*, maintained in temperature-controlled rooms under a 12 h light-dark cycle.

Treatments

For experiments involving glycolipid treatment, α-GalCer (KRN-7000; Kirin Brewery, Gunma, Japan) and the synthetic α-GalCer analogues DB06-9 and SKC08-27 were reconstituted in dimethyl sulfoxide (DMSO) at 1 mg/ml as stock. Analogues were designed and synthesised by Steven A. Porcelli (Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA). The molecular structures of the glycolipids are presented in **Figure 5.1**. Stock concentrations of glycolipids were diluted in saline with Tween-20 for a final dosage of 2 ug (in 0.5% DMSO and 0.5% Tween 20) and administered into mice via intraperitoneal injection. This dosage was obtained from previously published data from our laboratory [72]. A vehicle control consisted of 0.5% DMSO and 0.5% Tween-20 in saline. For experiments quantifying liver toxicity, α-GalCer, analogues or vehicle was administered in naïve WT mice and humanely euthanised after 24 h. For experiments exploring the role of activating iNKT cells after stroke, α-GalCer, analogues or vehicle was

administered into mice at 2 h following MCAO stroke surgery and humanely euthanised at 24 h after surgery for endpoint analysis.

Serum cytokine and alanine aminotransferase (ALT)

To obtain sera, mice were euthanised and whole blood was taken via cardiac puncture. Blood was allowed to clot for 25 min at RT and centrifuged at 2.4 × *g* for 5 min for sera collection. For lungs, the left lobe was homogenised in 700 µl of cOmplete, EDTA-free Protease Inhibitor Cocktail (Sigma). Lung homogenates were centrifuged at 6000 × *g* for 10 min at 4ºC. Sera and lung supernatant were collected and stored at -80ºC until analysis. A mouse IFN-γ (AN-18) and IL-10 OptEIA ELISA set (BD Biosciences) were used as per manufacturer's protocol to quantify cytokine levels in the serum and lung. For assessment of liver injury, ALT levels in the sera was determined by Monash Medical Centre Pathology.

Culturing bacteria and testing antibacterial immunity induced by α-GalCer analogues

A bacterial culture was prepared by inoculating 10 ml of Luria-Bertani (LB) broth with non-pathogenic *Escherichia coli* and incubated at 37ºC overnight on a shaker. From the overnight culture, 1 ml was added to fresh LB broth and incubated for 2 h or until a concentration of 3×10^8 CFU/ml was obtained. Bacteria were pelleted and resuspended in saline and 3×10^7 CFU was intraperitoneally injected into mice. At 2 h following injection of bacteria, α-GalCer, α-GalCer analogue or vehicle was administered. Mice were then humanely euthanised at 8 h post-infection for collection of spleen and lung for bacteriological analysis.

List of other techniques used as described in chapter 2:

- 30 min, 60 min tMCAO and pMCAO (pg 35)
- Preclinical MRI (pg 36)
- Brain infarct quantification (pg 37)

- Bacteriological analysis (pg 37)
- Enumeration of circulating leukocytes (pg 38)
- Flow cytometry of whole lung (pg 38)
- Statistics (pg 40)

Results

Role of pulmonary iNKT cells in the post-stroke lung

To investigate the effect of stroke on pulmonary iNKT cells, we performed flow cytometry on lung tissue and analysed the number, activation, and cytokine production of iNKT cells at 24 h following stroke. In this chapter, we used the three pre-established models of MCAO to achieve strokes of varying severities by adjusting the time of arterial occlusion: 30 min tMCAO (mild model); 60 min tMCAO (moderate model); and pMCAO (severe model) (as described in chapters 2 and 3). In chapter 3, we showed a significant reduction in numbers of lymphocytes in the lung at 24 h following all 3 models of stroke. Despite this, there were no changes in the number of pulmonary iNKT cells after all severities of stroke (**Figure 5.2A**). Although no change in iNKT cell number was detected in the post-stroke lung, an alteration in function may be possible. Thus, we examined the expression of CD69 to assess iNKT cell activation and found an increase in the proportion of $CD69^+$ iNKT cells following all models of stroke compared to sham-surgery control (**Figure 5.2B**). However, the activation of pulmonary iNKT cell after stroke was not accompanied by changes in the production of IFN-γ (**Figure 5.2C**) and IL-10 (**Figure 5.2D**) by iNKT cells after all severities of stroke.

Deficiency of iNKT cells prevents the loss of pulmonary lymphocytes

We next examined the role of iNKT cells in the host antibacterial defence at 24 h after stroke. We achieved this by comparing the lung immune cell populations between WT and Jα18 deficient mice, which we confirmed lack iNKT cells (**Figure 5.3A**). Wildtype and Jα18 deficient mice underwent the 60 min tMCAO model as no discernible changes were seen in pulmonary iNKT cell number and function between stroke severities. In WT mice, there was a reduced number of overall lymphocytes in the lung of mice that underwent 60 min tMCAO

compared to mice that underwent sham surgery (**Figure 5.3B**). The loss of lymphocytes in the lung of WT post-stroke mice was predominantly attributed to the significant loss of $CD4^+$ T cells (**Figure 5.3C**), but not of $CD8^+$ T cells (**Figure 5.3D**) or B cells (**Figure 5.3E**). However, this reduction in pulmonary lymphoid populations after stroke were not seen in Jα18 deficient mice that underwent 60 min tMCAO compared to sham-operated Jα18 deficient mice (**Figure 5.3B-E**). Similar to the findings in chapter 3, there were no changes to number of $CD11b^+$ cells (**Figure 5.3F**), neutrophils (**Figure 5.3G**) or monocytes (**Figure 5.3H**) in the lung of sham- and 60 min tMCAO-operated mice for both WT and Jα18 deficient mice. These findings suggest that iNKT cells play a role in reducing $CD4^+$ T cell abundance in the lung after stroke.

Mice deficient in iNKT cells are not protected from post-stroke lung infection

Numerous clinical and experimental studies have provided evidence for systemic changes after stroke, including reduced spleen cellularity and decreased circulating leukocytes [117, 125, 128, 174]. Therefore, we investigated whether the lack of iNKT cells alters these parameters after stroke and whether Jα18 deficient mice are more protected from post-stroke infection. Similar to previous findings, there was a reduction in spleen weights (**Figure 5.4A**) and a loss of circulating leukocytes (**Figure 5.4B**) at 24 h post-stroke in WT mice that underwent 60 min tMCAO when compared to sham-operated WT mice. This was associated with spontaneous lung infection in these mice (**Figure 5.4C**). In Jα18 deficient mice, there was no change in spleen weight between sham-operated animals and 60 min tMCAO-operated animals (**Figure 5.4A**). It is noteworthy that, although there is a statistically significant difference between sham-operated WT and Jα18 deficient mice, this is likely attributed to the smaller size of Jα18 mice despite being age-matched. Nevertheless, the stroke-induced reduction of spleen size in WT is not seen in Jα18 deficient mice, suggesting iNKT cells may have a role in splenic atrophy after stroke (**Figure 5.4A**). In contrast to WT, circulating

leukocyte numbers did not change in Jα18 deficient mice between sham and 60 min tMCAO operated animals (**Figure 5.4B**) and may suggest that Jα18 deficient mice are less immune compromised after stroke and therefore have reduced risk of infection. However, there was no change in lung infection in Jα18 deficient mice that underwent 60 min tMCAO compared to sham operated Jα18 deficient mice (**Figure 5.4C**). Importantly, deficiency of iNKT cells did not alter the extent of cerebral infarction in post-stroke mice (**Figure 5.4D**). Overall, these data suggest that iNKT cells do not protect or exacerbate post-stroke lung infection. However, as the abundance and function of iNKT cells remain unchanged after stroke, there may be an opportunity to stimulate iNKT cells to induce an antibacterial immunity to reduce post-stroke infection.

Treatment of α-GalCer reduces post-stroke infection

A potent and specific activator of iNKT cells is α-GalCer [89]. To investigate whether iNKT cells can be stimulated to reduce post-stroke infection, we administered a single dose of α-GalCer at 2 h post-stroke surgery and then assessed bacterial infection in the lung and other immune parameters at 24 h following stroke onset (**Figure 5.5A**). In vehicle-treated mice, post-stroke mice had a significantly increased presence of lung bacteria compared to sham-operated mice (**Figure 5.5B**). This coincided with the characteristic signs of post-stroke immunosuppression where spleen weights (**Figure 5.5C**) and circulating leukocyte numbers (**Figure 5.5D**) decreased following 60 min tMCAO in vehicle-treated mice. Treatment with α-GalCer in mice that underwent 60 min tMCAO could significantly reduce the presence of bacteria in the lungs compared to its vehicle-treated counterpart. While α-GalCer treatment could not prevent splenic atrophy after stroke, there was a significant decrease in spleen weight in 60 min tMCAO mice compared to sham-surgery controls in the α-GalCer treatment groups (**Figure 5.5C**). However, in stroke mice, α-GalCer treatment could significantly prevent the

decrease in spleen weight when compared to vehicle treatment. (**Figure 5.5C**). In the circulation, there was a significantly reduced number of circulating leukocytes in α-GalCer treated sham-operated mice compared to its vehicle-treated counterpart (**Figure 5.5D**). Furthermore, there was no significant difference in circulating leukocyte numbers in between vehicle and α-GalCer-treated mice that underwent 60 min tMCAO (**Figure 5.5D**). In the context of stroke, cerebral inflammation dictates the extent of brain infarction [114, 175-177]. Therefore, with immunomodulatory therapy in stroke, it is feasible that stimulation of immunity may promote cerebral inflammation and exacerbate brain infarction. Here, we show that there was no significant difference in infarct volume between vehicle and α-GalCer treated mice after 60 min tMCAO (**Figure 5.5E**). Overall, these data suggest that α-GalCer can significantly reduce the level of post-stroke lung infection and partially improve aspects of stroke-induced immunosuppression without exacerbating cerebral injury.

α-GalCer induces pulmonary and systemic production of IFN-γ

α-GalCer induces the robust production of IFN-γ and therefore we assessed whether reduced infection rates were associated with cytokines levels in the lung. In our sham-operated animals, we confirmed that α-GalCer treatment increased lung IFN-γ at 24 h post-stroke (**Figure 5.6A**). However, IFN-γ levels in the lung did not increase to the same extent in mice that underwent 60 min tMCAO and treated with α-GalCer, and IFN-γ levels remained similar to that of vehicle-treated 60 min tMCAO-operated mice (**Figure 5.6A**). Despite the lack of change in IFN-γ levels in the lungs of α-GalCer-treated post-stroke mice, we examined whether levels of IL-10 are reduced after α-GalCer treatment in post-stroke mice to possibly explain the decreased infection seen in this cohort. IL-10 levels in the lung increased after stroke in vehicle treated mice that underwent 60 min tMCAO when compared to sham-operated mice treated with vehicle (**Figure 5.6B**). However, α-GalCer treatment after stroke could prevent this

increase as lung IL-10 was significantly lower between vehicle and α-GalCer treatment in mice that underwent 60 min tMCAO (**Figure 5.6B**). It is important to note that lungs were not perfused prior to cytokine analysis, and therefore the cytokine levels presented here may be a representation of both systemic and lung cytokine levels. Indeed, was there a dramatic increase in serum IFN-γ following α-GalCer treatment in sham-operated animals at 8 h post-surgery compared to its vehicle counterpart (**Figure 5.6C**). However, while the extent of this increase was significantly lower in α-GalCer-treated stroke mice compared to α-GalCer-treated sham-operated animals, serum IFN-γ remained significantly elevated in comparison to vehicle-treated mice that underwent 60 min tMCAO (**Figure 5.6C**). These findings were reflected at 24 h post-stroke as serum IFN-γ remained elevated in α-GalCer-treated mice that underwent sham surgery, but to a lesser degree in α-GalCer treated mice that underwent 60 min tMCAO (**Figure 5.6D**). As such, our data suggests that α-GalCer treatment can reduce the occurrence of post-stroke lung infection, potentially via inducing rapid and systemic production of IFN-γ and reducing pulmonary IL-10.

The protection against lung infection conferred by α-GalCer is not seen in iNKT cell deficient mice

To confirm that the protective effects of α-GalCer is dependent on iNKT cells, we induced 60 min tMCAO in Jα18 deficient mice and treated with α-GalCer or vehicle. α-GalCer could not reduce bacterial load in the lung after 60 min tMCAO (**Figure 5.7A**). Furthermore, α-GalCer did not improve the stroke-associated characteristics of immunosuppression as there were no changes to spleen weights (**Figure 5.7B**) or circulating leukocyte counts (**Figure 5.7C**) between vehicle-treated and α-GalCer-treated Jα18 deficient mice. This data suggests that α-GalCer reduces post-stroke lung infection by activating iNKT cells.

Analogues of α-GalCer induce rapid antibacterial immunity

Previous studies with α-GalCer dissuade from its use due to its conflict in inducing both Th1 and Th2 immune responses. To overcome this conflicting role, we synthesised two analogues of α-GalCer, DB06-9 and SKC8-27 (**Figure 5.1**) and assessed their capacity to induce antibacterial immunity. Here we hypothesise that DB06-9 and SKC8-27 would induce differing cytokine profiles due to the difference in structure. Within 6 h of administration into naïve mice, DB06-9, and SKC8-27 increased production of IFN-γ in the lung (**Figure 5.8A**) and sera (**Figure 5.8B**) comparable to that in mice treated with α-GalCer. With a strong capability to induce IFN-γ in naïve mice, α-GalCer and analogues were next administered into mice following 2 h of pre-established *E. coli* infection. By 6 h following glycolipid or vehicle injection (i.e. 8 h post-infection with *E. coli*), mice treated with DB06-9 or SKC8-27 were equally effective as α-GalCer in reducing *E. coli* from the spleen (**Figure 5.8C**). It should be noted that another well-reported drawback of using α-GalCer is liver injury [113]. We believe that the side effects of α-GalCer may be avoided while still maintaining antibacterial effects by using α-GalCer analogues. At 8 h following injection of α-GalCer, DB06-9 or SKC8-27, the extent of liver toxicity (as measured by sera ALT) remained at baseline levels (**Figure 5.8D**). By 24 h following α-GalCer treatment in naïve mice, serum ALT was significantly elevated in comparison to naïve and vehicle-treated mice (**Figure 5.8E**). Liver toxicity was also evident in mice treated with DB06-9 and SKC8-27 as serum ALT was similar to that in α-GalCer treated animals (**Figure 5.8E**). Nonetheless, these experiments show that the analogues DB06-9 and SKC8-27 are as effective as α-GalCer in inducing antibacterial immunity in naïve animals.

Analogues of α-GalCer do not reduce post-stroke infection

We next tested the efficacy of α-GalCer analogues in reducing infection in post-stroke animals. At 2 h following surgery, post-stroke mice were treated with either vehicle, DB06-9

or SKC8-27 and bacterial load at 24 h was assessed (**Figure 5.9A**). Interestingly, vehicle treated post-stroke mice did not acquire infection to the extent of previous experiments (as indicated by dotted line) (**Figure 5.9B**). As a result, there was no difference in bacterial load in the lungs post-stroke mice treated with analogue DB06-9 or SKC8-27 compared to vehicle-treated stroke mice (**Figure 5.9B**). In mice treated with vehicle, spleen weights from post-stroke mice did not change in comparison to its sham counterpart (**Figure 5.9C**). Treatment of post-stroke mice with DB06-9 had no impact on spleen size compared to post-stroke vehicle-treated mice (**Figure 5.9C**). However, while spleen weight decreased after stroke in SKC8-27-treated mice, this was still significantly greater in comparison to post-stroke mice treated with vehicle (**Figure 5.9C**). Circulating leukocyte numbers decreased after stroke in vehicle-treated mice compared to vehicle-treated mice that underwent sham surgery (**Figure 5.9D**). Furthermore, treatment with either analogue DB06-9 or SKC8-27 after stroke also had decreased circulating leukocyte levels in respect to vehicle-treated post-stroke mice (**Figure 5.9D**). To ensure that infarct volume was not exacerbated by analogue treatment after stroke, brain infarct volume was measured in post-stroke mice. Brain infarct volume was unchanged by DB06-9 or SKC8-27 treatment after stroke when compared to vehicle treatment (**Figure 5.9E**). Furthermore, we monitored the development of brain oedema using MRI over 4 days in post-stroke mice treated with vehicle, DB06-9, or SKC8-27 (**Figure 5.10**). We showed that DB06-9 or SKC8-27 treatment did not delay the resolution of brain oedema in the first 4 days following stroke (**Figure 5.10B** and **C**). Overall, we show that both DB06-9 and SKC8-27 could not reduce post-stroke lung infection. However, it should be noted that the presence of bacteria in the lung in vehicle-treated post-stroke mice was lower in these experiments than expected.

Infection challenge after stroke

Due to the low presence of lung bacteria in vehicle-treated post-stroke mice in the experiments described above (**Figure 5.9B**), we attempted to mimic post-stroke infection by injecting bacteria after surgery. Before using the analogues, we first tested whether α-GalCer is still effective in reducing bacterial load in this model of post-stroke infection. In a previous study, we provide evidence of bacterial dissemination from the gut that peaks approximately 3 h post-stroke [153]. In that study, we demonstrate that the primary source of bacteria in the post-stroke lung originated from the gut [153]. Furthermore, other studies detected the presence of *Escherichia coli* in the lungs and blood of post-stroke mice [125, 158]. Therefore, we administered *E. coli* (a common gut microbe) intraperitoneally at 3 h post-surgery to mimic the dissemination of bacteria from the gut, then evaluated whether α-GalCer treatment at 2 h post-surgery could reduce the presence of bacteria in the spleen and lung (**Figure 5.11A**). While bacteria counts were higher in the spleen of sham-operated mice treated with α-GalCer compared to vehicle, it was no different to stroke mice after α-GalCer treatment (**Figure 5.11B**) Similarly, in the lung, sham-operated mice treated with vehicle had unusually little bacteria, and treatment with α-GalCer could not lower the presence of bacteria in post-stroke mice (**Figure 5.11C**).

Discussion

Infection is a major cause of post-stroke mortality that is largely contributed by a shift in immunity that impairs antibacterial responses [125, 178]. Previous studies from our laboratory demonstrated this shift is due to the ability of hepatic iNKT cells to rapidly respond to distant brain injury [72]. However, since post-stroke infection predominantly occurs in the lung, we hypothesised that lung bacterial defence is compromised by the immunomodulatory effects of pulmonary iNKT cells, resulting in increased lung infection. In this study, our data suggests that there are no discernible changes to iNKT cell number and function in the lung after stroke. Furthermore, susceptibility to post-stroke infection was not exacerbated or reduced in iNKT cell deficient mice, suggesting that iNKT cells do not play a role in post-stroke infection. Despite this, we demonstrate that boosting IFN-γ production by activating iNKT cells with α-GalCer could reduce post-stroke infection. We next showed that the α-GalCer analogues, DB06-9, and SKC8-27, could effectively enhance immunity to combat infection to the same extent as α-GalCer in a simple model of *E. coli* infection. However, neither analogue could reduce lung infection in post-stroke mice as effectively as α-GalCer. As such, this study suggests that enhancing iNKT cell responses using glycolipids may offer an alternative target for therapeutics to reduce post-stroke infection.

Differential roles of lung and liver iNKT cells

To the best of our knowledge, there is limited research that explores the role of iNKT cells in lung immunity after stroke. In this study, while pulmonary iNKT cells had increased expression of the activation marker CD69, this increase was modest, and we could not find any changes to pulmonary iNKT cell number or cytokine production after stroke. It is known that iNKT cells make up 40% of hepatic lymphocytes in mice and are positioned with the capability

of sensing disruptions to homeostasis and respond accordingly [73, 74]. In contrast, iNKT cells only account for approximately 5% of resident lymphocytes in the lung [73, 171, 179]. Therefore, it is somewhat unsurprising that we found that pulmonary iNKT cells only play a limited role in lung immunity during post-stroke infection. We suggest that hepatic iNKT cells, rather than pulmonary iNKT cells, are primarily responsible for systemic changes in immunity after stroke. Indeed, a previous study demonstrated that a single, systemic dose of α-GalCer promoted lymphocyte expansion in the liver over a 7-day period, while thymic and splenic lymphocyte cell numbers remained unchanged [180], showing that the majority of immune changes following α-GalCer treatment occur within the liver. This is further supported by our previous study that showed regional specific activation of iNKT cells that responds to brain injury by systemically dampening immunity via the release of IL-10 [72].

In this study, Jα18 deficient mice (on a C57Bl/6 background) were not protected nor at greater risk to post-stroke infection compared to wildtype mice. However, a previous study from our laboratory demonstrated that CD1d deficient mice (on a BALB/c background) that are deficient in all NKT cells had higher post-stroke mortality [72]. This may suggest that variant NKT cells (type II NKT cells) play a larger role in post-stroke infection, while iNKT cell play a secondary role. Additionally, the discrepancies in findings may also be due to background strain differences between these studies. It is well documented that BALB/c mice are more susceptible to infectious complications compared to mice on a C57Bl/6 background [110]. It was later determined that BALB/c mice have a Th2-dominant immunity, while C57Bl/6 mice have a Th1-dominant immunity, meaning that BALB/c mice are less capable of combatting infection compared to C57Bl/6 mice [111, 112]. In NKT deficient mice on a BALB/c background, the high mortality after stroke suggests these mice had further impaired antibacterial immunity and succumbed to post-stroke infection. To support this, the use of antibiotic prophylaxis could improve the survival rate of NKT cell deficient BALB/c mice [72].

On the other hand, iNKT cell deficiency in C57Bl/6 mice used in the current study had no mortality nor differences in bacterial load in the lung. This suggest that C57Bl/6 mice are still capable of combatting post-stroke lung infection even in the absence of iNKT cells and therefore have a secondary role.

While iNKT cells are clearly more abundant in the liver compared to the lung, the composition of iNKT cell subsets in these organs also differ. There are currently five iNKT cell subsets that are defined by their expression of various transcription factors and cytokines: NKT1 cells express high amounts of T-bet and secrete high amounts of IFN-γ and IL-4 upon TCR stimulation; NKT2 cells express promyelocytic leukemia zinc finger protein (PLZF) and release IL-4 and IL-13; NKT 17 cells express retinoid-related orphan receptor gamma t (RORγt) and release IL-17; follicular helper NKT (NKT_{FH}) cells express B cell lymphoma 6 protein (BCL-6) and release IL-21; and NKT10 cells that express E4 promoter-binding protein 4 (E4BP4) and release IL-10 [73]. In the liver, the populations of iNKT cells are comprised of predominantly NKT1 cells, whereas the lung iNKT cell populations are made up of NKT1and enriched with NKT2 and NKT17 cells [179, 181, 182]. Therefore, there may be a tissue-specific difference in iNKT cell responses following α-GalCer stimulation. In this study, while no changes to pulmonary iNKT cell numbers were observed, we did not measure the proportions of different iNKT cell subsets in the lung. We hypothesise that the proportion of NKT1 cells decreases while an increase in NKT2 cells occurs in the lungs after stroke, shifting pulmonary immunity toward a Th2-skewed response and therefore increasing susceptibility to infection. Future studies to differentiate and quantify pulmonary iNKT cell subsets may shed light on the immune environment in the lung after stroke.

In this study, α-GalCer treatment successfully reduced the bacterial load in the lung following stroke. We suggest that the systemic antibacterial immunity is provided by hepatic iNKT cells, potentially due to a much higher frequency of iNKT cells in the liver. Additionally,

the hepatic iNKT cell population primarily consists of NKT1 cells [73, 183], and are therefore capable of inducing a potent IFN-γ response that exudes from the liver into the periphery. In support of this, we show that α-GalCer treatment significantly increased both IFN-γ in the lung and sera, however it is unclear if IFN-γ originates from hepatic iNKT cells. Our data suggests that further in-depth tests are required to test whether hepatic iNKT cells are the main producers of systemic IFN-γ that we detected. This can be tested by isolating leukocytes from the liver and lung and comparing IFN-γ production by iNKT cells following *in vitro* α-GalCer stimulation. The findings of this future experiment will reveal if changes to systemic immunity induced by α-GalCer and glycolipid-based therapies are predominately dictated by hepatic iNKT cells.

Evaluation of DB06-9 and SKC8-27

As stroke outcomes can be dependent on immune or inflammatory responses, and iNKT cells have been shown to control post-stroke infection, modulating their responses with a highly specific activator could be a potential therapeutic. Targeting iNKT cells through α-GalCer is an attractive option due to the specificity of α-GalCer to activate iNKT cell, and the ability of iNKT cells to induce various types of immune responses [76]. However, while the effects of α-GalCer are well-documented to induce production of large quantities of IFN-γ and IL-4, this consequently induces conflicting immune profiles [104]. In our study, α-GalCer was still able to reduce post-stroke lung infection, however we continued to test synthetic analogues of α-GalCer in an attempt to find a compound with more distinct immunological effects. Similar to α-GalCer, we found that the two analogues, DB06-9 and SKC8-27, also successfully induced an IFN-γ response and antibacterial effects when naïve mice were challenged with *E. coli*. Surprisingly, DB06-9 and SKC8-27 were unable to confer protection against infection in the context of stroke and suggest that these analogues may induce immune conflicting cytokines,

such as IL-4, but this was not assessed in our study. This emphasises the need for further research into the mechanisms behind the polarisation of iNKT cells by glycolipids to facilitate the development of novel therapeutics with targeted effects. Earlier work indicates that the polarisation of iNKT cell responses involve the binding avidity between the CD1d-glycolipid-TCR complex of the antigen presenting cell and iNKT cell, respectively [97-99]. However, current consensus behind iNKT cell polarity is the location of antigen loading and association of lipid rafts with CD1d [94, 102]. Intracellular loading of glycolipid onto CD1d and presentation with the localisation of cholesterol-rich lipid rafts to the plasma membrane near the CD1d-glycolipid complex has been strongly associated to induce a greater Th1 response [102]. Conversely, rapid and direct loading of glycolipid onto CD1d without the requirement of intracellular loading and the absence of lipid raft association to the CD1d-glycolipid complex has been better associated with a Th2-skewed immune response. Indeed, a previous study showed that SKC8-27 had relatively similar lipid raft association to α-GalCer, which induced robust IFN-γ and IL-4 production [102], while little is known about DB06-9. Overall, mechanisms to iNKT cell polarisation by glycolipids require further elucidation to identify potential glycolipids with more precise immunological effects.

It should be noted that the vehicle-treated post-stroke mice had lower infection rates (compared to previous experiments) when testing our analogues, which may have impacted our statistical analysis. Due to this, we attempted to model post-stroke infection by injecting *E. coli* to more effectively test the capacity of α-GalCer analogues in reducing post-stroke infection. We previously demonstrated a breakdown in gut barrier integrity by 3 h following stroke, allowing for translocation of gut microbes into peripheral organs such as the lungs [153]. Therefore, as a preliminary study, post-stroke mice were infected with *E. coli* (a common gut microbe) interperitoneally at the timepoint that gut barriers are compromised (3 h post-stroke) and then treated with α-GalCer. Unexpectantly, sham-operated animals infected with

E. coli and treated with α-GalCer had significantly higher bacterial load in the spleen compared to vehicle-treated counterpart. Furthermore, in post-stroke mice treated with *E. coli*, α-GalCer was unable to lower bacterial load in the lungs at 7 h post-stroke, which conflicts with previous results showing α-GalCer could reduce bacterial load in the lung at 24 h after stroke. This finding may be due to the temporal cytokine production profile of iNKT cells when activated by α-GalCer. While IFN-γ and IL-4 are known to be produced following iNKT cell stimulation with α-GalCer, their release occurs at differing timepoints. Peak IL-4 is detected at 1.5 h, while peak IFN-γ occurs at 18 h following α-GalCer treatment in mice [100, 113]. Therefore, the 5 h timepoint following α-GalCer (7 h post-stroke) may be too acute for an effective IFN-γ response to clear *E. coli* infection following stroke. Therefore, future experiments to test whether α-GalCer can reduce this model of post-stroke *E. coli* infection will be assessed at the 24 h timepoint. If this is achieved, next steps would involve treating with DB06-9 and SKC8-27 using this model to give a better indication of the efficacy of these analogues to treat post-stroke infection.

Another factor that requires consideration in our study is the pathogenic organism in post-stroke infection. The use of *E. coli* in this experiment is further justified by other studies that found *E. coli* in blood and lung cultures from post-stroke mice [125, 158]. However, using *E. coli* in the present study and in future studies will prompt an *E.coli*-specific immune response that may not be mediated by iNKT cells. Furthermore, *E. coli* infection may not be representative in patients as the infective organism in post-stroke lung infection is unknown. This is especially evident as several studies could not identify the causative organism in a large portion of patients with stroke diagnosed with pneumonia [140, 166, 184]. Indeed, no one organism is solely responsible for pneumonia and can be caused by a number of microbes including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and even various viruses [185]. Additionally, findings from our previous study demonstrate that the

causative agents of post-stroke bacterial pneumonia may be contributed by microbes from the gut as more than 70% of detected microbes in infected patients with stroke were known to be commensal bacteria that commonly reside in the gut, including *Enterococcus* spp and *Morganella morganii* [153]. Therefore, inoculation of post-stroke mice with other microbes that are associated with pneumonia should be considered when testing the efficacy of glycolipid-based therapies.

Roadblocks to translation of glycolipid-based therapies

There is a multitude of issues that impede the translation of α-GalCer or glycolipid-based therapies into the clinic. These include 1) liver toxicity, 2) difference in the abundance of iNKT cells between mice and humans, or amongst humans and 3) longevity of iNKT cell responses after glycolipid treatment. Firstly, α-GalCer is known to cause liver toxicity in mice [113]. Indeed, our analogues cause similar levels of liver injury as measured by serum ALT levels in glycolipid-treated mice. The mechanisms of α-GalCer-induced liver injury involves uptake of free α-GalCer and presentation by hepatic $CD31^+$ endothelial cells to iNKT cells [186, 187]. This induces production of TNF-α by hepatic lymphocytes and upregulate expression of Fas ligand by iNKT cells, causing hepatic injury [113, 186, 187]. A method to avoid liver toxicity is by pulsing antigen presenting cells with α-GalCer. One study showed that administering dendritic cells loaded with α-GalCer compared to free α-GalCer could prevent lethal liver injury in mice [180, 186]. However, iNKT cells are more abundant in mice where they constitute up to 40% of hepatic lymphocytes and 2% of circulating lymphocytes, while humans have considerably fewer iNKT cells where they account for up to 5% of hepatic lymphocytes and only 0.5% of lymphocytes in circulation [73, 188, 189]. Therefore, the occurrence of α-GalCer-induced toxicity in humans has been reported to be minimal [190]. There is little known about the relative abundance of iNKT cells within non-human primates,

however the abundance of iNKT cells in F344 inbred rats are reported to be more reflective of humans compared to that of mice [191, 192].

In contrast, while the low abundance of iNKT cells may be optimal to prevent liver toxicity, the lower number of iNKT cells in humans may also negatively impact on the effectiveness of glycolipid therapy. This was especially notable in cancer patients where there is evidence suggesting these patients had a lower abundance of circulating iNKT cells and a relatively weaker immunological response to α-GalCer [190]. In the case of patients with stroke, we previously showed that the number of circulating iNKT cells did not change over the course of 90 days following stroke compared to both hospital and healthy human controls [109]. Due to this, the likelihood of patients with stroke that fail to respond glycolipid therapy is less likely and therefore may still be a viable option to treat post-stroke infection.

Lastly, while the administration of α-GalCer induces rapid (within hours) and robust iNKT cell activation, it is followed by downregulation of TCR [193, 194] and therefore iNKT cells become less responsive to further TCR stimulation by α-GalCer [195]. This is followed by apoptosis of a large portion of iNKT cells by 10 days, while the remainder of iNKT cells display an anergic phenotype that persists for one month after initial stimulation [196]. This makes repeated doses of α-GalCer less effective. While this may be a hindrance in diseases such as cancer, it may be desirable in the case of combatting post-stroke infection. It is crucial to ensure the immune enhancement after glycolipid treatment is not prolonged to reduce the risk of exacerbating brain injury following stroke. Furthermore, post-stroke immunosuppression is thought to be the body's natural response to prevent the development of autoimmune responses to central nervous system (CNS) antigens (such as myelin basic protein and glial fibrillary acidic protein) that leak into the periphery after stroke [197, 198]. Therefore, caution must be taken when considering stimulating immunity using α-GalCer based therapies as this may increase the risk of developing autoimmunity to CNS antigens.

In this study, while we demonstrate that pulmonary iNKT cells do not alter lung immunity or post-stroke lung infection, the iNKT cells can still be activated with α-GalCer to invoke an immune response capable of reducing lung infection. We demonstrated that the α-GalCer analogues DB06-9 and SKC8-27 could induce a strong IFN-γ response and antibacterial immunity that is on par with α-GalCer, although neither analogue could reduce post-stroke lung infection in mice. Importantly, we also ensure that treatment with these analogues do not cause further ischemic brain damage over a longer period. Ultimately, this study suggests that targeting iNKT cells to reduce post-stroke infection remains an attractive therapeutic avenue that merits further investigation. As detailed throughout this chapter, further studies should explore 1) changes to iNKT cell subsets in the lung after stroke, 2) the differences in response to α-GalCer between pulmonary iNKT cells and hepatic iNKT cells, 3) the mechanisms of iNKT cell polarisation by α-GalCer analogues, and 4) the causative microbes in post-stroke infection.

Figures

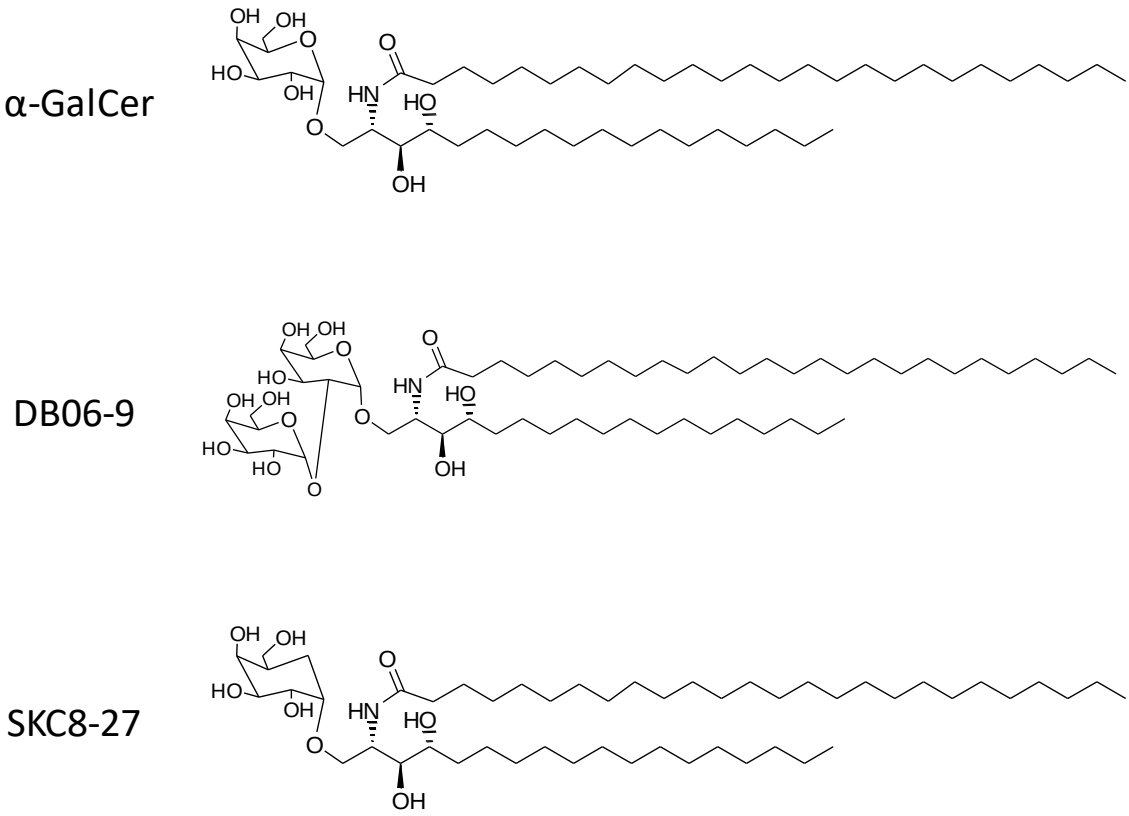


Figure 5.1 The molecular structures of α-GalCer, DB06-9 and SKC8-27.

α-GalCer (top) is made up of a sugar group that is glycosidically bound to a 18 carbon (C) sphingosine base chain and a 26C fatty acyl chain. The analogues DB06-9 (middle) differs by an addition of another sugar group, while SKC8-27 (bottom) differs by the substitution of the oxygen (O) to C in the sugar group. Analogues were synthesised and provided by Steven A. Porcelli (Albert Einstein College of Medicine, USA)

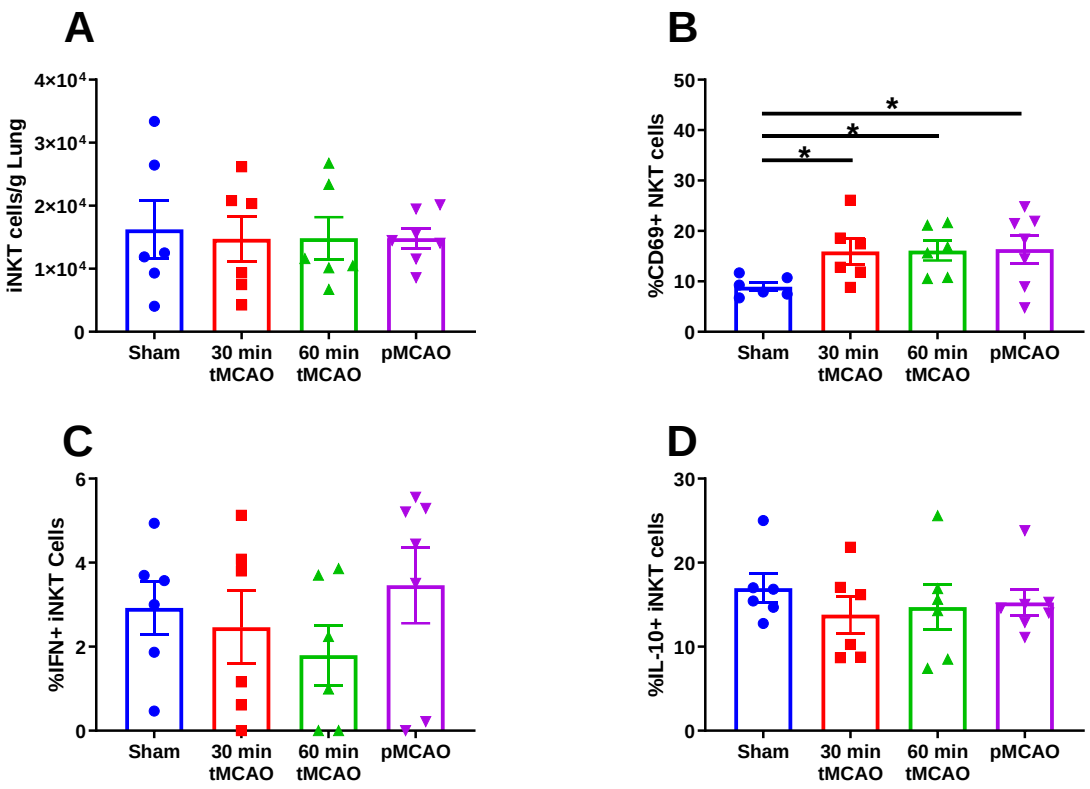


Figure 5.2 Pulmonary iNKT cells remain unchanged after stroke.

Lungs from post-surgery mice were dissected out and processed for flow cytometry and iNKT cell number (A) and presence of CD69 (B) were quantified. Intracellular IFN-γ (C) and IL-10 (D) production from pulmonary iNKT cells were also measured. Data are presented as mean ± SEM, where n≤6, *p<0.05, one-way ANOVA with Holm-Sidak's multiple comparisons test.

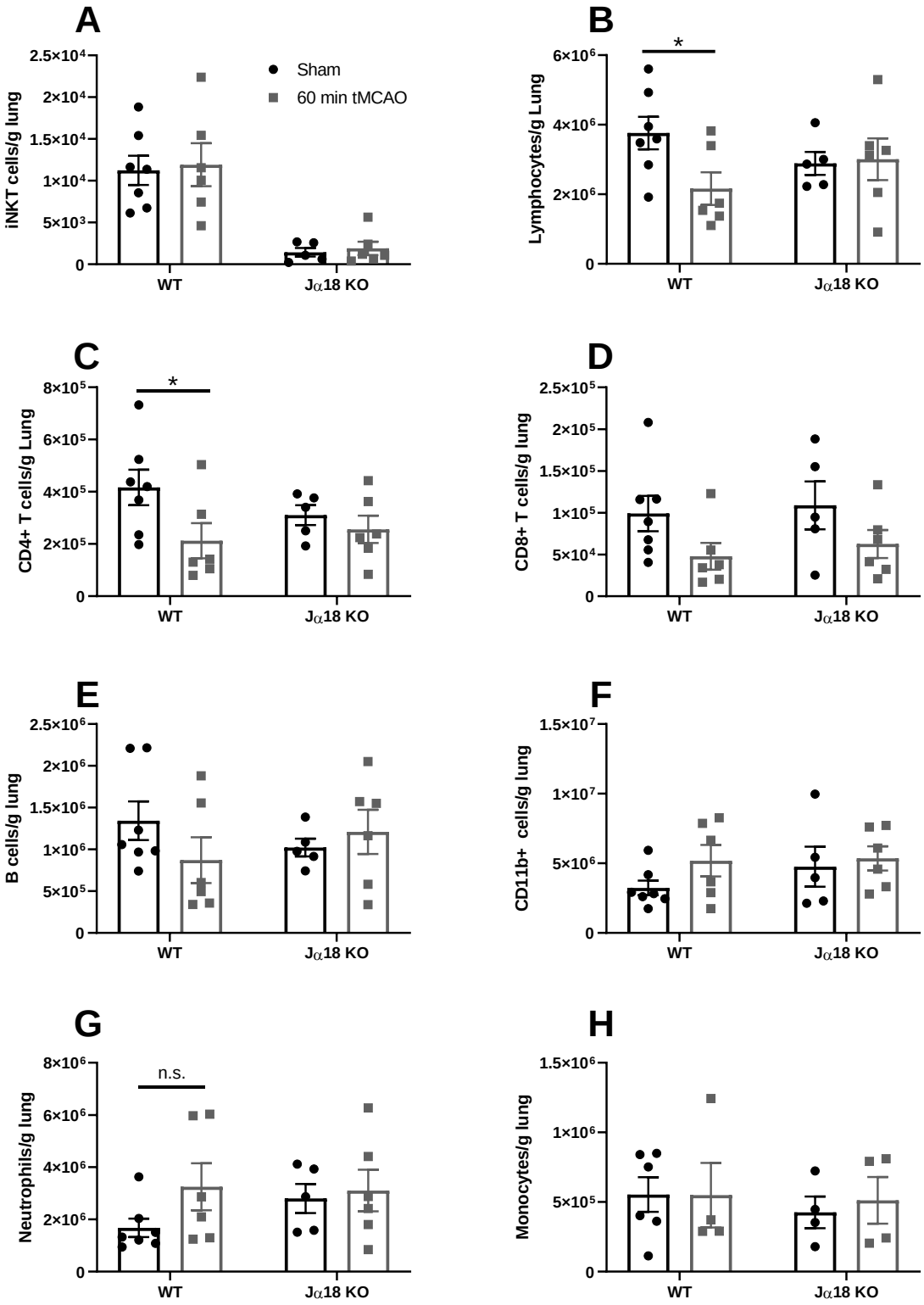

A
iNKT cells/g lung
2.5×10⁴
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
Sham
60 min tMCAO
WT
Jα18 KO
B
Lymphocytes/g Lung
6×10⁶
4×10⁶
2×10⁶
0
*
WT
Jα18 KO
C
CD4+ T cells/g Lung
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
*
WT
Jα18 KO
D
CD8+ T cells/g lung
2.5×10⁵
2×10⁵
1.5×10⁵
1×10⁵
5×10⁴
0
WT
Jα18 KO
E
B cells/g lung
2.5×10⁶
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
WT
Jα18 KO
F
CD11b+ cells/g lung
1.5×10⁷
1×10⁷
5×10⁶
0
WT
Jα18 KO
G
Neutrophils/g lung
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
n.s.
WT
Jα18 KO
H
Monocytes/g lung
1.5×10⁶
1×10⁶
5×10⁵
0
WT
Jα18 KO

Figure 5.3 iNKT cell deficiency preserves CD4+ T cell populations in the lung after stroke.

Sham or stroke (60 min tMCAO) surgery was performed in wildtype (WT) or iNKT cell deficient mice (Jα18 KO). Lungs from post-surgery mice were processed flow cytometric analysis for iNKT cells (A) and lymphocytes (B), including CD4+ T cells (C), CD8+ T cells (D) and B cells (E). Pulmonary myeloid cell populations including CD11b+ cells (F), neutrophils (G) and monocytes (H) were also quantified. Data are presented as mean ± SEM, where n<6, n.s. denotes no statistical significance, *p<0.05, **p<0.01, one-way ANOVA with Holm-Sidak's multiple comparisons test.

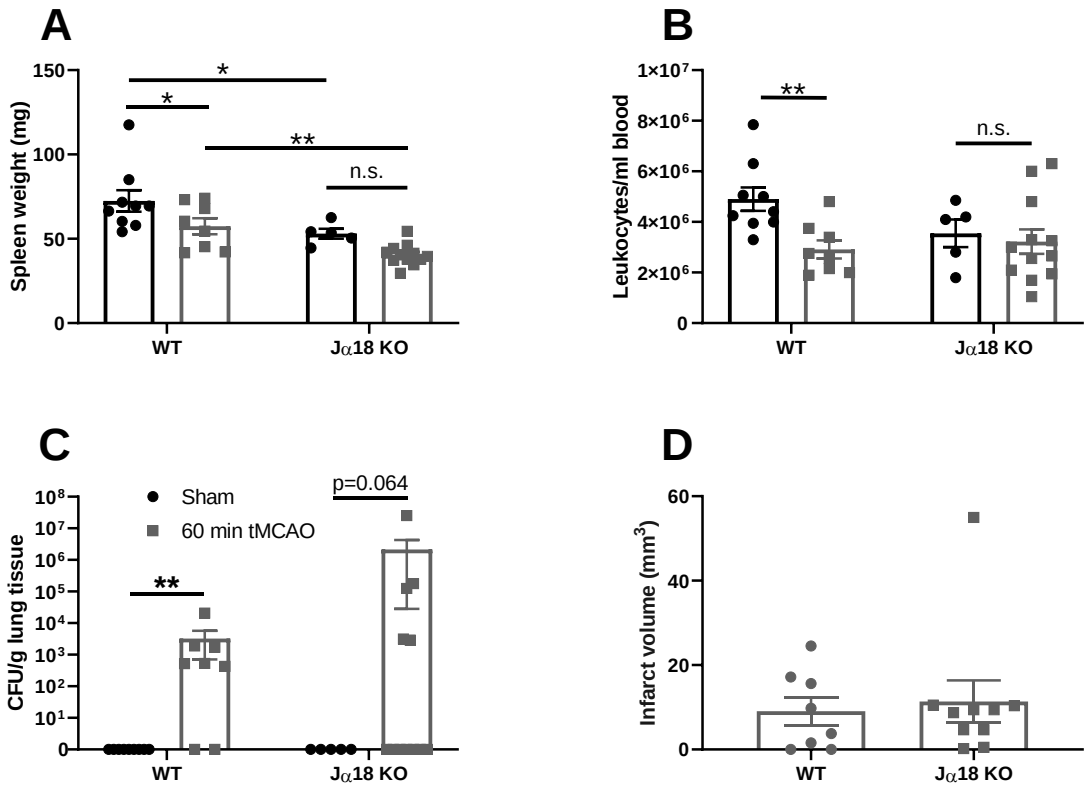


Figure 5.4 iNKT cell deficient mice are neither protected or more prone to post-stroke lung infection.

Sham or stroke (60 min tMCAO) surgery was performed in wildtype (WT) or iNKT cell deficient mice (Jα18 KO) and euthanised at 24 h post-surgery. Spleens were weighed (A) and circulating leukocyte counts were enumerated (B) to observe signs of post-stroke immunosuppression. The lung was taken for bacteriological analysis (C) and brains were processed, sectioned and stained with 0.01% thionin to visualise infarct volume (D). Data are presented as mean ± SEM, where n=5-9 for sham-operated mice and n=8-12 for stroke-operated mice, n.s. denotes no statistical significance, *p<0.05, **p<0.01 using a Kruskal-Wallice test with Dunn's multiple comparisons for bacteriological analysis data or a one-way ANOVA with Holm-Sidak's multiple comparisons test for normally distributed data.

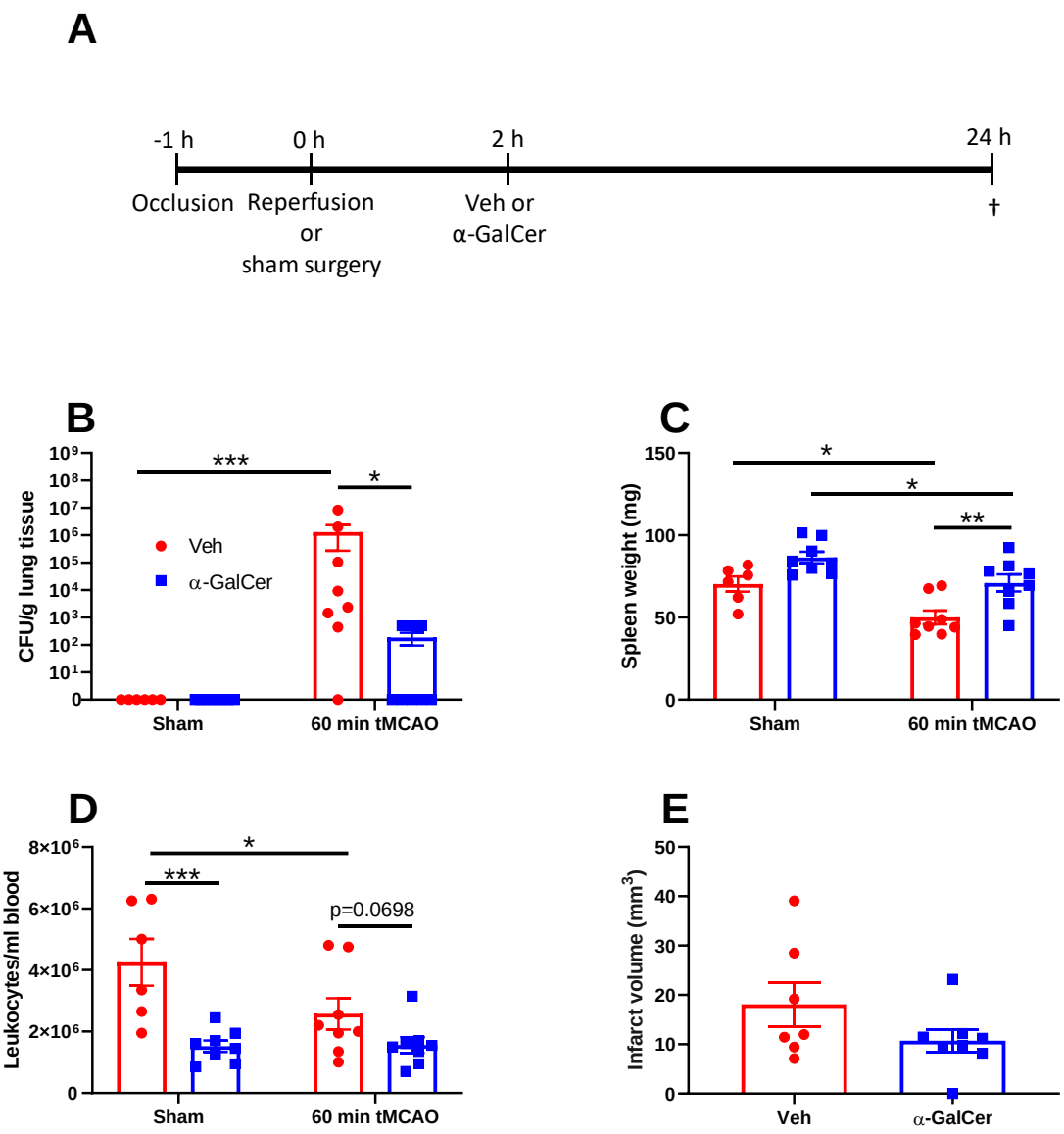

A
-1 h
0 h
2 h
24 h
Occlusion
Reperfusion or sham surgery
Veh or α-GalCer
†
B
CFU/g lung tissue
Veh
α-GalCer
***
*
Sham
60 min tMCAO
C
Spleen weight (mg)
*
*
**
Sham
60 min tMCAO
D
Leukocytes/ml blood
*
***
p=0.0698
Sham
60 min tMCAO
E
Infarct volume (mm3)
Veh
α-GalCer

Figure 5.5 Treatment with α-GalCer reduces post-stroke lung infection.

Mice underwent sham or stroke (60 min tMCAO) surgery and were injected with vehicle (0.5% DMSO and 0.5% Tween-20 in saline) or 2 μg of α-GalCer (in 0.5% DMSO and 0.5% Tween-20 in saline) at 2 h post-surgery and subsequently euthanised at 24 h (A). Lungs were taken for bacteriological analysis (B), spleens were weighed (C) and circulating leukocyte numbers were enumerated (D). Brains of post-stroke animals were also taken to quantify brain infarct (E). Data are presented as mean ± SEM, where n=6-8, n.s. denotes no statistical significance, *p<0.05, **p<0.01, ***p<0.001 using a Kruskal-Wallice test with Dunn's multiple comparisons between groups in bacteriological analysis or one-way ANOVA with Holm-Sidak's multiple comparisons test for normally distributed data.

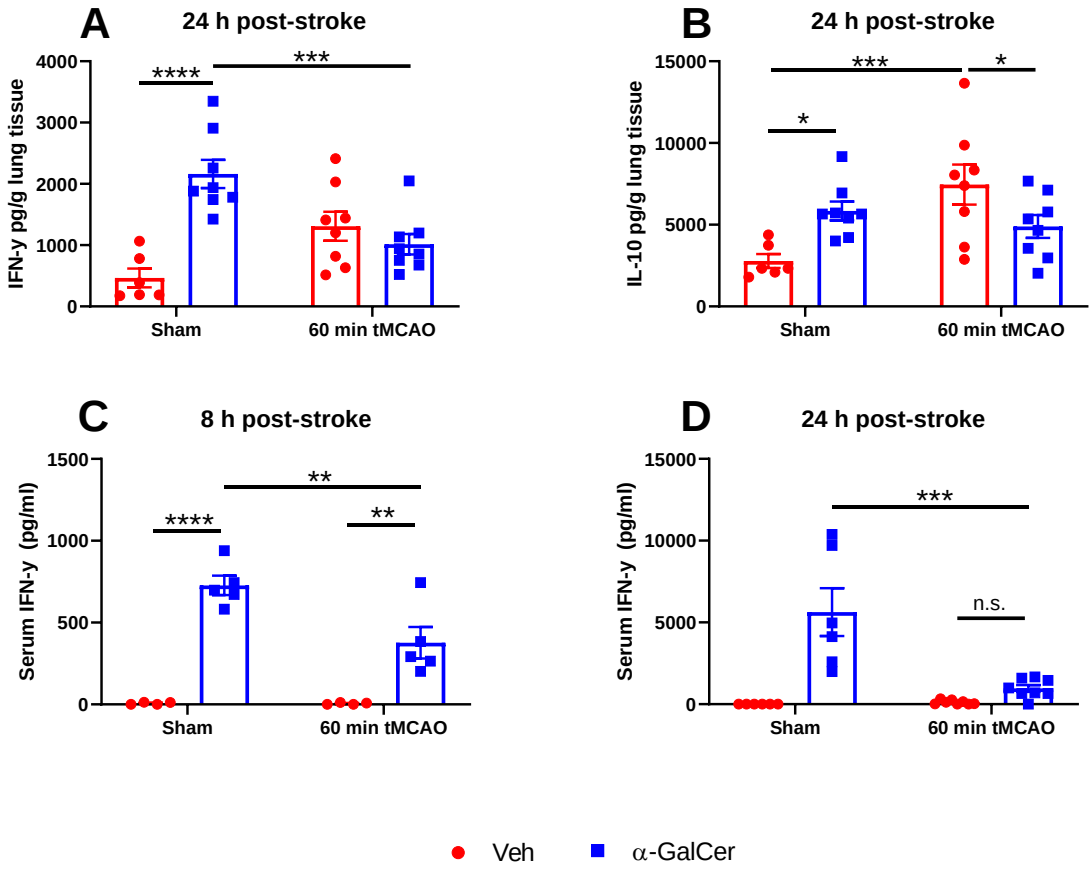


Figure 5.6 α-GalCer treatment enhances systemic IFN-γ production and lowers lung IL-10

Mice underwent sham or stroke (60 min tMCAO) surgery and injected with vehicle (0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of α-GalCer (in 0.5% DMSO and 0.5% Tween-20 in saline) at 2 h post-surgery and euthanised at 8 or 24 h. The left lobe of the lung was processed to determine IFN-γ (A) and IL-10 (B) levels at 24 h post-stroke using an ELISA. Serum levels of IFN-γ was also measured at 8 h (C) and 24 h (D) following stroke. Data are presented as mean ± SEM, where n=5 for 8 h timepoints and n>6 for 24 h timepoints, n.s. denotes no statistical significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 using a one-way ANOVA with Holm-Sidak's multiple comparisons test for normally.

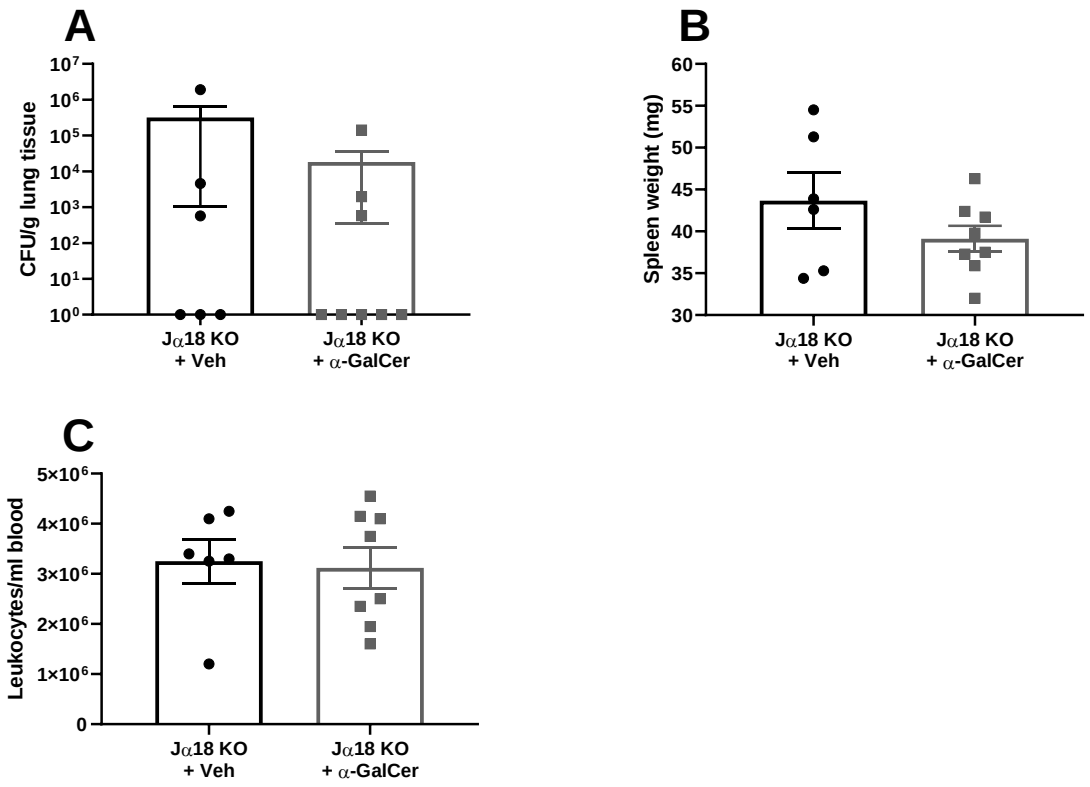


Figure 5.7 α-GalCer treatment does not protect iNKT cell deficient mice from bacterial lung infection.

Mice deficient in iNKT cells (Jα18 KO) mice underwent stroke surgery (60 min tMCAO) and were treated with either vehicle (veh, 0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of α-GalCer (in 0.5% DMSO and 0.5% Tween-20 in saline) at 2 h post-surgery and euthanised at 24 h post-stroke. Lungs were taken for bacteriological analysis (A), spleens weighted (B) and circulating leukocyte numbers enumerated (C). Data are presented as mean ± SEM, where n>5, Kruskal-Wallice test with Dunn's multiple comparisons between groups in bacteriological analysis or one-way ANOVA with Holm-Sidak's multiple comparisons test for normally distributed data.

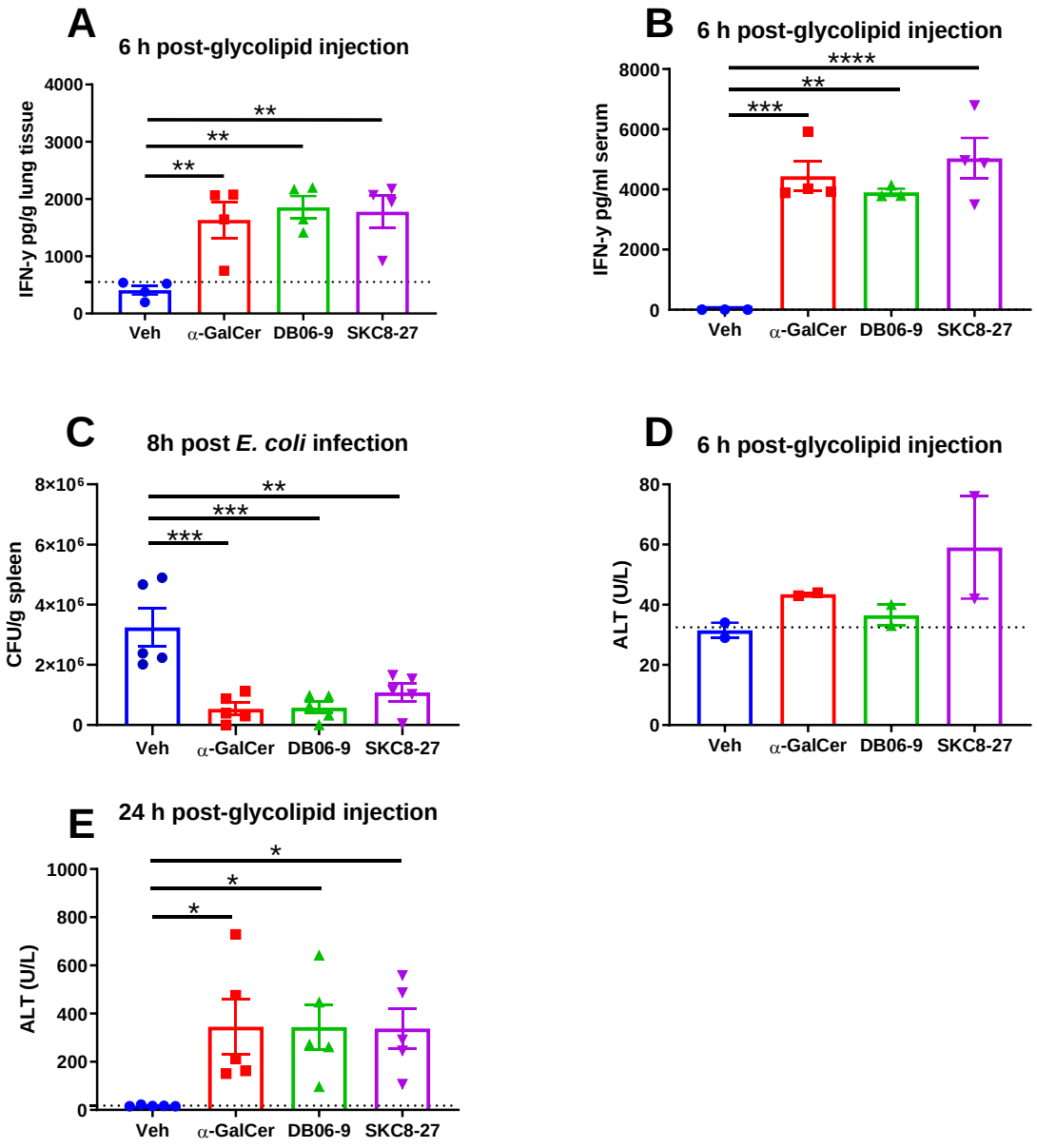

A
6 h post-glycolipid injection
IFN-γ pg/g lung tissue
4000
3000
2000
1000
0
**
**
**
Veh
α-GalCer
DB06-9
SKC8-27
B
6 h post-glycolipid injection
IFN-γ pg/ml serum
8000
6000
4000
2000
0
****
**
***
Veh
α-GalCer
DB06-9
SKC8-27
C
8h post E. coli infection
CFU/g spleen
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
**
***
***
Veh
α-GalCer
DB06-9
SKC8-27
D
6 h post-glycolipid injection
ALT (U/L)
80
60
40
20
0
Veh
α-GalCer
DB06-9
SKC8-27
E
24 h post-glycolipid injection
ALT (U/L)
1000
800
600
400
200
0
*
*
*
Veh
α-GalCer
DB06-9
SKC8-27

Figure 5.8 The structural analogues of α-GalCer, DB06-9 and SKC8-27, enhance antibacterial immunity.

Mice were treated with either vehicle (veh, 0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of the glycolipids (in 0.5% DMSO and 0.5% Tween-20 in saline) α-GalCer, DB06-9, or SKC8-27 via i.p. injection. At 6 h post-injection, mice were euthanised via cardiac puncture and lung (A) and serum (B) IFN-γ was measuring using an ELISA. To test the antibacterial effects of α-GalCer and analogues, the same dosage of veh, α-GalCer, DB06-7 or SKC8-27 was administered 2 h following *E. coli* infection. At 8 h following *E. coli* infection, mice were humanely euthanised and the spleen taken for bacteriological analysis (C). To measure liver toxicity, mice were treated with veh, α-GalCer, DB06-9, or SKC8-27 and euthanised at 6 or 24 h later for serum collection. Sera were run to measure the presence of ALT at 6 h (D) or 24 h (E) following glycolipid treatment. Dotted lines represent the level of infection in vehicle-treated stroke mice from Figure 5.5B. Data are presented as mean ± SEM, where n>3, *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallice with Dunn's multiple comparisons test for comparison between groups in bacteriological analyses or one-way ANOVA with Holm-Sidak's multiple comparisons test for normally distributed data.

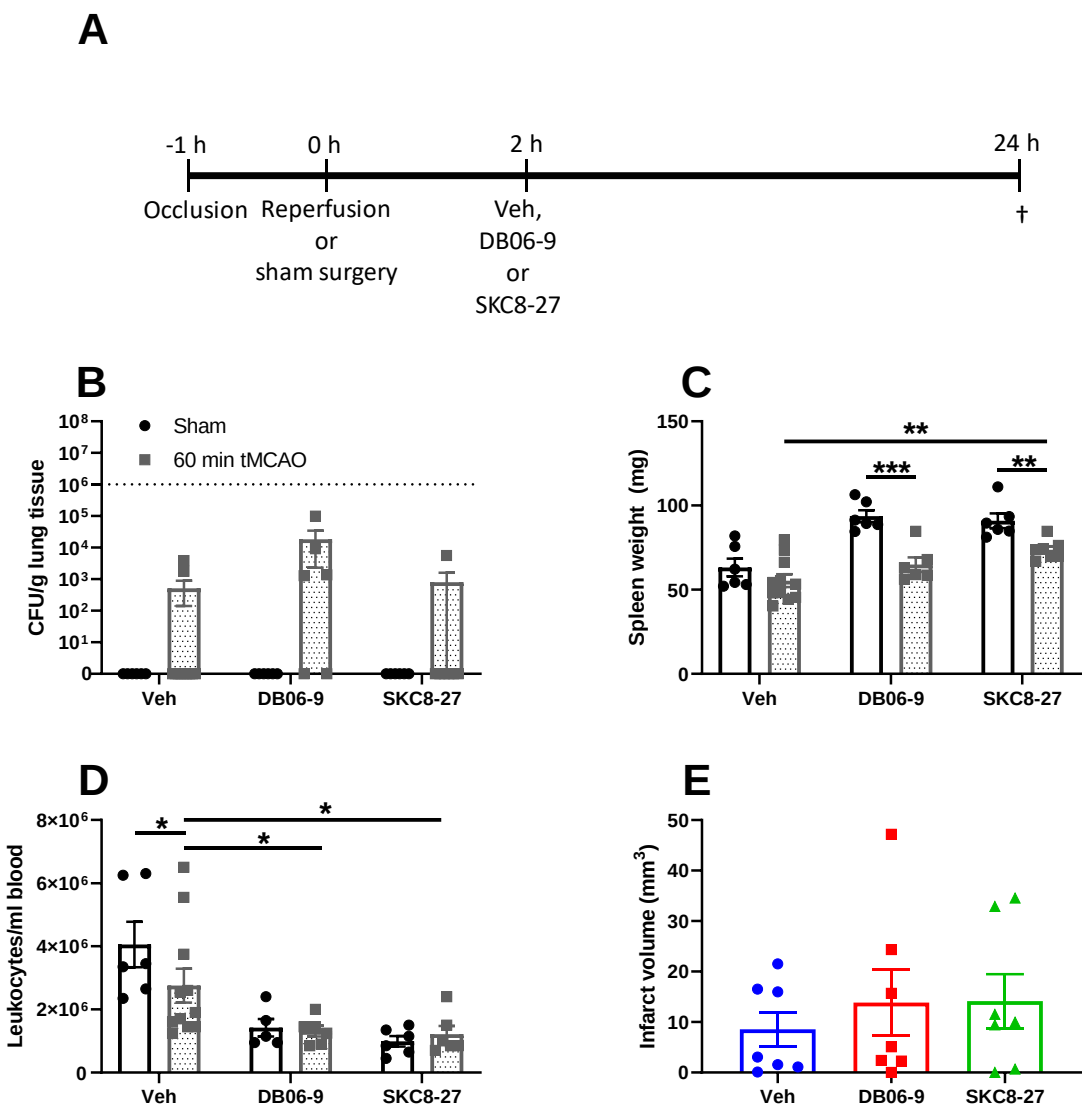
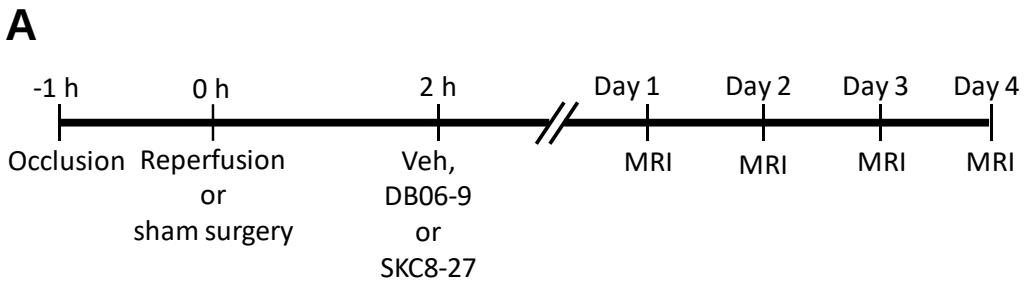
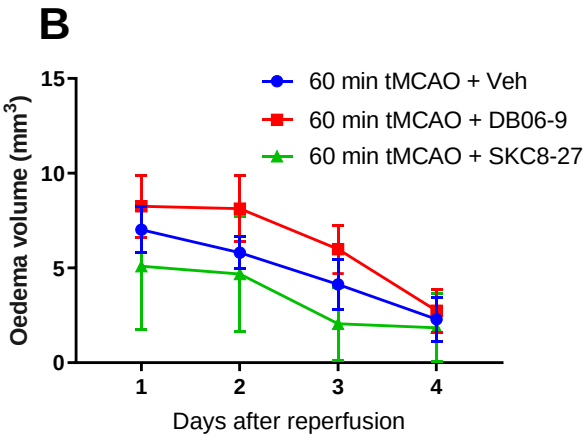

A
-1 h
0 h
2 h
24 h
Occlusion
Reperfusion or sham surgery
Veh, DB06-9 or SKC8-27
†
B
Sham
60 min tMCAO
CFU/g lung tissue
Veh
DB06-9
SKC8-27
C
Spleen weight (mg)
**
***
**
Veh
DB06-9
SKC8-27
D
Leukocytes/ml blood
*
*
*
Veh
DB06-9
SKC8-27
E
Infarct volume (mm3)
Veh
DB06-9
SKC8-27

Figure 5.9 Analogue treatment after stroke does not reduce post-stroke lung infection.

Mice underwent either sham or stroke (60 min tMCAO) surgery and were treated with either vehicle (veh, 0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of the glycolipids (in 0.5% DMSO and 0.5% Tween-20 in saline) DB06-9 or SKC8-27 via i.p. injection at 2 h post-surgery and euthanised at 24 h post-surgery (A). Upon euthanisation, lungs were taken for bacteriological analysis (B), spleens weighed (C), and circulating leukocytes enumerated (D). Brains were slowly frozen over liquid nitrogen, sliced at 30 µm sections and stained with 0.1% thionin to visualise and quantify infarct volume (E). Data are presented as mean ± SEM, where n>6, *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallice with Dunn's multiple comparisons test for comparison between groups in bacteriological analyses or one-way ANOVA with Holm-Sidak's multiple comparisons test for normally distributed data.


A
-1 h
0 h
2 h
Day 1
Day 2
Day 3
Day 4
Occlusion
Reperfusion or sham surgery
Veh, DB06-9 or SKC8-27
MRI
MRI
MRI
MRI


B
60 min tMCAO + Veh
60 min tMCAO + DB06-9
60 min tMCAO + SKC8-27
Oedema volume (mm3)
15
10
5
0
1
2
3
4
Days after reperfusion

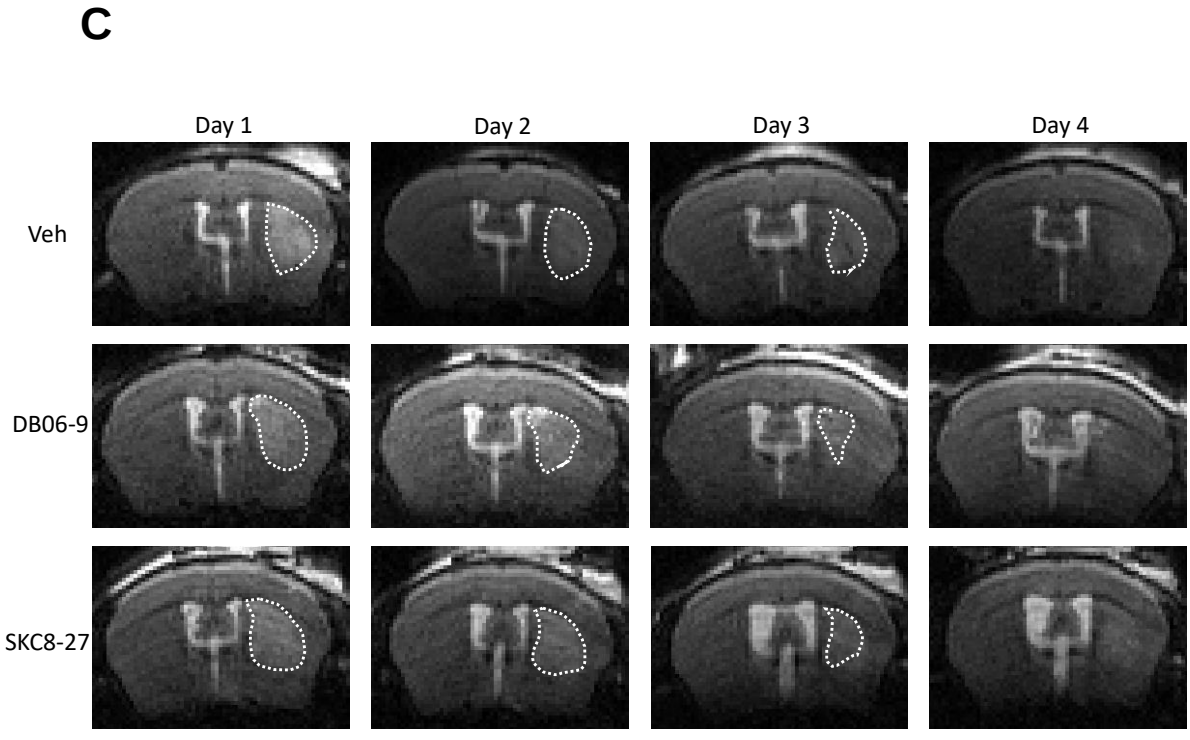

C
Day 1
Day 2
Day 3
Day 4
Veh
DB06-9
SKC8-27

Figure 5.10 Glycolipid treatment after stroke does not alter long-term recovery of cerebral oedema.

Mice underwent either sham or stroke (60 min tMCAO) surgery and were treated with either vehicle (veh, 0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of the glycolipids (in 0.5% DMSO and 0.5% Tween-20 in saline) DB06-9 or SKC8-27 via i.p. injection at 2 h post-surgery and underwent MRI daily for up to 4 days following stroke (A). MRI scans were analysed to determine oedema volume across 4 days (B) and representative images are depicted (C). Dotted lines in MRI scans outline areas of observable oedema. Data are presented as mean ± SEM, where n=4, and comparisons between groups performed using a student's T-test.

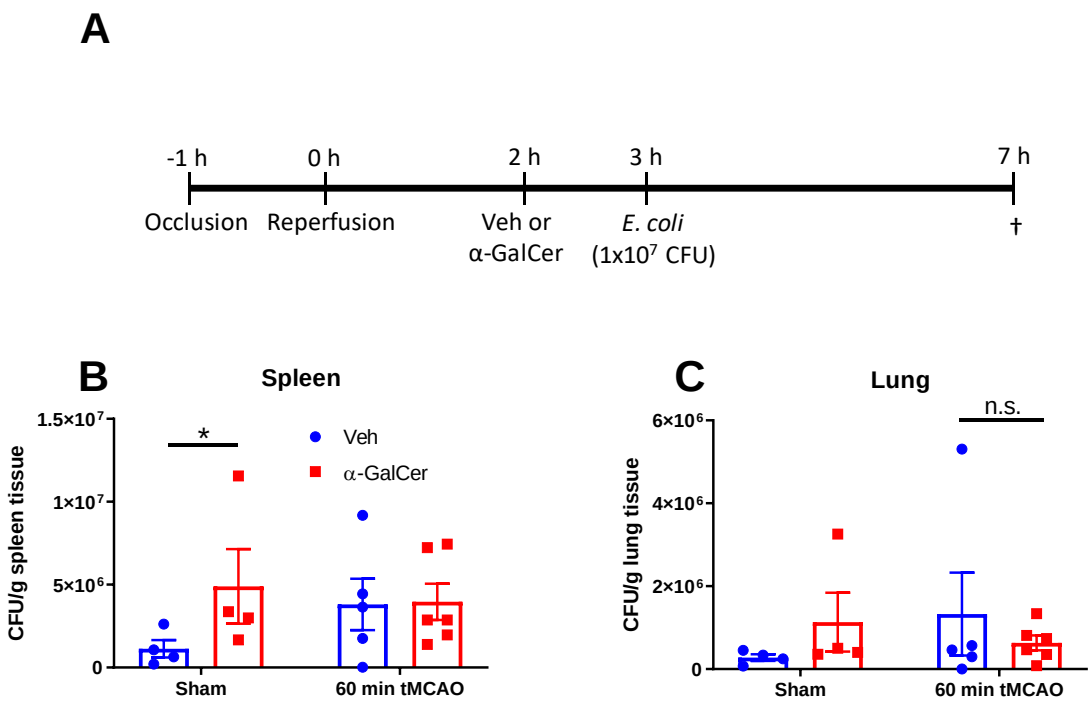


Figure 5.11 Treatment with α-GalCer could not reduce artificial infection at 7 h following stroke.

Mice underwent either sham or stroke (60 min tMCAO) surgery and were treated with either vehicle (veh, 0.5% DMSO and 0.5% Tween-20 in saline) or 2 µg of α-GalCer (in 0.5% DMSO and 0.5% Tween-20 in saline) at 2 h post-surgery, and then 1 × 10^7 CFU of *E. coli* via i.p. injection at 3 h post-surgery and euthanised at 7 h post-stroke (A). Upon euthanisation, bacterial analysis of the spleen (B) and lung (C) was performed. Data are presented as mean ± SEM, where n>4 for sham and n>5 for stroke, n.s. denotes no statistical significance, *p<0.05, Kruskal-Wallice with Dunn's multiple comparisons test for comparison between groups in bacteriological analyses.

Chapter Six

General discussion

For the past two decades, the focus of stroke research is no longer limited to the effect of ischaemic injury on the brain but has expanded to the impact of such brain insult on extra-cerebral cells and tissues. This shift in research efforts is attributed to the growing recognition that patients with stroke develop fatal infections. This observation highlights a notion that the interplay between the nervous system and immune system is disrupted following stroke and renders the host at risk of infections. However, the mechanisms behind this remains unclear. As such, this thesis attempts to address some of these outstanding questions.

Shedding light on the mechanisms to post-stroke infection

The complications that occur after stroke are conventionally associated with neurological deficits and disability. In patients, and particularly those who experienced a greater severity of stroke, neurological deficits may result in complications such as dysphagia and aspiration, and patients may become bedridden [199, 200]. These complications increase the likelihood of entry of bacteria from oral and gastric contents. As a result, the high incidence of post-stroke pneumonia was commonly reported to be contributed by symptoms of dysphagia and aspiration whilst the underlying biological mechanisms were largely overlooked. This led to the speculation that the incidence of post-stroke pneumonia could be reduced by improving oral hygiene or utilising nasogastric tubing (NGT) or mechanical ventilators in an attempt to bypass the swallowing deficiencies in patients [164-166, 201]. However, such interventions were proven ineffective [164] and dysphagia was found not to be an independent predictor for respiratory infections in several studies [140, 202, 203]. Additionally, the use of NGT and mechanical ventilators are even recognised as risk factors to infection during the acute phase following stroke [204-207]. Moreover, some studies identified that aspiration and dysphagia could not fully explain the high occurrence of post-stroke pneumonia, as approximately 50%

of patients with stroke that develop pneumonia were not dysphagic [208], and only 17% of patients with stroke that were dysphagic go on to develop pneumonia [202]. Furthermore, performing dysphagia screen and keeping patients "nil by mouth" (reducing oral intake of foods and medicine) does not prevent the occurrence of post-stroke pneumonia [203]. It is now recognised that the contributing factors that lead to the development of post-stroke pneumonia involve a multitude of mechanisms.

Evidence over the past two decades demonstrates that the increased susceptibility to infection is a result of immune impairment induced by stroke. Characteristics of post-stroke immune alterations are well documented in both clinical and experimental studies, including reduced spleen cellularity, loss of circulating leukocytes, as well as changes to various immune cells around different tissue compartments (as described in Chapter 1). These factors ultimately contribute to an immunosuppressed phenotype where host immune defence is compromised after stroke and becomes more prone to infections. While immunosuppression after stroke is apparent, the mechanisms remain poorly understood. Therefore, this thesis explored and expanded our understanding of the relationship between infarct volume and location on immunity and lung infection, the contribution of the SNS on post-stroke immunosuppression, and whether infection can be reduced by stimulating specific aspects of peripheral immunity. The main findings of this thesis were: 1) increased infarct size promotes greater susceptibility to infection; 2) location of cerebral infarction does not influence the susceptibility to infection; 3) the SNS may be a primary pathway to post-stroke infection but other pathways may have a greater role in severe forms of stroke; and 4) activating iNKT cells using α-GalCer in a therapeutic manner can reduce post-stroke infection.

Complexity of mechanisms behind post-stroke infection and immunosuppression

Stroke severity is well recognised as a major determinant of post-stroke infection, whereby we and others found that greater stroke severity comes an increased risk of acquiring pneumonia [117, 127, 143, 209-211]. A recent systematic review and meta-analysis demonstrated that approximately 1 in 10 patients with stroke will develop pneumonia during hospital care [204]. While it was shown that a severe stroke (as defined by National Institutes of Health Stroke Scale [NIHSS]>14) increases the risk of developing pneumonia to almost 40% [212], the reason for this association remains unclear. There is a plethora of studies that suggest the increased frequency of pneumonia following severe stroke is due to a higher occurrence of dysphagia and aspiration of oral, pharyngeal or gastric contents directly into the lungs [213-217]. As discussed above, these factors do contribute but do not fully explain the high incidence of pneumonia after severe stroke. It is conceivable that the severity of stroke dictates the extent of immunosuppression. Previous findings demonstrate that increased stroke severity exacerbate parameters that indicate suppressed immune function in humans, such as monocytic expression of human leukocyte antigen-DR (HLA-DR) and lipopolysaccharide-induced TNF-α production [143, 210], increased plasma IL-10 [109], and decreased plasma IgM and IgG concentration[127, 218]. Data from this thesis are consistent with findings that show the loss of circulating lymphocytes after stroke occurs in a severity dependent manner [117, 139, 210, 219]. However, these data do not explain why the lung is a specific site for infection. Therefore, we examined the immune cell composition of the lung following various ischemic stroke models in mice in Chapter 3, as well as cytokine immune environment in Chapter 5. Our data demonstrates that there is a reduction of lymphocyte-neutrophil ratio (caused by the loss of lymphocytes) in the lungs that occurs with increased cerebral infarct

volume. Specifically, mice that underwent the most severe form of ischemic stroke (and highest infarct volume) showed the greatest reduction of lymphocyte-neutrophil ratio in the lung at 24 h after stroke onset. In addition, we showed in Chapter 5 that the post-stroke lung had 3-fold greater amount of the immunosuppressive cytokine, IL-10, strongly suggesting that the overall lung immune defence is compromised after stroke.

The underlying mechanisms of how cerebral infarct size influences systemic immunity is unclear. A leading hypothesis is that activation of the SNS and excess catecholamines release control the degree of immunosuppression after stroke. While we know that catecholamines (namely NA) are elevated after stroke [120, 122], few studies stratify stroke severity with catecholamine levels and therefore it is unclear whether greater stroke severity is related to increased catecholamine output. Using baroreflex sensitivity index (a measurement to assess the relationship between changes in arterial blood pressure and heart rate) as a surrogate indicator for ANS activity, one study found that patients with greater stroke severity had lower baroreflex sensitivity and were at higher risk of developing post-stroke infection. This study suggests that increased stroke severity impairs ANS activity, and therefore implies an alteration of SNS function [220]. In support of this, increased infarct size has been previously associated with ANS alterations in a small cohort of patients with stroke as characterised by cardiac arrhythmias and increased blood pressure [221]. Assessing the relationship between stroke severity and catecholamine levels may allow for further insight into the role of the SNS in the susceptibility to infection after stroke. Revealing this causal relationship potentially creates an opportunity to use NA levels as a biomarker to identify patients at risk of post-stroke infection or develop agents to block or inhibit sympathetic signalling to reduce infection after stroke.

In our mouse model of ischemic stroke with various severities, we assessed plasma catecholamine levels within the first 3 h following stroke onset (Chapter 4). The catecholamines involved in the generation and metabolism of NA were measured in an attempt

to capture increases in sympathetic activity immediately after stroke. Only at 3 h following severe stroke was there a significant elevation in plasma NA and DA and therefore our data suggests that the levels of circulating catecholamines are influenced by stroke severity. This coincided with a 92% infection rate in mice that underwent a severe stroke (Chapter 3). As such, there may be potential in using cerebral infarct size and plasma catecholamine levels together as a predictive marker to post-stroke infection and allow clinicians to stratify and manage patients differently. However, we did not measure plasma catecholamine levels beyond the 3 h post-stroke timepoint and therefore further studies exploring the dynamics of plasma catecholamine release may shed further light on the involvement of SNS dysfunction and post-stroke immunosuppression.

An alternative method we utilised to demonstrate a role for SNS dysfunction in mediating post-stroke immunosuppression involved inducing stroke localising to the insular cortex. The insular cortex has traditionally been implicated in the regulation of the SNS [222]. Cerebral infarctions involving the insular cortex have been associated with SNS dysfunction as measured by changes in heart rate variability parameters and cardiac outcomes [223-227]. Whether infarction of the insular cortex alters catecholamine release is less clear as some studies show increase with normetanephrine levels (NA metabolite) with insular cortex involvement [146, 228], while others showed no changes to circulating NA [145]. Furthermore, the suggestion of insular cortex involvement on post-stroke immunosuppression and infection are controversial. The discrepancy in the literature may be due to the differential roles of the right insular cortex versus the left insular cortex, where electric stimulation of the right insular cortex increases heart rate and diastolic blood pressure, whilst stimulation of the left insular cortex lowers heart rate in rodents [222]. Alternatively, the inconsistencies in findings can be explained by the fact that stroke with infarct involving the insular cortex in humans are usually larger infarcts [229]. This suggests that infarct size rather than location is more important in

SNS dysfunction and a better predictor of post-stroke infection. Findings from Chapter 3 of this thesis support this notion as infarction of the insular cortex did not increase the frequency of infection. Moreover, infarction of other brain regions was not associated with post-stroke infection in our retrospective analysis in both experimental and clinical stroke.

A leading hypothesis in which SNS dysfunction induces immunosuppression is by activation of β-adrenergic receptors by NA. Our results show that blocking β-adrenergic receptors (using PPL) could not reduce infection following severe experimental stroke, while others have shown PPL is effective in reducing infection following less severe models of stroke [72, 125, 139]. Furthermore, we show that pharmacologically blocking Adrb2 was effective in reducing infection following moderate stroke compared to severe stroke (Chapter 4). Therefore, our study also suggests mechanisms independent of β_2-adrenergic-receptor-mediated suppression of immunity are at play in severe forms of stroke. One possible mechanism not assessed in this thesis is the HPA axis. Markers of HPA axis activity include elevations in cortisol, but these were not assessed in our studies. Increased serum cortisol has been frequently reported in patients with stroke during the acute phase and has also been associated with greater stroke severity and larger infarctions [230]. Regarding the role of cortisol in post-stroke immunosuppression, increased serum cortisol has recently been correlated with lymphopenia in a small cohort of patients [231]. Similar findings were echoed in experimental stroke where glucocorticoid receptor blockade prevented the loss of circulating lymphocytes [125, 139]. Interestingly, elevation in serum cortisol was only detected following extensive infarction in this study [139] and supports our hypothesis that other mechanisms contribute to the susceptibility to infection following severe stroke.

Along with dysregulated SNS and HPA axis activity after stroke, other factors that contribute to post-stroke immunosuppression and infection have been described throughout this thesis. These include immune exhaustion after stroke [152] and the breakdown of gut

barriers allowing for the dissemination of gut microbes into the periphery [153]. However, it is clear from this thesis that activation of these mechanisms may be dependent on stroke severity. For example, in Chapter 3, immunosuppression in mice that underwent mild-moderate stroke recovered from immunosuppression (as evidenced by recovered spleen size, circulating leukocyte counts and absence of bacteria in lung tissue) by 2 days after stroke, while mice that underwent severe stroke had persistent characteristics of immunosuppression even at 4 days after stroke. Therefore, it is unsurprising that "one-size-fits-all" treatments in clinical trials (such as in trials of antibiotic prophylaxis and β-blocker treatment) have found little success as they do not consider the possible alternative or additional pathways involved in varying degrees of stroke severity.

Strategies to reduce infection in patients with stroke

Currently, antibiotics following the diagnosis of infection are the gold standard treatment for post-stoke infection. However, not all classes of antibiotics are effective at reducing post-stroke infection in a therapeutic manner [156] and treatment at this stage may be too late as existing infection decreases the rate of recovery, worsens functional outcome, increases the likelihood of recurring stroke and extends hospital stay for the patient [232]. Additionally, there is an alarming rise of antibiotic-resistant strains of bacteria that discourages the widespread use of antibiotics [233]. At present, there are no new or emerging therapies that can effectively reduce post-stroke infection. Targeting mechanisms that initiate immune suppression hold strong promise as novel avenues of treatment. Additionally, identifying biomarkers that indicate reduce immunity after stroke may allow for early intervention in patients at high risk of developing to infections. Measuring parameters of dysphagia and aspiration may be an option, however as abovementioned, the validity of this is questionable

as this does not fully explain the frequency of post-stroke infection. Furthermore, there are difficulties in diagnosing aspiration as a large portion of patients with stroke experience silent aspiration [215]. As such, studies have combined other biomarkers to generate a post-stroke pneumonia prediction model, including NIHSS, dysphagia, heart rate, circulating leukocyte count, and HLA-DR expression on leukocytes [212, 234, 235]. Patients with an NIHSS score >14 at admission, pulse rate >111 at admission and lower circulating leukocyte counts had a 40% chance of acquiring pneumonia after stroke [212]. However, while early detection of patient at high risk of developing infection will allow for early interventions, some data suggests that early treatment with antibiotics is still ineffective. In the STRAWINSKI clinical trial, procalcitonin (PCT; an early marker of bacterial infections) was used to predict the occurrence of post-stroke infection in patients [236]. Significantly elevated PCT was detected in patients with stroke-associated pneumonia and sepsis. However, using antibiotic prophylaxis in patients with elevated PCT could not reduce the occurrence of post-stroke pneumonia [236]. This study and many other clinical trials put it beyond doubt that PAT is ineffective in reducing post-stroke pneumonia and emphasises the urgent need for alternative therapeutics to prevent post-stroke infection [160, 161, 163, 236-238].

Given that a multitude of pathways may be responsible for post-stroke infection, the search for new effective therapeutics will prove difficult. Care must be taken to ensure that enhancing antibacterial immunity after stroke does not exacerbate the local brain inflammatory response to increase brain injury. For example, systemic inflammation is known to occur within the first few hours after stroke and this is followed by systemic immunosuppression [239, 240]. As such, patients may benefit in receiving therapeutics that regulate systemic inflammation at early timepoints (within 12 h post-stroke) to prevent further exacerbation of brain infarct, while immunostimulatory compounds could be administered at timepoints where infection is most common (at 1-3 days following stroke in humans). Therefore, there is a risk of further

increasing systemic inflammation and cerebral infarct volume if immunomodulatory therapies are administered too early. In our study, α-GalCer treatment at 2 h post-stroke did not exacerbate brain infarction and did confer protection against the development of post-stroke lung infection following a model of moderate stroke. To the best of our knowledge, α-GalCer is currently the only experimental therapeutic compound that is effective in reducing post-stroke infection. In this thesis we tested two other analogues of α-GalCer but conclude that the parent molecule remains better at inhibiting lung infection after stroke. However, it is important to keep in mind that we have only tested α-GalCer in the transient MCAO model of ischemia and reperfusion stroke injury in mice. Given that the majority of patient do not receive thrombolytic therapy after stroke, it is critical that α-GalCer be tested in a model of stroke without reperfusion, such as pMCAO. Furthermore, to potentially translate our experimental research findings to clinical settings, we would also need to test α-GalCer on other models of stroke, as well as on other non-primate species. Nevertheless, the findings of our study indicate that α-GalCer, through specific activation of iNKT cells, is a promising immunomodulating therapy that can reduce the development of infection after stroke and ultimately improve stroke patient outcomes.

Limitations and future directions

There are some limitations to the studies performed in the thesis that should be considered. Our analysis of post-stroke immunosuppression has been limited to macroscopic changes to major immune organs and the presence of immune cells in peripheral sites such as the blood, lung, liver and spleen. Functional analyses of immune cells were not investigated in this thesis but are of importance. For example, the expression of HLA-DR by monocytes from patients is associated with the severity of stroke and is a predictive marker to infection [143,

210, 241]. Neutrophils isolated from post-stroke animals have shown decreased ability for migration towards potent chemoattractants and bacterial ligands [36]. In this thesis, while infection was prominent after experimental stroke, no changes to the frequency of myeloid cell populations in the lung of post-stroke mice were observed. This may suggest functional impairments in these cells that hinder bacterial clearance in the lung. Further studies are required to determine functional changes of myeloid cell populations that impede the clearance of bacteria in the lung after stroke.

As previously mentioned, we did not measure parameters of other mechanisms to post-stroke infection and immunosuppression, including markers of HPA axis activity, gut barrier breakdown, or immune exhaustion. Additionally, the relationship between the changes of these peripheral biological systems have not been connected to changes that occur in the brain following stroke. Given the complexity of stroke progression and the various biological changes that occur after onset, working towards establishing an accurate timeline of mechanistic events are required for identification of potential interventions and treatment windows. However, due to the time constraints of a PhD candidature, these investigations are beyond the scope of this project. Future studies may need to study these mechanisms in unison and profile the dynamics of these systems throughout the acute phase after stroke.

The intraluminal MCAO model is a well-established, clinically relevant and most frequently used model of stroke. It closely resembles the manifestations of human stroke as most occlusions occur within the territory of the MCA [242]. However, MCAO in rodents often induce larger infarcts compared to humans. Infarction of the hypothalamus region that is seen following severe stroke (≥120 min of occlusion) in rodents is uncommon in humans [242-244]. This suggests the need to validate experimental findings by performing alternative models of stroke, as well as inducing stroke in non-human primates. Another model of ischemic stroke is the photothrombotic (PT) model that involves systemic injection of a photosensitive dye (i.e.

Rose Bengal) and subsequent illumination through the intact skill to activate platelets for aggregation and occlude a specific cerebrovascular region of the brain [245, 246]. This model has advantages of being highly reproducible and enables control of infarct localisation, although it is unknown whether injection of photosensitive dyes can influence systemic immunity [242]. Alternatively, craniectomy models of ischemic stroke require direct occlusion of cerebral blood vessels via cauterisation or clamping to cause focal ischemic injury [247, 248]. However, this model involves partial craniectomy that exposes the brain to the external environment and impacts on intracranial pressure [242]. Furthermore, little is known of whether these models produce similar systemic immune changes as seen in the clinical settings.

Other limitations of our studies are related to experimental design. Firstly, MCAO was performed on healthy mice in our studies. However, in humans, patients with stroke usually experience other comorbidities, including diabetes, smoking and other heart diseases [249]. Therefore, the MCAO model used in this thesis does not perfectly model human disease as the effects of other comorbidities were not studied. Secondly, our experiments were performed on young mice and therefore the effects of stroke and aging on immunity and infection were not assessed. This is of critical importance as stroke most frequently occurs in the aged population and advanced age is linked to worsened stroke severity and increased risk to infection [154, 250]. Future studies will be required to investigate the mechanisms to post-stroke infection in an aged population. Thirdly, we chose to exclude female mice from our study due to the neuroprotective role of oestrogen [251, 252]. However, stroke is a sexually dimorphic disease [253] and therefore future studies that explore mechanisms of post-stroke infections will be required in both female and males. Lastly, infection of the lung was determined by culturing lung homogenate on bacterial plates. This method of quantification cannot detect the presence of all bacteria, especially those that require specific growth factors. We attempted to overcome this by performing 16S qPCR (bacterial specific ribosomal RNA) to quantify the presence of

all bacteria, however these reflected the results found with bacterial culturing (Chapter 3) and thus we did not further pursue this in the latter thesis chapters. Furthermore, we cannot exclude the possibility that viruses are the causative pathogen in post-stroke that infection. This may be of particular interest as many clinical studies were unable to determine the causative bacteria that contributes to pneumonia in their patient cohorts [166, 184], potentially suggesting the involvement of unculturable microbes, including viruses. Previous work from our lab used high throughput 16S rRNA gene amplicon sequencing identify the genera of bacteria that infiltrated the lung following stroke in mice [153]. Use of tools to analyse genomic sequencing may be required to quantify and identify microbes that cause post-stroke pneumonia in humans. However, collection of tissues for such analysis in patients with stroke may be limited to blood, urine and sputum and therefore may not be fully indicative of the causative microbial population(s) in post-stroke pneumonia.

As the mechanisms to post-stroke infection involve the SNS, the impact of the MCAO surgery on SNS activity should be noted. The intraluminal MCAO model that was used in this thesis is performed by temporarily occluding the common carotid artery, inserting a filament into the common carotid artery and proceeding it up the internal carotid artery to occlude blood flow into the middle cerebral artery. The baroreceptor in the carotid artery senses low blood pressure and induces an increase in blood pressure by activating the SNS. As such, the temporary occlusion of common carotid artery may activate baroreceptors to increase blood pressure. Subsequently, this increase in blood pressure activates depressor receptors in the aorta and sequesters sympathetic activity [254, 255]. This may explain the absence of sympathetic activity within the first 3 h following stroke in our study. We attempted to control for such surgery stress by comparing all findings from post-stroke mice with sham-operated mice, and not naïve mice. Future studies may require using other stroke models, such as the photothrombotic stroke model, especially when exploring SNS changes after stroke.

The findings of this thesis may also be applied in other fields independent to stroke that involve sterile cerebral injury. Patients that experience severe traumatic brain injury (TBI) are more likely to acquire pneumonia [256-258]. Similar to stroke, this susceptibility to pneumonia following TBI has been attributed to suppression in peripheral immunity [259-261], impairments in intestinal permeability [262], and SNS dysfunction [263-265]. Therefore, the manifestations of TBI-induced infection may share similar aetiologies to stroke. Our findings such as reduced pulmonary lymphocyte-neutrophil ratio with increasing cerebral injury, involvement of additional pathways following severe cerebral injury, and the use of α-GalCer and iNKT cell activation as therapeutic avenues, may also be applicable to post-TBI infection.

Concluding remarks

This thesis describes the effect of various severities of ischemic stroke, as well as the contribution of SNS activation on post-stroke immunity and infection. Our findings demonstrate that size of cerebral infarction, but not infarct location, increases the risk of post-stroke lung infection. Higher post-stroke infection with more severe strokes also coincides with impaired lung immunity as characterised by decreased lymphocyte-neutrophil ratio and increased IL-10. Mice that underwent severe stroke had significantly increased levels of circulating NA at 3 h post-stroke, suggesting sympathetic activation mediates post-stroke infection. However, pharmacological inhibition of Adrb2 could reduce post-stroke infection in mild-moderate forms of stroke but proved insufficient in more severe stroke. This data suggests that other mechanisms that are independent to SNS dysfunction may come into play to induce infection following a severe stroke. As an alternative treatment method, we investigated whether stimulating an antibacterial immunity following stroke could prevent the development of lung infection. We show that specifically activating the iNKT cell with α-GalCer was

extremely effective in reducing post-stroke infection while not exacerbating brain infarction. Overall, targeting the iNKT cell is a viable method to reduce this major complication that plagues patients with stroke.

In addition, we also highlight the major obstacles obstructing the translation and discovery of new therapeutics to post-stroke infection. We identify that the diversity and complexity of mechanisms that contribute to post-stroke infection ebb and flow over the acute phase following the onset of stroke, thus creating challenges in developing targeted therapeutics. A schematic of the hypothesised changes to these mechanisms is proposed in **Figure 6.1**. Determining the optimal point in time for drug administration will dictate the success of therapeutics in reducing infection after stroke.

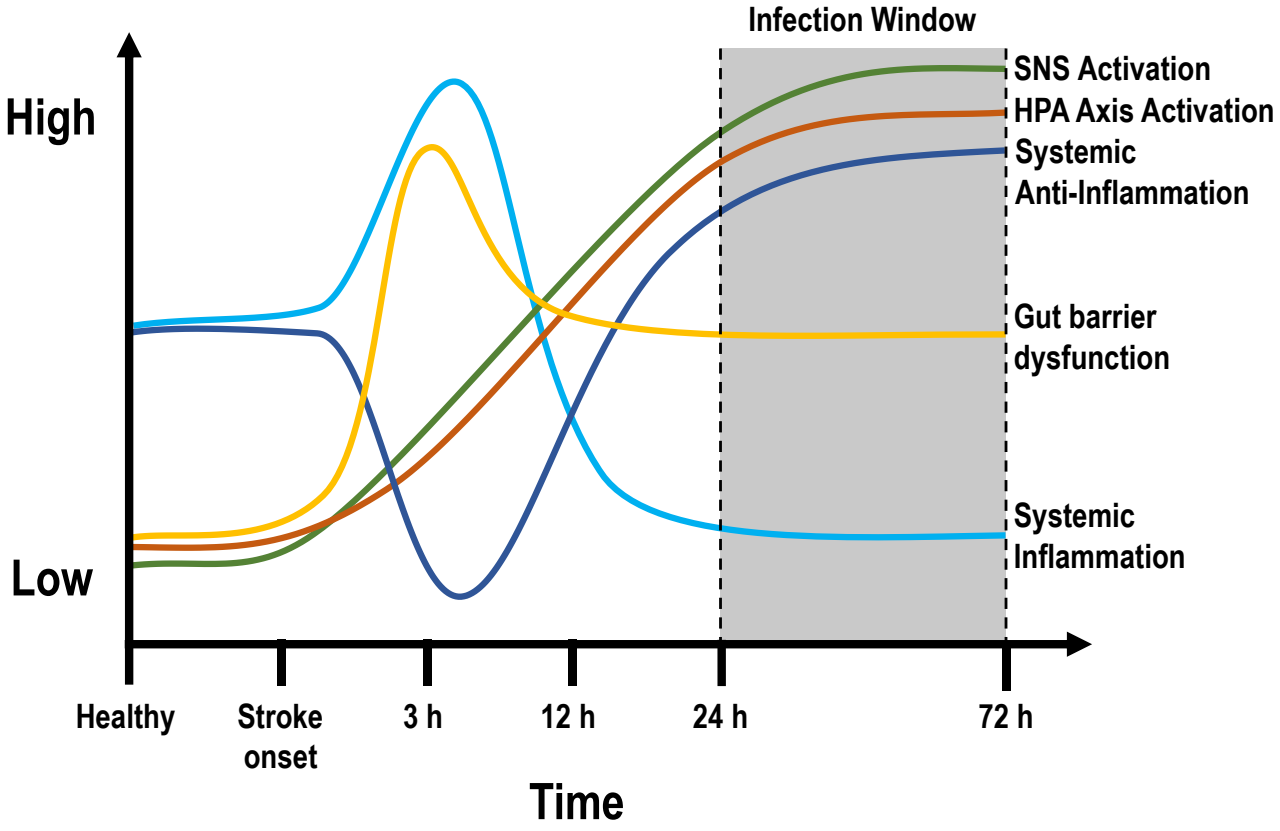


Figure 6.1 Hypothesised temporal profile of mechanisms to post-stroke infection.

The mechanisms of post-stroke infection are diverse, complex, and peak at varying times during the acute phase after stroke. Following the onset of stroke, systemic inflammation increases and peaks at approximately 4 h following stroke and gradually declines by 24 h and stays low until 72 h (light blue). In contrast, systemic anti-inflammation decreases, is at its lowest by 4 h following stroke. Anti-inflammation then peaks at 24 h and remains high by 72 h post-stroke (dark blue). This may be induced by activation of the SNS (green) and HPA axis (orange) that gradually increases over 72 h following stroke. Finally, gut barrier dysfunction peaks at 3 h following stroke, allowing for dissemination of intestinal microbes into the periphery, and may remain partially compromised (yellow). These mechanisms ultimately contribute to the susceptibility to post-stroke infection.

References

1. Goldstein, D.S., G. Eisenhofer, and I.J. Kopin, *Sources and significance of plasma levels of catechols and their metabolites in humans.* Journal of Pharmacology and Experimental Therapeutics, 2003. **305**(3): p. 800-811.
2. Czura, C. and K. Tracey, *Autonomic neural regulation of immunity.* Journal of internal medicine, 2005. **257**(2): p. 156-166.
3. Tracey, K.J., *Reflex control of immunity.* Nature Reviews Immunology, 2009. **9**(6): p. 418-428.
4. Scanzano, A. and M. Cosentino, *Adrenergic regulation of innate immunity: a review.* Frontiers in pharmacology, 2015. **6**: p. 171.
5. Bellinger, D.L. and D. Lorton, *Autonomic regulation of cellular immune function.* Autonomic Neuroscience, 2014. **182**: p. 15-41.
6. Bylund, D.B., et al., *International Union of Pharmacology nomenclature of adrenoceptors.* Pharmacological reviews, 1994. **46**(2): p. 121-136.
7. Wallukat, G., *The β-adrenergic receptors.* Herz, 2002. **27**(7): p. 683-690.
8. Guimarães, S. and D. Moura, *Vascular adrenoceptors: an update.* Pharmacological reviews, 2001. **53**(2): p. 319-356.
9. Krief, S., et al., *Tissue distribution of beta 3-adrenergic receptor mRNA in man.* The Journal of clinical investigation, 1993. **91**(1): p. 344-349.
10. Ackerman, K., et al., *Noradrenergic sympathetic innervation of the spleen: III. Development of innervation in the rat spleen.* Journal of neuroscience research, 1987. **18**(1): p. 49-54.
11. Felten, D., et al., *Noradrenergic and peptidergic innervation of lymphoid tissue.* Journal of immunology (Baltimore, Md.: 1950), 1985. **135**(2 Suppl): p. 755s-765s.

12. Calvo, W., *The innervation of the bone marrow in laboratory animals.* American journal of anatomy, 1968. **123**(2): p. 315-328.

13. Maryanovich, M., S. Takeishi, and P.S. Frenette, *Neural regulation of bone and bone marrow.* Cold Spring Harbor perspectives in medicine, 2018. **8**(9).

14. Trotter, R.N., et al., *Transneuronal mapping of the CNS network controlling sympathetic outflow to the rat thymus.* Autonomic Neuroscience, 2007. **131**(1-2): p. 9-20.

15. Ding, X., et al., *Panicle-Shaped Sympathetic Architecture in the Spleen Parenchyma Modulates Antibacterial Innate Immunity.* Cell reports, 2019. **27**(13): p. 3799-3807. e3.

16. Felten, D.L., et al., *Sympathetic innervation of lymph nodes in mice.* Brain research bulletin, 1984. **13**(6): p. 693-699.

17. Fink, T. and E. Weihe, *Multiple neuropeptides in nerves supplying mammalian lymph nodes: messenger candidates for sensory and autonomic neuroimmunomodulation?* Neuroscience letters, 1988. **90**(1-2): p. 39-44.

18. Callaway, E.M., *Transneuronal circuit tracing with neurotropic viruses.* Current opinion in neurobiology, 2008. **18**(6): p. 617-623.

19. Denes, A., et al., *Central autonomic control of the bone marrow: multisynaptic tract tracing by recombinant pseudorabies virus.* Neuroscience, 2005. **134**(3): p. 947-963.

20. Sivaraj, K.K. and R.H. Adams, *Blood vessel formation and function in bone.* Development, 2016. **143**(15): p. 2706-2715.

21. Itkin, T., et al., *Distinct bone marrow blood vessels differentially regulate haematopoiesis.* Nature, 2016. **532**(7599): p. 323-328.

22. Kusumbe, A.P., et al., *Age-dependent modulation of vascular niches for haematopoietic stem cells.* Nature, 2016. **532**(7599): p. 380-384.

23. Powell, N.D., et al., *Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via β-adrenergic induction of myelopoiesis.* Proceedings of the National Academy of Sciences, 2013. **110**(41): p. 16574-16579.

24. Hattori, K., et al., *Plasma elevation of stromal cell–derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells.* Blood, The Journal of the American Society of Hematology, 2001. **97**(11): p. 3354-3360.

25. Petit, I., et al., *G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4.* Nature immunology, 2002. **3**(7): p. 687-694.

26. Méndez-Ferrer, S., et al., *Haematopoietic stem cell release is regulated by circadian oscillations.* Nature, 2008. **452**(7186): p. 442-447.

27. Cano, G., et al., *Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing.* Journal of Comparative Neurology, 2001. **439**(1): p. 1-18.

28. Casale, T.B. and M. Kaliner, *Demonstration that circulating human blood cells have no detectable alpha1-adrenergic receptors by radioligand binding analysis.* Journal of allergy and clinical immunology, 1984. **74**(6): p. 812-818.

29. Kavelaars, A., *Regulated expression of α-1 adrenergic receptors in the immune system.* Brain, behavior, and immunity, 2002. **16**(6): p. 799-807.

30. Van Der Voort, C.R., et al., *Noradrenaline induces phosphorylation of ERK-2 in human peripheral blood mononuclear cells after induction of α1-adrenergic receptors.* Journal of neuroimmunology, 2000. **108**(1-2): p. 82-91.

31. Muthu, K., et al., *Adrenergic modulation of cytokine release in bone marrow progenitor-derived macrophage following polymicrobial sepsis.* Journal of neuroimmunology, 2005. **158**(1-2): p. 50-57.

32. Scanzano, A., et al., *Adrenergic modulation of migration, CD11b and CD18 expression, ROS and interleukin-8 production by human polymorphonuclear leukocytes.* Inflammation Research, 2015. **64**(2): p. 127-135.

33. Mantovani, A., et al., *Neutrophils in the activation and regulation of innate and adaptive immunity.* Nature Reviews Immunology, 2011. **11**(8): p. 519-531.

34. Hummel, I.B., et al., *Dissociations in the effects of β2-adrenergic receptor agonists on cAMP formation and superoxide production in human neutrophils: support for the concept of functional selectivity.* PLoS One, 2013. **8**(5).

35. Nielson, C., *Beta-adrenergic modulation of the polymorphonuclear leukocyte respiratory burst is dependent upon the mechanism of cell activation.* The Journal of Immunology, 1987. **139**(7): p. 2392-2397.

36. Nicholls, A.J., et al., *Activation of the sympathetic nervous system modulates neutrophil function.* Journal of leukocyte biology, 2018. **103**(2): p. 295-309.

37. Ağaç, D., et al., *The β2-adrenergic receptor controls inflammation by driving rapid IL-10 secretion.* Brain, behavior, and immunity, 2018. **74**: p. 176-185.

38. Nakamura, A., et al., *Regulation of tumour necrosis factor and interleukin-6 gene transcription by β2-adrenoceptor in the rat astrocytes.* Journal of neuroimmunology, 1998. **88**(1-2): p. 144-153.

39. Guereschi, M.G., et al., *Beta2-adrenergic receptor signaling in CD 4+ F oxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner.* European journal of immunology, 2013. **43**(4): p. 1001-1012.

40. Suzuki, K., et al., *Adrenergic control of the adaptive immune response by diurnal lymphocyte recirculation through lymph nodes.* Journal of Experimental Medicine, 2016. **213**(12): p. 2567-2574.

41. Nakai, A., et al., *Control of lymphocyte egress from lymph nodes through β2-adrenergic receptors.* Journal of Experimental Medicine, 2014. **211**(13): p. 2583-2598.

42. Scheiermann, C., et al., *Adrenergic nerves govern circadian leukocyte recruitment to tissues.* Immunity, 2012. **37**(2): p. 290-301.

43. Estrada, L.D., D. Ağaç, and J.D. Farrar, *Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8+ T-cell effector function.* European journal of immunology, 2016. **46**(8): p. 1948-1958.

44. Araujo, L.P., et al., *The Sympathetic Nervous System Mitigates CNS Autoimmunity via β2-Adrenergic Receptor Signaling in Immune Cells.* Cell reports, 2019. **28**(12): p. 3120-3130. e5.

45. Kolmus, K., J. Tavernier, and S. Gerlo, *β2-Adrenergic receptors in immunity and inflammation: stressing NF-κB.* Brain, behavior, and immunity, 2015. **45**: p. 297-310.

46. Murray, D.R., et al., *Sympathetic and immune interactions during dynamic exercise. Mediation via a beta 2-adrenergic-dependent mechanism.* Circulation, 1992. **86**(1): p. 203-213.

47. Chida, Y., et al., *Do stress-related psychosocial factors contribute to cancer incidence and survival?* Nature clinical practice Oncology, 2008. **5**(8): p. 466-475.

48. Andersen, B.L., et al., *Stress and immune responses after surgical treatment for regional breast cancer.* JNCI: Journal of the National Cancer Institute, 1998. **90**(1): p. 30-36.

49. Bellinger, D.L., et al., *Sympathetic modulation of immunity: relevance to disease.* Cellular immunology, 2008. **252**(1-2): p. 27-56.

50. Cole, S.W., et al., *Sympathetic nervous system regulation of the tumour microenvironment.* Nature Reviews Cancer, 2015. **15**(9): p. 563-572.

51. Cole, S.W. and A.K. Sood, *Molecular pathways: beta-adrenergic signaling in cancer.* Clinical cancer research, 2012. **18**(5): p. 1201-1206.

52. De Giorgi, V., et al., *Treatment with β-blockers and reduced disease progression in patients with thick melanoma.* Archives of internal medicine, 2011. **171**(8): p. 779-781.

53. Grytli, H.H., et al., *Use of β-blockers is associated with prostate cancer-specific survival in prostate cancer patients on androgen deprivation therapy.* The Prostate, 2013. **73**(3): p. 250-260.

54. Lamkin, D.M., et al., *Chronic stress enhances progression of acute lymphoblastic leukemia via β-adrenergic signaling.* Brain, behavior, and immunity, 2012. **26**(4): p. 635-641.

55. Powe, D.G., et al., *Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival.* Oncotarget, 2010. **1**(7): p. 628.

56. Bucsek, M.J., et al., *β-adrenergic signaling in mice housed at standard temperatures suppresses an effector phenotype in CD8+ T cells and undermines checkpoint inhibitor therapy.* Cancer research, 2017. **77**(20): p. 5639-5651.

57. Cao, L., N.M. Filipov, and D.A. Lawrence, *Sympathetic nervous system plays a major role in acute cold/restraint stress inhibition of host resistance to Listeria monocytogenes.* Journal of neuroimmunology, 2002. **125**(1-2): p. 94-102.

58. Cao, L., C.A. Hudson, and D.A. Lawrence, *Acute cold/restraint stress inhibits host resistance to Listeria monocytogenes via β1-adrenergic receptors.* Brain, behavior, and immunity, 2003. **17**(2): p. 121-133.

59. Miura, T., et al., *Effect of 6-hydroxydopamine on host resistance against Listeria monocytogenes infection.* Infection and immunity, 2001. **69**(12): p. 7234-7241.

60. Seeley, E.J., et al., *Noradrenergic neurons regulate monocyte trafficking and mortality during gram-negative peritonitis in mice.* The Journal of Immunology, 2013. **190**(9): p. 4717-4724.

61. Pál, E. and T. Tabira, *Autonomic Regulation of Experimental Autoimmune Encephalomyelitis: The Role of Interferon-γ.* Neuroimmunomodulation, 2002. **10**(2): p. 80-84.

62. Simonini, M.V., et al., *Increasing CNS noradrenaline reduces EAE severity.* Journal of Neuroimmune Pharmacology, 2010. **5**(2): p. 252-259.

63. Chelmicka-Schorr, E., et al., *The β-adrenergic agonist isoproterenol suppresses experimental allergic encephalomyelitis in Lewis rats.* Journal of neuroimmunology, 1989. **25**(2): p. 203-207.

64. Wiegmann, K., et al., *β-Adrenergic agonists suppress chronic/relapsing experimental allergic encephalomyelitis (CREAE) in Lewis rats.* Journal of neuroimmunology, 1995. **56**(2): p. 201-206.

65. Nance, D.M. and V.M. Sanders, *Autonomic innervation and regulation of the immune system (1987–2007).* Brain, behavior, and immunity, 2007. **21**(6): p. 736-745.

66. Taketo, M., et al., *FVB/N: an inbred mouse strain preferable for transgenic analyses.* Proceedings of the National Academy of Sciences, 1991. **88**(6): p. 2065-2069.

67. Chruscinski, A.J., et al., *Targeted disruption of the β2 adrenergic receptor gene.* Journal of Biological Chemistry, 1999. **274**(24): p. 16694-16700.

68. Sanders, V.M., et al., *Adaptive immunity in mice lacking the β2-adrenergic receptor.* Brain, behavior, and immunity, 2003. **17**(1): p. 55-67.

69. Emeny, R.T., D. Gao, and D.A. Lawrence, *β1-adrenergic receptors on immune cells impair innate defenses against Listeria.* The Journal of Immunology, 2007. **178**(8): p. 4876-4884.

70. Lin, R., et al., *Chronic treatment in vivo with β-adrenoceptor agonists induces dysfunction of airway β2-adrenoceptors and exacerbates lung inflammation in mice.* British journal of pharmacology, 2012. **165**(7): p. 2365-2377.

71. Starr, J.B., D.L. Tirschwell, and K.J. Becker, *Increased infections with β-blocker use in ischemic stroke, a β2-receptor mediated process?* Neurological Sciences, 2017. **38**(6): p. 967-974.

72. Wong, C.H., et al., *Functional innervation of hepatic iNKT cells is immunosuppressive following stroke.* Science, 2011. **334**(6052): p. 101-105.

73. Crosby, C.M. and M. Kronenberg, *Tissue-specific functions of invariant natural killer T cells.* Nature Reviews Immunology, 2018. **18**(9): p. 559-574.

74. Syn, W.K., et al., *Accumulation of natural killer T cells in progressive nonalcoholic fatty liver disease.* Hepatology, 2010. **51**(6): p. 1998-2007.

75. Subleski, J.J., et al., *The split personality of NKT cells in malignancy, autoimmune and allergic disorders.* Immunotherapy, 2011. **3**(10): p. 1167-1184.

76. Matsuda, J.L., et al., *CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system.* Current opinion in immunology, 2008. **20**(3): p. 358-368.

77. Albacker, L.A., et al., *Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity.* Nature medicine, 2013. **19**(10): p. 1297-1304.

78. Jyonouchi, S., et al., *Invariant natural killer T cells in children with eosinophilic esophagitis.* Clinical & Experimental Allergy, 2014. **44**(1): p. 58-68.

79. Cantorna, M.T., J. Zhao, and L. Yang, *Vitamin D, invariant natural killer T-cells and experimental autoimmune disease.* Proceedings of the Nutrition Society, 2012. **71**(01): p. 62-66.

80. Siegmann, N., et al., *Invariant Natural Killer T (iNKT) Cells Prevent Autoimmunity, but Induce Pulmonary Inflammation in Cystic Fibrosis.* Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology, 2013. **34**(1): p. 56-70.

81. Das, R., et al., *NKTT320, a novel monoclonal antibody activates the immuno-stimulatory functions of human invariant natural killer T cells.* Cancer Research, 2014. **74**(19 Supplement): p. 653-653.

82. Lindau, D., et al., *The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells.* Immunology, 2013. **138**(2): p. 105-115.

83. Hawley, K., et al., *Macrophage p38 mitogen-activated protein kinase activity regulates invariant natural killer T-cell responses during Borrelia burgdorferi infection.* Journal of Infectious Diseases, 2012. **206**(2): p. 283-291.

84. Juno, J.A., et al., *Elevated expression of LAG-3, but not PD-1, is associated with impaired iNKT cytokine production during chronic HIV-1 infection and treatment.* Retrovirology, 2015. **12**(1): p. 17.

85. Romagnani, S., *Th1/th2 cells.* Inflammatory bowel diseases, 1999. **5**(4): p. 285-294.

86. Neurath, M.F., S. Finotto, and L.H. Glimcher, *The role of Th1/Th2 polarization in mucosal immunity.* Nature medicine, 2002. **8**(6): p. 567-573.

87. Skapenko, A., et al., *The role of the T cell in autoimmune inflammation.* Arthritis research & therapy, 2005. **7**(2): p. S4.

88. Walker, J.A. and A.N. McKenzie, *T H 2 cell development and function.* Nature Reviews Immunology, 2018. **18**(2): p. 121.

89. Chang, Y.-J., et al., *Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids.* Proceedings of the National Academy of Sciences, 2007. **104**(25): p. 10299-10304.

90. Kawano, T., et al., *CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides.* Science, 1997. **278**(5343): p. 1626-1629.

91. Natori, T., et al., *Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge Agelas mauritianus.* Tetrahedron, 1994. **50**(9): p. 2771-2784.

92. Yamaguchi, Y., et al., *Enhancing effects of (2S, 3S, 4R)-1-O-(α-d-galactopyranosyl)-2-(N-hexacosanoylamino)-1, 3, 4-octadecanetriol (KRN7000) on antigen-presenting function of antigen-presenting cells and antimetastatic activity of KRN7000-pretreated antigen-presenting cells.* Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 1996. **8**(10-11): p. 399-407.

93. Kawano, T., et al., *Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells.* Proceedings of the National Academy of Sciences, 1998. **95**(10): p. 5690-5693.

94. Im, J.S., et al., *Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation.* Immunity, 2009. **30**(6): p. 888-898.

95. Zajonc, D.M., et al., *Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor.* Nature immunology, 2005. **6**(8): p. 810-818.

96. McCarthy, C., et al., *The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation.* The Journal of experimental medicine, 2007. **204**(5): p. 1131-1144.

97. Laurent, X., et al., *Relationships between Th1 or Th2 iNKT Cell Activity and Structures of CD1d-Antigen Complexes: Meta-analysis of CD1d-Glycolipids Dynamics Simulations.* PLoS computational biology, 2014. **10**(11): p. e1003902.

98. Li, X., et al., *Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant.* Proceedings of the National Academy of Sciences, 2010. **107**(29): p. 13010-13015.

99. Ly, D., et al., *An α-galactosylceramide C20: 2 N-acyl variant enhances anti-inflammatory and regulatory T cell-independent responses that prevent type 1 diabetes.* Clinical & Experimental Immunology, 2010. **160**(2): p. 185-198.

100. Sullivan, B.A., et al., *Mechanisms for glycolipid antigen-driven cytokine polarization by Vα14i NKT cells.* The journal of immunology, 2010. **184**(1): p. 141-153.

101. Oki, S., et al., *The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells.* Journal of Clinical Investigation, 2004. **113**(11): p. 1631.

102. Arora, P., et al., *A rapid fluorescence-based assay for classification of iNKT cell activating glycolipids.* Journal of the American Chemical Society, 2011. **133**(14): p. 5198-5201.

103. Carreño, L.J., N.A. Saavedra-Ávila, and S.A. Porcelli, *Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents.* Clinical & translational immunology, 2016. **5**(4): p. e69.

104. Chennamadhavuni, D., et al., *Dual Modifications of α-Galactosylceramide Synergize to Promote Activation of Human Invariant Natural Killer T Cells and Stimulate Anti-tumor Immunity.* Cell chemical biology, 2018. **25**(5): p. 571-584. e8.

105. Oleinika, K., et al., *CD1d-dependent immune suppression mediated by regulatory B cells through modulations of iNKT cells.* Nature communications, 2018. **9**(1): p. 1-17.

106. Crosby, C.M. and M. Kronenberg, *Invariant natural killer T cells: front line fighters in the war against pathogenic microbes.* Immunogenetics, 2016. **68**(8): p. 639-648.

107. Kawakami, K., et al., *Critical role of Vα14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection.* European journal of immunology, 2003. **33**(12): p. 3322-3330.

108. Minagawa, S., et al., *Activation of natural killer T cells by α-galactosylceramide mediates clearance of bacteria in murine urinary tract infection.* The Journal of urology, 2005. **173**(6): p. 2171-2174.

109. Wong, C.H., et al., *Prolonged activation of invariant natural killer T cells and TH2-skewed immunity in stroke patients.* Frontiers in Neurology, 2017. **8**.

110. Chiodini, R. and C. Buergelt, *Susceptibility of Balb/c, C57/B6 and C57/B10 mice to infection with Mycobacterium paratuberculosis.* Journal of comparative pathology, 1993. **109**(4): p. 309-319.

111. Fornefett, J., et al., *Comparative analysis of humoral immune responses and pathologies of BALB/c and C57BL/6 wildtype mice experimentally infected with a highly virulent Rodentibacter pneumotropicus (Pasteurella pneumotropica) strain.* BMC microbiology, 2018. **18**(1): p. 45.

112. Watanabe, H., et al., *Innate immune response in Th1-and Th2-dominant mouse strains.* Shock, 2004. **22**(5): p. 460-466.

113. Biburger, M. and G. Tiegs, *α-Galactosylceramide-induced liver injury in mice is mediated by TNF-α but independent of Kupffer cells.* The Journal of Immunology, 2005. **175**(3): p. 1540-1550.

114. Shim, R. and C.H. Wong, *Ischemia, immunosuppression and infection—tackling the predicaments of post-stroke complications.* International journal of molecular sciences, 2016. **17**(1): p. 64.

115. Connolly, E.S., et al., *Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia.* Neurosurgery, 1996. **38**(3): p. 523-532.

116. Zhang, S.R., et al., *IL-33 modulates inflammatory brain injury but exacerbates systemic immunosuppression following ischemic stroke.* JCI insight, 2018. **3**(18).

117. Shim, R., et al., *Stroke Severity, and Not Cerebral Infarct Location, Increases the Risk of Infection.* Translational stroke research, 2019: p. 1-15.

118. Bassi, A., et al., *Cardiac autonomic dysfunction and functional outcome after ischaemic stroke.* European journal of neurology, 2007. **14**(8): p. 917-922.

119. Xiong, L., et al., *Autonomic dysfunction predicts clinical outcomes after acute ischemic stroke: a prospective observational study.* Stroke, 2018. **49**(1): p. 215-218.

120. Eşref, A., et al., *Identifying autonomic nervous system dysfunction in acute cerebrovascular attack by assessments of heart rate variability and catecholamine levels.* Journal of neurosciences in rural practice, 2015. **6**(02): p. 145-150.

121. McCulloch, L., et al., *Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence.* F1000Research, 2019. **8**.

122. Myers, M.G., et al., *Plasma norepinephrine in stroke.* Stroke, 1981. **12**(2): p. 200-204.

123. Mizuno, K., et al., *β2-Adrenergic receptor stimulation inhibits LPS-induced IL-18 and IL-12 production in monocytes.* Immunology letters, 2005. **101**(2): p. 168-172.

124. Wirth, T., et al., *The sympathetic nervous system modulates CD4+ Foxp3+ regulatory T cells via noradrenaline-dependent apoptosis in a murine model of lymphoproliferative disease.* Brain, behavior, and immunity, 2014. **38**: p. 100-110.

125. Prass, K., et al., *Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper*

cell type 1–like immunostimulation. The Journal of experimental medicine, 2003. **198**(5): p. 725-736.

126. Grailer, J.J., et al., *Induction of M2 regulatory macrophages through the β2-adrenergic receptor with protection during endotoxemia and acute lung injury.* Journal of innate immunity, 2014. **6**(5): p. 607-618.

127. McCulloch, L., C.J. Smith, and B.W. McColl, *Adrenergic-mediated loss of splenic marginal zone B cells contributes to infection susceptibility after stroke.* Nature Communications, 2017. **8**.

128. Offner, H., A. Vandenbark, and P. Hurn, *Effect of experimental stroke on peripheral immunity: CNS ischemia induces profound immunosuppression.* Neuroscience, 2009. **158**(3): p. 1098-1111.

129. Liu, Q., et al., *Brain Ischemia Suppresses Immunity in the Periphery and Brain via Different Neurogenic Innervations.* Immunity, 2017. **46**(3): p. 474-487.

130. Dziedzic, T., et al., *Beta-blockers reduce the risk of early death in ischemic stroke.* Journal of the neurological sciences, 2007. **252**(1): p. 53-56.

131. Sykora, M., et al., *β-Blockers, Pneumonia, and Outcome After Ischemic Stroke Evidence From Virtual International Stroke Trials Archive.* Stroke, 2015. **46**(5): p. 1269-1274.

132. Barer, D., et al., *Low dose β blockade in acute stroke ("BEST" trial): an evaluation.* British medical journal (Clinical research ed.), 1988. **296**(6624): p. 737.

133. Maier, I.L., et al., *Effect of Beta-blocker therapy on the risk of infections and death after acute stroke-a historical cohort study.* PloS one, 2015. **10**(2): p. e0116836.

134. Westendorp, W.F., et al., *Pre-stroke use of beta-blockers does not lower post-stroke infection rate: an exploratory analysis of the preventive antibiotics in stroke study.* Cerebrovascular Diseases, 2016. **42**(5-6): p. 506-511.

135. Courties, G., et al., *Glucocorticoids regulate bone marrow B lymphopoiesis after stroke.* Circulation research, 2019. **124**(9): p. 1372-1385.

136. Lambert, G.W. and I.H. Jonsdottir, *Influence of voluntary exercise on hypothalamic norepinephrine.* Journal of Applied Physiology, 1998. **85**(3): p. 962-966.

137. Smolich, J.J., et al., *Greater sympathoadrenal activation with longer preventilation intervals after immediate cord clamping increases hemodynamic lability at birth in preterm lambs.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2017. **312**(6): p. R903-R911.

138. Eisenhofer, G., et al., *Differences in the neuronal removal of circulating epinephrine and norepinephrine.* The Journal of Clinical Endocrinology & Metabolism, 1990. **70**(6): p. 1710-1720.

139. Mracsko, E., et al., *Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke.* Brain, behavior, and immunity, 2014. **41**: p. 200-209.

140. Westendorp, W.F., et al., *Post-stroke infection: a systematic review and meta-analysis.* BMC neurology, 2011. **11**(1): p. 110.

141. Offner, H., et al., *Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages.* The Journal of Immunology, 2006. **176**(11): p. 6523-6531.

142. Deng, Q.-W., et al., *Blocking sympathetic nervous system reverses partially stroke-induced immunosuppression but does not aggravate functional outcome after experimental stroke in rats.* Neurochemical research, 2016. **41**(8): p. 1877-1886.

143. Haeusler, K.G., et al., *Cellular immunodepression preceding infectious complications after acute ischemic stroke in humans.* Cerebrovascular Diseases, 2008. **25**(1-2): p. 50-58.

144. Akıl, E., et al., *Identifying autonomic nervous system dysfunction in acute cerebrovascular attack by assessments of heart rate variability and catecholamine levels.* Journal of neurosciences in rural practice, 2015. **6**(2): p. 145.

145. Harms, H., et al., *Influence of stroke localization on autonomic activation, immunodepression, and post-stroke infection.* Cerebrovascular Diseases, 2011. **32**(6): p. 552-560.

146. Smith, K.E., et al., *Changes in plasma catecholamine levels after insula damage in experimental stroke.* Brain research, 1986. **375**(1): p. 182-185.

147. De Raedt, S., A. De Vos, and J. De Keyser, *Autonomic dysfunction in acute ischemic stroke: an underexplored therapeutic area?* Journal of the neurological sciences, 2015. **348**(1-2): p. 24-34.

148. Grassi, G., et al., *Comparison between reproducibility and sensitivity of muscle sympathetic nerve traffic and plasma noradrenaline in man.* Clinical Science, 1997. **92**(3): p. 285-289.

149. Holmes, C., G. Eisenhofer, and D.S. Goldstein, *Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection.* Journal of Chromatography B: Biomedical Sciences and Applications, 1994. **653**(2): p. 131-138.

150. Raffel, D.M., et al., *First-in-Human Studies of [18F] Fluorohydroxyphenethylguanidines: Positron Emission Tomography Radiotracers for Quantifying Regional Cardiac Sympathetic Nerve Density*. Circulation: Cardiovascular Imaging, 2018. **11**(12): p. e007965.

151. Kruyt, N.D., et al., *Door-to-needle time and the proportion of patients receiving intravenous thrombolysis in acute ischemic stroke: uniform interpretation and reporting.* Stroke, 2013. **44**(11): p. 3249-3253.

152. Liesz, A., et al., *DAMP Signaling is a Key Pathway Inducing Immune Modulation after Brain Injury.* The Journal of Neuroscience, 2015. **35**(2): p. 583-598.

153. Stanley, D., et al., *Translocation and dissemination of commensal bacteria in post-stroke infection.* Nature medicine, 2016. **22**(11): p. 1277-1284.

154. Wen, S.W., et al., *Advanced age promotes colonic dysfunction and gut-derived lung infection after stroke.* Aging cell, 2019: p. e12980.

155. Atgié, C., F.o. D'Allaire, and L.J. Bukowiecki, *Role of β1-and β3-adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes.* American Journal of Physiology-Cell Physiology, 1997. **273**(4): p. C1136-C1142.

156. Smith, C.J., et al., *Antibiotic class and outcome in post-stroke infections: an individual participant data pooled analysis of VISTA-Acute.* Frontiers in neurology, 2019. **10**: p. 504.

157. Hetze, S., et al., *Superiority of preventive antibiotic treatment compared with standard treatment of poststroke pneumonia in experimental stroke: a bed to bench approach.* Journal of Cerebral Blood Flow & Metabolism, 2013. **33**(6): p. 846-854.

158. Meisel, C., et al., *Preventive antibacterial treatment improves the general medical and neurological outcome in a mouse model of stroke.* Stroke, 2004. **35**(1): p. 2-6.

159. Chamorro, A., et al., *The Early Systemic Prophylaxis of Infection After Stroke Study A Randomized Clinical Trial.* Stroke, 2005. **36**(7): p. 1495-1500.

160. Kalra, L., et al., *Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial.* The Lancet, 2015. **386**(10006): p. 1835-1844.

161. Langhorne, P., *Preventive antibiotics reduce infections but not mortality in adults with acute stroke.* Evidence-based nursing, 2010. **13**(1): p. 17-17.

162. van de Beek, D., et al., *Preventive antibiotics for infections in acute stroke: a systematic review and meta-analysis.* Archives of neurology, 2009. **66**(9): p. 1076-1081.

163. Westendorp, W.F., et al., *The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial.* The Lancet, 2015.

164. Lam, O.L., et al., *Effect of oral hygiene interventions on opportunistic pathogens in patients after stroke.* American journal of infection control, 2013. **41**(2): p. 149-154.

165. Nakajoh, K., et al., *Relation between incidence of pneumonia and protective reflexes in post-stroke patients with oral or tube feeding.* Journal of internal medicine, 2000. **247**(1): p. 39-42.

166. Walter, U., et al., *Predictors of pneumonia in acute stroke patients admitted to a neurological intensive care unit.* Journal of neurology, 2007. **254**(10): p. 1323-1329.

167. Chamorro, Á., et al., *The immunology of acute stroke.* Nature Reviews Neurology, 2012. **8**(7): p. 401-410.

168. Shim, R. and C.H. Wong, *Complex interplay of multiple biological systems that contribute to post-stroke infections.* Brain, behavior, and immunity, 2018.

169. Liew, P.X., W.-Y. Lee, and P. Kubes, *iNKT cells orchestrate a switch from inflammation to resolution of sterile liver injury.* Immunity, 2017. **47**(4): p. 752-765. e5.

170. Wang, H. and K.A. Hogquist, *Wait, wait… ok now go in: iNKT cells resolve liver inflammation.* Immunity, 2017. **47**(4): p. 609-610.

171. Thanabalasuriar, A., et al., *iNKT cell emigration out of the lung vasculature requires neutrophils and monocyte-derived dendritic cells in inflammation.* Cell reports, 2016. **16**(12): p. 3260-3272.

172. Nakamatsu, M., et al., *Role of interferon-γ in Vα14+ natural killer T cell-mediated host defense against Streptococcus pneumoniae infection in murine lungs.* Microbes and infection, 2007. **9**(3): p. 364-374.

173. Kinjo, Y., et al., *Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria.* Nature immunology, 2011. **12**(10): p. 966.

174. Vahidy, F.S., et al., *Acute splenic responses in patients with ischemic stroke and intracerebral hemorrhage.* Journal of Cerebral Blood Flow & Metabolism, 2016. **36**(6): p. 1012-1021.

175. Vahedi, K., et al., *Early decompressive surgery in malignant infarction of the middle cerebral artery: a pooled analysis of three randomised controlled trials.* The Lancet Neurology, 2007. **6**(3): p. 215-222.

176. Abramov, A.Y., A. Scorziello, and M.R. Duchen, *Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation.* The Journal of neuroscience, 2007. **27**(5): p. 1129-1138.

177. Yilmaz, G. and D.N. Granger, *Leukocyte recruitment and ischemic brain injury.* Neuromolecular medicine, 2010. **12**(2): p. 193-204.

178. Römer, C., et al., *Blocking Stroke-Induced Immunodeficiency Increases CNS Antigen-Specific Autoreactivity But Does Not Worsen Functional Outcome after Experimental Stroke.* The Journal of Neuroscience, 2015. **35**(20): p. 7777-7794.

179. Scanlon, S.T., et al., *Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation.* Journal of Experimental Medicine, 2011. **208**(10): p. 2113-2124.

180. Osman, Y., et al., *Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide.* European journal of immunology, 2000. **30**(7): p. 1919-1928.

181. Kastenmüller, W., et al., *A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread.* Cell, 2012. **150**(6): p. 1235-1248.

182. Hill, T.M., et al., *Border patrol gone awry: lung NKT cell activation by Francisella tularensis exacerbates tularemia-like disease.* PLoS pathogens, 2015. **11**(6).

183. Lee, W.-Y., et al., *Invariant natural killer T cells act as an extravascular cytotoxic barrier for joint-invading Lyme Borrelia.* Proceedings of the National Academy of Sciences, 2014. **111**(38): p. 13936-13941.

184. Vargas, M., et al., *Clinical Consequences of Infection in Patients With Acute Stroke Is It Prime Time for Further Antibiotic Trials?* Stroke, 2006. **37**(2): p. 461-465.

185. Mizgerd, J.P., *Inflammation and Pneumonia: Why Are Some More Susceptible than Others?* Clinics in chest medicine, 2018. **39**(4): p. 669-676.

186. Hasegawa, H., et al., *Liver Injury After Invariant NKT Cell Activation by Free Alpha-galactosylceramide and Alpha-galactosylceramide-loaded Dendritic Cells.* Anticancer research, 2016. **36**(7): p. 3667-3672.

187. Nakagawa, R., et al., *Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice.* The Journal of Immunology, 2001. **166**(11): p. 6578-6584.

188. Krovi, S.H. and L. Gapin, *Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates.* Frontiers in immunology, 2018. **9**: p. 1393.

189. Lee, P.T., et al., *Distinct functional lineages of human Vα24 natural killer T cells.* The Journal of experimental medicine, 2002. **195**(5): p. 637-641.

190. Giaccone, G., et al., *A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors.* Clinical Cancer Research, 2002. **8**(12): p. 3702-3709.

191. Krystle, K., et al., *Conservation of molecular and cellular phenotypes of invariant NKT cells between humans and non-human primates.* Immunogenetics, 2019. **71**(7): p. 465-478.

192. Monzon-Casanova, E., et al., *Direct identification of rat iNKT cells reveals remarkable similarities to human iNKT cells and a profound deficiency in LEW rats.* European journal of immunology, 2013. **43**(2): p. 404-415.

193. Uldrich, A.P., et al., *NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge.* The Journal of Immunology, 2005. **175**(5): p. 3092-3101.

194. Sullivan, B.A. and M. Kronenberg, *Activation or anergy: NKT cells are stunned by α-galactosylceramide.* The Journal of clinical investigation, 2005. **115**(9): p. 2328-2329.

195. Iyoda, T., et al., *Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation.* International immunology, 2010. **22**(11): p. 905-913.

196. Parekh, V.V., et al., *Glycolipid antigen induces long-term natural killer T cell anergy in mice.* The Journal of clinical investigation, 2005. **115**(9): p. 2572-2583.

197. Becker, K.J., et al., *Autoimmune responses to the brain after stroke are associated with worse outcome.* Stroke, 2011. **42**(10): p. 2763-2769.

198. Planas, A.M., et al., *Brain-derived antigens in lymphoid tissue of patients with acute stroke.* The Journal of Immunology, 2012. **188**(5): p. 2156-2163.

199. Langhorne, P., et al., *Medical complications after stroke a multicenter study.* Stroke, 2000. **31**(6): p. 1223-1229.

200. Vernino, S., et al., *Cause-Specific Mortality After First Cerebral Infarction A Population-Based Study.* Stroke, 2003. **34**(8): p. 1828-1832.

201. Langdon, P., A. Lee, and C. Binns, *High incidence of respiratory infections in 'nil by mouth'tube-fed acute ischemic stroke patients.* Neuroepidemiology, 2009. **32**(2): p. 107-113.

202. Brogan, E., et al., *Respiratory infections in acute stroke: nasogastric tubes and immobility are stronger predictors than dysphagia.* Dysphagia, 2014. **29**(3): p. 340-345.

203. Phan, T.G., et al., *Stroke severity versus Dysphagia Screen as driver for post-stroke pneumonia.* Frontiers in neurology, 2019. **10**.

204. Badve, M.S., et al., *Frequency of post-stroke pneumonia: Systematic review and meta-analysis of observational studies.* International Journal of Stroke, 2019. **14**(2): p. 125-136.

205. Brogan, E., et al., *Dysphagia and factors associated with respiratory infections in the first week post stroke.* Neuroepidemiology, 2014. **43**(2): p. 140-144.

206. Dziewas, R., et al., *Pneumonia in acute stroke patients fed by nasogastric tube.* Journal of Neurology, Neurosurgery & Psychiatry, 2004. **75**(6): p. 852-856.

207. Dziewas, R., et al., *Do nasogastric tubes worsen dysphagia in patients with acute stroke?* BMC neurology, 2008. **8**(1): p. 28.

208. Katzan, I.L., et al., *The effect of pneumonia on mortality among patients hospitalized for acute stroke.* Neurology, 2003. **60**(4): p. 620-625.

209. Matz, K., et al., *Post-stroke pneumonia at the stroke unit–a registry based analysis of contributing and protective factors.* BMC neurology, 2016. **16**(1): p. 107.

210. Hug, A., et al., *Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection.* Stroke, 2009. **40**(10): p. 3226-3232.

211. Urra, X., et al., *Neuroanatomical correlates of stroke-associated infection and stroke-induced immunodepression.* Brain, behavior, and immunity, 2017. **60**: p. 142-150.

212. Kuo, Y.-W., et al., *Risk stratification model for post-stroke pneumonia in patients with acute ischemic stroke.* European Journal of Cardiovascular Nursing, 2019: p. 1474515119889770.

213. Arnold, M., et al., *Dysphagia in acute stroke: incidence, burden and impact on clinical outcome.* PloS one, 2016. **11**(2).

214. Minnerup, J., et al., *The impact of lesion location and lesion size on poststroke infection frequency.* Journal of Neurology, Neurosurgery & Psychiatry, 2010. **81**(2): p. 198-202.

215. Perry, S.E., et al., *The Dysphagia in Stroke Protocol reduces aspiration pneumonia in patients with dysphagia following acute stroke: a clinical audit.* Translational stroke research, 2019. **10**(1): p. 36-43.

216. Power, M.L., et al., *Predicting aspiration after hemispheric stroke from timing measures of oropharyngeal bolus flow and laryngeal closure.* Dysphagia, 2009. **24**(3): p. 257-264.

217. Yuan, M.-z., et al., *Risk factors for lung infection in stroke patients: a meta-analysis of observational studies.* Expert review of anti-infective therapy, 2015. **13**(10): p. 1289-1298.

218. Liesz, A., et al., *Acquired immunoglobulin G deficiency in stroke patients and experimental brain ischemia.* Experimental neurology, 2015. **271**: p. 46-52.

219. Liesz, A., et al., *The spectrum of systemic immune alterations after murine focal ischemia: immunodepression versus immunomodulation.* Stroke, 2009. **40**(8): p. 2849-2858.

220. Sykora, M., et al., *Baroreflex sensitivity is associated with post-stroke infections. An open, prospective study.* Journal of the neurological sciences, 2019. **406**: p. 116450.

221. Sander, D. and J. Klingelhöfer, *Stroke-associated pathological sympathetic activation related to size of infarction and extent of insular damage.* Cerebrovascular Diseases, 1995. **5**(6): p. 381-385.

222. Oppenheimer, S.M., et al., *Cardiovascular effects of human insular cortex stimulation.* Neurology, 1992. **42**(9): p. 1727-1727.

223. Chen, P.-L., T.B. Kuo, and C.C. Yang, *Parasympathetic activity correlates with early outcome in patients with large artery atherosclerotic stroke.* Journal of the neurological sciences, 2012. **314**(1-2): p. 57-61.

224. Colivicchi, F., et al., *Prognostic implications of right-sided insular damage, cardiac autonomic derangement, and arrhythmias after acute ischemic stroke.* Stroke, 2005. **36**(8): p. 1710-1715.

225. Constantinescu, V., et al., *Cortical lateralization and cardiac autonomic control. Insights from insular stroke and epilepsy.* Archive of Clinical Cases, 2017. **4**(3).

226. Meyer, S. and M. Strittmatter, *Left insular stroke is associated with adverse cardiac outcome.* Neurology, 2006. **67**(6): p. 1103-1104.

227. Strittmatter, M., et al., *Location-dependent patterns in cardio-autonomic dysfunction in ischaemic stroke.* European neurology, 2003. **50**(1): p. 30-38.

228. Walter, U., et al., *Insular stroke is associated with acute sympathetic hyperactivation and immunodepression.* European Journal of Neurology, 2013. **20**(1): p. 153-159.

229. Kodumuri, N., et al., *The association of insular stroke with lesion volume.* Neuroimage: Clinical, 2016. **11**: p. 41-45.

230. Christensen, H., G. Boysen, and H.H. Johannesen, *Serum-cortisol reflects severity and mortality in acute stroke.* Journal of the neurological sciences, 2004. **217**(2): p. 175-180.

231. Zierath, D., et al., *Cortisol is more important than metanephrines in driving changes in leukocyte counts after stroke.* Journal of Stroke and Cerebrovascular Diseases, 2018. **27**(3): p. 555-562.

232. Aslanyan, S., et al., *Pneumonia and urinary tract infection after acute ischaemic stroke: a tertiary analysis of the GAIN International trial.* European Journal of Neurology, 2004. **11**(1): p. 49-53.

233. Yan, L., et al., *Etiologic Diagnosis and Clinical Treatment of Multiple Drug-Resistant Bacteria Infection in Elderly Patients with Stroke-Associated Pneumonia After Neurosurgery.* Cell biochemistry and biophysics, 2015. **71**(2): p. 731-734.

234. Hoffmann, S., et al., *Stroke-induced immunodepression and dysphagia independently predict stroke-associated pneumonia–The PREDICT study.* Journal of Cerebral Blood Flow & Metabolism, 2017. **37**(12): p. 3671-3682.

235. Westendorp, W.F., et al., *Development and internal validation of a prediction rule for post-stroke infection and post-stroke pneumonia in acute stroke patients.* European stroke journal, 2018. **3**(2): p. 136-144.

236. Ulm, L., et al., *The randomized Controlled STraWinSKi Trial: Procalcitonin-Guided antibiotic Therapy after Stroke.* Frontiers in neurology, 2017. **8**.

237. Chamorro, Á., X. Urra, and A.M. Planas, *Infection after acute ischemic stroke a manifestation of brain-induced immunodepression.* Stroke, 2007. **38**(3): p. 1097-1103.

238. Tziomalos, K., et al., *Prophylactic antibiotic treatment in severe acute ischemic stroke: the Antimicrobial chemopRrophylaxis for Ischemic STrokEIn MaceDonIa–Thrace Study (ARISTEIDIS).* Internal and emergency medicine, 2016. **11**(7): p. 953-958.

239. Offner, H., et al., *Experimental stroke induces massive, rapid activation of the peripheral immune system.* Journal of Cerebral Blood Flow & Metabolism, 2006. **26**(5): p. 654-665.

240. Chapman, K.Z., et al., *A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke.* Journal of Cerebral Blood Flow & Metabolism, 2009. **29**(11): p. 1764-1768.

241. Zhang, D., et al., *A decrease of human leucocyte antigen-DR expression on monocytes in peripheral blood predicts stroke-associated infection in critically-ill patients with acute stroke.* European journal of neurology, 2009. **16**(4): p. 498-505.

242. Sommer, C.J., *Ischemic stroke: experimental models and reality.* Acta neuropathologica, 2017. **133**(2): p. 245-261.

243. Gerriets, T., et al., *Complications and pitfalls in rat stroke models for middle cerebral artery occlusion: a comparison between the suture and the macrosphere model using magnetic resonance angiography.* Stroke, 2004. **35**(10): p. 2372-2377.

244. Kwiecien, T.D., C. Sy, and Y. Ding, *Rodent models of ischemic stroke lack translational relevance… are baboon models the answer?* Neurological research, 2014. **36**(5): p. 417-422.

245. Watson, B.D., et al., *Induction of reproducible brain infarction by photochemically initiated thrombosis.* Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 1985. **17**(5): p. 497-504.

246. Kim, G.W., T. Sugawara, and P.H. Chan, *Involvement of oxidative stress and caspase-3 in cortical infarction after photothrombotic ischemia in mice.* Journal of Cerebral Blood Flow & Metabolism, 2000. **20**(12): p. 1690-1701.

247. Tamura, A., et al., *Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion.* Journal of Cerebral Blood Flow & Metabolism, 1981. **1**(1): p. 53-60.

248. Dirnagl, U., *Rodent models of stroke*. Vol. 12. 2010: Springer.

249. Boehme, A.K., C. Esenwa, and M.S. Elkind, *Stroke risk factors, genetics, and prevention.* Circulation research, 2017. **120**(3): p. 472-495.

250. Wen, S.W. and C.H. Wong, *Aging-and vascular-related pathologies.* Microcirculation, 2019. **26**(2): p. e12463.

251. Bushnell, C.D., *Oestrogen and stroke in women: assessment of risk.* The Lancet Neurology, 2005. **4**(11): p. 743-751.

252. Sohrabji, F. and M. Williams, *Stroke neuroprotection: oestrogen and insulin-like growth factor-1 interactions and the role of microglia.* Journal of neuroendocrinology, 2013. **25**(11): p. 1173-1181.

253. Turtzo, L.C. and L.D. McCullough, *Sex-specific responses to stroke.* Future neurology, 2010. **5**(1): p. 47-59.

254. Cechetto, D.F., et al., *Autonomic and myocardial changes in middle cerebral artery occlusion: stroke models in the rat.* Brain research, 1989. **502**(2): p. 296-305.

255. Dorrance, A.M. and G. Fink, *Effects of stroke on the autonomic nervous system.* Comprehensive Physiology, 2011. **5**(3): p. 1241-1263.

256. Alharfi, I.M., et al., *Infection rates, fevers, and associated factors in pediatric severe traumatic brain injury.* Journal of neurotrauma, 2014. **31**(5): p. 452-458.

257. Doran, S.J., et al., *Early or late bacterial lung infection increases mortality after traumatic brain injury in male mice and chronically impairs monocyte innate immune function.* Read Online: Critical Care Medicine| Society of Critical Care Medicine, 2020. **48**(5): p. e418-e428.

258. Sharma, R., et al., *Infections after a traumatic brain injury: The complex interplay between the immune and neurological systems.* Brain, behavior, and immunity, 2019.

259. Ritzel, R.M., et al., *Chronic alterations in systemic immune function after traumatic brain injury.* Journal of neurotrauma, 2018. **35**(13): p. 1419-1436.

260. Schwulst, S.J., et al., *Traumatic brain injury-induced alterations in peripheral immunity.* The journal of trauma and acute care surgery, 2013. **75**(5): p. 780.

261. Vermeij, J.-D., et al., *Traumatic brain injury in rats induces lung injury and systemic immune suppression.* Journal of neurotrauma, 2013. **30**(24): p. 2073-2079.

262. Bansal, V., et al., *Traumatic brain injury and intestinal dysfunction: uncovering the neuro-enteric axis.* Journal of neurotrauma, 2009. **26**(8): p. 1353-1359.

263. Yang, Y., et al., *Acute traumatic brain injury induces CD4+ and CD8+ T cell functional impairment by upregulating the expression of PD-1 via the activated sympathetic nervous system.* Neuroimmunomodulation, 2019. **26**(1): p. 43-57.

264. de Oliveira Manoel, A.L., et al., *Beta-blockers in Traumatic Brain Injury.* 2019.

265. Woiciechowsky, C., et al., *Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury.* Nature medicine, 1998. **4**(7): p. 808-813.

Appendix

Appendix 1 – Animal monitoring sheet

Appendix 2 – Clinical signs severity scoring chart

Appendix 3 – Antibodies used for flow cytometry

Appendix 4 – Flow cytometry gating strategies

Appendix 1 – Animal monitoring sheet

ANIMAL MONITORING SHEET

Project #		**Mouse Strain**	
Investigator		**Mouse number**	

CLINICAL OBSERVATION	**DATE**																		
Hours/Day after MCAO																			
Activity																			
Alertness																			
Body weight (g and score)																			
Breathing																			
Coat																			
Movement																			
MCAO-related symptoms (For signs 1, also record # per minute)																			
Laser Doppler microtip insertion-related symptoms																			
Comments																			
INITIALS:																			

Signature of Chief Investigator

Date

Appendix 2 – Clinical signs severity scoring chart

CLINICAL SIGNS SEVERITY SCORE

SIGNS	0	1	2	3
Activity	normal	isolated, abnormal posture	huddled/inactive OR overactive	moribund OR fitting
Alertness	normal	dull or depressed	little response to handling	unconscious
Body weight (record weight and score)	normal weight and growth rate	reduced growth rate	chronic weight loss >15% OR failure to grow	acute weight loss>10% chronic weight loss 15% OR failure to grow
Breathing	normal	rapid, shallow	rapid, abdominal breathing	laboured, irregular, skin blue
Coat	normal	coat rough	unkempt; wounds, hair thinning	bleeding or infected wounds, or severe hairloss or self mutilation
Movement	normal	slight incoordination OR abnormal gait	incoordinated OR walking on tiptoe OR reluctance to move	staggering OR limb dragging OR paralysis
MCAO-related symptoms	normal spontaneous movements	circling towards the right (#/min)	animal spinning on its spinal axis (#/min)	crouched on all fours and unresponsive to external stimuli
Laser Doppler microtip insertion-related symptoms	normal		Sutured removed by mouse with the skull exposed or inflammation (wound redness or discharge) sighted	

SPECIAL HUSBANDRY REQUIREMENTS**

Mice are required to be housed individually post surgery.

EUTHANASIA/HUMANE EXPERIMENTAL ENDPOINT CRITERIA**

CLINICAL SIGN	ACTION
2 or 3 in one or more categories	Humanely killed
Stage 1 and do not improve (continue to have reduced weight or no decrease in circling revolution numbers)	Humanely killed

** as approved by the AEC, relevant to each specific situation

SCIENTIFIC MEASURES (ie data or tissues to be collected as part of the experimental use)

(eg animals that are killed should be weighed and have their bodies placed in labelled bags and refrigerated)

Reference: Morton, D.B. (1997) A scheme for the recognition and assessment of adverse effects in animals. In: Developments in animal and veterinary sciences, 27. *Animal Alternatives, Welfare and Ethics*. pp 235-240. Eds van Zutphen, L.F.M. and Balls, M. Elsevier Science B.V.

Appendix 3 – Antibodies used for flow cytometry

Myeloid cell stain

Target	Fluorophore	Clone	Stock conc (mg/ml)	Working dilution	Company
CD45	PE	30-F11	0.2	1 in 300	eBioscience
CD11b	FITC	M1/70	0.5	1 in 300	BioLegend
Ly6C	APC Cy7	AL-21	0.2	1 in 300	BD Pharmingen
Ly6G	BV510	1A8	0.2	1 in 300	BioLegend
F4/80	PE-Cy7	BM8	0.2	1 in 300	eBioscience
7AAD					BioLegend
Fc receptor block	-	2.4G2	0.5	1 in 100	BD Biosciences

Lymphoid cell stain

Ab	Fluorophore	Clone	Stock conc (mg/ml)	Working dilution	Company
CD45	PE	30-F11	0.2	1 in 300	eBioscience
CD3e	APC	145-2C11	0.2	1 in 300	BD Biosciences
CD4	PE-Cy7	RM4-5	0.2	1 in 300	eBioscience
CD8a	FITC	53-67	0.5	1 in 300	BD Biosciences

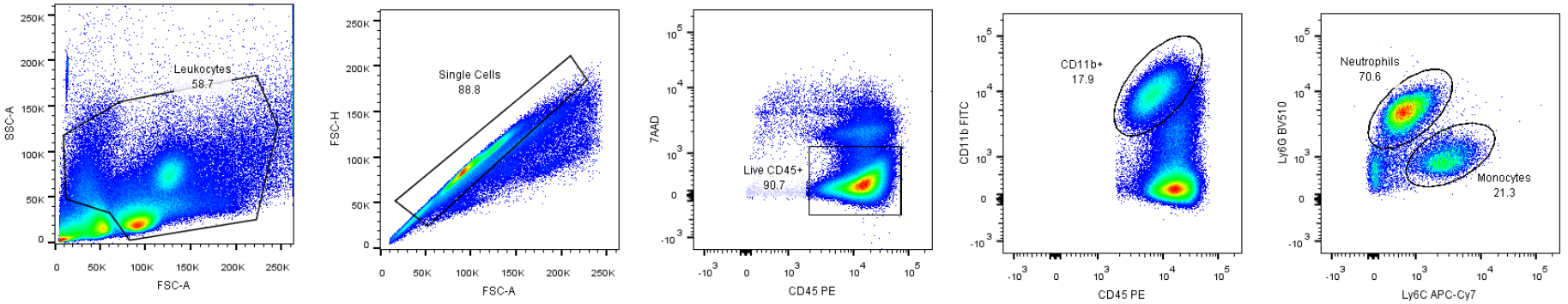
B220	e450	RA33B2	0.2	1 in 300	eBioscience
7AAD					Biolegend
Fc receptor block		2.4G2	0.5	1 in 100	BD Biosciences

iNKT cell stain

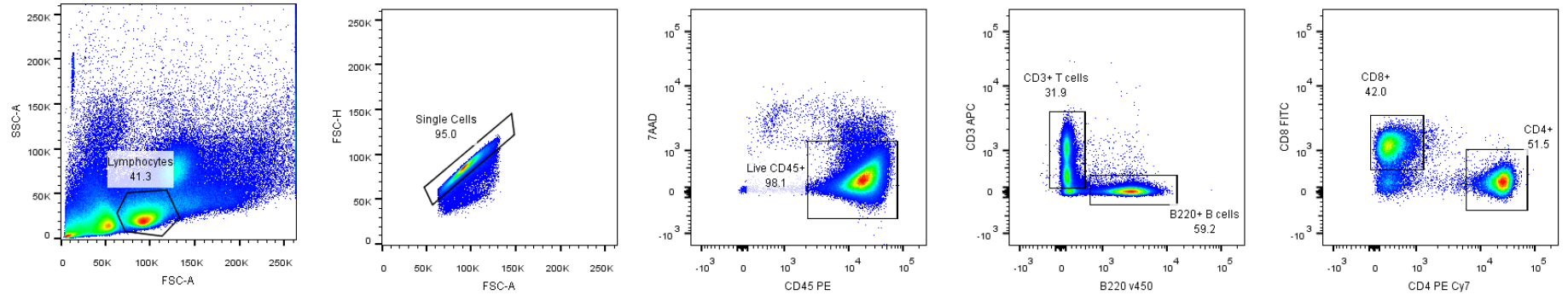
Ab	**Fluorophore**	**Clone**	**Stock conc (mg/ml)**	**Working dilution**	**Company**
CD45	APC eFluor780	30-F11	0.2	1 in 300	Invitrogen
TCR-β	APC	H57-597	0.2	1 in 300	BD Pharmingen
PBS-56-loaded CD1d-tetramer	PE		1.3	1 in 300	NIH Tetramer facility
CD69	FITC	H1.2F3	0.5	1 in 300	BD Pharmingen
IFN-γ	PE Cy7	XMG1.2	0.2	1 in 300	eBioscience
IL-10	PerCP-Cy5.5	JES5-16e3	0.2	1 in 300	eBiosciences
live/dead stain	v450			1 in 1000	eBiosciences

Appendix 4 – Flow cytometry gating strategies

1. Myeloid stain



2. Lymphoid stain



3. iNKT cell stain