



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Treatment with IgM-enriched intravenous immunoglobulins (IgM-IVIg) enhances clearance of stroke-associated bacterial lung infection

Citation for published version:

McCulloch, L, Harris, A, Malbon, A, Daniels, M, Younas, M, Grainger, JR, Allan, SM, Smith, CJ & McColl, BW 2022, 'Treatment with IgM-enriched intravenous immunoglobulins (IgM-IVIg) enhances clearance of stroke-associated bacterial lung infection', *Immunology*. <https://doi.org/10.1111/imm.13553>

Digital Object Identifier (DOI):

[10.1111/imm.13553](https://doi.org/10.1111/imm.13553)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Immunology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.





Treatment with IgM-enriched intravenous immunoglobulins (IgM-IVIg) enhances clearance of stroke-associated bacterial lung infection

Journal:	<i>Immunology</i>
Manuscript ID	IMM-2022-6994.R2
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	<p>McCulloch, Laura; University of Edinburgh, Centre for Inflammation Research Harris, Alison; University of Edinburgh, UK Dementia Research Institute Malbon, Alexandra; The University of Edinburgh Royal Dick School of Veterinary Studies, R(D)SVS Daniels, Michael; University of Edinburgh, UK Dementia Research Institute Younas, Mehwish; University of Manchester, Division of Neuroscience and Experimental Psychology Grainger, John; The University of Manchester, MCCIR/FLS Allan, Stuart; University of Manchester, Faculty of Biology, Medicine and Health Smith, Craig; Salford Royal NHS Foundation Trust, Greater Manchester Comprehensive Stroke Centre McColl, Barry; University of Edinburgh, UK Dementia Research Institute</p>
Key Words:	B Cell, Immunotherapy, Antibodies

Treatment with IgM-enriched intravenous immunoglobulins (IgM-IVIg) enhances clearance of stroke-associated bacterial lung infection

Laura McCulloch^{1*} 0000-0002-1396-6643, Alison J. Harris² 0000-0002-4114-783X, Alexandra Malbon³ 0000-0002-5144-3333, Michael J. D. Daniels² 0000-0001-7489-5626, Mehwish Younas^{4, 8} 0000-0002-3054-1997, John R. Grainger^{5, 8} 0000-0002-4052-5923, Stuart M. Allan^{4, 8} 0000-0001-9646-4456, Craig J. Smith^{6, 7, 8} 0000-0002-9078-9919 and Barry W. McColl^{2*} 0000-0002-0521-9656

¹ Centre for Inflammation Research, University of Edinburgh, Edinburgh, EH16 4TJ, ² UK Dementia Research Institute, University of Edinburgh, Edinburgh, EH16 4SB, UK, ³ Easter Bush Pathology, The Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Easter Bush Campus, Roslin, Midlothian, EH25 9RG, ⁴ Division of Neuroscience and Experimental Psychology, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, M13 9PT, ⁵ Lydia Becker Institute of Immunology and Inflammation, Division of Immunology, Immunity to Infection and Respiratory Medicine, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PT, ⁶ Greater Manchester Comprehensive Stroke Centre, Manchester Centre for Clinical Neurosciences, Manchester Academic Health Science Centre, Salford Royal NHS Foundation Trust, Salford, M6 8HD, ⁷ Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PT, ⁸ Geoffrey Jefferson Brain Research Centre, Manchester Academic Health Science Centre, Northern Care Alliance NHS Group, University of Manchester, Manchester,

*Corresponding authors laura.mcculloch@ed.ac.uk barry.mccoll@ed.ac.uk

KEYWORDS: Stroke, Immune Suppression, Pneumonia, Intravenous Immunoglobulins, B cell, macrophage

Conflict of Interest

The authors indicate that they have no potential competing interests.

Acknowledgements

We thank staff in the University of Edinburgh biological research facility on the Edinburgh Bioquarter campus for animal husbandry and technical support, in particular Will Mungall who performed all of the i.v. tail vein injections of treatment. We thank staff in the QMRI Flow Cytometry and cell sorting facility (University of Edinburgh) for their support in generating the flow cytometry data and staff at Shared University Research facilities (SURF; University of

1 Edinburgh) for their help with processing, embedding and assistance with slide scanning and Easter Bush Pathology
2 Laboratory (University of Edinburgh) for cutting and H&E staining of lung sections. Graphical abstract created with
3 Biorender.
4
5
6

7 **Funding Information**

8

9

10 This work was funded by a grant from the Medical Research Council (Grant Number MR/R001316/1). L.M. is supported
11 by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant Number
12 220755/Z/20/Z). J.R.G is funded by a Senior Fellowship awarded by The Kennedy Trust for Rheumatology Research.
13
14 B.W.M., S.M.A. and C. J. S. receive funding from Leducq Foundation Transatlantic Network of Excellence, Stroke-
15 IMPaCT (Grant Number 19CVD01), and B.W.M receives funding from the UK Dementia Research Institute which
16 receives its funding from the Medical Research Council, Alzheimer's Society, and Alzheimer's Research UK.
17
18
19
20
21
22

23 **Author contributions**

24

25

26 Conceptualization, L.M., S.M.A., C.J.S., and B.W.M.; methodology, L.M., A.J.H., A.M., M.J.D.D., S.M.A., C.J.S. and
27 B.W.M.; validation, L.M., A.J.H., A.M., M.J.D.D., M.Y., J.R.G., S.M.A., C.J.S. and B.W.M.; formal analysis, L.M.,
28 A.J.H., A.M., and B.W.M.; investigation, L.M., A.J.H., A.M., and M.J.D.D.; resources, L.M., J.R.G., S.M.A., C.J.S.,
29 and B.W.M.; data curation, L.M., A.J.H., A.M., and B.W.M. writing – original draft, L.M. and B.W.M.; writing –
30 review & editing, L.M., A.J.H., A.M., M.J.D.D., M.Y., J.R.G., S.M.A., C.J.S. and B.W.M. ; visualization, L.M., A.J.H.,
31 A.M., and B.W.M. ; supervision, L.M., S.M.A., C.J.S., and B.W.M; project administration, L.M., J.R.G., S.M.A., C.J.S.,
32 and B.W.M.; funding acquisition, L.M., J.R.G., S.M.A., C.J.S and B.W.M.
33
34
35
36
37
38
39
40
41

42 **PERMISSION TO REPRODUCE**

43

44

45 The authors provide full permission to reproduce content of the article where relevant.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Post-stroke infection is a common complication of stroke that is associated with poor outcome. We previously reported that stroke induces an ablation of multiple sub-populations of B cells and reduces levels of IgM antibody, which coincides with the development of spontaneous bacterial pneumonia. The loss of IgM after stroke could be an important determinant of infection susceptibility and highlights this pathway as a target for intervention.

We treated mice with a replacement dose of IgM-enriched intravenous immunoglobulin (IgM-IVIg) prior to and 24 h after middle cerebral artery occlusion (MCAO) and allowed them to recover for 2 d or 5 d post-surgery. Treatment with IgM-IVIg enhanced bacterial clearance from the lung after MCAO and improved lung pathology but did not impact brain infarct volume. IgM-IVIg treatment induced immunomodulatory effects systemically, including rescue of splenic plasma B cell numbers and endogenous mouse IgM and IgA circulating immunoglobulin concentrations that were reduced by MCAO. Treatment attenuated MCAO-induced elevation of selected pro-inflammatory cytokines in the lung. IgM-IVIg treatment did not increase the number of lung mononuclear phagocytes or directly modulate macrophage phagocytic capacity but enhanced phagocytosis of *Staphylococcus aureus* bioparticles *in vitro*.

Low dose IgM-IVIg contributes to increased clearance of spontaneous lung bacteria after MCAO likely via increasing availability of antibody in the lung to enhance opsonophagocytic activity. Immunomodulatory effects of IgM-IVIg treatment may also contribute to reduced levels of damage in the lung after MCAO. IgM-IVIg shows promise as an antibacterial and immunomodulatory agent to use in the treatment of post-stroke infection.

INTRODUCTION

The incidence and prevalence of stroke is continually increasing, however improvements in hyper-acute stroke care have also led to increased survival rates leading to estimates that the number of stroke survivors within the UK will double in the next 20 years-[1, 2]. However, recovery of stroke survivors is often compromised by physical disability, mental health issues and cognitive impairment [3, 4]. Infection is also a serious complication of stroke, affecting up to 30 % of patients, and infections in both the acute and chronic phase of stroke recovery are associated with increased mortality and disability, and cognitive decline [5-8]. Indeed, the impact of infection on stroke recovery is so extensive that preventing complications of stroke, such as pneumonia, was listed in the top 10 priorities for stroke research in a recent report from the Stroke Priority Setting Partnership (SPSP)[9].

Ischaemic stroke induces rapid immune suppression which is thought to contribute to infection risk in patients [10, 11]. Our previous studies identified an early loss of B cells, including spleen marginal zone (MZ) B cells, after experimental stroke with an associated reduction in circulating concentration of IgM antibodies, and the development of spontaneous bacterial pneumonia [12]. Reduced IgM concentrations were similarly measured in blood samples from patients within one week of ischaemic stroke, compared to controls of similar age and sex distribution [12, 13]. MZ B cell production of IgM provides crucial early defence against systemic infection, in particular infections caused by encapsulated bacteria [14, 15], which are implicated in pneumonia complicating stroke [16]. Therefore, reductions in B cells and their production of antibody may be an important determinant of infection susceptibility after stroke.

Intravenous immunoglobulin (IVIg) is a therapeutic agent prepared from serum pooled from thousands of healthy blood donors. Low (replacement) doses of IVIg intended to restore natural circulating antibody concentrations, can be used to treat both primary and secondary immunodeficiencies [17, 18]. At higher doses, IVIg has potent anti-inflammatory properties and is used in the treatment of autoimmune diseases including Kawasaki disease, Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy (CIDP) [19, 20]. IgM is the most potent antibacterial antibody isotype as its multi-valent structure results in high antigen avidity and lipopolysaccharide (LPS)-neutralising abilities and potent agglutination and complement fixing properties [21-23]. IVIg has been used in experimental animal models to treat bacterial pneumonias [24, 25]. Furthermore, IgM-enriched preparations of intravenous immunoglobulin (IgM-IVIg) showed improved efficacy in reducing bacteraemia in animal models of pneumonia and sepsis [26, 27] and have progressed to clinical trials with improved outcome reported in specific patient sub-groups [28-30]. Given the

antibacterial properties of IgM, and the depleted circulating IgM concentration in patients after acute ischaemic stroke, IgM-IVIg may be a useful therapeutic agent for pneumonia complicating stroke.

Here we show that treatment with IgM-IVIg enhanced the clearance of bacteria and reduced infection-associated pathology in the lung of animals after experimental stroke. Treatment modulated stroke-induced immune alterations, including a rescue of plasma B cells and immunoglobulins systemically and a reduction in lung pro-inflammatory cytokine concentration. IgM-IVIg treatment increases total antibody concentration via the provision of human antibodies, which likely contributes to enhanced phagocytosis of bacteria by increasing opsonisation, as we have demonstrated *in vitro*. This suggests that both direct effect of IgM-IVIg on bacteria and indirect effects modulating the immune environment, contribute to the reduced infectious burden in IgM-IVIg treated mice after MCAO. In summary, these data demonstrate that the loss of B cell-associated antibacterial immune defence is an important contributing factor to the onset of post-stroke infection, as targeted therapeutic intervention to replace B cell function reduces infectious burden. These studies provide proof of concept for the use of IgM-IVIg as an adjunct therapeutic strategy to lessen the impact of pneumonia in patients after stroke.

MATERIALS AND METHODS

Animals

Male 8–12-week-old C57BL/6J mice (Charles River Laboratories, UK) were used in all experiments. Mice were maintained under specific pathogen free (SPF) conditions and a standard 12 h light/dark cycle with unrestricted access to food and water. Mice were housed in individually ventilated cages in groups of up to 6 and were acclimatized for a minimum of 1 week before procedures. All animal experiments were carried out under the authority of a UK Home Office Project Licence in accordance with the ‘Animals (Scientific procedures) Act 1986’ and Directive 2010/63/EU and were approved by the University of Edinburgh’s Animal Welfare and Ethics Review Board. Experimental design, analysis and reporting followed the ARRIVE 2.0 guidelines [31]. The IMPROVE guidelines were followed to refine procedures and improve animal welfare after MCAO [32].

Experimental stroke model

Middle cerebral artery occlusion (MCAO) surgery was performed under isoflurane anaesthesia induced at 3.5% and maintained at 1.4% with O₂ (200 ml/min) and N₂O (400 ml/min) by insertion of a 6-0 nylon monofilament with a 2-mm silicone coated tip (210 µm diameter; Doccol) through the external carotid artery and advanced through the internal carotid artery to occlude the MCA. The filament was withdrawn after 30 min to allow reperfusion, the neck wound sutured and the animals recovered. Topical local anaesthetic (Lidocaine 4%) was applied to the wound. For sham surgery, the filament was advanced to the MCA and immediately retracted. Sham-operated animals remained anaesthetized for 30 min and recovered as above to ensure total time under anaesthetic was the same as MCAO-operated animals. Core body temperature was maintained at 37±0.5 °C throughout the procedure with a feedback controlled heating blanket (Harvard Apparatus). Animals were recovered for 2 or 5 d then euthanised using a rising concentration of CO₂ followed by exsanguination.

Treatment

IgM-IVIg (Pentaglobin; Biotest) was aliquoted neat and given to animals at 250 mg/ kg body weight. HSA (Biotest) was given to animals at 50 mg/ kg. Dilution of control was advised by manufacturer to replicate the use of HSA in clinical trials, where a 5 x lower concentration is used to mimic the foaming, colour and viscosity of IgM-IVIg and therefore assists with protecting blinding when used as a placebo. Treatments were numbered and randomly assigned to animals allowing the surgeon to be blinded to the treatment throughout procedures and welfare assessment during recovery. Treatment was administered intravenously via the tail vein; All animals were given first dose at 8am prior to MCAO or sham surgeries which were carried out throughout the morning and again at 24 h post-surgery.

Tissue harvests

Tissue harvests were carried out in a laminar flow cabinet under aseptic conditions. Following euthanasia, the peritoneal cavity was carefully opened and a 23G needle with a 1cm length of sterile polyethylene tubing (Thermo Fisher) over tip was inserted through the diaphragm. Dulbecco's Phosphate Buffered Saline (800 µl; dPBS; Thermo Fisher) was flushed into the pleural cavity then retrieved using a 1 ml syringe and repeated twice more to obtain a pleural cavity lavage sample. A cardiac blood sample, anti-coagulated with 3.8% w/v tri-sodium citrate, was taken. Half of the sample was

1 stored on ice for bacteriological analysis, the remaining half was spun at 400 x G for 10 min, plasma removed and stored
2 at -80 °C for analysis of soluble mediators. A clean 23G needle with polyethylene tubing was inserted into the trachea
3 and tied in place. dPBS (800 µl) was flushed into the lungs and retrieved using a 1 ml syringe to obtain a bronchial-
4 alveolar lavage (BAL) sample. BAL samples were centrifuged at 800 x G for 10 min and supernatant stored as BAL
5 fluid (BALF). Lungs, heart and thymus were removed from the thoracic cavity as one, with the needle still tied in the
6 trachea. The inferior lobe of the right lung was tied off and removed into a gentleMACS™ C tube (Miltenyi Biotech)
7 containing 1 ml of sterile dPBS for analysis of bacterial load. The middle and superior lobes of the right lung were tied
8 off and removed into a cryovial and snap frozen on dry ice. 800 µl of 4% buffered paraformaldehyde (PFA) was used
9 to inflate the left lung lobe via a needle in the trachea and intact heart, thymus, left lung were dropped into a bijou tube
10 of 4 % PFA. Tissues were immersion fixed for 24 h. The left lung lobe was then dissected from remaining tissue,
11 processed and routinely embedded in paraffin blocks for histology. Next, the spleen was dissected, weighed and halved.
12 Half was snap frozen in a cryovial over dry ice for immunostaining whilst the other half was taken into a bijou tube and
13 stored on ice for flow cytometry. Finally, the brain was removed and frozen in cold isopentane over dry ice and stored
14 in a bijou tube at -80 °C.

15 Flow cytometry of the lungs at 2 d post sham or MCAO surgery was carried out in a separate cohort of animals. Animals
16 were euthanised in a rising concentration of CO₂ followed by exsanguination. Pleural cavity lavage was taken as
17 previously described. BAL was also taken as above, however the lungs were flushed three times with 800 µl dPBS to
18 maximise cellular harvest. The entire lung was dissected into 1 ml cold RPMI 1640 medium (RPMI; Thermo Fisher).
19 Samples were stored on ice until processed for flow cytometry.

20 Exclusion criteria

21 Post-hoc exclusions of tissues for analysis were applied as follows. Animals that did not have infarcts affecting the
22 cortex and striatum after MCAO surgery were excluded from the study as [this indicated a technically](#) unsuccessful
23 MCAO (n=5; 3 HSA, 2 IgM-IVIg). For lung pathology, lungs that were not fully inflated before fixation and showed
24 crush artefact were excluded from analysis (2 d Sham IgM-IVIg n=1; MCAO HSA n=1; 5 d HSA n=1; IgM-IVIg n=3).
25 Plasma samples from failed cardiac punctures were excluded from multiplex ELISA analysis (2 d Sham IgM-IVIg n=2;
26 MCAO HSA n=1). BALF samples which had low/ undetectable levels of protein were excluded from multiplex ELISA
27 analysis (2 d Sham HSA n= 2; IgM-IVIg n=3; MCAO HSA n=2; MCAO IgM-IVIg n=1). Samples were excluded from
28

1 analysis of spleen flow cytometry where staining of one or more antibodies in the cocktail failed (2 d Sham HSA n= 1;
2 IgM-IVIg n=1; MCAO HSA n=2; MCAO IgM-IVIg n=1). Lung samples with cell harvest $< 1 \times 10^7$ total cells or where
3 staining of one or more antibodies in the cocktail failed were excluded from flow cytometry analysis failed (2 d Sham
4 HSA n= 2; IgM-IVIg n=1; MCAO HSA n=2; MCAO IgM-IVIg n=1).
5
6
7
8

9 **Infarct quantification**

10
11
12
13
14
15 Coronal cryosections (20 μm) were taken at 400 μm intervals from fresh frozen brains and stained with Harris
16 Haematoxylin stain-acidified (CellPath) and Eosin Y (CellPath) (H&E). Briefly, sections were hydrated by soaking for
17 2 min each in 100% > 95% > 80% > 70% ethanol, then distilled water. Slides were stained with haematoxylin for 5 min,
18 washed in distilled water, immersed in Scott's tap water (CellPath) for 30 s followed by a 30 s wash in distilled water.
19 Sections were then stained with eosin for 3 min and washed with distilled water. Sections were dehydrated by soaking
20 for 2 min in 70% > 80% > 95% > 100% ethanol, fixed for 5 min in xylene, then coverslips were applied using DPX
21 mountant (Merck). Sections were scanned on an Axio Scan.Z1 (Zeiss) and visualized using Zeiss Zen 3.2 blue edition
22 software. Sections identified by neuroanatomical landmarks at 8 specific coronal levels for quantification as described
23 in [33]. The infarcted area ~~for each region (cortex, striatum/pallidum, hippocampus, thalamus and other)~~ at each of the
24 8 coronal levels was quantified using Fiji (U. S. National Institutes of Health, USA <https://imagej.nih.gov/ij/>) and
25 summed to give a total for each section. These values were plotted against distance from rostral pole and area under the
26 curve calculations performed in Prism (GraphPad Prism v9) to calculate infarct volume.
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 **Quantifying lung bacterial load**

42
43
44
45 The inferior lobe of the right lung was homogenised in 1 ml sterile dPBS using a gentleMACS (Miltenyi Biotech). In a
46 laminar flow hood, lung homogenates and blood, collected as described above, were serially diluted from neat to 10^{-6}
47 in sterile dPBS and dilutions plated onto Columbia agar with horse blood for a general bacteria screen or MaConkey's
48 agar to assess ~~gut-derived enteric or gram negative~~ bacteria. Plates were incubated at 37 °C for 48 h and bacterial colonies
49 counted.
50
51
52
53
54
55
56
57
58
59
60

Lung pathology

Sections (4 μm) were cut from inflated left lungs embedded in paraffin wax blocks. Sections were dewaxed in xylene and then stained with H&E as above. Pathology was assessed by a board-certified veterinary pathologist, who was blinded to treatment groups, using scoring criteria described in **Supplementary Fig. 2**.

Multiplex ELISA

Human and mouse immunoglobulins and mouse cytokines were measured in plasma samples and BALF using MILLIPLEX® multiplex assays. Coded samples were randomised across plates for analysis to ensure the researcher was blinded to treatment group. The MILLIPLEX®MAP Human Isotyping Magnetic Bead Panel- Isotyping Multiplex Assay (HGAMMAG-301K-06, Merck) was used to measure IgG1, IgG2, IgG3, IgG4, IgA and IgM. Plasma samples were diluted 1/ 16,000 before adding to plate. Total protein concentration of BALF was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher). BALF volume equivalent to 25 μg of protein was added to each well to normalise across groups accounting for any effects of imbalances in protein concentration due to treatments. MILLIPLEX®MAP Mouse Isotyping Magnetic Bead panel (MGAMMAG-300K, Merck) was used to measure IgG1, IgG2a, IgG2b, IgG3, IgA, IgM. Many samples had concentrations of IgG2a below the detection range of the standard curve and so results for this analyte are not reported. Plasma samples were added to plate at a 1/25,000 dilution and BALF samples were added to plate as previous. MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MCCYTOMAG-70K, Merck) was used to measure GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-12(p40), IL-33 and TNF α . Many samples had concentrations of IL-1 β below the detection range of the standard curve and so results for this analyte were not reported. Plasma samples were added to plate at a 1/10 dilution and BALF samples were added to plate as previous. In all assays, samples were assayed as single replicates and all samples, standards and quality controls were prepared in accordance with the manufacturer's instructions. Samples were incubated with beads on a plate for 1 h (isotyping assay) at room temperature or overnight (cytokine assay) at 4° C and washes carried out using a magnetic plate washer. Plates were analysed using a Magpix™ Luminex® machine and Luminex xPonent® software version 4.2, with a sample volume of 50 μl per well and a minimum of 50 events counted per sample.

Flow cytometry

Spleens were disrupted with a syringe plunger and passed through a 100 μ M cell sieve. Red blood cells were lysed with red blood cell lysing buffer Hybri-MaxTM (Sigma) before counting live cells with trypan blue (Sigma) exclusion. Splenocytes were plated at 2 million cells/well and incubated with Zombie UV (Biolegend) for dead cell discrimination, then Fc blocked with normal mouse serum (Thermo Fisher) and TruStain FcX α mouse CD16/32 (Biolegend) before staining with antibody cocktails. B cell subsets and other lymphoid and myeloid populations were identified using combinations of the following anti-mouse antibodies: CD3 PerCP-Cy5.5, CD3 BV785, CD11b BV711, CD11c AF488, CD20 PerCP-Cy5.5, CD24 PerCP-Cy5.5, CD43 PE-Cy7, CD45 BV510, CD64 PE, I-A/I-E (MHCII) BV785, IgD BV711, IgM BV421, Ly6C APC-Cy7, Ly6G PE-Cy7, NKp46 PE/Dazzle594 (all Biolegend); CD19 BUV395, CD21/35 PE-CF594, CD23 BB515, SiglecF AF647 (all Becton Dickinson); CD5 APC, CD93 APC, CD138 PE (all Miltenyi Biotec).

Lungs were removed from RPMI, chopped with scissors and 2 ml of enzyme cocktail [0.8 mg/ml Collagenase V (Sigma), 0.625 mg/ml Collagenase D (Roche), 1 mg/ml Dispase (Gibco), 30 μ g/ml DNase (Roche) in RPMI] added before 45 min incubation at 37°C on a shaking incubator. Digested lung homogenate was passed through a 100 μ m cell strainer. Red blood cells were lysed with red blood cell lysing buffer Hybri-MaxTM (Sigma) before counting live cells with trypan blue (Sigma) exclusion. Lung cells were plated at 2 million cells/well and incubated with Zombie UV (Biolegend) for dead cell discrimination, then Fc receptors blocked with normal mouse serum (Thermo Fisher) and TruStain FcX α mouse CD16/32 (Biolegend) before staining with antibody cocktails. B cell subsets and other lymphoid and myeloid populations were identified using combinations of the following anti-mouse antibodies: CD3 BV785 or PerCP-Cy5.5, CD11b BV711, CD20 BV421, CD43 PE-Cy7, CD45 BV510, CD64 PE, Ly6C APC-Cy7, Ly6G PE-Cy7, MHC II (I-A/ I-E) BV785 or APC-Cy7, NKp46 PE/ Dazzle 594 (all Biolegend); CD5 APC (Miltenyi Biotec), CD11c BUV395 and Siglec F AF647 (all Becton Dickinson).

Cells were fixed using a formaldehyde-based fixation buffer (Biolegend) and analysed within 48 h on a 5-laser LSR Fortessa (Becton Dickinson). Data analysis was performed on Flowjo (Treestar), with FSCA/FSCH doublet exclusion and dead cell exclusion. Spleens were weighed then divided into two in order to perform both flow cytometry and IHC. The weight of each half as a proportion of the total weight was used to convert the cell count for flow cytometry into an estimated total for the whole spleen as follows:

1 Absolute cell count = [population as % of live leukocytes x trypan blue total cell count x (total spleen weight/ weight of
1 spleen used for cell isolation)].
2
3
4

84 **Spleen immunohistochemistry**

9

10 Acetone fixed 6 µm spleen sections were blocked with normal goat serum (Sigma). For detection of germinal centres,
11 sections were labelled with rat anti-mouse B220 (Biolegend RA3-6B2) followed by goat anti-rat AF594 secondary
12 antibody (Thermo Fisher). Sections were washed and subsequently incubated with AF647-conjugated lectin peanut
13 agglutinin (PNA; Thermo Fisher) in the presence of sodium azide and bovine serum albumin overnight at 4°C.
14
15
16
17
18
19

23 ***In vitro* phagocytosis assay**

24

25 Bone marrow single cell suspensions were made by flushing cells from the femurs and tibias of C57Bl/6J mice into
26 sterile dPBS. Cells were resuspended in RPMI containing 10% foetal bovine serum (Thermo Fisher) and 1% Penicillin-
27 Streptomycin (Thermo Fisher) and 25% conditioned media from L929 cells and incubated at 37°C with 5% CO₂.
28 Cultures were supplemented with fresh medium at 24 h. After 7 d, differentiated bone marrow-derived macrophages
29 (BMDM) were transferred to a 96 well plate at 5 x10⁵ cells/ ml and incubated for 24 h prior to use in assay. Cells were
30 either untreated or given treatment 0.25 mg/ml or 2 mg/ ml IgM-IVIg, control 0.05 mg/ml or 0.4 mg/ml HSA and
31 returned to incubator for 4 h. After incubation with treatment, cells were either “washed” in PBS with fresh RPMI
32 replaced or left “unwashed” and proceeded directly to next step of assay. Plate was inserted into an IncuCyte® S3 Live-
33 Cell Analysis System (Sartorius) for a single phase-contrast read in order to determine cell count. 10 µM Cytochalasin
34 D was added to negative control wells for 10 min, then pHrodo® Red *Staph. aureus* BioParticles® (Thermo Fisher)
35 were diluted 1:4 in RPMI and 10 µl added to each well of cells. Plates were then returned to the IncuCyte® and imaged
36 every 5 min for 2.5 h on both phase-contrast and ‘Red’ channels (400 milliseconds exposure time). Cell-by-cell analysis
37 was performed on Incucyte® analysis software, using a mask to automatically identify (1) all BMDM, using phase
38 contrast and (2) “red objects” i.e. BMDM that have phagocytosed pHrodo® Red bioparticles. Thresholds for size and
39 fluorescence were adjusted during a pilot experiment, to eliminate non-specific signal while maximising detection of
40 phagocytosing cells, with visual inspection of multiple FOVs to check sensitivity and accuracy. Integrated density of
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 red fluorescent signal was measured at each time point and was normalised to the total number of macrophages present
2 in the well at the beginning of assay.
3
4
5
6
7

8 **Experimental design and statistical analysis**

9
10 Sample sizes were estimated from previous data on reduced IgM concentration and reduced splenic MZ B cells after
11 experimental stroke [12] using power analysis carried out on InVivoStat software (<http://invivostat.co.uk/>) to be
12 sensitive to a 35% effect of experimental stroke at 80% statistical power at a 5 % significance level. Animals were ear
13 notched for identification and randomised to experimental groups (sham or MCAO surgery and treatment) using a
14 computer-based random number generator (<https://www.randomizer.org/>) and treatments were administered in a blinded
15 manner and allocation of treatment was concealed throughout study. Animals were given an experimental identifier and
16 all samples were analysed using this coded identifier. Data was unblinded for analysis after experimental work was
17 complete.
18
19
20
21
22
23
24
25
26

27 For normally distributed data, differences were tested using unpaired Student's t-test or 2- way analysis variance
28 (ANOVA) with Tukey multiple comparison test. Results were displayed as mean \pm standard deviation. When data were
29 non-normally distributed, log transformation was used to normalise data sets and data were then analysed as previous.
30 Non-parametric tests, such as Mann-Whitney test, were used to analyse data produced from scoring systems and results
31 were displayed as violin plots. Data were analysed using GraphPad Prism. In all experiments, values of $P \leq 0.05$ were
32 accepted as statistically significant.
33
34
35
36
37
38
39
40
41
42
43
44

45 **Data availability**

46 Data supporting the findings of these studies are available from authors upon reasonable request.
47
48
49
50
51
52

53 **RESULTS**

54 **Low dose IgM-IVIg enhances clearance of spontaneous bacterial infection after experimental stroke**

55
56 To determine if preventing a stroke-induced loss of immunoglobulins is an effective therapeutic strategy to limit
57 stroke-associated bacterial pneumonia, animals were given a low dose of IgM-IVIg (250 mg/kg) prior to experimental
58
59
60
61

1 stroke induced by transient MCAO or sham surgery and at 24 h post-surgery. At 2 d post-MCAO there was a trend for
2 mice treated with IgM-IVIg to have reduced general (blood agar; **Fig. 1a**,) and gut-derived enteric and gram negative
3 bacteria (MacConkey agar; **Fig. 1b**) lung bacterial burdens in comparison to HSA treated mice. By 5 d post-MCAO,
4 both general (**Fig. 1c**) and gut-derived (**Fig. 1d**) lung bacterial burdens were significantly reduced in mice treated with
5 IgM-IVIg. Spontaneous bacterial pneumonia after stroke is known to be associated with stroke severity [34]. As
6 expected, treatment with IgM-IVIg at a low/replacement dose, did not alter brain infarct volume size at 2 d or 5 d post-
7 MCAO (**Supplementary Fig. 1a-d**). This confirms that reduced bacterial counts were a direct effect of the treatment
8 and not due to alterations in primary stroke pathology.

9 Lung pathology was assessed using a scoring system that accounted for perivascular edema, inflammation in the
10 perivascular and peribronchiolar regions, macrophages in the alveolar spaces and macrophages/ hypercellularity in the
11 interstitium (**Supplementary Fig. 2**). Representative images of whole left lung slide scans used to assess pathology in
12 sham and MCAO-operated animals treated with IgM-IVIg or HSA are shown in Figure 1E. Consolidation with
13 clustering of inflammatory cells can be observed in both treatment groups at 2d post MCAO (arrows; Figure 1E). At
14 2 d post-surgery, a low level of lung pathology was seen in sham-operated animals, regardless of treatment. Sham
15 animals only scored under the categories for perivascular edema and a homeostatic level of macrophages in the
16 perivascular space. Previous studies have shown exposure to inhaled isoflurane can enhance the development of
17 neurogenic pulmonary edema and therefore may be causative of the low levels of edema seen in sham animals [35]. At
18 2 d post-MCAO, lung pathology scores were significantly increased in HSA treated animals, whereas in IgM-IVIg
19 treated animals there was no difference to sham-operated controls (**Fig. 1 e, f**). A trend for reduced lung pathology
20 scores in IgM-IVIg treated animals was also observed at 5 d after MCAO (**Fig. 1 e, g**).

21 Associations between lung pathology and bacterial burden, or stroke severity, were explored using these data. There
22 was an association between lung pathology and bacterial burden at 2 d and 5 d post MCAO (**Fig. 1 h, i**) and between
23 lung pathology and infarct volume at 2 d post MCAO in HSA treated animals. The association between lung pathology
24 and bacterial burden was not observed in IgM-IVIg treated animals but there was an association between lung pathology
25 and infarct volume at 2 d post MCAO. Consistent with previous data showing that IgM-IVIg did not alter brain infarct
26 volume, this indicates that IgM-IVIg treatment modulates the association between bacterial burden and lung pathology
27 observed in HSA treated controls. Furthermore, as bacterial burden and lung pathology were altered to different degrees
28 in IgM-IVIg treated animals, some lung pathology may be driven by the stroke itself, in addition to that caused by
29 infection.

1 Although high-dose IVIg treatment is typically associated with inhibitory immunoreceptor signalling, IVIg have a range
2 of known immunomodulatory properties, including the potential to also trigger activating Fc receptor pathways. There
3 was a possibility that this treatment could have detrimental effects on recovery from stroke (e.g. if harmful hyper-
4 immune responses were triggered). No differences in general welfare score (**Supplementary Fig. 1e, f; Supplementary**
5 **Table 1**) or neurological deficit (Bederson Score; **Supplementary Fig. 1g, h; Supplementary Table 1**) were observed
6 at either time point. Treatment with IgM-IVIg did result in higher body weight loss 2 d after both sham and stroke
7 surgery (**Supplementary Fig. 1i**) however this normalised by 5 d post-stroke (**Supplementary Fig. 1j**). Weight loss
8 data was only analysed from animals that survived to endpoint. As mortality was reduced in IgM-IVIg treated animals
9 at 2 d and 5 d post-MCAO we can conclude that reduced body weight at 2 d in IgM-IVIg treated animals was not
10 associated with reduced survival. There was no difference in mortality between treatment groups at 2 d post-stroke,
11 however reduced mortality was seen in IgM-IVIg treated mice at 5 d (**Supplementary Fig. 1k**).

12 These data demonstrate that low dose IgM-IVIg enhances the clearance of spontaneous bacterial infection after
13 experimental stroke. This confirms that stroke-induced deficits to B cell function are important in conferring
14 susceptibility to bacterial pneumonia and intervention strategies targeting B cell pathways after stroke may be of
15 important therapeutic benefit. IgM-IVIg treatment also led to reduced lung pathology in comparison to HSA treated
16 animals with equivalent bacterial burden or stroke severity. Furthermore, although an early increase in weight loss is
17 seen in IgM-IVIg treated animals, there were no adverse effects of IgM-IVIg treatment on primary stroke pathology,
18 welfare or mortality.

29 **Distribution of human immunoglobulins in IgM-IVIg treated animals**

30 We next wanted to determine that IgM-IVIg treatment was present both systemically, and within the lung, after
31 intravenous administration. As IgM-IVIg is pooled from human donors, multiplex ELISA to detect human
32 immunoglobulins can be used to distinguish the presence of IgM-IVIg from endogenously produced mouse
33 immunoglobulin. Human immunoglobulin subsets IgM, IgG1, IgG2, IgG3, IgG4 and IgA were significantly increased
34 in the plasma of animals receiving IgM-IVIg treatment at 2 d post MCAO or sham surgery (**Fig. 2a-f**). Low levels of
35 some immunoglobulin isotypes were detected in HSA treated animals, indicating a mild cross reactivity of assay with
36 endogenous mouse immunoglobulin, however the presence of human immunoglobulins were clearly detected above this
37 background in IgM-IVIg treated animals (**Fig. 2a-f**). Human immunoglobulins were also detected in BALF, although

at relatively lower concentrations than detected in plasma (**Fig. 2g-l**). The proportion of IgM, IgA and total IgG detected in plasma and BALF was compared to the original IgM-IVIg treatment. There was proportionally less IgM in the BALF than was detected in the plasma or in the original IgM-IVIg treatment (**Fig. 2m**). These data demonstrate that IgM-IVIg-derived human immunoglobulins can be detected both systemically, and within the lung, after intravenous administration. However there may be relatively less access of human IgM to the lung compartment than other immunoglobulin isotypes.

Low dose IgM-IVIg rescues splenic plasma B cells and endogenous antibody production after experimental stroke

Experimental stroke is known to result in lymphopenia, and in particular a loss of B cells and circulating antibodies, which is thought to contribute to the onset of spontaneous bacterial pneumonias [12]. Furthermore, IgM signalling via the Fc μ receptor can have autocrine effects on B cell development and activation [36]. We therefore characterised the effects of experimental stroke on B cell subpopulations and endogenous mouse immunoglobulins and determined if these were altered by treatment with IgM-IVIg. Flow cytometry was used to detect splenic B cells (**Fig. 3a**), 2 d after experimental MCAO or sham surgery and treatment with HSA or IgM-IVIg. A reduction in spleen weight was observed 2 d after MCAO as previously reported [12] (**Supplementary Fig. 3a**). Treatment with IgM-IVIg did not alter spleen weight 2 d or 5 d after MCAO suggesting no generalised effect of treatment on systemic immune cellularity (**Supplementary Fig. 3a, b**). In confirmation, MCAO-induced reduction in total spleen leukocytes was also unaltered by treatment with IgM-IVIg (**Fig. 3b**). Furthermore, total CD19⁺ splenic B cells were reduced by MCAO, but unaffected by treatment with IgM-IVIg (**Fig. 3c**).

We next determined if IgM-IVIg affected B cell sub-populations within the CD19⁺ splenic B cell pool. Experimental stroke resulted in a reduction in the proportion of antibody secreting CD138⁺ plasma cells and plasmablasts. Treatment with IgM-IVIg rescued plasma B cell abundance after MCAO (**Fig. 3d**). In contrast, the proportion of T1 transitional B cells was unaffected by stroke, but reduced in animals treated with IgM-IVIg (**Fig. 3e**). T2/T3 transitional B cells, naïve B cells, pooled GC and memory B cells, marginal zone B cells and innate-like B1 B cells were unaffected by treatment with IgM-IVIg (**Supplementary Fig. 3c-g**). There was a trend for more mature germinal centres (GC) to be present in spleens of IgM-IVIg treated animals 2 d after either MCAO or sham surgery (**Supplementary Fig. 3h, i**).

1 Multiplex ELISA was used to measure endogenous mouse immunoglobulins in plasma. Treatment with IgM-IVIg
2 restored circulating concentration of mouse IgM (**Fig. 3f**) and IgA (**Fig. 3g**) in animals 2 d after MCAO, whereas
3 concentrations of mouse IgG1, 2b and 3 (**Fig. 3h, i, j**) were unaffected by treatment.
4
5
6

7 In summary, treatment with IgM-IVIg in experimental stroke prevented the MCAO-induced reduction in plasma B cells
8 abundances and circulating IgM and IgA antibody concentrations, reductions in transitional B cells, and had no effect
9 on other antibody secreting cellular subsets (B1 and MZ B cells). Taken together, these data suggest treatment with
10 IgM-IVIg may drive T1 B cells into the GC response producing increased output of plasma B cells and a rescue of
11 endogenous mouse IgM and IgA circulating antibody concentration.
12
13
14
15
16
17
18
19
20

21 **Low dose IgM- IVIg does not modulate lung B cells or endogenous immunoglobulins in the lung after** 22 **experimental stroke** 23 24

25 As experimental stroke results in spontaneous bacterial pneumonia, we next investigated if similar effects of MCAO
26 and IgM-IVIg treatment occurred in antibody secreting B cell populations and immunoglobulin concentrations within
27 the lung. Flow cytometry was used to detect antibody-secreting B cell populations in the lung (**Fig. 4a**). MCAO induced
28 an increase in the number of leukocytes in the lungs of mice at 2 d and this was not altered by treatment with IgM-IVIg
29 (**Fig. 4b**). In contrast to splenic B cell numbers (**Fig. 3c**), total CD20⁺ B cells were not reduced by MCAO but were
30 reduced by IgM-IVIg treatment in both sham and experimental stroke animals (**Fig. 4c**). Furthermore, MHC II⁺ mature
31 plasma cells in the lung increased after MCAO, most likely in response to bacterial infection (**Fig. 4d**). A trend for lower
32 levels of plasma cells was seen in the lungs of IgM-IVIg treated mice after MCAO, but not sham, surgery (**Fig. 4d**). As
33 there was no effect in sham animals, this may reflect the lower levels of bacterial infection in IgM-IVIg treated animals
34 after MCAO, rather than a direct effect of IgM-IVIg on plasma cell numbers. Innate-like B1 cells can rapidly produce
35 antibody in response to infection and are an additional and important source of antibody in the lung [37]. B1 B cells
36 were unaltered by either MCAO or IgM-IVIg (**Fig. 4e**).
37
38
39
40
41
42
43
44
45
46
47
48
49
50

51 We next examined the effects of MCAO and IgM-IVIg treatment on endogenous mouse immunoglobulin concentrations
52 in the lung. Despite a MCAO-induced increase in plasma cells within the lung, there was no proportional increase in
53 mouse IgM, IgA, IgG1, IgG2b or IgG3 in the BALF (**Fig. 4f, g, h, i, j**). There was also no additional effect of treatment
54 with IgM-IVIg, with the exception of reduced concentration of IgG3 in sham-operated animals only (**Fig. 4j**). This
55
56
57
58
59
60

1 contrasts with circulating immunoglobulin concentrations where reduced levels of IgM and IgA after MCAO are
2 restored by treatment with IgM-IVIg (**Fig. 3f, g**).
3
4

5 These data demonstrate the differential effects of MCAO and IgM-IVIg on endogenous mouse cellular and
6 immunoglobulin profiles within the lung versus systemically, as measured in the spleen. Whereas systemically, MCAO
7 reduces B cell numbers, antibody-secreting plasma cell numbers and circulating IgM and IgA, there was no reduction
8 in B cells or antibody concentrations observed in the lung. There was an increase in plasma cells in the lung after MCAO
9 but this was not accompanied by an increase in immunoglobulin concentrations which may indicate some MCAO-
10 induced functional deficits. Furthermore, treatment with IgM-IVIg prevented a loss of splenic plasma cell numbers and
11 IgM and IgA concentration, but has no effect on lung plasma cells or immunoglobulins. Despite the lack of effect of
12 IgM-IVIg treatment on mouse immunoglobulin concentration in the lung, there is an increase in total antibody due to
13 the presence of human immunoglobulin in the BALF (**Fig. 2 g-l**) from the IgM-IVIg.
14
15
16
17
18
19
20
21
22
23
24
25
26
27

28 **Effect of low dose IgM-IVIg on lung mononuclear phagocytes**

29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Antibodies are capable of directly lysing bacteria however they can also aid clearance by coating the surface of bacteria enhancing uptake by phagocytes in a process known as opsonophagocytosis [38]. Therefore, we next examined the effects of MCAO and IgM-IVIg treatment on lung mononuclear phagocytes. Flow cytometry was used to detect myeloid cells in the lung (**Fig. 5a**). The lung contains two major populations of macrophages, the alveolar macrophage and the interstitial macrophage, although heterogeneity exists within these broad categorisations [39]. Alveolar macrophages, which are the majority population, were not affected by MCAO or by treatment with IgM-IVIg (**Fig. 5b**). In contrast, interstitial macrophages were reduced after MCAO and further reduced after treatment with IgM-IVIg (**Fig. 5c**). Interstitial macrophages can be repopulated from blood-derived monocytes [39]. There was no difference in total monocyte numbers, or Ly6C^{lo} and Ly6C^{hi} functionally distinct monocyte subsets in the lung after MCAO or IgM-IVIg treatment (**Fig. 5d, e, f**), suggesting that a lack of precursors was not responsible for the reduction in interstitial macrophages. MCAO also resulted in increased numbers of neutrophils (**Supplementary Fig. 4a**) and reduced the number of eosinophils in the lung (**Supplementary Fig. 4b**). Lung T cells, dendritic cells and NK cells were unaffected by either MCAO or IgM-IVIg treatment (**Supplementary Fig. 4c, f**).

1 We next used multiplex ELISA to profile the cytokine environment in the lung after MCAO and treatment with IgM-
2 IVIg. MCAO induced an increase in GM-CSF (**Fig. 5g**) and TNF α (**Fig. 5h**) concentrations in the BALF, likely a
3 response to post-stroke bacterial pneumonia. Treatment with IgM-IVIg reduced the concentrations of these
4 inflammatory cytokines. Stroke also increased the concentration of IL-33 in the BALF, however this was unaffected by
5 IgM-IVIg treatment (**Supplementary Fig. 5a**). There were no significant differences in concentrations of IL-6, IFN γ ,
6 IL-1 α , IL-2, IL-4, IL-10 and IL-12. However IL-6 and IFN γ showed the same trend of a MCAO-induced increase which
7 was blunted after treatment with IgM-IVIg (**Supplementary Fig. 5b-h**).

8 We next wanted to understand if IgM-IVIg might influence macrophage function, notably, anti-bacterial phagocytic
9 activity given that lung macrophages will be exposed to elevated exogenous immunoglobulin levels after IgM-IVIg
10 treatment (**Fig. 2**). Macrophages express multiple Fc receptors which can have both activating and inhibitory effects
11 [40], therefore it is plausible that IgM-IVIg could exert immunomodulatory effects on the macrophages themselves
12 which alters their phagocytic capabilities. Furthermore, the presence of intravenous immunoglobulins at high doses can
13 inhibit phagocytosis via saturating macrophage Fc receptors [41]. To distinguish direct immunomodulatory effects of
14 IgM-IVIg on macrophage phagocytosis from the effects on opsonisation induced uptake of bacteria, we incubated bone
15 marrow-derived macrophages (BMDM) with IgM-IVIg for 4 h. IgM-IVIg was then washed from cells and replaced
16 with media, or left in the well with cells, and pHrodo® Red *S. aureus* BioParticles® were added. Phagocytosis of pHrodo
17 bioparticles was then measured over 2.5 h. A low dose of IgM-IVIg (0.25 mg/ml) enhanced phagocytosis of pHrodo
18 bioparticles in comparison to control cells that were pre-treated with media only, whereas a high dose of IgM-IVIg (2
19 mg/ml) inhibited phagocytosis (**Fig. 5g**). Low (0.05 mg/ml) or high (0.25 mg/ml) dose of HSA did not alter phagocytosis
20 above baseline. In contrast, when IgM-IVIg was washed from cells prior to the addition of pHrodo bioparticles, no
21 effect of IgM-IVIg at low or high dose were observed (**Fig. 5h**). The density of internalised pHrodo bioparticles per
22 cell was calculated at 90 min after the addition of pHrodo across three independent experiments. This confirmed low
23 dose IgM-IVIg significantly increased phagocytosis of pHrodo bioparticles when present throughout the assay, however
24 this effect was lost when IgM-IVIg was washed prior to incubation with bioparticles (**Fig. 5i, j**). In conclusion, these
25 data show that reduced bacterial load in the lungs of IgM-IVIg treated mice is not associated with an increased
26 abundance of mononuclear phagocytes within the lung. Certain pro-inflammatory cytokines elevated after MCAO were
27 dampened after treatment with IgM-IVIg. Furthermore, IgM-IVIg does not directly alter the capabilities of macrophages
28 to phagocytose bacteria *in vitro*, but instead at low dose (but not high dose), enhances phagocytosis via bacterial
29 opsonisation.

DISCUSSION

We describe here the effects of low dose of IVIg (i.e. antibody replacement dose), enriched for IgM in the context of stroke-associated infection. These data provide the first evidence that targeting B cell pathways after stroke is beneficial in reducing post-stroke infection, highlighting the role of B cell and antibody deficits in post-stroke infection susceptibility. Our key *in vivo* findings are that IgM-IVIg enhances the clearance of bacterial infection and reduces lung pathology and inflammatory cytokines levels alongside preventing loss of systemic B cell composition and antibody levels following experimental stroke. We also observed that IgM-IVIg enhanced bacterial opsonophagocytosis by macrophages *in vitro*, a potential mechanism contributing to enhanced bacterial clearance *in vivo*. Infections are a frequent complication of stroke, affecting up to 30% of stroke patients, and independently affect functional outcome and survival when experienced during the acute and chronic phase of stroke recovery [5-8]. Bacterial pneumonias have the greatest impact on clinical outcome and are associated also with further complications such as recurrent stroke [42, 43]. Currently there are no available treatments to prevent pneumonia as prophylactic antibiotic treatment after stroke had no effect on pneumonia incidence or clinical outcome in randomised trials [44, 45]. In recent years we have learned that ischemic stroke induces early changes to systemic immune function including atrophy of secondary lymphoid organs, reduced numbers of lymphocytes [10, 12, 46-49] and functional deficiencies in B cell [12], T cell [46], NKT cell [50] and monocyte [51-53] populations. Preventing some of the immunosuppressive effects of stroke, or selectively restoring immune function may provide alternative or adjunct therapies to reduce infection incidence and improve outcome and long-term survival after stroke. However, infiltration of certain immune cell populations into the brain and associated inflammatory responses are known to exacerbate brain injury [54]. Therefore any attempts to enhance immune function to prevent infection must be targeted to prevent unfavourable effects on the developing stroke lesion and processes influencing repair and recovery.

There are 5 main classes of immunoglobulins; IgG, IgM, IgA, IgD and IgE, which all have different properties and functions [55]. In this study we have used an IgM-enriched preparation of IVIg, Pentaglobin™, which contains 72% IgG, 12% IgM and 16% IgA. This is similar to the proportion of antibodies naturally occurring in serum [55], unlike conventional IVIG which is comprised of >96% IgG antibodies. IgM is known to be the most potent antibacterial immunoglobulin due to its highly effective agglutination and complement fixation properties [56]. Immunosuppressed patients with selective IgM deficiency often present with recurring respiratory infections [57]. Those with agammaglobulinemia, where production of all immunoglobulin isotypes is impaired, who were given IgG only immunoglobulin replacement therapy had recurrent bacterial lung infections that resulted in chronic lung disease in

1 some [58]. *In vitro* assays showed Pentaglobin™ had a greater opsonic activity against *Pseudomonas aeruginosa*,
2 *Staphylococcus aureus* and *Escherichia coli*, in comparison to standard IVIg [22, 27] which are some of the common
3 bacterial species that cause pneumonia in stroke patients [59]. Our data, confirm and expand on these findings,
4 demonstrating that at low dose this effect is solely due to opsonisation of bacteria and does not directly influence
5 macrophage phagocytic capacity. However at high dose, the presence of IgM-IVIg actually inhibits this process. In
6 addition to its use in infection prevention, conventional IVIg has also been used at high doses in experimental stroke,
7 where it had immunomodulatory and anti-inflammatory properties and reduced infarct size via suppressing TLR
8 responses to damage-associated molecular patterns (DAMPs) and suppressing inflammasome-mediated and
9 complement-mediated neuronal cell death [60-65]. Therefore it is unlikely that an IVIg immunomodulatory approach
10 after stroke will worsen injury in the brain. In this study, stroke infarct size was unaffected by treatment with low dose
11 IgM-IVIg (**Supplementary Fig. 1**), which is consistent with the use of low dose IVIg in replacing circulating antibody
12 in immunosuppressed patients and not for its anti-inflammatory properties. This allows us to confirm that effects on
13 bacterial load are a direct consequence of the anti-bacterial properties of IgM-IVIg and not due to altering stroke severity.
14 To our knowledge, there have been no previous studies that target B cell- and antibody-mediated immune defence
15 pathways as a means to improve post stroke infection and avoid exacerbation of brain injury. In this study, low dose
16 IgM-IVIg was given prior to stroke to provide proof of concept data that antibody replacement after stroke could meet
17 both these criteria. However as the beneficial effects of IgM-IVIg treatment on infection were greatest at 5 d post
18 experimental stroke, it is likely that treatment at delayed time points after stroke could also improve post stroke infection.
19 These data show treatment with IgM-IVIg does not exacerbate brain injury and reduces bacterial infection, and supports
20 future pre-clinical studies to find a treatment regimen where timing and dosage could be optimised to confers both anti-
21 infective and brain reparative properties.

22 Potential harmful effects of IgM-IVIg treatment in the context of cerebrovascular disease also require consideration.
23 IVIg is known to increase serum viscosity and one of the reported adverse effects in a minority population is
24 thromboembolic events, including stroke [66]. Often IVIg is given as a continuous infusion to minimise effects on blood
25 viscosity. In this study, IgM-IVIg was given as a single bolus pre-surgery and again at 24 h. Treatment had no adverse
26 effects on animal welfare except for a small increase in body weight loss which normalised by 5 d (**Supplementary**
27 **Fig. 1**). Mortality rates were similar between groups at 2 d and improved in IgM-IVIg treated animals at 5 d post
28 experimental stroke (**Supplementary Fig. 1**). Our data, together with previous studies, further substantiate the
29 favourable preclinical safety profile of IVIg treatment in stroke.

1 Low dose IVIg is used as an anti-infective agent in immunosuppressed patients rather than for its immunomodulatory
2 properties reported at high doses. However even at these low doses we saw immunomodulatory effects on plasma B
3 cells and antibodies in the spleen (**Fig. 3**). This is in agreement with reports that show IVIg can induce proliferation and
4 immunoglobulin production in B cells from immunosuppressed patients [67]. Furthermore, IgM-IVIg has β -
5 propiolactone added which reduces complement fixation and prevents aggregation in the circulation reducing systemic
6 adverse effects such as immune complex deposition [68]. β -propiolactone also reduces binding of immunoglobulins to
7 monocyte Fc receptors reducing the immunomodulatory effects on mononuclear phagocytes [69].
8

9 Although we have demonstrated important B cell immunomodulatory effects even at this low dose of IgM-IVIg, the
10 main benefits of treatment may be exerted by increasing systemic and lung antibody concentrations, via the provision
11 of human immunoglobulins and the rescue of systemic mouse immunoglobulin concentrations, which in turn improves
12 pathogen opsonisation and clearance. In the lung there was no effect of treatment with IgM-IVIg on plasma B cell
13 number or antibody secretion. It is possible that this is due to the reduced access of exogenous human IgM to the lung
14 compartment versus concentrations that are present in the circulation (**Fig. 2**) as signalling via the Fc μ receptor on B
15 cells is known to confer signals for survival and activation [36]. Furthermore, IgM-IVIg rescued the MCAO-induced
16 loss of the plasma B cell pool in the spleen. There may be no observed effects of treatment as there was no comparable
17 drop in lung plasma B cells after experimental stroke. Indeed lung plasma B cells increased after MCAO, likely in
18 response to the bacterial infection. The increase in plasma B cells observed in the lung after MCAO was not associated
19 with an increase in endogenous mouse immunoglobulins demonstrating that although B cell populations persist in the
20 lung, they may be functionally impaired. Despite this, total lung antibodies were increased by the provision of IgM-
21 IVIg (**Fig. 2**) and we demonstrated *ex vivo* that these have the capacity to improve phagocytosis via opsonisation of
22 bacteria. In addition, the mice treated with IgM-IVIg showed lower pathology scores. This may be in part due to the
23 reduced bacterial loads present in these animals (**Fig. 1a-d**), however bacterial load was not significantly associated
24 with pathology score in IgM-IVIg treated animals in contrast to the association observed in controls (**Fig. 1 h, i**). This
25 may be in part due to the reduced presence of pro-inflammatory cytokines such as GM-CSF and TNF α which are known
26 to be associated with exacerbated pathology in other respiratory lung diseases, such as SARS-CoV2, and have therefore
27 been proposed as targets for clinical intervention [70, 71].
28

29 Although these data suggest targeting stroke-associated deficits in circulating IgM concentration may be a novel
30 therapeutic strategy to reduce post-stroke pneumonia, it is important to acknowledge some caveats of the current study

1 and highlight the need for next-stage pre-clinical trials to address important translation-relevant questions. In this study
2 we have used young, male animals whereas stroke patients are more likely to be elderly with complicating comorbidities
3 and have alterations in immune function and infection susceptibility. Further studies are necessary to elucidate the
4 efficacy of IgM-IVIg treatment in mixed sex, aged and co-morbid animals. However, our recent analysis of two
5 independent cohorts of stroke patients show reduced circulating IgM concentration in comparison to age matched
6 controls in two independent cohorts of stroke patients suggesting targeting IgM deficits post stroke may be of benefit.
7 Additionally, in this study, IgM-IVIg was given prior to experimental stroke which would not be possible in a clinical
8 setting. Our data shows that the IgM-IVIg did not prevent bacterial infection, but instead enhanced its clearance with a
9 significant reduction in lung bacterial burden in IgM-IVIg treated animals not apparent until 5 d post MCAO. The
10 temporal profile of this treatment effect suggests that IgM-IVIg given after stroke will also have bioactivity at the
11 appropriate time window to mediate therapeutic benefit. Further studies examining dose and frequency are necessary
12 to optimise treatment regimen post-stroke.

13 In conclusion, these data provide evidence that stroke-induced impairments on B cells and antibodies are important
14 determinants of post-stroke infection susceptibility and targeting these pathways with IgM-IVIg offers potential as a
15 safe and effective method to reduce bacterial load and pathology in the lung after stroke. Further preclinical studies are
16 warranted to optimise dosing regimen, including in combination with antibiotics, and at delayed time points after MCAO
17 and in aged and co-morbid animals, to better understand the translational potential of IgM-IVIg as an anti-infective
18 agent for stroke patients. Modulation of dose may be able to harness direct systemic anti-bacterial and
19 neuroimmunomodulatory properties to result in a treatment with dual benefits by preventing infection and reducing
20 harmful aspects of brain inflammation in stroke patients.

REFERENCES

1. Patel, A., et al., *Executive summary Part 2: Burden of Stroke in the next 20 years and potential returns from increased spending on research*, S. Association, Editor. 2017.
2. Stroke Association, *State of the Nation: Stroke statistics*. 2018.
3. Bowen, A. and P. Patchick, *Cognitive rehabilitation and recovery after stroke.*, in *Behavioural consequences of stroke*. 2014, Springer-Verlag: New Your.
4. Adamson, J., A. Beswick, and S. Ebrahim, *Is stroke the most common cause of disability?* . *Journal of Stroke and Cerebrovascular Diseases*, 2004. **13**: p. 171-177.
5. Westendorp, W., et al., *Post-stroke infection: A systematic review and meta-analysis*. *BMC Neurology*, 2011. **11**(1): p. 110.
6. 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.
7. Learoyd, A.E., et al., *Infections Up to 76 Days After Stroke Increase Disability and Death*. *Translational Stroke Research*, 2017.
8. Elkind, M.S.V., et al., *Infection as a Stroke Risk Factor and Determinant of Outcome After Stroke*. *Stroke*, 2020. **51**(10): p. 3156-3168.
9. Association, S., *The Stroke Priority Setting Partnership results for investment*. 2021.
10. Vogelgesang, A., et al., *Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke*. *Stroke*, 2008. **39**.
11. 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.
12. 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**: p. 15051.
13. McCulloch, L., et al., *Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence*. *bioRxiv*, 2019: p. 587881.
14. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens*. *Immunity*, 2001. **14**(5): p. 617-29.
15. Zouali, M. and Y. Richard, *Marginal zone B-cells, a gatekeeper of innate immunity*. *Frontiers in Immunology*, 2011. **2**.
16. Kishore, A.K., et al., *Microbiological Etiologies of Pneumonia Complicating Stroke*. *A Systematic Review*, 2018.
17. Wood, P., et al., *Recognition, clinical diagnosis and management of patients with primary antibody deficiencies: a systematic review*. *Clin Exp Immunol*, 2007. **149**(3): p. 410-23.
18. Kaveri, S.V., S. Lacroix-Desmazes, and J. Bayry, *The antiinflammatory IgG*. *N Engl J Med*, 2008. **359**(3): p. 307-9.
19. Sibénil, S., et al., *Intravenous immunoglobulins in autoimmune and inflammatory diseases: a mechanistic perspective*. *Ann N Y Acad Sci*, 2007. **1110**: p. 497-506.
20. Nimmerjahn, F. and J.V. Ravetch, *Anti-Inflammatory Actions of Intravenous Immunoglobulin*. *Annual Review of Immunology*, 2008. **26**(1): p. 513-533.
21. Ehrenstein, M.R. and C.A. Notley, *The importance of natural IgM: scavenger, protector and regulator*. *Nature Reviews Immunology*, 2010. **10**(11): p. 778-786.
22. Garbett, N.D., C.S. Munro, and P.J. Cole, *Opsonic activity of a new intravenous immunoglobulin preparation: Pentaglobin compared with sandoglobulin*. *Clin Exp Immunol*, 1989. **76**(1): p. 8-12.
23. McCabe, W.R., et al., *Immunization with rough mutants of Salmonella minnesota: protective activity of IgM and IgG antibody to the R595 (Re chemotype) mutant*. *J Infect Dis*, 1988. **158**(2): p. 291-300.
24. Li, J., et al., *Effect of intravenous immunoglobulin on the function of Treg cells derived from immunosuppressed mice with Pseudomonas aeruginosa pneumonia*. *PLoS One*, 2017. **12**(5): p. e0176843.
25. Diep, B.A., et al., *IVIg-mediated protection against necrotizing pneumonia caused by MRSA*. *Sci Transl Med*, 2016. **8**(357): p. 357ra124.
26. Lachmann, R.A., et al., *Immunoglobulin M-enriched intravenous polyclonal immunoglobulins reduce bacteremia following Klebsiella pneumoniae infection in an acute respiratory distress syndrome rat model*. *Exp Lung Res*, 2004. **30**(4): p. 251-60.
27. Rossmann, F.S., et al., *In vitro and in vivo activity of hyperimmune globulin preparations against multiresistant nosocomial pathogens*. *Infection*, 2015. **43**(2): p. 169-75.

- 1 28. Rodríguez, A., et al., *Effects of high-dose of intravenous immunoglobulin and antibiotics on survival for severe*
2 *sepsis undergoing surgery*. Shock, 2005. **23**(4): p. 298-304.
- 3 29. Tugrul, S., et al., *The effects of IgM-enriched immunoglobulin preparations in patients with severe sepsis*
4 *[ISRCTN28863830]*. Crit Care, 2002. **6**(4): p. 357-62.
- 5 30. Welte, T., et al., *Efficacy and safety of trimodulin, a novel polyclonal antibody preparation, in patients with*
6 *severe community-acquired pneumonia: a randomized, placebo-controlled, double-blind, multicenter, phase II*
7 *trial (CIGMA study)*. Intensive care medicine, 2018. **44**(4): p. 438-448.
- 8 31. Percie du Sert, N., et al., *The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research*. Journal
9 of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and
10 Metabolism, 2020. **40**(9): p. 1769-1777.
- 11 32. Percie du Sert, N., et al., *The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements Of in Vivo*
12 *Experiments)*. J Cereb Blood Flow Metab, 2017. **37**(11): p. 3488-3517.
- 13 33. Osborne, K.A., et al., *Quantitative assessment of early brain damage in a rat model of focal cerebral ischaemia*.
14 Journal of neurology, neurosurgery, and psychiatry, 1987. **50**(4): p. 402-410.
- 15 34. Katzan, I.L., et al., *The effect of pneumonia on mortality among patients hospitalized for acute stroke*.
16 Neurology, 2003. **60**(4): p. 620-625.
- 17 35. Kandatsu, N., et al., *Opposing Effects of Isoflurane and Sevoflurane on Neurogenic Pulmonary Edema*
18 *Development in an Animal Model*. Anesthesiology, 2005. **102**(6): p. 1182-1189.
- 19 36. Nguyen, T.T.T., et al., *slgM-Fc μ R Interactions Regulate Early B Cell Activation and Plasma Cell Development*
20 *after Influenza Virus Infection*. J Immunol, 2017. **199**(5): p. 1635-1646.
- 21 37. Baumgarth, N., et al., *B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of*
22 *the protective response to influenza virus infection*. J Exp Med, 2000. **192**(2): p. 271-80.
- 23 38. Lu, L.L., et al., *Beyond binding: antibody effector functions in infectious diseases*. Nature reviews. Immunology,
24 2018. **18**(1): p. 46-61.
- 25 39. Liegeois, M., et al., *The interstitial macrophage: A long-neglected piece in the puzzle of lung immunity*. Cell
26 Immunol, 2018. **330**: p. 91-96.
- 27 40. Williams, M., et al., *The function of Fc γ receptors in dendritic cells and macrophages*. Nature Reviews
28 Immunology, 2014. **14**(2): p. 94-108.
- 29 41. Nagelkerke, S.Q., et al., *Inhibition of Fc γ R-mediated phagocytosis by IVIg is independent of IgG-Fc sialylation*
30 *and Fc γ RIIb in human macrophages*. Blood, 2014. **124**(25): p. 3709-3718.
- 31 42. Smith, C.J., et al., *Diagnosis of Stroke-Associated Pneumonia: Recommendations From the Pneumonia in Stroke*
32 *Consensus Group*. Stroke, 2015. **46**(8): p. 2335-40.
- 33 43. Ji, R., et al., *Interrelationship among common medical complications after acute stroke: pneumonia plays an*
34 *important role*. Stroke, 2013. **44**(12): p. 3436-44.
- 35 44. Kalra, L., et al., *Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia*
36 *(STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial*. The
37 Lancet, 2015. **386**(10006): p. 1835-1844.
- 38 45. Westendorp, W.F., et al., *The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label*
39 *masked endpoint clinical trial*. The Lancet, 2015. **385**(9977): p. 1519-1526.
- 40 46. Prass, K., et al., *Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated*
41 *by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation*. J Exp Med, 2003.
42 **198**.
- 43 47. Liesz, A., et al., *Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke*.
44 Nat Med, 2009. **15**(2): p. 192-199.
- 45 48. Offner, H., et al., *Splenic Atrophy in Experimental Stroke Is Accompanied by Increased Regulatory T Cells and*
46 *Circulating Macrophages*. The Journal of Immunology, 2006. **176**(11): p. 6523-6531.
- 47 49. Brait, V.H., et al., *Importance of T Lymphocytes in Brain Injury, Immunodeficiency, and Recovery after Cerebral*
48 *Ischemia*. Journal of Cerebral Blood Flow & Metabolism, 2012. **32**(4): p. 598-611.
- 49 50. Wong, C.H.Y., et al., *Functional Innervation of Hepatic iNKT Cells Is Immunosuppressive Following Stroke*.
50 Science, 2011. **334**(6052): p. 101-105.
- 51 51. Hoffmann, S., et al., *Stroke-induced immunodepression and dysphagia independently predict stroke-associated*
52 *pneumonia – The PREDICT study*. Journal of Cerebral Blood Flow & Metabolism, 2016.
- 53 52. Urra, X., et al., *Monocytes Are Major Players in the Prognosis and Risk of Infection After Acute Stroke*. Stroke,
54 2009. **40**(4): p. 1262-1268.
- 55
- 56
- 57
- 58
- 59
- 60

- 1 53. Römer, C., et al., *Blocking Stroke-Induced Immunodeficiency Increases CNS Antigen-Specific Autoreactivity But*
2 *Does Not Worsen Functional Outcome after Experimental Stroke*. The Journal of Neuroscience, 2015. **35**(20):
3 p. 7777-7794.
- 4 54. Anrather, J. and C. Iadecola, *Inflammation and Stroke: An Overview*. Neurotherapeutics, 2016. **13**(4): p. 661-
5 670.
- 6 55. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins*. J Allergy Clin Immunol, 2010.
7 **125**(2 Suppl 2): p. S41-52.
- 8 56. Klimovich, V.B., *IgM and its receptors: Structural and functional aspects*. Biochemistry (Moscow), 2011. **76**(5):
9 p. 534-549.
- 10 57. Chovancova, Z., et al., *Selective IgM Deficiency: Clinical and Laboratory Features of 17 Patients and a Review*
11 *of the Literature*. J Clin Immunol, 2017. **37**(6): p. 559-574.
- 12 58. Hodkinson, J.P., et al., *Low IgA and IgM Is Associated with a Higher Prevalence of Bronchiectasis in Primary*
13 *Antibody Deficiency*. J Clin Immunol, 2017. **37**(4): p. 329-331.
- 14 59. Armstrong, J.R. and B.D. Mosher, *Aspiration pneumonia after stroke: intervention and prevention*. The
15 Neurohospitalist, 2011. **1**(2): p. 85-93.
- 16 60. Arumugam, T.V., et al., *Intravenous immunoglobulin (IVIg) protects the brain against experimental stroke by*
17 *preventing complement-mediated neuronal cell death*. Proceedings of the National Academy of Sciences of
18 the United States of America, 2007. **104**(35): p. 14104-14109.
- 19 61. Walberer, M., et al., *Intravenous Immunoglobulin Reduces Infarct Volume but Not Edema Formation in Acute*
20 *Stroke*. Neuroimmunomodulation, 2010. **17**(2): p. 97-102.
- 21 62. Fann, D.Y.-W., et al., *Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated*
22 *neuronal death in ischemic stroke*. Cell death & disease, 2013. **4**(9): p. e790-e790.
- 23 63. Tunik, S., et al., *The effects of intravenous immunoglobulin on cerebral ischemia in rats: An experimental study*.
24 Toxicology and Industrial Health, 2013. **32**(2): p. 229-234.
- 25 64. Lok, K.Z., et al., *Intravenous immunoglobulin (IVIg) dampens neuronal toll-like receptor-mediated responses in*
26 *ischemia*. Journal of neuroinflammation, 2015. **12**: p. 73-73.
- 27 65. Chen, X., et al., *Combination Therapy with Low-Dose IVIg and a C1-esterase Inhibitor Ameliorates Brain*
28 *Damage and Functional Deficits in Experimental Ischemic Stroke*. Neuromolecular medicine, 2018. **20**(1): p.
29 63-72.
- 30 66. Dalakas, M.C. and W.M. Clark, *Strokes, thromboembolic events, and IVIg: Rare incidents blemish an excellent*
31 *safety record*. Neurology, 2003. **60**(11): p. 1736-1737.
- 32 67. Bayry, J., et al., *Intravenous immunoglobulin induces proliferation and immunoglobulin synthesis from B cells*
33 *of patients with common variable immunodeficiency: A mechanism underlying the beneficial effect of IVIg in*
34 *primary immunodeficiencies*. Journal of Autoimmunity, 2011. **36**(1): p. 9-15.
- 35 68. Stephan, W., *[Elimination of complement fixation of gamma-globulin by chemical modification with beta-*
36 *propiolactone]*. Z Klin Chem Klin Biochem, 1969. **7**(3): p. 282-6.
- 37 69. Jungi, T.W., et al., *Effect of various treatments of gamma-globulin (IgG) for achieving intravenous tolerance on*
38 *the capacity to interact with human monocyte Fc receptors. A comparative study*. Vox Sang, 1986. **51**(1): p. 18-
39 26.
- 40 70. Huang, C., et al., *Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China*. Lancet
41 (London, England), 2020. **395**(10223): p. 497-506.
- 42 71. Bonaventura, A., et al., *Targeting GM-CSF in COVID-19 Pneumonia: Rationale and Strategies*. Frontiers in
43 immunology, 2020. **11**: p. 1625-1625.
- 44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1 Low dose IgM-IVIg enhances clearance of spontaneous bacterial infection after experimental stroke

Bacterial load (log[CFU/ml]) in the inferior lobe of the right lung in IgM-IVIg (○) or human serum albumin (HSA; ●) treated mice plated on blood agar at (A) 2d or (C) 5d after MCAO or sham surgery and plated on MacConkey agar at (B) 2 d or (D) 5 d after MCAO or Sham surgery (Sham HSA n=12; Sham IgM-IVIg n=11; MCAO HSA n=11; MCAO IgM-IVIg n=13). (E) Representative haematoxylin and eosin stained sections of the left lung 2 d after sham or MCAO surgery in IgM-IVIg or HSA treated mice. Arrows denote inflammatory cluster. Pathology score of left lung at (F) 2 d or (G) 5 d after sham or MCAO surgery in IgM-IVIg (○) or HSA (●) treated mice from scoring criteria detailed in Supplementary Fig 2 (Sham HSA n=12; Sham IgM-IVIg n=9; MCAO HSA n=10; MCAO IgM-IVIg n=10). The association between lung pathology score and lung bacterial load (log [CFU/ml]) at (H) 2 d and (I) 5 d post MCAO in IgM-IVIg (○) or HSA (●) treated mice. The association between lung pathology and stroke infarct volume at (J) 2 d and (K) 5 d post MCAO in HSA (●) and IgM-IVIg (○) treated mice. Data show data points with mean ± S.D; * P<0.05; (A, C) two-way ANOVA with Tukey's multiple comparison test; (B, D) unpaired t-test. (F, G) Violin plots with Mann-Whitney test. (H-K) Simple linear regression with Pearson's correlation.

Figure 2 Distribution of human immunoglobulins in IgM-IVIg treated animals

Presence of IgM-IVIg in the circulation was detected by measuring concentration of human immunoglobulin isotypes (A) IgM, (B) IgG1, (C) IgG2, (D) IgG3, (E) IgG4 and (F) IgA in plasma before treatment (baseline) with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=11; Sham IgM-IVIg D2 n=10; MCAO HSA D2 n=10; MCAO IgM-IVIg D2 n=13). Presence of IgM-IVIg in the lung was detected by measuring concentration of human (G) IgM, (H) IgG1, (I) IgG2, (J) IgG3, (K) IgG4 and (L) IgA in bronchial alveolar lavage fluid (BALF) in animals treated with HSA (●) or IgM-IVIg (○) after 2 d recovery from sham or MCAO surgery (Sham HSA n=9; Sham IgM-IVIg n=8; MCAO HSA n=9; MCAO IgM-IVIg n=12). (M) The proportion of human IgM, IgG and IgA reported to be present in IgM-IVIg in comparison to proportion detected in plasma and BALF. Data show data points with mean ± S.D; * P<0.05; ** P<0.01; *** P<0.001; (A-L) two way ANOVA with Tukey's multiple comparison test.

Figure 3 Low dose IgM-IVIg rescues splenic plasma B cells and endogenous antibody production after experimental stroke

(A) Gating strategy used to define spleen B cell subsets by flow cytometry. (B) Total live leukocytes and (C) total CD19⁺ B cells measured by flow cytometry of spleens from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery. (D) The proportion of CD138⁺ plasma cells and plasmablasts and (E) CD138⁺CD43⁺CD21⁺CD23⁺CD93⁺ T1 Transitional B cells within the CD19⁺ total B cell population mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=11; Sham IgM-IVIg n=10; MCAO HSA n=9; MCAO IgM-IVIg n=12). Circulating concentration of endogenously produced mouse immunoglobulins (F) IgM, (G) IgA, (H) IgG1, (I) IgG2a, (J) IgG2b and (K) IgG3 in plasma in animals treated with HSA (●) or IgM-IVIg (○) after 2 d recovery from sham or MCAO surgery (Sham HSA n=11; Sham IgM-IVIg n=10; MCAO HSA n=10; MCAO IgM-IVIg n=13). Data show data points with mean ± S.D; * P<0.05; ** P<0.01; *** P<0.001, (B-K) 2 way ANOVA with Tukey's multiple comparison test.

Figure 4 Low dose IgM-IVIg does not modulate lung B cells or immunoglobulins after experimental stroke

(A) Gating strategy used to define lung B cell subsets by flow cytometry. (B) Total live lung leukocytes, (C) CD20⁺ B cells (D) CD20⁺MHC II⁻ plasma B cells and (E) CD20⁺MHC II⁺ CD43⁺CD5^{+/-} B1 B cells measured by flow cytometry of lung single cell suspensions from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=6; Sham IgM-IVIg n=7; MCAO HSA n=7; MCAO IgM-IVIg n=10). Concentration of endogenously produced mouse immunoglobulins (F) IgM, (G) IgA, (H) IgG1, (I) IgG2b and (J) IgG3 and in bronchial alveolar lavage fluid (BALF) in animals treated with HSA (●) or IgM-IVIg (○) after 2 d recovery from sham or MCAO surgery (Sham HSA n=9; Sham IgM-IVIg n=8; MCAO HSA n=9; MCAO IgM-IVIg n=12). Data show data points with mean ± S.D; * P<0.05; (B-J) two way ANOVA with Tukey's multiple comparison test.

Figure 5 Effect of low dose IgM- IVIg on lung mononuclear phagocytes

(A) Gating strategy used to define lung immune cell subsets by flow cytometry. (B) Total CD45⁺SiglecF⁺CD11c⁺ alveolar macrophages, (C) total CD45⁺CD11b⁺CD64⁺MHCII⁺ interstitial macrophages, (D) total CD45⁺CD11b⁺CD64⁺MHC II⁻ monocytes and monocytes distinguished by (E) low expression of Ly6C or (F) high expression of Ly6C measured by flow cytometry of lung single cell suspensions from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=6; Sham IgM-IVIg n=7; MCAO HSA n=7; MCAO IgM-IVIg n=10). Concentration of (G) GM-CSF (H) TNF α measured by multiplexed ELISA of BALF from mice treated with HSA (●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=9; Sham IgM-IVIg n=8; MCAO HSA n=8; MCAO IgM-IVIg n=11). Phagocytosis of pHrodo[®] Red S. aureus BioParticles[®] (fluorescent density/ cell) measured over 2.5 h in co-culture with bone marrow-derived macrophages (BMDM) stimulated for 4 h with IgM-IVIg (0.25 mg/ml and 2 mg/ml) or HSA (0.05 mg/ml and 0.4 mg/ml) where treatments are (I) present during phagocytosis assay or (J) washed off prior to the addition of pHrodo[®] Red S. aureus BioParticles[®], with cytochalasin D (10 μ M) as a negative control. Data show technical replicates from one experiment. (K) Representative image of phagocytosis of pHrodo[®] Red S. aureus BioParticles[®] by BMDM stimulated for 4 h with IgM-IVIg (0.25 mg/ml and 2 mg/ml), HSA (0.4 mg/ml) or cytochalasin D (10 mM) where treatments are present during phagocytosis assay or washed off prior to the addition of pHrodo[®] Red S. aureus BioParticles[®]. (L) pHrodo[®] Red S. aureus BioParticles[®] density/ cell measured after 90 min incubation with bone marrow-derived macrophages stimulated for 4 h with IgM-IVIg (0.25 mg/ml and 2 mg/ml) or HSA (0.05 mg/ml and 0.4 mg/ml) where treatments are present during phagocytosis assay or washed off prior to the addition of pHrodo[®] Red S. aureus BioParticles[®].

Data show data points with mean \pm S.D; * P<0.05; ** P<0.01; *** P<0.001; P<0.0001; (B-H, L) two way ANOVA with Tukey's multiple comparison test; (K) Scale bar 400 μ M.

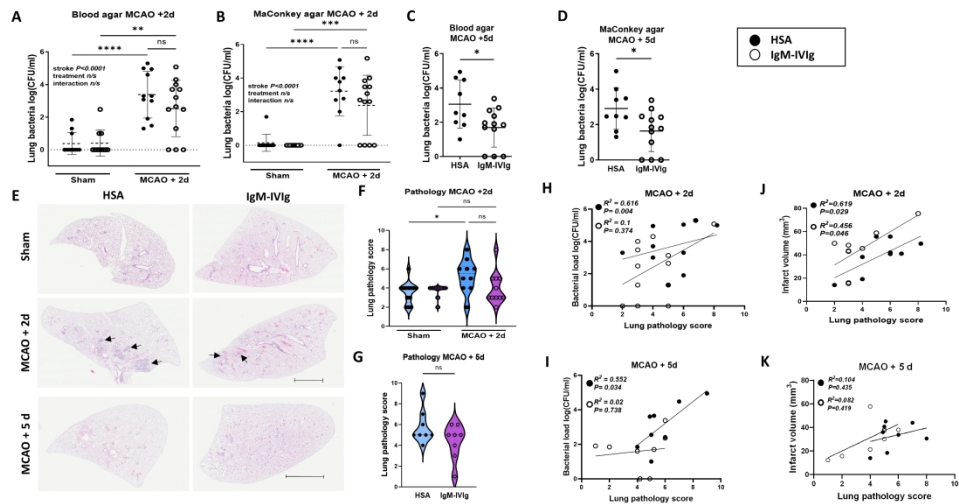


Figure 1 Low dose IgM-IVIg enhances clearance of spontaneous bacterial infection after experimental stroke

338x190mm (300 x 300 DPI)

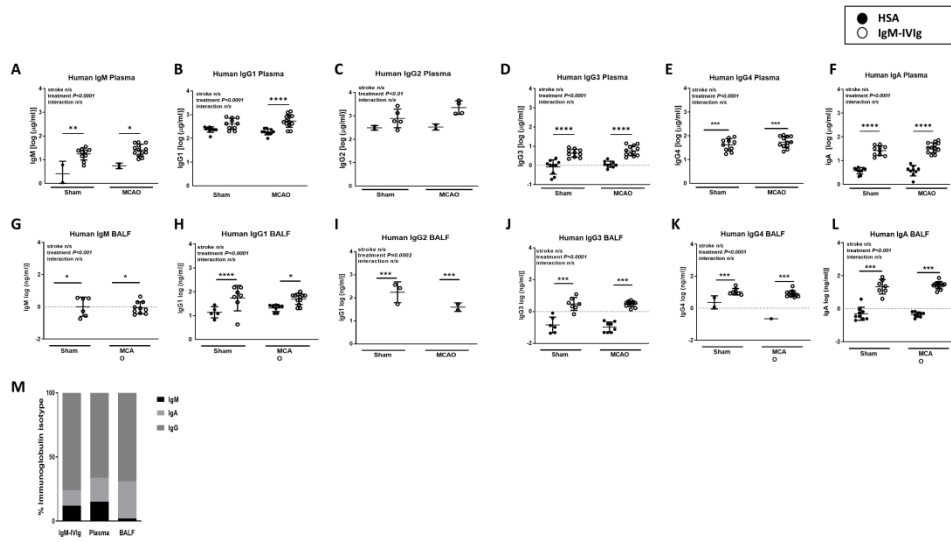


Figure 2 Distribution of human immunoglobulins in IgM-IVIg treated animals

338x190mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

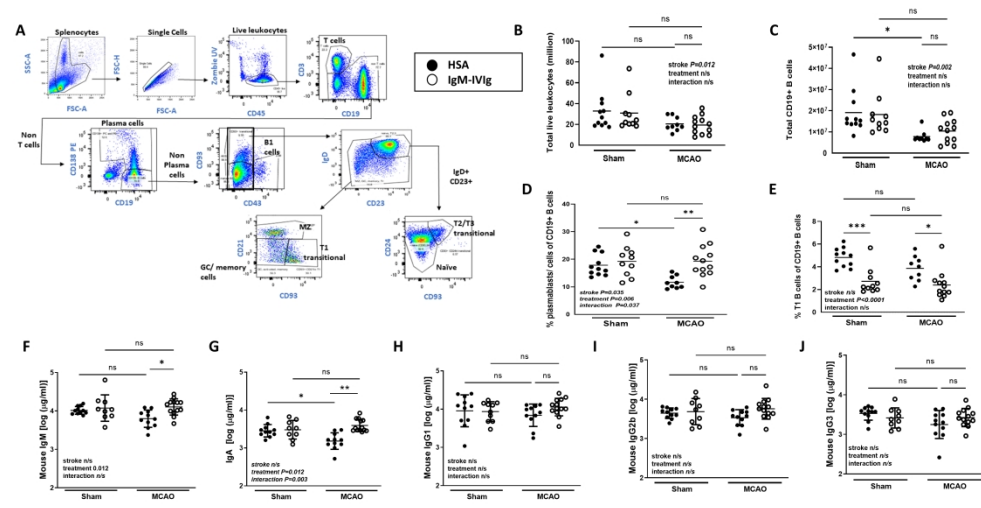


Figure 3 Low dose IgM-IVIg rescues splenic plasma B cells and endogenous antibody production after experimental stroke

338x190mm (300 x 300 DPI)

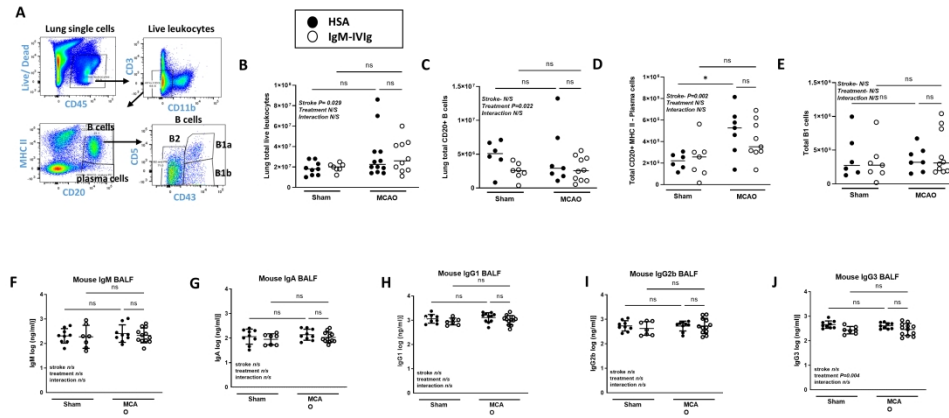


Figure 4 Low dose IgM- IVIg does not modulate lung B cells or immunoglobulins after experimental stroke

338x190mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

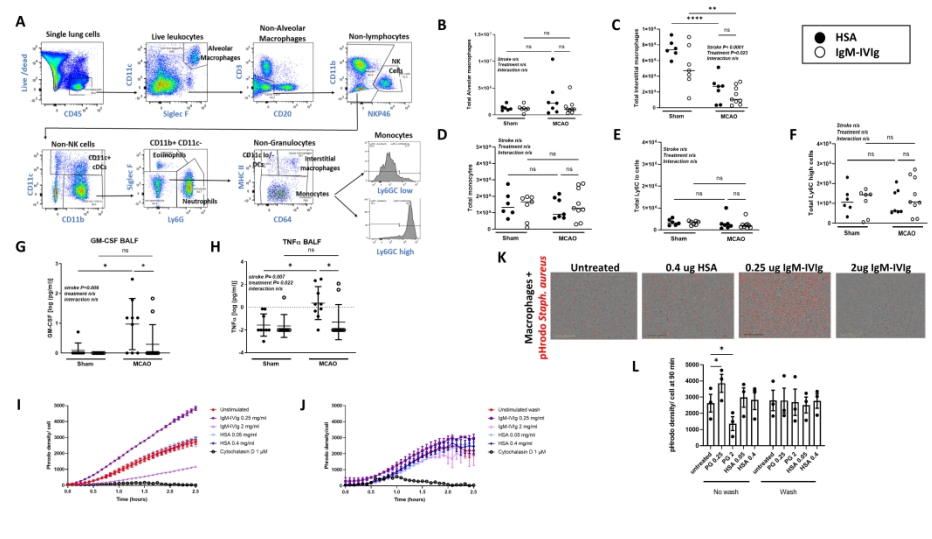
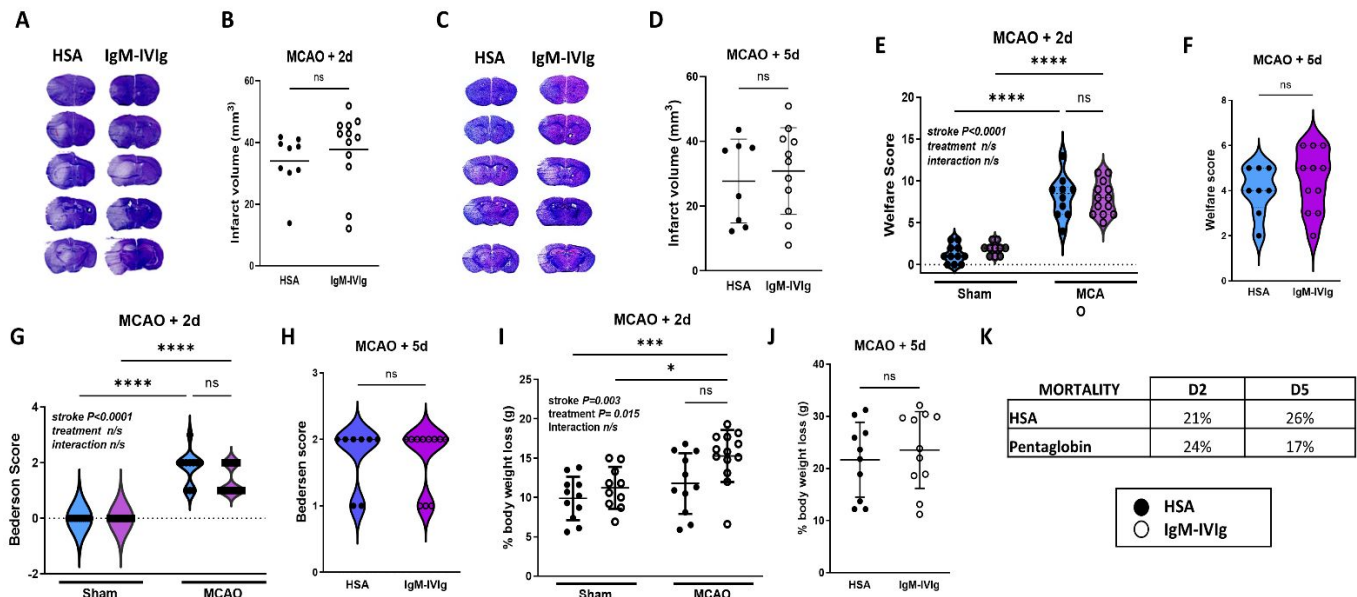


Figure 5 Effect of low dose IgM- IVIg on lung mononuclear phagocytes

338x190mm (300 x 300 DPI)



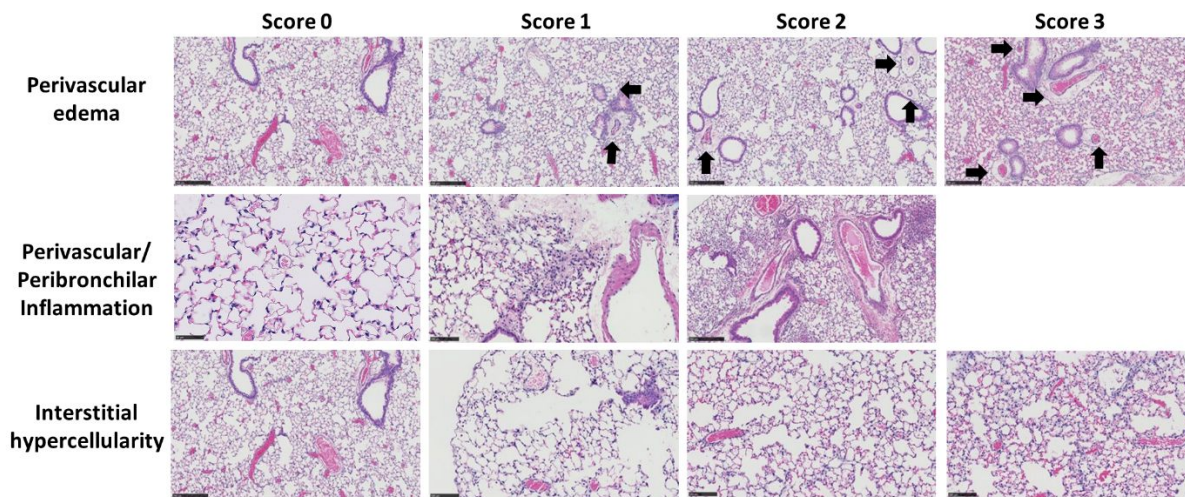
Supplementary Figure 1 Low dose IgM-IVIg does not modulate primary stroke pathology

Infarct volume (mm³) in the brains of animals treated with human serum albumin (HSA; ●) or IgM-IVIg (○) at (A, B) 2 days or (C, D) 5 days post MCAO. Treatment with IgM-IVIg did not affect welfare score at (E) 2 days or (F) 5 days after MCAO. (G) Animals treated with IgM-IVIg had a higher percentage reduction in bodyweight 2 days post sham or MCAO surgery. (H) However at 5 days post MCAO there was no effect of treatment with IgM-IVIg on percentage body weight loss. Treatment with IgM-IVIg did not affect Bedersen score at (I, J) 2 days or (K, L) 5 days after MCAO. (K, L) Treatment with IgM-IVIg had no effect on mortality at 2 days after MCAO however at 5 days post MCAO, mortality was lower in mice treated with IgM-IVIg (B, D, G, H, K, L). Data show data points with mean \pm S.D; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; (B, D, H, L) unpaired t-test. (G, K) two way ANOVA with Tukey's multiple comparison test; (E, F, I, J) Violin plots with Mann-Whitney

	Score	Criteria
Coat appearance	0	Normal
	1	Piloerection
	2	Severe piloerection
Grooming	0	Normal
	1a	Mild impairment
	1b	Excessive
Ocular/ Nasal discharge	2	None
	0	None
	1	Discharge present
Posture	0	Normal
	1	Mild hunching
	2	Severe hunching
Vocalising	0	Normal
	1	Mild/ intermittent
	2	Persistent/ distressed
Respiratory Rate	0	Normal
	1	Mildly depressed
	2	Severely depressed
General exploratory behaviour	0	Normal
	1a	Subdued
	1b	Excessive
	2	No peer interaction/ exploration
Neurological Deficit (Bederson Score)	0	Normal
	1	Torso flexion upon tail lift
	2	Circling
	3	Severe circling/ leaning
	4	No movement
Responsiveness	0	Spontaneous movement
	1	Responsive to touch
	2	Unresponsive
Tremors	0	None
	1	Intermittent
	2	Persistent
Convulsions/ Seizure activity	0	No
	1	Yes
Barrel-rolling	0	No
	1	Yes

Supplementary Table 1 Welfare scoring of animals post-surgery

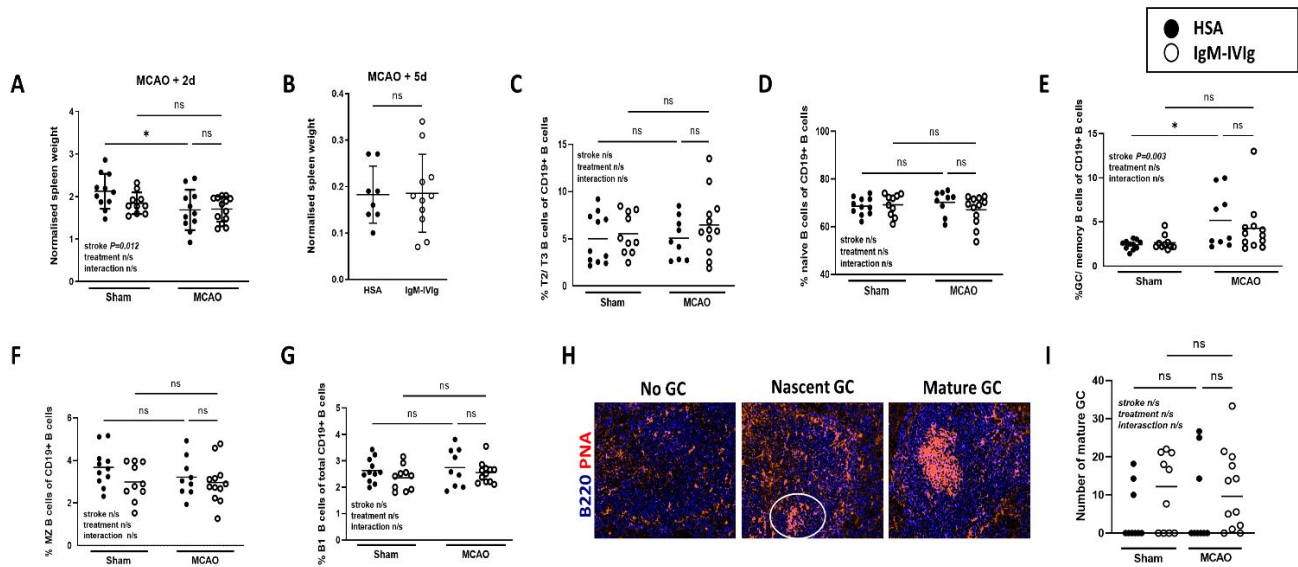
Scoring criteria to assess the welfare of animals as they recover from sham or MCAO surgery. The categories of "Responsiveness", "Tremors", "Convulsions/ Seizure activity" and "Barrel-rolling" reflect humane end points and animal that score in these categories would be culled for welfare.



	Score	Criteria
Perivascular edema	0	Absent
	1	< 25% vascular spaces
	2	25- 75% vascular spaces
	3	> 75 %vascular spaces
Perivascular/ peribronchiolar acute inflammation	0	Absent
	1	< 5 neutrophils per high powered field of view (HPF)
	2	> 5 neutrophils per HPF and/ or neutrophils in perivascular spaces extending to peribronchiolar spaces
	3	Numerous neutrophils throughout
Goblet cell metaplasia of bronchioles	0	Absent
	1	Few goblet cells present in 1 or 2 bronchiolar profiles
	2	Large numbers of goblet cells present
Eosinophilic macrophages in alveolar spaces	0	Absent
	1	< 25% of alveolar spaces
	2	> 25% of alveolar spaces
Presence of macrophages in interstitium	0	Interstitia thin and/ or rare macrophages presents
	1	< 25% hypercellular areas with macrophages
	2	25-50% hypercellular areas with macrophages
	3	> 50% hypercellular areas with macrophages

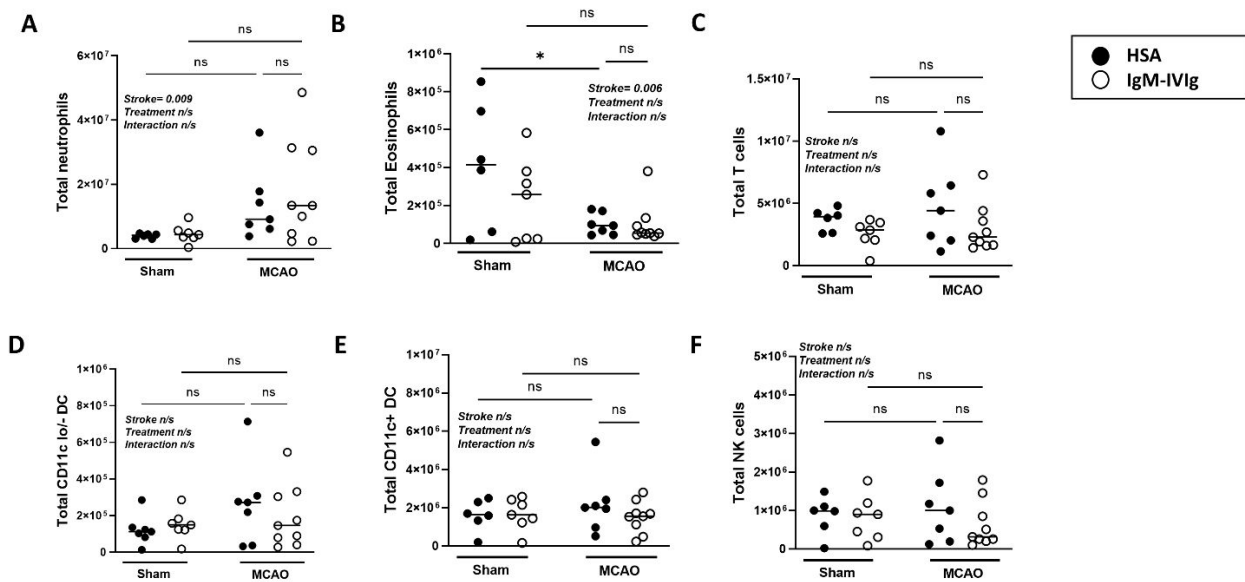
Supplementary Figure 2 Criteria for scoring lung pathology

Scoring system used to grade the extent of pathology in lung from animals treated with HSA or IgM-IVIG 2 days and 5 days after sham or MCAO surgery (Figure 1). Goblet cell hyperplasia was not detected in any animals. All animals had <25% alveolar spaces with macrophages present and scored 1. Inflammation did not reach a score of 3 in any animals. Scores in sham-operated animals mainly consisted of edema and some with mild inflammation. Scale bars 50 μ m.



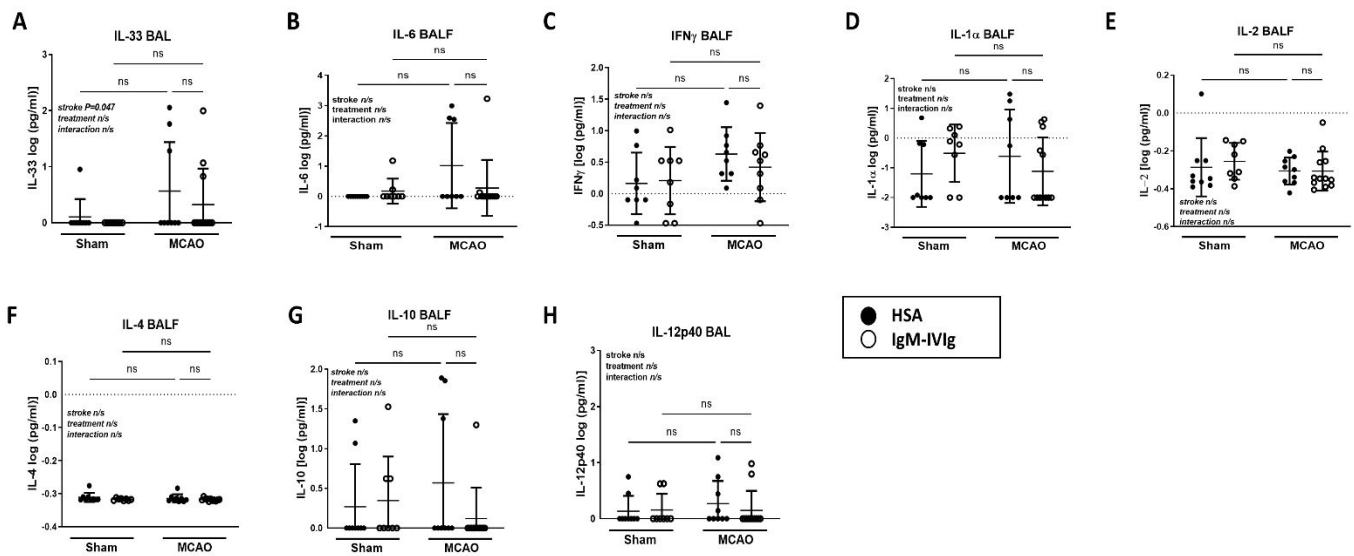
Supplementary Figure 3 Splenic B cell responses to IgM-IVIg

Spleen weight, normalised to total body weight, is significantly reduced by stroke (A) but there is no effect of treatment with IgM-IVIg at (A) 2 d or (B) 5 d after sham or MCAO surgery. Percentage of (C) IgD⁺CD23⁺CD24⁺CD93⁺ T2 transitional B cells (D) IgD⁺CD23⁺CD23⁺CD24⁺CD93⁻ naïve B cells (E) CD23⁻CD21⁻CD93⁻ GC and memory B cells (F) CD19⁺CD23⁻CD21⁺CD93⁻ marginal zone B cells and (G) CD93⁻CD43⁺ B1 B cells within the total CD19⁺ B cell population measured by flow cytometry of spleens from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery. Gating strategy in Figure 3A (Sham HSA n=11; Sham IgM-IVIg n=10; MCAO HSA n=9; MCAO IgM-IVIg n=12). (H) Immunolabelling of germinal centers (GC) using peanut agglutinin (PNA; orange) and DAPI staining of nuclei (blue) in spleens to identify areas of white pulp with no GC, nascent GC and mature GC. (I) Number of mature GC per half spleen section in spleens from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=9; Sham IgM-IVIg n=10; MCAO HSA n=9; MCAO IgM-IVIg n=12). Data show data points with mean ± SD; (A, B, C, D, E, G) two way ANOVA with Tukey's multiple comparison test



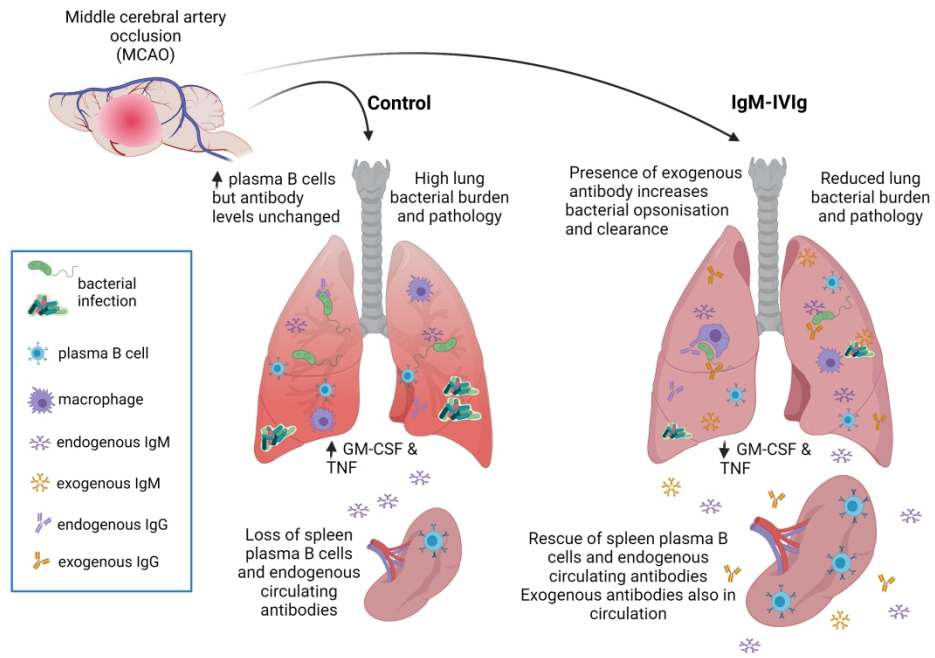
Supplementary Figure 4 Effect of IgM-IVIg on lung immune cell subsets

Total (A) CD11b⁺SiglecF⁻LygG⁺ neutrophils, (B) CD11b⁺SiglecF⁺LygG⁻ eosinophils, (C) CD3⁺CD20⁻ T cells, (D) CD11b⁺SiglecF⁻Ly6G⁻CD64⁺MHC II⁺ DC (E) CD11b⁺CD11c⁺ DC and (F) CD11b⁻NKP46⁺ NK cells measured by flow cytometry of lung single cell suspensions from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery. For gating strategy see Figure 4A. (Sham HSA n=6; Sham IgM-IVIg n=7; MCAO HSA n=7; MCAO IgM-IVIg n=10). Data show data points with mean \pm S.D; * P<0.05; (A-F) two way ANOVA with Tukey's multiple comparison test.



Supplementary Figure 5 Effect of IgM-IVIg on lung immune cell subsets

Concentration of (A) IL-1 α (B) IL-2 (C) IL-4 (D) IL-6 (E) IL-10 (F) IL-12p40 (G) IL-33 and (H) IFN γ measured by multiplexed ELISA of BALF from mice treated with human serum albumin (HSA; ●) or IgM-IVIg (○) and after 2 d recovery from sham or MCAO surgery (Sham HSA n=9; Sham IgM-IVIg n=8; MCAO HSA n=8; MCAO IgM-IVIg n=11). Data show data points with mean \pm S.D; (A-H) two way ANOVA with Tukey's multiple comparison test.



Graphical abstract

645x452mm (118 x 118 DPI)