Innate immune anti-inflammatory response in human spontaneous intracerebral haemorrhage

Anan Shtaya PhD FRCS[1,2], Leslie R Bridges FRCPath[1,3], Rebecca Williams MSc[4], Sarah Trippier MSc[4], Liqun Zhang MD[4], Anthony C Pereira FRCP[1,4], James AR Nicoll FRCPath[5], Delphine Boche PhD[5] and Atticus H Hainsworth PhD[1,4]

[1]Molecular and Clinical Sciences Research Institute, St. George's, University of London, London, UK.

[2]Wessex Spinal Unit, University Hospital Southampton NHS Foundation Trust, Southampton, UK.

[3]Department of Cellular Pathology, St George's University Hospitals NHS Foundation Trust, London, UK.

[4]Neurology Department, St George's University Hospitals NHS Foundation Trust, London, UK.

[5]Clinical Neurosciences, Clinical & Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK.

Corresponding Author:

Mr Anan Shtaya PhD, FRCS (SN)

Wessex Spinal Unit, University Hospital Southampton NHS Foundation Trust, Southampton,

UK

E-mail Anan.Shtaya@uhs.nhs.uk

Dr A H Hainsworth ahainsworth@sgul.ac.uk

Running title: Anti-inflammatory process in ICH

For social media: Twitter (@StGeorgesUni, @STGNeuro, @StGeorgesTrust)

Cover Title: Anti-inflammatory process in ICH

Supplementary Methods

Primary Antibodies

The *CD163* primary antibody is a clinical diagnostic reagent (NCL-L-CD163; Leica-Novocastra Biosystems, Newcastle-upon-Tyne, UK). It is a murine monoclonal IgG1, Clone 10D6. Immunogen was a recombinant protein corresponding to domains 1 to 4 of the N-terminal region of the human CD163 molecule.

CD68 (mouse monoclonal IgG3, Clone PG-M1, M087601-2, Dako-Agilent Technologies LDA UK Limited Stockport, Cheshire, UK). This is also a clinical diagnostic antibody, which labels COS-1 and WOP cells transfected with CD68 cDNA. Unlike other CD68 antibodies, which label both macrophages and myeloid cells, the PG-M1 antibody detects a fixative-resistant epitope on the macrophage-restricted form of the CD68 antigen. This antibody has been used extensively by our laboratory and others. [16,39]

TMEM119 (ab185333, Abcam, Cambridge, UK) is a rabbit polyclonal IgG raised against the C-terminal of microglia-specific transmembrane protein TMEM119. The immunogen is a recombinant peptide corresponding to human TMEM119 aa150 to the C-terminus. This antibody has recently been used by another group to label microglia in human brain material.[40]

CD206 (ab64693, Abcam, Cambridge, UK) is a rabbit polyclonal IgG. The immunogen is a synthetic peptide conjugated to KLH derived from within residues 1400 to the C-terminus of the human Mannose Receptor CD206. CD206 is a 175-kDa transmembrane protein, expressed by macrophages, and is widely recognized as a "M2" microglial marker.[41]

Primary antibodies were selected for specificity in human brain tissue based on manufacturers' data, and used according to the manufacturers' instructions. For each antibody, a range of titres (usually 10-fold) were examined and a titre selected based on signal/background for known cell labelling patterns.

Table I. Test for Associations of Neuropathological Markers with Age

	Iba1	TMEM119	CD68	CD163	CD206
Spearman r	0.1715	0.1757	0.2666	0.09201	-0.09119
95% C.I.	-0.3345 to 0.6007	-0.3306 to 0.6034	-0.2428 to 0.6608	-0.4043 to 0.5464	-0.5724 to 0.4368
P (2-tailed)	0.4963	0.4857	0.2849	0.7165	0.7360
Number of XY Pairs	18	18	18	18	16

Data shown are Spearman's rho for association between the extent of labelling (AF%) with each neuropathological marker and age at death. Iba1 data are from our previous study (Shtaya et al. 2019) shown for comparison.

Table II. Test for Associations of Neuropathological Markers with Sex

	Iba1	TMEM119	CD68	CD163	CD206
Spearman r	0.09885	0.1208	0.09885	-0.07688	0.1230
95% C.I.	-0.3985 to 0.5513	-0.3796 to 0.5665	-0.3985 to 0.5513	-0.5357 to 0.4169	-0.4104 to 0.5936
P (2-tailed)	0.6964	0.6330	0.6964	0.7617	0.6806
Number of XY Pairs	18	18	18	18	16

Data shown are Spearman's rho for association between the extent of labelling (AF%) with each neuropathological marker and sex (female=1, male=2). Iba1 data are from our previous study (Shtaya et al. 2019) shown for comparison.

Table III. Test for Associations of Neuropathological Markers with Location of ICH

	Iba1	TMEM119	CD68	CD163	CD206
Spearman r	-0.1666	0.1614	0.1393	0.002604	-0.2950
95% C.I.	-0.6091 to 0.3549	-0.3596 to 0.6057	-0.3792 to 0.5912	-0.4905 to 0.4944	-0.7097 to 0.2715
P (2-tailed)	0.5204	0.5337	0.5920	0.9940	0.2825
Number of XY Pairs	17	17	17	17	15

Data shown are Spearman's rho for association between the extent of labelling (AF%) with each neuropathological marker and anatomical ICH location. Locations was coded as: frontal cortex=1, parietal cortex=2, temporal cortex=3, occipital cortex=4, basal ganglia=5, thalamus=6. Iba1 data are from our previous study (Shtaya et al. 2019) shown for comparison.

Table IV. Test for Association of Blood Monocyte Counts with Age, in Living ICH Patients

	1-2 days post-ICH	3-5 days post-ICH	7-10 days post-ICH
Spearman r	-0.2395	0.1627	-0.04091
95% C.I.	-0.7076 to 0.3747	-0.4414 to 0.6653	-0.5909 to 0.5350
P (2-tailed)	0.4262	0.5914	0.8947
Number of XY Pairs	13	13	13

Data shown are Spearman's rho for association between blood monocyte counts (cells/ml) and age in years, within each time-period post-ICH.

Table V. Test for Association of Blood Monocyte Counts with Hematoma Volume (mm^3), in Living ICH Patients

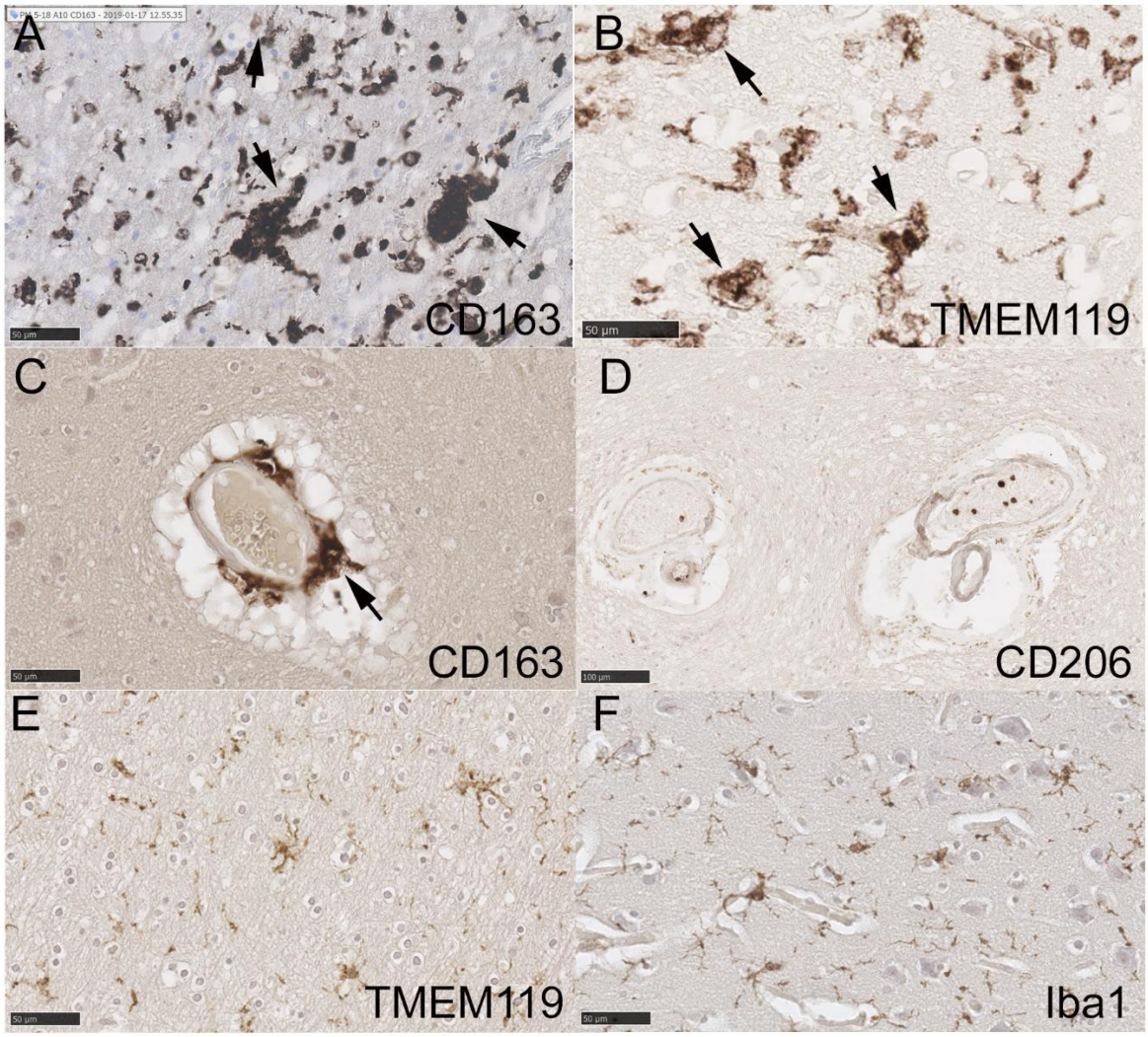
	1-2 days post-ICH	3-5 days post-ICH	7-10 days post-ICH
Spearman r	0.03240	0.4727	0.4232
95% C.I.	-0.5411 to 0.5853	-0.1239 to 0.8183	-0.1844 to 0.7968
P (2-tailed)	0.9167	0.1039	0.1495
Number of XY Pairs	13	13	13

Data shown are Spearman's rho for association between blood monocyte counts (cells/ml) and hematoma volume in mm^3, within each time-period post-ICH.

Table VI. Test for Association of Blood Monocyte Counts with Clinical Outcome (modified Rankin scale, 0-6), in ICH Patients

	1-2 days post-ICH	3-5 days post-ICH	7-10 days post-ICH
Spearman r	-0.3189	0.5217	0.3194
95% C.I.	-0.7481 to 0.2983	-0.05944 to 0.8387	-0.2979 to 0.7483
P (2-tailed)	0.3091	0.0685	0.2965
Number of XY Pairs	13	13	13

Data shown are Spearman's rho for association between blood monocyte counts (cells/ml) and clinical outcome, according to the modified Rankin scale (range 0-6), within each time-period post-ICH.



Supplementary Figure I. Immunohistochemical labelling of microglia-macrophage markers in human brain tissue from people who died post ICH and from control brains. A, B: giant microglial cells that were positive for CD163 (A) or TMEM119 (B) were seen post-ICH. Arrows show examples. C-F, Control brains. C: CD163 positive cells were seen primarily in perivascular spaces, with the appearance of perivascular macrophages (arrow). D: CD206 positive cells were rarely or never seen in the parenchyma, but were seen in blood remaining within the lumen of blood vessels. E, F: in control brains TMEM119 positive cells (panel E) were seen with a similar morphology and distribution to Iba1 (F). Haematoxylin nuclear counterstain (blue). Scale bars 100 µm in panel D, all others 50 µm.

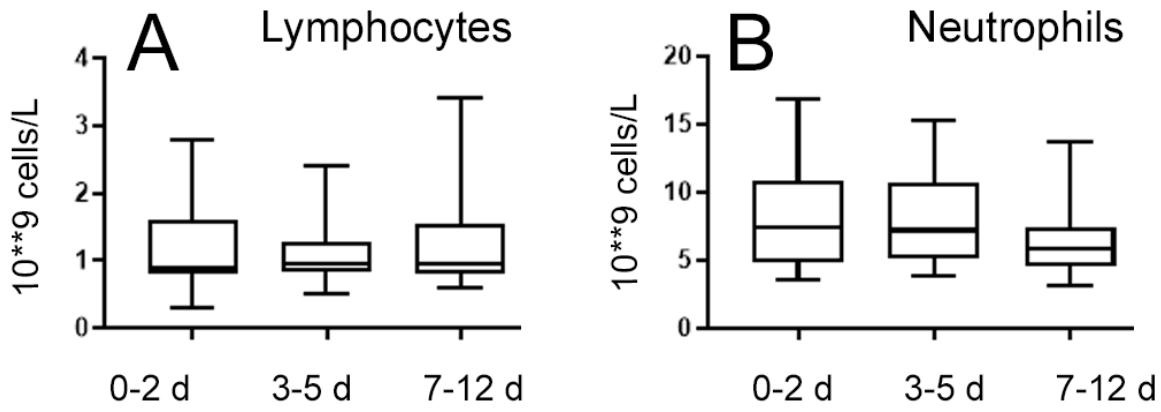


Figure II. Temporal course of peripheral blood cell counts in patients with sICH. A) Blood lymphocyte counts ($x10^9$ cells/L). No significant differences were detected, Kruskal-Wallis test P=0.99, H= 0.02. B) Blood neutrophil counts ($x10^9$ cells/L). No significant differences were detected, Kruskal-Wallis test P=0.28, H= 2.6. Box-whisker plots show median, IQR and full range.

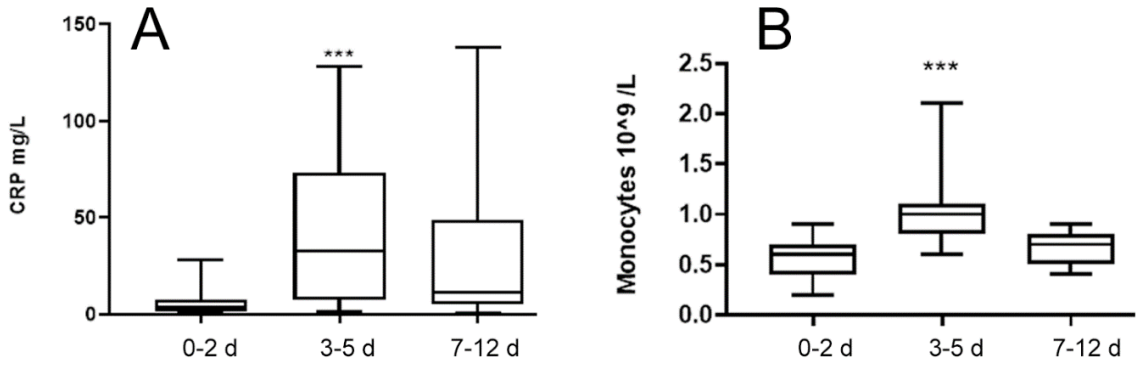


Figure III. Temporal course of peripheral blood CRP concentration and monocyte counts in all ICH patients, including patients who did not have data at all three-time intervals. A) Plasma CRP concentration (mg/L) was significantly elevated at days 3-5 following sICH, relative to days 0-2 (***P=0.0008, H= 14.3, Kruskal-Wallis test), before declining at days 7-12. B) Blood monocyte counts ($x10^9$ cells/L) increased significantly from days 0-2 to days 3-5 (*** P<0.001, H= 26.6 Kruskal-Wallis test). Box-whisker plots show median, IQR and full range.