Innate immune anti-inflammatory response in human spontaneous intracerebral haemorrhage

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Running title: Anti-inflammatory process in ICH

Title word count: 9

Manuscript word count: 3109

Number of figures: 3

Number of tables: 3

Number of references: 38

Supplementary manuscript: 1

For social media: Twitter (@StGeorgesUni, @STGNeuro, @StGeorgesTrust, @CentreWessex, @UHSFT, @SotonNeuro, @unisouthampton)

Cover Title: Anti-inflammatory process in ICH

Key Words

intracerebral hemorrhage, inflammation, innate immunity, microglia, monocytes, macrophages.

Abstract

Background and Purpose-Spontaneous intracerebral haemorrhage (sICH) is a common form of haemorrhagic stroke, with high mortality and morbidity. Pathophysiological mechanisms in sICH are poorly understood and treatments limited. Neuroinflammation driven by microglial-macrophage activation contributes to brain damage post-sICH. We aim to test the hypothesis that an anti-inflammatory ("repair") process occurs in parallel with neuroinflammation in clinical spontaneous intracerebral haemorrhage (sICH).

Methods- We performed quantitative analysis of immunohistochemical markers for microglia and macrophages (Iba1, CD68, TMEM119, CD163, CD206) in brain tissue biospecimens 1-12 days post sICH and matched control cases. In a parallel, prospective group of patients, we assayed circulating inflammatory markers (C-reactive protein, total white cell and monocyte count) over 1-12 days following sICH.

Results- In 27 supra-tentorial sICH cases (n=27, median, [IQR] age: 59, [52-80.5], 14F/13M) all microglia-macrophage markers increased post-sICH, relative to control brains. Anti-inflammatory markers (CD163, CD206) were elevated alongside pro-inflammatory markers (CD68, TMEM119). CD163 increased progressively post-sICH (15.0-fold increase at 7-12 days, p<0.001). CD206 increased at 3-5 days (5.2-fold, p<0.001) then returned to control levels at 7-12 days. The parenchymal immune response combined brain-derived microglia (TMEM119-positive) and invading monocyte-derived macrophages (CD206-positive). In a prospective sICH patient cohort (n=26, age 74, [66-79], NIHSS on admission: 8, [4-17]; 14F/12M) blood CRP concentration and monocyte density (but not WBC) increased post-sICH. CRP increased from 0-2 to 3-5 days (8.3-fold, p=0.020) then declined at 7-12 days. Monocytes increased from 0-2 to 3-5 days (1.8-fold, p<0.001) then declined at 7-12 days.

Conclusions- An anti-inflammatory pathway, enlisting native microglia and blood monocytes, occurs alongside neuroinflammation post-sICH. This novel pathway offers therapeutic targets and a window of opportunity (3-5 days post-sICH) for delivery of therapeutics via invading monocytes.

Non-standard Abbreviations and Acronyms

sICH: Spontaneous intracerebral haemorrhage

ICH: Intracerebral haemorrhage

IQR: Interquartile range

NIHSS: National Institutes of Health Stroke Scale

CRP: C-reactive protein

WBC: Total white cell count

GCS: Glasgow Coma Scale

mRS: Modified Rankin score

Introduction

Spontaneous intracerebral haemorrhage (sICH) is the most common form of haemorrhagic stroke, with poor prognosis and limited treatment options [1, 2]. Haemorrhage causes primary brain injury due to mass effect and physical disruption of brain parenchyma. Secondary brain damage then results from neuroinflammatory reactions and release of clot constituents [3]. It has been suggested that infiltrating white blood cells (WBC) play a role in secondary injury after ICH [4, 5].

Microglia are part of the innate immune system, providing a stable microenvironment for functionality of the central nervous system [6, 7]. Following sICH, cytokines and other cytotoxic mediators attract and activate microglia [6, 8]. Activated microglia can exacerbate damage to the brain parenchyma by activating pro-inflammatory (sometimes termed M1) pathways [9-11]. Recently, activation of anti-inflammatory (M2) brain repair pathways have been reported in animals [8, 12].

We recently reported greatly increased microglia-macrophage activation around the haematoma in well-characterized tissue from sICH cases[13] using a pan-selective marker (Iba1). This prompted two questions. First, are these immune cells primarily neuroinflammatory or is there an anti-inflammatory component? Second, do blood-derived macrophages contribute to this immune response? Both questions have implications for therapy, the first in developing drugs to manipulate microglial function, the second as a possible route for delivering therapeutic agents to brain tissue.

Here we examined brain tissue from subjects who died 0-12 days following supratentorial sICH, using microglia-macrophage markers specific for pro-inflammatory (CD68, TMEM119) and anti-inflammatory cells (CD163, CD206). We compared blood markers of inflammation (CRP, WBC and monocyte counts) over a similar timescale, in prospective sICH patients.

Material and Methods

Human tissue

The post-mortem study includes spontaneous supra-tentorial haemorrhage cases (n=27, M = 13, F = 14, age range 19-90 years old, median = 59 years old) (Table 1). Tissue blocks (1-2 blocks per case) were taken at the border of the haemorrhage, and further samples (1-2 blocks per case) distant from bleed, defined as contralateral similar anatomical regions. If not available, a different lobe within the same hemisphere (n=17) was examined. A group of control subjects (n=16) deceased due to non-neuropathological cause (M=11, F=5, range 26-60, median 51 years old) were also examined in the same anatomical regions (Table 1).

Recruitment of patients with sICH

This was a prospective study of patients aged ≥18 years with sICH admitted to our tertiary regional stroke service in whom informed consent was provided by the patient/legal representative. sICH was defined as a spontaneous, nontraumatic, abrupt onset appropriate clinical symptoms and signs (e.g. focal neurologic deficit) that was associated with a focal brain parenchymal haematoma visible on neuroimaging. Cases of ICH due to malignancy-associated coagulopathy, dural venous sinus thrombosis, vascular malformations, aneurysm rupture, tumours or hemorrhagic transformation of a recent ischemic stroke were excluded. Patients' demographics (Table 2), Glasgow Coma Scale (GCS) score, admission National Institutes of Health Stroke Scale (NIHSS), 30-day case-mortality, 3-month follow up modified Rankin score (mRS), WBC and monocyte concentration were also recorded. ICH volume was calculated from the presenting computerized tomography (CT) using the ABC/2 technique. This is a validated bedside method for measuring ICH volume from the CT head scan [14, 15]. In the ABC/2 method, A = greatest hemorrhage diameter by CT, B = diameter perpendicular to A, C = the approximate number of CT slices with hemorrhage multiplied by the slice thickness.

Patients with WBC, C-Reactive Protein (CRP) and monocytes results within 3-time intervals (results within 2 days, 3-5 days and 7-12 days) were included to allow data analysis in line with our immunohistochemistry study. An average was taken if more than one result was available during the time interval.

Ethics approval

All aspects of this study were approved by the UK National Research Ethics Service, as part of the NHS Health Research Authority. For the neuropathology study, ethical approval was provided by BRAIN UK (Research Ethics Committee South Central Hampshire B, reference 14/SC/0098) for post-mortem cases from St George's University Hospitals NHS Foundation Trust, North Bristol NHS Trust, and University Hospitals Plymouth NHS Trust. The cases provided by the Oxford Brain Bank and University of California, Irvine had ethical approval, reference 12/EM/0028, from Health Authority Service, NRES Committee East Midlands-Derby.

Our sICH study titled "Inflammation after intracerebral haemorrhage: understanding the pathophysiology to enhance brain repair" received ethical approval from Health Research Authority (London and South East Research Ethics Committee, REC reference 18/LO/1892, protocol number18.0037, IRAS project ID 241340).

Immunohistochemistry

Sections of formalin-fixed paraffin embedded tissue were processed for hematoxylin-eosin (H&E) and immunohistochemistry as described previously [13, 16]. Briefly, sections (6µm) were de-waxed and processed for standard immunohistochemical labelling. Endogenous peroxidase activity was blocked by exposure to H_2O_2 (3% v/v, aqueous solution) for 10 min. After high-pressure heat-induced antigen retrieval (30s, 125oC, in pH7.8 Tris-citrate buffer), non-specific binding was blocked with PBS supplemented with Triton-X100 (0.1%) and BSA (3%) (PBT-

BSA) for 60min at room temperature and sections were incubated with the following primary antibodies, diluted in PBT-BSA at 4°C overnight (see Supplementary file for further details). CD68 (mouse monoclonal, clone PG-M1, 1:800, M087601-2, Dako-Agilent Technologies LDA UK Limited Stockport, Cheshire, UK), TMEM119 (1:1000, rabbit polyclonal ab185333, Abcam, Cambridge, UK), CD163 (mouse monoclonal, clone 10D6, 1:800, NCL-L-CD163, Leica-Novocastra Biosystems Newcastle Ltd, Newcastle-upon-Tyne, UK), CD206 (1:2000, rabbit polyclonal ab64693, Abcam, UK). Antibody labelling was visualised using a peroxidase-conjugated secondary reagent (Envision® kit, K500711, Dako-Agilent Technologies LDA, UK) and diaminobenzidine (DAB) chromagen, then counterstained for nuclear chromatin with Mayer's hematoxylin. As a negative control neighbouring sections were treated with irrelevant primary antibody (rabbit anti-sheep IgG; BD-Pharmingen).

Microglia-macrophage quantification

CD68, TMEM119, CD163 and CD206 immunolabelled slides were digitized at x20 magnification using a slide scanner (Hamamatsu WEB). From the scanned slide, ten images from the peri-haematoma area were digitally acquired using NDP View software (Hamamatsu WEB) and analysed with Image J (Version 1.51j8, Wayne Rasband, NIH, USA). MaxEntropy macro filter was exclusively applied to threshold the images. Labelled area fraction (%AF) is reported as 100x (number of pixels positive for each marker)/total number of pixels.

The number of CD163 positive cells in the vessel wall were counted in 5 cases known to have died within day 1 post ICH and compared with 5 matched control brains .

Morphology assessment

In sICH scanned sections, areas of maximal changes, defined as high cell density areas, were identified adjacent to the haematoma region. The morphology of microglia/macrophage labelled cells was assessed using the zoom in function of NDP.view2 Viewing software

(Hamamatsu WEB) at 40x magnification. For comparison, similar areas in the same section were examined distant from the haematoma and in sections from control cases. We utilized our previously published method of assessment of microglia/macrophages in terms of identifying resting microglia as having a small oval cell body and branched processes, activated microglia as being swollen ramified cells characterized by a larger, denser cell body with shorter, stouter processes, amoeboid as being spherical in shape, lacking processes and containing numerous phagocytic vacuoles and giant microglia as being large cells with two or more nuclei [13].

Statistical analysis

Descriptive analysis was performed to assess the normality of the data. For some of the analyses, the sICH post-mortem cases were separated according to the time interval from haemorrhage to death as follows: 0-2 days (n=7); 3-5 days (n=7) and 5-12 days (n=5).

The data were non-parametric and Kruskal-Wallis test with Dunn's multiple comparison post-hoc test was performed to analyse %AF load of microglia-macrophages comparing the peri-haematoma area of sICH, with distant from bleed region in sICH and controls. Furthermore, we analysed the temporal course of microglia/macrophages %AF load difference in sICH compared with control subjects.

The sICH patients' WBC, CRP and monocyte counts were compared over time using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test was performed. Spearman's correlation was used to analyse the relationship between monocyte counts and patients' age, sex, sICH volume and outcome.

GraphPad Prism software was used to perform the statistical analysis, with p value considered significant when <0.05.

Results

We studied brain tissue samples from 27 supratentorial sICH donors (median age 59, range 19-90 years; 14F/13M) and 16 control cases without CNS injury (median 51, range 26-60, 5F/11M). Demographics and clinical data are provided in Table 1.

Anti-inflammatory process alongside inflammation after sICH

We used two established anti-inflammatory markers, CD163 and CD206[8, 17-19]. From day 3-5 post-ICH clusters of amoeboid CD163-positive cells were present in perivascular spaces and abundantly scattered in the parenchyma, apparently distant from the blood vessels (Figure 1A, B). Giant microglia [13] that were CD163-positive were common in the peri-haematoma region from day 5 post sICH (examples shown in supplementary figure S1). No CD163-positive ramified microglia were observed. In control brains, sparse CD163 positive macrophages were seen, mainly in perivascular spaces (figure S1).

The CD163 labelling pattern was similar to that of the standard neuropathological marker for inflammatory microglia-macrophages, CD68. At days 0-2 post-sICH, perivascular macrophages and a few microglia were CD68-positive, similar to control tissue samples. CD68 labelling increased significantly at days 7-12 post-sICH, (6-fold; Kruskal-Wallis test with Dunn's multiple comparison post-hoc test, H (df=3, N=33) =17.91, p=0.002; Figure 1D). This pattern of CD68 labelling agrees with that obtained using the pan-selective microglia-macrophage marker Iba1 (in our previous study[13]). The extent of CD163 labelling increased not only in peri-haematoma but also in haematoma-distant regions compared to control cases (Figure 1E). The extent of CD163 labelling increased progressively with time post-sICH, with

modest but significant elevation at 0-2 days, and progressive, substantial increases at 3-5 and 7-12 days post-sICH (15-fold increase at 7-12 days relative to 0-2 days, Kruskal-Wallis test H (df:3, N:33) =28.29, p<0.0001; Figure 1F). In a subset of patients who died within 1-day post-sICH, the median number of CD163 positive cells in the wall of blood vessels was significantly higher than in control cases (13.3 vs 4.2 cells/vessel, n=5, 5, respectively; p=0.004, Mann Whitney test).

CD206 positive cells were absent from control brains (though monocytes within blood vessel lumina were positive as expected; see figure S1). In sICH, the extent of CD206 labelling increased in the peri-haematoma region but not in haematoma-distant regions (Figure 2C). By day 1 post sICH, some CD206-positive cells were present in brain parenchyma near blood vessels (Figure S1 J). The extent of CD206 labelling was elevated at days 3-5 (5.2-fold, Kruskal-Wallis H (df:3, N=34) =17.06, p=0.001) but returned to control levels at days 7-12 (Figure 2D).

Blood-derived monocytes as well as native microglia contribute to the innate immune response post-sICH

TMEM119 labels microglia but not macrophages or monocytes, whereas CD206 labels monocytes and monocyte-derived macrophages but not microglia. Cell labelling was abundant for both these markers following sICH (examples in Figure 2A, B).

In control brains, TMEM119 labelled ramified microglia, with a similar pattern to that observed with Iba1[13]. Following sICH, TMEM119-positive microglia were evident in the peri-haematoma region from day 1 onwards and manifested a primarily amoeboid morphology, less commonly a fused microglial morphology and occasionally giant multinucleated cells [13] at days 7-12 post sICH (figure S1). The extent of TMEM119 labelling was significantly elevated in peri-haematoma regions compared to control cases, but not in areas distant from the

haematoma (Figure 2E). Extent of TMEM119 labelling was modestly elevated (2-fold) compared to controls at 3-5 days and 7-12 days post-sICH (Figure 2F). In the 3-5-day time-period, the extent of TMEM119 was significantly elevated (Kruskal-Wallis test, H (df:3, N:34) =11.03, p=0.02) and similar in magnitude to CD206 (Figure 2D, F).

All immunohistochemical markers showed no significant association with age at death, sex or brain anatomical location of ICH (Tables S1-3).

Peripheral blood markers confirm the time-course of microglia-macrophage activation

To test whether circulating blood biomarkers support a contribution of blood monocytes to the brain tissue response to sICH, we prospectively enrolled 26 sICH patients (median [IQR] age: 74, [66-79], NIHSS on admission: 8, [4-17]; 14F/12M; Table 2) who had blood tests at different time intervals following sICH (days 0-2, days 3-5 and days 7-12). Thirteen patients had blood samples from all three time periods (median age 76 y, [65-83.5], 6F/7M; details in Table 2). Plasma CRP concentration increased significantly from days 0-2 to days 3-5 (Kruskal-Wallis test H(df:2, N:38)=12.6, p=0.002), before declining at days 7-12 (p=0.390; Figure 3A). Blood monocyte counts were increased significantly at days 3-5 (Kruskal-Wallis H(df:2, N=39)=18.97, p<0.0001) relative to days 0-2 but not at days 7-12 (Figure 3B). By contrast, total WBC count, lymphocyte count and neutrophil count did not vary significantly over time (Figure 3C, Figure S2). The proportion of monocytes (as a fraction of total WBCs) was significantly higher at days 3-5 compared to days 0-2 or days 7-12 post sICH (Kruskal-Wallis H (df:2, N=39) =10.14, p=0.006; Figure 3D). Considering the blood data of all patients, including those who did not have data at all three-time intervals, gave similar results (supplementary figure S3). No significant correlations were identified between monocyte counts and age, haematoma volume or clinical outcome, within each of the time-periods 1-2, 3-5 and 7-10 days post-ICH (supplementary tables S4-6).

Discussion

We examined subtype-specific microglia-macrophage markers in neuropathological tissue from people who died up 12 days following supratentorial spontaneous ICH. The main findings are as follows. The anti-inflammatory marker CD163 increased progressively over time, through 1-2, 3-5 and 7-12 days post-ICH, alongside the inflammatory marker CD68. Another anti-inflammatory marker CD206 also increased to peak at 3-5 days, then declined at 7-12 days. Elevation of both CD206 and TMEM119 (specific for blood monocyte-derived cells, and brain-derived microglia, respectively) was similar at 3-5 days. Blood samples from living ICH patients showed elevated monocyte counts and CRP at 3-5 days post-ICH, consistent with a blood-derived contribution to the innate immune response to ICH. Our findings are summarized in Table 3.

The timescale for macrophage-microglial activation that we observed with the standard diagnostic marker CD68 agreed with our previous data obtained with Iba1 [13]. The timescale was also in accord with experimental animal studies of ICH[8, 12]. Together, these observations suggest a general increase in microglial-macrophage activation, beginning by 3-5 days post-sICH and progressing through 7-12 days.

CD163, the haptoglobin-hemoglobin receptor[20], and CD206, also known as the mannose receptor [21], have both been classified as anti-inflammatory markers [8, 17-19]. Our findings of elevated immunolabelling for CD163 and CD206 close to the haematoma support an anti-inflammatory process following sICH, coincident with neuroinflammation. This may act as a negative feedback mechanism to limit bystander damage, but may also have a role in tissue repair after sICH. CD163 and CD206 were localised to the peri-hematoma area, consistent with previous reports that these markers were absent from undamaged tissue [21, 22]. Elevation of

CD163 levels in CSF following ICH or SAH have been reported by other groups [23-25]. One prior study of ICH[17] examined peri-haematoma tissue, surgically excised at the time of the haematoma evacuation, from patients who survived ICH and were selected for surgery (whereas our study is of ICH patients who died within 12 days post-ICH). Despite the different clinical settings, they reported western data showing a progressive increase in CD163 abundance, continuing to increase beyond 72 h post-ICH (B-H Liu et al. 2015)[17] in agreement with our findings (Fig 1). Experimental studies in a large species with gyrencephalic brain structure revealed substantial CD163 expression 1-3 days after an ICH-like challenge (autologous blood injection)[26].

The decline in CD206 (an "M2" marker) 7-12 days post-ICH may reflect either a decline in anti-inflammatory cells with maintained accumulation of inflammatory ("M1") cells, or conversion of anti-inflammatory cells to inflammatory phenotype. To discriminate these alternatives requires a prospective study in an appropriate animal model. Based on these findings with CD163 and CD206, we speculate that the anti-inflammatory pathway may offer novel treatment targets in sICH.

TMEM119 specifically labels microglia [27] and not infiltrating macrophages [27, 28] whereas CD206 is a marker specific for blood monocytes and monocyte-derived macrophages but does not identify microglia [29, 30]. Considering our data for TMEM119 and for CD206, it appears there are substantial contributions not only from brain-derived microglia (TMEM119-positive) but also from blood monocyte-derived macrophages (CD206-positive) in the response to sICH. CD206 labelling reached a peak at 3-5 days, whereas TMEM119 remained elevated through 7-12 days, with approximately equal extent of labelling by the two markers (Figure 2D, F). CD68 and CD163 by contrast both continued to rise from 3-5 through 7-12 days (Figure 1D, F). These

results confirm a substantial contribution from monocyte efflux, alongside activation of native microglia, in response to sICH.

We previously reported giant Iba1-positive microglial cells in sICH [13]. In the present study we found giant microglia cells positive for CD163 and TMEM119. Giant microglia have been described in a mouse model of ICH and were associated with improved outcome [12].

For all the neuropathological markers studied, we observed no significant associations with age, sex or location of ICH (Tables S1-S3). This is a relatively modest cohort, reflecting the heterogeneity dependent on human tissue access. We cannot exclude a shift in microglial function with ageing [31]. A larger neuropathological cohort will be required to confidently assess potential effects of age and other demographic factors.

Our blood biomarker results are consistent with the hypothesis that systemic inflammatory processes occur in parallel with brain microglia-macrophage activation in response to sICH. Our data from blood samples showed specific augmentation of the circulating monocyte population (Figure 3B, D) providing the potential to contribute to the concomitant peak of CD206 labelling in brain tissue at days 3-5 (Figure 2D). Previous studies observed that high monocyte counts [32-34] and rising blood CRP concentration [35] in the early phase post-sICH (days 1-3) were correlated with poor outcomes (worsening neurological deficit and haematoma expansion) [35-37]. It appears likely that blood monocyte-derived macrophages contribute to both inflammatory and anti-inflammatory pathways in sICH brain tissue. We and others reported that neutrophils infiltrate into and around the haematoma as early as 1-day post haemorrhage [4, 5, 13]. These may cause direct injury by releasing reactive oxygen species or inflammatory proteases with additional BBB disruption [4, 5, 13]. Neutrophils also facilitate the recruitment of monocytes to the haematoma which may influence functional outcome [38]. The monocyte influx

into brain tissue appears to be transitory, with a "window of opportunity" 3-5 days after sICH. We speculate that invading monocytes offer a potential delivery route for therapeutics in clinical sICH.

Conclusion: our data support an anti-inflammatory microglia-macrophage response in human sICH, alongside conventional neuroinflammation. Neuropathological findings suggest that, in addition to microglia, blood-derived monocytes contribute to the local response to sICH, with a timescale confirmed by blood biomarkers. Our results provide new pathological insight into the non-adaptive immune response to sICH in humans, and widen the scope for therapeutic intervention for this common, disabling condition.

Acknowledgement

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

Data availability

Researchers can apply for access to anonymized data from the present study for well-defined research questions that are in line with the overall research agenda for the cohort. Please contact the corresponding author.

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Figure Legends

Figure 1. Anti-inflammatory process alongside inflammation in human ICH.

Cells immunolabelled with the anti-inflammatory marker CD163 were absent in the parenchyma at day 1 post-ICH (A) but abundant at 5 days (B). Haematoma is marked with asterisks (*). Scale bars: 1 mm, for insets 50 µm.

The inflammatory marker CD68 (C) (n=30) and CD163 (E) (n=30) both increased in peri-haematoma tissue, and CD163 also increased in tissue distant from the bleed (n=17), relative to control brains (n=14). CD68 (D) and CD163 (F) both increased progressively through 3-5 and 7-12 days post-sICH (B, D).

Scatter dot plots show individual cases. Horizontal lines indicate median and IQR. Asterisks above dot clusters show significant difference from control brains, *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Local microglia and invading monocytes contribute equally to the innate immune response in ICH.

A, B. CD206 which labels blood monocyte-derived macrophages (A), and TMEM119 which is specific for brain-derived microglia (B) were both abundant at day 5 post-ICH. Asterisks mark the haematoma. Scale bars: 1 mm, for insets 50 µm.

CD206 (n=30) was significantly elevated peri-haematoma relative to tissue distant from the bleed, and also relative to control tissue (C) (n=15). CD206 was elevated at 3-5 days post-ICH relative to control tissue, but not at 7-12 days (D). TMEM119 (n=30) was significantly elevated peri-haematoma relative to control tissue (E) (n=14) and showed a modest but significant elevation at 3-5 days and remained elevated through 7-12 days (F).

Scatter dot plots show individual cases. Horizontal lines indicate median and IQR. Asterisks above dot clusters show significant difference from control brains, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Temporal course of peripheral blood inflammatory markers in prospective patients with sICH. A) Plasma CRP concentration (mg/L) was significantly elevated at days 3-5 following sICH, relative to days 0-2, before declining at days 7-12. Each line indicates an individual patient. B) Blood monocyte counts (10^9 cells/L) increased significantly from days 0-2 to days 3-5. C) Total WBC count (10^9 cells/L) did not change significantly over time. Scatter dot plots show individual cases. Horizontal lines indicate median and IQR. D) The ratio of monocytes to total WBCs was significantly higher at days 3-5 post sICH compared to days 0-2 and days 7-12 post sICH. box-whisker plot shows median, IQR and full range, *, p<0.05, ** p<0.01, *** p<0.001 relative to 0-2 days post-ICH.

Table 1. Demographic data of post mortem sICH and control cases studied.

Study Number	ICH or Control	Sex (F/M)	Age (years)	Location of sICH	Time from ICH to death (days)	Relevant past medical history
1	ICH	M	86	Lobar	NA	AD
2	ICH	F	90	Deep	NA	AD, HTN
3	ICH	M	82	Lobar	NA	AD, IHD
4	ICH	F	82	Deep	NA	AD
5	ICH	M	90	Lobar	NA	None
6	ICH	M	76	Lobar	NA	AD, CAA
7	ICH	M	90	Lobar	NA	AD
8	ICH	M	90	Lobar	NA	AD
9	ICH	F	75	Deep	2	None
10	ICH	F	40	Deep	2	Leukaemia
11	ICH	M	52	Deep	4	None
12	ICH	F	79	Lobar	12	None
13	ICH	F	56	Deep	12	None
14	ICH	F	52	Deep	1	None
15	ICH	F	58	Deep	1	None
16	ICH	M	59	Lobar	7	HTN
17	ICH	M	78	Lobar	10	None
18	ICH	M	30	Lobar	3	HTN

19	ICH	F	55	Deep	5	HTN
20	ICH	F	19	Lobar	8	Pneumonia
21	ICH	F	53	Lobar	3	None
22	ICH	F	53	Lobar	1	None
23	ICH	F	27	Lobar	< 1	Drug user, Seizures
24	ICH	F	52	Deep	3	COPD, IHD
25	ICH	M	65	Deep	3	Alcohol, Leukaemia
26	ICH	M	49	Lobar	3	Alcohol HTN
27	ICH	M	65	Deep	< 1	HTN, DMII
28	Control	M	36	Not applicable		
29	Control	M	56	Not applicable		
30	Control	M	56	Not applicable		
31	Control	M	56	Not applicable		
32	Control	M	41	Not applicable		
33	Control	M	59	Not applicable		
34	Control	F	26	Not applicable		
35	Control	M	56	Not applicable		
36	Control	M	51	Not applicable		
37	Control	F	48	Not applicable		
38	Control	F	42	Not applicable		
39	Control	M	60	Not applicable		
40	Control	M	58	Not applicable		
41	Control	M	51	Not applicable		
42	Control	F	51	Not applicable		
43	Control	F	51	Not applicable		

Abbreviations. sICH: Spontaneous intracerebral haemorrhage, M: Male, F: Female, NA: Not available, AD: Alzheimer's disease, HTN: Hypertension, CAA: Cerebral amyloid angiopathy,

COPD: Chronic obstructive pulmonary disease, IHD: Ischaemic heart disease, DMII: Diabetes mellitus type 2.

Table 2. Prospectively recruited sICH patients

Patient number	Age/sex	Location of ICH	Volume of ICH (ml)	IVH (Y/N)	Pre-Morbid mRS	mRS on admission	NIHSS on admission	mRS at 3 months
001*	71/M	Lobar	84	No	0	5	NA	5
003*	80/F	Deep	58	Yes	3	5	24	6
004	76/M	Deep		Yes	0	3	6	2
005*	83/F	Lobar	1.5	No	4	5	8	5
006	87/F	Lobar		No	1	5	NA	4
007	54/F	Deep		No	0	4	8	1
008	79/M	Deep		No	0	3	2	1
009	74/M	Lobar		Yes	1	4	26	4
010*	63/F	Lobar	50	Yes	3	5	NA	6
011*	84/F	Deep	19	Yes	2	5	22	4
012*	69/M	Deep	15	Yes	0	4	17	4
013*	66/F	Deep	1	No	4	5	9	4
014	41/F	Deep		No	0	2	2	0
015	68/F	Lobar		No	3	5	23	6
016*	45/M	Deep	11	Yes	0	4	10	2
017	66/M	Deep		No	2	4	4	2
018	58/F	Deep		No	0	4	15	2
019*	86/F	Lobar	9	No	0	4	6	4
020*	86/M	Lobar	24	No	0	3	0	3
021	74/F	Lobar		No	1	4	7	1
022*	77/M	Lobar	15	No	1	2	4	1
023*	77/M	Deep	29	Yes	1	5	21	6
024	69/F	Deep		No	0	4	12	3

| 025 | 76/M | Lobar | | No | 1 | 2 | NA | 2 |
| 026* | 64/M | Deep | 1.5 | No | 0 | 2 | 1 | 0 |

Abbreviations ICH: Intracerebral hemorrhage, NIHSS: National Institutes of Health Stroke Scale, mRS: Modified Rankin score, NA: Not available.

* patients with 3-time points blood results. The rest of the patients did not have all 3 time points studied due to death, repatriation to local hospital or discharge home. Patient 002 withdrew from the study. ICH volume was only estimated for patients with 3-time points blood results.

Table 3. Temporal course of microglial-macrophage markers following sICH

	Control brain	0-2 days	3-5 days	7-12 days	Marker description
Iba1	+	++	++	+++	Pan microglia-macrophage marker. Expressed by pro-inflammatory and anti-inflammatory cells.
CD68	+	+	++	++	Standard diagnostic marker for activated microglia-macrophages, indicating phagocytic activity.
CD163	+	+	++	+++	Selective for anti-inflammatory cells. Labels macrophages and microglia.
CD206	+	+	++	++	Selective for anti-inflammatory cells. Labels monocytes and macrophages.
TMEM119	+	++	++	++	Specific for microglia (not macrophages).
Blood WBC count	NA	+	+	+	
Blood monocytes	NA	+	++	+	
Blood CRP	NA	+	++	+	

Semiquantitative representation of the data of all microglia-macrophages' markers and description of the markers. Categories for the extent of marker abundance (% area fraction). 0: absent; + <3%; ++ <10%; +++ >10%. Abbreviations. sICH: spontaneous intracerebral hemorrhage, WBC: white blood cells, PMN: polymorphonuclear cell, CRP: C-reactive protein, NA: not available.