Stroke

CLINICAL AND POPULATION SCIENCES

Innate Immune Anti-Inflammatory Response in Human Spontaneous Intracerebral Hemorrhage

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BACKGROUND AND PURPOSE: Spontaneous intracerebral hemorrhage (sICH) is a common form of hemorrhagic 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 sICH.

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

RESULTS: In 27 supratentorial sICH cases (n=27, median [interquartile range] age: 59 [52–80.5], 14F/13M) all microglia-macrophage markers increased post-sICH, relative to control brains. Anti-inflammatory markers (CD163 and CD206) were elevated alongside proinflammatory markers (CD68 and TMEM119). CD163 increased progressively post-sICH (15.0-fold increase at 7–12 days, $P<0.001$). CD206 increased at 3 to 5 days (5.2-fold, $P<0.001$) then returned to control levels at 7 to 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], National Institutes of Health Stroke Scale on admission: 8 [4–17]; 14F/12M) blood CRP concentration and monocyte density (but not white blood cell) increased post-sICH. CRP increased from 0 to 2 to 3 to 5 days (8.3-fold, $P=0.020$) then declined at 7 to 12 days. Monocytes increased from 0 to 2 to 3 to 5 days (1.8-fold, $P<0.001$) then declined at 7 to 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.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: immunity ■ inflammation ■ macrophages ■ microglia ■ monocytes

Spontaneous intracerebral hemorrhage (sICH) is the most common form of hemorrhagic stroke, with poor prognosis and limited treatment options.[1,2] Hemorrhage 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 (WBCs) 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

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For Sources of Funding and Disclosures, see page 3622.

Nonstandard Abbreviations and Acronyms

CRP	C-reactive protein
ICH	intracerebral hemorrhage
sICH	spontaneous intracerebral hemorrhage
WBC	white blood cell

brain parenchyma by activating proinflammatory (sometimes termed M1) pathways.[9–11] Recently, activation of anti-inflammatory (M2) brain repair pathways has been reported in animals.[8,12]

We recently reported greatly increased microglia-macrophage activation around the hematoma in well-characterized tissue from sICH cases[13] using a pan-selective marker (Iba1). This prompted 2 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 to 12 days following supratentorial sICH, using microglia-macrophage markers specific for proinflammatory (CD68 and TMEM119) and anti-inflammatory cells (CD163 and CD206). We compared blood markers of inflammation (CRP [C-reactive protein], WBC, and monocyte counts) over a similar timescale in prospective sICH patients.

METHODS

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.

Human Tissue

The postmortem study includes spontaneous supratentorial hemorrhage 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 hemorrhage, and further samples (1–2 blocks per case) distant from bleed, defined as contralateral similar anatomic 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 anatomic 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 (eg, focal neurological deficit) that was associated with a focal brain parenchymal hematoma visible on neuroimaging. Cases of ICH due to malignancy-associated coagulopathy, dural venous sinus thrombosis, vascular malformations, aneurysm rupture, tumors, or hemorrhagic transformation of a recent ischemic stroke were excluded. Patients' demographics (Table 2), Glasgow Coma Scale score, admission National Institutes of Health Stroke Scale, 30-day case-mortality, 3-month follow-up modified Rankin Scale score, WBC, and monocyte concentration were also recorded. ICH volume was calculated from the presenting computed tomography using the ABC/2 technique. This is a validated bedside method for measuring ICH volume from the computed tomography head scan.[14,15] In the ABC/2 method, A=greatest hemorrhage diameter by computed tomography, B=diameter perpendicular to A, and C=the approximate number of computed tomography slices with hemorrhage multiplied by the slice thickness.

Patients with WBC, CRP, and monocytes results within 3 time intervals (results within 2, 3–5, 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 postmortem 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 Hemorrhage: 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 (6 μm) were dewaxed and processed for standard immunohistochemical labeling. Endogenous peroxidase activity was blocked by exposure to H_2O_2 (3% v/v, aqueous solution) for 10 minutes. After high-pressure heat-induced antigen retrieval (30 seconds, 125°C, in pH7.8 Tris-citrate buffer), nonspecific binding was blocked with PBS supplemented with Triton-X100 (0.1%) and BSA (3%; PBT-BSA) for 60 minutes at room temperature, and sections were incubated with the following primary antibodies, diluted in PBT-BSA at 4°C overnight (see Data Supplement for further details). CD68 (mouse monoclonal, clone PG-M1, 1:800, M087601-2, Dako-Agilent Technologies LDA UK Limited Stockport, Cheshire, United

Table 1. Demographic Data of Postmortem sICH and Control Cases Studied

Study number	ICH or control	Sex (F/M)	Age, y	Location of sICH	Time from ICH to death, d	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	Leukemia
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, Leukemia
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		

AD indicates Alzheimer disease; CAA, cerebral amyloid angiopathy; COPD, chronic obstructive pulmonary disease; DMII, diabetes type 2; F, female; HTN, hypertension; IHD, ischemic heart disease; M, male; NA, not available; and sICH, spontaneous intracerebral hemorrhage.

Kingdom), TMEM119 (1:1000, rabbit polyclonal ab185333, Abcam, Cambridge, United Kingdom), CD163 (mouse monoclonal, clone 10D6, 1:800, NCL-L-CD163, Leica-Novocastra Biosystems Newcastle Ltd, Newcastle-upon-Tyne, United Kingdom), CD206 (1:2000, rabbit polyclonal ab64693, Abcam, United Kingdom). Antibody labeling was visualized

Table 2. Prospectively Recruited sICH Patients

Patient number	Age/sex	Location of ICH	Volume of ICH, mL	IVH (Y/N)	Premorbid mRS	mRS on admission	NIHSS on admission	mRS at 3 mo
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

ICH indicates intracerebral hemorrhage; mRS, modified Rankin Scale; NA, not available; NIHSS, National Institutes of Health Stroke Scale; and sICH, spontaneous intracerebral hemorrhage.

*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.

using a peroxidase-conjugated secondary reagent (Envision kit, K500711, Dako-Agilent Technologies LDA, United Kingdom) and diaminobenzidine chromagen, then counterstained for nuclear chromatin with Mayer hematoxylin. As a negative control neighboring sections were treated with irrelevant primary antibody (rabbit anti-sheep IgG; BD-Pharmingen).

Microglia-Macrophage Quantification

CD68, TMEM119, CD163, and CD206 immunolabeled slides were digitized at ×20 magnification using a slide scanner (Hamamatsu WEB). From the scanned slide, 10 images from the perihematoma area were digitally acquired using NDP View software (Hamamatsu WEB) and analyzed with Image J (Version 1.51j8, Wayne Rasband, NIH, United States). MaxEntropy macro filter was exclusively applied to threshold the images. Labeled area fraction (%AF) is reported as 100× (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 hematoma region. The morphology of microglia/macrophage labeled cells was assessed using the zoom in function of NDP.view2 Viewing software (Hamamatsu WEB) at ×40 magnification. For comparison, similar areas in the same section were examined distant from the hematoma and in sections from control cases. We used 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 2 or more nuclei.[13]

Statistical Analysis

Descriptive analysis was performed to assess the normality of the data. For some of the analyses, the sICH postmortem cases were separated according to the time interval from hemorrhage

to death as follows: 0 to 2 days (n=7); 3 to 5 days (n=7), and 5 to 12 days (n=5).

The data were nonparametric, and Kruskal-Wallis test with Dunn multiple comparison post hoc test was performed to analyze %AF load of microglia-macrophages comparing the perihematoma area of sICH, with distant from bleed region in sICH and controls. Furthermore, we analyzed 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 the Kruskal-Wallis test with Dunn multiple comparison post hoc test was performed. The Spearman correlation was used to analyze 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 2 established anti-inflammatory markers, CD163 and CD206.[8,17–19] From day 3 to 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 and 1B). Giant microglia[13] that were CD163 positive were common in the perihematoma region from day 5 post-sICH (examples shown in Figure I in the Data Supplement). No CD163-positive ramified microglia were observed. In control brains, sparse CD163-positive macrophages were seen, mainly in perivascular spaces (Figure I in the Data Supplement).

The CD163 labeling pattern was similar to that of the standard neuropathological marker for inflammatory microglia-macrophages, CD68. At days 0 to 2 post-sICH, perivascular macrophages and a few microglia were CD68 positive, similar to control tissue samples. CD68 labeling increased significantly at days 7 to 12 post-sICH (6-fold; Kruskal-Wallis test with Dunn multiple comparison post hoc test, H [df=3, N=33]=17.91, *P*=0.002; Figure 1D). This pattern of CD68 labeling agrees with that obtained using the pan-selective microglia-macrophage marker Iba1 (in our previous study[13]). The extent of CD163 labeling increased not only in perihematoma but also in hematoma-distant regions compared with control cases (Figure 1E). The extent of CD163 labeling increased progressively with time post-sICH, with modest but significant elevation at 0 to 2 days, and progressive, substantial increases at 3 to 5 and 7 to 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 versus 4.2 cells/vessel, n=5, 5, respectively; *P*=0.004, Mann-Whitney test).

CD206-positive cells were absent from control brains (although monocytes within blood vessel lumina were positive as expected; see Figure I in the Data Supplement). In sICH, the extent of CD206 labeling increased in the perihematoma region but not in hematoma-distant regions (Figure 2C). By day 1 post-sICH, some CD206-positive cells were present in brain parenchyma near blood vessels (Figure ID in the Data Supplement). The extent of CD206 labeling was elevated at days 3 to 5 (5.2-fold, Kruskal-Wallis H [df:3, N=34]=17.06, *P*=0.001) but returned to control levels at days 7 to 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 labeling was abundant for both these markers following sICH (examples in Figure 2A and 2B).

In control brains, TMEM119 labeled ramified microglia, with a similar pattern to that observed with Iba1.[13] Following sICH, TMEM119-positive microglia were evident in the perihematoma 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 to 12 post-sICH (Figure I in the Data Supplement). The extent of TMEM119 labeling was significantly elevated in perihematoma regions compared with control cases but not in areas distant from the hematoma (Figure 2E). Extent of TMEM119 labeling was modestly elevated (2-fold) compared with controls at 3 to 5 days and 7 to 12 days post-sICH (Figure 2F). In the 3- to 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 and 2F).

All immunohistochemical markers showed no significant association with age at death, sex, or brain anatomic location of ICH (Tables I through III in the Data Supplement).

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

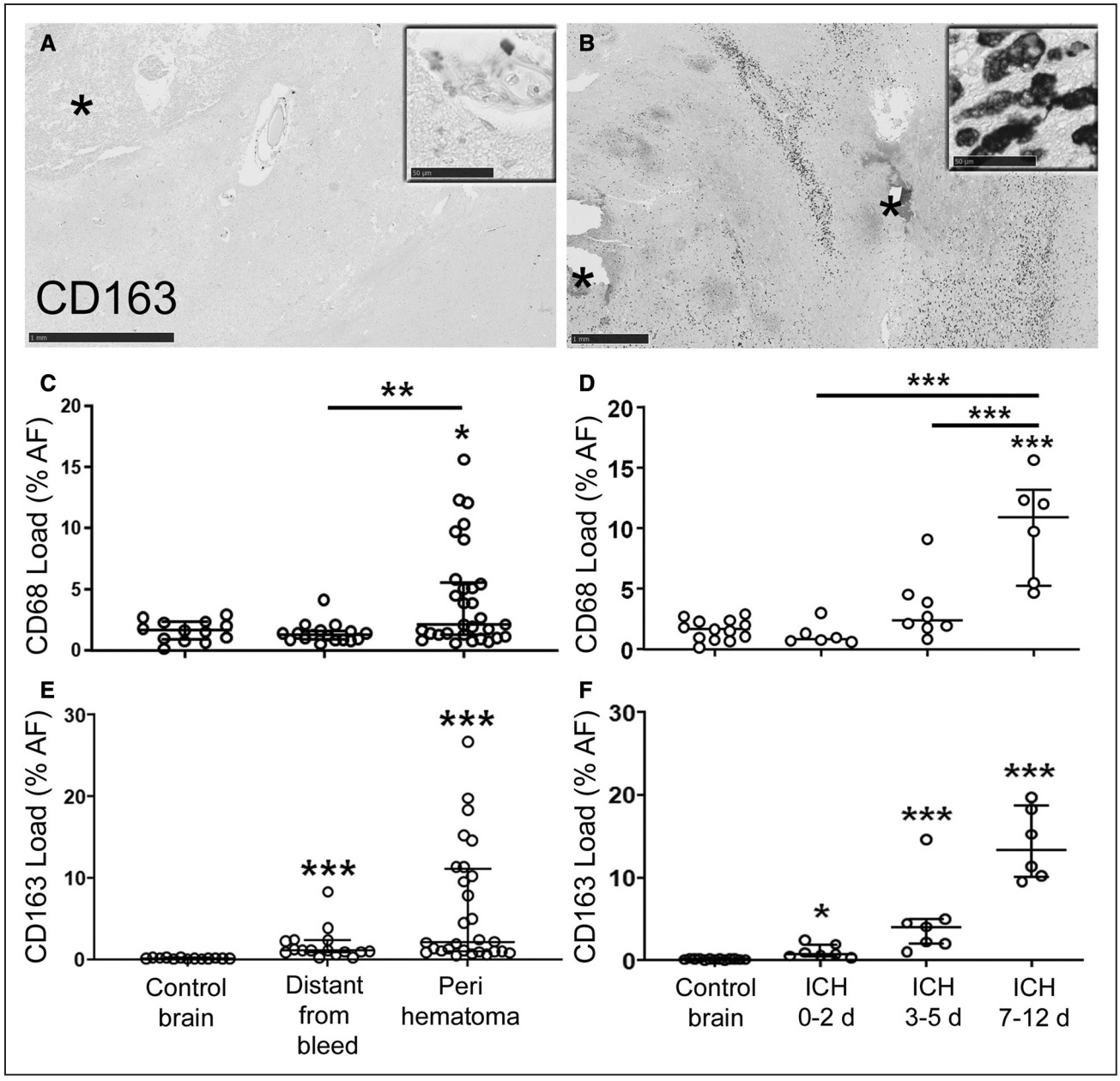

Figure 1. Anti-inflammatory process alongside inflammation in human intracerebral hemorrhage (ICH).
Cells immunolabeled with the anti-inflammatory marker CD163 were absent in the parenchyma at day 1 post-ICH (**A**) but abundant at 5 d (**B**). Hematoma is marked with asterisks (*). Scale bars: 1 mm, for insets 50 µm. The inflammatory marker CD68 (**C**; n=30) and CD163 (**E**; n=30) both increased in perihematoma 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 d post-sICH (**B** and **D**). Scatter dot plots show individual cases. Horizontal lines indicate median and interquartile range (IQR). Asterisks above dot clusters show significant difference from control brains, *P<0.05, **P<0.01, and ***P<0.001.

patients (median [interquartile range] age: 74 [66–79], National Institutes of Health Stroke Scale 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 3 time periods (median age 76 years [65–83.5], 6F/7M; details in Table 2). Plasma CRP concentration increased significantly from days 0 to 2 to days 3 to 5 (Kruskal-Wallis test H [df:2, N:38]=12.6, P=0.002), before declining at days 7 to 12 (P=0.390; Figure 3A). Blood monocyte counts were increased significantly at days 3 to 5 (Kruskal-Wallis H [df:2, N=39]=18.97, P<0.0001) relative to days 0 to 2 but not at days 7 to 12 (Figure 3B). By contrast, total WBC count, lymphocyte count, and neutrophil count did not vary significantly over time (Figure 3C, Figure II in the Data Supplement). The proportion of monocytes (as a fraction of total WBCs) was significantly higher at days 3 to 5 compared with days 0 to 2 or days 7 to 12 post-sICH (Kruskal-Wallis H [df:2, N=39]=10.14, P=0.006; Figure 3D). Considering

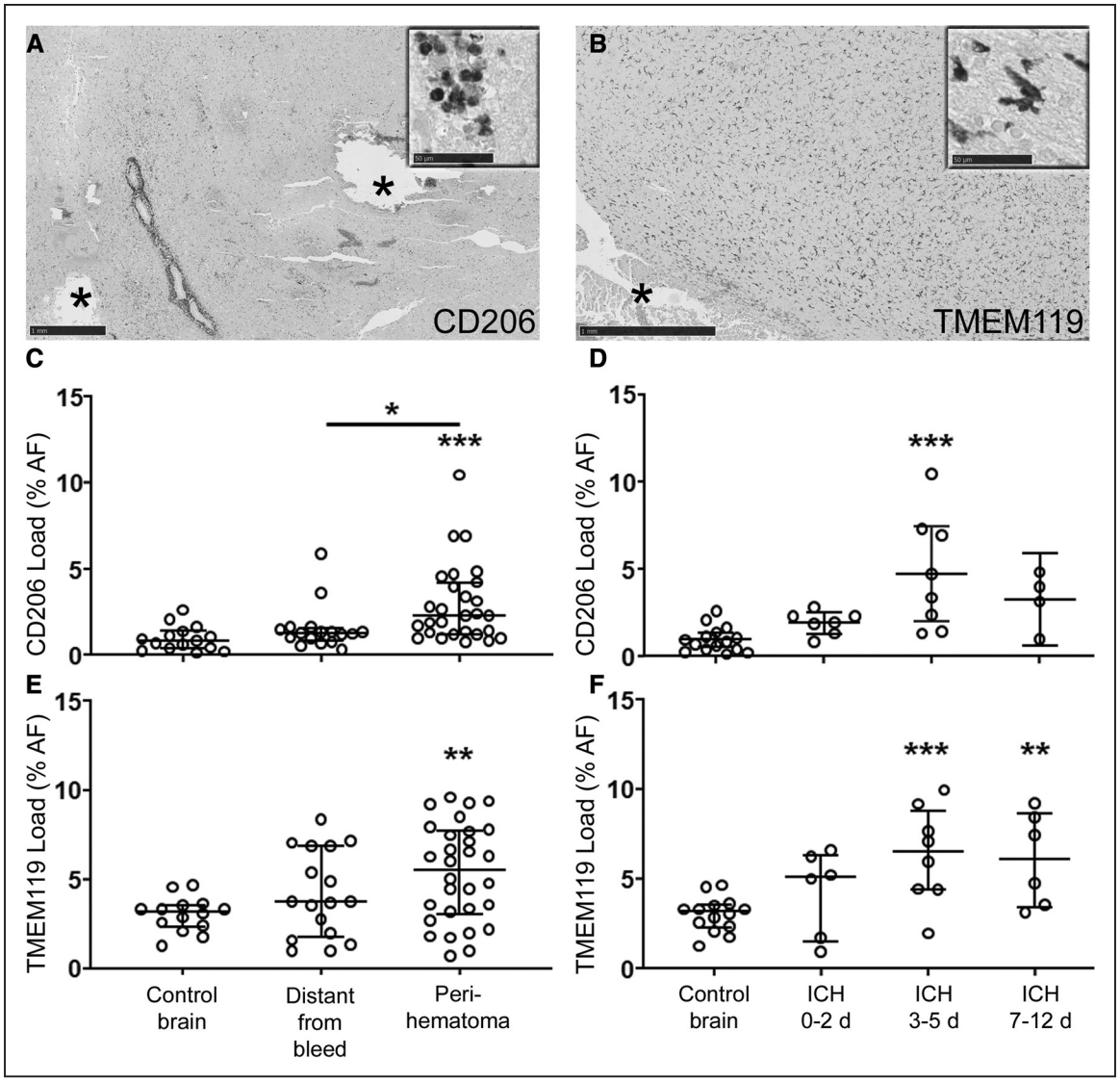

Figure 2. Local microglia and invading monocytes contribute equally to the innate immune response in intracerebral hemorrhage (ICH).

A and **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 hematoma. Scale bars: 1 mm, for insets 50 µm. CD206 (n=30) was significantly elevated perihematoma relative to tissue distant from the bleed and also relative to control tissue (**C**; n=15). CD206 was elevated at 3–5 d post-ICH relative to control tissue, but not at 7–12 d (**D**). TMEM119 (n=30) was significantly elevated perihematoma relative to control tissue (**E**; n=14) and showed a modest but significant elevation at 3–5 days and remained elevated through 7–12 d (**F**). Scatter dot plots show individual cases. Horizontal lines indicate median and interquartile range (IQR). Asterisks above dot clusters show significant difference from control brains, **P*<0.05, ***P*<0.01, and ****P*<0.001.

the blood data of all patients, including those who did not have data at all 3 time intervals, gave similar results (Figure III in the Data Supplement). No significant correlations were identified between monocyte counts and age, hematoma volume, or clinical outcome, within each of the time periods 1 to 2, 3 to 5, and 7 to 10 days post-ICH (Tables IV through VI in the Data Supplement).

DISCUSSION

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

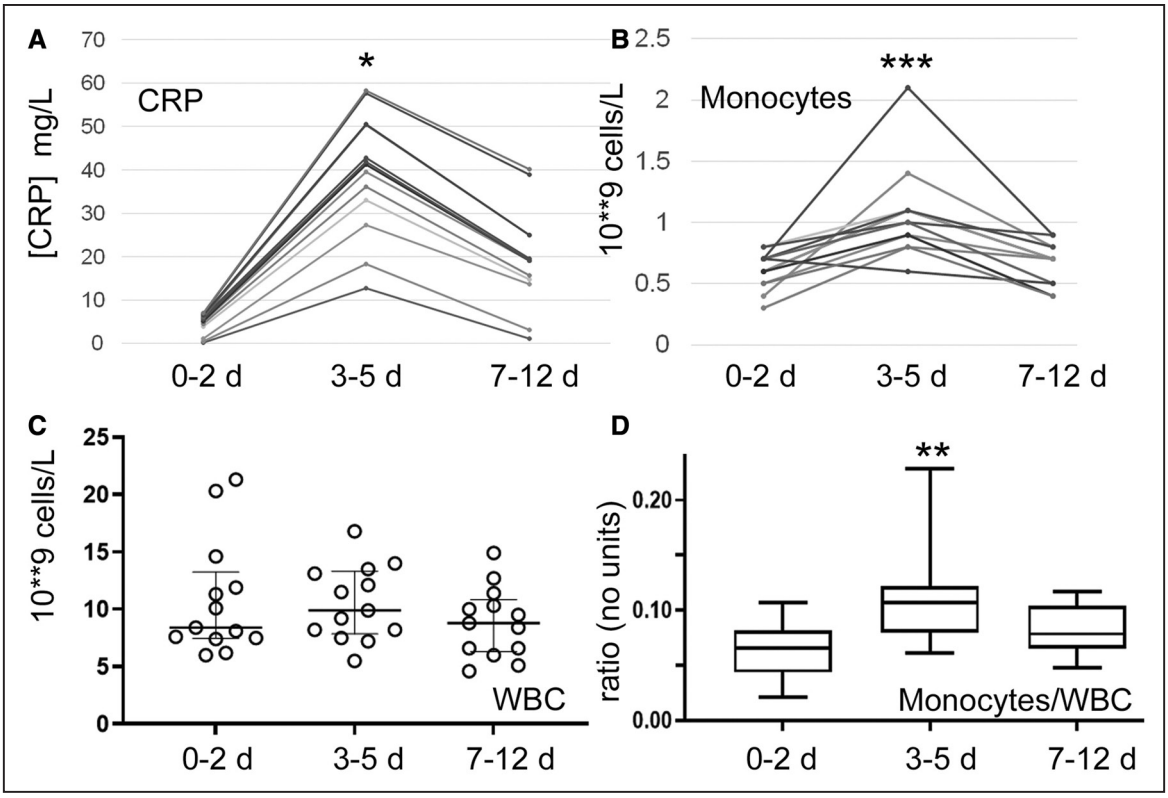

Figure 3. Temporal course of peripheral blood inflammatory markers in prospective patients with spontaneous intracerebral hemorrhage (sICH).

A, Plasma C-reactive protein (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 white blood cell (WBC) count (10^9 cells/L) did not change significantly over time. Scatter dot plots show individual cases. Horizontal lines indicate median and interquartile range (IQR). **D**, The ratio of monocytes to total WBCs was significantly higher at days 3–5 post-sICH compared with days 0–2 and days 7–12 post-sICH. Box-whisker plot shows median, IQR, and full range, *P<0.05, **P<0.01, and ***P<0.001 relative to 0–2 d post-ICH.

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 to 5 days post-sICH and progressing through 7 to 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 immunolabeling for CD163 and CD206 close to the hematoma 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 localized to the perihematoma area, consistent with previous reports that these markers were absent from undamaged tissue.[21,22] Elevation of CD163 levels in cerebral spinal fluid following ICH or SAH has been reported by other groups.[23–25] One prior study of ICH[17] examined perihematoma tissue, surgically excised at the time of the hematoma evacuation, from patients who survived ICH and were selected for surgery (whereas our study is of patients with ICH 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 hours post-ICH (Liu et al[17] 2015) in agreement with our findings (Figure 1). Experimental studies in a large species with gyrencephalic brain structure revealed substantial CD163 expression 1 to 3 days after an ICH-like challenge (autologous blood injection).[26]

The decline in CD206 (an M2 marker) 7 to 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

Table 3. Temporal Course of Microglial-Macrophage Markers Following sICH

	Control brain	0–2 d	3–5 d	7–12 d	Marker description
Iba1	+	++	++	+++	Pan microglia-macrophage marker. Expressed by proinflammatory 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%; and +++ >10%. CRP indicates C-reactive protein; NA, not available; PMN, polymorphonuclear cell; sICH, spontaneous intracerebral hemorrhage; and WBC, white blood cells.

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 labeling reached a peak at 3 to 5 days, whereas TMEM119 remained elevated through 7 to 12 days, with approximately equal extent of labeling by the 2 markers (Figure 2D and 2F). CD68 and CD163 by contrast both continued to rise from 3 to 5 through 7 to 12 days (Figure 1D and 1F). 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 I through III in the Data Supplement). This is a relatively modest cohort, reflecting the heterogeneity dependent on human tissue access. We cannot exclude a shift in microglial function with aging.[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 and 3D) providing the potential to contribute to the concomitant peak of CD206 labeling in brain tissue at days 3 to 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 hematoma 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 hematoma as early as 1-day post hemorrhage.[4,5,13] These may cause direct injury by releasing reactive oxygen species or inflammatory proteases with additional blood–brain barrier disruption.[4,5,13] Neutrophils also facilitate the recruitment of monocytes to the hematoma which may influence functional outcome.[38] The monocyte influx into brain tissue appears to be transitory, with a window of opportunity 3 to 5 days after sICH. We speculate that invading monocytes offer a potential delivery route for therapeutics in clinical sICH.

CONCLUSIONS

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.

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Disclosures

None.

Supplemental Materials

Expanded Materials and Methods
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REFERENCES

1. van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. *Lancet Neurol*. 2010;9:167–176. doi: 10.1016/S1474-4422(09)70340-0
2. Wilkinson DA, Pandey AS, Thompson BG, Keep RF, Hua Y, Xi G. Injury mechanisms in acute intracerebral hemorrhage. *Neuropharmacology*. 2018;134(pt B):240–248. doi: 10.1016/j.neuropharm.2017.09.033
3. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. *Lancet Neurol*. 2012;11:720–731. doi: 10.1016/S1474-4422(12)70104-7
4. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. *Prog Neurobiol*. 2010;92:463–477. doi: 10.1016/j.pneurobio.2010.08.001
5. Wang J, Doré S. Inflammation after intracerebral hemorrhage. *J Cereb Blood Flow Metab*. 2007;27:894–908. doi: 10.1038/sj.jcbfm.9600403
6. Dudvarski Stankovic N, Teodorczyk M, Ploen R, Zipp F, Schmidt MHH. Microglia-blood vessel interactions: a double-edged sword in brain pathologies. *Acta Neuropathol*. 2016;131:347–363. doi: 10.1007/s00401-015-1524-y
7. Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science*. 2005;308:1314–1318. doi: 10.1126/science.1110647
8. Lan X, Han X, Li Q, Yang QW, Wang J. Modulators of microglial activation and polarization after intracerebral haemorrhage. *Nat Rev Neurol*. 2017;13:420–433. doi: 10.1038/nrneurol.2017.69
9. Zhang Z, Liu P, Zhou D, Zhang L, Lei H. Intracerebral hemorrhage (ICH) evaluation with a novel magnetic induction sensor: a preliminary study using the Chinese head model. *Biomed Mater Eng*. 2014;24:3579–3587. doi: 10.3233/BME-141184
10. Wu H, Zhang Z, Hu X, Zhao R, Song Y, Ban X, Qi J, Wang J. Dynamic changes of inflammatory markers in brain after hemorrhagic stroke in humans: a postmortem study. *Brain Res*. 2010;1342:111–117. doi: 10.1016/j.brainres.2010.04.033
11. Liesz A, Middelhoff M, Zhou W, Karcher S, Illanes S, Veltkamp R. Comparison of humoral neuroinflammation and adhesion molecule expression in two models of experimental intracerebral hemorrhage. *Exp Transl Stroke Med*. 2011;3:11. doi: 10.1186/2040-7378-3-11
12. Wei J, Wang M, Jing C, Keep RF, Hua Y, Xi G. Multinucleated giant cells in experimental intracerebral hemorrhage. *Transl Stroke Res*. 2020;11:1095–1102. doi: 10.1007/s12975-020-00790-4
13. Shtaya A, Bridges LR, Esiri MM, Lam-Wong J, Nicoll JAR, Boche D, Hainsworth AH. Rapid neuroinflammatory changes in human acute intracerebral hemorrhage. *Ann Clin Transl Neurol*. 2019;6:1465–1479. doi: 10.1002/acn3.50842
14. Khan M, Baird GL, Elias R, Rodriguez-Srednicki J, Yaghi S, Yan S, Collins S, Thompson BB, Wendell LC, Potter NS, et al. Comparison of intracerebral hemorrhage volume calculation methods and their impact on scoring tools. *J Neuroimaging*. 2017;27:144–148. doi: 10.1111/jon.12370
15. Kothari RU, Brott T, Broderick JP, Barsan WG, Sauerbeck LR, Zuccarello M, Khoury J. The ABCs of measuring intracerebral hemorrhage volumes. *Stroke*. 1996;27:1304–1305. doi: 10.1161/01.str.27.8.1304
16. Hainsworth AH, Minett T, Andoh J, Forster G, Bhide I, Barrick TR, Elderfield K, Jeevahan J, Markus HS, Bridges LR. Neuropathology of white matter lesions, blood-brain barrier dysfunction, and dementia. *Stroke*. 2017;48:2799–2804. doi: 10.1161/STROKEAHA.117.018101
17. Liu B, Hu B, Shao S, Wu W, Fan L, Bai G, Shang P, Wang X. CD163/Hemoglobin oxygenase-1 pathway regulates inflammation in hematoma surrounding tissues after intracerebral hemorrhage. *J Stroke Cerebrovasc Dis*. 2015;24:2800–2809. doi: 10.1016/j.jstrokecerebrovasdis.2015.08.013
18. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol*. 2010;11:889–896. doi: 10.1038/ni.1937
19. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3:23–35. doi: 10.1038/nri978
20. Dennis C. Haemoglobin scavenger. *Nature*. 2001;409:141. doi: 10.1038/35051680
21. Galea I, Palin K, Newman TA, Van Rooijen N, Perry VH, Boche D. Mannose receptor expression specifically reveals perivascular macrophages in normal, injured, and diseased mouse brain. *Glia*. 2005;49:375–384. doi: 10.1002/glia.20124
22. Boche D, Perry VH, Nicoll JA. Review: activation patterns of microglia and their identification in the human brain. *Neuropathol Appl Neurobiol*. 2013;39:3–18. doi: 10.1111/nan.12011
23. Roy-O'Reilly M, Zhu L, Atadja L, Torres G, Aronowski J, McCullough L, Edwards NJ. Soluble CD163 in intracerebral hemorrhage: biomarker for perihematomal edema. *Ann Clin Transl Neurol*. 2017;4:793–800. doi: 10.1002/acn3.485
24. Thomas AJ, Ogilvy CS, Griessenauer CJ, Hanafy KA. Macrophage CD163 expression in cerebrospinal fluid: association with subarachnoid hemorrhage outcome. *J Neurosurg*. 2018;131:47–53. doi: 10.3171/2018.2.JNS172828
25. Galea J, Cruickshank G, Teeling JL, Boche D, Garland P, Perry VH, Galea I. The intrathecal CD163-haptoglobin-hemoglobin scavenging system in subarachnoid hemorrhage. *J Neurochem*. 2012;121:785–792. doi: 10.1111/j.1471-4159.2012.07716.x
26. Liu R, Cao S, Hua Y, Keep RF, Huang Y, Xi G. CD163 expression in neurons after experimental intracerebral hemorrhage. *Stroke*. 2017;48:1369–1375. doi: 10.1161/STROKEAHA.117.016850
27. Satoh J, Kino Y, Asahina N, Takitani M, Miyoshi J, Ishida T, Saito Y. TMEM119 marks a subset of microglia in the human brain. *Neuropathology*. 2016;36:39–49. doi: 10.1111/neup.12235
28. Bonham LW, Sirkis DW, Yokoyama JS. The transcriptional landscape of microglial genes in aging and neurodegenerative disease. *Front Immunol*. 2019;10:1170. doi: 10.3389/fimmu.2019.01170
29. Chang CF, Wan J, Li Q, Renfroe SC, Heller NM, Wang J. Alternative activation-skewed microglia/macrophages promote hematoma resolution in experimental intracerebral hemorrhage. *Neurobiol Dis*. 2017;103:54–69. doi: 10.1016/j.nbd.2017.03.016
30. Durafourt BA, Moore CS, Zammit DA, Johnson TA, Zaguia F, Guiot MC, Bar-Or A, Antel JP. Comparison of polarization properties of human adult microglia and blood-derived macrophages. *Glia*. 2012;60:717–727. doi: 10.1002/glia.22298
31. Ritzel RM, Patel AR, Pan S, Crapser J, Hammond M, Jellison E, McCullough LD. Age- and location-related changes in microglial function. *Neurobiol Aging*. 2015;36:2153–2163. doi: 10.1016/j.neurobiolaging.2015.02.016
32. Walsh KB, Sekar P, Langefeld CD, Moomaw CJ, Elkind MS, Boehme AK, James ML, Osborne J, Sheth KN, Woo D, et al. Monocyte count and 30-day

case fatality in intracerebral hemorrhage. *Stroke*. 2015;46:2302–2304. doi: 10.1161/STROKEAHA.115.009880
33. Adeoye O, Walsh K, Woo JG, Haverbusch M, Moomaw CJ, Broderick JP, Kissela BM, Kleindorfer D, Flaherty ML, Woo D. Peripheral monocyte count is associated with case fatality after intracerebral hemorrhage. *J Stroke Cerebrovasc Dis*. 2014;23:e107–e111. doi: 10.1016/j.jstrokecerebrovasdis.2013.09.006
34. You S, Zhong C, Zheng D, Xu J, Zhang X, Liu H, Zhang Y, Shi J, Huang Z, Cao Y, et al. Monocyte to HDL cholesterol ratio is associated with discharge and 3-month outcome in patients with acute intracerebral hemorrhage. *J Neurol Sci*. 2017;372:157–161. doi: 10.1016/j.jns.2016.11.022
35. Di Napoli M, Godoy DA, Campi V, Masotti L, Smith CJ, Parry Jones AR, Hopkins SJ, Slevin M, Papa F, Mogoanta L, et al. C-reactive protein in intracerebral hemorrhage: time course, tissue localization, and prognosis. *Neurology*. 2012;79:690–699. doi: 10.1212/WNL.0b013e318264e3be
36. Di Napoli M, Parry-Jones AR, Smith CJ, Hopkins SJ, Slevin M, Masotti L, Campi V, Singh P, Papa F, Popa-Wagner A, et al. C-reactive protein predicts hematoma growth in intracerebral hemorrhage. *Stroke*. 2014;45:59–65. doi: 10.1161/STROKEAHA.113.001721
37. Bernstein JE, Savla P, Dong F, Zampella B, Wiginton JG 4th, Miulli DE, Wacker MR, Menoni R. Inflammatory markers and severity of intracerebral hemorrhage. *Cureus*. 2018;10:e3529. doi: 10.7759/cureus.3529
38. Sansing LH, Harris TH, Kasner SE, Hunter CA, Kariko K. Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. *Acta Neurochir Suppl*. 2011;111:173–178. doi: 10.1007/978-3-7091-0693-8_29
39. Fernando MS, Simpson JE, Matthews F, Brayne C, Lewis CE, Barber R, Kalaria RN, Forster G, Esteves F, Wharton SB, et al; MRC Cognitive Function and Ageing Neuropathology Study Group. White matter lesions in an unselected cohort of the elderly: molecular pathology suggests origin from chronic hypoperfusion injury. *Stroke*. 2006;37:1391–1398. doi: 10.1161/01.STR.0000221308.94473.14
40. Swanson MEV, Murray HC, Ryan B, Faull RLM, Dragunow M, Curtis MA. Quantitative immunohistochemical analysis of myeloid cell marker expression in human cortex captures microglia heterogeneity with anatomical context. *Sci Rep*. 2020;10:11693. doi: 10.1038/s41598-020-68086-z
41. Bellamri N, Viel R, Morzadec C, Lecureur V, Joannes A, de Latour B, Llamas-Gutierrez F, Wollin L, Jouneau S, Vernhet L. TNF-α and IL-10 control CXCL13 expression in human macrophages. *J Immunol*. 2020;204:2492–2502. doi: 10.4049/jimmunol.1900790