Harmful neutrophil subsets in patients with ischemic stroke

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Association with disease severity

David Weisenburger-Lile, MD, Yuan Dong, PhD, Marion Yger, MD, Gaëlle Weisenburger, MD,
Giulia Frasca Polara, MD, Thomas Chaigneau, MS, Riccardo Zapata Ochoa, MS, Beatrice Marro, MD,
Bertrand Lapergue, MD, PhD, Sonia Alamowitch, MD, and Carole Elbim, MD, PhD

Correspondence
Dr. Carole Elbim
carole.elbim@upmc.fr

Abstract

Objective
To better understand the functional state of circulating neutrophils in patients with ischemic stroke (IS) for planning future clinical trials.

Methods
We analyzed by flow cytometry activation state of circulating neutrophils and the distribution of neutrophil peripheral subsets in 41 patients with acute IS less than 6 hours before admission and compared them with 22 age-matched healthy controls.

Results
Our results demonstrated continuous basal hyperactivation of circulating neutrophils during acute IS, characterized by lower L-selectin expression and higher CD11b expression at the cell surface, increased ROS production by neutrophils, and greater circulating levels of neutrophil elastase. Neutrophil hyperactivation was associated with deregulation of the equilibrium between apoptotic and necrotic. Patients also had higher percentages than controls of the overactive senescent ($CXCR4^{bright}/CD62L^{dim}$) neutrophil subset and increased percentage of neutrophils with a reverse transendothelial migration ($CD54^{high}CXCR1^{low}$) phenotype. Importantly, neutrophil alterations were associated with the clinical severity of the stroke, evaluated by its NIH Stroke Scale score.

Conclusion
Altogether, our results indicate that during acute IS, the inflammatory properties of circulating neutrophils rise, associated with the expansion of harmful neutrophil subsets. These changes in neutrophil homeostasis, associated with disease severity, may play an instrumental role by contributing to systemic inflammation and to the blood-brain barrier breakdown. Our findings highlight new potential therapeutic approaches of stroke by rebalancing the ratio of senescent to immunosuppressive neutrophils or decreasing reverse neutrophil transmigration or both.

Glossary

AAD = amino-actinomycin D; **ANOVA** = analysis of variance; **APC** = allophycocyanine; **BBB** = blood-brain barrier; **DAMP** = danger-associated molecular pattern; **fMLP** = N-formylmethionyl-leucyl-phenylalanine; **HC** = healthy control; **HE** = hydroethidine; **HMGB** = high-mobility group box; **IS** = ischemic stroke; **LPS** = lipopolysaccharide; **MMP** = matrix metalloproteinase; **MPO** = myeloperoxidase; **NET** = neutrophil extracellular trap; **NIHSS** = NIH Stroke Scale; **NLR** = Nod-like receptor; **PBS** = phosphate buffered saline; **PMN** = polymorphonuclear neutrophil; **ROS** = reactive oxygen species; **rTEM** = reverse transendothelial migration; **sJAM-C** = soluble JAM-C; **TNF** = tumor necrosis factor; **TLR** = Toll-like receptor.

Cerebral ischemia elicits a strong inflammatory response involving the transmigration of several subsets of leukocytes, including neutrophils.[1] Murine models of ischemic stroke (IS) reveal that neutrophils are the first cells recruited into the brain, minutes after stroke, accompanied by local production of cytokines or chemokines and facilitated by a very early ischemia-induced breakdown in the blood-brain barrier (BBB).[2,3] Massive infiltration of neutrophils into ischemic brain tissue has also been documented in human stroke patients.[4] Although animal stroke models suggest that depleting circulating neutrophils before or at the onset of stroke reduces infarct size, human drug trials aimed at preventing potentially harmful subacute neutrophil adhesion and infiltration into the brain parenchyma poststroke have not improved patient recovery.[5,6] The failure of these trials might be partially explained by the undiscriminating nature of these approaches: the anti-inflammatory potential of some circulating neutrophil subsets means that globally blocking neutrophil access to the brain might not be a viable therapeutic approach. A better understanding of the functional state of circulating neutrophils in patients with IS is therefore crucial for planning future clinical trials targeting postischemic inflammation.

Neutrophils, the most abundant circulating leukocytes, play a key role in the immune defense against bacterial and fungal pathogens. They are rapidly recruited from blood to sites of infection, where they kill microorganisms via a number of different mechanisms including phagocytosis, production of reactive oxygen species (ROS), release of granular proteins, neutrophil extracellular traps (NETs), a process called NETosis. However, in case of inappropriate and/or excessive activation, neutrophils play also a key role in the induction or promotion of inflammation-induced tissue injury.[7] Recent studies point to the existence of heterogenous populations of mature and immature neutrophils. In fact, diverse subsets of neutrophils differ markedly in their proinflammatory activity. Senescent neutrophils, an overly active subset, express elevated levels of CXCR4,[8] whereas the immunosuppressive CD16^{bright}/CD62L^{dim} subset exhibits reduced proinflammatory properties.[9] Recent studies also demonstrate that neutrophils can migrate back into the circulation, in a process referred to as reverse transendothelial migration (rTEM).[10]

Contradictory results on the phenotype and functions of circulating neutrophils in patients with IS have been reported. Some authors report fewer oxidative bursts and NET production,[11] whereas others suggest increased degranulation and ROS production.[12] Some analyzed neutrophil functions 24–48 hours after IS, although it is now clear that inflammatory pathways are involved in the first hours.[1] None has investigated the distribution of the senescent, immunosuppressive, and rTEM neutrophil subsets or their consequences on the course of IS.

Our study, performed in whole blood to avoid artifacts due to isolation procedures,[13] aimed to characterize fully the phenotypes and functions of human peripheral neutrophils at different stages of acute IS. We analyzed neutrophil activation state and the distribution of 3 neutrophil subsets, the senescent subset, the immunosuppressive subset, and the rTEM subset in the aim of addressing the right targets for planning future clinical trials. We investigated potential correlations between neutrophil parameters and clinical markers of disease progression.

Methods

Study design

This study enrolled 41 patients with acute IS hospitalized in the stroke unit of Saint-Antoine Hospital, Pitié-Salpêtrière Hospital (both Paris, France), and Foch Hospital (Suresnes, France) during the acute phase of brain ischemia. Patients were included if they had had a stroke less than 6 hours before admission, regardless of the NIH Stroke Scale (NIHSS) score, were aged ≥18 years, and showed no signs of infection at the admission. All patients were examined by neurologists, admitted to the stroke unit, and treated according to accepted stroke guidelines.[14] We prospectively collected the following data for each, with a structured questionnaire: vascular risk factors (age, sex, presence of atrial fibrillation, hypertension, diabetes, hypercholesterolemia, previous stroke history, and treatment); stroke severity assessed by the NIHSS at admission (day 0), day 2, and day 7; and time from symptom onset to brain imaging and sampling. The modified Ranking scale (mRs) was used for functional disability/dependence evaluations at 3 months. Standard stroke evaluation included brain imaging (MRI performed for 37 patients and CT performed for 4 patients when MRI was contraindicated), blood sampling (especially neutrophil and lymphocyte counts), electrocardiography for at least 48 hours, transthoracic echocardiography, and vessel imaging by ultrasound, MR angiography, or angio-CT. We prospectively collected stroke information including location of ischemia in the brain, presence and location of

occlusion, and etiology according to the Trial of Org 10172 in Acute Stroke Treatment classification. IS volumes were measured on diffusion sequences acquired on a 3-Tesla MRI instrument at admission (and not measured for patients with contraindications to brain MRI). A semiautomatic segmentation procedure by Oléa Sphere postprocessing software was used for analysis.

The 22 age-matched recruited healthy controls (HCs), previously presented,[16] with blood biochemical and hematologic values within the normal range were recruited from the Unit of Neurology of Memory and Language, Centre Hospitalier Sainte Anne (Paris, France). HCs were close members of the family of patients followed in the memory clinic. As previously described,[16] HCs had no memory complaint or cognitive deficit. In addition, we did not include HCs with a history of IS.

Exclusion criteria in the HC and patient groups included acute or chronic inflammatory conditions and treatment with corticosteroid or nonsteroidal anti-inflammatory drugs, both of which could modulate neutrophil functions.

Whole blood was sampled, kept on ice, and transported immediately to the laboratory for neutrophil analysis. Importantly, all patients were sampled at inclusion (day 0, D0), within the first 6 hours after IS symptom onset; 14 also had samples taken on D2 and D7.

Standard protocol approvals, registrations, and patient consents

The Ethics Committee of Saint-Antoine Hospital approved the study, and all patients or their proxies provided written informed consent before participating began. The HCs were recruited as part of their participation in the prospective longitudinal IMABio3 study (PHRC-0054-N 2010), which was approved by the Institutional Review Committee of Pitié-Salpêtrière Hospital.

Determination of surface molecule expression on resting neutrophils

Heparinized whole-blood samples (500 μL) were kept on ice and stained with allophycocyanine (APC)-anti-human CD62L (BD Biosciences, San Jose, CA) and PE-anti-human CD11b (clone ICRF44, BD Biosciences) antibodies, as previously reported.[15] The senescent $CXCR4^{bright}/CD62L^{dim}$ neutrophil subset and the immunosuppressive $CD16^{bright}/CD62L^{dim}$ neutrophil subset were investigated as previously described,.[16] To investigate the rTEM neutrophil subset, samples kept on ice were incubated for 45 minutes with FITC-anti-human CD81 (BD Biosciences) and PE-anti-CD54 (BD Biosciences) antibodies. The red blood cells were then lysed with BD FACS lysing solution and resuspended with Cell Fix (BD Biosciences).[16]

Measurement of neutrophil oxidative burst

Superoxide anion (O_2^-) production by neutrophils was measured with a flow cytometry–based assay derived from the hydroethidine (HE) oxidation technique, as previously described.[16] After loading with HE for 15 minutes, whole-blood samples were incubated for 45 minutes at 37°C with phosphate buffered saline (PBS) or either of the following Toll-like receptor (TLR) agonists: Pam_3CSK_4 or lipopolysaccharide (LPS), or tumor necrosis factor (TNF)-α, as previously reported.[22] Samples were then treated with PBS or 10^{-6} M N-formylmethionyl-leucyl-phenylalanine (fMLP) for 5 minutes.[22] Erythrocytes were lysed before analysis by flow cytometry.

Measurement of neutrophil apoptosis and necrosis

Spontaneous neutrophil cell death in whole blood was quantified with annexin V and the impermeant nuclear dye 7-amino-actinomycin D (7-AAD). Whole-blood samples (500 μL) were incubated with APC-anti-CD15 antibody (clone HI98, BD Biosciences), FITC-annexin V, and 7-AAD (BD Biosciences), as previously described, and analyzed by flow cytometry.[16]

Flow cytometry analysis

Cells were analyzed with a Gallios flow cytometer and the data with Kaluza software (Beckman Coulter). To determine neutrophil expression of surface molecules and ROS production, forward and side scatter were used to identify the granulocyte population and to gate out other cells and debris. The purity of the gated cells was assessed using FITC- or PE-conjugated CD3, CD45, CD14, and CD15 antibodies. Ten thousand events were analyzed per sample, and the fluorescence pulses were amplified by 4-decade logarithmic amplifiers. In all cases, unstained cells were run, and the photomultiplier settings were adjusted so that the unstained cell population appeared in the lower left-hand corner of the fluorescence display. In the multicolor analysis, single-cell controls were used to optimize signal compensation. All the results were obtained with the use of a constant photomultiplier gain value. To measure cell death in whole blood by flow cytometry, neutrophils were identified as $CD15^{high}$ cells, and 2×10^5 events were analyzed per sample.

Measurement of intravascular NETosis

NETs are networks of DNA, histone, and proteolytic enzymes produced by activated neutrophils, including myeloperoxidase (MPO), citrullinated histone 3, and elastase. Serum NET levels were quantified by detecting MPO-DNA complexes in serum samples. To prepare the DNA standard, NETs from a healthy subject were produced and isolated, and DNA was quantified in NET samples with PicoGreen. NET-associated MPO-DNA complexes were then quantified, also as previously described.[17]

Measurement of soluble pro- and anti-inflammatory mediators

Whole-blood samples were centrifuged for 15 minutes at 1,000g within 30 minutes of collection. Soluble chemokine (IL-8) and cytokines (IL-1β, IL-6, TNF-α, G-CSF, IL-18, IL-22,

IL-10, and transforming growth factor-β) were detected with Luminex assays (Luminex Performance Assays, R&D Systems). High-mobility group box-1 (HMGB1) and elastase were detected from serum by ELISA.

Statistics

Statistical analyses were performed with Rstudio 1.0.143 software. All tests were 2 tailed, with a significance level of α = 0.05. When a parametric test was used, normality of distribution was tested with the Shapiro-Wilk test. Differences between groups were assessed with the χ^2 test or analysis of variance (ANOVA), followed by the Tukey post hoc test, as appropriate. ANOVA, adjusted for age, was used to compare neutrophil markers between the IS groups and controls (with age as a covariate). Bonferroni correction was used for multiple comparisons. Linear partial correlation analysis, with adjustment for covariates, identified correlations.

Data availability statement

Data not provided will be available upon request.

Results

Participants' characteristics

The table summarizes the clinical characteristics of the 41 patients with IS and 22 HCs. Patients were included in 2 steps: the results from the first set of 27 patients led us to include a second set of 14 patients for complementary analyses. The 2 patient cohorts did not differ significantly for baseline characteristics, including risk factors. Patients with IS and HCs did not differ significantly for age or sex, but risk factors were significantly more frequent in the IS than the HCs. The proportion of etiologies for cerebral infarction observed in patients appears similar to recent data.[18] We observed 4 brainstem strokes (9.8%), 13 cortical strokes (31.7%), 11 subcortical strokes (26.8%), and 13 cortical and subcortical strokes (31.7%). Seven patients had hemorrhagic transformation. Five patients died within 7 days of onset. As expected,[19] patients with IS had higher polymorphonuclear neutrophil (PMN) counts than HCs and higher PMN/lymphocyte ratios.

Highly activated circulating neutrophils in patients with acute stroke

We analyzed neutrophil activation in the 27 patients with IS included in the first set of patients and in the 22 HCs. Neutrophil activation is associated with the modulation of surface molecules, i.e., decreased L-selectin (CD62L) and increased β2-integrins (i.e., CD11b/CD18), through either stimulus-induced shedding or translocation from intracellular granules. As figure 1, A and B show, resting neutrophils from patients on D0 expressed less CD62L and more CD11b than HCs. Because neutrophil degranulation can also result in rapid release of neutrophil serine proteases from azurophilic granules into the extracellular medium and circulation, we measured the level of circulating neutrophil elastase. Consistently with

previous data,[20] serum neutrophil elastase levels were significantly higher in patients with IS than HCs (figure 1C).

Measuring ROS production by neutrophils, we observed it was higher in unstimulated neutrophils from whole blood (PBS-incubated sample) from patients than from HCs (figure 1D). We then assessed whether the higher constitutive ROS production by patient neutrophils was associated with higher production capacity in response to various activating stimuli. We have previously reported that neutrophils in whole blood produce minimal ROS in response to a single stimulus.[16] We therefore studied neutrophil oxidative bursts in response to the bacterial peptide fMLP after priming with TNF-α or various TLR agonists. ROS production by nonprimed neutrophils (sample preincubated with PBS and stimulated with fMLP) was significantly higher in patients than in HCs (figure 1E), and under the different priming conditions, the significantly higher ROS production in patients with IS (figure 1E) demonstrates their neutrophil hyperreactivity.

Among the patients included in our study, 14 were also sampled on D2 and D7. Except for CD62L expression, which returned to normal levels on those days, no markers of neutrophil activation were modulated from D0 during the course of acute IS (figure 1, F–J). No significant differences among any of the neutrophil activation markers were observed between patients with or without subsequent stroke-associated infections or patients with or without IS risk factors (such as hypertension, diabetes, and hyperlipidemia) (data not shown).

These results reflect basal hyperactivation of patients' circulating neutrophils during acute IS, associated with their hyperreactivity to various stimuli by ROS production.

Deregulation of neutrophil death in patients with acute stroke

Neutrophils constitutively undergo apoptosis, and this process is critical for the resolution of inflammation. Neutrophil death by necrosis, inversely, induces the release of cytotoxic mediators that can damage adjacent healthy tissue. Measuring the percentage of neutrophils undergoing spontaneous apoptosis and necrosis showed that the percentage of apoptotic neutrophils at D0 was significantly lower in patients with IS and tended to return to normal values during the course of IS (figure 2, A and B). The percentage of necrotic neutrophils, however, was rising at D0, and the increase became significant from D2 (figure 2, C and D). This deregulation of the balance between apoptosis and necrosis, combined with basal neutrophil hyperactivation, may play a role in establishing inflammatory processes in these patients.

Neutrophils can undergo NETosis, an alternative form of programmed cell death associated with the release of decondensed chromatin lined with granular components that creates fibrous structures serving as NETs. These NETs can entangle pathogens but also contribute to both venous and arterial thrombosis.[21] Patients with IS had lower levels of

Table Characteristics of patients with ischemic stroke and HCs participating in the study

	HC (n = 22)	Patient set 1 (n = 27)	Patient set 2 (n = 14)	p Value, set 1 vs HC	p Value, set 2 vs HC	p Value, set 1 vs set 2
Sex (male), (n) [%]	6 [35.3]	14 [51.8]	8 [57.14]	0.36	0.29	0.99
Age, yr, (mean) [min–max]	68.1 [22–89]	72.6 [18–96]	71.1 [40–92]	0.20	0.54	0.59
History of: (n) [%]						
Diabetes	1 [4.54]	6 [22.2]	1 [5.89]	0.11	0.99	0.69
High blood pressure	2 [11.8]	21 [77.8]	10 [71.4]	0.001	0.001	0.71
Dyslipidemia	4 [23.5]	14 [51.9]	2 [14.3]	0.02	0.99	0.69
Myocardial infarction	0	9 [33.3]	4 [28.6]	0.002	0.02	0.99
Atrial fibrillation	0	8 [29.6]	4 [28.6]	0.006	0.02	0.99
Stroke	0	6 [22.2]	1 [7.14]	0.026	0.39	0.39
NIHSS D0 (mean) [min–max]	—	9.15 [0–21]	8.64 [1–23]	—	—	0.64
Etiologies/TOAST (n) [%]						
Thromboembolism	—	6 [22.2]	2 [14.3]	—	—	0.69
Cardioembolism	—	11 [40.7]	4 [28.6]	—	—	0.51
Lacunar stroke	—	1 [3.7]	2 [14.3]	—	—	0.54
Other specific cause	—	3 [7.4]	3 [21.4]	—	—	0.64
Undetermined cause	—	6 [22.2]	3 [21.4]	—	—	0.99
Occlusion at inclusion (n) [%]	—	16 [59.3]	6 [42.8]	—	—	0.51
Volume of cerebral infarct (mL) (mean) [min–max]	—	23.92 [0.04–240.8]	16.17 [0–63.8]	—	—	0.99
Treatment at acute stage (n) [%]	—					
IV Thrombolysis	—	17 [62.9]	5 [35.7]	—	—	0.115
Endovascular treatment	—	9 [33.3]	4 [28.6]	—	—	0.99
Time from symptom onset to blood sample (min) (mean) [min–max]	—	129 [45–240]	186 [95–340]	—	—	0.09
Previous treatment by statins	2 [11.7]	9 [33.3]	3 [21.4]	0.158	0.636	0.494
Previous treatment by antithrombotic agents	0	8 [29.6]	4 [28.6]	0.006	0.017	0.99
Neutrophil count (G/L) (mean) [min–max]	3.15 [2.32–4.80]	6.1 [2.37–14.40]	7.85 [2.33–15.3]	0.007	0.002	0.22
Lymphocyte count (G/L) (mean) [min–max]	1.68 [1.06–2.15]	1.48 [0.53–3.15]	1,52 [0.57–3.26]	0.43	0.55	0.91
Ratio PMN/lymphocyte (mean) [min–max]	2.09 [1.37–4.36]	5.63 [1.04–23.34]	7.70 [1.04–26.84]	0.023	0.024	0.40

Abbreviations: HC = healthy control; NIHSS = NIH Stroke Scale; TOAST = Trial of Org 10172 in Acute Stroke Treatment.
Sex, risk factors, and type of treatment were compared with the χ^2 test. The Mann-Whitney test was used to compare quantitative variables.
We considered all thrombolyses and all mechanical thrombectomies.

circulating NETosis-derived products in their blood samples at inclusion than HCs (figure 2E). This difference persisted at D2 and D7 (figure 2F).

Patients with or without either infection or IS risk factors did not differ significantly for neutrophil apoptosis, necrosis, or intravascular NETosis (data not shown).

Expansion of the overactive senescent neutrophil subset in patients with acute stroke

The phenotypes and functions of neutrophils change over time from their release into the blood (as fresh neutrophils) until their disappearance from the circulation (as aged neutrophils). Aged or senescent CXCR4^{high} neutrophils express lower levels of CD62L and higher levels of β2-integrins and produce ROS at a higher rate

Figure 1 Hyperactivation of circulating neutrophils from patients with ischemic stroke
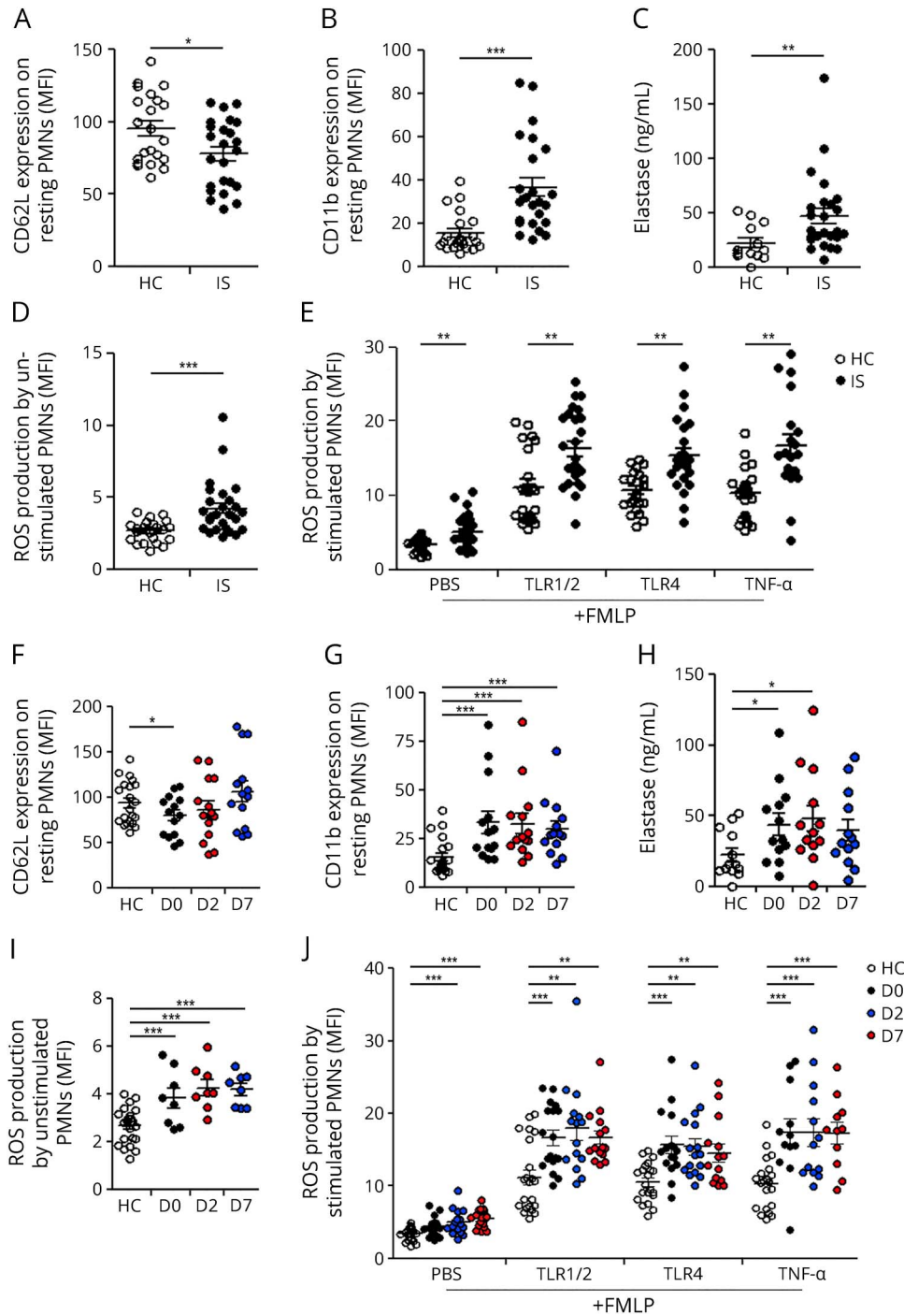

(A–E) Adhesion molecules expression, polymorphonuclear neutrophil (PMN) elastase, and ROS production were studied in patients with IS at inclusion. Expression of CD62L (A) and CD11b (B) at the surface of resting PMNs was studied on whole-blood samples kept on ice and incubated with anti-CD62L and anti-CD11b monoclonal antibodies. Results are expressed as mean fluorescence intensity (MFI). (C) Circulating levels of PMN elastase were quantified by ELISA; results are expressed as ng/mL. (D and E) ROS production by unstimulated PMNs was studied after treatment of whole-blood samples for 50 minutes with PBS; results are expressed as MFI (D). ROS production by stimulated PMNs was measured after pretreatment of whole-blood samples for 45 minutes with PBS, Pam_3CSK_4 (TLR1/2 agonist, 1 µg/mL), or LPS (TLR4 agonist, 10 ng/mL), or TNF-α (TNF, 5 ng/mL) and incubation for 5 minutes with fMLP (10^{-6} M); results are expressed as MFI (E). (F–J) Follow-up of surface expression of CD62L (F), surface expression of CD11b (G), circulating levels of PMN elastase (H), and ROS production by unstimulated (I) and stimulated PMNs (J) during the first week after IS symptoms. All samples came from age-matched HCs, (n = 22) and patients with IS at day 0 (n = 27), day 2 (n = 14), and day 7 (n = 14). Values are mean ± SEM. *Significantly different from controls $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, adjusted for age. D0 = day 0; D2 = day 2; D7 = day 7; fMLP = N-formylmethionyl-leucyl-phenylalanine; HC = healthy control; IS = ischemic stroke; LPS = lipopolysaccharide; ROS = reactive oxygen species; SEM = standard error of mean; TLR = Toll-like receptor; TNF = tumor necrosis factor.

than the total circulating pool.[8] Conversely, in contexts of acute inflammation[9] and healthy aging,[22] an immunosuppressive subset of CD16^{bright}/CD62L^{dim} neutrophils show decreased adhesion properties and less ROS production. Changes in the phenotypic and functional features of patients' neutrophils might thus be related to an imbalance between these 2 neutrophil subsets. The senescent CXCR4^{bright}/CD62L^{dim} neutrophil subset expanded in patients with IS at D0 (figure 3, A and C), whereas the percentage of neutrophils in the CD16^{bright}/CD62L^{dim} immunosuppressive subset did not differ between patients with IS and HCs (figure 3, B and D). Accordingly, patients with IS had a higher ratio of senescent to immunosuppressive subsets than HCs (figure 3, E). Similar results were observed in patients with IS at D2 and D7 (figure 3, F–H). Moreover, PMN subsets did not differ significantly between patients with or without infection or IS risk factors (data not shown). These results reflect the expansion of the senescent subset in patients with IS may lead to greater PMN oxidative burst.

Figure 2 Deregulation of neutrophil death in patients with ischemic stroke
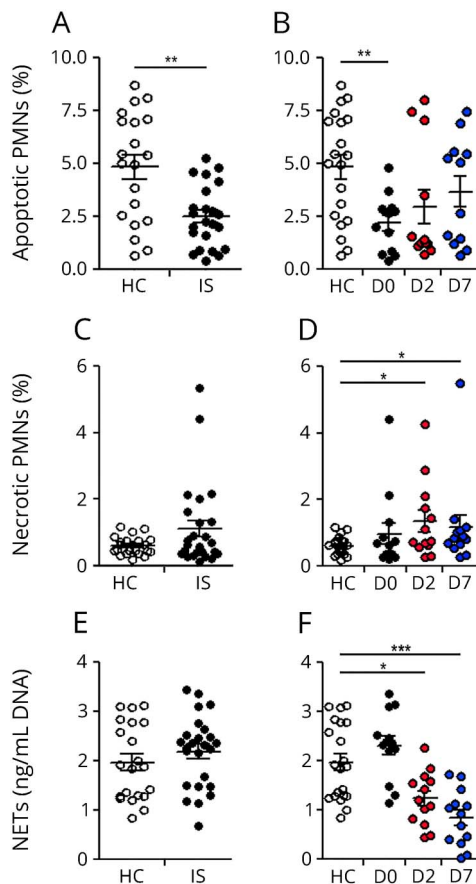
(A–D) Polymorphonuclear neutrophil (PMN) death was measured immediately after sampling by staining with annexin V and 7-AAD. Results are expressed as the percentages of apoptotic (A and B) and necrotic (C and D) PMNs. (A and C) Values of PMN death in patients with IS at inclusion. (B and D) Follow-up of PMN death during the first week after IS symptoms. (E and F) Circulating NETs were quantified by MPO-DNA complex ELISA; results are expressed as ng/μL DNA. Values of circulating NETs in patients with IS at inclusion (E). Follow-up of intravascular NETosis during the first week after IS symptoms (F). All samples came from age-matched healthy controls (HCs, n = 22) and patients with IS at day 0 (n = 27), day 2 (n = 14), and day 7 (n = 14). Values are mean ± SEM. *Significantly different from controls $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, adjusted for age. AAD = amino-actinomycin D; D0 = day 0; D2 = day 2; D7 = day 7; HC = healthy control; IS = ischemic stroke; MPO = myeloperoxidase; NET = neutrophil extracellular trap; SEM = standard error of mean.

Increased levels of soluble JAM-C were associated with an increase in the rTEM neutrophil subset in patients with acute stroke

JAM-C is an adhesion molecule expressed by endothelial cells that plays a role in tight junction formation, leukocyte adhesion, and transendothelial migration. Elastase-induced proteolytic cleavage of endothelial JAM-C leading to soluble JAM-C (sJAM-C) was recently reported to be instrumental in promoting neutrophil rTEM in vivo.[23] Measuring the circulating levels of JAM-C in our first cohort of 27 patients with IS showed a higher level at D0 than in HCs (figure 4A); values returned to normal at D2 (figure 4B). As expected,[23] increased sJAM-C levels were

associated with an increased circulating level of neutrophil elastase (figure 4C).

We thus performed a complementary study in a second cohort of 14 patients with IS to investigate the percentage of circulating rTEM neutrophils and its association with the sJAM-C level. The clinical characteristics of this second cohort did not differ from that of the main cohort (table), nor did their sJAM-C level on D0 (421 ± 20 pg/mL and 463 ± 49 pg/mL for the patient set 1 and set 2, respectively, $p = 0.77$), which was similarly significantly higher than in HCs. As previously reported,[23] a small population of neutrophils with an rTEM phenotype (CD54^{high}, CXCR1^{low}) was consistently found in HCs, equivalent to 1% of all circulating neutrophils. In patients with IS, we could also identify a distinct population of cells with an rTEM phenotype; its percentage was significantly higher in patients than in HCs (figure 4, D and E), and the increase of this percentage was associated with increased levels of sJAM-C (figure 4F). Taken together, these results demonstrate the expansion of the rTEM subset in IS migration.

Neutrophil abnormalities are associated with the severity of acute IS

We then investigated whether the neutrophil abnormalities observed in patients with IS might be associated with stroke severity. Patients were dichotomized according to the NIHSS score at inclusion into minor-to-moderate (NHISS score ≤12) and severe stroke (NHISS score >12) categories. Basal neutrophil activation at baseline was greater in severe than in minor-to-moderate strokes, as shown by patients' lower CD62L expression (figure 5A), higher circulating neutrophil elastase levels (figure 5C), and higher neutrophil production of ROS (figure 5D), although CD11b expression did not differ significantly between the groups (figure 5B). These phenotypic and functional properties of neutrophils from the severe stroke group were associated with a higher percentage of neutrophils in the senescent hyperactive subset (figure 5E), but not in the immunosuppressive subset (figure 5F). Finally, the sJAM-C level was higher in the severe stroke group (figure 5G). In contrast, the percentage of apoptotic (figure 5H) and necrotic neutrophils (data not shown) and the intravascular NETosis level (data not shown) did not differ between the patient groups. Higher NIHSS scores at D0 were associated with decreased expression of CD62L (figure 5I), increased ROS production by both unstimulated (figure 5J) and LPS-primed neutrophils (figure 5K), and an increase in the percentage of the senescent hyperactive subset (figure 5L), the level of sJAM-C (figure 5M), and the percentage of rTEM neutrophils (figure 5N). Increased NIHSS score at D2 was also associated with increased ROS production by LPS-primed neutrophils (data not shown). The correlations were adjusted for age. We found no correlation between neutrophil parameters and the infarct volume at inclusion and the presence of macroangiopathy, the occurrence of hemorrhagic transformation, the use of antithrombotic treatment, or the use of thrombolysis or endovascular treatment (data not shown).

Figure 3 Impaired homeostasis of the circulating neutrophils in patients with ischemic stroke
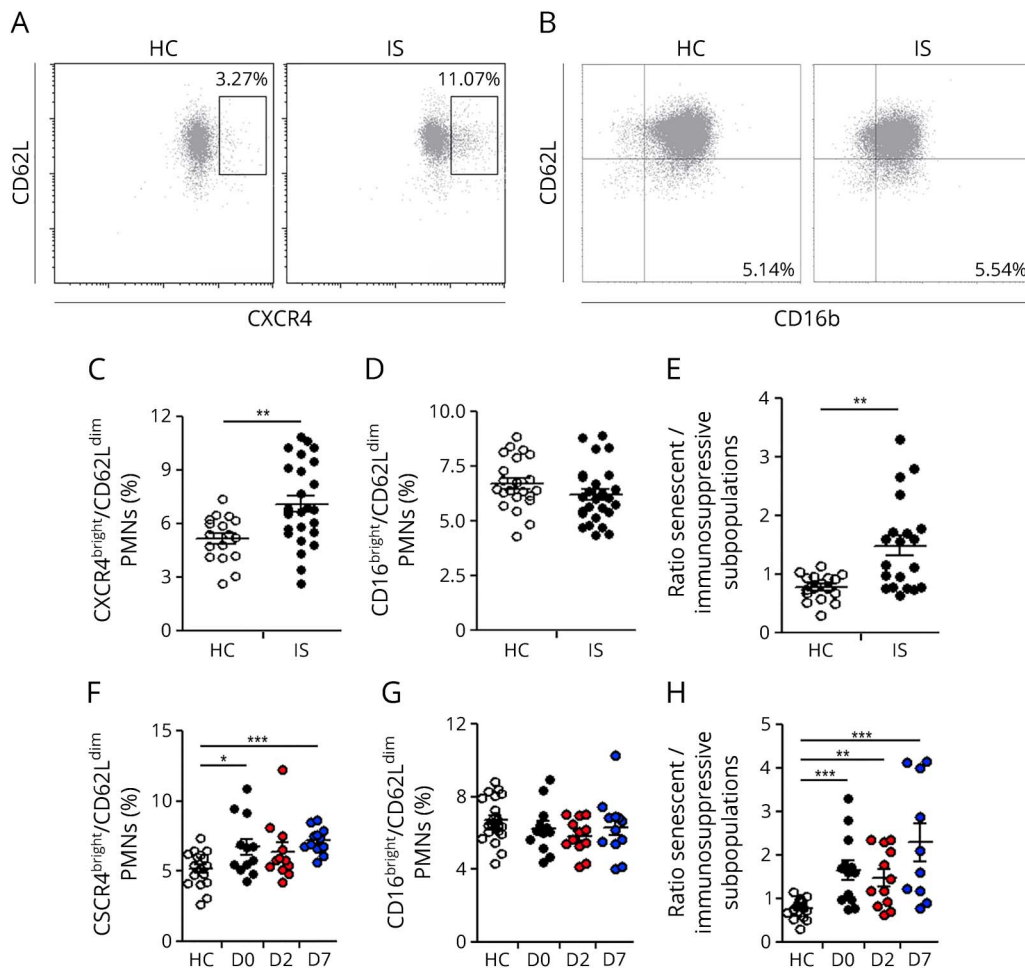
(A–E) Analysis of senescent and immunosuppressive polymorphonuclear neutrophil (PMN) subsets in patients with IS at inclusion. Whole-blood samples were incubated for 45 minutes at 4°C with Pe-Cy7-anti-human CXCR4, PE-anti-human CD11b, and APC-antihuman CD62L (A and B) or with FITC-anti-human CD16, PE-anti-human CD11c, Pe-Cy7-antihuman CD11b, and APC-anti-human CD62L (C and D) antibodies. (A) Representative dot plots of the PMN phenotype according to CXCR4 and CD62L expression in a HC (left) and a patient with IS (right). (B) Representative dot plots of the PMN phenotype according to CD16 and CD62L expression in an HC (left) and a patient with IS (right). (C) Percentages of the CXCR4^{bright}/CD62L^{dim} senescent PMN subset. (D) Percentages of the CD16^{bright}/CD62L^{dim} immunosuppressive PMN subset. (E) Ratio between the senescent and the immunosuppressive PMN subsets. (F–H) Follow-up of senescent PMN subset (F), immunosuppressive PMN subset (G), and ratio between the senescent and the immunosuppressive PMN subsets (H) during the first week after IS symptoms. All samples came from age-matched HCs, (n = 22) and patients with IS at day 0 (n = 27), day 2 (n = 14), and day 7 (n = 14). Values are mean ± SEM. *Significantly different from controls $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, adjusted for age. D0 = day 0; D2 = day 2; D7 = day 7; HC = healthy control; IS = ischemic stroke; SEM = standard error of mean.

Considered together, these results strongly suggest that the impaired homeostasis of circulating neutrophils associated with neutrophil hyperactivation is correlated with the clinical severity of acute stroke disease progression.

Proinflammatory environment in patients with acute stroke

Ischemic cerebral tissue rapidly induced the release of cytokines, chemokines, and danger-associated molecular patterns (DAMPs), including HMGB1.[24] Because cytokines[25] and HMGB1[26] strongly regulate neutrophil activity, we investigated patients' peripheral proinflammatory environment. Levels of classic proinflammatory (IL-1β, IL-6, IL-8, and TNFα) and anti-inflammatory (IL-10 and transforming growth factor-β) cytokines were similar in the IS

patient and HC groups (figure 6A). Serum levels of G-CSF and IL-22, a bifunctional cytokine with both proinflammatory and protective functions, were significantly lower in the IS than the HC group. Inversely, serum levels of the proinflammatory cytokine IL-18 were higher in the patients with IS (figure 6A), as were circulating HMGB1 levels (figure 6, B and C), which were associated with the clinical NIHSS score on D0 (figure 6D). Taken together, these results demonstrate higher circulating levels of the proinflammatory cytokine IL-18 and of the DAMP HMGB-1 in patients with IS.

We then investigated correlations between neutrophil parameters and proinflammatory mediator levels. Neutrophil markers were not correlated with cytokine levels. In

Figure 4 Patients with ischemic stroke have higher levels of soluble JAM-C and of PMN reverse transmigration
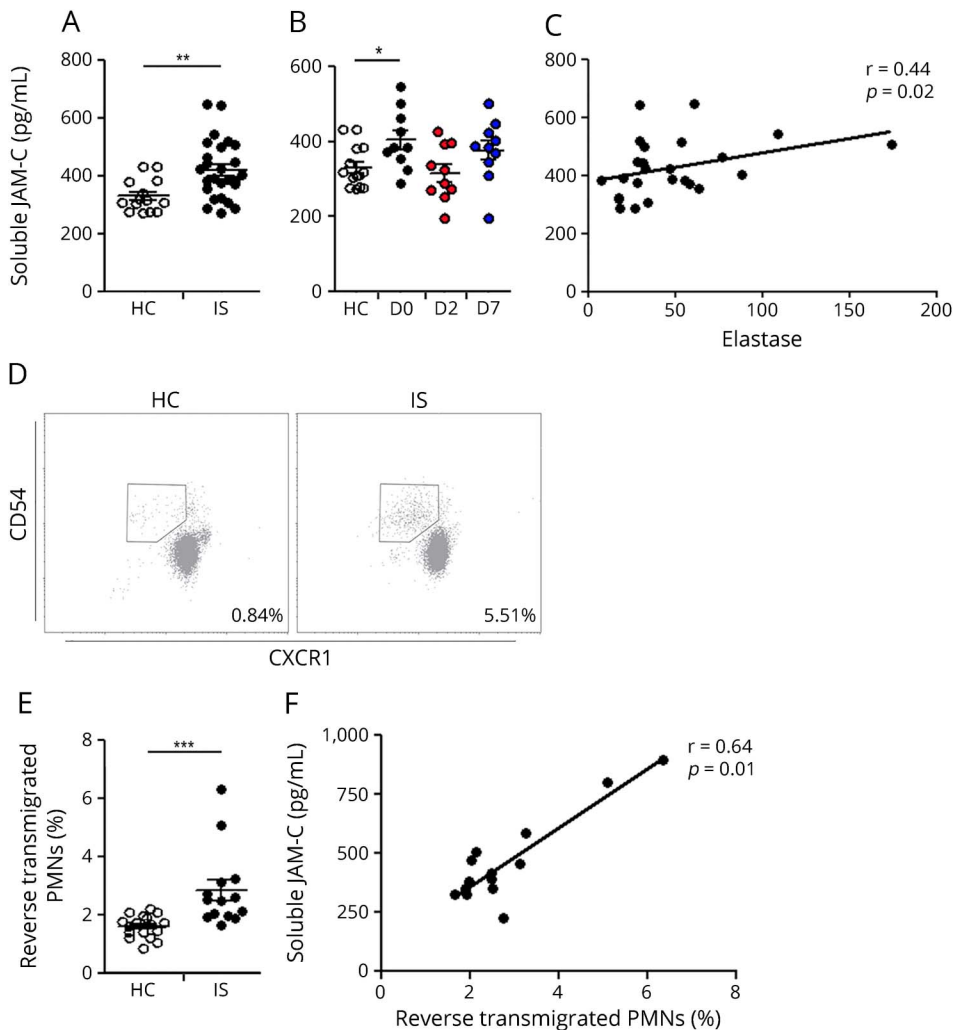
(A and B) sJAM-C was quantified by ELISA; results are expressed as pg/mL. (A) Values of sJAM-C in age-matched HCs, (n = 22) and patients with IS at inclusion (n = 27) (patients included in set 1). (B) Follow-up of sJAM-C levels during the first week after IS symptoms in 10 patients. (C) Correlation between the levels of sJAM-C and of circulating PMN elastase. (D and E) Quantification of rTEM PMNs in patients with IS included in set 2. Whole-blood samples were incubated for 45 minutes at 4°C with FITCanti-human CD181 and PE-anti-human CD54 antibodies. (D) Representative dot plots of the PMN phenotype according to CXCR4 and CD62L expression in an HC (left) and a patient with IS (right). (E) Percentages of the rTEM PMN subset (CD54^{high}, CXCR1^{low}) in age-matched HCs (n = 17) and patients with IS at inclusion (n = 14). (F) Correlation between the percentage of rTEM PMN subset and the level of sJAM-C in patients with IS included in the set 2. *Significantly different from controls $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, adjusted for age. D0 = day 0; D2 = day 2; D7 = day 7; HC = healthy control; IS = ischemic stroke; PMN = polymorphonuclear neutrophil; rTEM = reverse transendothelial migration; sJAM-C = soluble JAM-C.

contrast, the increased serum HMGB1 level was associated with decreased CD62L expression on resting neutrophils (figure 6E), increased CD11b expression (figure 6F), and increased ROS production by primed neutrophils (figure 6G). These findings suggest that HMGB1 might preactivate neutrophils in vivo.

Discussion

In the present study, phenotypes and functions of peripheral neutrophils from patients less than 6 hours after IS have been extensively investigated. Our results demonstrated continuous basal hyperactivation of circulating neutrophils during acute IS, characterized by lower L-selectin expression and higher CD11b expression at the cell surface, increased ROS production by neutrophils, and greater circulating levels of neutrophil elastase. The release of this soluble cytotoxic molecule might induce vascular damage that aggravates ischemia or diffuses into the brain parenchyma and causes

tissue injury. Neutrophil hyperactivation was associated with deregulation of the equilibrium between apoptotic and necrotic PMNs, which may be a supplemental factor promoting acute ischemic inflammation. Furthermore, the lack of difference in neutrophil changes according to the presence or absence of risk factors for stroke, such as type 2 diabetes and hypertension, suggests that these abnormalities might be directly linked to IS.

Activated neutrophils may contribute to vascular inflammation during IS through neutrophil-induced BBB impairment. The BBB forms a protective barrier around the brain, with the essential function of maintaining brain homeostasis. During ischemia and subsequent reperfusion, its integrity is disrupted, which results in the leakage of blood-borne proteins into brain tissue. Loss of BBB integrity correlates with poorer long-term outcomes in patients with IS.[27] ROS, including those produced extracellularly by neutrophils,[28] have been reported to induce BBB breakdown.[29] In particular, ROS activate matrix

**Figure 5** Neutrophil hyperactivation is associated with disease severity
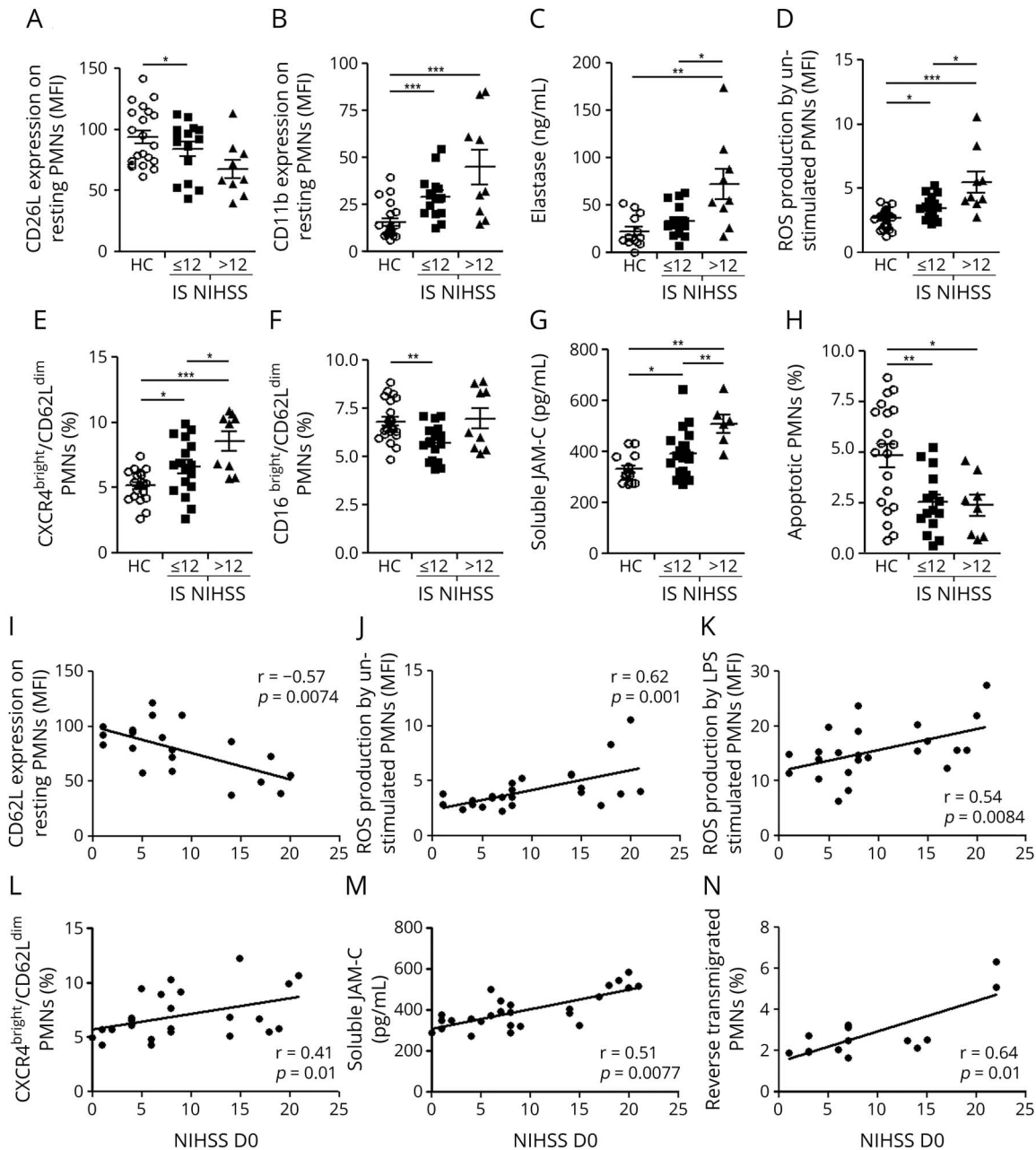
(A and B) Expression of CD62L (A) and CD11b (B) at the surface of resting polymorphonuclear neutrophils (PMNs). Results are expressed as MFI. (C) Circulating levels of neutrophil elastase were quantified by ELISA. (D) ROS production by unstimulated PMNs; results are expressed in MFI. (E) Percentages of the CXCR4^{bright}/CD62L^{dim} senescent PMN subset. (F) Percentages of the CD16^{bright}/CD62L^{dim} immunosuppressive PMN subset. (G) sJAM-C was quantified by ELISA; results are expressed as pg/mL (H) PMN apoptosis was measured immediately after sampling by staining with annexin V and 7-AAD. Results are expressed as percentages of apoptotic PMNs. All measurements came from patients with minor-to-moderate stroke (IS NHISS ≤ 12, n = 17) and patients with severe stroke (IS NHISS > 12, n = 10) at the inclusion. Values are mean ± SEM. Statistical significance as determined by the nonparametric Mann-Whitney test is indicated. *Significantly different $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Correlation between the NIHSS score at inclusion and CD62L expression at the PMN surface (I), ROS production by unstimulated PMNs (J), ROS production by LPS-stimulated PMNs (K), percentage of the CXCR4^{bright}/CD62L^{dim} senescent PMN subset (L), sJAM-C level (M), and percentage of rTEM PMNs (N). AAD = amino-actinomycin; D0 = day 0; IS = ischemic stroke; LPS = lipopolysaccharide; MFi = mean fluorescence intensity; NIHSS = NIH Stroke Scale; PMN = polymorphonuclear neutrophil; ROS = reactive oxygen species; SEM = standard error of mean; sJAM-C = soluble JAM-C.

metalloproteinase (MMP) activation, which in turn plays a key role in BBB breakdown by disrupting the basement membrane of the neurovascular unit.[30] Among the various MMPs, MMP-9 (gelatinase B) is thought to be central to BBB impairment. Neutrophils are the major source of MMP-9 after IS, and neutrophil-derived MMP-9 has been shown to be important in postischemic BBB disruption, leukocyte infiltration, and brain damage.[29] BBB disruption triggers inflammatory response and infiltration of immune cells including neutrophils, which are in turn associated with increased neuronal cell death through the

Figure 6 Proinflammatory mediators in patients with ischemic stroke
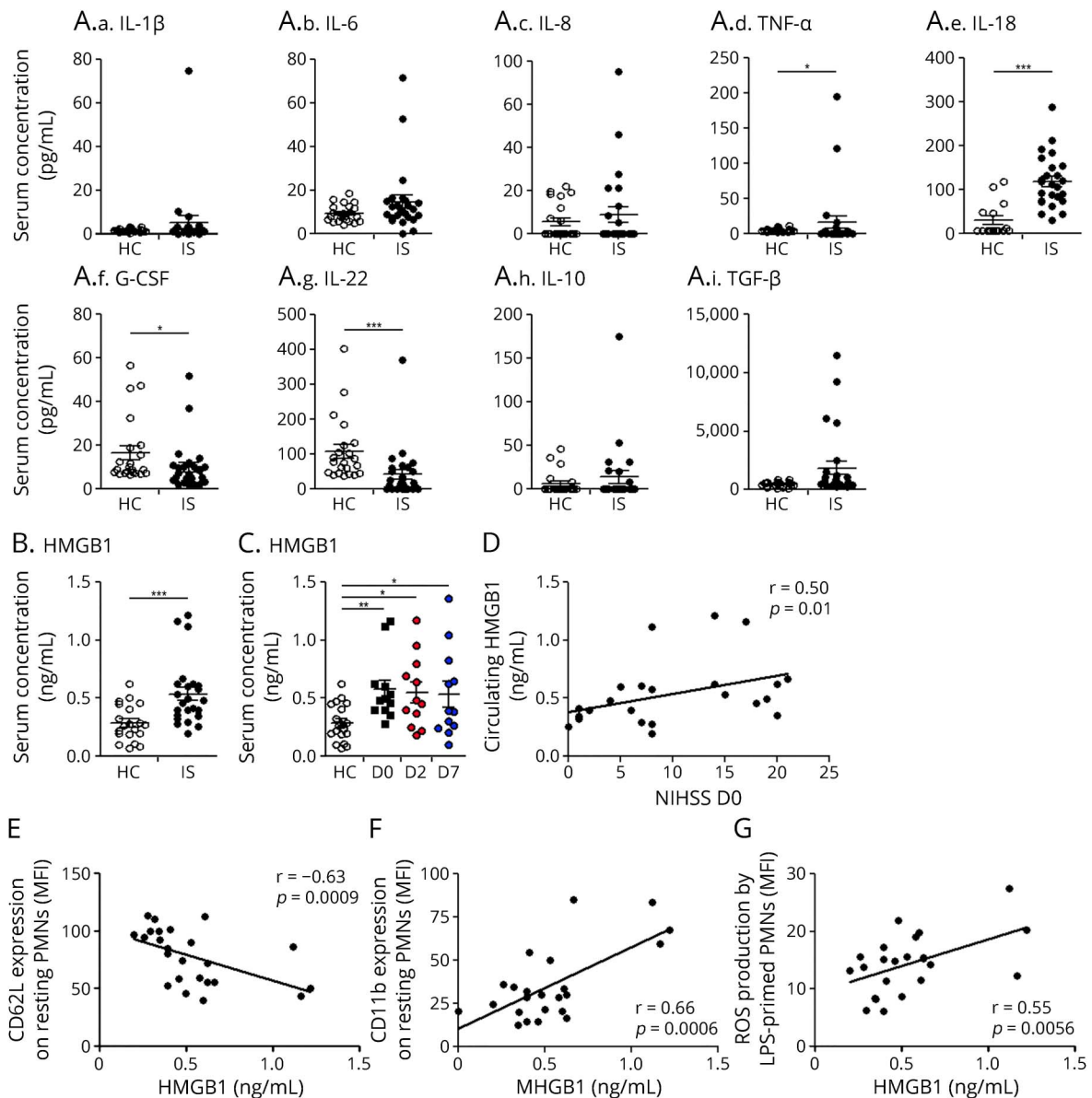
(A) Circulating levels of pro- and anti-inflammatory cytokines were measured with Luminex assays in samples from controls (HCs, n = 22) and patients with IS at day 0 (n = 27). (B and C) Circulating levels of HMGB1 were measured in serum by ELISA. (B) Comparative analysis of circulating levels of HMGB1 between age-matched HCs (n = 22) and patients with IS (IS) at day 0 (n = 27). (C) Follow-up of circulating levels of HMGB1 in patients with IS at day 0, 2, and 7. Values are mean ± SEM. *Significantly different from controls $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, adjusted for age. Correlation between circulating levels of HMGB1 at day 0 and the NIHSS score at day 0 (D). (E–G) Correlation between circulating levels of HMGB1 at day 0 and PMN activation markers at day 0: PMN surface expression of CD62 (E) and of CD11b (F) and ROS production by LPS-stimulated PMNs (G). D0 = day 0; D2 = day 2; D7 = day 7; HC = healthy control; HMGB = high-mobility group box; IS = ischemic stroke; LPS = lipopolysaccharide; NIHSS = NIH Stroke Scale; PMN = polymorphonuclear neutrophil; ROS = reactive oxygen species; SEM = standard error of mean.

release of neurotoxic substances and secondary injuries. As senescent neutrophils in the circulation have been reported to rapidly migrate to the inflammatory site,[31] we speculate that the expansion of the senescent neutrophil subset observed in patients with IS might result in enhanced migration of these hyperactive cells in the brain. Both the percentage of senescent neutrophils and the parameters of neutrophil hyperactivation were correlated with disease severity measured by the NIHSS score. We did not, however, find any correlation with the infarct volume at inclusion. In fact, a limitation of our study is related

to the fact that the measured volume matches the ischemic core and does not include the volume of potentially reversible ischemia (penumbra). It has been suggested that inflammatory processes take place in the penumbral area and contribute to potential enlargement of the infarct.[32] Furthermore, the measured volumes on diffusion do not match with the final volume, especially in treated patients.[33,34] A potential association between neutrophil abnormalities and the progression of damage in the penumbra and the final ischemic volume have to be explored in further studies. In addition, we had a relatively limited number of

patients, and our observations must be corroborated in larger cohorts.

Despite neutrophil hyperactivation, intravascular NETosis decreased in patients with IS starting at D2 after IS. This discrepancy with previous data[35] might be related to the different techniques used to study NETosis. As chromatin constitutes the backbone of NETs, and MPO is present in the NETs, we adopted complexes of nucleosomes and MPO as the definition of NET remnants, as suggested,[17] and quantified serum NET levels by detecting the MPO-DNA complexes. The mixing of the nuclear components with the content of azurophilic granules is a critical step in NETosis and enables the formation of complexes between nucleosomes and MPO. In contrast, in a previous study performed in patients with IS,[35] 3 markers were evaluated: plasma cell-free DNA, nucleosomes, and citrullination of histone 3, all only indirect markers of NETs. The decrease in intravascular NETosis observed in our study could be due to the decreased capacity of stimulated PMNs to produce NETs[11] and to the presence of NETs in thrombotic occlusions from patients with IS.[36]

The occurrence of neutrophil reverse transmigration after ischemia/reperfusion injury has previously been reported in animal models.[23] Our findings describe an increased percentage of neutrophils with an rTEM (CD54^{high}CXCR1^{dim}) phenotype in human IS. The recent report that rTEM neutrophils show an activated morphology with unaltered antimicrobial activity suggests that they might be deleterious if reinfiltrated into remote organs.[37] Moreover, rTEM neutrophils are relatively rigid, a physical characteristic that might delay their passage through the tissue microvasculature and prolong contact with the sinusoids. These cells could become mechanically entrapped in the microvasculature of major organs (most likely in the pulmonary circulation). We thus postulate that a larger population of functionally primed, recirculating neutrophils in patients with IS, with a predilection for mechanical or adhesive entrapment in the vasculature, could contribute to distant organ damage and multiorgan failure. As these rTEM neutrophils have been characterized by their prolonged survival and enhanced oxidative burst,[38] their expansion in patients with IS might explain, at least in part, the higher ROS production and the lower rate of neutrophil apoptosis in our cohort. Furthermore, because rTEM neutrophils are reported to inhibit T-cell proliferation,[9] we can speculate that they might contribute to the T lymphopenia observed during the stroke-induced immunodepression phase.

Consistent with data suggesting that sJAM-C may serve as an indirect marker of neutrophil rTEM,[23,38] higher levels of this adhesion molecule in patients with IS associated with the percentage of rTEM neutrophils. On the other hand, sJAM-C also acts as a potent proangiogenic molecule with chemotactic action for endothelial cells, and it induces their formation of vascular tubes in vitro.[39] After ischemic injury, angiogenesis increases the number of new collateral conduits, enhancing the blood supply and improving ischemic brain function.[40] Despite this potential beneficial action of sJAM-C, we clearly observed that the sJAM-C level was higher in the severe stroke group.

As previously reported,[41,42] we observed higher circulating levels of the proinflammatory cytokine IL-18 and of the DAMP HMGB-1 in patients with IS than in age-matched HCs. IL-18 production requires activation of the inflammasome, a group of multimeric protein complexes that sense stress signals, activate caspase-1, and thus cause its proteolytic cleavage. Notably, IS increases the expression and activation of the Nod-like receptor (NLR) pyrin domain containing 1 and 3 (NLRP1 and NLRP3) inflammasome proteins and both IL-1β and IL-18 in neurons.[43] IL-18[44] and HMGB1[26] enhance neutrophil functions, including priming neutrophils for oxidative burst and degranulation. In turn, activated neutrophils produce IL-18. In addition, necrotic cells, including necrotic neutrophils, passively release HMGB1. Of interest, we found that the percentage of necrotic neutrophils was higher in patients with IS than HCs. These processes together might thus maintain a vicious cycle. We also observed that increased plasma levels of HMGB-1 were associated with markers of neutrophil activation.

The phenotypic and functional neutrophil changes that accompany acute IS may also be related to the expansion of the overactive aged neutrophil subpopulation. A recent study showed that neutrophil aging is regulated by the microbiota.[8] Recent studies report gut dysbiosis in patients with IS.[45,46] Commensal microbiota affects IS outcome in mice with ischemic brain injury by impairing immune homeostasis in the small intestine, thus increasing IL-17$^+$ γδ T cells and decreasing regulatory T cells (Tregs).[47] Of note, γδ T cells, a major lymphocyte population with innate immune features, can aggravate ischemic brain injury by secreting IL-17 and generating chemotactic signals for peripheral myeloid cells such as neutrophils.[48] In contrast, Tregs are known to inhibit neutrophil functions[49]: in rodent IS models, adoptively transferred Tregs exert a protective effect by modulating peripheral neutrophils and thus prevent proteolytic damage to the BBB.[50] Alterations in the microbiota could contribute both to expansion of senescent neutrophils and changes in neutrophil activation in patients with IS. We can speculate that modulation of the microbiome might affect disease progression by limiting neutrophil aging and reduction production of neutrophil-derived cytotoxic molecules.

We demonstrated that homeostasis of circulating neutrophils is impaired during acute IS. These findings need to be replicated in an independent cohort of patients in which a meticulous definition of neutrophil subsets will be performed. The expansion of aged neutrophils might play a role in poststroke systemic inflammation and BBB disruption, and rTEM neutrophils might further contribute to the propagation of systemic inflammation. Our findings highlight new potential

therapeutic approaches of stroke based on neutrophil modulation, in particular, by rebalancing the ratio of senescent to immunosuppressive neutrophils or decreasing reverse neutrophil transmigration or both. Further studies are needed, however, to determine whether these changes might serve as a predictive indicator of IS.

Author contributions

C. Elbim conceived the study, designed the experiments, and supervised the project. D. Weisenburger-Lile and Y. Dong conducted the experiments and analyzed the data. D. Weisenburger-Lile, M. Yger, G.F. Polara, and B. Lapergue were involved in patient recruitment and characterization and in the data analysis. G. Weisenburger-Lile, T. Chaigneau, and R. Zapata Ochoa participated in the data analysis. D. Weisenburger-Lile, S. Alamowitch, and C. Elbim wrote the manuscript. All authors read and approved the content of the final manuscript.

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Disclosure

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Publication history

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