Defining cerebral leukocyte populations in local ischemic blood samples from patients with hyperacute stroke

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Abstract
In acute stroke, neuroinflammation can nowadays be analyzed by local cerebral aspiration of pial-ischemic blood during mechanical thrombectomy. Recently, Shaw et al. reported on differences in leukocyte subpopulations within the occluded cerebrovascular compartment. In their study, a main proportion of granulocytes was lost during isolation. By immediate analysis, we found a reproducible increase in absolute local granulocytes without variations in absolute lymphocyte and monocyte numbers. Flow-cytometric phenotyping confirmed a high proportion of granulocytes and a local shift towards CD4⁺ T cells. Thus, immediate analysis appears to be critical to observe distinct local responses of leukocytes to acute ischemic stroke.

Keywords
Acute ischemic stroke, flow cytometry, immune cells, leukocytes, T cells

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In acute experimental stroke, there is strong evidence that infiltrating immune cells in concert with platelets exert deleterious effects within the ischemic brain.¹ In human acute ischemic stroke, the method of local cerebral aspiration of arterial blood from the occluded ischemic compartment only recently has opened an avenue to investigate cellular and molecular processes of the acute stroke immune response. These unique samples are obtained from within the collateral circulation distal to an embolic occlusion and, importantly, before recanalization through mechanical thrombectomy (MT) is achieved.²,³ Thereby, we observed a robust local infiltration of granulocytes during hyperacute human stroke.²,⁴ In parallel, the Blood and Clot Thrombectomy Registry and Collaboration (BACTRAC) collected the clot and arterial blood during standard of care thrombectomy for laboratory analysis in which leukocytes are isolated and then cryopreserved.³,⁵ Recently, Shaw et al. reported on flow-cytometry analysis of 16 stroke patients from this biobank. Their analysis uncovered differences in lymphocyte subsets within the intracranial arterial blood compared to the systemic circulation, but the observed granulocyte fraction was exceedingly low.⁵ We here report on a shift in leukocyte subsets sampled directly from the secluded cerebral vasculature before MT. In this cohort of 36 stroke patients, we performed immediate flow-cytometry analysis of these whole blood samples conserving all leukocyte subpopulations.

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From July 2020 to May 2021, we prospectively observed large-vessel occlusion (LVO) stroke patients undergoing emergency MT. Local microcatheter aspiration within the cerebral ischemic arterial compartment and the cervical internal carotid artery (systemic control) was performed as previously described. A cohort of patients meeting all *a priori* defined inclusion criteria comprised 43 patients.

![Figure 1](image)

**Figure 1.** Analysis of leukocyte populations within systemic vs local ischemic blood samples of stroke patients. (a) Gating strategy. (b) Counting of absolute granulocytes, monocytes and lymphocytes numbers. (c) Percentage composition of the lymphocyte population. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). Data are given as mean and SD. Gaussian distribution was tested using the D’Agostino-Pearsons Test followed by a Wilcoxon signed-rank Test or a paired Student’s *t* Test. *P*-values <0.05 (two-sided) were considered statistically significant.
Due to the time-critical flow-cytometry work-up, only 36 patients admitted during regular working hours could be analyzed. In brief, Citrate-Phosphate-Dextrose-Adenine (CPDA) 1-anticoagulated whole blood samples of the different arterial regions were immediately stained and analyzed by flow-cytometry using the Multitest™ 6-color TBNK reagent (†#644611, BD Biosciences, Heidelberg, Germany) and BD Trucount Tubes (†#340334, BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. We calculated absolute cell numbers using the bead population of the Trucount Tubes. Samples were analyzed using a FACS Lyric (BD Biosciences, Heidelberg, Germany) and evaluated through FACSuite Software V1.4 (BD Biosciences, Heidelberg Germany) and FlowJo V10.8.1 (TreeStar). Ethical approval was obtained by the local ethics committee of the University of Würzburg, Germany (approval #135/17). All patients or their legal representatives provided written informed consent.

Flow-cytometry based analysis of leukocyte populations (Figure 1(a)) revealed a significant increase in absolute granulocyte numbers in ischemic blood samples when compared to intraindividual systemic controls (systemic 5394 ± 2540 vs ischemic 5901 ± 2706, p = 0.0403). No difference could be observed in absolute monocyte and lymphocyte numbers between the sampling locations (Figure 1(b)). Detailed immune phenotyping of lymphocytes, represented as percent of gated lymphocytes, uncovered an overrepresentation of CD3⁺ T cells under occlusion condition (systemic 69.56 ± 12.00 vs ischemic 72.45 ± 11.12, p = 0.003). Especially percentages of CD4⁺ T cells were significantly increased within the ischemic compartment (systemic 46.99 ± 12.03 vs ischemic 50.96 ± 12.81, p = 0.0001), while percentages of CD8⁺ T, B and NK cells concomitantly decreased in these probes (Figure 1(c)).

To date, the recent study of Shaw and colleagues and our present work are the first studies using flow-cytometry of gated lymphocytes, uncovered an overrepresentation of CD3⁺ T cells under occlusion condition (systemic 69.56 ± 12.00 vs ischemic 72.45 ± 11.12, p = 0.003). Especially percentages of CD4⁺ T cells were significantly increased within the ischemic compartment (systemic 46.99 ± 12.03 vs ischemic 50.96 ± 12.81, p = 0.0001), while percentages of CD8⁺ T, B and NK cells concomitantly decreased in these probes (Figure 1(c)).

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