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RESEARCH ARTICLE

# Hyperacute immune responses associate with immediate neuropathology and motor dysfunction in large vessel occlusions

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## Abstract

**Objective**: Despite successful endovascular therapy, a proportion of stroke patients exhibit long-term functional decline, regardless of the cortical reperfusion. Our objective was to evaluate the early activation of the adaptive immune response and its impact on neurological recovery in patients with large vessel occlusion (LVO). **Methods**: Nineteen (13 females, 6 males) patients with acute LVO were enrolled in a single-arm prospective cohort study. During endovascular therapy (EVT), blood samples were collected from pre and post-occlusion, distal femoral artery, and median cubital vein (controls). Cytokines, chemokines, cellular and functional profiles were evaluated with immediate and follow-up clinical and radiographic parameters, including cognitive performance and functional recovery. **Results**: In the hyperacute phase (within hours), adaptive immune activation was observed in the post-occlusion intra-arterial environment (post). Ischemic vascular tissue had a significant increase in T-cell-related cytokines, including IFN-γ and MMP-9, while GM-CSF, IL-17, TNF-α, IL-6, MIP-1a, and MIP-1b were decreased. Cellularity analysis revealed an increase in inflammatory IL-17+ and GM-CSF+ helper T-cells, while natural killer (NK), monocytes and B-cells were decreased. A correlation was observed between hypoperfused tissue, infarct volume, inflammatory helper, and cytotoxic T-cells. Moreover, helper and cytotoxic T-cells were also significantly increased in patients with improved motor function at 3 months. **Interpretation**: We provide evidence of the activation of the inflammatory adaptive immune response during the hyperacute phase and the association of pro-inflammatory cytokines with greater ischemic tissue and worsening recovery after successful reperfusion. Further characterization of these immune pathways is warranted to test selective immunomodulators during the early stages of stroke rehabilitation.

## Introduction

Recent advancement in stroke intervention has established endovascular therapy (EVT) as the standard of care for acute ischemic stroke due to large vessel occlusion (LVO). While 90% of LVO patients achieve near complete reperfusion after EVT, at least 40% still suffer a substantial disability.[1] Ischemia and reperfusion can lead to continued injury and dysfunction at the arteriolar and capillary levels even after recanalization. Moreover, continued infarct growth is also attributed to the activation of several mechanisms and inflammatory pathways.[2,3]

The initial intrinsic ischemic injury leads to cellular dysfunction, which is primarily caused by an insufficient energy supply resulting in hemostatic disruption and accumulation of cell-damaging species, eventually leading to excitotoxicity.[4] These events are followed by the activation of brain-resident microglia, recruitment of peripheral immune cells, and induction of a local inflammatory response within the ischemic core and penumbra.[4,5] Consequently, this inflammatory cascade leads to secondary neuropathological changes within the ischemic tissue. However, it remains unclear how this inflammatory process affects the immune responses involved in modulating recovery in stroke patients.[6,7]

Previous studies have reported an early activation of the immune response as a result of these ischemic changes.[8–10] Initial studies detected CD4 and CD8 T-cells in the cerebral spinal fluid (CSF) and interleukin (IL)-17A and IL-17$^{+}$ helper T-cells in venous blood within 24 h to 7 days, respectively.[11,12] Similarly, ischemic brain tissue removed during necropsy were observed to have elevated levels of IL-17-producing cells within 2 days of the infarct.[13] Although growing evidence shows that an acute ischemic injury to the brain tissue results in modulating adaptive immune system responses,[8,9] there is a lack of knowledge on when and how this immune system profile can impact neurological recovery.

In the present study, we sought to investigate the adaptive immune profile modulated within hours after injury at the ischemic vascular territory and evaluate its impact on neurological recovery in acute ischemic stroke patients due to LVO.

## Methods

### Study design and participants

This is a single-arm prospective study with a cohort of consecutive stroke patients enrolled after approval from the Institutional Review Board (IRB #201908789). The patients or their surrogates (legally authorized persons) were provided written informed consent. The inclusion criteria included 18+ years of age, anterior circulation acute ischemic stroke due to proximal LVO, diagnosed within 24 h from last known well (LKW) time, and undergoing EVT. Exclusion criteria included posterior circulation stroke, pregnancy, history of leukemia, mental illness, degenerative neurological diseases (Alzheimer's disease, dementia, and others), use of immunosuppressive medication, use of steroids in the last 3 months, and pre-morbid modified Rankin Score (mRS) >2. Acute LVO stroke diagnosis was based on clinical presentation and radiological evidence of LVO on computed tomography (CT) angiography. The management decisions were made by the clinical team in accordance with the current American Heart Association (AHA) guidelines.[14] All the patients underwent CT perfusion prior to EVT and a magnetic resonance imaging (MRI) study 24 h post-EVT for the evaluation of ischemic volumes before and after treatment.

### Data elements

Patient demographics, clinical, and radiological parameters were obtained as part of a prospective database and were interpreted by a clinical research team blinded to the immunological data. Information collected on the patient's demographics included age, gender, and race. Risk factors, comorbid conditions, and other parameters including hypertension (HTN), hyperlipidemia, diabetes mellitus (DM), carotid artery disease (CAD), atrial fibrillation, chronic kidney disease (CKD), history of smoking, body mass index (BMI), National Institute of Health Stroke Scale (NIHSS) were collected at baseline and follow-up. Use of intravenous tissue plasminogen activator (tPA), and stroke time metrics including time from last known well (LKW) to hospital arrival and LKW to reperfusion. Imaging parameters prior to reperfusion included cerebral blood flow (CBF), cerebral blood volume (CBV), mean transit time (MTT), and time to peak contrast concentration (TTP)/$T_{max}$ were obtained using the automatic post-processing CT perfusion RAPID software (iSchemaView, Menlo Park, CA). $T_{max}$ 4, 6, 8, and 10 s were used as a surrogate of hypoperfused tissue to estimate the penumbra. MRI at 24 h was used to estimate the final infarct volume and evaluate hemorrhagic complications (defined by ECASS III classification) within 24 h after the intervention (Fig. 4A).[15] Procedural characteristics included the degree of revascularization, as determined by the modified thrombolysis in cerebral infarction (mTICI) score. Successful reperfusion was defined as mTICI score of 2b/3.[16] All the neuroimaging data were interpreted by two independent investigators (SOG, DQO), blinded to the immunological data.

### Neurological recovery outcomes

Functional outcome was assessed by a board-certified vascular neurologist (SOG) using the modified Rankin Score (mRS) for the motor function at discharge and 3 months follow-up.[17] The mRS scale assigns a score from 0 (no symptoms) to 6 (deceased) to establish the functional status of patients after stroke. Patients whose 3-month follow-up scores were lower than their discharge mRS were labeled as "improved." Patients whose mRS did not change over the period were labeled as "no change," while those who had an increased mRS score were labeled as "declined".

Cognitive outcomes were assessed by the Montreal Cognitive Assessment (MoCA), a 30-point brief screening scale used to assess cognitive impairment.[18] MoCA evaluates memory, language, attention and concentration, orientation to time and space, visuo-constructional abilities, executive functions, conceptual thinking, and calculation. It was administered during hospitalization within 3 days from LKW as a baseline, and at the 3 months follow-up by a neuropsychologist blinded to the clinical and immunological data (KM). Cognitive impairment was defined as a MoCA score of <26.[18,19]

## Interventional and sampling protocol

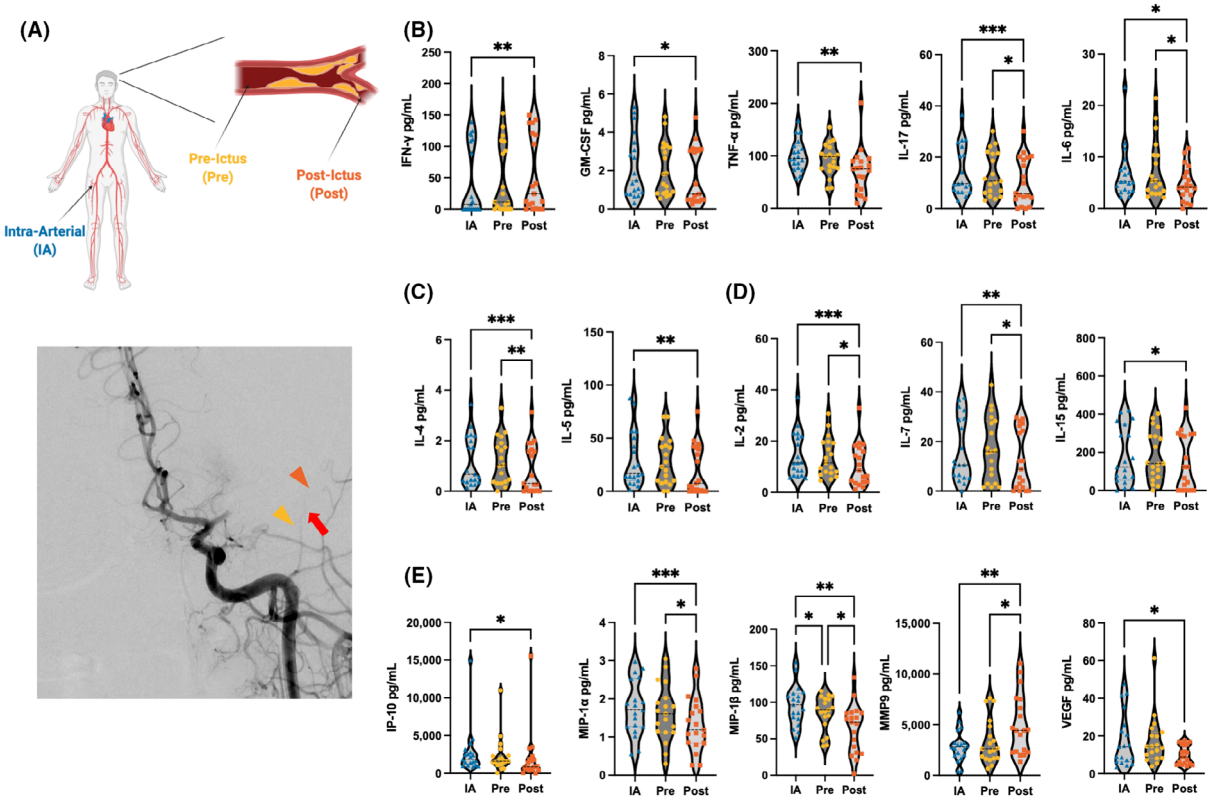
Arterial blood samples were collected by the neurointerventionalist during the standard EVT procedure. Briefly, a sheath is placed through the femoral artery, accessed via groin puncture, while the patient is under conscious sedation or general anesthesia. A diagnostic angiogram is first performed to confirm the site of intracranial occlusion. An intermediate aspiration catheter is then placed into the distal intracranial portion of the internal carotid artery (ICA) proximal to the clot. Next, a microcatheter is guided from the femoral artery across the ICA and beyond the occlusion site in the large intracranial vessel. 1–3 mL of blood is suctioned back to confirm the position of the catheter using a 3 mL syringe. This withdrawn blood is usually discarded; however, we collected this blood for our study as post-clot samples (Post). A 3–5 mL of arterial blood proximal to the occlusion was also collected simultaneously as a pre-clot sample (Pre). A standard EVT procedure is then proceeded using a stent-retriever (sometimes concomitant contact aspiration was also performed) to retrieve the clot. The pre-and post-clot samples were only collected during the first pass of the EVT procedure to avoid any contamination and to identify the initial immune response near the ischemic penumbra after an LVO. Intra-arterial (IA) control samples (3–5 mL) were also collected from the femoral artery sheath right after the guiding catheter was pulled out (Fig. 1A). Lastly, blood samples (3–5 mL) from the median cubital vein were collected as intravenous (IV) controls by the anesthesiologist at the same time as the femoral artery sample was obtained.

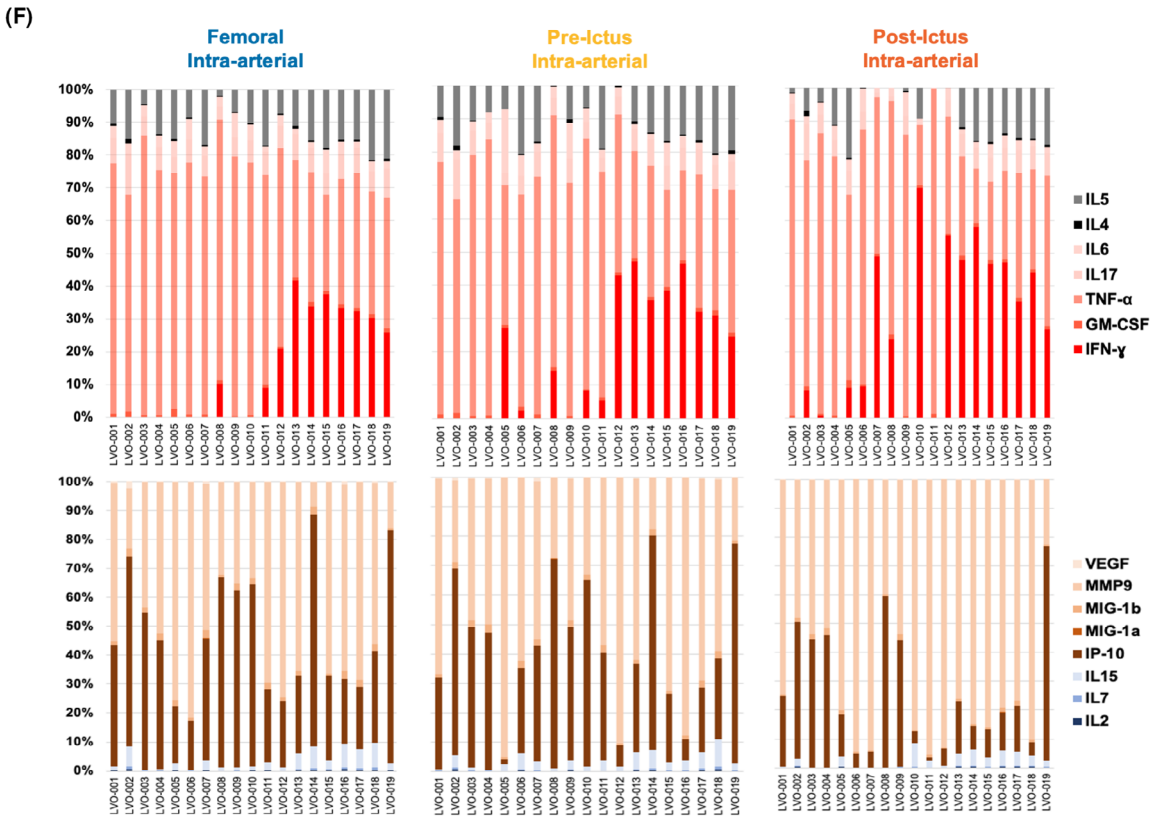
## Cytokine and chemokine quantification

Plasma was isolated by centrifugation of blood for 10 min at room temperature and was stored at −80°C. BioPlex Pro Human Immunotherapy panel 20-Plex assay (Bio-Rad Laboratories) with a 96-well plate was used for the analysis. The 20-plex assay contains beads conjugated with monoclonal antibodies to granulocyte-colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-2, -4, -5, -6, -7, -8, -10, -13, -15, -17A, -18, IFN-γ-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), monokine-induced by interferon-gamma (MIG), macrophage inflammatory protein-1-alpha (MIP-1a) and 1-beta (MIP-1b), regulated on activation, normal T-cell expressed and secreted (RANTES), and tumor necrosis factor-alpha (TNF-α). Standard enzyme-linked immunosorbent assays (ELISAs) were used to quantify IL-12, vascular endothelial growth factor (VEGF-165, Tonbo Bioscience), and matrix metalloproteinases-9 (MMP-9, R&D Systems). The concentration of cytokines and chemokines (pg/mL) was determined based on the best fit of a standard curve (12 points) for mean fluorescence intensity versus concentration. All the analyses were performed according to the manufacturer's instructions.

Furthermore, we quantified the post-clot environment for neuronal survivability. The patient's plasma was used to generate 5% conditioned media and was co-cultured with murine cortical cells (neurons and glia) for 7 days.[20] Immunofluorescence was performed, and image quantification was assessed per well using four random independent images averaged for each patient sample site.

**Figure 1.** Altered immune microenvironment within hours after stroke onset. We quantified 22 cytokines, growth factors, and cerebrovascular injury-related proteins in the plasma of hyper-acute stroke patients. Arterial plasma was isolated after an average of 7.5 h post-ischemic injury downstream of clot (Post), upstream of clot (Pre), and distant from clot (IA). Representative angiography of an M1-MCA occlusion (red arrow). The location of pre-clot arterial sampling (yellow arrowhead) and post-clot arterial sampling (orange arrowhead) is shown (A). Post-clot arterial sampling is not visible due to angiography methodology. In (B–F), plasma proteins were quantified using the Bioplex Immunotherapy Assay or ELISA-based quantification. Inflammation-related protein, IFN-γ was significantly increased in post-clot plasma, relative to the intra-arterial control site. Inflammatory cytokines, GM-CSF, TNF-α, IL-17, and IL-6 were decreased relative to the control site (B). Anti-inflammatory proteins IL-4 and IL-5 are decreased in post-ictus plasma (C). Pro-survival IL-2, IL-7, and IL-15 were significantly decreased relative to the control site (D). Macrophage-associated proteins IP-10, MIP-1a, and MIP-1b were also found to be significantly decreased relative to the control site. Vascular injury-related protein MMP9 was found to be increased, while VEGF was decreased relative to the control site (E). Relative comparison of inflammatory and anti-inflammatory cytokines in their respective locations (F). All samples were tested in duplicate and concomitantly assayed with manufacturer-provided standards. Parametric data were graphed as violin plots with individual data points and analyzed by repeated measures one-way ANOVA with Tukey's multiple comparisons test. A $p$-value of ≤0.05 was considered significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



## Leukocyte survey

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Bioscience AB). PBMC cellularity was quantified using hemocytometer counts, and viability was assessed by trypan blue exclusion. An aliquot of the cells was used for an immediate survey of leukocytes and intracellular cytokine quantification. Leukocyte survey assay was performed as described previously but tailored for blood isolated during the recanalization procedure.[21] PBMCs were washed twice with phosphate-buffered saline (PBS), and non-specific antibody binding was blocked using FcR blocking reagent (Miltenyi Biotec). Cells were labeled with GhostDye (Tonbo Bioscience) and fluorescent flow cytometry antibodies, including cluster designation (CD)45-BV786, CD8-BV510, CD66b-BV421, CD4-Percp5.5, CD3-FITC, CD19-PE-Cy7, CD11b-PE-Cy5, CD161-PE, CD14-A700, and CD11c-APC. Subsequently, cells were incubated with antibodies in the absence of light for 30 min at 4°C. Next, cells were washed with fluorescence-activated cell sorting (FACS) buffer and fixed with 1% paraformaldehyde (PFA, Electron Microscopy Science). Flow cytometry data were acquired within 30 min on a Becton Dickinson LSR II (BD Bioscience) using FACS Diva 8.0.1 at the Flow Cytometry Core at the University of Iowa's Carver College of Medicine, and data were analyzed using Flow Jo (v10) software. Absolute counts were extrapolated using the hemocytometer counts and percentages of specific leukocyte subpopulations surveyed according to the gating strategy (Fig. 2A).

## Statistical analysis

For all experiments, including ELISA, FACS, and in vitro imaging, data analysis was performed by technicians blinded to test conditions. For cytokine concentration, repeated measures one-way ANOVA with Tukey's multiple comparison test was applied, and data is displayed as violin plots for concentrations (GraphPad Prism). For 2D and polyfunctional analysis, repeated measures two-way ANOVA with Tukey's multiple comparison test was applied, and data was graphed as mean ± SD or as a heat map (GraphPad Prism). For all cellularity (non-parametric) data, Friedman's one-way ANOVA with Dunn's multiple comparison, and data are displayed as median with range (GraphPad Prism). All testing was conducted using alpha ≤0.05 as our significance cutoff.

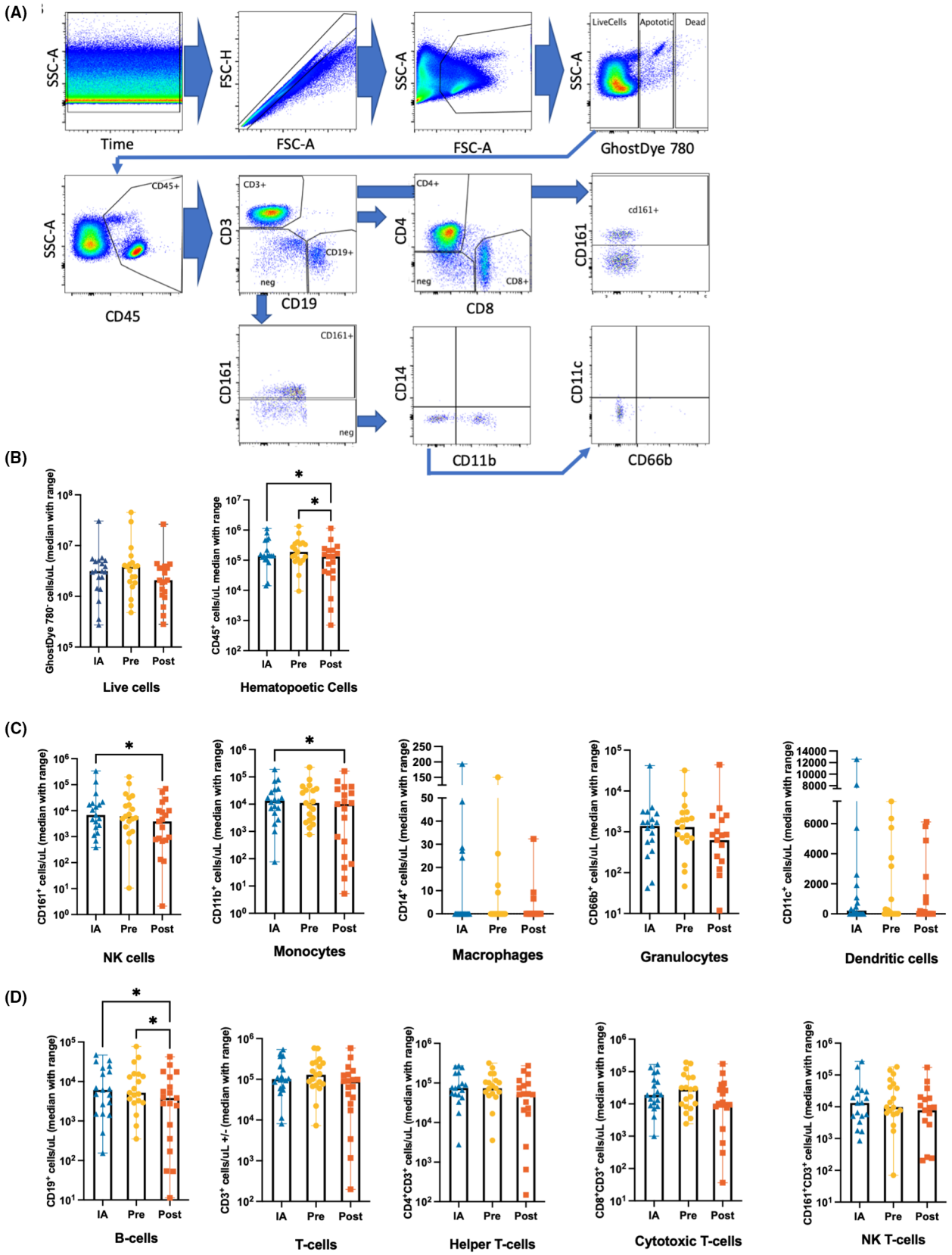
We employed a machine learning algorithm called Least Absolute Shrinkage and Selection Operator (LASSO) to detect clinically relevant immune correlates.[22] This allowed us to overcome the problematic high dimensionality versus the small sample size analysis. There is no well-known value for the LASSO penalty in the clinical context; thus, we estimated the penalty parameter using 10-fold cross-validation. Many of the estimated effects of the different cell types, interleukins, and cytokines were penalized to zero, thus narrowing down the coefficients and leading to a more parsimonious model selection mechanism. The LASSO algorithm automatically selects the strongest predictive variables; therefore, we considered cytokines to be significantly associated with the clinical outcome of interest if the LASSO model coefficient was non-zero, which included all CT perfusion parameters, final stroke volume on MRI, functional outcome, and cognitive outcome. We then performed a repeated-measures ANOVA and post hoc Tukey test to compare the LASSO-selected coefficients across the different sample points (IA, Pre, and Post). Finally, these coefficients were plotted against a univariate trend line, and their specific Pearson correlation coefficient (r) is shown. All LASSO-based analyses were performed using R software version 4.0.0 (2020-04-24). In addition, a simple linear regression analysis was incorporated, and an $r \geq 0.7$ and an alpha = 0.05 were used as our significance cutoff.

# Results

A total of 19 (13 females; 6 males) patients were recruited with a median age of 68 (59–81) years. Of these, the majority (17; 89.4%) were whites. Hypertension (11; 57.9%) was the most common comorbid condition, followed by atrial fibrillation (7; 36.8%), diabetes mellitus (4; 21.1%), and coronary artery disease (4; 21.1%). The median NIHSS at the time of admission was 16 (12.5–19). While IV-tPA was given to 6 (31.6%), mTICI of ≥2b was achieved for all the

**Figure 2.** Decreased Monocytes, NK, and B-cells distal to the clot. Immediately after sampling, blood was processed and stained, and major adaptive and innate immune cell subsets were quantified using flow cytometry. We qualified our flow data by using a gating strategy that focused on events within the laminar flow, removed doublets, and focused on live (GhostDye $780^{-}$) cells (A). Blood isolated from the blood downstream of the clot (Post) was found to have a decrease in hematopoietic ($CD45^{+}$) cells relative to blood upstream of the clot (B). Within the innate immune cell populations, only natural killer (CD161+), and monocytes (CD11b+) cells were found to be decreased in number (C). Within the adaptive immune cell populations, only B-cells (CD19+) were found to be decreased in number (D). All three sample sites of each patient were assayed together immediately after the acquisition of blood. Nonparametric data graphed as median with range and analyzed using Friedman's test with Dunn's multiple comparison test correction. A *p*-value of ≤0.05 was considered significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



patients. There were 7 (36.8%) patients who were transferred to acute rehabilitation, 4 (21.1%) to skilled nursing, 3 (15.8%) were discharged to home, and 4 (21.0%) had in-hospital mortality. At 90 days, 10 (52.6%) patients had an mRS of 0–2, and 8 (42.1%) were categorized as mRS 4–6, while one patient was lost to follow-up. The average in-hospital baseline and 90-day follow-up MoCA scores were 20.5 (IQR: 11–22) and 25 (IQR: 21–26), respectively. Table 1 shows the demographics, clinical characteristics, and time metrics for the cohort.

**Table 1.** Demographic, clinical, imaging, and interventional characteristics.

| | |
|---|---|
| Demographics | |
| Age, years, median (IQR) | 68 (59–81) |
| Sex, male, *n* (%) | 6 (31.6) |
| Race, *n* (%) | |
| Black | 2 (10.5) |
| White | 17 (89.5) |
| Body mass index, median (IQR) | 24.43 (22.4–31.07) |
| Comorbidities, *n* (%) | |
| Hypertension | 11 (57.9) |
| Atrial fibrillation | 7 (36.8) |
| Diabetes mellitus | 4 (21.1) |
| Coronary artery disease | 4 (21.1) |
| Tobacco use | 4 (21.1) |
| Hyperlipidemia | 3 (15.8) |
| Chronic kidney disease | 1 (5.3) |
| Stroke characteristics | |
| Size, median, $cm^3$ (min–max) | 36.6 (6.7–65.8) |
| Occlusion location | |
| Intracerebral artery, *n* (%) | 3 (15.8) |
| Middle cerebral artery, *n* (%) | 16 (84.2) |
| Cerebral blood flow <30%, mL/s, (IQR) | 11 (0–33) |
| $T_{max}$ 6 s (IQR) | 129 (78–162) |
| Thrombolysis in Cerebral Infarction score, *n* (%) | |
| Grade 0 | 0 |
| Grade 1 | 0 |
| Grade 2a | 0 |
| Grade 2b | 6 (31.5) |
| Grade 3 | 13 (68.5) |
| Clinical presentation and time metrics, median (IQR) | |
| NIHSS at admission | 16 (12.5–19) |
| Door-to-groin time | 61 (45–80) |
| Last-known-well-to-groin time | 330 (160–650) |
| Last-known-well-to-reperfusion time | 380 (176–664) |
| Treatment | |
| Intravenous rtPA, *n* (%) | 6 (31.6) |
| No of passes (≥2) | 11 (57.8) |
| Outcomes | |
| Modified Rankin score, *n* (%) | At discharge |
| 90 days | |
| Grade 0 | 0 |
| Grade 1 | 3 (15.8) |
| Grade 2 | 3 (15.8) |
| Grade 3 | 4 (21.05) |
| Grade 4 | 3 (15.8) |
| Grade 5 | 2 (10.5) |
| Grade 6 | 4 (21.05) |
| Modified Rankin scale dichotomized, *n* (%) | At discharge |
| 90 days | |
| Grade 0–3 | 13 (68.4) |
| Grade 4–6 | 6 (31.6) |
| Montreal cognitive assessment score, median (IQR) | |
| In hospital | 20.5 (11.5–22) |
| At 90 days | 25 (21–26) |
| Hemorrhagic transformation, *n* (%) | |
| HI1 | 2 (10.5) |
| HI2 | 6 (31.6) |
| PH1, PH2 | 0 |
| Post care, *n* (%) | |
| Home | 3 (15.8) |
| Acute rehabilitation | 7 (36.8) |
| Skilled nursing facility | 4 (21.1) |
| Death | 4 (21.1) |

| | |
|---|---|
| Outcomes | |
| Modified Rankin score, *n* (%) | At 90 days |
| Grade 0 | 5 (26.3) |
| Grade 1 | 3 (15.8) |
| Grade 2 | 1 (5.3) |
| Grade 3 | 1 (5.3) |
| Grade 4 | 2 (10.5) |
| Grade 5 | 2 (10.5) |
| Grade 6 | 4 (21.05) |
| Modified Rankin scale dichotomized, *n* (%) | At 90 days |
| Grade 0–3 | 10 (52.6) |
| Grade 4–6 | 8 (42.1) |
| Montreal cognitive assessment score, median (IQR) | |
| In hospital | |
| At 90 days | |
| Hemorrhagic transformation, *n* (%) | |
| HI1 | |
| HI2 | |
| PH1, PH2 | |
| Post care, *n* (%) | |
| Home | |
| Acute rehabilitation | |
| Skilled nursing facility | |
| Death | |

(*Continued*)

## Hyperacute post-occlusion plasma exhibits a unique inflammatory environment

Analysis of pro-inflammatory cytokines within the post-occlusion plasma environment revealed a significant increase in post-clot IFN-γ (IA: 45.75 pg/mL vs. Post: 55.43 pg/mL, $p = 0.0035$) and a decrease in GM-CSF (IA: 2.219 pg/mL vs. Post: 1.742 pg/mL, $p = 0.0106$), TNF-α (IA: 101.5 pg/mL vs. Post: 70.83 pg/mL, $p = 0.0056$), IL-17 (IA: 14.61 pg/mL and Pre: 13.40 pg/mL vs. Post: 10.55 pg/mL, $p = 0.0006$ and $p = 0.0120$, respectively), and IL-6 (IA: 6.828 pg/mL and Pre: 7.501 pg/mL vs. Post: 4.786 pg/mL, $p = 0.0412$ and $p = 0.0342$, respectively) (Fig. 1B). Analysis of cytokines from type 2 helper T-cells (Th2) including IL-4 (IA: 1.208 pg/mL and Pre: 1.213 pg/mL vs. Post: 0.8832 pg/mL, $p = 0.0010$ and $p = 0.0075$, respectively) and IL-5 (IA: 29.91 pg/mL vs. Post: 20.13 pg/mL, $p = 0.0025$) were also found to be decreased in the post-clot environment (Fig. 1C). Pro-survival cytokines including IL-2 (IA: 14.64 pg/mL and Pre: 13.69 pg/mL vs. Post: 10.78 pg/mL, $p = 0.0005$ and $p = 0.01$, respectively), IL-7 (IA: 16.67 pg/mL and Pre: 16.96 pg/mL vs. Post: 12.54 pg/mL, $p = 0.0015$ and $p = 0.0296$, respectively), and IL-15 (IA:

179.4 pg/mL vs. Post: 145.9 pg/mL, $p = 0.0457$) were significantly decreased in the post-clot environment (Fig. 1D). Chemokines involved in tissue injury and innate immune activation revealed a decrease in IP-10 (IA: 2,737 pg/mL vs. Post: 1,948 pg/mL, $p = 0.0248$), MIP-1α (IA: 1.748 pg/mL and Pre: 1.622 pg/mL vs. Post: 1.329 pg/mL, $p = 0.0004$ and $p = 0.0110$, respectively), MIP-1β (IA: 96.14 pg/mL and Pre: 84.65 pg/mL vs. Post: 64.35 pg/mL, $p = 0.0097$ and $p = 0.048$, respectively) and a concomitant increase in MMP-9 (IA: 2,870 pg/mL and Pre: 3,317 pg/mL vs. Post: 4,989 pg/mL, $p = 0.0019$ and $p = 0.0338$, respectively), post-clot (Fig. 1E). We also observed a decrease in VEGF (IA: 17.85 pg/mL vs. Post: 10.07 /mL, $p = 0.0408$) (Fig. 1E). Other plasma proteins including IL-8, IL-10, MCP-1, RANTES, and MIG were similar across all sampling sites (Fig. S1). Overall comparative analysis revealed a greater percentage of inflammatory markers (red-tinted) relative to Th2 markers (gray-tinted) in the post-occlusion plasma, as well as a decrease in tissue injury markers (brown-tint) relative to pro-survival markers (blue-tint) (Fig. 1F).

## Hyperacute post-occlusion endovascular environment exhibits decreased cellularity in monocytes, natural killer-, and B-cells

Using FACS and a flow gating strategy that removes non-laminar flow artifacts, doublets, and dead cells; innate, and adaptive immune cell subsets were quantified (Fig. 2A). We observed a decrease in hematopoietic cells ($CD45^+$, IA: $1.4 \times 10^5$ and Pre: $1.8 \times 10^5$ vs. Post: $1.3 \times 10^5$, $p = 0.0449$ and $p = 0.0449$, respectively) (Fig. 2B). Within the innate subsets, natural killer (NK, IA: $6.8 \times 10^3$ vs. Post: $3.8 \times 10^3$, $p = 0.0175$), and monocytes were found to be decreased ($CD11b^+$, IA: $1.3 \times 10^4$ vs. Post: $1.0 \times 10^4$, $p = 0.0283$) (Fig. 2C). B-cells were also found to be decreased in the post-clot environment ($CD19^+$, IA: $6.1 \times 10^3$ and Pre: $5.2 \times 10^3$ vs. Post: $3.8 \times 10^3$, $p = 0.0449$ and $p = 0.0105$, respectively, Fig. 2D). We also compared intravenous and intra-arterial blood samples within a subset of our patients to exclude vasculature-specific variations and did not observe any significant differences (Fig. S2).

## Increase in post-occlusion inflammatory helper T-cells

Functional analyses of adaptive immune cells (Fig. 3A) using one-dimensional paired analysis revealed that there was a significant increase in CD4 T-cells producing GM-CSF (IA: 14.36% vs. Post: 18.35%, $p < 0.0001$) and IL-17 (IA: 1.105%, and Pre: 1.215 vs. Post: 1.736%, $p = 0.0013$ and $p = 0.0170$, respectively) distal to the clot (Fig. 3B). Two-dimensional analysis observed an increase in GM-$CSF^+$IFN-$\gamma^-$ (IA: 5.02% and Pre: 4.88% vs. Post: 7.03%, $p = 0.0003$ and $p = 0.0108$, respectively) and IFN-$\gamma^+$GM-$CSF^+$ (IA: 9.11% vs. Post: 11.33%, $p = 0.0009$) helper T-cells distal to the clot. While IFN-$\gamma^-$GM-$CSF^-$ $CD4^+$ T-cells (IA: 72.20% vs. Post: 67.81%, $p = 0.0062$) were found to be decreased (Fig. 3C). Similarly, we observed an increase in IFN-$\gamma^-$IL-$17^+$ CD4 T-cells (IA: 0.97% and Pre: 1.06 vs. Post: 1.53%, $p = 0.0017$ and $p = 0.0183$, respectively) in the post-clot environment (Fig. 3C). Polyfunctional analysis, (number of cells producing three or more cytokines), revealed a consistent increase in CD4 T-cells with GM-$CSF^+$IFN-$\gamma^+$IL-$10^+$IL-$17^-$ (Post: 0.280% vs. IA: 0.211%, $p = 0.038$) and GM-$CSF^+$IFN-$\gamma^+$IL-$17^+$ (Post: 0.415% vs. IA: 0.243%, $p = 0.001$) in the post-clot environment (Fig. 3D). Two-dimensional analysis of the cytotoxic T-cell subset revealed an increase in IFN-$\gamma^+$GM-$CSF^+$ CD8 T-cells (Post: 13.83% vs. IA: 10.40%, $p = 0.005$) distal to the occlusion with a concomitant reduction in IFN-$\gamma^-$GM-$CSF^-$ CD8 T-cells (Post: 33.27% vs. IA: 38.62%, $p = 0.003$) (Fig. S3). In summary, arterial sampling reveals an increased presence of inflammatory helper T-cells within hours after ischemic injury.

## Neuroimaging biomarkers and immune correlates in hyper-acute stroke

Analysis between molecular immune mediators and clinical and radiological biomarkers revealed that B-cells ($CD19^+$) correlated with several perfusion parameters. Plasma proteins associated with inflammation also correlated with imaging biomarkers; MCP-1 was inversely correlated with perfusion at $T_{max}$ 4 s ($r = -0.65$, $p = 0.018$) and $T_{max}$ 6 s ($r = -0.7$, $p = 0.017$, Fig. 4A). Similarly, we also found that the pre-occlusion RANTES was inversely associated with MoCA during hospitalization ($r = -0.7$, $p = 0.017$) and at 3 months ($r = -0.78$, $p = 0.017$) (Fig. 4B, bottom row). B-cells within the femoral arterial environment were found to correlate with perfusion at $T_{max}$ 4 s ($r = 0.73$, $p = 0.0067$) and $T_{max}$ 6 s ($r = 0.63$, $p = 0.027$), while B-cells within the post-clot environment correlated with $T_{max}$ perfusion at 4 s ($r = 0.6$, $p = 0.0024$) and 6 s ($r = 0.7$, $p = 0.015$) (Fig. 4C). Moreover, CD4 T-cells within the femoral arterial environment were strongly correlated with the infarct volume ($r = 0.68$, $p < 0.002$). In contrast, post-clot inflammatory CD4 T-cells were inversely correlated with perfusion at $T_{max}$ 6 s ($r = -0.77$, $p = 0.005$). (Fig. 4D). Inflammatory CD8 T-cells from the post-clot were also found to correlate with infarct volume ($r = 0.7$, $p = 0.0013$) (Fig. 4E). In summary, within hours after ischemia, we were able to detect several adaptive immune responses that correlated with neuroimaging biomarkers of brain injury and pathology.

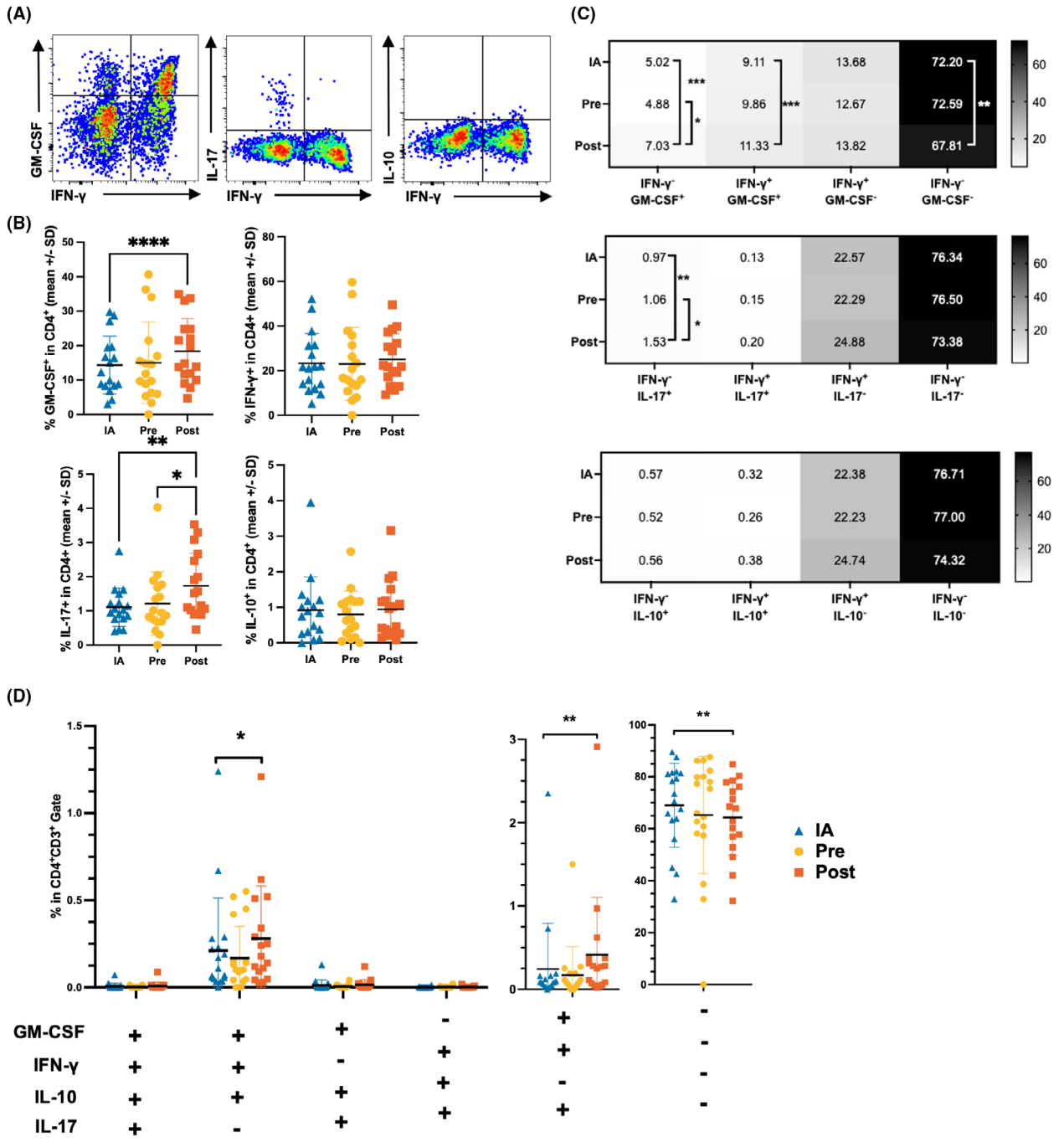



**Figure 3.** Increase in multiple pro-inflammatory helper T-cells in hyperacute post-occlusion blood. Immediately following blood processing, intracellular cytokine production was measured by labeling with fluorescent antibodies specific to indicated protein and quantified using flow cytometry. Representative dot plots indicating the intensity of cytokine levels for GM-CSF, IFN-γ, IL-17, and IL-10 are shown (A). Single-dimensional analysis revealed an increase in GM-CSF$^+$ CD4 and IL-17$^+$ CD4 T-cells in the post-occlusion samples (B). Two-dimensional analysis reveals an increase in IFN-γ$^+$GM-CSF$^+$ CD4 T-cells in the post-occlusion samples (C). Three-dimensional analysis reveals an increase in GM-CSF$^+$IFN-γ$^+$IL-17$^+$ and GM-CSF$^+$IFN-γ$^+$IL-10$^+$ CD4 T-cells in the post-occlusion samples (D). All three sample sites of each patient were assayed together immediately after the acquisition of blood. Parametric data were graphed as mean ± SD and analyzed by repeated measures one-way ANOVA, Tukey's multiple comparisons test.

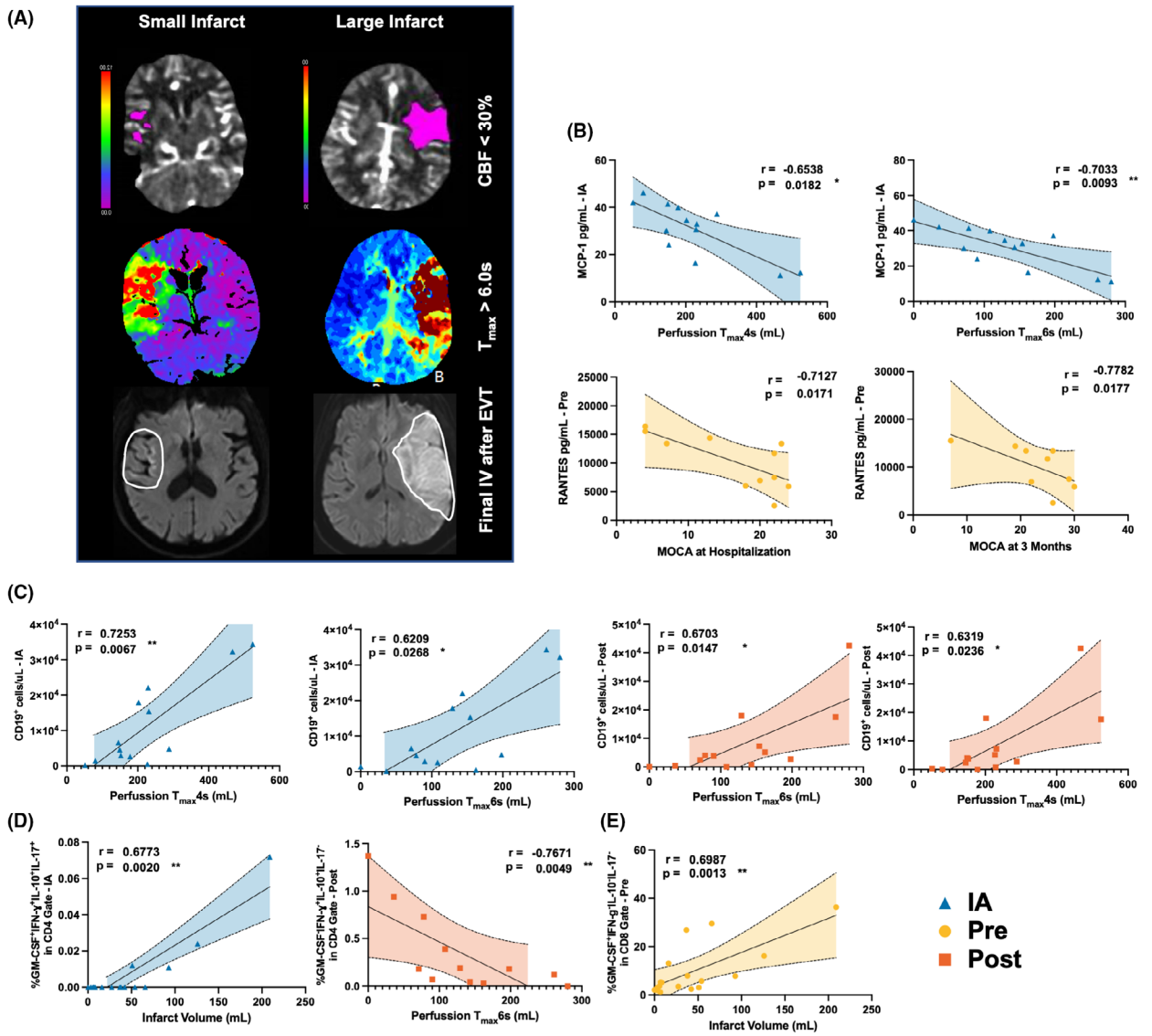



**Figure 4.** Clinically relevant immune correlates in hyper-acute stroke patients. Correlation analysis between biological (cellularity, cytokine, and function profiles) and clinical (perfusion, infarct volume, cognitive and motor function) parameters were performed. (A) Representative neuroimaging of patients with small (left column) and large (right column) ischemic strokes. Perfusion imaging showing CBF < 30% (top row) and $T_{max}$ >6 s (second row); and DVI-MRI (bottom row) after EVT is shown. Tissue damage-associated proteins (MCP-1) located in the femoral arterial sample (IA) were found to correlate with perfusion negatively (B top row), while RANTES were associated with motor function at 3 months (B, bottom row). B-cell cellularity was found to correlate with perfusion in the intra-arterial and post-occlusion locations positively (C). Post-occlusion inflammatory CD4 T-cells were found to correlate with $T_{max}$ 6 s perfusion negatively and femoral arterial samples correlate with infarct volume positively (D). Pre-occlusion inflammatory CD8 T-cells were found to correlate with infarct volume (E). Statistical analysis for (B–E) was performed using the LASSO method, and an *r*-value of >0.7 and *p*-value ≤0.05 was considered significant.

## Distinct adaptive immune profile in patients with improved functional recovery

Patterns on neurological recovery are illustrated in Figure 5A. In patients with declining motor performance, we observed a significant increase in pre-occlusion IL-15 compared to both the no change or improving groups (declined: 2416 pg/mL vs. no change: 664.4 pg/mL and improved: 885.6 pg/mL, $p = 0.005$ and $p = 0.010$, respectively). In contrast, patients with a decline in performance were found to have lower IP-10 levels in the post-occlusion environment (declined: 39.60 pg/mL vs. no change: 286.7 pg/mL and improved: 171.2 pg/mL, $p = 0.0.0014$ and $p = 0.05$, respectively, Fig. 5B).

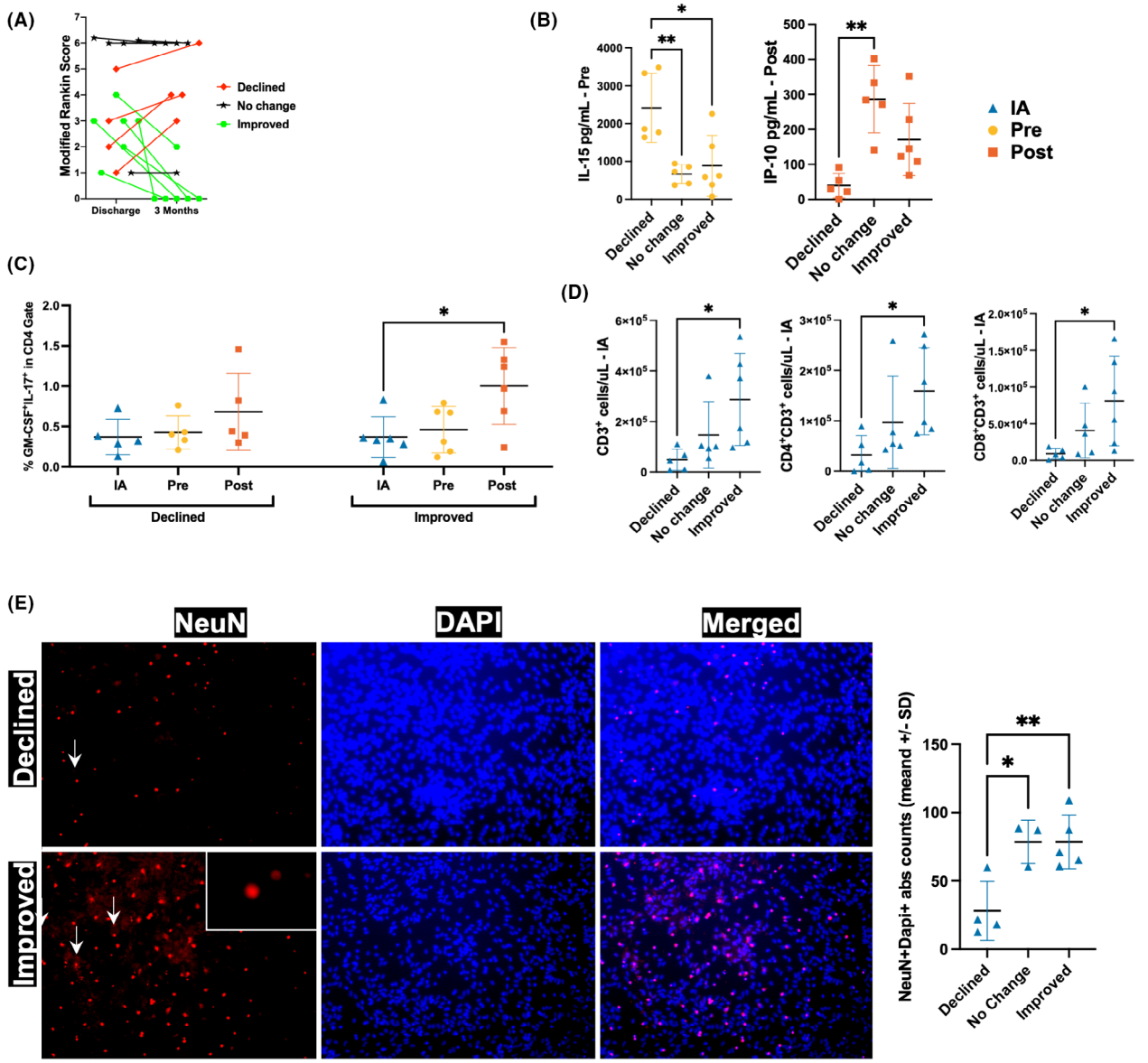



**Figure 5.** Decreased adaptive cellularity and increased plasma neurotoxicity during hyperacute stroke associates with poor long-term recovery. At 3 months, motor function recovery is distinct and stratified into those whose performance declines as revealed by an increase in mRS scores (Declined), those who maintain function and no change in mRS scores (No change), and those who improve and had a decrease in mRS scores (Improved, A). Motor function-dependent separation of patients reveals an increase in pre-occlusion IL-15 and a decrease in post-occlusion IP-10 in patients with poor functional recovery (B). Conversely, improving patients have an increased quantity of GM-CSF$^{+}$IL-17$^{+}$ CD4 T-cells in the post-occlusion sample relative to the distal control sample (IA), which is absent in declining patients (C). Declining patients exhibit a loss of both helper and cytotoxic T-cell cellularity in the distal arterial sample, as compared to recovering patients (D). After in vitro culture with patient plasma (5%) from declining patients, murine brain cultures exhibit a decrease in viable neurons (NeuN$^{+}$DAPI$^{+}$, arrows) as compared to brain cells cultured with the plasma of improving patients. Representative immunofluorescence image (left) and cumulative data (right). Quantification was assessed per well using four random independent images averaged for each patient sample site (E). Red = NeuN and blue = DAPI. Parametric data were graphed as mean ± SD and analyzed by repeated measures one-way ANOVA, Tukey's multiple comparisons test.

Functional analysis revealed that in patients with marked improvement in functional recovery, GM-CSF$^{+}$IL-17$^{+}$ helper T-cells were more abundant (×2.7) post-occlusion, as compared to distal femoral sample (Post: 1.003 vs. IA: 0.369, $p$ = 0.035). This significant increase was found to be absent in patients with declining recovery (Fig. 5C). There was a significant increase in intra-arterial CD3$^{+}$ T-cells in patients with improved functional recovery

(declined: $4.8 \times 10^4/\mu L$ vs. improved: $2.8 \times 10^5/\mu L$, $p = 0.03$). This increase was composed of both helper CD4 (declined: $3.2 \times 10^4/\mu L$ vs. improved: $1.5 \times 10^5/\mu L$, $p = 0.04$) and cytotoxic CD8 T-cells (declined: $9.2 \times 10^3/\mu L$ vs. improved: $8.1 \times 10^4/\mu L$, $p = 0.04$) (Fig. 5D).

With the observation that patients with poor motor function recovery exhibited an altered plasma environment (Fig. 5B), we proceeded to test the hypothesis that the post-occlusion endovascular environment affects neuronal survivability, thereby modulating functional recovery. Using patient plasma, we generated 5% conditioned media and co-cultured murine cortical cells (neurons and glia) with patient plasma for 7 days.[12] Murine cortical brain cultures treated with plasma from patients with declining motor function exhibited an overall lower number of neurons present when compared to those treated with the plasma of patients with improved motor function recovery (declined: 28 vs. improved: 78.6, $p = 0.009$, Fig. 5E). In summary, we have detected a unique inflammatory signature present at intake that delineates motor functional trajectories. In addition, the loss of viable neurons in cultures with plasma from declining patients revealed a possible mechanistic insight into motor function trajectories after a stroke.

## Discussion

This study observed that after an acute stroke due to LVO, a specific set of adaptive immune responses were observed within hours of ischemia. Our analysis reveals changes in (i) local levels of the inflammatory cytokines surrounding the ischemic territory, (ii) a significant increase in inflammatory helper T-cells and a decrease in NK, B-cells, and monocytes in the post-occlusion environment, and (iii) distinct cytokines and immune cells profile that associates with poor motor recovery at 3 months.

Currently, there are two types of treatments that attempt to mitigate additional stroke-induced neuropathology. The first approach involves using pharmacological (rtPA) or mechanical intervention (EVT) that targets the ischemia-inducing clot. However, despite the use of these interventions, only a portion (<50%) of patients exhibit a reduction in brain injury and cognitive/functional recovery.[23] Second, neuroprotective approaches such as therapeutic hypothermia and transcranial magnetic stimulation have demonstrated limited clinical efficacy.[24,25] As of yet, there are no approved therapies that target the post-stroke neuropathology-inducing immune response. Several approaches, including shifting the systemic immune response to an anti-inflammatory state or inhibiting immune cell trafficking into the brain non-selectively, have failed.[26,27] Not all neurons are alike; similarly, not all immune responses are comparable in their role. Thus, it is highly warranted that further investigations into the individual pathways of the immune response proceed with the utmost fervor.

The role of the adaptive immune response and its initiation of secondary neuro-injury is most granular in stroke animal models. Recombinase activating gene (RAG) knock-out mice (devoid of B and T-cells) reconstituted with wild-type T-cells were found to develop increased neuropathology after the ischemic injury.[28] Repletion of B-cell knock-out or wild-type mice with B-cells after induction of ischemic injury resulted in improved motor function and reduced infarct volume.[20,29] Relative to controls, infiltration by cytotoxic T-cells and B-cells into ischemic parenchymal territories can be seen as early as 3 h post-stroke, and helper T-cells as early as 24 h.[30] Similarly, cytotoxic T-cell knock-out mice exhibit reduced infarct volumes and improved neurological outcomes indicating a neuropathological role. Cytotoxic effector molecules that play a role in ischemic injury include IFN-γ,[31] perforin,[32] and granzyme.[33] Blocking IFN-γ and its downstream target inflammatory chemokine (IP-10) has been shown to decrease ischemia-induced neurodegeneration.[34]

Our study aimed to build upon this knowledge by evaluating the immune responses in the ischemic vascular territory in humans presenting with an LVO ischemic stroke within hours (median: 6.33 h). Intra-arterial sampling revealed an early increase in IFN-γ, a pleiotropic cytokine mainly produced by T-cells. We found a bi-modal expression of IFN-γ and its significance is a focus of ongoing studies.[35] We also observed an increase in the plasma level of MMP-9, a molecule involved in blood-brain barrier breakdown (BBB), leukocyte extravasation, and T-cell activation.[36] It is likely that the increase in intra-arterial post-clot MMP-9 stems from the parenchymal sources and indicates active leukocyte recruitment into the infarct tissue.[37,38] Moreover, in agreement with Kes et al., and Ferrarese et al.,[39,40] we also observed an increase in the overall production of IL-6 in the arterial environment—relative to the control group in Kes et al., however, we noted a decrease in the IL-6 distal to the occlusion (post-clot) when compared with femoral artery samples. We speculate that these findings are reflective of a systemic immune response to the ischemic injury. IL-6 is implicated in the production of growth-associated protein 43 (GAP-43), neurotrophin-3 (NT-3), transient expression of brain-derived neurotrophic factor (BDNF), and spontaneous neurological recovery.[41] Furthermore, with regard to TNF-α, we also observed a decrease in the post-clot environment relative to the intra-arterial environment. While previous studies have shown no significant differences in TNF-α levels among cases and controls and at

much lower levels than we observed, there was a gradual increase in the TNF-α levels within the subsequent days after the ischemic insult.[39,42] We infer that our elevated findings reflect the spatial proximity to the occlusion site and the hyper-acute time window. Taken together, our findings indicate an early and enhanced inflammatory response proximal to ischemic brain injury.

Several immune molecules exhibit a robust correlation with radiological parameters, potentiating their use as biomarkers. We observed that MCP-1 in the peripheral circulation was tightly correlated with brain tissue perfusion volumes. MCP-1 is released by monocyte/macrophage and is involved in the early inflammatory response by facilitating cell trafficking and blood–brain barrier disruption.[43,44] The observations in our study may reflect a peripheral systemic innate immune response to the ischemic brain injury rather than the local. On the other hand, B-cells were found to correlate with tissue perfusion at all sampled locations. B-cells are implicated as both disease-ameliorating and/or disease-promoting in post-stroke recovery.[20,29,45] However, the timing and level of B-cell activation may invoke their respective functional role. Our study further expands on this understanding by revealing that ischemic injury and reperfusion alter B-cell cellularity even within hours after stroke onset. It is interesting to note our study showed a positive relationship between polyfunctional (GM-CSF+IFN-g+IL10+IL17+) CD4 T-cells, inflammatory (GM-CSF+) CD8 T-cells, and neuropathology (infarct volume). While these associations are mere biomarkers of severity, they may be implicated in the initial pathway leading to secondary brain injury after reperfusion. However, further research is necessary to better understand the mechanism of these interactions. In our pilot study, we also observed that several immune mediators were associated with functional motor recovery at 3 months. We found an increased level of plasma IL-15 in individuals with declining functional performance. This observation is reflected in a pre-clinical study by Li et al., which showed that astrocyte-derived IL-15 promoted the accumulation of cytotoxic CD8 and NK T-cells in the brain of ischemic mice and further augmented brain injury.[46] In contrast, CD4 T-cells displayed a more enigmatic profile as those individuals with improved functional outcomes were found to have a higher ratio of inflammatory GM-$CSF^{+}IL\text{-}17^{+}$ CD4 T-cells in the post-clot environment as compared to the patients with declining motor function. At this moment, we speculate that this may be due to the active trafficking of inflammatory cells into the brain parenchyma in these patients with poor outcomes. However, further studies are warranted to confirm this association and to better understand the pathway as a potential therapeutic target.

Finally, using an in vitro system, we gained a possible mechanistic insight into why certain individuals exhibit poor functional recovery, even though all the patients underwent successful endovascular reperfusion. Culturing murine cortical cells with patient-derived conditioned media revealed a loss of neurons in patients with declining motor function compared to those who improved. These results may indicate the presence of a neurotoxic protein within the arterial vasculature, which may produce further secondary injury and drive poor functional recovery. However, the origin (neurological or immunological) of this protein(s) is still to be defined. Given its presence within the arterial system and considering our recent finding, we strongly speculate it is of immunological origin, which will be the focus of our future studies.

Despite the novelty of our findings, results must be interpreted with caution, given the limitations of it being a pilot study. It must be noted that our study is modest in size and thus falls under the category of hypothesis discovery. While we strengthened our analysis using a machine learning methodology that addresses high-dimensional data and avoids multiple comparisons with low sample size; initial associations are exploratory and will need further confirmatory studies. Moreover, this study was performed using standard immunological tools, thus making our observations accessible to a broader range of clinical/research laboratories. Our study was not powered to study the effects of thrombolytic therapy (tPA) on the cellular or molecular components of the immune system. However, several studies have postulated its role in proinflammatory mechanism (complement activation, increased proinflammatory cytokines, and BBB disruption), while other suggest that tPA attenuate the immune response (increase anti-inflammatory cytokines, decrease circulating monocytes, and lymphocytes) after an ischemic injury.[47–49] Although the benefits of tPA in the acute phase outweigh the risks, the interaction between tPA and both innate and adaptive immune responses remains to be elucidated. Finally, our MoCA evaluation was performed only during the acute and subacute phases, thus we acknowledge that long-term cognitive testing is warranted to fully appreciate the prolonged implications of adaptive immune cell activation.

In summary, this study characterizes the hyperacute environment within the ischemic vascular region. The current understanding of the immune processes involved in human brain stroke biology is limited. Our study provided proof of concept for the immediate immune response after an ischemic brain injury. Recently, immunomodulation has shown to be effective in several other diseases and has been the focus of research on stroke. Fingolimod decreases the circulating lymphocytes by inhibiting their egress from the spleen and lymph

nodes, and was observed to increase the clinical outcomes and decrease infarct volumes when co-administered with tPA.[50,51] Similarly, minocycline mediates anti-inflammatory effects and inhibiting MMP-9 was noted to improves functional outcomes and decrease the NIHSS in AIS patients.[52] T-cell mediated inflammation has been recently implicated in modulating ischemic stroke; Poly-YE (immunomodulator) has been shown to be neuroprotective by regulating T-cell mediated inflammation in animal stroke models.[53] Our study reveals an increase in inflammatory cytokines (IFN-γ, MMP-9, IL-17+ helper T-cells) that can be targeted for early therapeutic intervention. The recent advances in EVT may provide access to specific target molecules during the hyperacute ischemic phase and should be tested in future large-scale studies.

## Conclusion

Our study provides evidence of the earliest changes in the inflammatory adaptive immune response after an ischemic brain injury and its potential involvement in modulating post-stroke neurological recovery. The impact of this analysis is two-fold. First, by characterizing the earliest adaptive immunological events after a stroke, we open the door for further research in an attempt to identify specific immune cells/effectors that may be targeted in order to modulate neuro-recovery. Second, by revealing early immune biomarkers that associate with long-term functional trajectories, clinicians may use these changes as guides in the immediate and long-term post-operative care of stroke patients and potentiate functional recovery in those patients predicted to have poor outcomes.

## Author Contributions

S.O.G, N.J.K., and S.B.O. designed the research; S.O.G, M.F., D.Q.O., C.Z., and A. M. R. recruited and consented the patients; K.H., S.B.O., K.M., and D.T. performed the research; P.T.E., V.O.T, and N.J.K. contributed new reagents/analytical tools; A.D., M.F., and S.B.O. analyzed data; S.O.G, M.F., and S.B.O. wrote the manuscript.

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## Conflict of Interest

The authors have no financial conflicts of interest relevant to this study.

## Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author, [SBO]. The data are not publically available due to restrictions [e.g., they contain information that could compromise the privacy of research participants].

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Altered immune micro-environment in hyper-acute stroke plasma. Quantification of proteins in the plasma of stroke patients. Parametric data were graphed as violin plots.

**Figure S2**. Intra-arterial vs. intravenous cellularity. Comparison of intra-arterial and intra-venous cellularity.

**Figure S3**. Subtle increase in inflammatory CD8 T-cells distal to occlusion in the hyper-acute phase of stroke. Similar analysis as performed on helper T-cells shows only a minute change in cytotoxic T-cells. Representative dot plot of cytotoxic data (A). One-dimensional analysis of inflammatory cytokine production by cytotoxic T-cells (B). Two-dimensional analysis (C) and polyfunctional analysis (D).

 