Microglia are prominent producers of inflammatory cytokines during the hyperacute phase of ischemic stroke

Supplemental Information

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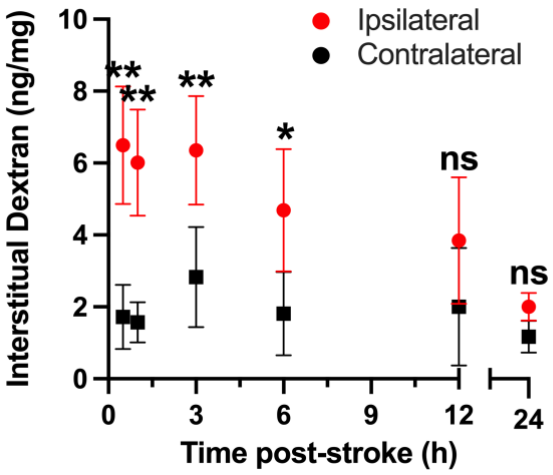
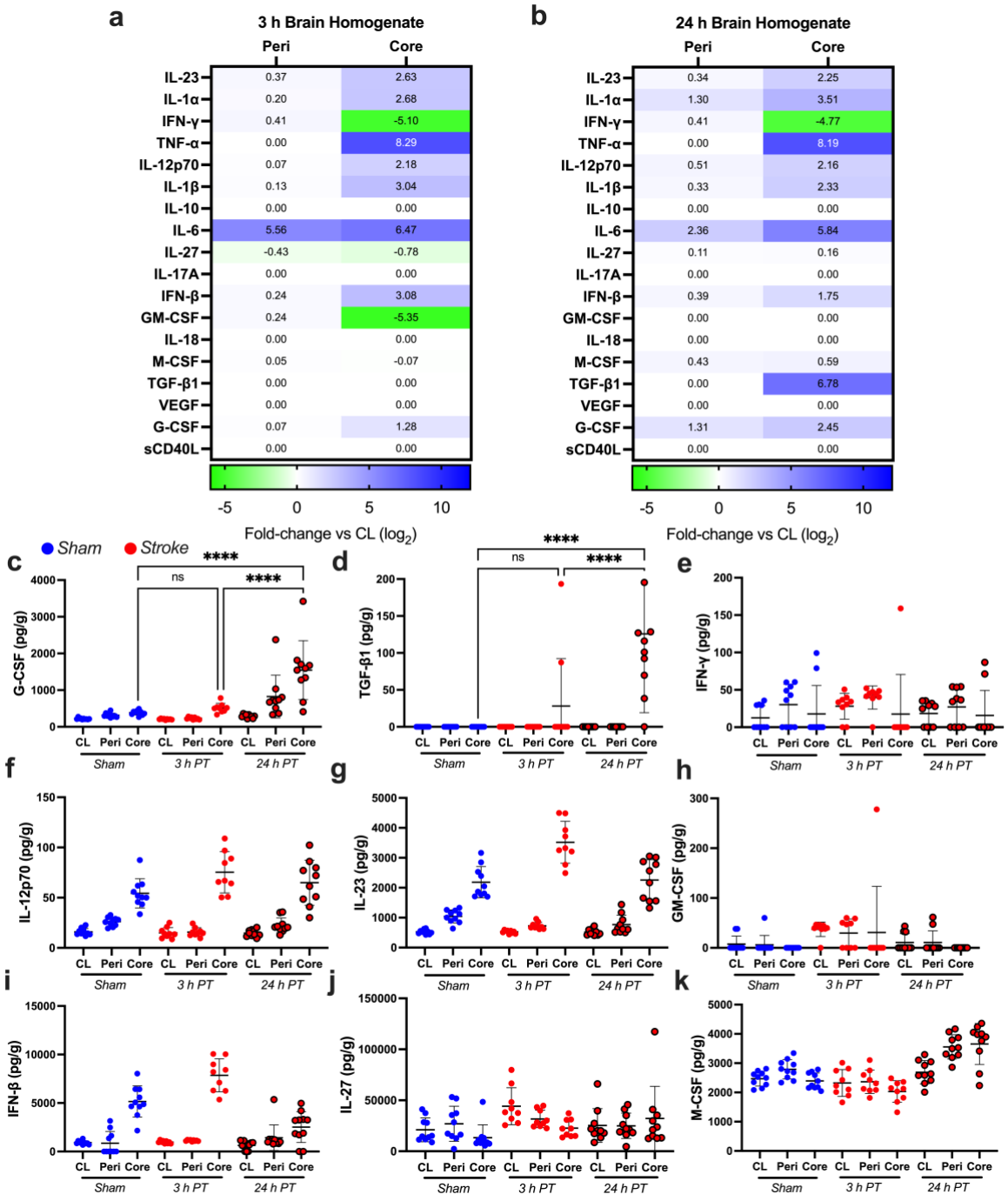
Supplemental Table 1

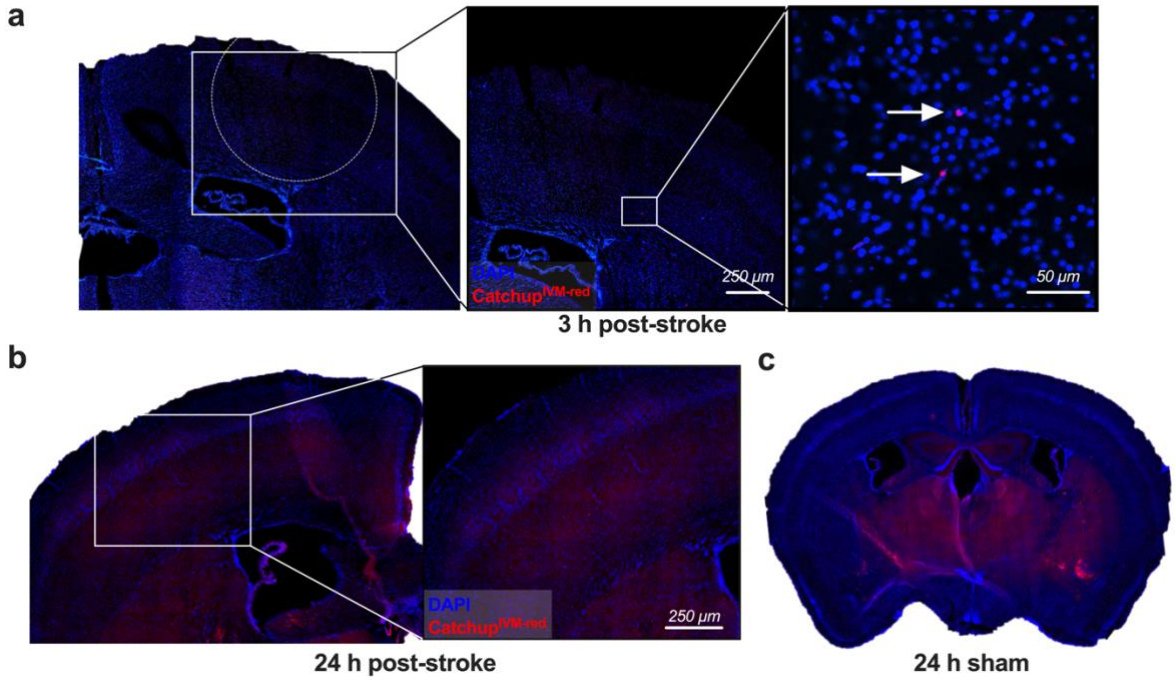
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies (clone)		
Rabbit α-laminin polyclonal	Abcam	ab11575
Rat α-Ly6C/Ly6G (RB6-8C5)	BioXCell	BE0075
Rat IgG2b isotype control (LTF-2)	BioXCell	BE0090
Arginase 1 (A1exF5) cFluor BYG781	Cytek Biosciences	RC-00073
CD11b (M1/70) BUV805	ThermoFisher	368-0112-80
CD140b (APB5) PECy7	ThermoFisher	25-1402-82
CD19 (1D3) BUV496	Cytek Biosciences	364-0193-80
CD206 (MR6F3) Alexa Fluor 700	ThermoFisher	6-2061-82
CD3 Epsilon (145-2C11) PECy5	Cytek Biosciences	55-0031-U025
CD4 (GK1.5) BV711	BD	563050
CD45 (30-F11) BUV395	Cytek Biosciences	363-0451-80
CD49b (HMα2) BV421	BD	740030
CD8 (53-6.7) BV480	Cytek Biosciences	414-0081-80
CD80 (16-10A1) BV510	BioLegend	104741
CD86 (GL-1) eFluor450	ThermoFisher	48-0862-80
CX3CR1 (SA011F11) PE-Fire 700	BioLegend	149051
EGR2 (erongr2) VioB515	Miltenyi Biotec	130-114-365
F4/80 (BM8) APC-Fire 810	BioLegend	123165
GLAST-1 (ACSA-1) APC	Miltenyi Biotec	130-123-641
GPIbβ Dylight 649	Emfret	X649
IL-1α (ALF-161) PE	BioLegend	503203
IL-1β pro-form (NJTEN3) FITC	ThermoFisher	11-7114-82
IL-6 (MP5-20F3) eFluor450	ThermoFisher	48-7061-82
iNOS (CXNFT) BV421	Cytek Biosciences	404-5920-80
Ly-6C (HK1.4) BV570	BioLegend	128030
Ly6G (1A8) BUV563	BD	612921
MHC Class II (M5/114.15.2) Spark Blue 550	BioLegend	107661
NeuN (EPR12763) Alexa Fluor 568	Abcam	ab207282
TNF-α (MP6-XT22) PE Dazzle 594	BioLegend	506345
Chemicals		
BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit	BD	555028
Evans Blue	Merck	E2129
GolgiPlug (containing Brefeldin A)	BD	555029
Rose Bengal	Merck	330000
Thionin acetate salt	Merck	861340
Critical commercial assays		
Adult Brain Dissociation Kit, mouse and rat	Miltenyi Biotec	130-107-677
LEGENDplex™ Mouse Inflammation Panel	BioLegend	740446
LEGENDplex™ Mouse Proinflammatory Chemokine Panel	BioLegend	740007



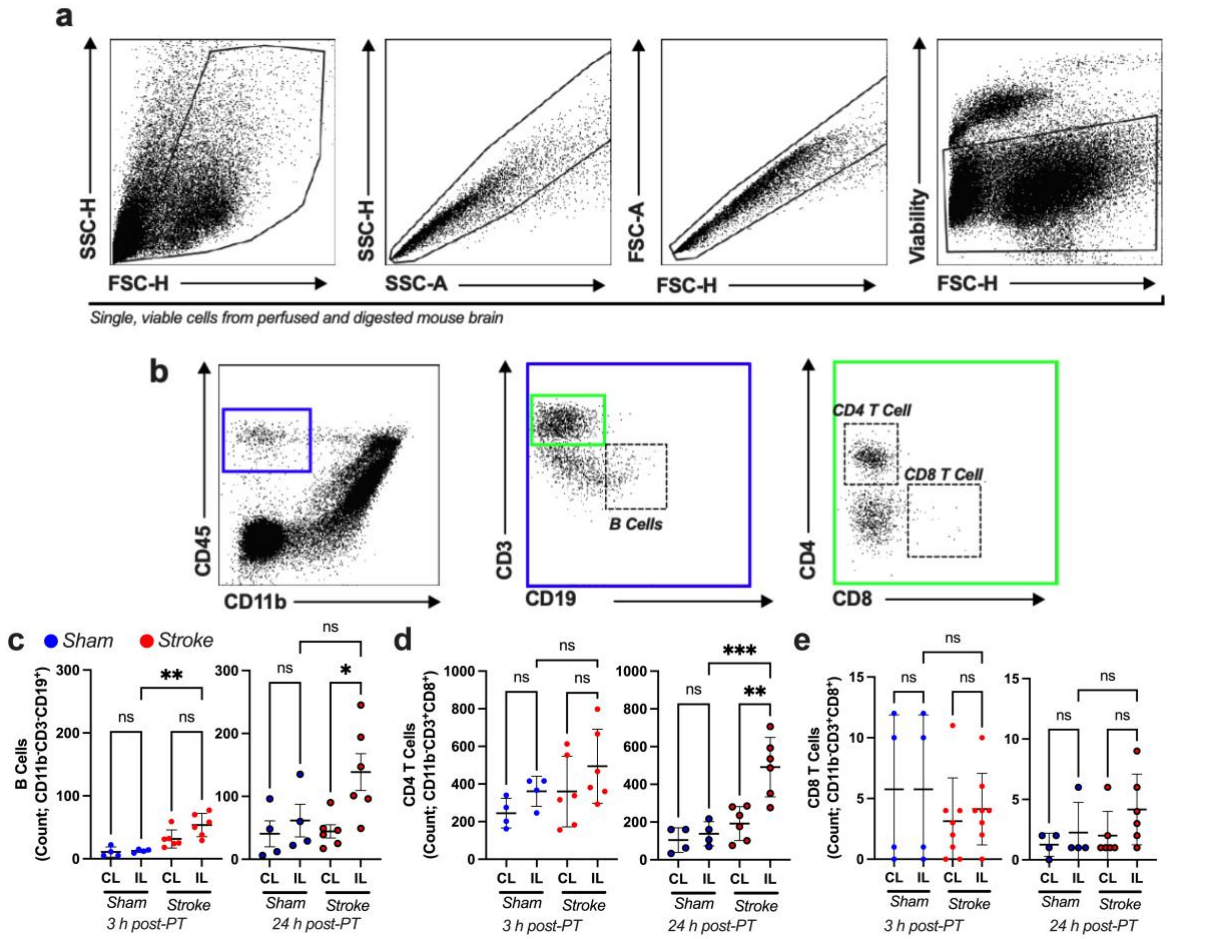
Supplemental Figure 1: ***Blood brain barrier integrity following stroke.*** Mice were subjected to photothrombotic (PT) stroke, and after intravenous injection of 70 kDa dextran (Rhodamine B) at intervals (0.5, 1, 3, 6, 12 and 24 h), brains were perfused and collected. Brain interstitial dextran was quantified by Rhodamine B fluorescence in brain homogenate, using a standard curve (n=5/timepoint). Graphs are presented as mean ± SD. A multiple unpaired t-test was performed for between contralateral and ipsilateral groups at each timepoint. *$p < 0.05$, **$p < 0.01$, ns: no significance



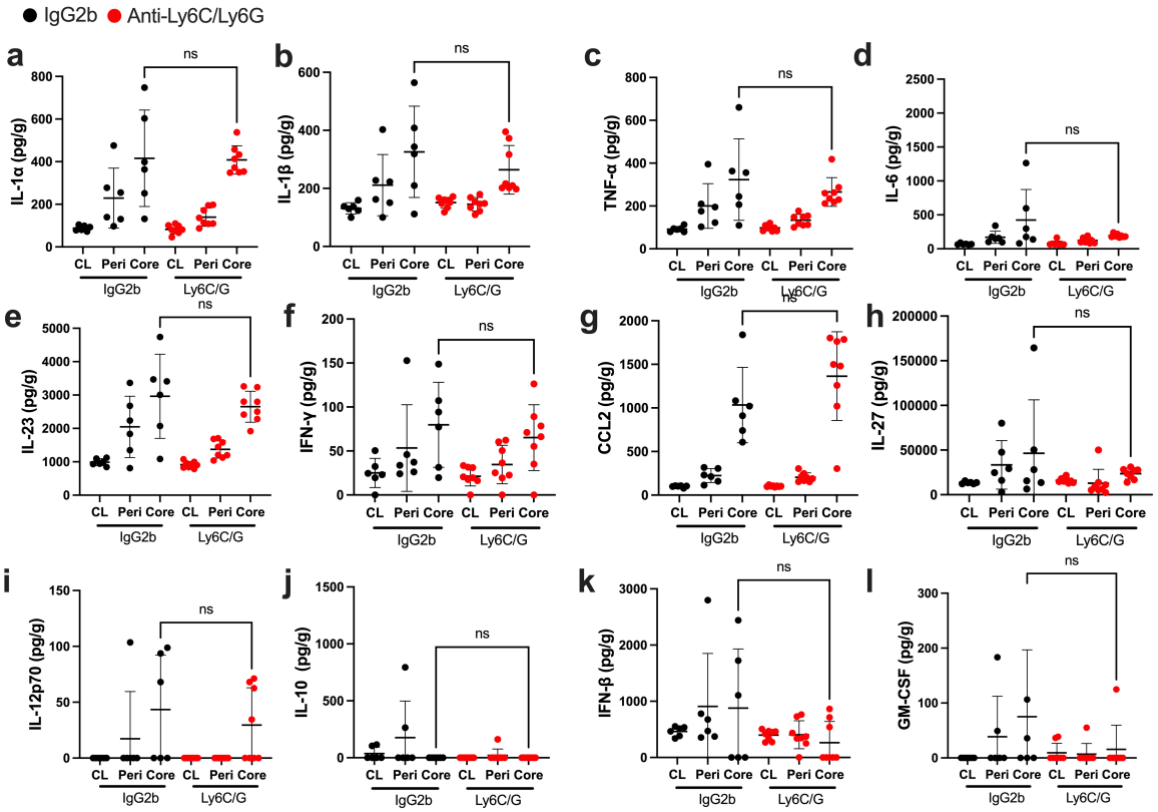
Supplemental Figure 2: ***Cytokine profile of brain homogenate after photothrombotic stroke.*** Mice were subjected to photothrombotic (PT) stroke for 3 or 24 h, and perfused brains were homogenized, and supernatant was analyzed using LEGENDplex panels. Analytes taken from brains at **a.** 3 h and **b.** 24 h post-stroke were assessed, and regions (core-infarct and peri-infarct tissue) were compared to contralateral control, presented as log_2 fold-change. Concentration (pg/g of brain tissue) of **c.** G-CSF, **d.** TGF-β1, **e.** IFN-γ, **f.** IL-12p70, **g.** IL-23, **h.** GM-CSF, **i.** IFN-β, **j.** IL-27 and **k.** M-CSF in regions of the brain, 3 and 24 h post-stroke (sham n=10, 3 h PT n=9, 24 h PT n=10). ****$p < 0.0001$, ns: no significance



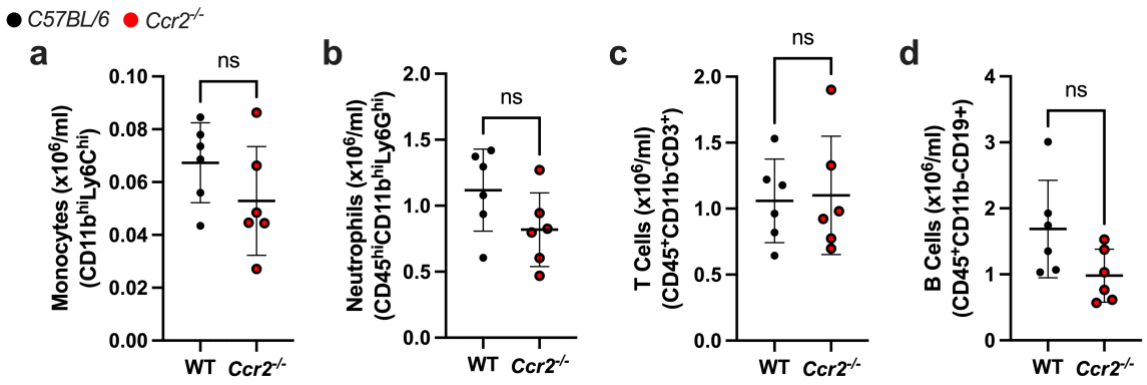
Supplemental Figure 3: ***Neutrophil infiltration into the brain after stroke or sham controls.*** Representative fluorescent image of a 30 µm brain section from perused Catchup$^{IVM\text{-}red}$ mice (neutrophil reporter; red, indicated by white arrows) with DAPI (blue). **a.** Images of the stroke infarct (indicated by white circle) 3 h post-stroke. Images of brains 24 h post-stroke in the **b.** contralateral tissue or **c.** sham control. Images were acquired using a Nikon AXR confocal microscope (a) or an Olympus VS120 slide scanner (b,c). Images are representative of 4 mice.



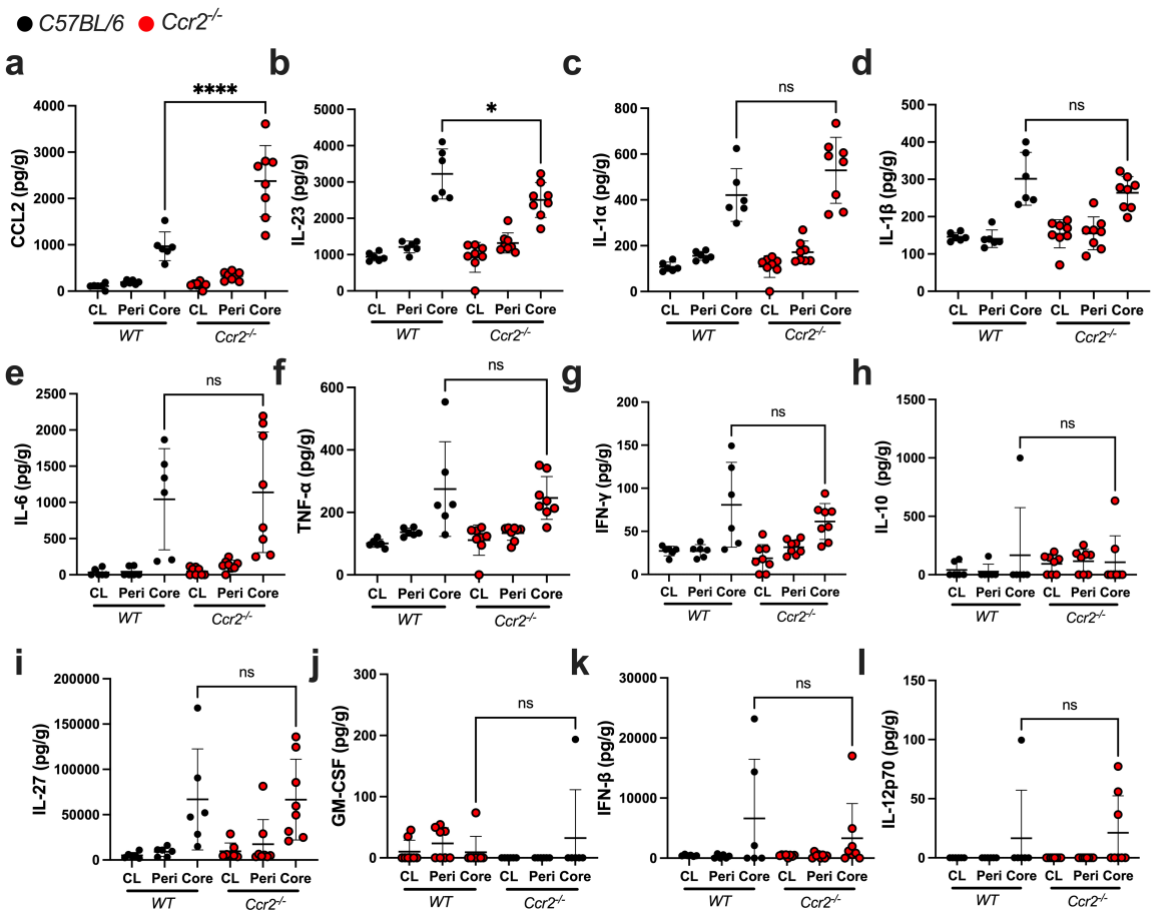
Supplemental Figure 4: ***Flow cytometry gating strategy and brain-lymphocyte assessment post-stroke.*** Mice were subjected to photothrombotic (PT) Stroke for 3 or 24 h, perfused brains were dissociated, and cells were stained for analysis by spectral flow cytometry. **a.** Gating strategy used throughout analysis to identify live (viability-negative) singlet cells. **b.** Gating strategy of lymphocytes ($CD45^{hi}CD11b^{lo}$) to identify B cells, CD4 T cells and CD8 T cells. Total count of **c.** B Cell ($CD45^{hi}CD11b^{lo}CD3^{-}CD19^{+}$), **d.** CD4 T cells ($CD45^{hi}CD11b^{lo}CD3+CD4^{+}$) and **e.** CD8 T cells ($CD45^{hi}CD11b^{lo}CD3^{+}CD8^{+}$) in contralateral (CL) or ipsilateral (IL) regions of the brain. Graphs are presented as mean ± SD. A one-way ANOVA was performed for analysis of multiple groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: no significance



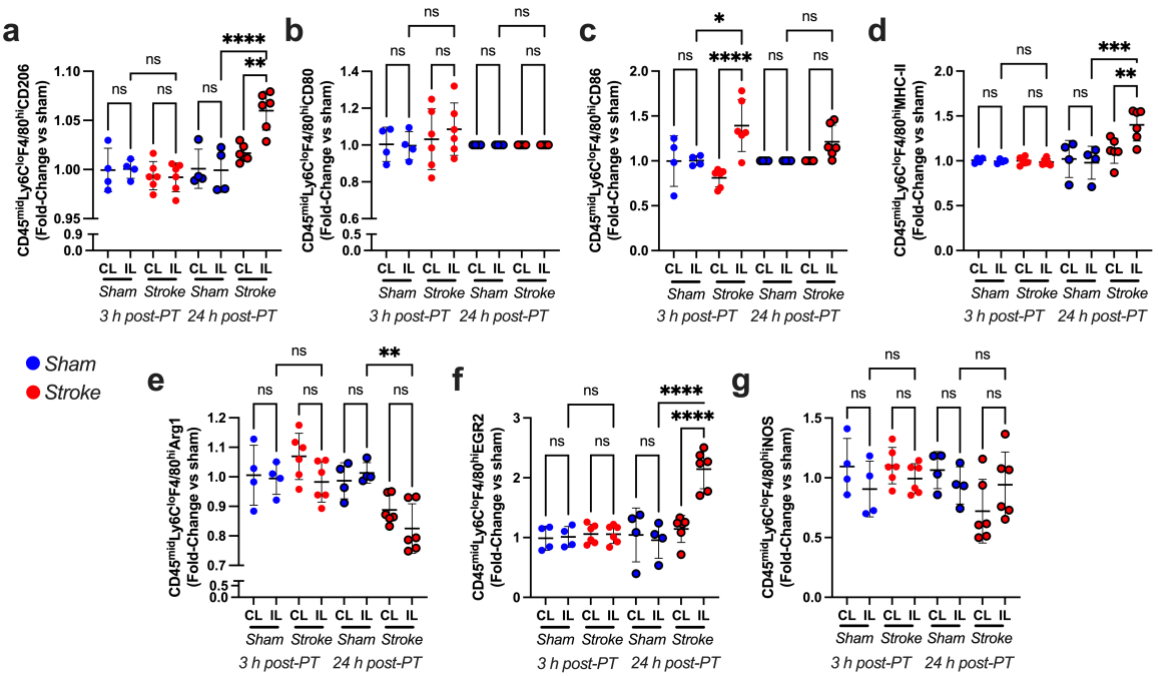
Supplemental Figure 5: ***Myeloid depletion did resolve brain inflammatory cytokine profile*****.** Peripheral myeloid cells were depleted using anti-Ly6C/Ly6G antibody (Ly6C/G; 10 µg/g) by intraperitoneal injection, day -1 and day 0 of photothrombotic (PT) stroke induction, or IgG2b (IgG) control. Perfused brains were homogenized, and supernatant was analyzed using a LEGENDplex cytokine panel. Raw concentration (per g of brain tissue) of **a.** IL-1α, **b.** IL-1β, **c.** TNF-α, **d.** IL-6, **e.** IL-23, **f.** IFN-γ, **g.** CCL2, **h.** IL-27, **i.** IL-12p70, **j.** IL-10, **k.** IFN-β, **l.** GM-CSF in regions of the brain 24 h post-stroke. A one-way ANOVA for analysis of multiple groups, and a Mann-Whitney test was performed when comparing blood analysis. ns: no significance



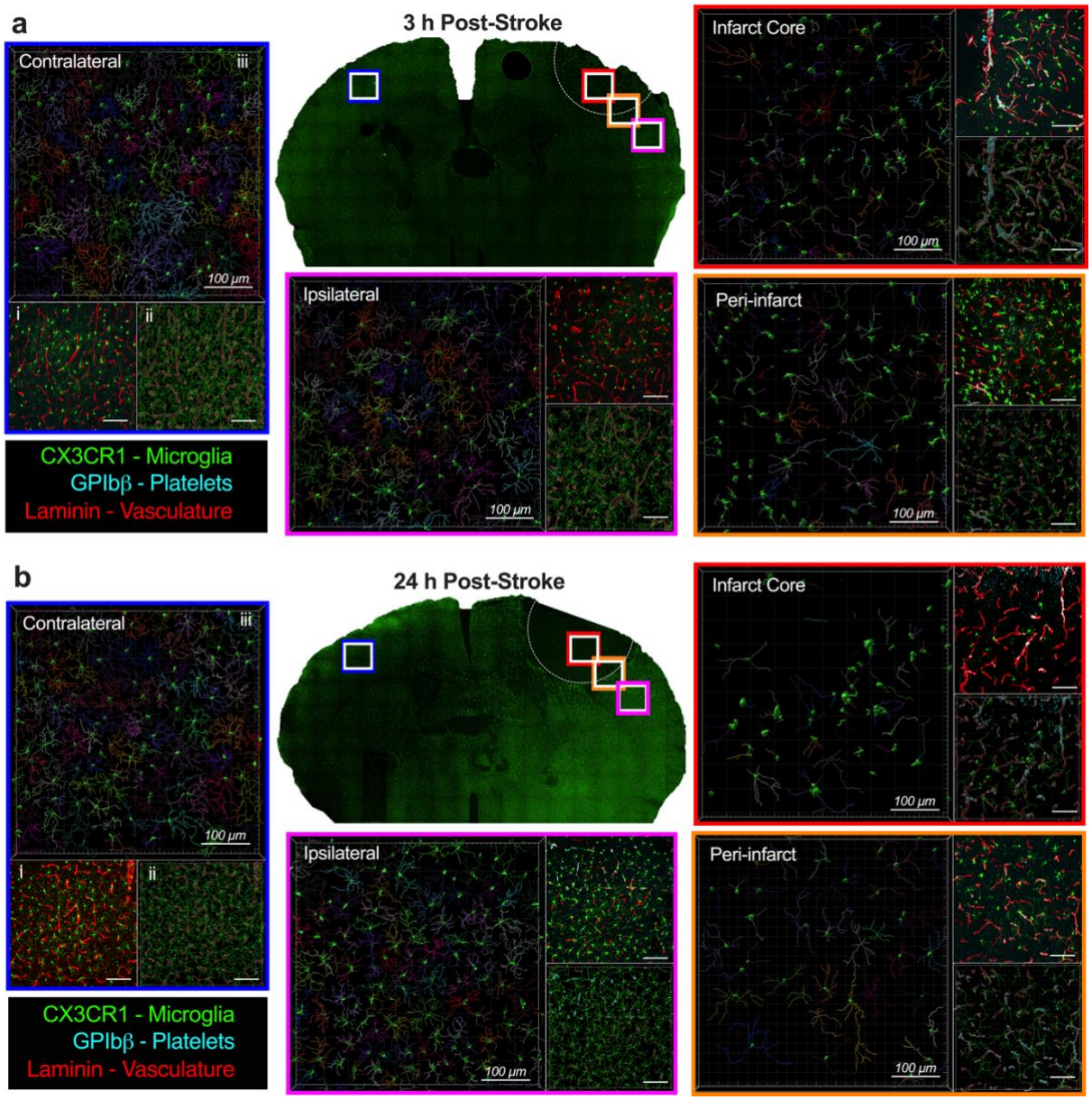
Supplemental Figure 6: ***CCR2 deficiency does not alter peripheral immune populations.*** Circulating blood of C57BL/6 (WT – wild type) or *CCR2$^{rfp/rfp}$* (*Ccr2$^{-/-}$*) mice was assessed for **a.** monocytes (CD45^{hi}CD11b^{hi}Ly6G^{-}Ly6C^{+}), **b.** neutrophils (CD45^{hi}CD11b^{hi}Ly6G^{+}), **c.** T cells (CD45^{hi}CD11b^{lo}CD3^{+}), and **d.** B cells (CD45^{hi}CD11b^{lo}CD3-CD19^{+}) by flow cytometry. Data was analyzed using an unpaired t-test. ns: no significance



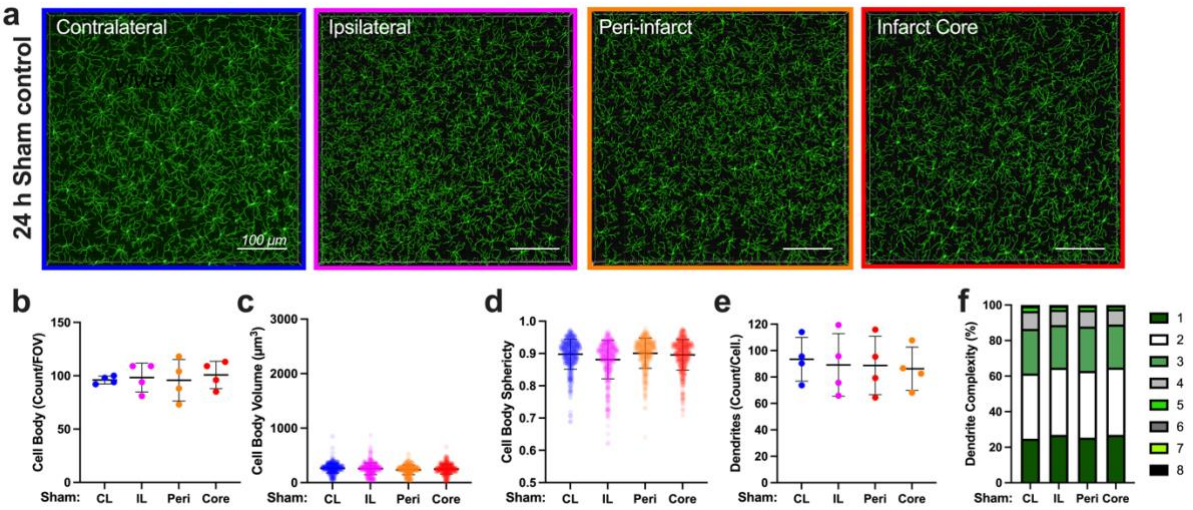
Supplemental Figure 7: ***Blockade of myeloid cell extravasation did not alter the inflammatory profile of the brain.*** C57BL/6 (WT – wild type) or *CCR2$^{rfp/rfp}$* (*Ccr2$^{-/-}$*) mice were subjected to photothrombotic (PT) stroke for 24 h, perfused brains were homogenized, and supernatant was analyzed using a LEGENDplex cytokine panel. Raw concentration (/g of brain tissue) of **a.** CCL2, **b.** IL-23, **c.** IL-1α **d.** IL-1β, **e.** IL-6, **f.** TNF-α, **g.** IFN-γ, **h.** IL-10, **i.** IL-27, **j.** GM-CSF, **k.** IFN-β and **l.** IL12p70 was assessed in the contralateral (CL), ipsilateral (IL) and core regions of the brain. A one-way ANOVA was used for analysis of multiple groups. *$p < 0.05$, ****$p < 0.0001$, ns: no significance



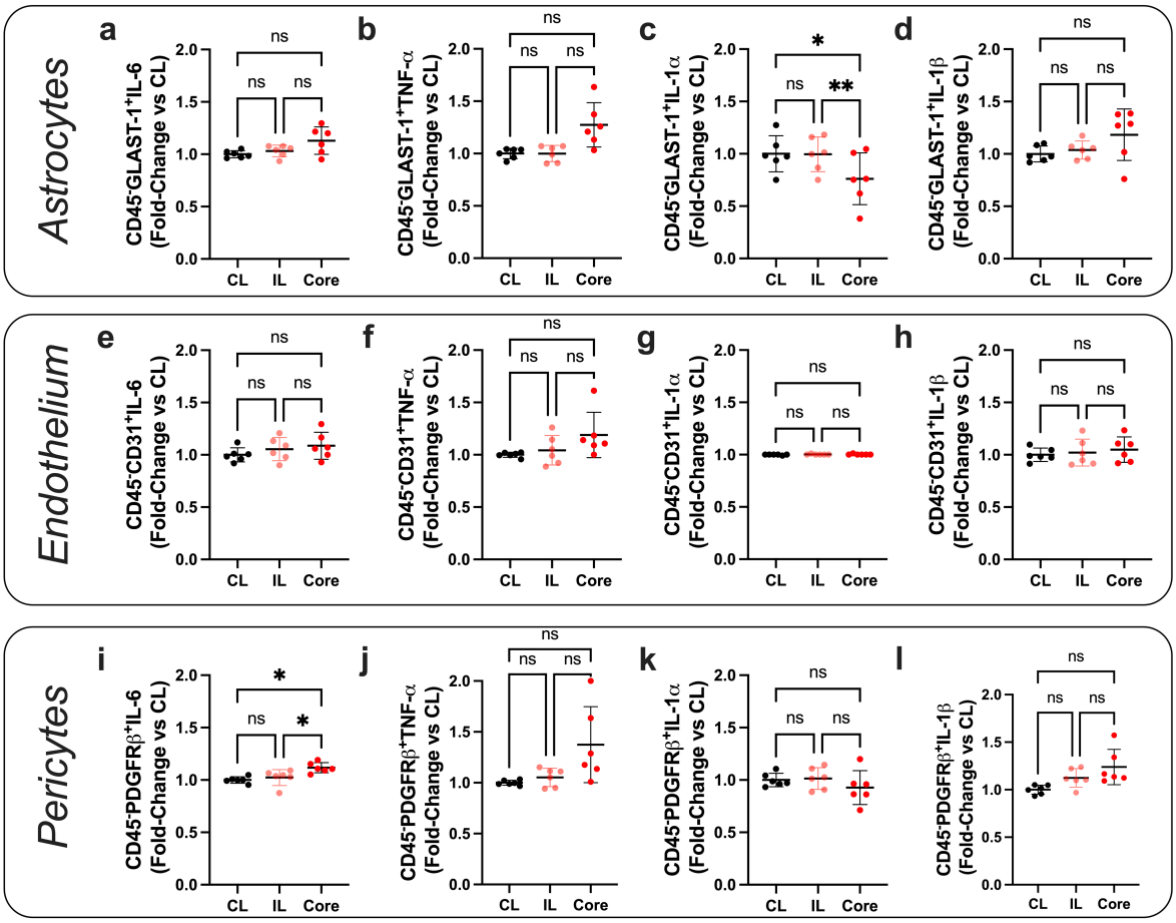
Supplemental Figure 8: ***Microglia display mild changes in identity markers in response to neuroinflammation.*** Mice were subjected to photothrombotic (PT) stroke or sham control for 3 or 24 h, perfused brains were dissociated, and cells were stained for analysis by spectral flow cytometry. Single, viable cells (*also in Figure S2*) were used to identify microglia ($CD45^{mid}CD11b^{+}Ly6G^{-}F4/80^{hi}Ly6C^{-}$). Median fluorescence intensity of **a.** CD206, **b.** CD80 **c.** CD86, **d.** MHC-II, **e.** Arg1, **f.** EGR2 and **g.** iNOS are displayed as fold-change versus sham control of the same region (IL – Ipsilateral, CL – contralateral; sham n=4, PT n=6). A one-way ANOVA was used for analysis of multiple groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: no significance



Supplemental Figure 9: ***Imaging microglia for morphological analysis in response to photothrombotic stroke*****.** *Cx3cr1*$^{gfp/+}$ mice were subjected to a photothrombotic (PT) stroke for **a.** 3 or **b.** 24 h, paraformaldehyde-perfused brains were snap-frozen. 30 µm sections were immunofluorescently stained with anti-GPIbβ (platelets; cyan) and anti-laminin (vasculature; red) to complement the microglia-reporter (green) and Z-stacks (0.5 µm intervals) were imaged using an Olympus FVMPE-RS two-photon microscope. Images were taken at specific regions across the brain, relative to stroke location. (i) Raw Z-compressed images are displayed, (ii) coupled with 3-D rendered images (using IMARIS v10), and then (iii) segmented microglia are displayed, which were used for morphological analysis. (n=5 mice)



Supplemental Figure 10: ***Imaging microglia for morphological analysis in sham control mice*****.** *Cx3cr1*$^{gfp/+}$ mice were subjected to sham surgery and after 24 h, paraformaldehyde-perfused brains were snap-frozen. 30 µm sections were mounted and Z-stacks (0.5 µm intervals) of microglia (green) were imaged using an Olympus FVMPE-RS two-photon microscope. Images were taken at specific regions across the brain, relative to locations acquired from mice with a stroke (Figure 5). **a.** 3-D rendered images (using IMARIS v10) were used for morphological analysis. CX3CR1^{+} microglia were assessed for **b.** count per field of view (FOV), **c.** cell body volume, **d.** cell body sphericity, **e.** dendrites per cell and **f.** dendrite complexity (n=5 mice; individual cells are plotted for volume and sphericity [≈n=400/region]). A repeated measures one-way ANOVA was used for analysis of multiple groups.



Supplemental Figure 11: ***Astrocytes, pericytes and endothelial cells have a limited inflammatory response to stroke.*** Microglia-reporter (*CX3CR1$^{gfp/+}$*) mice were subjected to photothrombotic (PT) stroke for 3 h, perfused brains were dissociated, and brain cells were cultured in the presence of GolgiPlug for 3 h, before staining for analysis by spectral flow cytometry. Astrocytes isolated from contralateral (CL), ipsilateral (IL) and core brain regions were assessed for the expression of **a.** IL-6, **b.** TNF-α, **c.** IL-1α and **d.** IL-1β (n=6). Endothelial cells isolated from CL, IL and core brain regions were assessed for the expression of **e.** IL-6, **f.** TNF-α, **g.** IL-1α and **h.** IL-1β (n=6). Pericytes isolated from CL, IL and core brain regions were assessed for the expression of **i.** IL-6, **j.** TNF-α, **k.** IL-1α and **l.** IL-1β (n=6). Data is presented as fold-change in median fluorescence intensity (MFI) versus the CL tissue. A repeated measures one-way ANOVA was used for analysis of multiple groups. *$p < 0.05$, **$p < 0.01$, ns: no significance