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ORIGINAL RESEARCH

Role of Polymorphonuclear Myeloid-Derived Suppressor Cells and Neutrophils in Ischemic Stroke

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BACKGROUND: Immune cells play a vital role in the pathology of ischemic stroke. Neutrophils and polymorphonuclear myeloid-derived suppressor cells share a similar phenotype and have attracted increasing attention in immune regulation research, yet their dynamics in ischemic stroke remain elusive.

METHODS AND RESULTS: Mice were randomly divided into 2 groups and intraperitoneally treated with anti-Ly6G (lymphocyte antigen 6 complex locus G) monoclonal antibody or saline. Distal middle cerebral artery occlusion and transient middle cerebral artery occlusion were applied to induce experimental stroke, and mice mortality was recorded until 28 days after stroke. Green fluorescent nissl staining was used to measure infarct volume. Cylinder and foot fault tests were used to evaluate neurological deficits. Immunofluorescence staining was conducted to confirm Ly6G neutralization and detect activated neutrophils and $CD11b^{+}Ly6G^{+}$ cells. Fluorescence-activated cell sorting was performed to evaluate polymorphonuclear myeloid-derived suppressor cell accumulation in brains and spleens after stroke. Anti-Ly6G antibody successfully depleted Ly6G expression in mice cortex but did not alter cortical physiological vasculature. Prophylactic anti-Ly6G antibody treatment ameliorated ischemic stroke outcomes in the subacute phase. Moreover, using immunofluorescence staining, we found that anti-Ly6G antibody suppressed activated neutrophil infiltration into parenchyma and decreased neutrophil extracellular trap formation in penumbra after stroke. Additionally, prophylactic anti-Ly6G antibody treatment reduced polymorphonuclear myeloid-derived suppressor cell accumulation in the ischemic hemisphere.

CONCLUSIONS: Our study suggested a protective effect of prophylactic anti-Ly6G antibody administration against ischemic stroke by reducing activated neutrophil infiltration and neutrophil extracellular trap formation in parenchyma and suppressing polymorphonuclear myeloid-derived suppressor cell accumulation in the brain. This study may provide a novel therapeutic approach for ischemic stroke.

Key Words: flow cytometry ■ ischemic stroke ■ myeloid-derived suppressor cells (MDSCs) ■ neutrophils ■ spleen

Stroke induces a cascade of immune responses.[1] After stroke, various immune cells engage rapidly in cross-talk with each other and modulate activation/suppression in response to ischemia.[2,3] Recruitment and infiltration of immune cells have double-sided effects, which may be both neuroprotective and detrimental after stroke.[4,5] Previous studies reported that peripheral immune cell invasion aggravated neuron injury induced by ischemic stroke,[6] but the dynamics of immune cell regulation in ischemic stroke have not been fully elucidated.

Neutrophils are a subpopulation of leukocytes and historically known as the first line of initial immunity. Neutrophils have several defensive mechanisms,[7] and recent studies pay increasing attention to the production of neutrophil extracellular traps (NETs).[8] Neutrophils are the first cells to migrate into the lesion area under

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CLINICAL PERSPECTIVE

What Is New?

- This study discusses the characteristics of neutrophils and polymorphonuclear myeloid-derived suppressor cells in mice ischemic stroke using both distal middle cerebral artery occlusion model and transient middle cerebral artery occlusion model.
- Anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration reduced both brain infiltration and blood circulation of neutrophils and polymorphonuclear myeloid-derived suppressor cells, reduced inflammatory responses, and ameliorated mice stroke outcomes.

What Are the Clinical Implications?

- The present study highlights the application of anti-Ly6G antibody therapy as a novel therapeutic approach for ischemic stroke.
- Our study suggests that polymorphonuclear myeloid-derived suppressor cells should be considered in clinical application of anti-Ly6G antibody therapy for ischemic stroke.

Nonstandard Abbreviations and Acronyms

dMCAO	distal middle cerebral artery occlusion
NETs	neutrophil extracellular traps
PMN-MDSC	polymorphonuclear myeloid-derived suppressor cell
tMCAO	transient middle cerebral artery occlusion

excess stimulation,[9] and high activation of neutrophils leads to DNA fibers release into the extracellular environment thus forming chromatin networks known as NETs.[10] NETs neutralize pathogens and exert a defensive role in immune response;[11] however, recent studies reported that the formation of NETs exacerbated ischemic brain damage,[12,13] and pharmaceutical modulation of NETs induced neuroprotection after stroke.[14] Furthermore, even without NET formation, neutrophils may form mechanical obstruction in brain capillaries that contributes to no-reflow in ischemic stroke, leading to an exacerbation of stroke outcomes.[15]

Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) have been well-studied in cancer immunopathology and immunotherapy.[16,17] Despite their phenotypical similarity to classically activated neutrophils, PMN-MDSCs are pathologically activated and immunosuppressive under natural circumstances.[18] A recent study reported that ischemic stroke induced a modulation of PMN-MDSCs and accelerated their infiltration into melanoma in cancer-bearing mice,[19] but the dynamics of PMN-MDSCs in ischemic stroke remain unclear. Our previous study observed that although no PMN-MDSCs were detected in the normal mouse brain, ischemic stroke induced PMN-MDSCs accumulation in the temporal and spatial regions of mice.[20] The underlying mechanisms of PMN-MDSCs warrant further investigation.

Elucidating the characteristics of immune cells and their roles in immune regulation in response to ischemic stroke may lead to novel therapeutic strategies. Both neutrophils and PMN-MDSCs express the surface marker Ly6G, and could be recognized by anti-Ly6G antibody. Therefore, to clarify whether neutrophils and PMN-MDSCs are involved in ischemic stroke pathophysiology, the current study applied the anti-Ly6G antibody to deplete neutrophils and PMN-MDSCs, and investigated the therapeutic effect of anti-Ly6G antibody administration on cerebral infarction.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

Seven-week-old male C57BL/6 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan) and randomly divided into 2 groups. Mice were raised under standard conditions (lights on: 8:00 AM to 8:00 PM, 23 °C, 40% humidity). All procedures were fully approved by the Ethics Committee for Animal Experiments of Osaka University Graduate School of Medicine (No. 30-048-009). A total of 202 mice were used in this study (pre, n=18; sham, n=6; distal middle cerebral artery occlusion [dMCAO], n=160; transient middle cerebral artery occlusion [tMCAO], n=18). For dMCAO model, 9 mice were excluded because of electrocoagulation-induced hemorrhage. For the tMCAO model, only mice with <30% of baseline control microperfusion during the first minute of occlusion were used in experiments. Six mice were excluded as failed to achieve the microperfusion reduction during the first 1 minute (n=4) or died in the first 24 hours after tMCAO (n=2).

Drug Administration

Anti-mouse Ly6G monoclonal antibody (Clone 1A8; Bio X Cell) was used to deplete neutrophils and PMN-MDSCs by intraperitoneal injection of 10 mg/kg twice at 2 days and then again 1 day before surgery. An intraperitoneal injection of 25 mg/kg was given once 2 days

before surgery only for preliminary flowcytometry experiments. Control mice were given the same amount of saline at the same time.

Distal Middle Cerebral Artery Occlusion

The dMCAO was conducted as previously reported.[21] Briefly, 7-week-old C57BL/6 male mice were rapidly anesthetized with 4% isoflurane and maintained using 1.5% isoflurane via a face mask; a 1-cm incision was made between the right ear and right eye, and then the temporal muscle was detached from the skull to identify the middle cerebral artery under the transparent skull. A high-speed drill (Leutor Mini Pen LP-120, Nihon Seimitsu Kikaku Kosaku Co., Ltd, Hyogo, Japan) was applied to thin out and remove the bone to expose the right middle cerebral artery. The middle cerebral artery was coagulated with electrocoagulation forceps at ≈1 mm distance proximal and distal to the bifurcation. After 30 seconds, when no spontaneous recanalization was observed, the temporal muscle was relocated to cover the hole, and skin wound was sutured. Body temperature was monitored by a rectal probe and maintained at 37.0±0.5 °C during the whole surgery. After the operation, mice recovered in their individual cages normally.

Transient Middle Cerebral Artery Occlusion

The tMCAO was performed as previously described.[22] Briefly, general anesthesia was performed using isoflurane with an open mask, and cortical cerebral blood flow was monitored by a laser-Doppler flowmetry during and until 10 minutes after operation. The right middle cerebral artery was occluded for 30 minutes with a suture followed by 24 hours of reperfusion. After the operation, mice recovered in their individual cages.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from penumbra tissues by mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA). The cDNA was prepared from 1 μg total RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Waltham, MA), then Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) was applied for real-time polymerase chain reaction. Relative mRNA expression was calculated using the comparative CT method using the QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems, Waltham, MA), and 36B4 served as endogenous control. Primer sequences were described as follows: interleukin-6 (IL-6): F: 5′-CCGGAGAGGAGACTTCACAG-3′, R: 5′-TCCACGATTTCCCAGAGAAC-3′. 36B4: F: 5′-TGTGTGTCTGCAGATCGGGT-3′, R: 5′-TGGATCAGCCAGGAAGGCCT-3′.

Cylinder Test and Foot Fault Test

A cylinder test and foot fault test were used to evaluate poststroke neurological deficits. For the cylinder test, mice were placed inside a transparent cylinder with a trifold mirror placed behind to ensure both forelimbs could be seen. Forelimb use for vertical exploration was evaluated by counting left forelimb contacts on the cylinder. Forelimb use was expressed as a ratio of right/left-sided independent forelimb use. For the foot fault test, mice were placed on an elevated gridded platform above the surface and allowed to walk for 5 minutes. A foot fault was counted when the left forefoot misstepped and fell through the space between the grids. The percentage of left foot faults was measured during the whole observation for statistical analysis.

Immunofluorescence Staining

Freshly isolated brains were covered and embedded in sufficient optimal cutting temperature compound on dry ice and then sectioned into 20 μm thick tissue sections. Sections were attached to slides and fixed with 1% paraformaldehyde at room temperature for 10 minutes, then incubated with 0.1% TritonX-100 at room temperature for 10 minutes. After 30 minutes of blocking with 10% donkey serum in PBS, slides were incubated with the following primary antibodies: anti-Ly6G (BD Bioscience, Clone 1A8; 1:500), 594-conjugated anti-lycopersicon esculentum lectin (LEL; Vector Laboratories; 1:1000), NeuroTrace 500/525 green fluorescent nissl stain (Thermo Fisher Scientific; 1:100), anti-myeloperoxidase (Abcam; 1:50), FITC-conjugated anti-rat CD11b (BD Bioscience, clone M1/70; 1:250), anti-histone H3 (citrulline R2+R8+R17) (Abcam; 1:250), anti-Iba-1 (ionized calcium binding adater molecule-1) (Wako; 1:250). Alexa Fluor 488-labeled donkey anti-mouse immunoglobulin G (Invitrogen), 647-labeled donkey anti-mouse immunoglobulin G (Invitrogen) and 647-labeled donkey anti-rabbit immunoglobulin G (Invitrogen) were used as secondary antibodies. Sections were counterstained with DAPI (4', 6-diamid ino-2-phenylindole) (Vector Laboratories), and a confocal laser-scanning microscope (Olympus FV3000) was used for visualization to avoid interference between channels and saturation.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay was performed on day 3 after dMCAO using NeuroTACS In Situ Apoptosis Detection Kit (Trevigen) according to the manufacturer's instruction but with a replacement of Strep-HRP/DAB visualization by a streptavidin Alexa Fluor 594 conjugate (Invitrogen). The number of NeuN/TUNEL double positive cells were counted for neuronal apoptosis analysis.

Infarct Volume Measurement

For brain infarct volume measurement, mouse brains were sectioned into 20-μm thick tissue sections every 400-μm interval. The infarct area was marked as a loss of nissl positive neurons. The infarct volume was calculated as: infarct volume=contralateral hemisphere volume−nonischemic ipsilateral hemisphere volume.

Immunofluorescence Staining Quantification

Quantification of immunofluorescence staining was conducted on 6 to 8 images which were obtained from 3 independent slides using 20× objective. For analysis of the cortex vasculature, an ImageJ macro was used to automatically process and analyze vascular numbers and vascular lengths within the image.[23] The

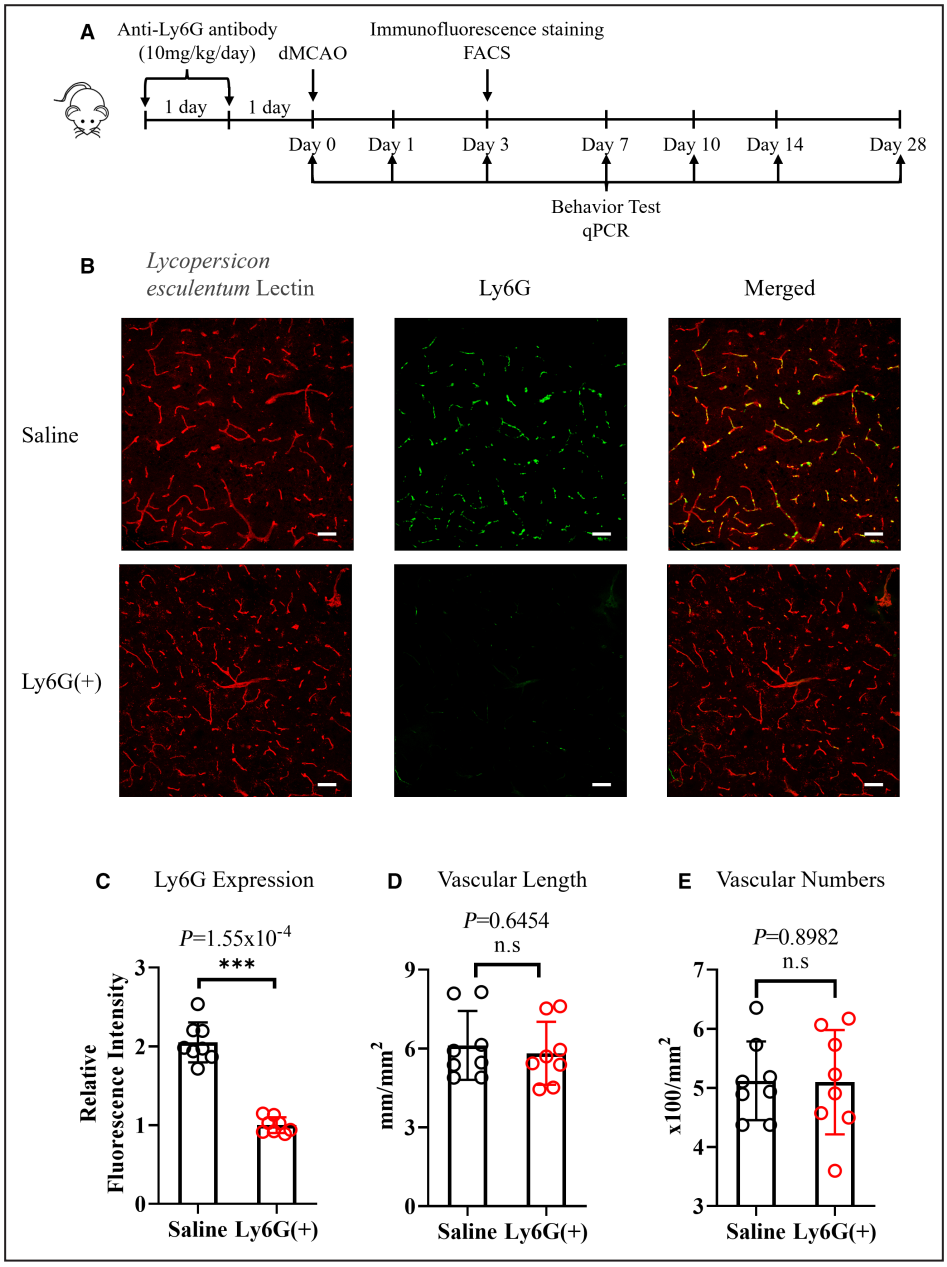

Figure 1. Ly6G (lymphocyte antigen 6 complex locus G) depletion did not alter the physiological vasculature of brain cortex in mice.

A, Schematic diagram of experimental procedures. Representative images (**B**) and quantification (**C**) of Ly6G immunofluorescence staining in brain cortex. n=8. **D**, Vascular length and (**E**) vascular numbers in brain cortex. Scale bar=50 μm. dMCAO indicates distal middle cerebral artery occlusion; FACS, fluorescence-activated cell sorting; Ly6G, lymphocyte antigen 6 complex locus G; and qPCR, quantitative polymerase chain reaction.

script for the macro is as follows: run("Clear Outside"); run("Make Binary"); run("Fill Holes"); run("Despeckle"); run("Skeletonize"); run("Analyze Skeleton (2D/3D)", "prune=none show"). For activated neutrophil quantification, only myeloperoxidase and CD11b double positive signal with nucleus counterstained by DAPI was defined as an activated neutrophil. Activated neutrophils are counted and calculated as cell numbers/area (mm^2). For activated microglia/macrophages quantification, Iba-1 positive microglia/macrophages were classified into 4 subgroups as ramified, intermediate, amoeboid, and round cells, according to a previously reported morphological grading scale.[24,25] Intermediate, amoeboid, and round microglial cells were counted and calculated as activated microglia/macrophages cell numbers/area (mm^2).

Brain Immune Cell Isolation

Brain immune cell isolation was performed as previously reported. Mice were deeply anesthetized using CO_2, then perfused with 20 mL of ice-cold HBSS (Wako). Brains were dissected into left (contralateral) and right (stroke) hemispheres. Each hemisphere was dissociated mechanically in RPMI 1640 (Wako) on ice. Immune cells were collected using the Percoll density gradient method. Finally, the collected cells were resuspended in 200 µL of stain buffer (fetal bovine serum) (BD Pharmingen) and used immediately for the fluorescence-activated cell sorting.

Blood and Spleen Cell Isolation

Whole blood was lysed using lysing buffer (BD Biosciences) to prepare a single-cell suspensions, then

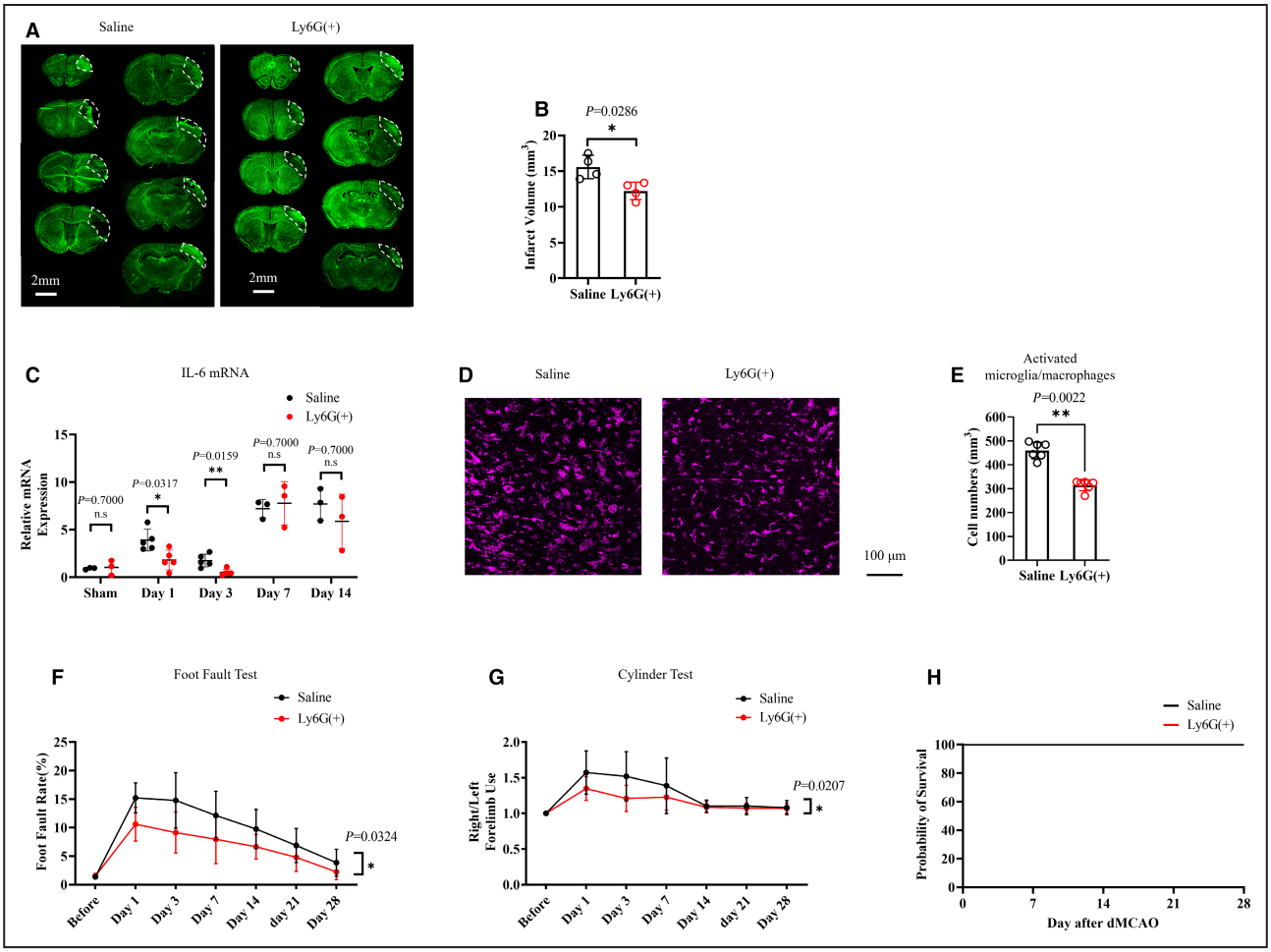

Figure 2. Prophylactic anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration ameliorated ischemic stroke outcomes in mice.

ARepresentative images (**A**) and quantification (**B**) of brain infarct volume on day 3 after stroke using green fluorescent nissl staining (n=4). **C**, Quantitative real-time polymerase chain reaction of interleukin-6 mRNA in mice penumbra before and 1, 3, 7, and 14 days after distal middle cerebral artery occlusion (n=3 for sham, day 7, and day 14; n=5 for day 1 and day 3). Representative immunofluorescence staining images (**D**) and quantification (**E**) of activated microglia/macrophage numbers in peri-infarct area on day 3 after stroke. n=6. Foot fault (**F**) and cylinder tests (**G**) of mice before and 1, 3, 7, 14, 21, and 28 days after distal middle cerebral artery occlusion. n=10. **H**, Kaplan–Meier curve of mouse survival after distal middle cerebral artery occlusion (n=10). dMCAO indicates distal middle cerebral artery occlusion; Il-6, intereleukin-6; and Ly6G, lymphocyte antigen 6 complex locus G.

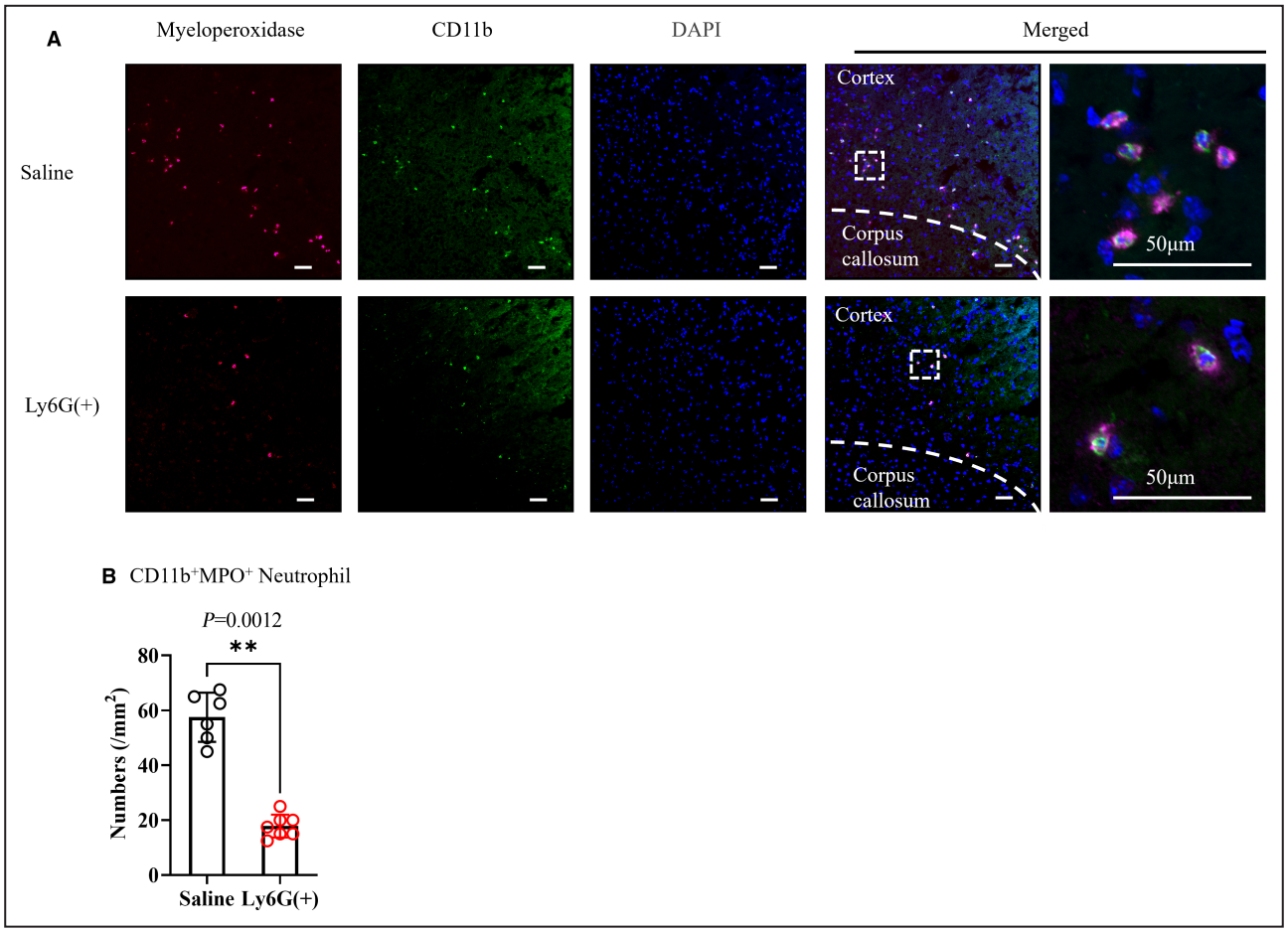

Figure 3. Prophylactic anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration suppressed activated neutrophil infiltration into brain parenchyma after stroke.
Representative images (**A**) and quantification (**B**) of CD11b$^+$ myeloperoxidase$^+$ neutrophils on day 3 after stroke (n=6). Scale bar=50 μm. Ly6G indicates lymphocyte antigen 6 complex locus G; DAPI, 4', 6-diamidino-2-phenylindole; and MPO, myeloperoxidase.

washed, resuspended in stain buffer (fetal bovine serum) at a concentration of 1×10^6 cells/mL. Spleens were mechanically dissociated and passed through 40-μm nylon cell strainers (BD Biosciences) to obtain a single-cell suspension, then lysed with red blood cell lysis buffer (BD Biosciences), washed, and resuspended in isolation buffer at a concentration of 1×10^7 cells/mL.

Fluorescence-Activated Cell Sorting

The cells were stained with allophycocyanin (APC)-Cy7-conjugated anti-CD45 (BD Pharmingen), phycoerythrin (PE)-conjugated anti-CD11b (BD Pharmingen), APC-conjugated anti-Ly6G (BD Pharmingen), BV421-conjugated anti-lymphocyte antigen 6 complex locus C (Ly6C) (BD Horizon), FITC Rat Anti-Mouse CD19 (Biolegend), BV421 Rat Anti-Mouse CD3 (BD Pharmingen), PE Rat Anti-Mouse CD4 (Biolegend) and APC Rat Anti-Mouse CD8a (BD Pharmingen). Dead cells and debris were gated out using forward light scatter, side light scatter, and 7-aminoactinomycin D (BD Biosciences, San Jose, CA). The fluorescence-activated cell sorting analysis was performed on a FACS Canto II flow cytometer and analyzed using FlowJo software (BD Biosciences).

Statistical Analysis

Comparisons between 2 groups were performed using Mann–Whitney test. Multiple group comparisons were performed using Kruskal–Wallis test with post hoc Dunn test for pairwise comparisons. Cylinder and foot fault test measurements were compared using 2-way repeated measurement ANOVA. Survival curves were determined with the Kaplan–Meier method and compared with the log-rank test. All data are presented as means± SD.

RESULTS

Ly6G Depletion Did Not Alter the Physiological Vasculature of Brain Cortex

To deplete neutrophils and PMN-MDSCs, C57BL/6 wild-type mice were randomly divided into 2 groups and

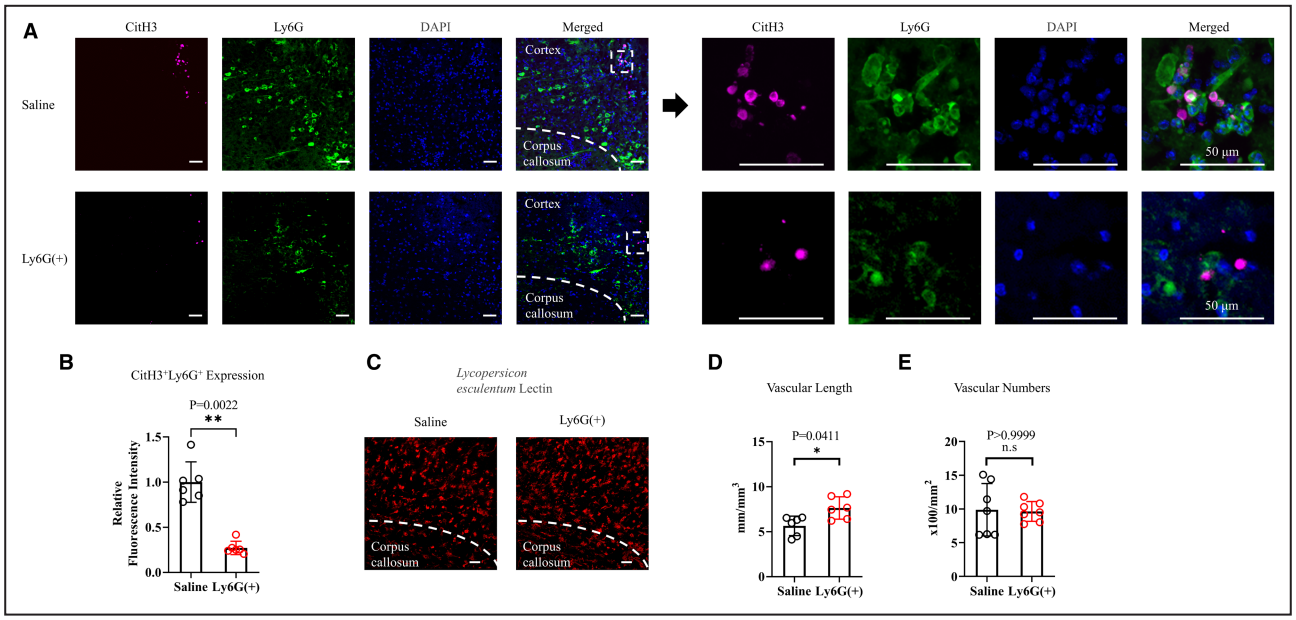

Figure 4. Prophylactic anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration decreased neutrophil extracellular trap formation in ischemic brain parenchyma.
Representative images (**A**) and (quantification (**B**) of citrullinated histone-3 expression and Ly6G expression on day 3 after stroke (n=6). Representative images (**C**) and quantification (**D**) of vascular length and vascular numbers (**E**) on day 3 after stroke (n=6). Scale bar=50 μm. CitH3 indicates citrullinated histone-3; DAPI, 4', 6-diamidino-2-phenylindole; and Ly6G, lymphocyte antigen 6 complex locus G.

intraperitoneally administrated twice with anti-Ly6G antibody (10 mg/kg per day) or saline before being subjected to experimental stroke (Figure 1A). LEL and Ly6G immunofluorescence costaining was applied to mice cortex tissues to confirm Ly6G depletion and analyze physiological vasculature (Figure 1B). We found that mice treated with anti-Ly6G antibody displayed significantly decreased Ly6G expression (Figure 1C). LEL staining was used to analyze the physiological microvascular structure in the cortex, and no significant differences were found in vascular length (Figure 1D) and vascular numbers (Figure 1E) between anti-Ly6G antibody-treated group and saline-treated group. These findings suggest that anti-Ly6G antibody successfully depleted Ly6G$^+$ cells, referring to neutrophils and PMN-MDSCs, without differing microvasculature of brain cortex under nonischemic condition.

Prophylactic Anti-Ly6G Antibody Treatment Ameliorates Ischemic Stroke Outcomes in Mice

To evaluate the effects of anti-Ly6G antibody on ischemic stroke outcomes, green fluorescent nissl staining was applied to mouse brain sections from day 3 after stroke (Figure 2A). The infarct area was indicated by a marked loss of nissl$^+$ neurons in cortex. We found that anti-Ly6G antibody-treated mice showed a decreased infarct volume in comparison with saline-treated mice (P=0.0286; Figure 2B). Double immunofluorescence staining of NeuN and TUNEL was also conducted to evaluate neuronal apoptosis (Figure S1A). We found that anti-Ly6G antibody-treated mice presented with significantly less NeuN$^+$TUNEL$^+$ cells in the peri-infarct area than the saline-treated mice (Figure S1B). To evaluate the effects of anti-Ly6G antibody on inflammatory responses in ischemic stroke, we applied quantitative real-time polymerase chain reaction to measure the IL-6 level in mice penumbra (Figure 2C). Consistent with previous studies,[26] IL-6 mRNA showed 2 peaks of elevation on day 1 and day 7 after stroke, respectively. Interestingly, significant differences were observed only in the acute phase (day 1 and day 3) but not in the chronic phase (day 7 and day 14), suggesting an anti-inflammatory effect of anti-Ly6G antibody treatment by inhibiting IL-6 mRNA elevation in acute ischemic stroke phase. As IL-6 expression is involved in microglia activation,[27,28] subsequently, we evaluated the activation of Iba-1 positive microglia/macrophages in peri-infarct area according to the morphological grading scale (Figure 2D). We found that activated microglia/macrophages cell numbers were significantly decreased in anti-Ly6G antibody-treated mice on day 3 after stroke (Figure 2E). To further measure the effects of anti-Ly6G antibody on long-term neurological deficits, a foot fault test (P=0.0324; Figure 2F) and cylinder test (P=0.0207; Figure 2G) were performed up to 28 days poststroke. No statistical differences were found between anti-Ly6G antibody- and saline-treated groups before experimental stroke. After stroke, anti-Ly6G antibody-treated mice showed significantly ameliorated neurological deficits in comparison with saline-treated

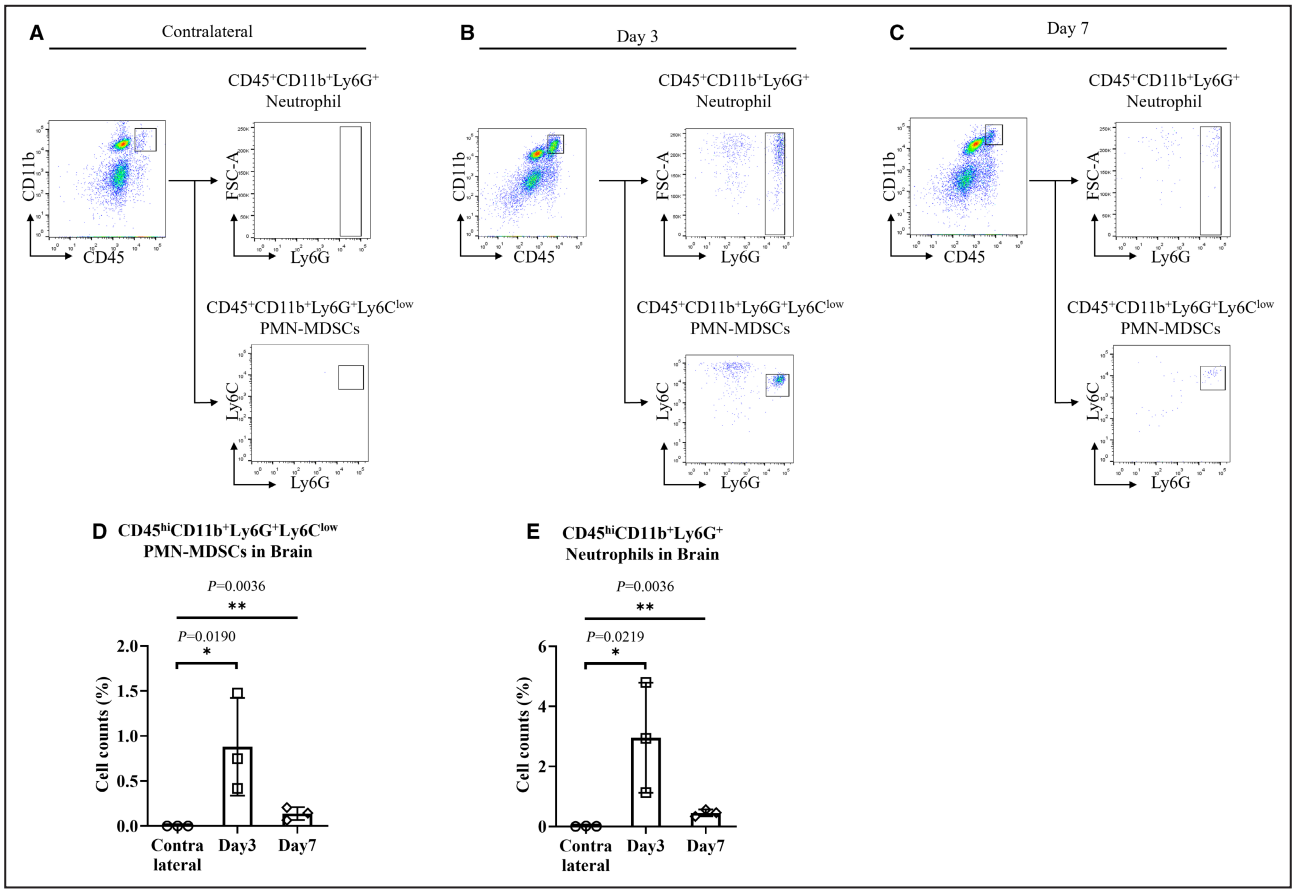

Figure 5. Gating strategy to identify CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low} polymorphonuclear myeloid derived suppressor cells and CD45^{hi}CD11b^{+}Ly6G^{+} neutrophils in brain.

A, Polymorphonuclear myeloid-derived suppressor cells and neutrophils were absent in the contralateral hemisphere on day 3 after distal middle cerebral artery occlusion. **B** and **C**, Polymorphonuclear myeloid-derived suppressor cells and neutrophils accumulated in the ischemic stroke hemisphere on day 3 (**B**) and day 7 (**C**) after distal middle cerebral artery occlusion. **D** and **E**, Quantification of polymorphonuclear myeloid-derived suppressor cells (**D**) and neutrophils (**E**) in the ischemic stroke hemisphere (n=3). FSC indicates forward scatter; Ly6C, lymphocyte antigen 6 complex locus C; Ly6G, lymphocyte antigen 6 complex locus G; and PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells.

mice up to day 7 poststroke. No mice died up to day 28 after dMCAO (Figure 2H). These findings indicate that prophylactic anti-Ly6G antibody treatment ameliorates ischemic stroke outcomes up to day 7 poststroke in mice.

Prophylactic Anti-Ly6G Antibody Treatment Suppressed Activated Neutrophil Infiltration into Parenchyma After Stroke

Activated neutrophils exert irreplaceable roles and are recruited rapidly to the impaired area in natural immune response. To clarify the protective effects of anti-Ly6G antibody, myeloperoxidase, as well as CD11b immunofluorescence costaining were used to detect activated neutrophil (Figure 3A). On day 3 after stroke, no myeloperoxidase^{+}CD11b^{+} signal was detected in the contralateral hemisphere parenchyma, implying no activated neutrophil infiltration in the contralateral hemisphere. Myeloperoxidase^{+}CD11b^{+} neutrophils accumulated around penumbra area of the ipsilateral hemisphere, in saline-treated mice, while anti-Ly6G antibody-treated mice displayed a decreased neutrophil accumulation around the penumbra area (Figure 3B), suggesting prophylactic anti-Ly6G antibody treatment suppressed infiltration of activated neutrophils in penumbra area after stroke.

Prophylactic Anti-Ly6G Antibody Treatment Decreased NET Formation in Ischemic Parenchyma

Strong activation of neutrophils leads to NET formation, indicating a detrimental aspect of neutrophil function.[29] Citrullination of histones has been demonstrated to be indispensable for NETs formation. Therefore, we applied citrullinated histone-3 as a marker of NETs formation in this study. Mouse brain sections of day 3 after stroke were costained with

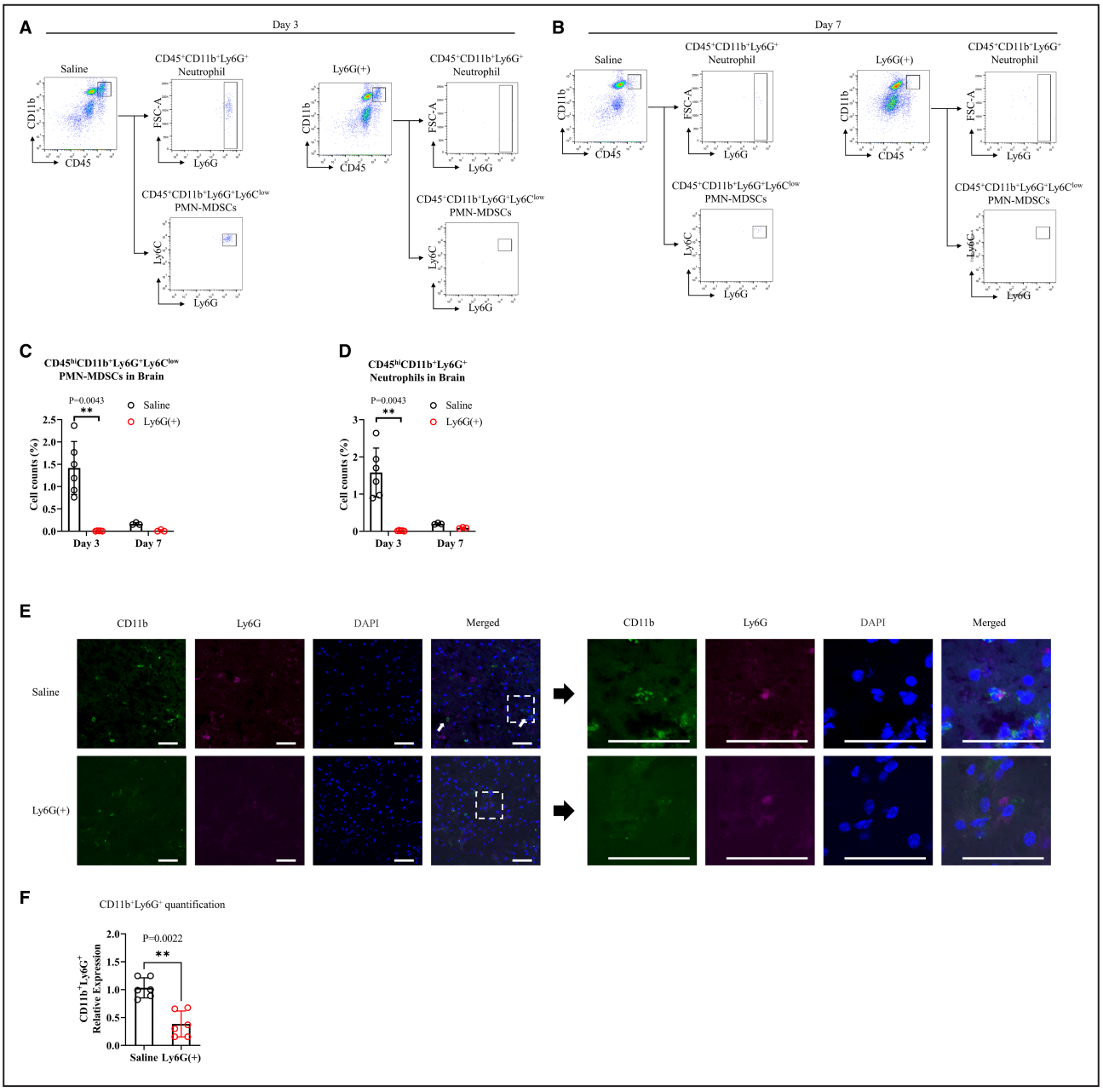

Figure 6. Prophylactic anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration decreased the accumulation of CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low} polymorphonuclear myeloid-derived suppressor cells and CD45^{hi}CD11b^{+}Ly6G^{+} neutrophils in the ischemic hemisphere.

A and **B**, Proportion of polymorphonuclear myeloid-derived suppressor cells and neutrophils in the ischemic hemisphere of anti-Ly6G antibody- and saline-treated mice on day 3 (**A**) and day 7 (**B**) after distal middle cerebral artery occlusion. **C** and **D**, Quantification of polymorphonuclear myeloid-derived suppressor cells (**C**) and neutrophils (**D**) in the ischemic stroke hemisphere of anti-Ly6G antibody- and saline-treated mice (n=5–6 for day 3, n=3 for day 7). **E** and **F**, Representative images (**E**) and quantification (**F**) of CD11b and Ly6G immunofluorescence costaining in peri-infarct areas of anti-Ly6G antibody- and saline-treated mice (n=6). Scale bar=50 μm. FSC indicates forward scatter; Ly6G, lymphocyte antigen 6 complex locus G; and PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells.

citrullinated histone-3, Ly6G, and DAPI (Figure 4A). We found that citrullinated histone-3^{+}Ly6G^{+} signal was significantly suppressed in penumbra of anti-Ly6G antibody-treated mice (Figure 4B), indicating a decreased NET formation by prophylactic anti-Ly6G antibody administration. Next, we applied LEL immunostaining to detect cortex vasculature under ischemic condition. LEL-marked vascular bed was significantly suppressed after stroke in both Ly6G antibody and saline groups (Figure 4C); however, penumbra of

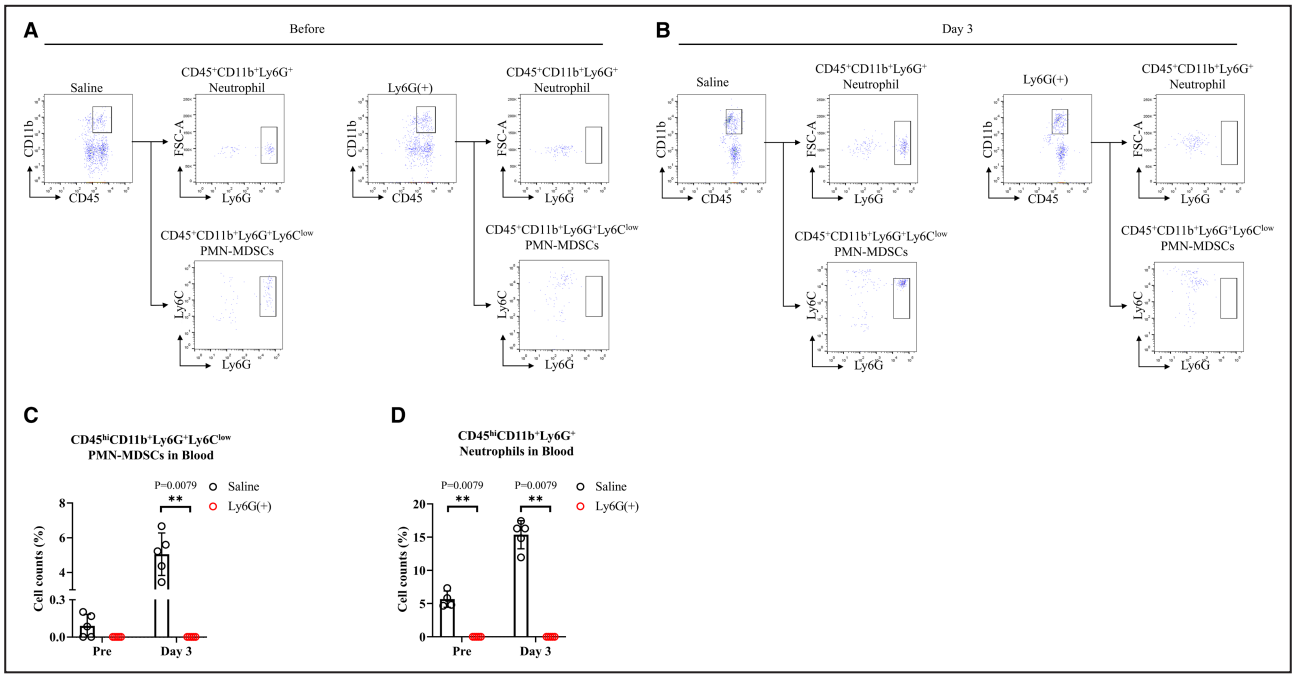

Figure 7. Proportion of polymorphonuclear myeloid-derived suppressor cells and neutrophils in blood.
A and **B**, Gating strategy to identify $CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low}$ polymorphonuclear myeloid-derived suppressor cells and $CD45^{hi}CD11b^{+}Ly6G^{+}$ neutrophils in blood of anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody- and saline-treated mice before (**A**) and day 3 after (**B**) distal middle cerebral artery occlusion. **C** and **D**, Quantification of polymorphonuclear myeloid-derived suppressor cells (**C**) and neutrophils (**D**) in blood of anti-Ly6G antibody- and saline-treated mice (n=4–5). FSC indicates forward scatter; Ly6G, lymphocyte antigen 6 complex locus G; and PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells.

anti-Ly6G antibody-treated mice displayed an attenuated destruction of vascular length (Figure 4D) but no statistical change in vascular numbers (Figure 4E) in comparison with saline-treated mice. A previous study reported that neutrophil obstruction in brain capillaries contributed to low reperfusion after ischemic stroke,[15] which could explain the attenuated suppression of vascular bed induced by Ly6G antibody administration. Our findings suggest that prophylactic Ly6G antibody administration protected ischemic stroke by decreasing NET formation and activated neutrophils numbers, which contributes to neutrophil obstruction in brain capillaries poststroke.

Prophylactic Anti-Ly6G Antibody Treatment Reduced $CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low}$ PMN-MDSC and $CD45^{hi}CD11b^{+}Ly6G^{+}$ Neutrophil Accumulation

Our previous study demonstrated that ischemic stroke raised PMN-MDSCs in brain. To investigate PMN-MDSC regulation in ischemic stroke up to subacute phase, we applied flow cytometry to analyze mice contralateral (Figure 5A) and ipsilateral hemispheres on day 3 (Figure 5B) and day 7 (Figure 5C) after dMCAO. There were little PMN-MDSCs and neutrophils in the contralateral hemisphere, and ischemic stroke caused an increase of PMN-MDSCs and neutrophils on day 3 after stroke, which slightly decreased on day 7 after stroke (Figure 5D and 5E). Next, we applied the same gated strategy to analyze the infiltration of PMN-MDSCs and neutrophils into brains from anti-Ly6G antibody- and saline-treated mice. Once intraperitoneal injection of anti-Ly6G antibody at 25 mg/kg per day on 2 days before surgery was adopted as positive control[30] for flow cytometry analysis in the preliminary stage (Figure S2). On day 3 (Figure 6A) and day 7 (Figure 6B) after stroke, anti-Ly6G antibody-treated mice showed a significantly decreased proportion of PMN-MDSCs (Figure 6C) and neutrophils (Figure 6D) compared with saline-treated mice. We also performed CD11b and Ly6G immunofluorescence costaining in mouse brains on day 3 after stroke. $CD11b^{+}Ly6G^{+}$ cells appeared in small amount in ischemic parenchyma, while prophylactic anti-Ly6G treatment significantly diminished the $CD11b^{+}Ly6G^{+}$ cells signal (Figure 6E and 6F). Subsequently, we evaluated the circulating PMN-MDSCs and neutrophils in blood before (Figure 7A) and after stroke (Figure 7B). Consistently, circulating PMN-MDSCs (Figure 7C) and neutrophils (Figure 7D) in blood increased on day 3 after stroke, while anti-Ly6G treatment significantly depleted both the circulating PMN-MDSCs and

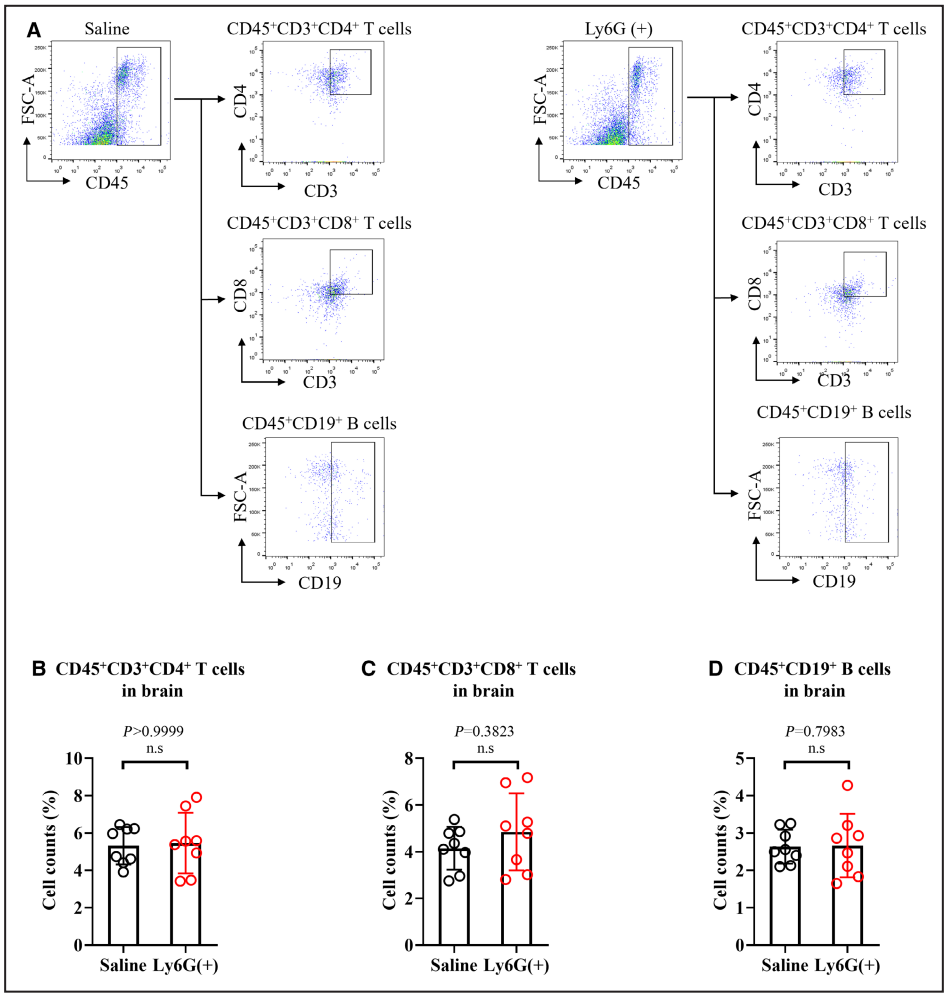

Figure 8. Proportion of CD4⁺ T cells, CD8⁺ T cells, and B cells in the ischemic hemisphere on day 3 after distal middle cerebral artery occlusion.
A, Gating strategy to identify CD4⁺ T cells (CD45⁺CD3⁺CD4⁺), CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), and B cells (CD45⁺CD19⁺) in the ischemic hemisphere of anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody- and saline-treated mice on day 3 after distal middle cerebral artery occlusion. **B** through **D**, Quantification of CD4⁺ T cells (**B**), CD8⁺ T cells (**C**), and B cells (**D**) in the brain of anti-Ly6G antibody- and saline-treated mice on day 3 after distal middle cerebral artery occlusion (n=8). FSC indicates forward scatter; and Ly6G, lymphocyte antigen 6 complex locus G.

neutrophils before and after stroke. Next, we conducted FACs using spleen from mice on day 3 after dMCAO to investigate the immune system regulation of PMN-MDSCs. Consistently, mice spleen showed an increased PMN-MDSC proportion to 11.19% on day 3 after stroke. Prophylactic anti-Ly6G antibody treatment significantly decreased the proportion of PMN-MDSCs to 2.47% (Figure S3). We also conducted tMCAO to trigger a more severe ischemic stroke in mice. In tMCAO model, PMN-MDSCs were elevated to 8.15% and were decreased to 1.92% by anti-Ly6G antibody treatment (Figure S4A). Moreover, tMCAO induced a markedly elevated mortality rate in comparison with dMCAO, and no statistically significant difference was found in the survival curve between the anti-Ly6G antibody- and saline-treated groups (*P*=0.2184; Figure S4B).

Prophylactic Anti-Ly6G Antibody Treatment Did Not Alter Lymphocyte Population

As lymphocytes play crucial roles in poststroke immune regulation,[31,32] next, we investigated the population of CD4⁺ T cells (CD45⁺CD3⁺CD4⁺), CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), and B cells (CD45⁺CD19⁺) in the brain on day 3 after stroke. No significant differences were found in the CD4⁺ T cell (Figure 8B), CD8⁺ T cell (Figure 8C), and B cell (Figure 8D) populations between saline- and anti-Ly6G antibody-treated mouse brains. We also measured the circulating lymphocytes in blood after stroke (Figure 9A). No significant differences were observed in the CD4⁺ T cells (Figure 9B), CD8⁺ T cells (Figure 9C), and B cells (Figure 9D) between saline- and

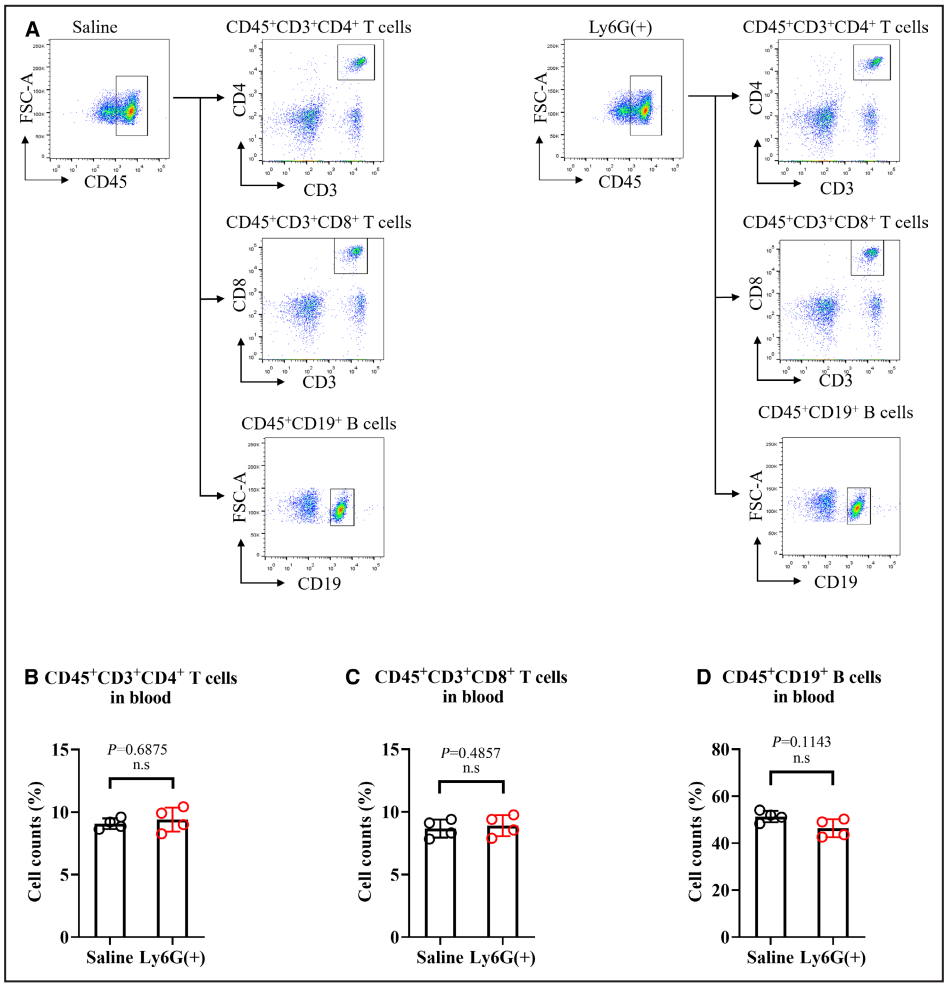

Figure 9. Proportion of CD4⁺ T cells, CD8⁺ T cells and B cells in blood on day 3 after distal middle cerebral artery occlusion.

A, Gating strategy to identify CD4⁺ T cells (CD45⁺CD3⁺CD4⁺), CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), and B cells (CD45⁺CD19⁺) in anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody- and saline-treated mouse blood on day 3 after distal middle cerebral artery occlusion. **B** through **D**, Quantification of CD4⁺ T cells (**B**), CD8⁺ T cells (**C**), and B cells (**D**) in anti-Ly6G antibody- and saline-treated mouse blood on day 3 after distal middle cerebral artery occlusion (n=4). FSC indicates forward scatter; and Ly6G, lymphocyte antigen 6 complex locus G.

anti-Ly6G antibody-treated mice blood after stroke. These findings suggest that prophylactic anti-Ly6G antibody treatment did not influence the lymphocyte populations in either the blood circulation or brain infiltration after ischemic stroke. Taken together, this work demonstrated that prophylactic anti-Ly6G antibody administration suppressed activated neutrophil infiltration and PMN-MDSCs accumulation induced by ischemic stroke and ameliorated poststroke neurological recovery up to the subacute phase (Figure 10).

DISCUSSION

Neutrophils and PMN-MDSCs exhibit a diverse repertoire of functions in stroke pathophysiology in spite of immune regulation.[33] This study used prophylactic anti-Ly6G antibody administration to successfully deplete neutrophils and PMN-MDSCs in mice, and found that prophylactic anti-Ly6G antibody administration reduced activated neutrophil infiltration and NET formation in parenchyma, inhibited inflammatory responses, and ameliorated ischemic stroke outcomes in the subacute phase.

Immune regulation and inflammatory responses play crucial roles in the pathologies and prognosis of ischemic stroke, exerting both protective and detrimental effects.[34,35] IL-6 is a crucial proinflammatory mediator in acute phase of ischemic stroke but becomes protective in chronic phase.[36,37] In patients with acute ischemic stroke within 72 hours after stroke onset, IL-6 was notably higher in the poor prognosis group.[38] But in the chronic ischemic stroke phase,

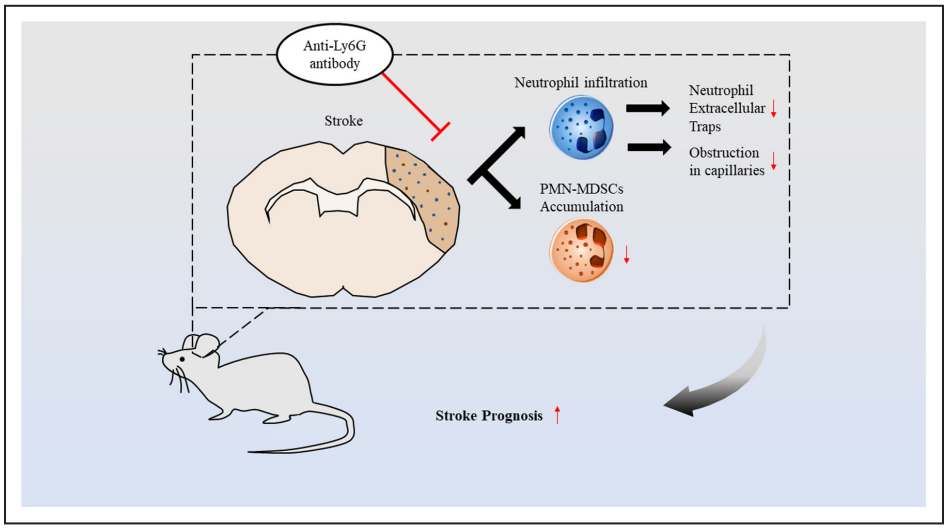

Figure 10. Prophylactic anti-Ly6G (lymphocyte antigen 6 complex locus G) antibody administration suppressed activated neutrophil infiltration and polymorphonuclear myeloid-derived suppressor cell accumulation induced by ischemic stroke and ameliorated poststroke neurological recovery up to the subacute phase.
Ly6G indicates lymphocyte antigen 6 complex locus G; and PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells.

IL-6 elevation benefits stroke recovery by promoting angiogenesis[26] and accelerating wound reparation.[39] In this study, we found an anti-inflammatory effect of anti-Ly6G antibody treatment by inhibiting IL-6 mRNA elevation in acute ischemic stroke phase. Further investigations are needed to discuss the inflammatory responses in both acute and chronic stroke phase. Immune cells, including neutrophils, PMN-MDSCs, and lymphocytes are usually restricted from migrating into brain parenchyma by blood brain barrier. Neutrophils appeared in small amounts around 12 to 24 hours after stroke, and overwhelmed the ischemic hemisphere 3 days poststroke and sustained this increase up to 7 days after the stroke occurred.[40] On the other hand, PMN-MDSCs were significantly increased at 3 days after stroke in the ischemic hemisphere.[20] The expression of PMN-MDSCs in the ischemic hemisphere is delayed compared with neutrophils, but both are present at about the same time thereafter. In this study, prophylactic anti-Ly6G antibody treatment ameliorated neurological function after stroke, which may involve both neutrophils and PMN-MDSCs.

We found no signal of myeloperoxidase$^+$CD11b$^+$ neutrophils and citrullinated histone-3, a reference marker of NET formation, in contralateral brain parenchyma. In ischemic hemisphere, we observed an infiltration of myeloperoxidase$^+$CD11b$^+$ neutrophils into ischemic area, accompanied with increased NET formation, which could be reduced by prophylactic anti-Ly6G antibody administration. Previous work found that neutrophil accumulation,[41,42] infiltration, and obstruction promoted capillary flow stagnation after experimental stroke,[43,44] and NET formation impaired revascularization and vascular remodeling after ischemic stroke,[45,46] in which NET levels in stroke thrombi were positively associated with poor prognosis of clinical stroke.[47] The present work found pretreatment with anti-Ly6G antibody ameliorated ischemic stroke outcomes in mice during the subacute phase, suggesting a protective effect of anti-Ly6G antibody against ischemic stroke.

Generally, MDSCs are composed of a heterogeneous population of immature myeloid cells that could be classified into 2 major subsets according to phenotype and morphology as polymorphonuclear (PMN-) and monocytic (M)-MDSCs. The PMN-MDSCs share a similar surface marker and morphology with neutrophils, but the roles of PMN-MDSCs in ischemic stroke remain debated. Our study elucidates the dynamics of PMN-MDSCs accumulation in the stroke hemisphere and found that it could be depleted by prophylactic anti-Ly6G antibody administration. However, the underlying mechanisms require further investigation.

This study has some limitations. Considering that it is not possible to use anti-Ly6G antibody to target neutrophils and PMN-MDSCs because of their essential roles in systematic immune regulation, it will be important for further studies to define specific pharmaceutical NET and PMN-MDSC targets to validate clinical stroke treatment. Additionally, studies are needed to examine the underlying mechanisms as to how PMN-MDSCs correlate with ischemic stroke, as the current study just examined the phenotype.

Overall, our study provides a novel insight into the application of anti-Ly6G antibody therapy for ischemic stroke.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Figures S1–S4

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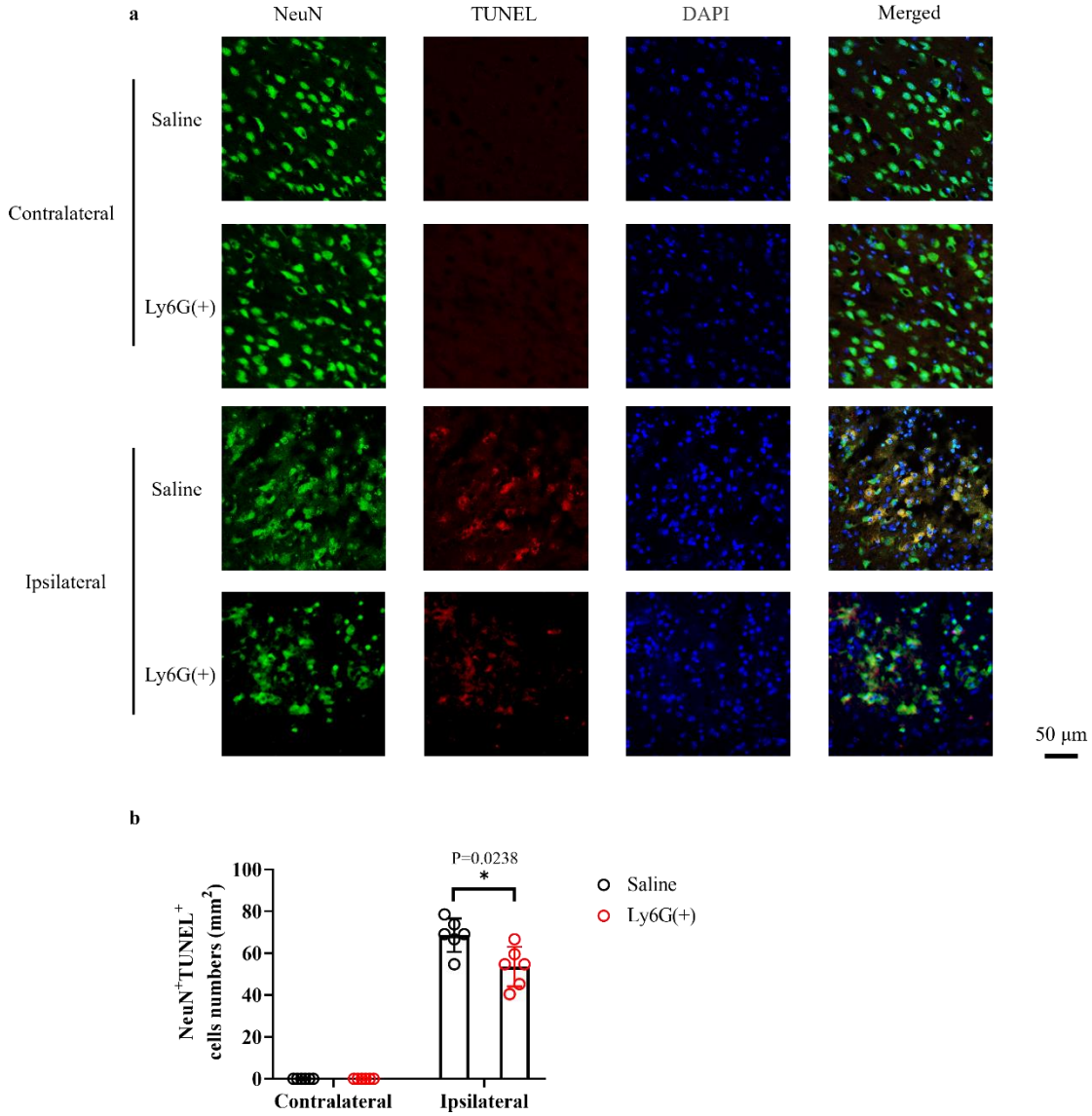
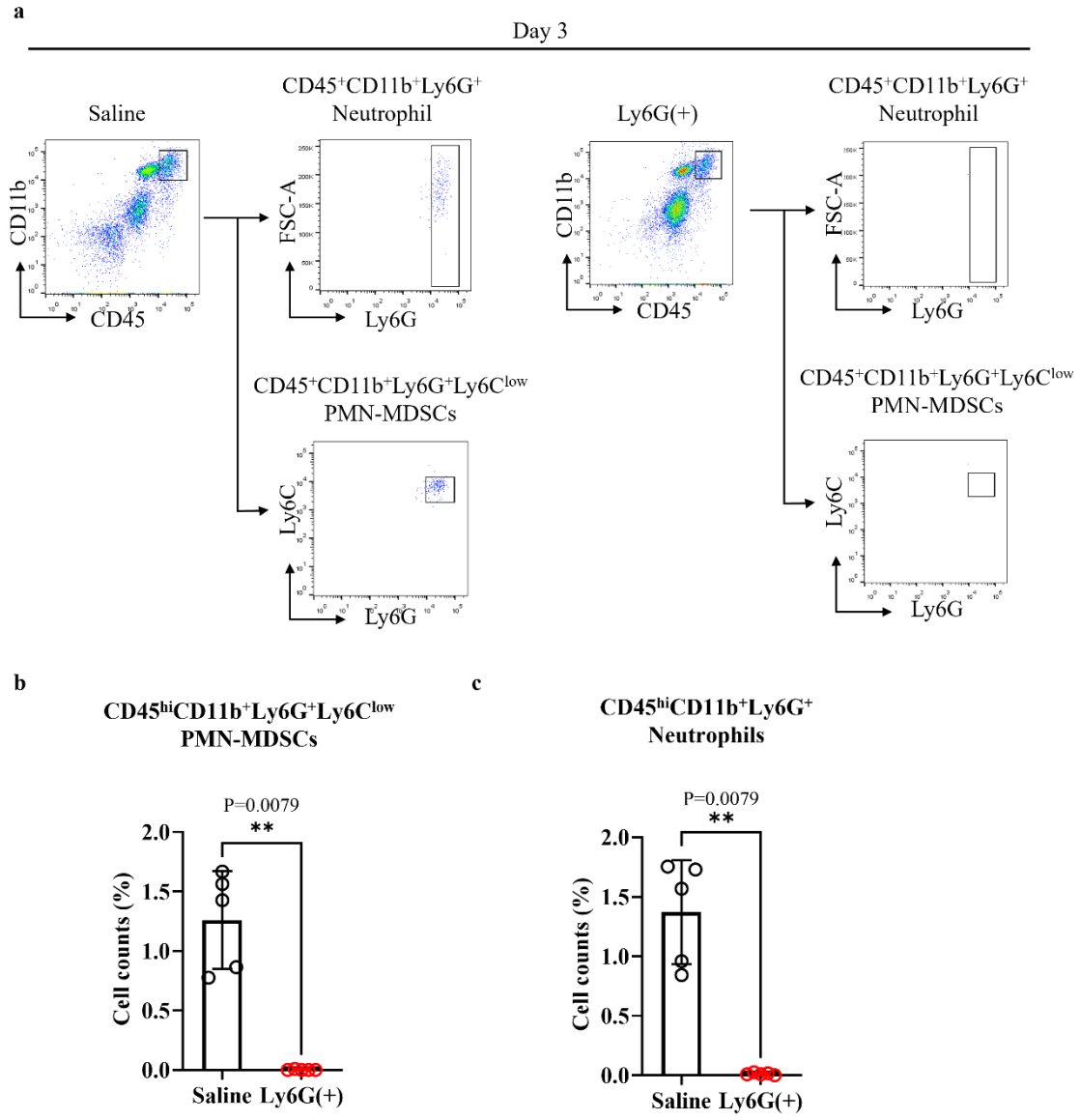
SUPPLEMENTAL MATERIAL

Figure S1. Neuronal apoptosis detected by NeuN and TUNEL co-immunofluorescence staining in the peri-infarct area of saline and anti-Ly6G antibody treated mice brain at Day 3 after distal middle cerebral artery occlusion (dMCAO).



(a) Representative immunofluorescence staining images and (b) quantification of $NeuN^+TUNEL^+$ cells numbers in each group. n = 6.

Figure S2. Once intraperitoneal injection of anti-Ly6G antibody at 25 mg/kg/day on 2 days prior to surgery diminished PMN-MDSCs and neutrophils elevation on Day 3 after dMCAO.



(a) Gating strategy and quantification of (b) $CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low}$ PMN-MDSCs and (c) $CD45^{hi}CD11b^{+}Ly6G^{+}$ neutrophils in mice brains on Day 3 after dMCAO, treated with saline or anti-Ly6G antibody at 25 mg/kg/day on 2 days prior to surgery. n = 5.

Figure S3. CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low} PMN-MDSCs were successfully depleted in spleen by prophylactic anti-Ly6G antibody administration on Day 3 after dMCAO.

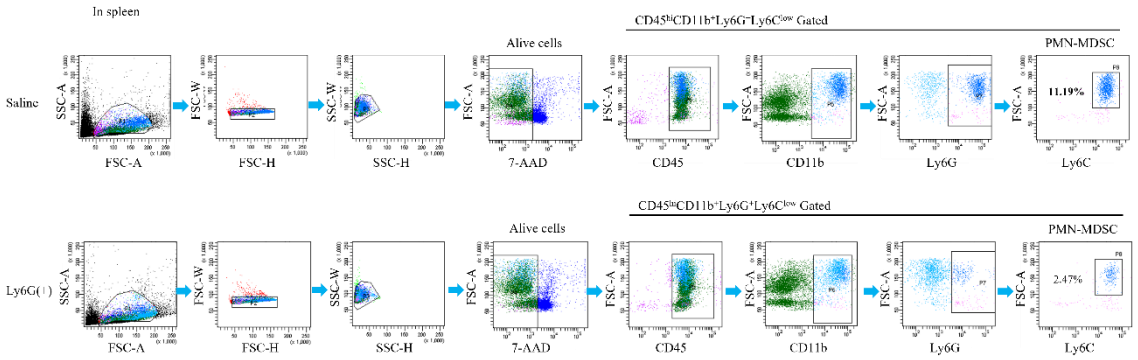
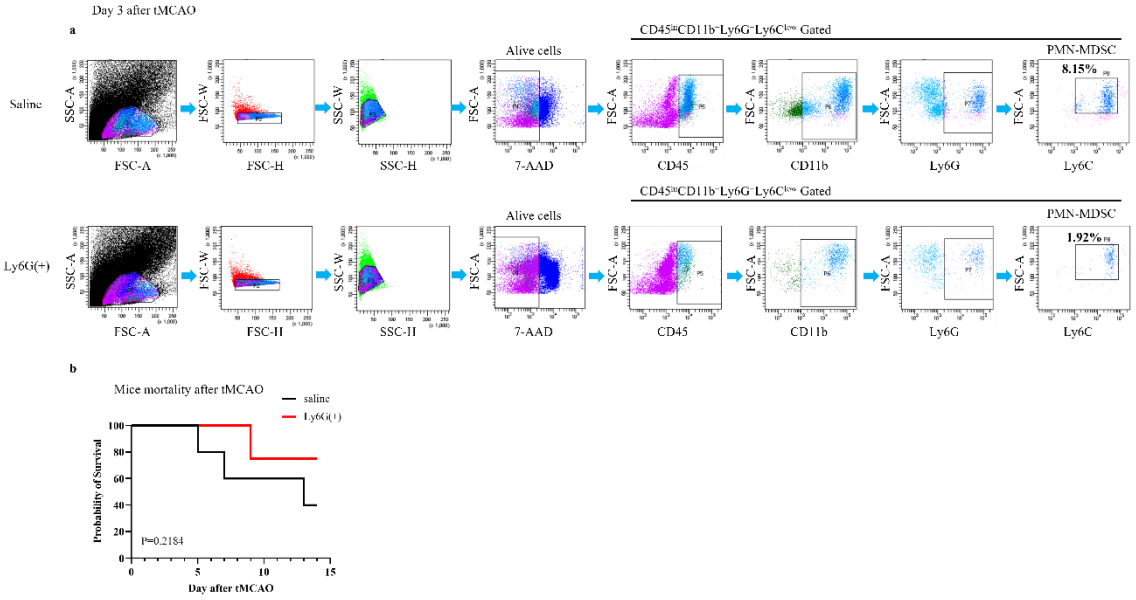


Figure S4. tMCAO was conducted to trigger a more severe ischemic stroke in mice.



(a) Prophylactic anti-Ly6G antibody administration successfully depleted $CD45^{hi}CD11b^{+}Ly6G^{+}Ly6C^{low}$ PMN-MDSCs in the stroke hemisphere after transient middle cerebral artery occlusion (tMCAO). (b) Kaplan Meier curve of mouse survival after tMCAO. n = 5.