Online Supplemental Material

Targeting neutrophil α9 improves functional outcomes after stroke in mice with obesity-induced hyperglycemia

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Running title: Role of α9β1 in ischemic stroke

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Methods

Mice

To generate neutrophil cell-specific α9 deficient mouse ($\alpha9^{fl/fl}$ Mrp8Cre $^{+/-}$), $\alpha9^{fl/fl}$ mouse was crossed with Mrp8Cre$^{+/-}$ mouse (**Figure S1A**). Littermates ($\alpha9^{fl/fl}$ Mrp8Cre $^{-/-}$) were used as controls. Mice were genotyped by PCR according to protocols from the Jackson laboratory. Mice were kept in standard animal house conditions with controlled temperature and humidity and had ad libitum access to standard chow diet and water. Both male and female mice, approximately

24-25 weeks which includes 20 weeks of high-fat diet (TD.06414, Envigo) feeding, were utilized. The University of Iowa Animal Care and Use Committee approved all the procedures and studies were performed according to the current Animal Research: Reporting of In Vivo Experiment guidelines (https://www.nc3rs.org.uk/arriveguidelines).

Filament model of cerebral ischemia

Focal cerebral ischemia was induced by transiently occluding the right middle cerebral artery as described.[10,14] We selected 30 mins of ischemia because in pilot studies ~50% of the obese mice subjected to 60 mins of ischemia died before 28 days (data not shown), making it difficult to study long-term functional outcomes. All surgeries were performed by the person blinded to the experimental groups. Mice were anesthetized with 1–1.5% isoflurane mixed with medical air. After a midline incision, the right common carotid artery was temporarily clamped and a silicon monofilament (Doccol, catalog# 702256PK5re) was inserted via the external carotid artery into the internal carotid artery up to the origin of the middle cerebral artery. Reperfusion was achieved by removing the filament after 30 minutes and opening the common carotid artery. Throughout the surgery, the body temperature of mice were maintained at 37°C ± 1.0 using a heating pad. Buprenorphine (0.1 mg/kg, SC) was administered as an analgesic agent every 6-12 hours for 48 hours post-surgery. Laser Doppler flowmetry (Perimed Instruments, Sweden) was used for each mouse to confirm the successful induction of ischemia and reperfusion. Animals having more than 70% reduction in the regional cerebral blood flow were included in the study. Mice with hemorrhagic transformations within 24 h of surgery and failure to doppler drop were excluded from the study (Table S5). The representative image for each group was selected based on the mean value.

MRI imaging, and infarct area quantification

MRI was performed on day 2 post-reperfusion as described.[10] Animals were anesthetized with isoflurane (2.5% induction, 1.2% maintenance) and placed in the bore of the 7.0 Tesla MRI (Agilent Technologies Inc., Santa Clara, CA, USA) with a two-channel receive-only surface coil. Following scout scans, high-resolution images were acquired with a 9-minute T2-weighted 2D fast spin-echo sequence oriented coronally. Imaging parameters included TR/TE = 6380 ms/83 ms, echo train length of 12, and 7 signal averages to achieve voxel resolution 0.10 mm x 0.10 mm x 0.50 mm with no gaps. The total imaging time for each animal was approximately 25 minutes. The area of infarction was quantified by the person blinded to the experimental groups, using NIH Image J software by outlining the zone with abnormally hyperintense regions in each brain slice, and the total infarct area was obtained by summation of the infarcted areas multiplied by the slice thickness. The corrected total infarct area (%) was calculated as: Corrected infarct area (%) = [area of the contralateral hemisphere- (area of ipsilateral hemisphere-area of infarct)]/ area of contralateral hemisphere X 100.

Functional assessment of neurological outcome:

All the functional assessment of neurological outcome was performed by the person blinded to the experimental groups. **Modified Neurological Severity Score (mNSS):** The mNSS rates neurological functioning with a minimum neurological score of 3, and a maximum of 18. A higher score indicated a better outcome. The mNSS included a composite score of six different tests, which were: spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch as previously described.[10] **Accelerated rotarod test:** The accelerated rotarod (Harvard Apparatus,

model#LE8205) was used to assess post-stroke motor coordination. For this, mice were trained for 3-5 days on rotarod rotating at 4 RPM so that animals may walk forward to keep balance. Training is considered complete when mice can stay on the rod rotating at 4 RPM for at least 1 minute. On the test day, mice were placed on the rod rotating at 4 RPM and then rotation was set in acceleration mode (4-40 RPM in 5 minutes). Latency to fall was recorded for each mouse.

Corner test: The corner was made by the edges of boards (2 opaque plexiglass boards, each with a dimension of 30x20x0.5 cm) at a 30-degree angle with a small gap at the joint between the two boards to encourage entry into the corner. The mouse was placed between the two angled boards facing halfway into the corner; the mouse reared forward and upward, stood up, then turned back to face the open end. A right/left turn was considered complete when the mouse turns with a greater than 90^{o} angle of the head when the mouse enters far enough into the corner that both vibrissae touch the corner boards with or without rearing/standing. A total number of 10 turns was registered. This test is used to detect sensorimotor dysfunction. The right turn ratio was calculated as: number of right turns/10. **Wire hanging test**: Wire hanging test was used to assess motor functions and grip strength. In this test, the animal was placed on wire mesh. The wire mesh was slowly inverted upside down, was held at an approximately 0.5–0.6 m height above a soft underlay to prevent the mouse from jumping down. The time until the animal falls was measured. If the animal did not fall up for to 300 seconds, it was removed from the wire mesh and considered as a cut-off time.

Laser speckle contrast imaging

To assess post-reperfusion local cerebral blood flow, we used a laser speckle contrast imager (moorFLPI-2 from Moor instruments), which provides real-time, high-resolution blood flow

images. Briefly, mice were anesthetized using isoflurane (2.5% induction, 1 % maintenance), an incision was made to provide access to the skull. Mineral oil was applied to avoid dryness. Speckle imaging was obtained using a temporal filter (250 frames, 10sec/frame) at 0.1 Hz at baseline, after middle cerebral artery occlusion, and 5, 30, 60, 120, and 180 min post-reperfusion. Mice were anesthetized at each time points. Blood fluxes were measured in the middle cerebral artery supplied region, and fluxes were expressed in arbitrary units using a 12-color palette.

Plasma insulin and blood glucose quantification

Plasma insulin levels were quantified by ELISA in overnight fasted mice after 20-weeks of chow diet or high-fat (HF) diet feeding as per the kit manufacturer's instruction (Sigma, catalog# EZRMI-13K). Blood glucose was measured in overnight fasted mice using glucometer.

Visceral fat and total body fat

Visceral fat (Mesenteric, retroperitoneal, perirenal and perigonadal) and total body fat (subcutaneous and visceral fat) were collected and weighed from lean and obese male mice.

Quantification of NETosis by Sytox Green

The bone-marrow derived neutrophils (2 X 10^6 per ml) were stimulated with cellular fibronectin (cFn; 20 µg/ml, Sigma, catalog# F2518), or fibronectin from plasma (pFn; 20 µg/ml, Sigma catalog# F2006) for 3 hours. Sytox Green (Thermofisher, catalog #S7020), a non-cell-permeant DNA binding dye, was added to the cells at a final concentration of 1 µM to detect extracellular DNA. Cell viability was monitored after incubation using the Trypan Blue to avoid participation

of dead cells for fluorescence. The plates were read in a fluorescence microplate reader with a filter setting of 485(excitation)/520 (emission). The U0126 (10 μM, Sigma, catalog# 662005) and GSK484 (10 μM, Sigma, catalog# SML1658,) were added 30 min before the addition of cFn or pFn.

Calcium Assay

The bone-marrow derived neutrophils (5 X 10^6 per ml) were loaded with 5 μm of Fura-2 AM and 4 mM probenecid for measuring cytosolic Ca^{2+} at Room Temperature (RT) for 45 mins. The Fura-2 AM (#F1221, ThermoFisher) and probenecid (#P36400, ThermoFisher) loaded neutrophils were centrifuged at 600g for 5 mins to remove the excessive extracellular dye. The neutrophils were resuspended in HBSS without Ca^{2+}. Neutrophils were incubated with pFn (20 μg/ml) or cFn (20 μg/ml) for 5 min. After incubation 1.3mM $CaCl_2$ was added and Ca^{2+} uptake was measured using a fluorometer at 340 and 380nm excitation and 509 nm emission for Fura-2 AM for 15 sec. An average of 15 sec was reported for Ca^{2+}. The cytosolic Ca^{2+} was measured using the following formula $[Ca^{2+}] = Kd\beta(R-Rmin)/(Rmax-R)$; where R=F340/F380, Rmax and Rmin are F340/F380 fluorescence ratios determined for Ca^{2+} bound and Ca^{2+} free forms of Fura-2, respectively. Kd is Ca^{2+} dissociation constants of Fura-2, and β=Fmin,380/Fmax,380, where Fmax,380 and Fmin,380 are fluorescence intensities for Ex=380 nm determined for Ca^{2+}bound and Ca^{2+}free form of Fura-2, respectively. The Fmin was obtained by quenching the signal by adding 4mM EGTA. The Fmax can be acquired by lysing the cells with TrionX-100.

Immunofluorescence

Immunostaining was performed in cryosections from the brain (48 hours post-reperfusion). All sections were subjected to heat-induced antigen retrieval and blocked with 5% normal goat

serum in Tris-buffered saline at room temperature (RT). Brain sections were washed thrice with PBS for 5 minutes and incubated overnight with primary antibodies for platelet (rat anti-mouse CD41; Bio-rad, catalog# MCA2245T), anti-fibrin(ogen) (1:400, Acris Antibodies, catalog# AP00766PU-N) at 4°C. After washing, sections were labeled with appropriate secondary antibodies [goat anti-rabbit IgG Alexa flour-546 (1:400, Invitrogen, catalog# A11010), and goat anti-rat IgG Alexa flour-546 (1:400, Invitrogen, catalog# A11003). Nuclei were stained using DAPI. Isotype-matched immunoglobulins were used as a negative control. Images were taken using Nikon Eclipse Ti-U inverted fluorescent microscope equipped with a 40x/0.75 and 20x/0.8 Plan Apo lens, cooled CCD camera and a Nis Elements imaging software (Nikon). ImageJ software (NIH ImageJ, USA) was used for all the quantifications. The number of total and partially occluded or occluded vessels in ipsilateral area of brain sections was counted by the person blinded to the experimental groups. The thrombotic index was calculated by dividing partially occluded or occluded vessels from a total number of vessels per field multiply by 100.

Flow cytometric analysis of integrin α9 and CD11b exposure on the neutrophils

After 6 hours of reperfusion, neutrophils were isolated from whole blood using density gradient centrifugation and resuspended in phosphate-buffered saline. Neutrophils were gated using neutrophil-specific Ly6G APC-conjugated clone 1A8 antibody (Biolegend, catalog# 127614) and stained with anti-mouse integrin α9 PE-conjugated antibody (R&D Systems catalog# FAB3827P) or CD11b PerCp-conjugated antibody (Biolegend, catalog# 101230) for 15 minutes in the dark at 4°C. After 15 minutes, the cells were fixed in 0.2% paraformaldehyde and analyzed on Becton Dickenson LSRII.

FeCl3 injury-induced carotid thrombosis

Thrombus formation in the carotid artery after the $FeCl_3$ injury was assessed by intravital microscopy as described.[9,14] Briefly, obese mice of both genotypes were anesthetized using 100-mg/kg ketamine and 10-mg/kg xylazine. Platelets (2.5×10^9 per kg) labeled with calcein green AM (3 μM, Thermo Fisher Scientific, catalog# C34852) were infused through the retro-orbital plexus. Infused platelets were isolated from age-matched donor mice of the respective genotype. The common carotid artery was carefully exposed and kept moist by super-fusion with warm (~37°C) saline. Whatman paper (0.5 x 1.5 mm) saturated with ferric chloride (7.5 % w/v) solution was applied topically for 90 seconds, and thrombus formation in the injured carotid vessel was monitored in real-time using a Nikon upright microscope (Plan Fluor 4X/0.2 objective), and thrombus growth was recorded using a high-speed electron-multiplying camera for 40 minutes or until occlusion occurred. The time to form an occlusive thrombus was considered as the time required for blood to stop flowing completely for >1 minute. Videos were evaluated offline using a Nikon computer-assisted image analysis program.

Tail bleeding assay

Tail-transection bleeding time was measured as described.[9] Briefly, male mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a heating pad warmed at 37°C, and a 3 mm segment of the tail was amputated with a sharp scalpel blade. The tail was immediately immersed in saline (at 37°C), and the time taken for the blood stream to stop for more than 30 seconds was defined as the bleeding time. If bleeding did not stop within 10 minutes, hemostasis was achieved by cauterizing the tail.

Western blot

Neutrophils and brain tissue proteins were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail. Peripheral neutrophils (collected 6 hours post-reperfusion), BM-derived neutrophils (15 min/2h post pFn or cFn stimulation), and brain cortical tissue (collected 48 hours post-reperfusion) from the infarcted and surrounding areas were homogenized in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing 0.1% SDS and 4% protease inhibitor (complete protease inhibitor cocktail, Roche, catalog# 11836153001). Samples were sonicated for a total of 30 seconds with 10-second gap. Tissue lysates were centrifuged at 14000×g for 20 min at 4°C, and supernatants were used to determine protein content (by Lowry method) and subsequent Western blot analysis. Total lysates were mixed with sample loading buffer (Novex by Life Technologies, catalog# NP0007) and heated at 95°C for 5 min. 20 µg of total protein was loaded per well, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 60 min with blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl pH 7.5, 0.05% Tween-20), membranes were incubated with: integrin α9 (1:1000, Abcam, catalog# ab140599), anti-fibrin(ogen) (1:5000, Acris Antibodies, catalog# AP00766PU-N), phospho-NF-κB p65 (Ser536) (1:1000, Cell Signaling Technologies, catalog# 3033S), NF-κB p65 (1:1000, Cell Signaling Technologies, catalog# 4764S), and anti-CD41 (1:1000, GeneTex, Catalog# GTX113758) Phospho ERK1/2 (1:1000, Cell Signaling Technologies, catalog# 4370), ERK1/2 (1:1000, Cell Signaling Technologies, catalog# 4695), and PAD-4 (Anti-PADI4 / PAD4 antibody from Abcam catalog# ab96758) at 4°C overnight, followed by appropriate secondary antibodies (polyclonal goat anti-rabbit IgG, Dako, catalog# P0448) conjugated to horseradish peroxidase (HRP). An enhanced chemiluminescence kit (Thermo Scientific, catalog# 34580) was used for Western blots. The

proteins were normalized with β-actin (anti-beta actin antibody from Abcam, catalog# ab8226) and respective total protein content. Densitometric analysis of the gels was done using ImageJ software.

ELISA assay for Cytokines, Elastase

After 6 hours of reperfusion, peripheral neutrophils were isolated from the whole blood using density gradient centrifugation. Neutrophils were lysed in the lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 2% Triton X-100) and were used for the determination of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and Elastase/ELA2 with commercially available mouse ELISA kits (R&D Systems, TNF-α catalog# MTA00B, IL-6 catalog# M6000B, IL-1β catalog#MLB00C, Elastase catalog# MELA20) according to the manufacturer's instructions. After 48 hours of reperfusion, cortical brain tissue was collected from the infarcted and surrounding areas and lysed in tissue lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 2% Triton X-100). Supernatants from brain homogenates were used to quantify IL-1β with commercially available mouse ELISA kits (IL-1β catalog# MLB00C, R&D Systems) according to the manufacturer's instructions.

ELISA for MPO-DNA complexes and DNase's activity

After 6 hours of reperfusion, plasma was collected for MPO-DNA complexes and DNase activity. For neutrophil MPO-DNA complexes: neutrophil supernatants were collected 3h post incubation with cFn or pFn. **MPO-DNA complexes:** An in-house ELISA was used to quantify MPO-DNA complexes with some modifications.[3] Briefly, after overnight coating with anti-MPO antibody (2 μg/ml; 0400-0002, Bio-Rad) at 4°C, a 96-wellplate was blocked with 2.5% bovine

serum albumin in PBS for 2 hours at 37°C temperature. The plate was subsequently washed before incubating for 90 minutes at 37°C with mouse plasma or activated neutrophil supernatants in blocking buffer. The plate was washed five times and then incubated for 90 minutes at 37°C with anti-DNA antibody (1:20; Cell Death detection ELISA, Sigma, catalog# 11544675001). After five washes, the plate was developed with ABTS substrate (Sigma, catalog# 10102946001). **DNase activity**: Plasma was diluted and analyzed using a DNase I assay kit according to the manufacturer instructions (Abcam, catalo# ab234056).

Bone marrow transplantation

Bone marrow transplantation (BMT) was performed at 16 weeks of HFD feeding. All mice were on the C57BL/6J background. Recipient mice were irradiated with 2 doses of 6.5-Gy at an interval of 4 hours between the first and second irradiations. Under sterile conditions, bone marrow cells were extracted from excised femurs and tibias of euthanized donor mice. Bone marrow cells were suspended in sterile PBS and injected (1×10^7 per mouse in 200 µl) into the retro-orbital venous plexus of lethally irradiated recipient mice. After transplantation, mice were maintained in sterile cages and fed autoclaved food and water ad libitum. BMT success was analyzed after 4 weeks by PCR to check presence of the genomic DNA (of the respective donor mice) in peripheral blood mononuclear cells from transplanted mice (not shown). Complete blood counts were obtained using automated veterinary hematology analyzer (ADVIA) to ascertain that BMT did not affect the number of BM-derived blood cells.

Tenascin C treatment: Tenascin C (LSBio, catalog# LS-G24580-10) was dissolved in water and administered once at the dose of 100 μg/kg, intravenously. Control mice received the same volume of vehicle.

Treatment with anti- integrin α9 antibody

Mice were randomly assigned and infused with either anti-α9 antibody (55A2C, 8 mg/kg, provided as a gift sample from Gene Techno Science Co., Ltd, Japan, dose converted from 200 μg/mouse of 25 g) or with control IgG isotype (8 mg/kg, Rockland antibodies and assays, catalog# 0007-0102-0005) intravenously, 5 minutes after the reperfusion.

Rigor and randomization

1) The experiment was designed to prospectively assessed a pre-defined group of mice with equal size with serial functional outcomes up to day 28, and we also consider mortality as a relevant outcome. 2) We defined inclusion/exclusion criteria before study initiation and did not exclude any mice after the data acquisition (Table S5). The group size is uneven due to differential attrition (mortality). 3) α9Neu-KO mice are mutant mice, which were genotyped before doing the surgery. The α9Neu-KO and littermate α9WT mice were not randomized. 4) In Figure 6, WT mice infused with the intervention (anti-α9 antibody 55A2C) were randomized. 5) For blinding, analysis was performed by another investigator unaware of the treatment/group allocation. 6) In vitro assays were done by different investigators for rigor and reproducibility. Western blotting was done by one investigator and quantification was done by another investigator.

Statistical analysis

Results are reported as mean ± SD except for the neurological scores (mNSS), and sensorimotor test (accelerated rotarod test, wire-hanging test and corner test) where median ± range was used. The number of experimental animals in each group was based on power calculations for the primary parameter (infarct area) with mean differences and standard deviations taken from pilot data at power 80% with an α of 0.05. For statistical analysis, GraphPad Prism software, version 9.1 was used. Shapiro-Wilk test was used to check normality, and Bartlett's test was used to check equal variance. The statistical significance was assessed using either unpaired t-test or one-way ANOVA followed by Dunnett's multiple comparisons test or two-way ANOVA followed by Holm-Sidak's multiple comparisons test (for normally distributed data), and Mann Whitney test (for not normally distributed data). $P<0.05$ was considered to be statistically significant.

Power calculation: The primary end point, which is infarct area (analyzed by T2-MRI), in neutrophil-specific α9 deficient mice was 16.9 (SD 7.3) compared with 24.1 (SD 6.6) in littermate control mice. The mean difference between groups was 7.2 % (approx. 30% reduction in infarct area). We used t-test to detect a reduction of 30% in infarct area between groups. At overall significance level of 0.05, the power to detect such a difference would be 80% with 15 mice in each group. We added 25% mice (4/group) in the sample size expecting high mortality because of pre-existing comorbidity obese-induced hyperglycemia.

Supplementary Tables

	Chow-fed $\alpha9^{WT}$	Chow-fed $\alpha9^{Neu\text{-}KO}$	P value (vs. Chow-fed $\alpha9^{WT}$)	High-fat diet-fed $\alpha9^{WT}$	High-fat diet-fed $\alpha9^{Neu\text{-}KO}$	P value (vs. High-fat diet-fed $\alpha9^{WT}$)
Plasma total cholesterol (mg/dL)	71.0 ± 8.9	70.9 ± 3.6	P=NS (Unpaired t -test)	238.5 ± 17.2	244.1 ± 61.3	P=NS (Unpaired t -test)
Plasma Triglycerides (mg/dL)	82.8 ± 22.9	76.3 ± 8.6	P=NS (Unpaired t -test)	114.5 ± 26.2	90.1 ± 38.7	P=NS (Unpaired t -test)

Supplementary Table 1: Total plasma levels of cholesterol and triglycerides were measured from each male mouse using enzymatic colorimetric assays according to the manufacturer's instructions. Values are expressed as mean ± SD. n= 6, 6, 6, 6. NS, non-significant.

	Chow-fed $\alpha9^{WT}$	Chow-fed $\alpha9^{Neu\text{-}KO}$	P value (vs. Chow-fed $\alpha9^{WT}$)	High-fat diet-fed $\alpha9^{WT}$	High-fat diet-fed $\alpha9^{Neu\text{-}KO}$	P value (vs. High-fat diet-fed $\alpha9^{WT}$)
WBC ($10^3/\mu L$)	9.2 ± 2.6	9.0 ± 1.3	P=NS (Unpaired t -test)	8.0 ± 3.7	9.6 ± 4.4	P=NS (Mann-Whitney test)
RBC ($10^6/\mu L$)	9.6 ± 2.3	8.7 ± 0.5	P=NS (Mann-Whitney test)	8.8 ± 1.2	10.0 ± 4.3	P=NS (Mann-Whitney test)
HGB (g/dL)	13.9 ± 3.2	12.6 ± 0.6	P=NS (Mann-Whitney test)	12.5 ± 1.5	11.8 ± 2.2	P=NS (Mann-Whitney test)
HCT (%)	48.1 ± 12.1	44.1 ± 2.5	P=NS (Mann-Whitney test)	44.8 ± 5.6	43.2 ± 7.5	P=NS (Mann-Whitney test)
PLT ($10^3/\mu L$)	1086 ± 115	950 ± 150	P=NS (Mann-Whitney test)	828 ± 104	873 ± 98	P=NS (Unpaired t -test)
Neutrophil ($10^3/\mu L$)	0.7 ± 0.2	0.6 ± 0.2	P=NS (Unpaired t -test)	0.4 ± 0.21	0.4 ± 0.23	P=NS (Unpaired t -test)
Monocytes ($10^3/\mu L$)	1.8 ± 0.9	1.6 ± 1.1	P=NS (Unpaired t -test)	1.1 ± 0.6	1.5 ± 0.9	P=NS (Mann-Whitney test)

Supplementary Table 2. Complete blood counts from chow-fed and high-fat diet-fed male mice were obtained using automated veterinary hematology analyzer (ADVIA). Values are expressed as mean ± SD. n=7, 7 mice for chow diet, 9, 9 for high-fat diet. NS, non-significant.

	High-fat diet-fed $\alpha9^{WT}$	High-fat diet-fed $\alpha9^{Neu\text{-}KO}$	P value (vs. high fat diet-fed $\alpha9^{WT}$)
Ischemia LDF (% of baseline)	24.1 ± 6.7	22.9 ± 3.9	P=NS (Unpaired t -test)

Supplementary Table 3: Laser Doppler Flowmetry (LDF) of male mice was similar among groups during and after ischemia. Values are expressed as mean ± SD. n= 8, 8. P=NS, non-significant.

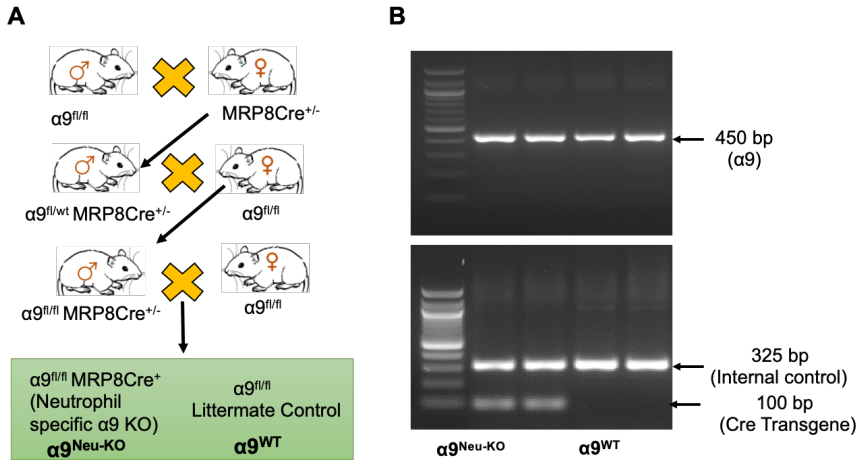
	Obese $\alpha9^{WT}$➔ Obese WT	Obese $\alpha9^{Neu\text{-}KO}$➔ Obese WT	P value (vs. Obese $\alpha9^{WT}$➔ Obese WT)	Obese $\alpha9^{WT}$➔ Obese Fn-EDA$^{+/+}$	Obese $\alpha9^{Neu\text{-}KO}$➔ Obese Fn-EDA$^{+/+}$	P value (vs. Obese $\alpha9^{WT}$➔ Obese Fn-EDA$^{+/+}$)
WBC ($10^3/\mu L$)	6.3 ± 2.3	7.1 ± 1.6	P=NS (Mann-Whitney test)	8.0 ± 3.3	6.3 ± 1.80	P=NS (Mann-Whitney test)
RBC ($10^6/\mu L$)	10.3 ± 1.4	10.3 ± 1.9	P=NS (Unpaired t -test)	9.9 ± 1.7	10.0 ± 1.5	P=NS (Mann-Whitney test)
HGB (g/dL)	14.2 ± 1.9	14.5 ± 2.5	P=NS (Unpaired t -test)	14.1 ± 2.1	14.2 ± 1.7	P=NS (Mann-Whitney test)
HCT (%)	53.6 ± 5.9	54.4 ± 8	P=NS (Unpaired t -test)	52.2 ± 7.6	48.6 ± 12.6	P=NS (Mann-Whitney test)
PLT ($10^3/\mu L$)	829 ± 231	1035 ± 331	P=NS (Unpaired t -test)	993 ± 482	938 ± 324	P=NS (Unpaired t -test)
Neutrophil ($10^3/\mu L$)	0.4 ± 0.4	0.3 ± 0.3	P=NS (Mann-Whitney test)	0.4 ± 0.3	0.6 ± 0.9	P=NS (Mann-Whitney test)
Monocytes ($10^3/\mu L$)	1.5 ± 0.8	1.9 ± 0.8	P=NS (Mann-Whitney test)	1.8 ± 0.8	2.0 ± 0.7	P=NS (Unpaired t -test)

Supplementary Table 4: Complete blood counts from obese BMT male mice were obtained using an automated veterinary hematology analyzer (ADVIA). Values are expressed as mean ± SD. n= 9, 10, 9, 10. NS, non-significant.

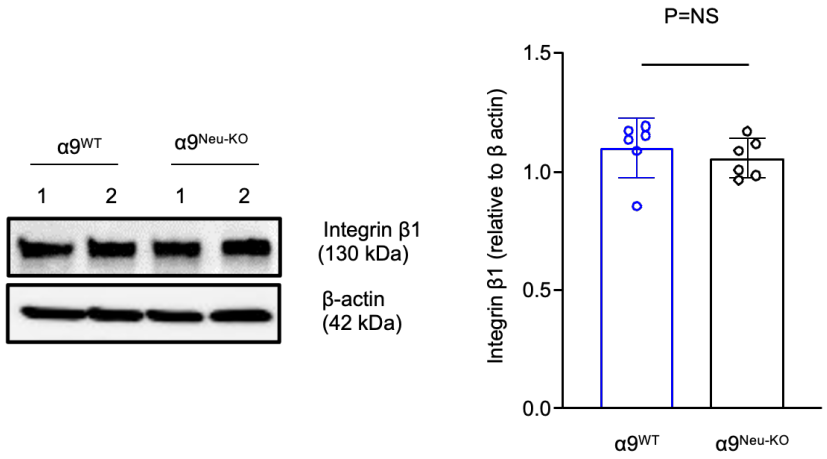
Figure details	Number of excluded animals (within 24 h after stroke)				Number of deaths (up to day 28)			
Figure 2 (Obese M)	$\alpha9^{WT}$ (N=19)		$\alpha9^{Neu\text{-}KO*}$ (N=19)		$\alpha9^{WT}$ (N=19)		$\alpha9^{Neu\text{-}KO}$ (N=19)	
	0		4		8		5	
Figure 6 (M + F)	Control Ig (N=14: 7M + 7F)		Anti-integrin α9 Ig (N=14: 7M+7F)		Control Ig (N=14: 7M + 7F)		Anti-integrin α9 Ig (N=14: 7M+7F)	
	0		0		2M		0	
Suppl- Fig 3 (Lean M)	$\alpha9^{WT}$ (N=12)		$\alpha9^{Neu\text{-}KO}$ (N=11)		$\alpha9^{WT}$ (N=12)		$\alpha9^{Neu\text{-}KO}$ (N=11)	
	1		1		1		0	
Suppl- Fig 4 (Lean F)	$\alpha9^{WT}$ (N=12)		$\alpha9^{Neu\text{-}KO}$ (N=11)		$\alpha9^{WT}$ (N=12)		$\alpha9^{Neu\text{-}KO}$ (N=11)	
	1		1		0		0	
Suppl- Fig 7 (Obese F)	$\alpha9^{WT}$ (N=11)		$\alpha9^{Neu\text{-}KO}$ (N=11)		$\alpha9^{WT}$ (N=11)		$\alpha9^{Neu\text{-}KO}$ (N=11)	
	0		1		1		0	
Figure 5 (Obese M)	$\alpha9^{WT}$ BM→ WT (N=15)	$\alpha9^{NeuKO}$BM → WT (N=15)	$\alpha9^{WT}$ BM→Fn-EDA$^{+/+}$ (N=15)	$\alpha9^{NeuKO}$BM→ Fn-EDA$^{+/+}$ (N=15)	$\alpha9^{WT}$ BM→ WT (N=15)	$\alpha9^{NeuKO}$BM → WT (N=15)	$\alpha9^{WT}$ BM →Fn-EDA$^{+/+}$ (N=15)	$\alpha9^{NeuKO}$BM→ Fn-EDA$^{+/+}$ (N=15)
	1	1	1	2	4	3	10	1

Supplementary Table 5: Number of animals excluded (due to hemorrhage and/or failure in doppler drop) and number of deaths in the study. M, Male; F, Female. * Two mice died during tMCAo surgery.

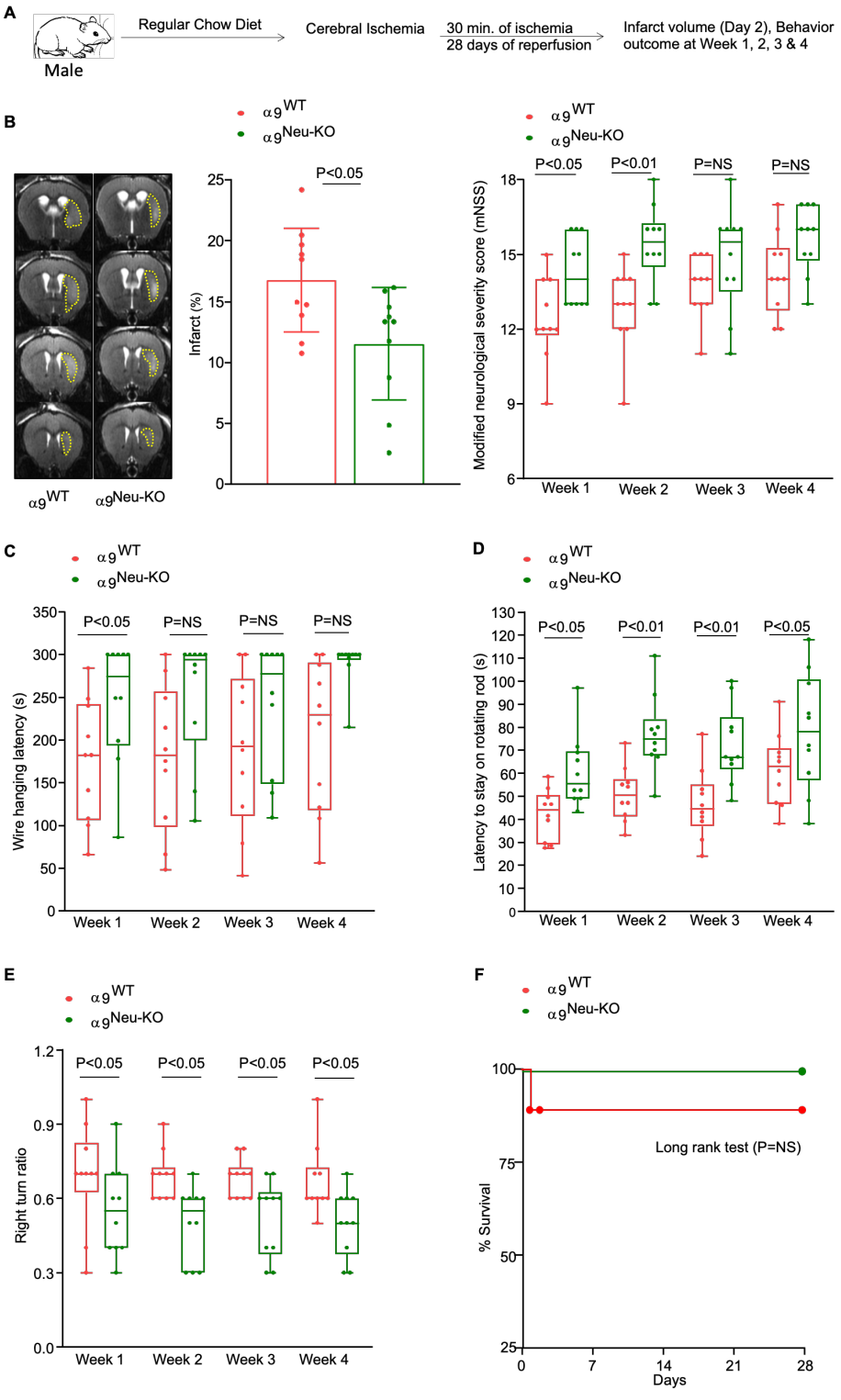
Supplementary figures



Supplemental Figure 1. (A) Breeding strategy used to generate neutrophil cell-specific $\alpha9^{-/-}$ mice. **(B)** Representative genomic PCR images confirming $\alpha9^{fl/fl}$ and MRP8Cre gene.

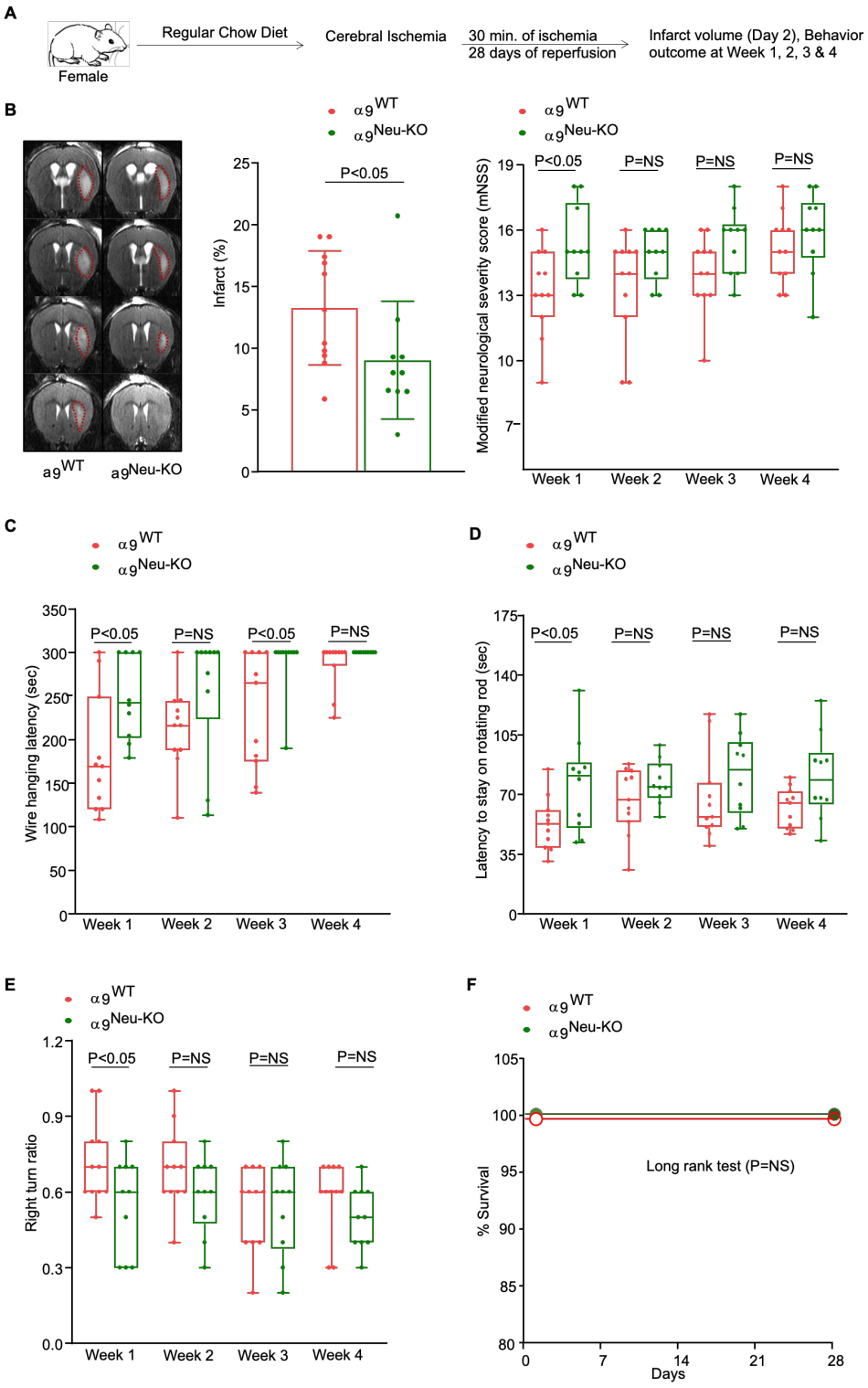


Supplemental Figure 2. **Integrin β1 level was comparable among genotypes. Left:** Representative immunoblot of integrin β1 from the bone-marrow-derived neutrophils of the $\alpha9^{WT}$ and $\alpha9^{Neu\text{-}KO}$ mice. #1 and #2 represent samples from two individual mice. β-actin was used as a loading control. n=6, 6. Data are mean ± SD. Statistical analysis: Mann-Whitney test. NS, non-significant.



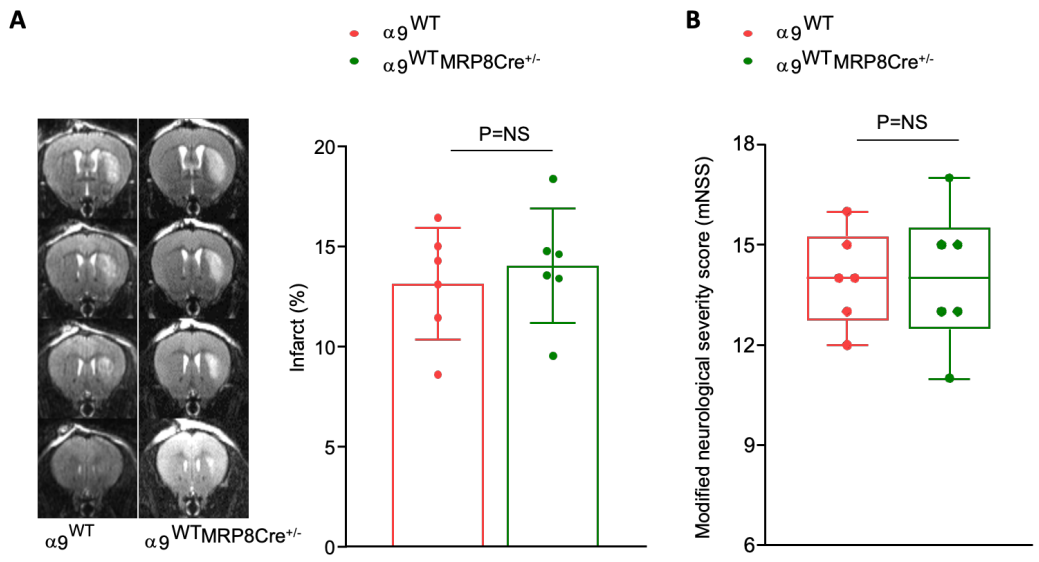
Supplemental Figure 3. **Neutrophil-specific deletion of α9 improves stroke outcome in male mice fed a regular chow diet. (A)** Schematic of experimental design. **(B)** Representative T2-MRI images from one mouse of each genotype on day 2 (left) and corrected mean infarct of each genotype (middle). White (demarcated by yellow dots) is the infarct area. n=10, 10. Modified

Neurological Severity Score (mNSS) in the same cohort of mice at weeks 1, 2, 3, and 4 (right) based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch (higher score indicates a better outcome). n=10, 10. **(C-E)** Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in the hanging-wire test **(C),** fall latency in the accelerated rota-rod test **(D),** and right turn ratio in the corner test **(E)**. n=10,10. **(F)** Survival (%) up to day 28. Data are mean ± SD (infarct) and median ± range (functional outcome). Statistical analysis: unpaired t-test (infarct), two-way ANOVA followed by Holm-Sidak multiple comparisons test (functional outcome). The comparison of survival curves was evaluated by log-rank (Mantel-Cox) test. NS, non-significant.

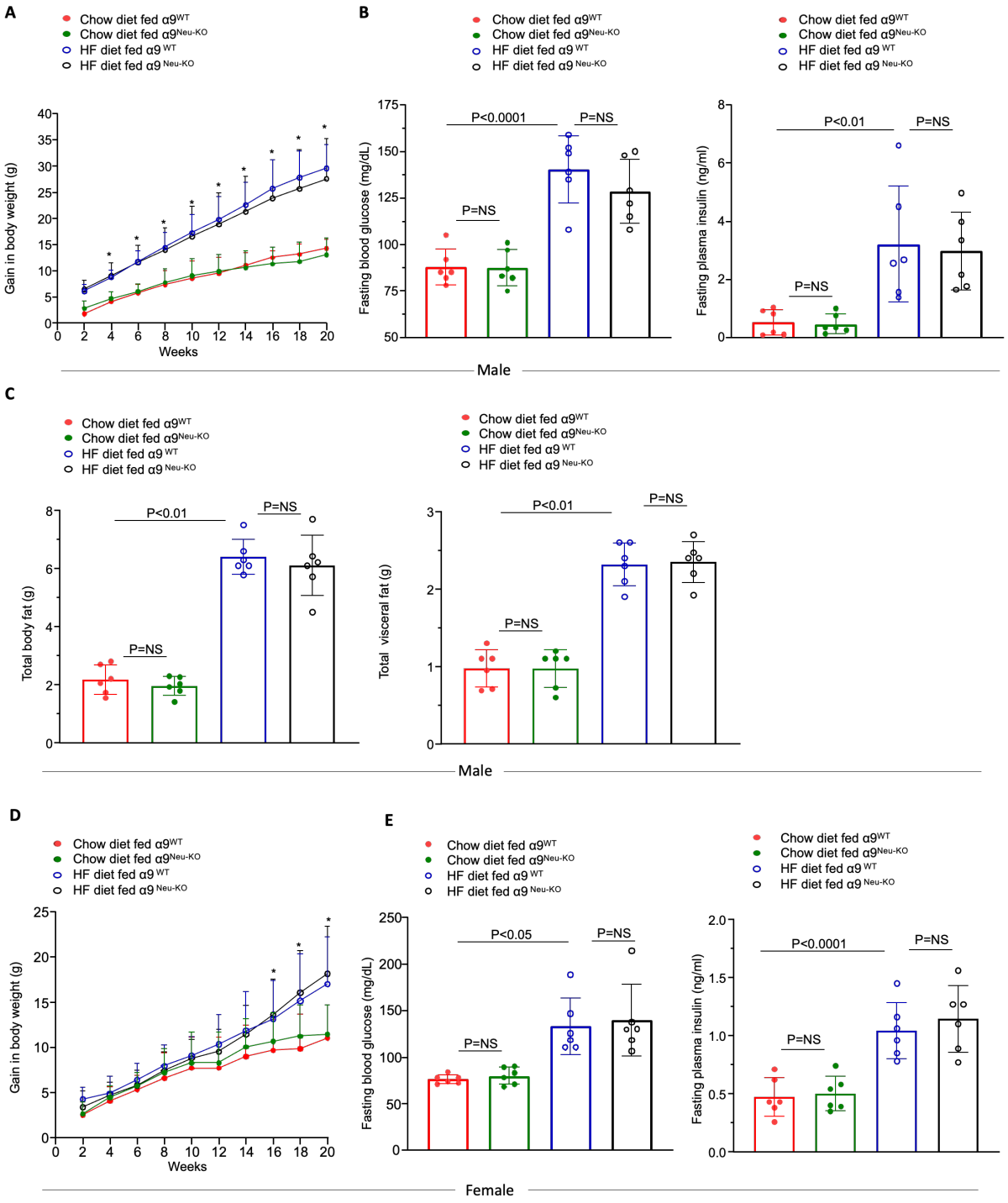


Supplemental Figure 4. Neutrophil-specific deletion of α9 improves stroke outcome in female mice fed a regular chow diet. (A) Schematic of experimental design. **(B)** Representative T2-MRI images from one mouse of each genotype on day 2 (left) and corrected mean infarct of each genotype (middle). White (demarcated by red dots) is the infarct area. n=11, 10. Modified Neurological Severity Score (mNSS) in the same cohort of mice at weeks 1, 2, 3,

and 4 (right) based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch (higher score indicates a better outcome). n=11, 10. **(C-E)** Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in the hanging-wire test **(C),** fall latency in the accelerated rota-rod test **(D),** and right turn ratio in the corner test **(E)**. n=11, 10. **(F)** Survival (%) up to day 28. Data are mean ± SD (infarct) and median ± range (functional outcome). Statistical analysis: unpaired t-test (infarct), Two-way ANOVA followed by Holm-Sidak multiple comparisons test (functional outcome). The comparison of survival curves was evaluated by log-rank (Mantel-Cox) test. NS, non-significant.

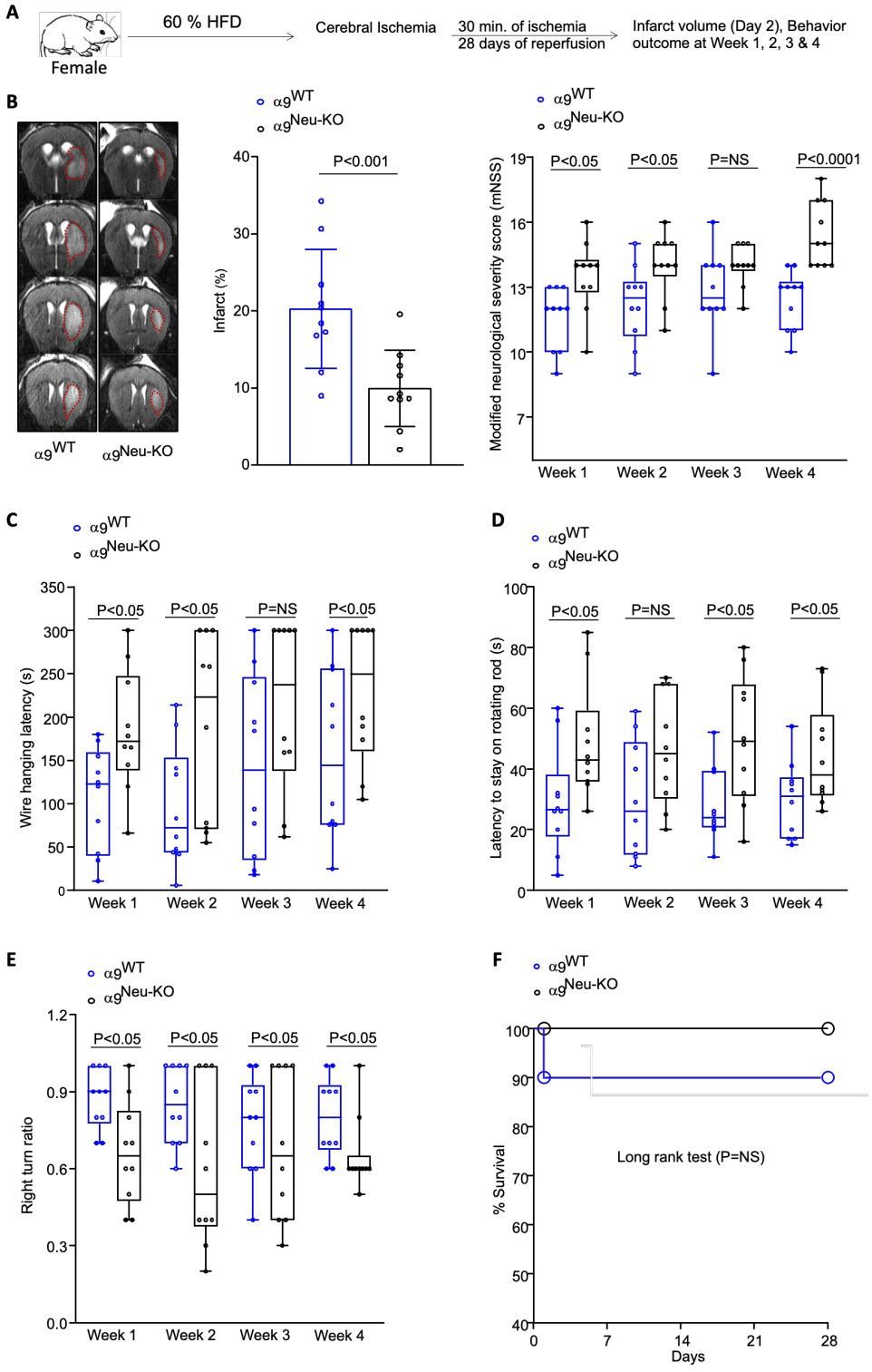


Supplemental Figure 5. MRP8Cre expression does not affect stroke outcome (A) Corrected mean infarct area of each genotype (male mice) on day 2 after 30 min MCAO in filament model. **(B)** Modified Neurological Severity Score (mNSS) on day 7. Data are mean ± SD (A) and median ± range, (B). n= 6, 6. Statistical analysis: unpaired t-test (A) and Mann Whitney test (B). NS, non-significant.



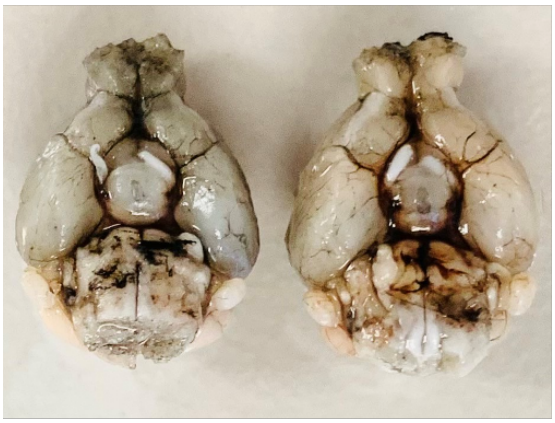
Supplemental Figure 6. **Body weight gain, visceral fat and total fat (for male), fasting blood glucose and plasma insulin levels were comparable among genotypes** Starting from the age of 5 weeks, male and female mice were fed on a regular chow or high-fat diet for 20-weeks (**A)** Body weight gain during regular chow and high-fat diet feeding in male mice. *P<0.05 vs. regular chow diet. **(B)** Glucose and insulin levels in the plasma from overnight fasted male mice. n=6, 6, 6, 6. **(C)** Total body fat and total visceral fat in male obese mice. n=6, 6, 6, 6. **(D)** Body

weight gain during regular chow and high-fat diet feeding in female mice. *P<0.05 vs. regular chow diet. **(E)** Glucose and insulin levels in the plasma from overnight fasted female mice. n=6, 6, 6, 6. Data represents mean ± SD. Statistical analysis: two-way ANOVA followed by Holm-Sidak multiple comparisons test. NS, non-significant.

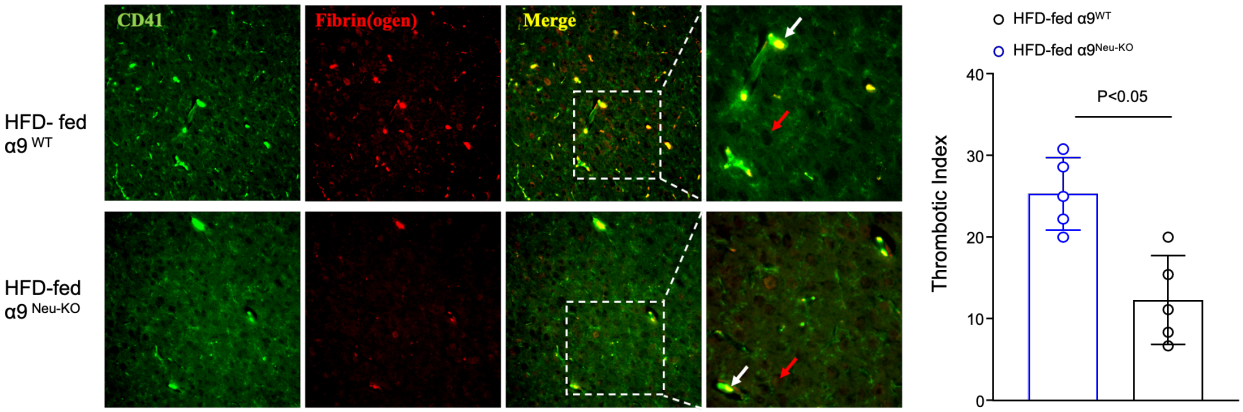


Supplemental Figure 7. Neutrophil-specific deletion of α9 improves stroke outcome in female obese mice. (A) Schematic of experimental design. **(B)** Representative T2-MRI images from one mouse of each genotype on day 2 (left) and corrected mean infarct of each genotype

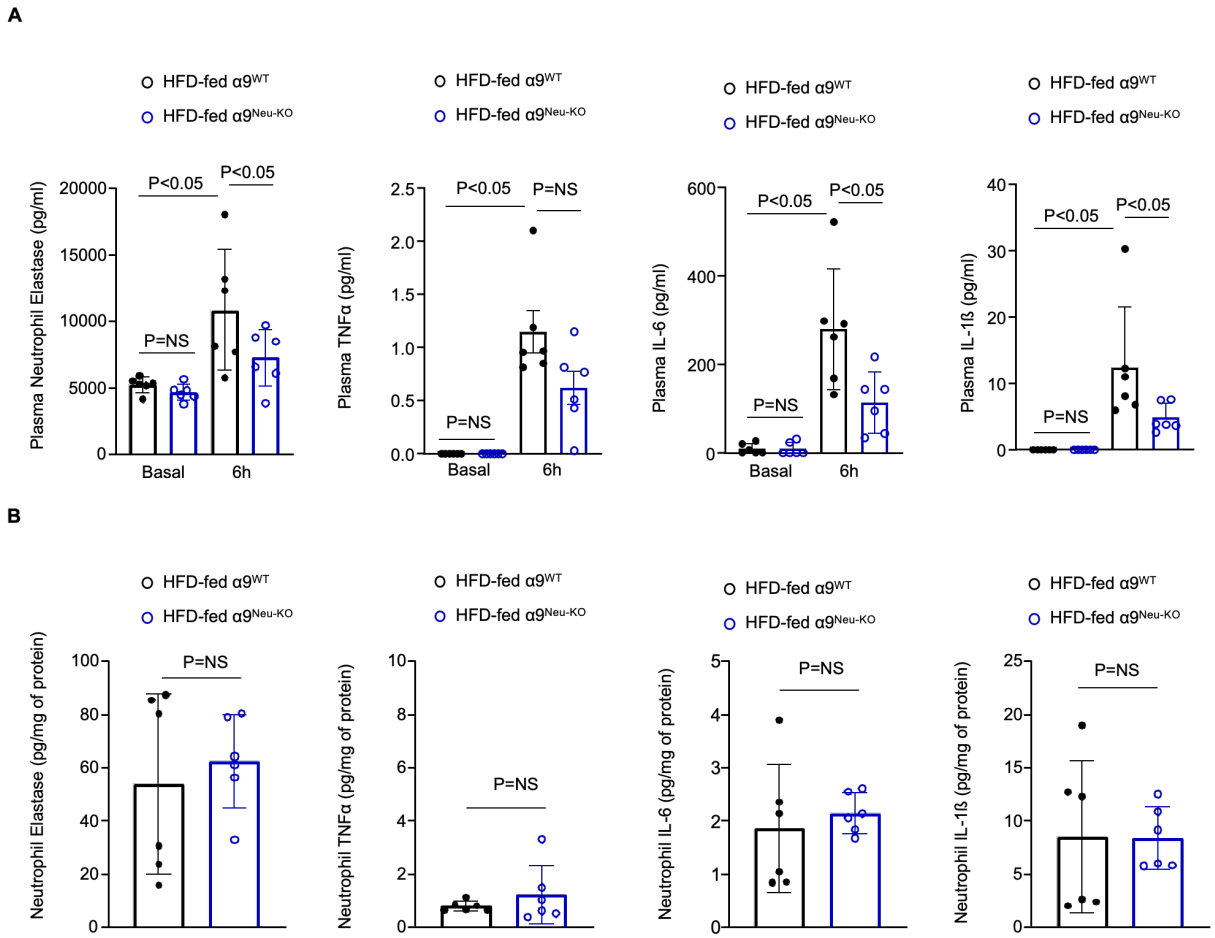
(middle). White (demarcated by red dots) is the infarct area. n=10, 10. Right: Modified Neurological Severity Score (mNSS) in the same cohort of mice at weeks 1, 2, 3, and 4 based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch (higher score indicates a better outcome). n=10, 10. **(C-E)** Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in the hanging-wire test **(C),** fall latency in the accelerated rota-rod test **(D),** and right turn ratio in the corner test **(E)**. n=10, 10. **(F)** Survival (%) up to day 28. Data are mean ± SD (infarct) and median ± range (functional outcome). Statistical analysis: unpaired t-test (infarct), Two-way ANOVA followed by Holm-Sidak multiple comparisons test (functional outcome). The comparison of survival curves was evaluated by log-rank (Mantel-Cox) test. NS, non-significant.



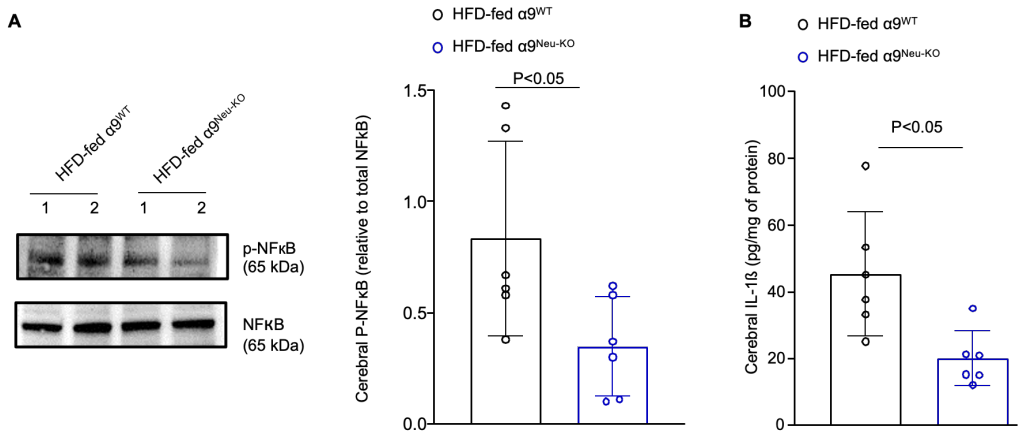
Supplemental Figure 8. Comparison of cerebrovascular anatomy of obese male mice. Anesthetized mice were given an intracardiac injection of India ink and were then exsanguinated according to approved animal protocol. Circle of Willis and bilateral posterior communicating arteries was comparable among groups indicating there are no strain-related differences in gross cerebrovascular anatomy.



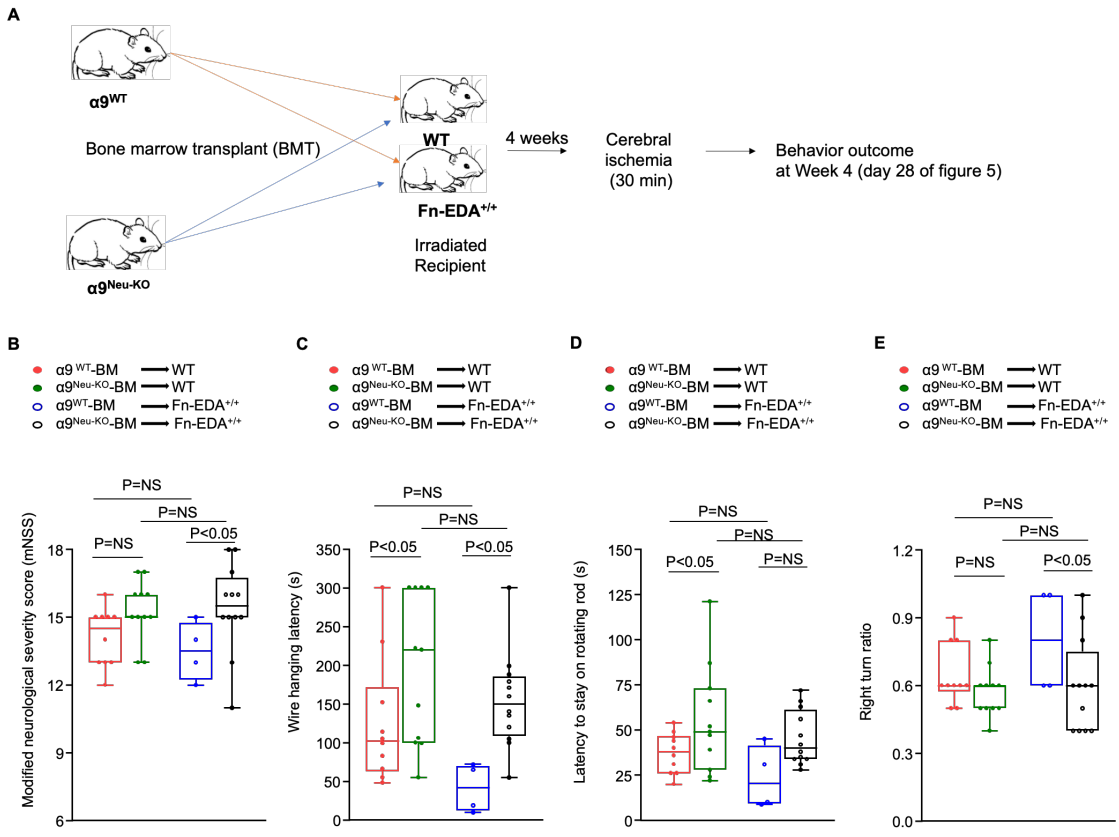
Supplemental Figure 9. Neutrophil-specific α9$^{-/-}$ obese mice exhibited reduced post-ischemic thrombosis. Left: Representative immunostaining images (20X) for platelet (CD41-positive, green) and fibrin(ogen) (red). Insert (dotted box) shows 40X image. White arrow indicates vessel with thrombi. Red arrow indicates empty vessel. Right: Thrombotic index, n=5, 5. Data are from male mice and are represented as mean ± SD. Statistical analysis: Mann-Whitney test. NS, non-significant.



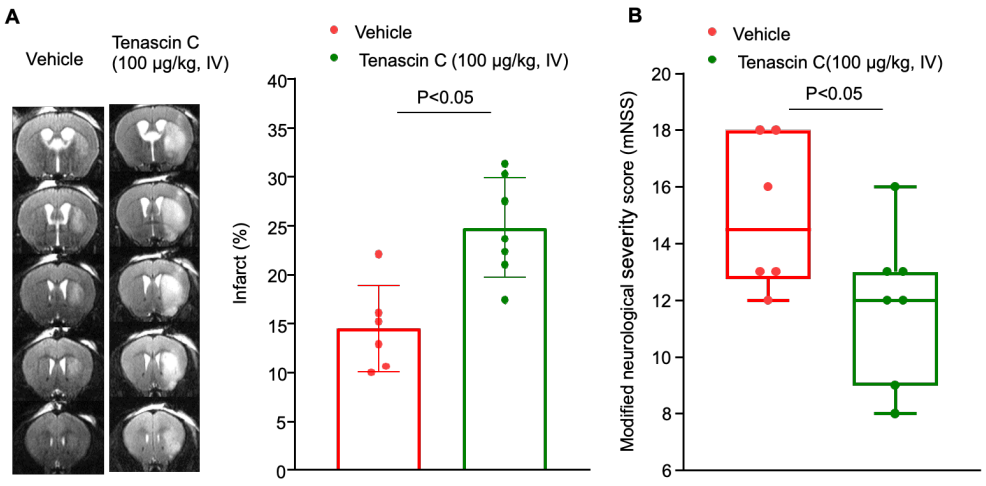
Supplemental Figure 10. Elastase and inflammatory cytokines at basal and after stroke onset. (A) Quantification of elastase, tumor necrosis factor- α (TNF-α), interleukin-1 β (IL-1β), and interleukin (IL-6) levels in plasma at basal and 6 hours post-reperfusion by ELISA. n=6, 6, 6, 6. **(B)** Basal level quantification of elastase, TNF-α, IL-1β, and IL-6 in the neutrophils by ELISA. n=6, 6**.** Data are from male obese mice and represented as mean ± SD. Statistical Analysis: Two-way ANOVA (A) and unpaired t-test (B). NS, not significant.



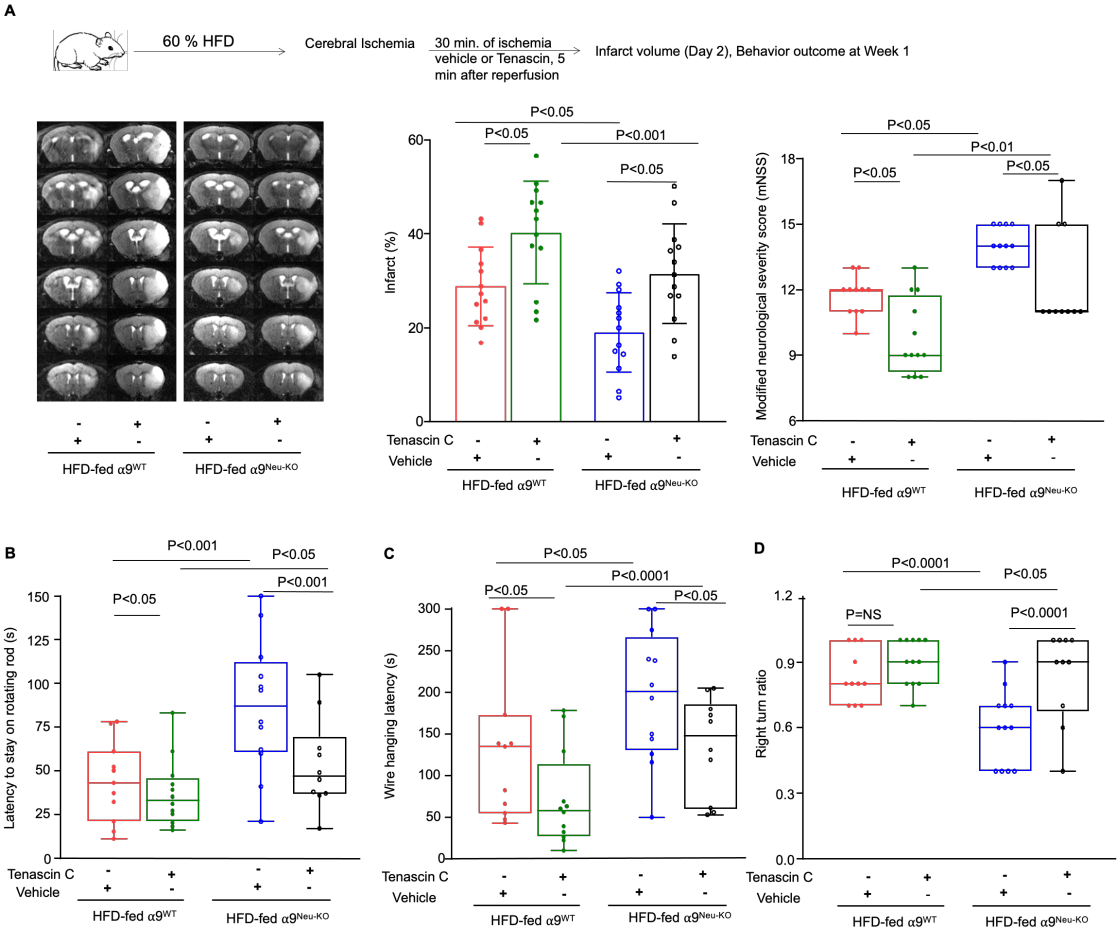
Supplemental Figure 11. Neutrophil-specific α9$^{-/-}$ obese mice exhibited reduced post-ischemic inflammation. (A). Representative immunoblots and densitometric analysis of nuclear factor κB p65 (NFκB p65) in the brain homogenates from the infarcted and peri-infarcted area on day 2 post 30 min ischemia. β-Actin was used as a loading control. n=6, 6. (**B)** Quantification of interleukin-1β (IL-1β) levels by ELISA in brain homogenates. n=6, 6. Data are from male obese mice and represented as mean ± SD. Statistical Analysis: unpaired t-test.



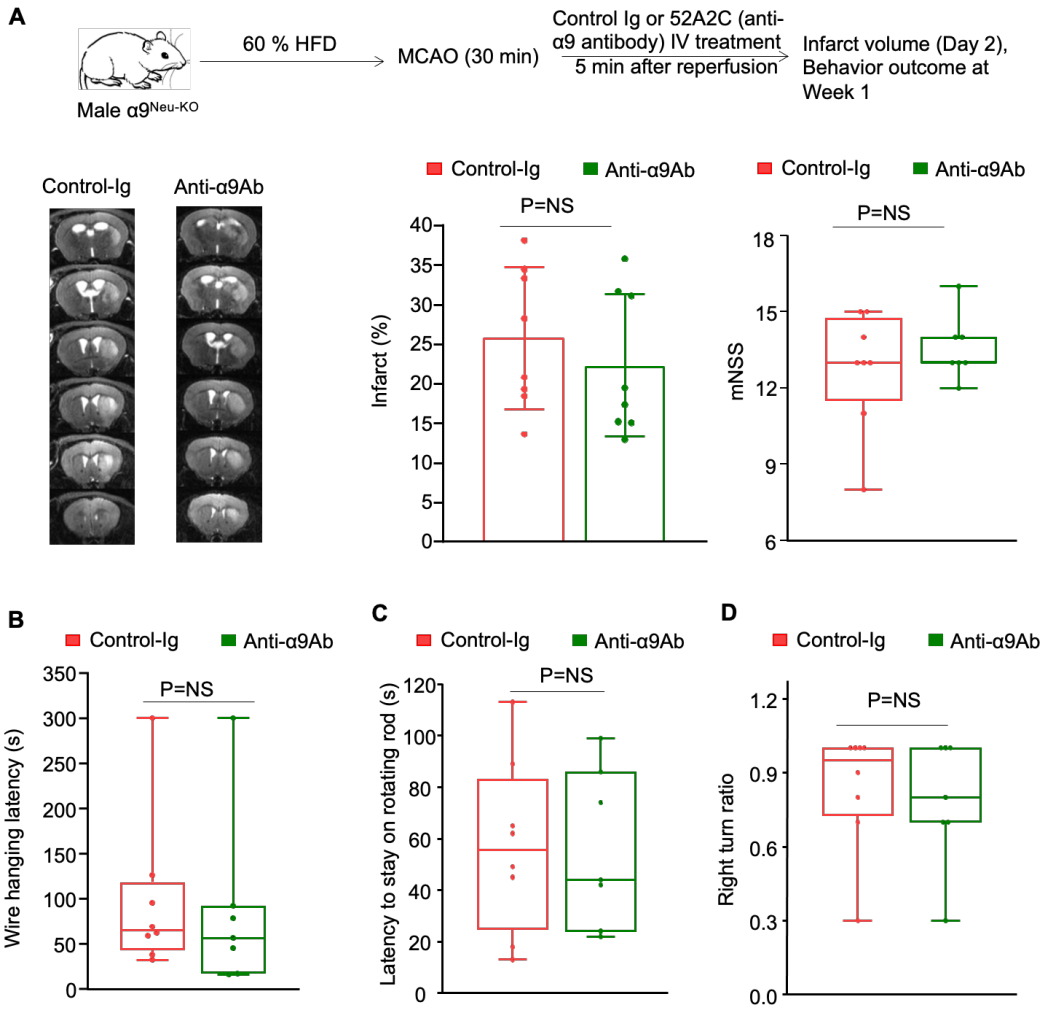
Supplemental Figure 12. Fn-EDA contributes to neutrophil cell-derived α9-mediated stroke exacerbation in obese mice. (A) Schematic of experimental design. **(B)** Modified Neurological Severity Score (mNSS) in the same cohort of mice at day 28 based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch. N=10, 11, 4, 12. (**C, D & E**) Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in hanging-wire test **(C),** fall latency in accelerated rota-rod test **(D)**, and right-turn ratio in corner test **(E)**. N=10, 11, 4, 12. Data are median ± range (functional outcome). It is important to note that n=4 in $\alpha9^{WT}$-BM→ Fn-EDA$^{+/+}$ mice because of high mortality observed in that group. Statistical analysis: two-way ANOVA followed by Holm-Sidak multiple comparisons test (B-E). NS, non-significant.



Supplemental Figure 13. Tenascin C (100 µg/kg, IV) infusion worsened stroke outcome in WT male mice fed a regular chow diet. (A) Left: Representative T2-MRI images from one mouse of each group on day 2. Right: Corrected mean infarct area of each group on day 2 after 30 min MCAO in filament model. **(B)** Modified Neurological Severity Score (mNSS) on day 7. Data are mean ± SD (A) and median ± range (B). n=6, 7. Statistical analysis: unpaired t-test (A) and Mann-Whitney test (B).



Supplemental Figure 14. Infusion of Tenascin C (100 µg/kg, IV) exacerbates stroke outcome in both α9^{WT} and α9^{Neu-KO} male high-fat diet-fed mice. (A) Top: Schematic of experimental design. Bottom (left): Representative T2-MRI images from one mouse of each genotype on day 2 and corrected mean infarct of each genotype. White is the infarct area. n=13, 13, 13, 13. Bottom (right): Modified Neurological Severity Score (mNSS) in the same cohort of mice at weeks 1based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch (higher score indicates a better outcome). n=11, 12, 12, 10. **(B-D)** Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in the hanging-wire test **(B),** fall latency in the accelerated rota-rod test **(C),** and right turn ratio in the corner test **(D)**. n=11, 12, 12, 10. Data are mean ± SD (Infarct) and median ± range (functional outcome). Statistical analysis: Two-way ANOVA followed by Holm-Sidak multiple comparisons test. NS, non-significant.



Supplemental Figure 15. Infarct size and functional outcome were comparable in anti-integrin α9 antibody and control Ig treated α9^{Neu-KO} high-fat diet-fed mice. (A) Top: Schematic of experimental design. Bottom (left): Representative T2-MRI images from one mouse of each genotype on day 2 and corrected mean infarct of each genotype. n=8, 8. Bottom (right): Modified Neurological Severity Score (mNSS) in the same cohort of mice at week 1 based on spontaneous activity, symmetry in the movement of 4 limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch (higher score indicates a better outcome). n=8, 7. **(B-D)** Sensorimotor recovery in the same cohort of mice as analyzed by motor strength in the hanging-wire test **(B),** fall latency in the accelerated rota-rod test **(C),** and right turn ratio in the corner test **(D)**. n=8, 7. Data are mean ± SD (infarct) and median ± range (functional outcome). Statistical analysis: unpaired t-test (infarct) and Mann-Whitney test (functional outcome). NS, non-significant.