Online Supplements

Targeting Myeloid-Cell Specific Integrin α9β1 Inhibits Arterial Thrombosis in Mice

Nirav Dhanesha[*1], Manasa K. Nayak[*1], Prakash Doddapattar[1], Manish Jain[1], Gagan D. Flora[1], Shigeyuki Kon[1], and Anil K. Chauhan[1]

[1]Department of Internal Medicine, Division of Hematology/Oncology, Iowa City, Iowa

[2]Department of Molecular Immunology, Faculty of Pharmaceutical Sciences, Fukuyama University, Hiroshima, Japan

*Both authors contributed equally to work.

Methods

Mice

$\alpha9^{fl/fl}$ mice have been described previously.[1] To generate myeloid-specific $\alpha9^{-/-}$ mice ($\alpha9^{fl/fl}$ LysMCre+), we crossed $\alpha9^{fl/fl}$ mice with LysMCre+ (hemizygous for Cre) mice (Figure S1). $\alpha9^{fl/fl}$ littermates were used as controls. Mice were genotyped by PCR according to protocols from the Jackson Laboratory, and as described previously.[1] Mice were kept in standard animal house conditions with controlled temperature and humidity and had ad libitum access to standard chow diet and water. All the mice used in the present study were on the C57BL/6J background. The University of Iowa Animal Care and Use Committee approved all the procedures.

$FeCl_3$ injury-induced carotid thrombosis

$FeCl_3$ injury-induced carotid thrombosis was assessed by intravital microscopy by the investigator blinded to the genotypes, as per previously published reports.[2,3] Briefly, 8- to 10-

week-old mice were anesthetized using 100-mg/kg ketamine and 10-mg/kg xylazine. Platelets ($2.5X10^9$/kg) were labeled with Calcein Green, AM (3 μM, Thermo Fisher Scientific, catalog#C34852) and were infused through the retro-orbital plexus. Infused platelets were isolated from adult (4-5 months) donor mice of the respective genotype. The common carotid artery was carefully exposed and kept moist by superfusion with warm (~37°C) saline. Whatman paper (0.5 X 1.0 mm^2) saturated with ferric chloride (7.5%) solution was applied topically for 3 minutes, and thrombus formation in the injured carotid vessel was monitored in real-time using a Nikon upright microscope with a Plan Fluor 4X/0.2 objective. Thrombus formation in real-time was recorded using a high-speed electron-multiplying camera for 30 minutes or until occlusion occurred. Videos were evaluated off-line using a Nikon computer-assisted image analysis program. The time to form stable occlusive thrombus was considered as the time required for blood to stop flowing entirely for one minute. The rate of thrombus growth over a period of two minutes was calculated by dividing the thrombus area at time (n) by the area of the same thrombus at time (0) (defined as the time point at which the thrombus diameter first reached 30 μm).

Laser injury-induced mesenteric artery thrombosis

Laser injury-induced mesenteric artery thrombosis was evaluated by the investigator blinded to the genotypes, using the Micropoint laser ablation system (Andor Technology), as per previously published reports.[3,4] In order to facilitate focusing of the laser and to minimize fat surrounding the arterioles, we used young mice (3 to 4 weeks [14-16 g] old males). Platelets ($1.5X10^9$/kg) were labeled with Calcein Green, AM (3 μM, Thermo Fisher Scientific, catalog#C34852) and were infused through the retro-orbital plexus. Infused platelets were isolated from adult (4-5

months) donor mice of the respective genotype. Mesenteric arterioles having a diameter of ~80 to 100 μm (with shear rates of ~1300-1800 s^{-1}) were used for the study. The specific illumination of the area of interest was carried out through the microscope eyepiece. The wavelength of light in the range of 365 to 400 nm with the maximum output of 50 to 500 μJ was used for illumination. The power and frequency of pulses were regulated by software and empirically defined. Thrombus growth in the injured vessel was monitored in real-time by using a Nikon upright microscope with a Plan Fluor 10X/0.3 objective, and thrombus formation over time was recorded using a high-speed EM camera for 3 to 4 minutes. In our experimental setup, in the laser injury model, the thrombus grows to its maximum size in ~1 minute and then gradually disintegrates over time. Videos were evaluated, and mean fluorescence intensity was calculated using a Nikon computer-assisted image analysis program.

Tail bleeding assay

Tail-transection bleeding time was measured as described.[3] Briefly, mice (approximately eight weeks of age) were anesthetized with 100-mg/kg ketamine and 10-mg/kg xylazine 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 stream of blood 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

Bone-marrow derived neutrophils from each genotype were isolated using a density-gradient centrifuge and lysed in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing

0.1% SDS and 4% proteinase inhibitor (complete protease inhibitor cocktail, Roche, catalog# 11836153001). Samples were sonicated for a total of 30 seconds with 10 seconds gap. Lysates were centrifuged at 14000×g for 20 min at 4°C and supernatants were used for the determination of 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: anti-Integrin alpha 9 antibodies [EPR9722] (1:1000, catalog# ab140599) at 4°C overnight, followed by appropriate secondary antibodies (polyclonal goat anti-rabbit IgG, Dako, catalog# P0448) conjugated to horseradish peroxidase (HRP). Enhanced chemiluminescence kit (Thermo Scientific, catalog# 34580) was used for Western blots. All blots were stripped and reanalyzed for the β-actin (anti-beta actin antibody from Abcam, catalog# ab8226) as a loading control. The intensity of the bands was measured by densitometry and quantified using NIH-Image J software.

Immunocytochemistry

Carotid arteries, along with the site of injury (~0.7 mm) were harvested after 30 minutes of 7.5% $FeCl_3$ application and processed for cryostat sectioning. Sections were fixed in 4% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1% Triton x 100 (in PBS). Following blocking with 5% BSA for 1 hour, samples were incubated with antibodies specific for RBC (TER119, Abcam, catalog# ab91113), Neutrophils (Ly6GB.2, Abcam, catalog# ab210204), and neutrophil elastase (Abcam, catalog# ab68672) overnight at 4° C. The samples

were washed and labeled with Alexa Fluor-568-conjugated appropriate secondary antibodies (Invitrogen) for 1hr at room temperature. Sections were counter-stained with Hoechst (5 μg/mL) before mounting, and images were acquired using an Olympus fluorescent microscope (BX51) equipped with a UPlanFLN 20x/0.5 NA objective lens and DP71 color CCD camera. Image analysis was done using ImageJ software. Ly6GB.2 positive neutrophils and neutrophil elastase positive NETs were counted manually. Staining and image acquisition was performed in parallel for the entire set. Measurements were obtained from 3-4 different sections, separated by 70 μm apart, and the mean was calculated per animal.

Neutrophil adoptive-transfer

Neutrophils were isolated from the bone-marrow of the donor male mice using density centrifugation method as described.[2] Neutrophils ($2X10^6$) dissolved in 200 μL saline were injected into the recipient male mice (8-10 weeks old) through the retro-orbital plexus 15 minutes before performing 7.5% $FeCl_3$ injury-induced carotid thrombosis as follows: $\alpha9^{fl/fl}$ mice neutrophils $\rightarrow\alpha9^{fl/fl}$LysMCre+ mice or $\alpha9^{fl/fl}$ LysMCre+ mice neutrophils$\rightarrow\alpha9^{fl/fl}$ mice.

Neutrophil induced platelet aggregation

Pooled neutrophils (n=4 mice) from each genotype (1×10^7 per ml) in HEPES-buffer supplemented with $CaCI_2$ 1.3 mM and $MgCl_2$ 1.0 mM and platelets (from the wild-type mice, 10×10^8 per ml) in Tyrode -Ca^{2+}-Mg^{2+} were suspended to obtain a final concentration of 6×10^6 neutrophils/ml and 2×10^8 platelets/ml. This platelet-neutrophil suspension was stirred (1200 rpm) at 37°C for 5 minutes in a Chrono-log Whole Blood/Optical Lumi-Aggregometer (model 700-2) before the addition of FMLP (1μM, Sigma, catalog# F3506). Aggregation was measured

as the percent change in light transmission, where 100% refers to transmittance through the blank sample.

Cathepsin G release assay

The samples were prepared as discussed above. Cathepsin G in the supernatant was measured using the commercially available kit (Lifespan Bio, Mouse CTSG / Cathepsin G ELISA Kit, catalog# LS-F7218-1).

NETosis assay

Freshly isolated neutrophils from bone marrow were seeded on poly-L lysine-coated coverslips (1 X 10^4 cells/coverslip). Cells were incubated for 60 minutes in a CO_2 incubator. NETosis assay was performed using thrombin (0.1 U/ml) activated platelets (1 X10^7) or TNF-α (40 ng/mL). Cells were incubated for 4 hours in a CO_2 incubator at 37 ^{0}C. 500 µl ice-cold PBS was added to stop the reaction, and the coverslips were placed on ice for 10 minutes. Coverslips were gently drained to discard liquid, and cells were fixed for 15 minutes in ice-cold PBS containing 2% paraformaldehyde at room temperature. The fixed cells were then washed with ice-cold PBS. For specific staining of extracellular nuclear structures, cells were then incubated with PlaNET Green (1:10 dilution: #PLANET-001, Sunshine antibodies) dye for 60 minutes at 4^{0}C. Coverslips were washed with PBS and mounted onto glass slides using a drop of mounting medium containing DAPI (Vector Labs, Cat. #H-1200.), prior to fluorescence microscopy analysis. Samples were analyzed using an Olympus BX51 microscope. For quantitation, two fields at 20x magnification were counted (coverslip edges were avoided). In total 400-600 neutrophils were

counted/field. Neutrophils releasing only extracellular structures (PlaNET Green-positive) were counted per field. Mean was calculated from 2 different fields per animal.

Monocyte depletion

Clodronate liposomes were purchased from the ClodronateLiposomes.org. Clodronate liposome (a single dose of 200 µl) was administered intravenously to induce monocyte depletion. 7.5% $FeCl_3$ injury-induced carotid thrombosis was performed 24 hours after the depletion.

Treatment with anti-integrin α9 antibody

Mice were infused with either anti-α9 antibody (55A2C, 200 µg/mouse, provided by Gene Techno Science Co., Ltd, Japan) or with control Ig isotype (200 µg/mouse, Rockland antibodies and assays, catalog# 610-4107-0500) intravenously, 30 minutes before the arterial thrombosis.

Statistical analysis

Data represent mean ± SEM and analyzed by Student's t-test and repeated measures ANOVA. For analysis, GraphPad Prism software (7.04) was used. Shapiro-Wilk test was used to check normality, and Bartlett's test was used to check equal variance. The results were considered significant at $P<0.05$.

References:

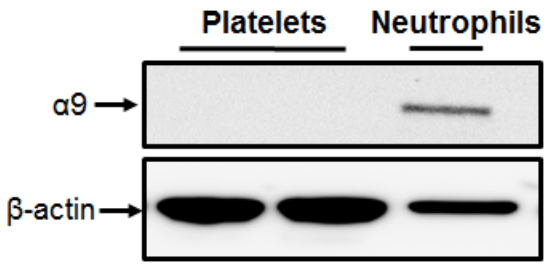
1. Huang XZ, Wu JF, Ferrando R, et al. Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. *Molecular and cellular biology*. 2000;20(14):5208-5215.
2. Dhanesha N, Ahmad A, Prakash P, Doddapattar P, Lentz SR, Chauhan AK. Genetic Ablation of Extra Domain A of Fibronectin in Hypercholesterolemic Mice Improves Stroke Outcome by Reducing Thrombo-Inflammation. *Circulation*. 2015;132(23):2237-2247.
3. Nayak MK, Dhanesha N, Doddapattar P, et al. Dichloroacetate, an inhibitor of pyruvate dehydrogenase kinases, inhibits platelet aggregation and arterial thrombosis. *Blood Adv*. 2018;2(15):2029-2038.
4. Prakash P, Nayak MK, Chauhan AK. P-selectin can promote thrombus propagation independently of both von Willebrand factor and thrombospondin1 in mice. *J Thromb Haemost*. 2016.

Table

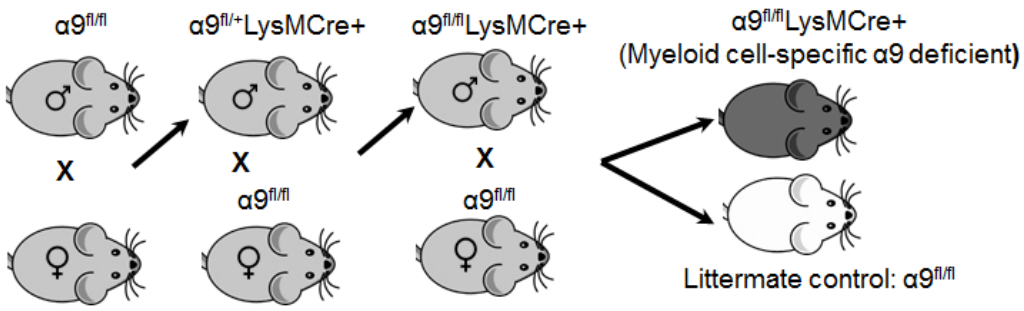
Table S1: Complete blood counts from 8-10 weeks old mice were obtained using an automated veterinary hematology analyzer (ADVIA). Value are expressed as mean ± SEM. N= 5-6 mice/group. P= Non-significant versus control $\alpha 9^{fl/fl}$ mice.

	$\alpha 9^{fl/fl}$	**$\alpha 9^{fl/fl}$LysMCre+**
WBC ($10^3/\mu L$)	13.1±0.9	11.8±0.4
RBC ($10^6/\mu L$)	10.2±0.2	10.5±0.1
HGB (g/dL)	15.9±0.2	16.4±0.2
HCT (%)	47.7±0.8	49.4±0.8
PLT ($10^3/\mu L$)	1074±26	1011±22
Neutrophil ($10^3/\mu L$)	0.5±0.1	0.4±0.1

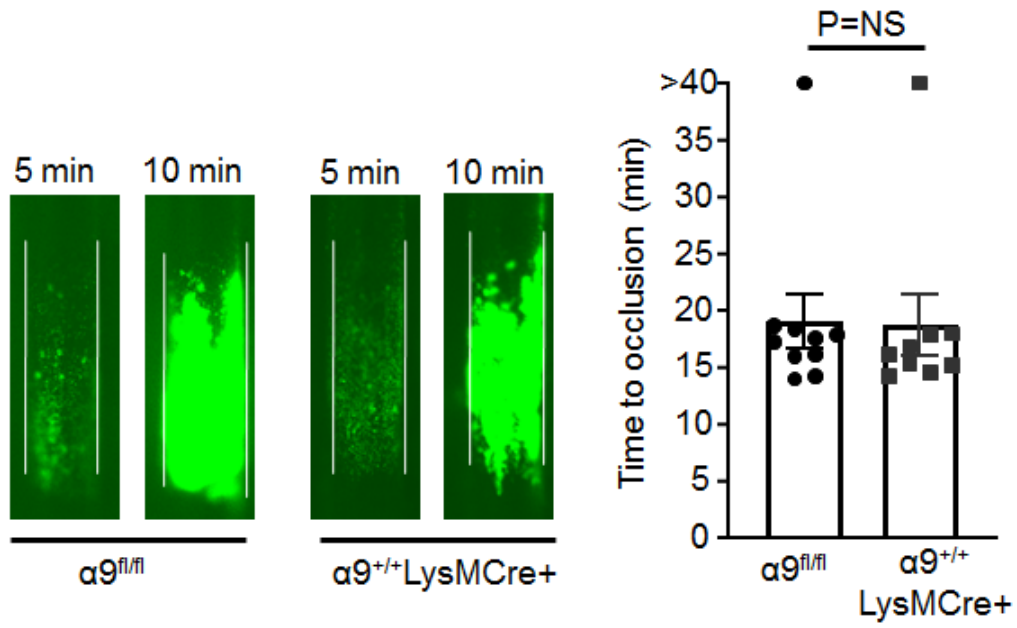
Figures



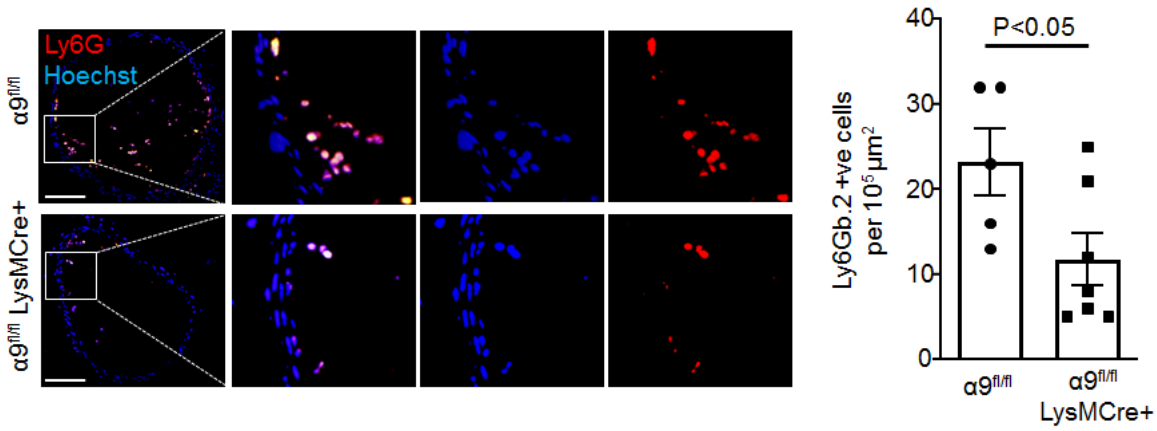
Supplementary Figure 1. Western blot analysis of α9 integrin from bone marrow-derived neutrophils and resting platelets from the wild-type mice.



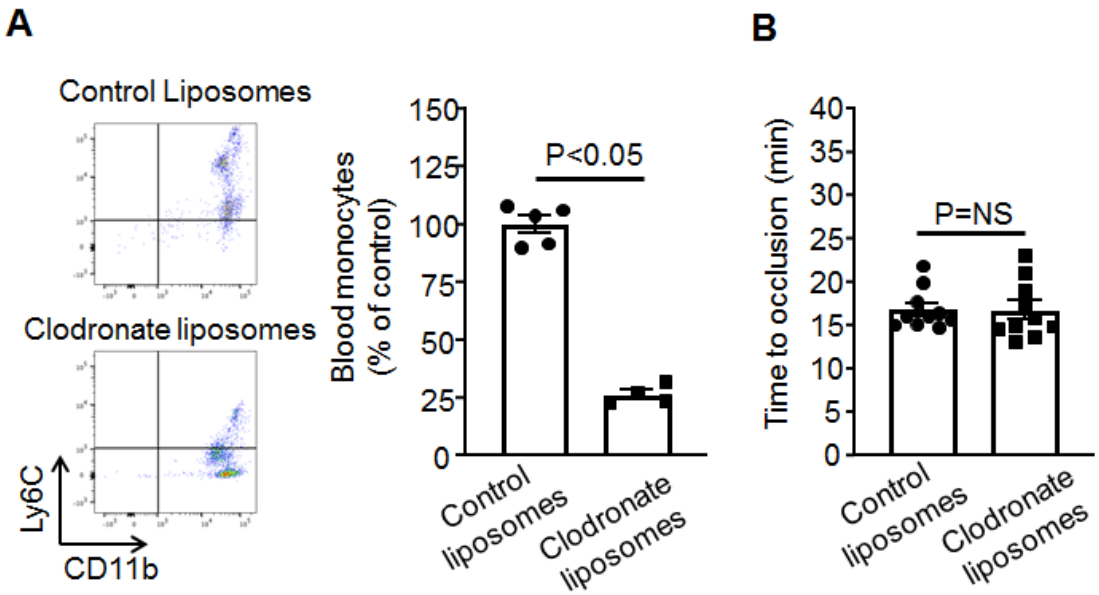
Supplementary Figure 2. Schematic showing generation of myeloid-specific α9 deficient mice.



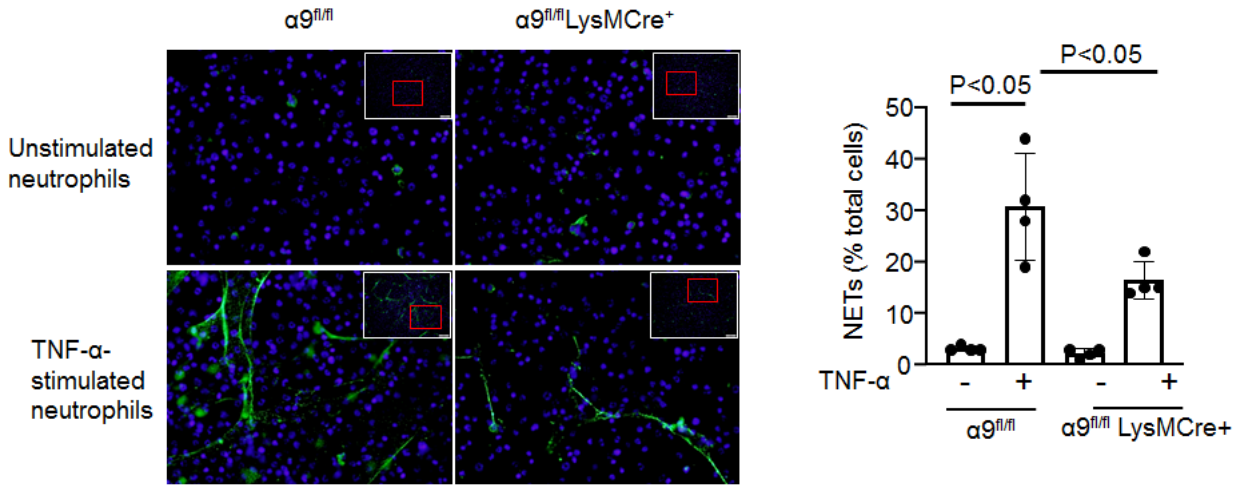
Supplementary Figure 3: The time to complete occlusion was comparable between $\alpha 9^{fl/fl}$ and $\alpha 9^{+/+}$LysMCre+ mice. The left panel shows representative microphotographs of carotid artery thrombus (7.5% FeCl3 injury) as visualized by intravital microscopy. Transfused platelets were labeled ex vivo with calcein green. White line delineates the arteries. The right panel shows time to stable occlusion (n=9-10 mice/group).



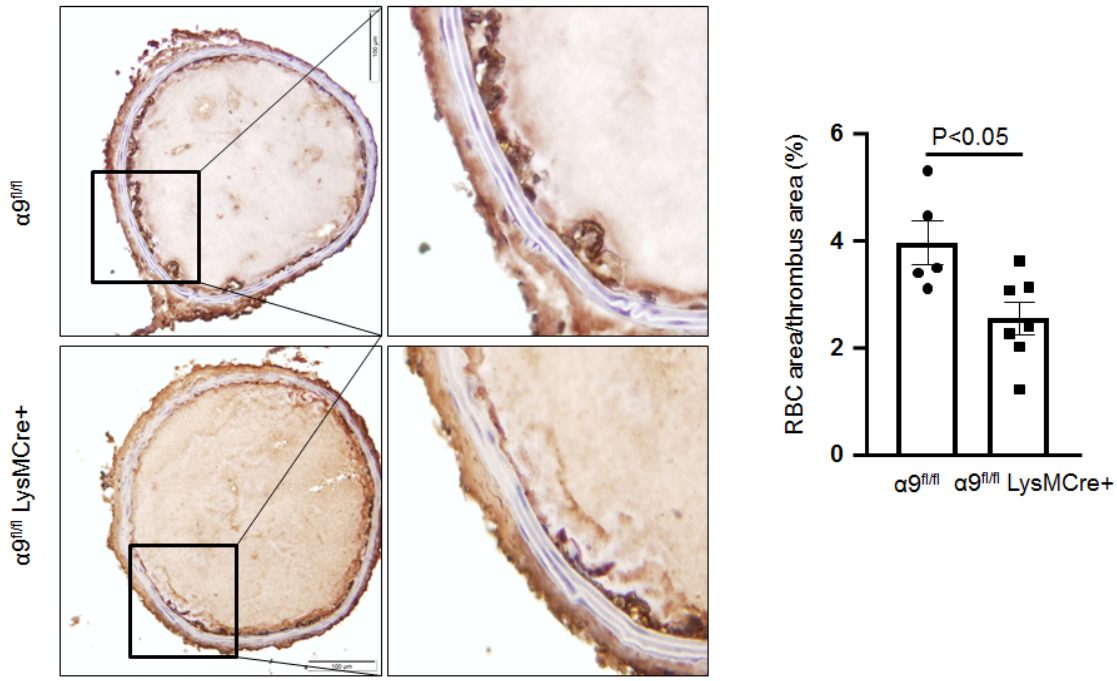
Supplementary Figure 4: Left panels show representative microphotographs of neutrophil (Ly6Gb.2 positive cells) at the site of $FeCl_3$ injury (7.5%). Scale bar; 100 μm. The right panel shows quantification (n=5-7 mice/group). Value for each mouse represents a mean from 3-4 serial sections (each section separated by ~70 μm).



Supplementary Figure 5: Monocyte depletion did not affect time to occlusion in $FeCl_3$ injury-induced thrombosis model. A. Left panels show representative dot plots displaying blood monocytes (Ly6C and CD11b double positive cells) 24 hours after the administration of control liposomes and clodronate liposomes. Right panel shows quantification. **B.** Time to occlusion after carotid artery thrombosis (7.5% $FeCl_3$ injury) in C57BL/6J WT mice treated with either control liposomes and clodronate liposomes.



Supplementary Figure 6. NET (neutrophil extracellular traps) assay was performed by stimulating neutrophils with TNF-α (40 ng/mL). Left panels show representative microphotographs of NETs stained with PlaNET Green (stains extracellular DNA, green), and counter-stained with Hoechst stain (stains nuclei, blue). Boxed region (lower magnification). Red insert in the boxed region is magnified and shown in the microphotographs. The right panel shows quantification of the percentage of cells releasing NETs (n=4 mice/group). Value for each mouse represents a mean from two experiments.



Supplementary Figure 7. Left panels show representative microphotographs of RBC (TER119-positive cells) at the site of $FeCl_3$ injury (7.5%) in an occlusive thrombus. Boxed region is magnified to show TER119-positive cells. The right panel shows quantification (n=5-7 mice/group).