Online Supplements

Pyruvate kinase M2 regulates Neutrophil Hyperactivation and promotes Cerebral Thrombo- inflammation: Therapeutic Implications for therapy in Acute Ischemic Stroke

Short title: Targeting PKM2 improves stroke outcome in mice.

Nirav Dhanesha,[1] Rakesh B. Patel,[1] Prakash Doddapattar,[1] Madankumar Ghatge,[1] Gagan D. Flora,[1] Manish Jain,[1] Daniel Thedens,[2] Heena Olalde,[3] Mariia Kumskova,[1] Enrique C. Leira,[3,4,5] Anil K. Chauhan[1]

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

[2]Department of Radiology, University of Iowa, Iowa City, Iowa, USA.

[3]Department of Neurology, University of Iowa, Iowa City, Iowa, USA

[4]Department of Neurosurgery, University of Iowa, Iowa City, Iowa, USA

[5]Department of Epidemiology, University of Iowa, Iowa City, Iowa, USA

MATERIALS AND METHODS

Mice

$PKM2^{fl/fl}$ strain was initially provided by Matthew G. Vander Heiden, MIT, Boston.[1] To generate myeloid cell-specific PKM2-deficient mouse ($PKM2^{fl/fl}$ $LysMCre^{+/-}$), $PKM2^{fl/fl}$ mouse was crossed with $LysMcre^{+/+}$ mouse (**Figure S1**). To generate myeloid-specific $PKM2^{-/-}$ mice on hyperlipidemic $Apoe^{-/-}$ background ($PKM2^{fl/fl}LysMCre^{+/-}Apoe^{-/-}$), $PKM2^{fl/fl}$ $Apoe^{-/-}$ mouse was crossed with $LysMCre^{+/-}Apoe^{-/-}$ mouse. Littermates $PKM2^{fl/fl}$ $LysMCre^{-/-}$ and $PKM2^{fl/fl}LysMCre^{-/-}Apoe^{-/-}$ mice were used as controls. 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 and studies were performed according to the current Animal Research: Reporting of *In Vivo* Experiment guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

Quantitative reverse transcription (RT) polymerase chain reaction (PCR)

WT mice were subjected to brain ischemia/reperfusion injury by transiently occluding the right middle cerebral artery for 60 minutes. Peripheral blood neutrophils were isolated (3, 6- and 23-hours post-reperfusion) using a density gradient centrifuge. Total RNA from the neutrophils was isolated using the RNeasy mini kit (Qiagen, catalog#74104). Total RNA (~400 ng) was reverse-transcribed using iScript™ Reverse Transcription Reagent Kit (BIO-RAD, catalog# 1708840). PCR amplification of the cDNA (100 ng) was performed with the Applied Biosystems 7900HT, and real-time PCR (total volume 20 µl) was done at the University of Iowa Core genomics division. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the comparative threshold cycle (ΔΔCT) method with values normalized to GAPDH. Primers for qRT-PCR for the indicated genes are included in Supplemental **Table 7**.

Filament stroke model

Focal cerebral ischemia was induced by transiently occluding the right middle cerebral artery for 30 or 60 minutes, as described [2,3]. All surgeries were performed by the person blinded to the experimental groups. Mice were anesthetized with isoflurane (1–1.5%) mixed with medical air. After midline incision, the right common carotid artery was temporarily ligated, and a filament (6.0 siliconized filament from Doccol Corp., catalog# 602256PK10) was inserted from the external carotid artery and advanced into the internal carotid artery up to the origin of the middle

cerebral artery. Reperfusion was achieved by removing the filament after 30 or 60 minutes. Throughout the surgery, the body temperature of the mice was 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 (rCBF) were included in the study.

Embolic stroke model

The embolic stroke model was performed as previously described [4,5]. Briefly, an autologous embolus clot was prepared using arterial blood was supplemented with human fibrinogen (2 mg/ml, Sigma, Catalog#F3879) and immediately clotted in a PE-50 tube for 4 hours at room temperature, followed by storage at 4°C overnight. On the following day, the clot was washed in PBS by several passages from a PE-10 tube and transferred to a modified PE-10 catheter. Animals were anesthetized with 1–1.5% isoflurane during the surgery. The catheter containing a single 15 mm fibrin-rich clot was then introduced into the external carotid artery and advanced to the internal carotid artery. After the embolization, the catheter was removed, and the external carotid artery was blocked by cauterization. Laser Doppler flow monitoring was used to confirm the induction of ischemia. Throughout the surgery, the body temperature of the mice was maintained at 37°C ± 1.0 using a heating pad. The right jugular vein was cannulated for the administration of rtPA (Cathflo from Genentech, 10 mg/kg, 10% volume by bolus, and remaining slow infusion for 30 minutes, 60 minutes post embolization). Buprenorphine (0.1 mg/kg, SC) was administered as an analgesic agent every 6-12 hours for 48 hours post-surgery.

Neutrophil extracellular traps assay

Peripheral neutrophils were isolated from the whole blood using density gradient centrifugation after 6 hours of reperfusion and seeded on poly-L lysine-coated coverslips (1 X 10^4 cells/coverslip). Cells were incubated for 60 minutes in a CO2 incubator. The NETs assay was performed using a suboptimal concentration of PMA (Phorbol 12-myristate 13-acetate, Sigma, Catalog# P8139, 10 ng/mL). Cells were incubated for 4 hours in a CO2 incubator at 37^0C. 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, Sunshine antibodies, catalog# PLANET-001) dye for 60 minutes at 4^0C. Coverslips were washed with PBS and mounted onto glass slides using a drop of mounting medium containing DAPI (Vector Labs, catalog#. #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.

MRI imaging, and infarct area quantification

MRI was performed on day-1 post-reperfusion as per previously described protocol [6]. Briefly, 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. 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 is as follows to correct for brain swelling due to edema. 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. **Bederson Scale:** The Bederson scale is a global neurological assessment that was developed to measure neurological impairments following stroke [7]. Neurological outcomes were assessed by an observer blinded to the experimental groups and were scored on a four-point scale as previously described [2]: 0, no observable neurological deficit (normal); 1, failure to extend left forepaw on lifting the whole body by the tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, leaning to the contralateral side at rest (severe); 4, no spontaneous motor activity. **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 indicates a better outcome. The mNSS includes a composite

score of six different tests, which are: spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, and responses to vibrissae touch as previously mentioned [8]. **Cylinder test:** The cylinder test [9] was used to assess forelimb use and rotation asymmetry. Mice were placed in a clear cylinder videotaped for 5 minutes. Forelimb use of the first contact against the wall after rearing and during lateral exploration was analyzed. The asymmetry index = (nonimpaired forelimb movement - impaired forelimb movement) / (nonimpaired forelimb movement + impaired forelimb movement + both movement).

Accelerated rotarod test: The accelerated rotarod (Harvard Apparatus, model#LE8205) was used to access post-stroke motor coordination. For this, mice were trained for 3-5 days on rotarod rotating at 4 RPM such 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^{o} 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, stand up, then turn 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 of 20 turns were recorded. This test is used to detect sensorimotor dysfunction. The right turn ratio was calculated as: number of right turns /20.

Mice that did not respond to the particular test (for example mice that did not move in either direction in corner test or severely affected mice not able to perform rotarod test) were not included in the particular analysis. All the mice that successfully completed the test were included in the analysis.

Laser speckle contrast imaging

To assess post-reperfusion 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, and 120-minute post-reperfusion. Blood fluxes were measured in the middle cerebral artery supplied region, and fluxes were expressed in arbitrary units using a 12-color palette.

Bone marrow transplantation

Bone marrow transplantation (BMT) was performed at 7-8 weeks of age as described [4]. All mice were on the C57BL/6J background. Recipient mice were irradiated with two 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 ($1 \text{ X } 10^7$) were suspended in sterile PBS and injected 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. We performed two different sets of BMT

experiments: 1) irradiated LDLr$^{-/-}$ mice reconstituted with BM from PKM2$^{fl/fl}$ donors (PKM2$^{fl/fl}$ BM→ LDLr$^{-/-}$ mice), 2) irradiated LDLr$^{-/-}$ mice reconstituted with BM from PKM2$^{fl/fl}$ LysMCre$^{+/-}$ donors (PKM2$^{fl/fl}$ LysMCre$^{+/-}$BM→ LDLr$^{-/-}$ mice). BMT success was analyzed after 4 weeks by PCR to check the 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 an automated veterinary hematology analyzer (ADVIA) to ascertain that BMT did not affect the number of BM-derived blood cells.

Immunofluorescence

Immunostaining was performed in paraffin-embedded sections from the brain (23 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 occluded vessels in brain sections was counted by the person blinded to the

experimental groups. The thrombotic index was calculated by dividing occluded vessels from a total number of vessels.

Immunohistochemistry

Brain cortical sections (obtained after 23 hours of reperfusion for neutrophils and after 3 days for macrophages) were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval as described [2]. Briefly, sections were blocked with 5% serum at room temperature (RT), from the species in which the secondary antibody was raised. Endogenous peroxidase activity was quenched with 0.1% hydrogen peroxide in methanol for 15 min. Sections were stained with primary antibodies for neutrophil (rat anti-mouse Ly6B.2; 1:100; Bio-rad catalog# MCA771G), and macrophages (anti-CD68 antibody Abcam catalog #ab125212) in the presence of 5% rabbit serum. After overnight incubation at 4˚C, slides were washed with PBS for 5 minutes and incubated with biotinylated secondary antibody for 1 hour at RT. Slides were then incubated with streptavidin-HRP for 40 minutes at RT, washed, and incubated with DAB substrate until color develops. Slides were then washed and counterstained with hematoxylin, mounted using an aqueous mounting medium, and examined under a light microscope (Olympus). For macrophage staining, Alexa fluor-tagged secondary antibody was used and imaging was done using fluorescent microscope (Olympus BX51). Incubation without primary antibodies and with isotype-matched immunoglobulins was used as a negative control for immunostaining.

Quantification: In four different regions of the infarct and surrounding area, extravascular neutrophils (400X magnification) were quantified by counting the immunoreactive cells (brown color staining). NIH Image J software (with the plugin for individual cell analysis) was used for

neutrophil quantification. Each data represents a mean of 16 fields from 4 serial sections (separated by 30 μm).

Co-immunoprecipitation

Cells were washed in PBS and spun down at 3000 rpm for 10min at 4^0 C. For nuclear extraction, nuclear and cytoplasmic fractions were isolated using the NE-PER (Nuclear and Cytoplasmic Extraction kit, Invitrogen), following the manufacturer's instructions. Nuclear extracts were then incubated for 30 min on ice and spun down at 12000 rpm for 20 min. Supernatant was precleared by incubation with protein A/G agarose beads (Santa Cruz, Catalog# sc-2003) (1:50) for 45 minutes at 4°C. The beads were pelleted down by centrifugation at 3000 rpm for 5 minutes, and the supernatant fraction was incubated with anti-PKM2 (1:50, Cell Signaling Technologies, catalog#3198S) antibody or anti-STAT3 (1:50, Cell Signaling Technologies, catalog#30835S) antibody or control IgG (1 μg) at 4°C overnight. Immunoprecipitated beads were then spun down at 3000 rpm for 3 min. Supernatant was collected as input and beads were washed with RIPA and used for immunoblotting experiments.

Western blot

Neutrophils, monocytes, and brain tissue proteins were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail. For nuclear extraction experiments, nuclear and cytoplasmic fractions were isolated using the NE-PER (Nuclear and Cytoplasmic Extraction kit, Invitrogen), following the manufacturer's instructions. Peripheral neutrophils (collected 6 hours post-reperfusion) and brain cortical tissue was (collected 23 hours post-reperfusion) from the infarcted and surrounding areas and 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 for the determination of protein content (by Lowry method) and subsequent Western blot analysis. For nuclear extraction experiments, nuclear and cytoplasmic fractions were isolated using the NE-PER (Nuclear and Cytoplasmic Extraction kit, Invitrogen), following the manufacturer's instructions. 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-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), phospho STAT3 (1:1000, Cell Signaling Technologies, catalog#9145S), STAT3 (1:1000, Cell Signaling Technologies, catalog#1260) and anti-CD41 (1:1000, GeneTex, Catalog# GTX113758) 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. Cytoplasmic and nuclear protein were normalized with GAPDH (1:1000, Cell Signaling Technologies, catalog#9131) and Lamin B1 (1:1000, Abcam, catalog# ab65986), respectively. Densitometric analysis of the gels was done using ImageJ software.

ELISA assay for Cytokines, Elastase, and NFkB p65 Transcription Factor Assay

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 (TNF-α catalog# MTA00B, IL-6 catalog# M6000B, IL-1β catalog#MLB00C, Elastase catalog# MELA20 all from R&D Systems) according to the manufacturer's instructions. NFkB p65 Transcription Factor Assay Kit was from Abcam (Catalog# ab133112). For brain cytokine quantification, after 23 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 for the determination of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) with commercially available mouse ELISA kits (TNF-α catalog# MTA00B, IL-6 catalog# M6000B, and IL-1β catalog#MLB00C, all from R&D Systems) according to the manufacturer’s instructions.

Flow cytometric analysis of Myeloperoxidase (MPO) in peripheral neutrophils

After 6 hours of reperfusion, peripheral neutrophils were isolated from the whole blood using density gradient centrifugation. Neutrophils were stained with anti-mouse MPO (1:50, ThermoFisher Catalog # BS-4943R) or 30 minutes at room temperature in phosphate-buffered saline supplemented with 5% fetal bovine serum. After 30 minutes, the cells were washed, stained with secondary antibody (goat anti-mouse IgG Alexa flour-546 (1:400, Invitrogen,

catalog# A11003), and subsequently fixed in 200 μL of 0.5% paraformaldehyde and analyzed on Becton Dickenson LSRII.

Quantification of glycolytic rate in neutrophils

The glycolytic rate in mouse neutrophils were assessed on Seahorse extracellular flux analyzer, XF96 (Sea-horse Bioscience, Chicopee, MA) at the University of Iowa core facility. The 400,000 neutrophils per well were suspended in seahorse media containing 5mM glucose, 1mM sodium pyruvate and 0.5mM HEPES at pH 7.4. The basal respiration values were noted for 15 mins before injecting the Rot/AA (0.5 μM). Then OCR and ECAR values were before and after (20 mins) the addition of PMA at 100 and 250 nM). Finally, 2-DG was injected, and values were measured for 40 mins. Based on these data, we calculated glycolytic proton efflux rate (glycoPER) and compensatory glycolysis.

ML-265 treatment

Formations of ML-265 (Cayman Chemicals Catalog# 13942) was prepared using 2.5% DMSO and 10% solutol and was administered once at the dose of 10,25 and 50 mg/kg, intraperitoneally. Control mice received the same volume of vehicle (2.5% DMSO and 10% solutol, intraperitoneally).

Statistical analysis

Results are reported as mean ± SEM except for the neurological scores (Bederson score and mNSS), and sensorimotor test (cylinder 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 Holm-Sidak's multiple comparisons test (for normally distributed data) and Mann Whitney test or Two-Way Repeated Measures ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparisons test (for not normally distributed data). $P<0.05$ was considered to be statistically significant.

References:

1. Israelsen WJ, Dayton TL, Davidson SM, et al. PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. *Cell*. 2013;155(2):397-409.
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. Dhanesha N, Prakash P, Doddapattar P, et al. Endothelial Cell-Derived von Willebrand Factor Is the Major Determinant That Mediates von Willebrand Factor-Dependent Acute Ischemic Stroke by Promoting Postischemic Thrombo-Inflammation. *Arterioscler Thromb Vasc Biol*. 2016;36(9):1829-1837.
4. Dhanesha N, Chorawala MR, Jain M, et al. Fn-EDA (Fibronectin Containing Extra Domain A) in the Plasma, but Not Endothelial Cells, Exacerbates Stroke Outcome by Promoting Thrombo-Inflammation. *Stroke*. 2019:STROKEAHA118023697.
5. Dhanesha N, Schnell T, Rahmatalla S, et al. Low-Frequency Vibrations Enhance Thrombolytic Therapy and Improve Stroke Outcomes. *Stroke*. 2020;51(6):1855-1861.
6. Dhanesha N, Vazquez-Rosa E, Cintron-Perez CJ, et al. Treatment with Uric Acid Reduces Infarct and Improves Neurologic Function in Female Mice After Transient Cerebral Ischemia. *J Stroke Cerebrovasc Dis*. 2018;27(5):1412-1416.
7. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986;17(3):472-476.
8. Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke*. 1995;26(4):627-634; discussion 635.
9. Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD, McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. *Exp Neurol*. 2004;187(1):94-104.

Patient #	Age	Sex	TICI score	Treatment	Site of tissue infarction within MCA territory
1	59	F	3	MT and No rtPA	Right insula, basal ganglia, corona radiata
2	73	F	2C	MT and No rtPA	Left basal ganglia
3	65	F	2B	MT and No rtPA	Left basal ganglia, scattered frontal, parietal and occipital
4	83	F	2B	MT and No rtPA	Right caudate, corona radiata, middle frontal gyrus and temporal gyrus.
5	66	M	3	MT and No rtPA	Left external capsule
6	69	M	2B	MT and No rtPA	small scattered left middle cerebral artery territory

Supplementary Table 1: Stroke patients' characteristics. MT: Mechanical thrombectomy

	PKM2$^{fl/fl}$	*PKM2*$^{fl/fl}$ *LysMCre*$^{+/-}$	P value (vs PKM2$^{fl/fl}$)	*PKM2*$^{fl/fl}$ *Apoe*$^{-/-}$	*PKM2*$^{fl/fl}$ *LysMCre*$^{+/-}$ *Apoe*$^{-/-}$	P value (vs PKM2$^{fl/fl}$ Apoe$^{-/-}$)
Plasma total cholesterol (mg/dL)	73.2±5.3	69.1±7.5	P=NS	216.1±11.1	232.7±15.6	P=NS
Plasma Triglycerides (mg/dL)	38.5±5.1	45.2±8.3	P=NS	38.6±3.4	41.21±5.0	P=NS
Body weight (g)	22.6±0.6	21.9±0.7	P=NS	23.4±0.7	22.9±1.1	P=NS

Supplementary Table 2. Total plasma cholesterol, plasma triglycerides concentrations were measured from each male mouse using enzymatic colorimetric assays according to the manufacturers' instructions. Values are expressed as mean ± SEM. N= 5 mice/group.

	PKM2$^{fl/fl}$	*PKM2$^{fl/fl}$ LysMCre$^{+/-}$*	P value (vs . PKM2$^{fl/fl}$)	*PKM2$^{fl/fl}$ Apoe$^{-/-}$*	*PKM2$^{fl/fl}$ LysMCre$^{+/-}$ Apoe$^{-/-}$*	P value (vs. PKM2$^{fl/fl}$ Apoe$^{-/-}$)
WBC (10^3/µL)	8.6±1.4	8.1±1.7	P=NS	9.2±3.1	9.4±2.1	P=NS
RBC (10^6/µL)	9.9±0.7	9.7±0.7	P=NS	10.3±0.7	10.3±0.4	P=NS
HGB (g/dL)	14.2±1.4	14.4±1.9	P=NS	16.2±0.9	16.2±0.6	P=NS
HCT (%)	50.8±3.8	49.3±3.7	P=NS	47.8±2.7	47.5±1.5	P=NS
PLT (10^3/µL)	1272±138	1161±76	P=NS	1037±38	964±37	P=NS
Neutrophil (10^3/µL)	0.4±0.2	0.5±0.2	P=NS	0.3±0.1	0.4±0.1	P=NS
Monocytes (10^3/µL)	6.5±0.9	7.1±1.9	P=NS	7.2±0.9	5.5±1.7	P=NS

Supplementary Table 3: Complete blood counts from 8-10 weeks old male mice were obtained using automated veterinary hematology analyzer (Advia). Values are expressed as mean ± SEM. N= 4-5 mice/group.

	PKM2$^{fl/fl}$ Apoe$^{-/-}$	*PKM2$^{fl/fl}$LysMCre$^{+/-}$ Apoe$^{-/-}$*	P value (vs PKM2$^{fl/fl}$ Apoe$^{-/-}$)
Ischemia LDF (% of baseline)	22.6±2.6	21.8±4.5	P=NS

Supplementary Table 4: Laser Doppler Flowmetry (LDF) was similar among groups during and after ischemia. Values are expressed as mean ± SEM. N= 4-5 male mice/group.

	Before ischemia		
	PO_2 mm Hg	**PCO_2 mm Hg**	**pH**
$PKM2^{fl/fl}$ $Apoe^{-/-}$	99.7 ± 21.2	55.7±8.0	7.412 ± 0.05
$PKM2^{fl/fl}LysMCre^{+/-}$ $Apoe^{-/-}$	85.1±16.1	53.4±12.0	7.404 ± 0.04
P value vs control	P=NS		
	After 60 min ischemia		
	PO_2 mm Hg	**PCO_2 mm Hg**	**pH**
$PKM2^{fl/fl}$ $Apoe^{-/-}$	98.3±21.0	70.2±21.5	7.381±0.03
$PKM2^{fl/fl}LysMCre^{+/-}$ $Apoe^{-/-}$	87.3±22.9	78.5±22.4	7.229±0.06
P value vs control	P=NS		

Supplementary Table 5. Physiological parameters were similar among groups during the procedure. N= 5 male mice/group. Values are expressed as mean ± SEM.

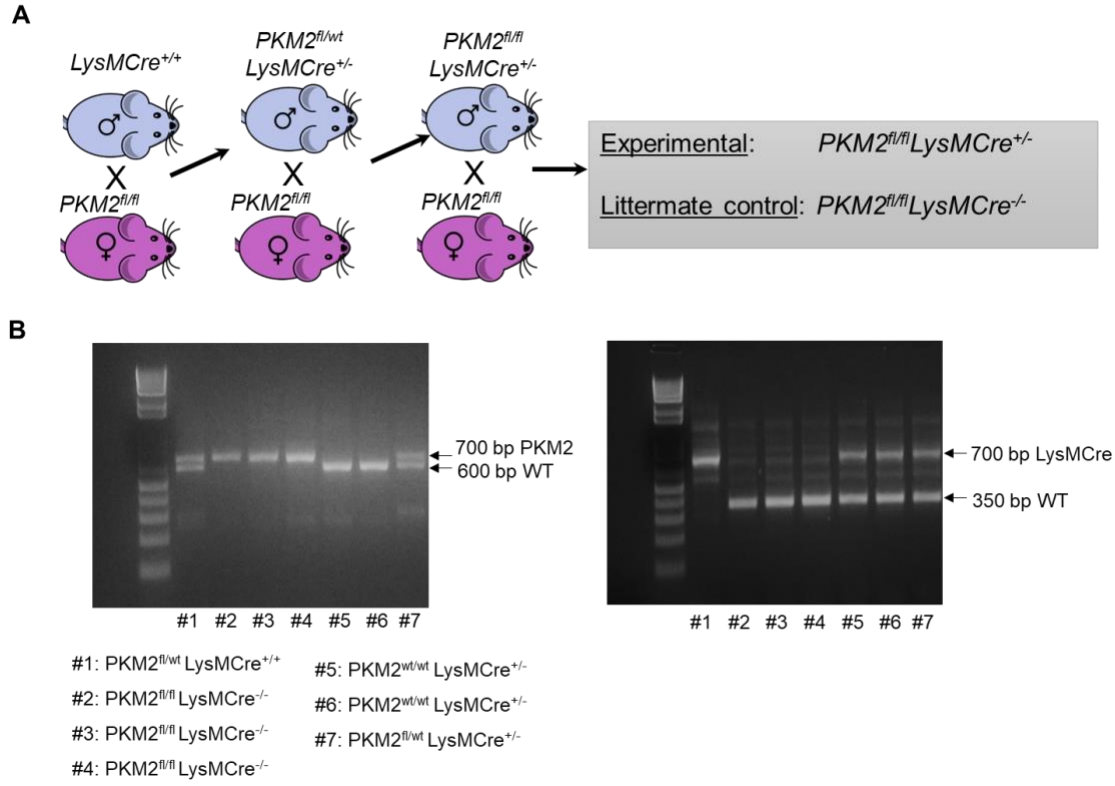
	$PKM2^{fl/fl}$ BM ➔ $LDLr^{-/-}$	*$PKM2^{fl/fl}$ $LysMcre^{+/-}$ BM ➔ $LDLr^{-/-}$*	P value (vs. control)
WBC (10^3/µL)	13.5±1.0	15.1±1.3	P=NS
RBC (10^6/µL)	9.5±0.1	9.6±0.3	P=NS
HGB (g/dL)	15.2±0.3	15.1±0.6	P=NS
HCT (%)	46.3±0.3	46.4±1.2	P=NS
PLT (10^3/µL)	841±35	993±64	P=NS
Neutrophil (10^3/µL)	0.7±0.2	0.5±0.1	P=NS
Monocytes (10^3/µL)	7.9±0.8	9.8±1.3	P=NS

Supplementary Table 6: Complete blood counts from 8- 10 weeks old mice were obtained using automated veterinary hematology analyzer (Advia). Value are expressed as mean ± SEM. N= 4 male mice/group.

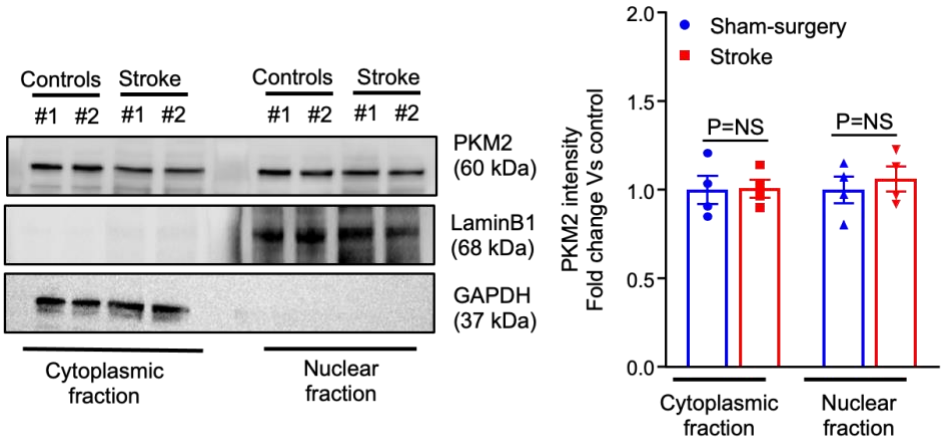
	Forward	*Reverse*
MPO	GAGCAGGACAAATACCGCACCA	AGAGAAGCCGTCCTCATACTCC
Elastase	CAGGAACTTCGTCATGTCAGCAG	AGCCATTCTCGAAGATCCGCTG
MMP9	GCTGACTACGATAAGGACGGCA	TAGTGGTGCAGGCAGAGTAGGA
HIF1α	CCTGCACTGAATCAAGAGGTTGC	CCATCAGAAGGACTTGCTGGCT
IL-1β	CACAGAAGCACCATCCAGT	AGATGCACACCCAGAAGTG
P65	CAGGTGTCTGCCTAGTCCTC	TTCCACACGTTTTCCTAAGC

Supplementary Table 7: Primer sequence

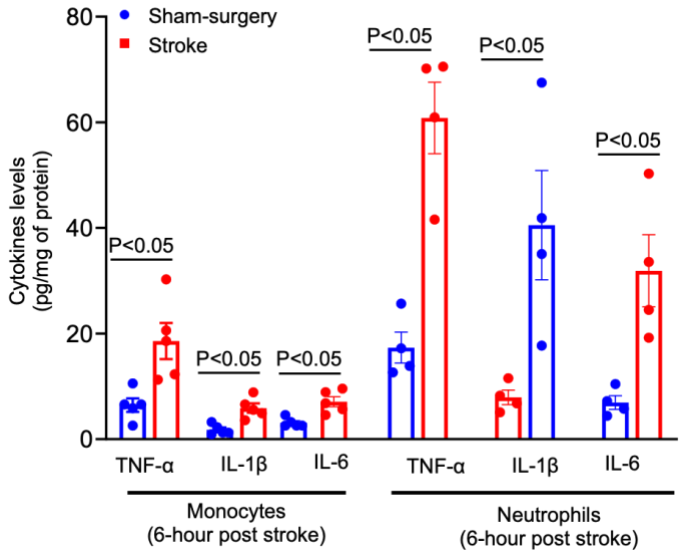
Supplementary Figures



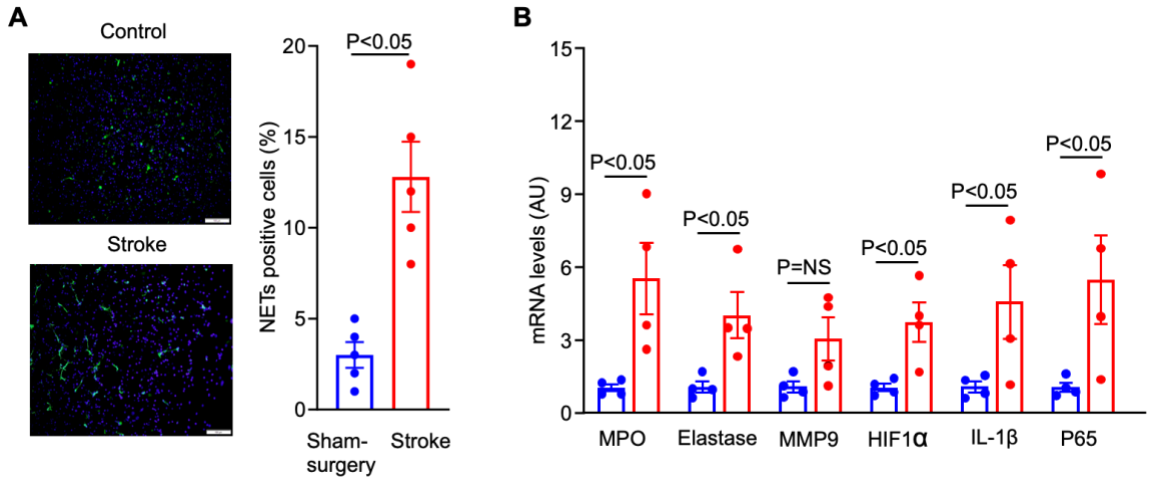
Supplemental Figure 1. (A) Breeding strategy used to generate myeloid cell specific PKM2$^{-/-}$ mice. **(B)** Representative genomic PCR images confirming PKM2$^{fl/fl}$ and LysMCre gene.



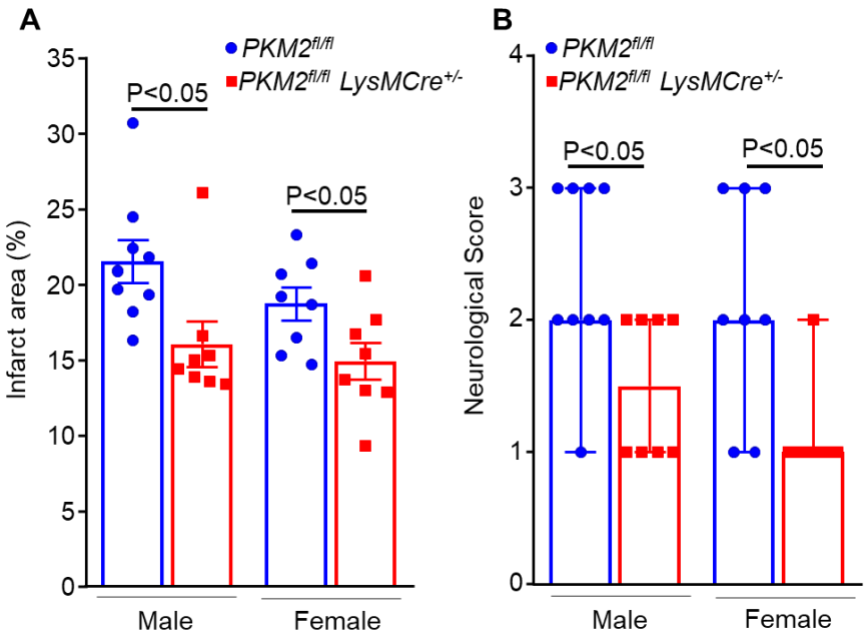
Supplemental Figure 2. Nuclear PKM2 levels were comparable in peripheral monocytes following a stroke vs. sham in wild-type mice. Left: Western blot analysis of PKM2 in the cytosolic and nuclear fraction from the peripheral monocytes of male mice. **Right:** The quantitative data of cytosolic and nuclear PKM2 intensity (normalized to the intensity LaminB1/GAPDH at each time point). Data are mean ± SEM, n=4. Statistical analysis: Two-Way Repeated Measures ANOVA (Kruskal-Wallis test) followed by Fisher's LSD test



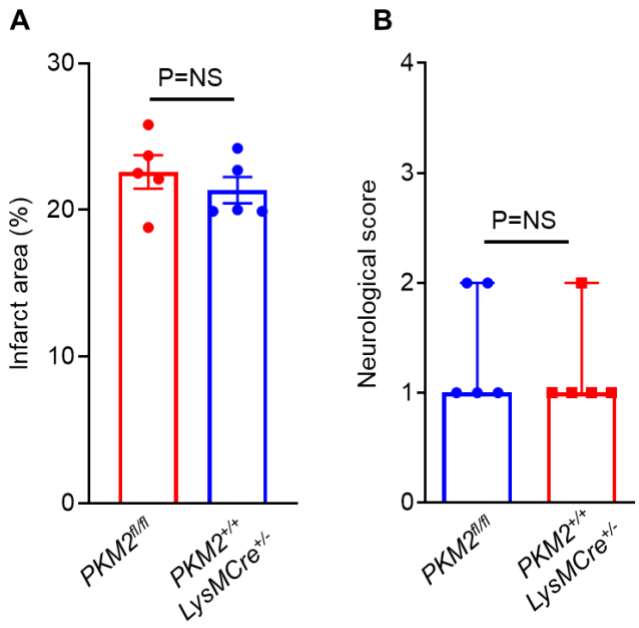
Supplemental Figure 3. Comparison of inflammatory cytokines in peripheral monocytes and neutrophils post stroke. Peripheral neutrophils and monocytes were isolated from the male WT mice that underwent 60 minutes of cerebral ischemia followed by 6 hours of reperfusion and from mice with sham surgery (control). TNF-α, IL-1β and IL-6 levels in monocytes and neutrophils isolated 6-hour post-reperfusion from each group as analyzed by ELISA. TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6. Data are mean ± SEM. Statistical analysis: unpaired t-test.



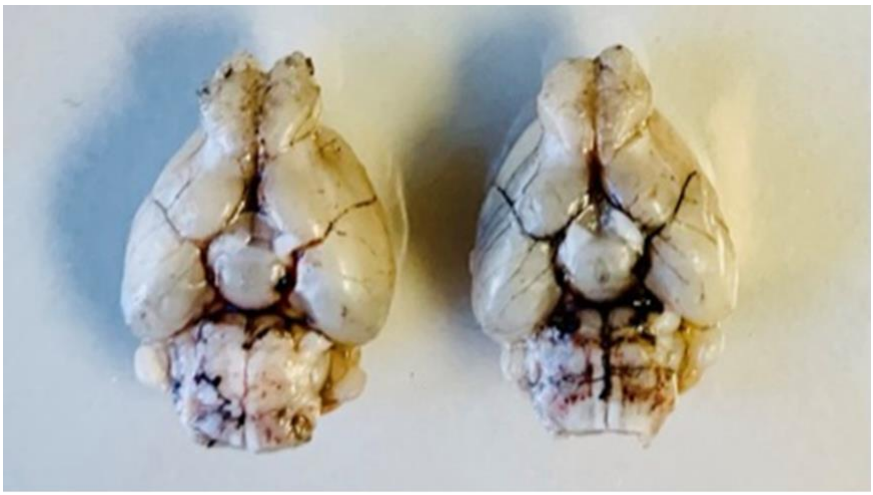
Supplemental Figure 4. Neutrophil hyperactivation in mice with acute ischemic stroke. Peripheral neutrophils were isolated from the male WT mice that underwent 60 minutes of cerebral ischemia followed by 6 hours of reperfusion and from mice with sham surgery (control). **(A)** Immunofluorescence analysis of NETs from the neutrophils isolated 6-hour post-reperfusion. Neutrophils were stimulated with suboptimal concentration of PMA (10 ng/mL) and NETs were visualized by SYTOX Green stain. Scale bar 100 µM. Quantification is shown on right. PMA, phorbol 12-myristate 13-acetate. **(B)** Gene expression analysis from the neutrophils isolated 6-hour post-reperfusion as analyzed by RT-PCR. MPO, myeloperoxidase; MMP9, matrix metallopeptidase 9; HIF1 α, Hypoxia-inducible factor 1-alpha. Data are mean ± SEM. Statistical analysis: unpaired t-test.



Supplemental Figure 5. PKM2 deficiency on myeloid cells improves stroke outcome (A) Corrected mean infarct area of each genotype after 1 hour of ischemia and 23 hours of reperfusion in filament model. (B) Bederson neurological Score on day 1. Data are mean ± SEM (A) and median ± range (B). Statistical analysis: unpaired t-test (A) and Mann Whitney test (B).

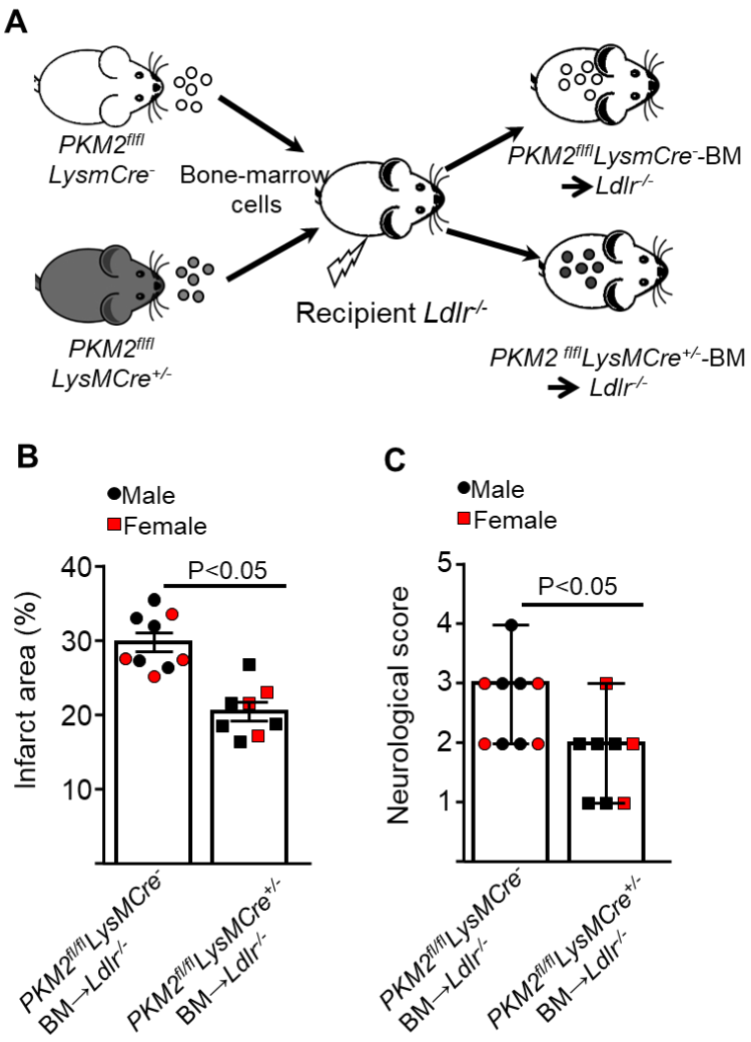


Supplemental Figure 6. LysMCre expression does not affect stroke outcome (A) Corrected mean infarct area of each genotype (male mice) after 1 hour of ischemia and 23 hours of reperfusion in filament model. (B) Bederson neurological Score on day 1. Data are mean ± SEM (A) and median ± range (B). Statistical analysis: unpaired t-test (A) and Mann Whitney test (B).

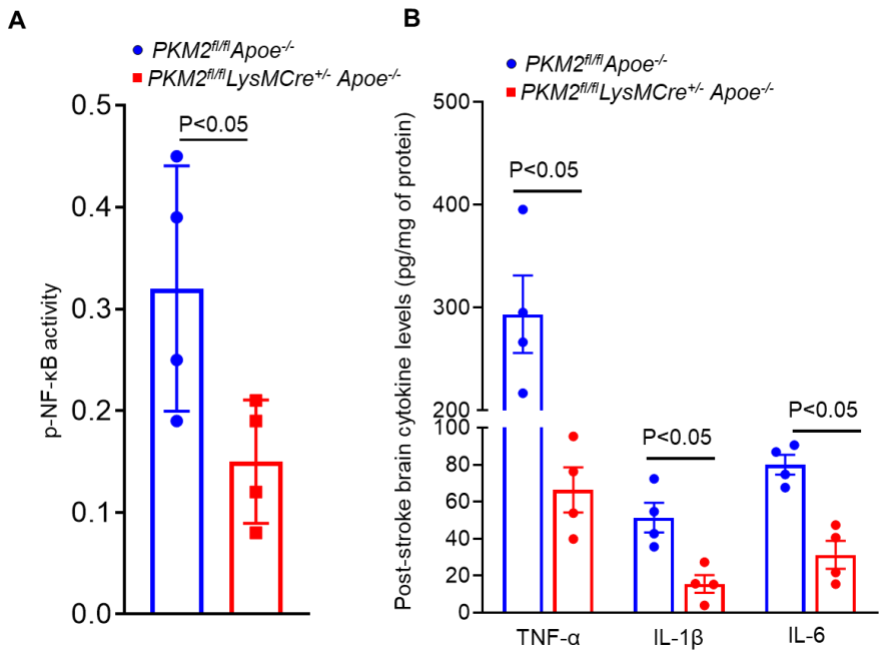


PKM2$^{fl/fl}$Apoe$^{-/-}$ *PKM2$^{fl/fl}$LysMCre$^{+/-}$ Apoe$^{-/-}$*

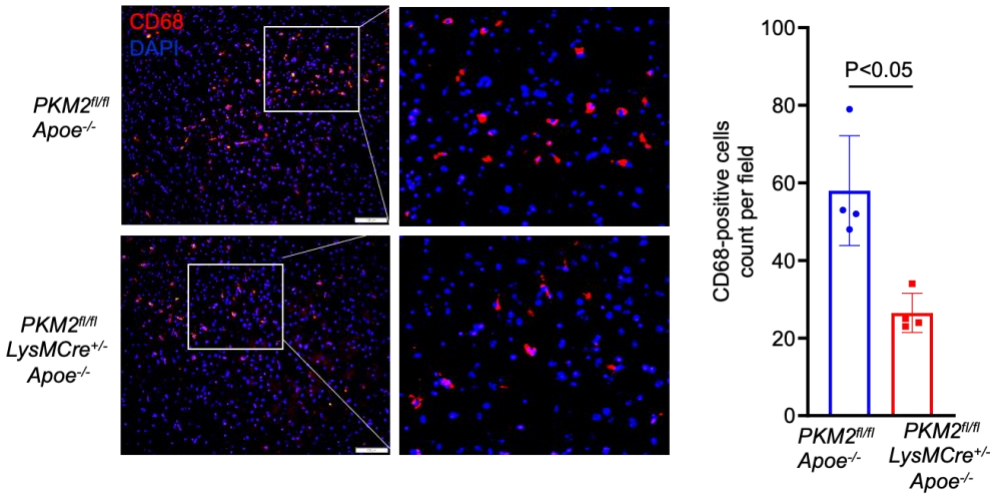
Supplemental Figure 7. Comparison of cerebrovascular anatomy. Anesthetized male mice were given an intracardiac injection of India ink and were then exsanguinated according to approved animal protocol. Circle of Willis and bilateral posterior communicating arteries was comparable among groups indicating there are no strain-related differences in gross cerebrovascular anatomy.



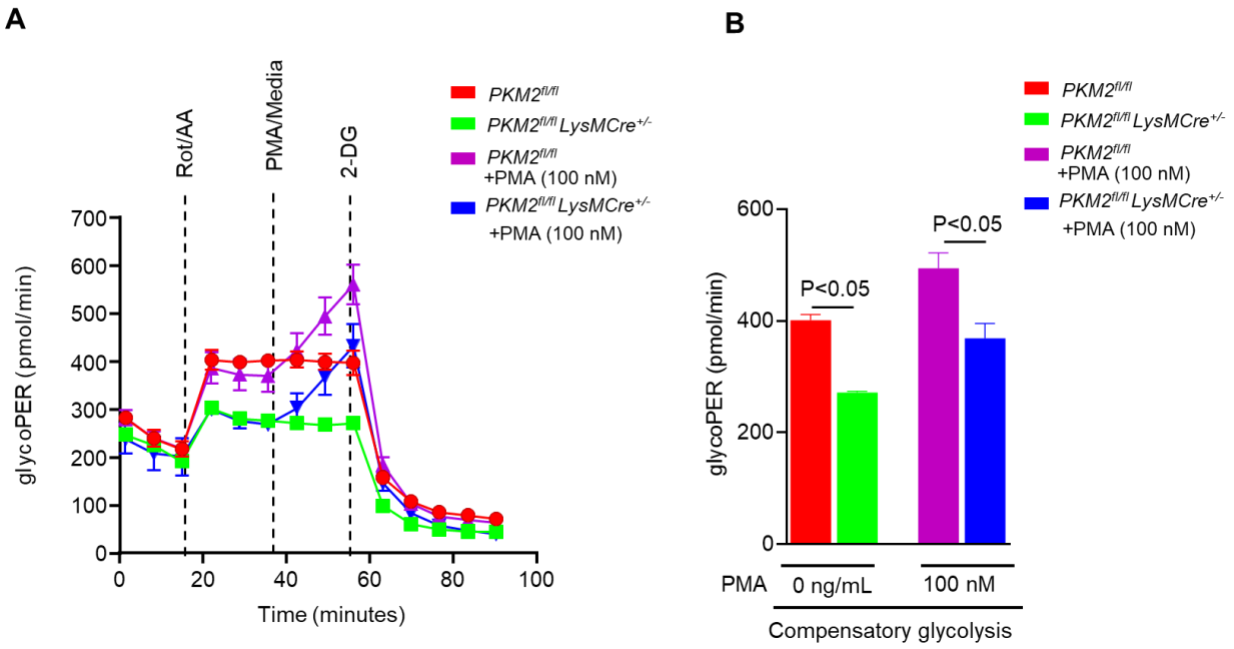
Supplemental Figure 8. Irradiated LDLr$^{-/-}$ mice transplanted with bone-marrow of PKM2$^{fl/fl}$ LysMCre$^{+/-}$ mice exhibit improved stroke outcome. (A). Schematic of experimental design. (**B)** Corrected mean infarct area of each genotype after 1 hour of ischemia and 23 hours of reperfusion in filament model. **(C)** Bederson neurological Score on day 1. Data are mean ± SEM (B) and median ± range (C). Statistical analysis: unpaired t-test (B) and Mann Whitney test (C).



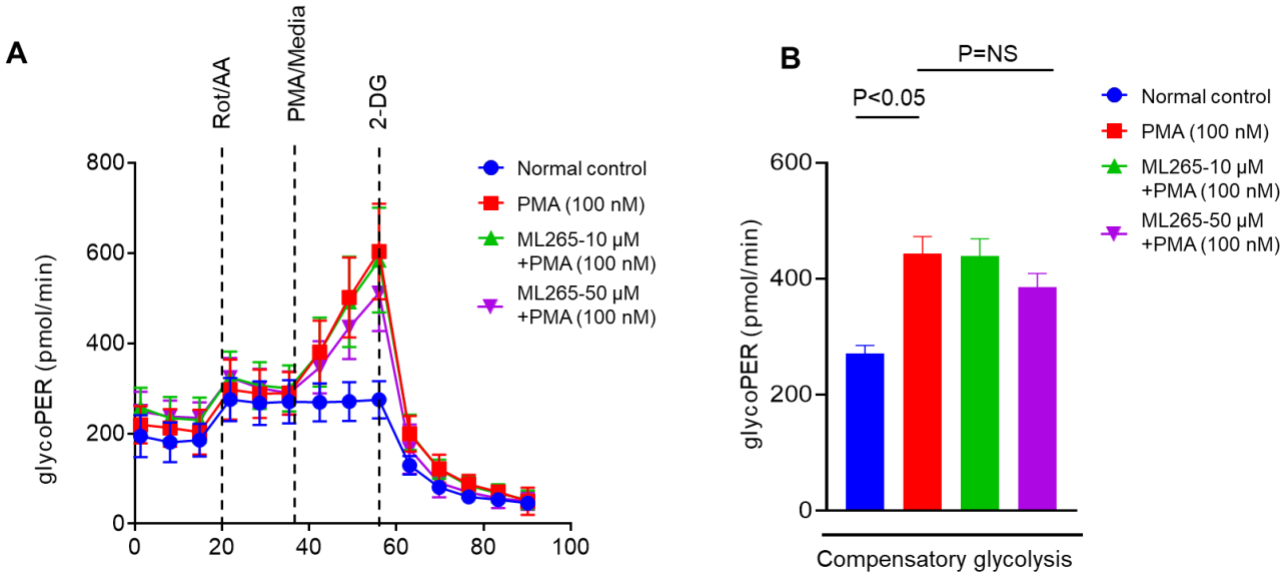
Supplemental Figure 9. (A) Phospho NF-κβ activity as analyzed by ELISA from the cell extracts of peripheral neutrophils, isolated 6 hours post-reperfusion in mice with stroke. (B) Cytokine levels within the infarct and peri-infarct regions of the perfused brain after 1 hour of ischemia and 23 hours of reperfusion in the filament model. Data are mean ± SEM. Statistical analysis: unpaired t-test (A & B).



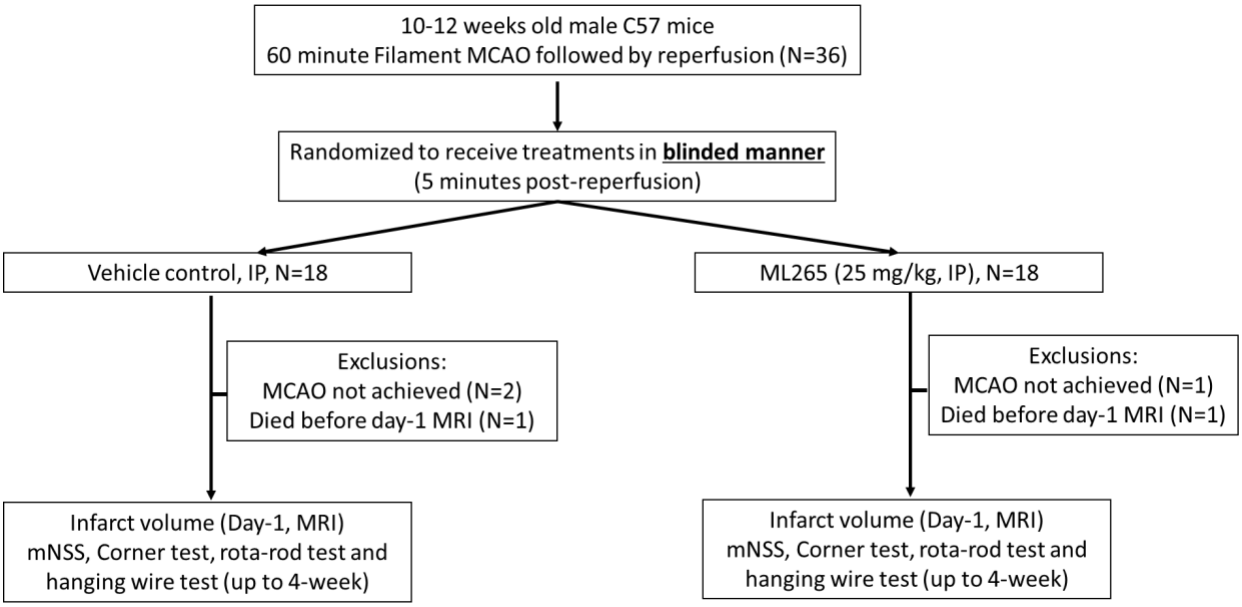
Supplemental Figure 10. Myeloid specific PKM2 deficient mice exhibited reduced brain macrophage content on day 3 following stroke Left: Representative immunostained images for macrophages (red CD68-positive cells) in infarcted brain regions. Boxed region (lower magnification). Inset in the boxed region (higher magnification) Right: Quantification. Data are from male mice and are mean ± SEM. Statistical analysis: unpaired t-test.



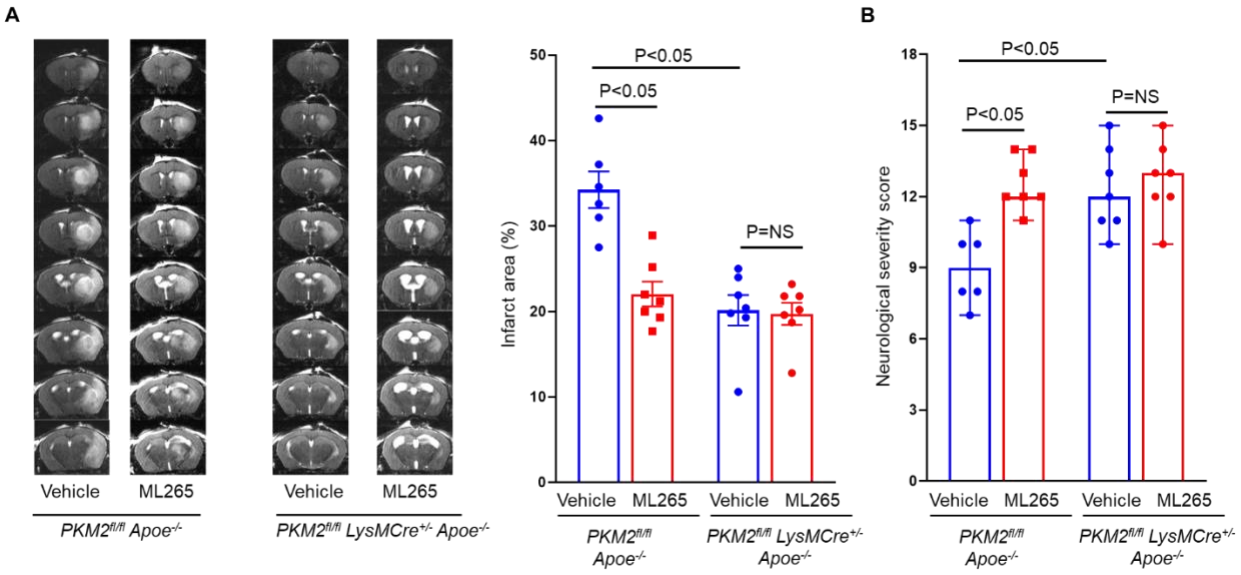
Supplemental Figure 11. Neutrophils from the myeloid specific PKM2 deficient mice exhibit reduced glycolytic rate. (A) Glycolytic proton efflux rate (glycoPER) levels in mouse neutrophils were assessed on Seahorse extracellular flux analyzer, XF96 (Sea-horse Bioscience, Chicopee, MA). The 400,000 neutrophils per well were suspended in seahorse media. The basal respiration values were noted for 15 mins before injecting the Rot/AA (0.5 µM) followed by addition of PMA at 100 nM. Finally, 2-DG was injected, and values were measured for 40 mins. Based on these data, we calculated glycolytic proton efflux rate (glycoPER) and compensatory glycolysis. **(B)** Compensatory glycolysis (glycoPER) levels in absence and in presence of PMA 100 nM (data derived from A). Data are from female mice and are mean ± SEM, n=7-8. Statistical analysis: Two-Way Repeated Measures ANOVA (Kruskal-Wallis test) followed by Fisher's LSD test (B).



Supplemental Figure 12. ML265 treatment did not change glycolytic rate in activated neutrophils from the WT mice. (A) Glycolytic proton efflux rate (glycoPER) levels in mouse neutrophils were assessed on Seahorse extracellular flux analyzer, XF96 (Sea-horse Bioscience, Chicopee, MA). The 400,000 neutrophils per well were pretreated with ML265 at 10 and 50 µM for 30 minutes and then suspended in seahorse media. The basal respiration values were noted for 15 mins before injecting the Rot/AA (0.5 µM) followed by addition of PMA at 100 nM. Finally, 2-DG was injected, and values were measured for 40 mins. Based on these data, we calculated glycolytic proton efflux rate (glycoPER) and compensatory glycolysis. **(B)** Compensatory glycolysis (glycoPER) levels in presence of PMA 100 nM or vehicle (data derived from A). Data are from female mice and are mean ± SEM, n=7-8. Statistical analysis: Two-Way Repeated Measures ANOVA (Kruskal-Wallis test) followed by Fisher's LSD test (B).



Supplemental Figure 13. Schematic of mouse study design used to evaluate effect of ML265 treatment on stroke outcome in WT mice.



Supplemental Figure 14. ML265 improves stroke outcomes via myeloid cell PKM2. (A) Left: Representative MRI from 1 mouse of each genotype on day 1. White is the infarct area. Right: Corrected mean infarct area of each genotype. **(B)**. Modified Neurological Severity Score (mNSS) on day 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. Data are from male mice and are mean ± SEM (A), median ± range (B). Statistical analysis: Two-Way Repeated Measures ANOVA (Kruskal-Wallis test) followed by Fisher's LSD test (A & B).