Supplemental Methods and Figures

Supplemental Methods

Evaluation of BBB permeability after ischemic stroke

Measurements of Evans Blue dye

Mice were intravenously injected with 4% Evans blue dye (4 mL/kg; Sigma-Aldrich)[1]. One hour later, mice were perfused transcardially with PBS, and ischemic hemispheres were weighted and placed in formamide for 72 hours. After centrifugation, optical density of supernatants was evaluated by spectrophotometry (Thermo Scientific, Waltham, MA) at 620 nm[2].

Measurements of FITC-dextran

FITC-dextran (MW = 40 KDa, Sigma-Aldrich; 50 μL of 100 mg/mL) was injected intravenously into mice and allowed to circulate for 2 minutes[3]. The brains were removed and fixed in 4% paraformaldehyde at 4°C overnight. Coronal brain sections (50 μm-thick) were incubated with goat anti-CD31 (AF3628, R&D Systems, Minneapolis, MN). The sections were then rinsed and incubated with Alexa Fluor 594-conjugated donkey anti-goat IgG (Invitrogen). Images were obtained using an Olympus FV1000 confocal microscope and an Olympus BX 63 microscope, and extravascular dextran fluorescence in tissue sections was quantified using ImageJ software.

Western blot analysis of IgG in microvessel-depleted brain cortex

Brain microvessels and capillary-depleted homogenates of brain cortex were prepared using dextran gradient centrifugation, as we previously described[3]. Samples were used for western blot analysis.

Behavioral measurements

Neurological deficits were assessed by investigators blinded to the experimental groups by beam walking test and forelimb force test at 24 hours after stroke as described[4, 5]. For the beam walking test, mice were placed on one end of a wooden beam (12-mm diameter, 1.2-m long and 45-cm high), and the total numbers of limb steps and the numbers of forelimb and hindlimb foot faults were recorded. The percentage of foot faults to total steps that occurred within 10 minutes was calculated. Before surgery, mice were trained for 3 days. For the forelimb force test, a digital grip strength meter (Bio-Seb, Vitrolles, France) was used to assess the maximum force exerted by an animal when the animal released its forepaws from a grid. The peak force was obtained in 6 trials for each mouse.

Western Blot Analysis

Western blot analysis was performed according to standard procedures[5, 6]. The following primary antibodies were used: rat anti-mouse Ly6G (1:1000, 551459, BD Biosciences, San Jose, CA), rabbit anti-H3Cit (1:1000, ab5103, Abcam), mouse anti-PAD4 (1:1000, 684202, BioLegend, San Diego, CA), rabbit anti-ZO-1 (1:1000, 617300, Invitrogen), rabbit anti-occludin (1:1000, ab167161, Abcam), rabbit anti-claudin5 (1:1000, ab15106, Abcam), rabbit anti-VE-cadherin (1:1000, ab33168, Abcam), rabbit anti-LRP-1 (1:1000, ab92544, Abcam), sheep anti-STING (1:1000, AF6516, R&D Systems), rabbit anti-H3 (1:1000, 9715), rabbit anti-cGAS (1:1000, 31659), rabbit anti-IRF-3 (1:1000, 4302), rabbit anti-phospho-IRF-3 (pIRF-3; 1:1000, 4947), rabbit anti-TBK1 (1:1000, 3504), rabbit anti-phospho-TBK1 (pTBK1; 1:1000, 5483), rabbit anti-GAPDH (1:1000, 5174), and rabbit anti-β-actin (1:1000, 4970, all from Cell Signaling Technology, Danvers, MA).

Immunohistochemistry

The brains were removed and 20-μm sections were prepared using a cryostat (Leica Microsystems Inc., IL). Sections were blocked with 1% BSA, 0.1% Triton-X and 5-10%

normal goat serum or normal donkey serum in PBS and were incubated with primary antibodies overnight at 4°C. Primary antibodies used were: rat anti-mouse Ly6G (1:200, 551459, BD Biosciences), rabbit anti-H3Cit (1:200, ab5103, Abcam), goat anti-CD31 (1:200, AF3628, R&D Systems), rabbit anti-fibrinogen (1:1000, AP00766PU-N, Acris Antibodies, San Diego, CA), rabbit anti-IFN-β (1:200, 32400-1, PBL Assay Science), rat anti-macrophage [RM0029-11H3] (1:200, ab56297, Abcam), goat anti-Iba1 (1:200, ab5076, Abcam), rabbit anti-LRP-1 (1:200, ab92544, Abcam), rabbit anti-cGAS (1:200, 31659), rabbit anti-STING (1:200, 13647), rabbit anti-pIRF-3 (1:200, 29047) and rabbit anti-IL-6 (1:200, 12912, all from Cell Signaling Technology). After washing with PBS, sections were incubated with species-specific fluorescent secondary antibodies in PBS for 1 hour at room temperature. The secondary antibodies used were Alexa Fluor 488-conjugated donkey anti-rat IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated donkey anti-goat IgG, Alexa Fluor 488-conjugated donkey anti-goat IgG (all from Invitrogen). DNA was stained with Hoechst 33342 (1:10,000, H3570, Invitrogen). For each animal, three fields from the peri-infarct cortex in each section were obtained under × 40 objective. Images were traced using ImageJ 1.46r software. The numbers of $ly6G^+$ neutrophils, $H3Cit^+$ neutrophils, $Iba1^+$ microglial cells, $pIRF3^+$, IFN-β^+ and $IL\text{-}6^+$ microglial cells, and $RM0029\text{-}11H3^+$ macrophages in the traced area were counted.

Determination of myeloperoxidase (MPO) activity

Cortical tissue was homogenized in 50 mM potassium phosphate buffer. Homogenates were centrifugated at 30,000 × g for 30 minutes at 4°C and suspended in 1% cetyltrimethylammonium bromide (Sigma-Aldrich) in potassium phosphate buffer. The suspensions were subjected to three freeze-thaw cycles in liquid nitrogen followed by 30 seconds of ultrasonic disruption. After centrifugation, 40 μL of supernatant was assessed

for MPO activity by adding 100 μL tetramethylbenzidine solution (Sigma-Aldrich). Absorbance at 450 nm was measured with a microplate reader.

References

1. Simao F, Ustunkaya T, Clermont AC, Feener EP. Plasma kallikrein mediates brain hemorrhage and edema caused by tissue plasminogen activator therapy in mice after stroke. Blood 2017;129:2280-2290.
2. Cai P, Luo H, Xu H, et al. Recombinant ADAMTS 13 Attenuates Brain Injury After Intracerebral Hemorrhage. Stroke 2015;46:2647-2653.
3. Xu H, Cao Y, Yang X, et al. ADAMTS13 controls vascular remodeling by modifying VWF reactivity during stroke recovery. Blood 2017;130:11-22.
4. Fan W, Dai Y, Xu H, et al. Caspase-3 modulates regenerative response after stroke. Stem Cells 2014;32:473-486.
5. Kang L, Yu H, Yang X, et al. Neutrophil extracellular traps released by neutrophils impair revascularization and vascular remodeling after stroke. Nat Commun 2020;11:2488.
6. Cao Y, Xu H, Zhu Y, et al. ADAMTS13 maintains cerebrovascular integrity to ameliorate Alzheimer-like pathology. PLoS Biol 2019;17:e3000313.

Supplemental Figure Legends

Supplemental Figure 1. Treatment with tPA increases neutrophil infiltration and H3Cit levels in the ischemic brain. (A) Representative images of Ly6G-positive neutrophils in the ischemic cortex at 24 hours after stroke in mice treated with vehicle or tPA. Bar = 50 μm**. (B)** Quantification of the numbers of $H3Cit^+$ neutrophils in the ischemic cortex at 24 hours after stroke in mice treated with vehicle or tPA, compared

with sham-operated mice (n = 6). Values are mean ± SD. *$P < 0.05$.

Supplemental Figure 2. Treatment with tPA upregulates PAD4 and LRP-1 and increases NET production. (A) Quantification of PAD4 expression in neutrophils isolated from ischemic mice treated with vehicle or 100 μg/ml tPA (n = 6). **(B)** Representative images of neutrophils isolated from ischemic mice. Neutrophils were treated with vehicle, 100 μg/ml tPA or tPA in combination with the PAD inhibitor Cl-amidine. Bar = 50 μm. **(C)** Quantification of LRP-1 expression in neutrophils isolated from ischemic mice treated with vehicle or 100 μg/ml tPA (n = 6). **(D)** Quantification of PAD4 expression in neutrophils isolated from ischemic mice treated with 100 μg/ml tPA or tPA in combination with the LRP antagonist RAP (n = 6). **(E)** Representative images of peripheral blood neutrophils isolated from sham-operated or ischemic mice. Neutrophils from sham-operated mice were treated with 100 μg/ml tPA. Neutrophils from ischemic mice were treated with vehicle, 100 μg/ml tPA or tPA in combination with RAP. Bar = 50 μm**.** Values are mean ± SD. *$P < 0.05$.

Supplemental Figure 3. Effects of RAP and tranexamic acid on tPA-induced increase in H3Cit in the ischemic brain. (A) Quantification of H3Cit levels in the ischemic cortex at 24 hours after stroke in mice treated with vehicle, tPA or tPA in combination with RAP (n = 5). **(B)** Quantification of H3Cit levels in the ischemic cortex at 24 hours after stroke in mice treated with vehicle, tPA or tPA in combination with tranexamic acid (TXA) (n = 5). Values are mean ± SD. *$P < 0.05$.

Supplemental Figure 4. DNase 1 treatment inhibits tPA-induced increase in H3Cit in the ischemic brain. (A) Quantification of H3Cit levels in the ischemic cortex at 24 hours after stroke in mice treated with vehicle, tPA, or tPA in combination with DNase 1 (n =

5). Values are mean ± SD. *$P < 0.05$.

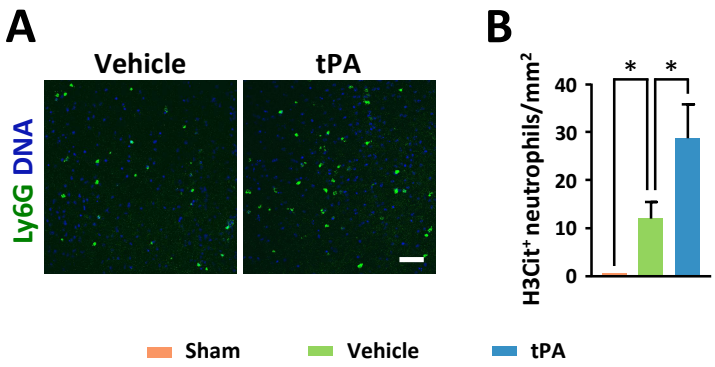
Supplemental Figure 5. DNase 1 reverses tPA-induced degradation of junctional proteins. (A-D) Quantification of ZO-1, occludin, claudin-5 and VE-cadherin in isolated brain microvessels at 24 hours after stroke in mice treated with vehicle, tPA, or tPA in combination with DNase 1 (n = 5). Values are mean ± SD. *$P < 0.05$.

Supplemental Figure 6. PAD4 deficiency inhibits tPA-induced H3Cit upregulation without affecting neutrophil infiltration. (A-B) Representative immunoblots and quantitative determinations of H3Cit levels in the ischemic cortex at 24 hours after stroke in WT and *Pad4*$^{-/-}$ mice treated with tPA (n = 5). **(C-D)** Representative immunoblots and quantitative determinations of the amount of neutrophil in the ischemic cortex at 24 hours after stroke in WT and *Pad4*$^{-/-}$ mice treated with tPA (n = 5). Values are mean ± SD. *$P < 0.05$.

Supplemental Figure 7. DNase 1 or PAD4 deficiency reduces the number of activated microglial cells in tPA-treated mice. (A-B) Representative confocal images of Iba1^{+} microglial cells and RM0029-11H3^{+} macrophages in the ischemic cortex 24 hours after stroke in WT mice treated with vehicle or tPA, tPA-treated *Pad4*$^{-/-}$ mice, and tPA-treated WT mice after treatment with DNase 1 or DNase 1 in combination with cGAMP. DNA was stained with DAPI (blue). Bar = 40 μm**.**

Supplemental Figure 8. PAD4 deficiency reduces pIRF3^{+}, IFN-β^{+} and IL-6^{+} microglial cells in tPA-treated mice with ischemia. (A-C) Representative confocal images of pIRF3^{+}, IFN-β^{+} and IL-6^{+} microglial cells (Iba1) in the ischemic cortex at 24 hours after stroke in WT and *Pad4*$^{-/-}$ mice treated with tPA. Bar = 20 μm**.**

Figure S1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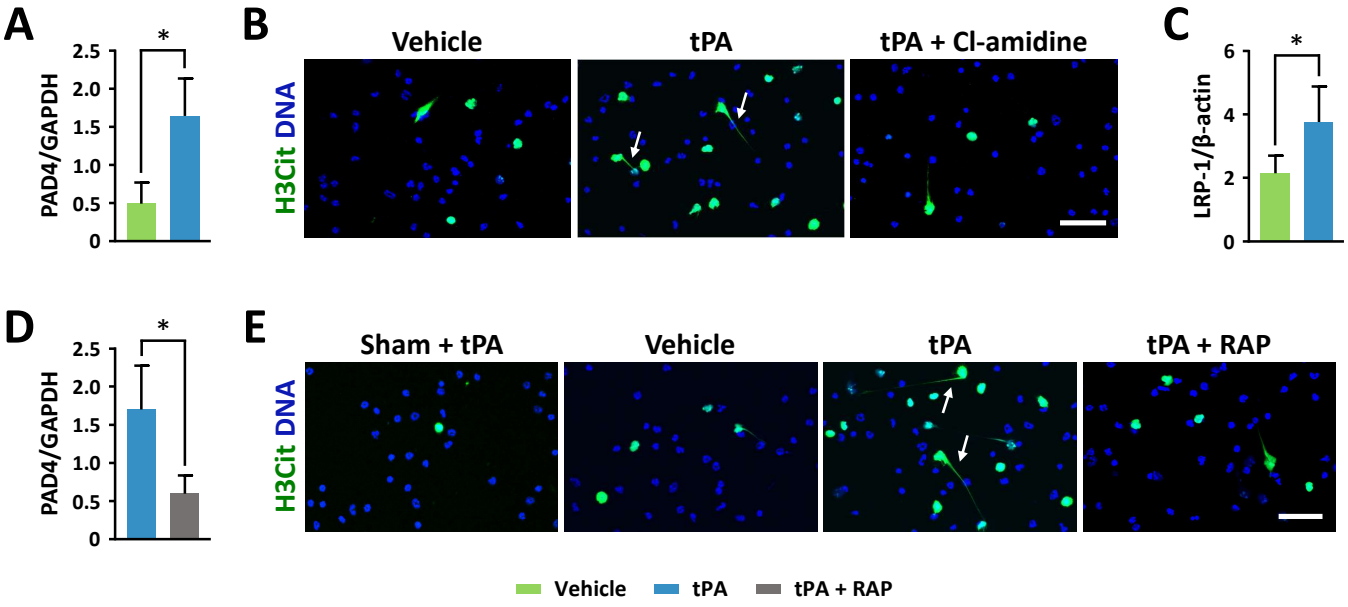
*
*
Sham
Vehicle
tPA

Figure S2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C
LRP-1/β-actin
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4
2
0
*
D
PAD4/GAPDH
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2.0
1.5
1.0
0.5
0
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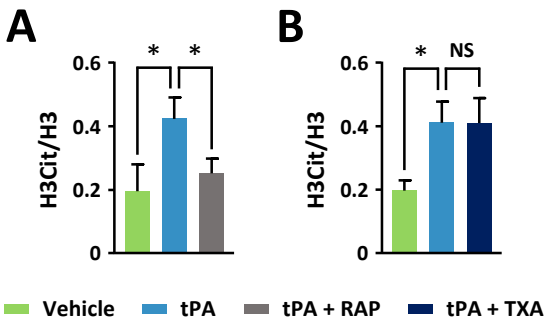
tPA
tPA + RAP

Figure S3



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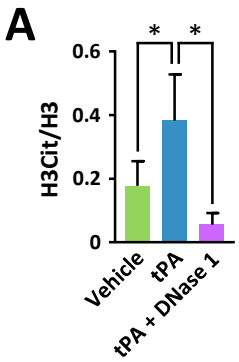

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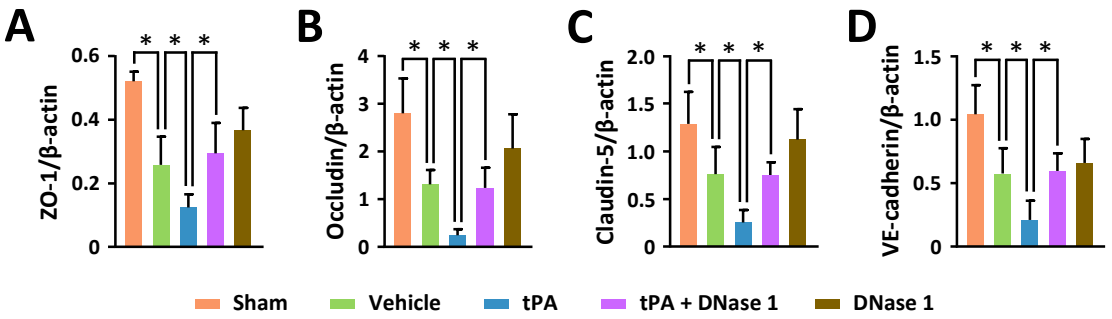
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C
Claudin-5/β-actin
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1.5
1.0
0.5
0
D
VE-cadherin/β-actin
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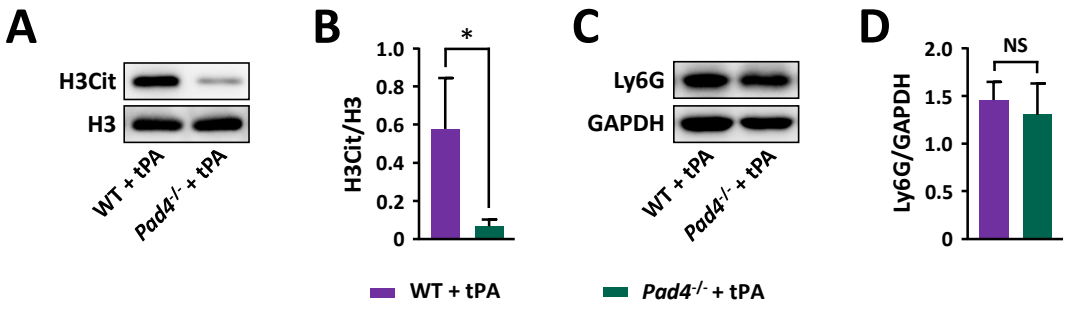
0.5
0
*
Sham
Vehicle
tPA
tPA + DNase 1
DNase 1

Figure S6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Pad4-/- + tPA
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0.2
0
*
C
Ly6G
GAPDH
WT + tPA
Pad4-/- + tPA
D
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1.0
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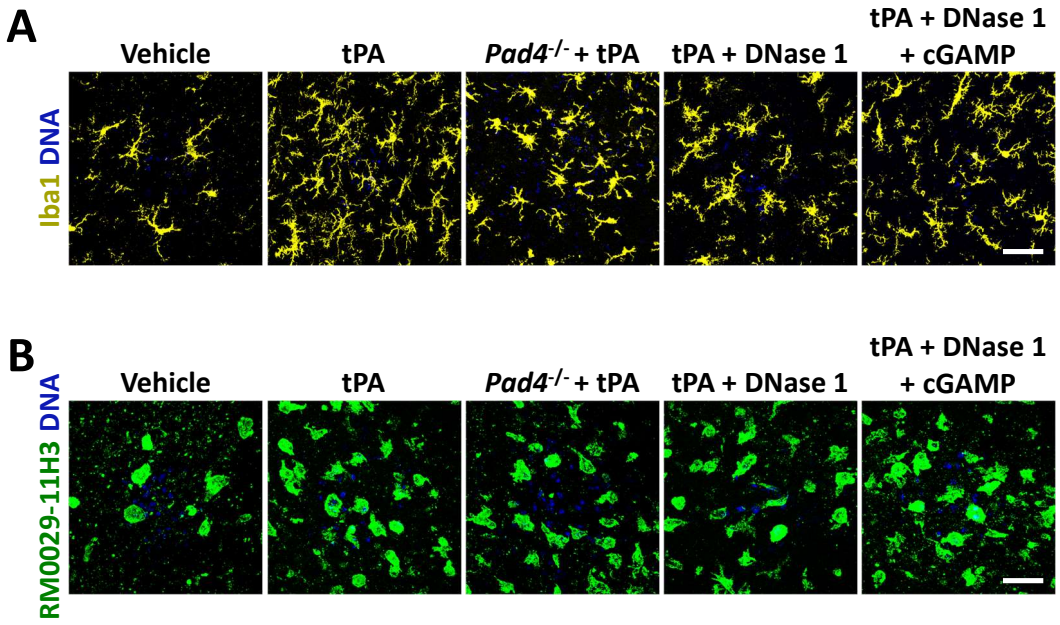
NS
WT + tPA
Pad4-/- + tPA

Figure S7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tPA + DNase 1
tPA + DNase 1 + cGAMP
Iba1 DNA
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tPA + DNase 1
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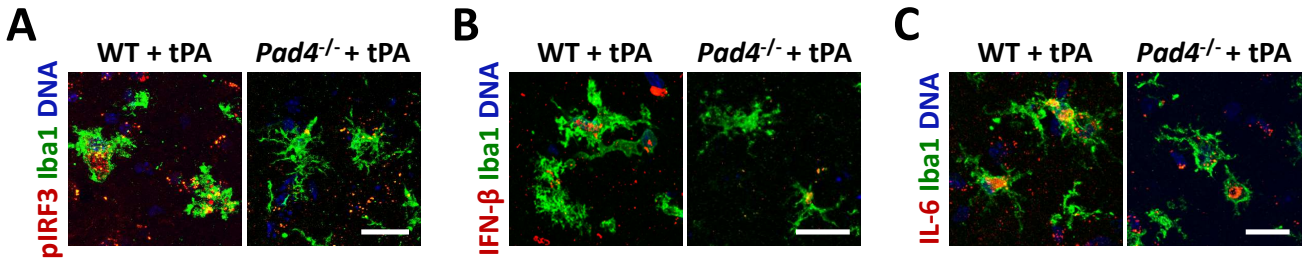
RM0029-11H3 DNA

Figure S8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pIRF3 Iba1 DNA
B
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IFN-β Iba1 DNA
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Pad4-/- + tPA
IL-6 Iba1 DNA