Neutrophil extracellular traps released by neutrophils impair revascularization and vascular remodeling after stroke

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Neovascularization and vascular remodeling are functionally important for brain repair after stroke. We show that neutrophils accumulate in the peri-infarct cortex during all stages of ischemic stroke. Neutrophils producing intravascular and intraparenchymal neutrophil extracellular traps (NETs) peak at 3–5 days. Neutrophil depletion reduces blood-brain barrier (BBB) breakdown and enhances neovascularization at 14 days. Peptidylarginine deiminase 4 (PAD4), an enzyme essential for NET formation, is upregulated in peri-ischemic brains. Overexpression of PAD4 induces an increase in NET formation that is accompanied by reduced neovascularization and increased BBB damage. Disruption of NETs by DNase 1 and inhibition of NET formation by genetic ablation or pharmacologic inhibition of PAD increases neovascularization and vascular repair and improves functional recovery. Furthermore, PAD inhibition reduces stroke-induced STING-mediated production of IFN-β, and STING knockdown and IFN receptor-neutralizing antibody treatment reduces BBB breakdown and increases vascular plasticity. Collectively, our results indicate that NET release impairs vascular remodeling during stroke recovery.
Stoke is one of the most common causes of long-term disability with limited therapeutic options. Recent evidence suggests that neovascularization after brain injury is functionally important for endogenous repair processes and blockade of angiogenic response worsens outcomes after cerebral ischemia. Therefore, promoting faster revascularization is of great therapeutic interest for treating a wide range of central nervous system diseases. To achieve this goal, an improved understanding is required of the mechanisms underlying vascular regrowth in the injured brain.

Neutrophils were traditionally considered as the first line of innate immune defense against microbes. In addition to playing a role in bacterial killing, activation of neutrophils causes the release of nuclear and granular contents to form extensive web-like structures of DNA (neutrophil extracellular traps, NETs). NETs contain double-stranded DNA, histone, and granule proteins including neutrophil elastase, cathepsin G, and myeloperoxidase (MPO). These NETs have been associated with autoimmune disorders, cardiovascular and pulmonary diseases, inflammation, and thrombosis. Blood-derived neutrophils and release of NETs have been identified in the brains of patients with ischemic stroke and in corresponding animal models. However, whether NETs contribute to favorable or poor outcomes during stroke recovery remains unclear. Furthermore, the role of neutrophils in cerebral ischemia has also been challenged. A recent study shows that both polymorphonuclear granulocytes and NETs were absent in the brain parenchyma after ischemic stroke in animals and humans.

The peri-infarct cortical areas have been previously shown to be critical for functional recovery in animal stroke model. In stroke patients, increased vascularization in these areas is also correlated with longer survival. In this study, we detect elevated levels of circulating DNA and show neutrophil-dependent NET formation inside the blood vessels and cerebral parenchyma in the peri-infarct cortical areas that peaked at 3–5 days after cerebral ischemia. We also demonstrate that target neutrophils and NETs improve cerebrovascular remodeling and functional recovery during the delayed phases after stroke.

Results

Neutrophils damage vascular remodeling after stroke. We subjected mice to cerebral ischemia and analyzed the brains at 1, 3, 5, 7, and 14 days. Using an anti-lymphocyte antigen 6 complex locus G (Ly6G) antibody, we found elevated levels of neutrophil that peaked at 3 days and persisted to at least 14 days in the ischemic cortex after stroke (Fig. 1a, c and Supplementary Fig. 1a), as reported. We then found a marked 5.3-fold increase in total content of the neutrophil enzyme MPO in the cortical areas at 3 days after stroke (Fig. 1d). Immunostaining showed that neutrophils were visualized throughout the peri-infarct cortex at 3 days but not after sham surgery (Fig. 1e). Neutrophils were observed inside blood vessels, adhering to vessels or migrating inside the parenchyma (Fig. 1f). The extravasation of neutrophils in the brains of ischemic mice was further confirmed using in-vivo multiphoton microscopy (Fig. 1g). Ly6G-labeled neutrophils abundantly infiltrated into the peri-infarct cortex at 3 days, whereas these cells were almost undetectable in the sham group. We also saw a higher percentage of circulating neutrophils in peripheral blood at 3 days after stroke as determined by flow cytometry (Fig. 1h).

Leukocyte infiltration contributes to blood–brain barrier (BBB) damage after brain injury by producing reactive oxygen species, proteases, and proinflammatory mediators. To investigate the role of neutrophils on cerebrovascular permeability during delayed phases after stroke, we depleted neutrophils in mice using an anti-Ly6G antibody for 14 days. Flow cytometry showed an 80% reduction in the number of blood neutrophils (Supplementary Fig. 2a, b) in mice treated with anti-Ly6G antibody. Peripheral blood counts also indicated that anti-Ly6G treatment reduced neutrophil counts in the blood by ~80% when compared with the isotype control group (Fig. 2a). While blood cell counts were also reduced (Fig. 2b), whereas red blood cell, platelet, monocyte, and lymphocyte counts were not significantly affected by anti-Ly6G treatment (Supplementary Fig. 2c-f), as reported. Correspondingly, immunostaining analysis indicated that the number of infiltrating neutrophils in the ischemic brain was significantly less in anti-Ly6G-treated mice than in control IgG-treated mice (Fig. 2c). Multiphoton microscopy analysis of intravenously (i.v.) injected fluorescein isothiocyanate (FITC)-dextran revealed a significant increase in vascular leakage in the peri-infarct cortical areas at 14 days after stroke compared with sham-operated brains (Fig. 2d, e), as we previously reported. Mice treated with anti-Ly6G antibody exhibited a 39.3% reduction in BBB permeability compared with mice treated with control IgG. Extravascular accumulation of circulating IgG was also reduced in the brains of mice treated with the anti-Ly6G antibody (Fig. 2f, g). We then investigated the effect of neutrophils on neovascularization; the total length of brain capillaries in the peri-infarct cortical areas was analyzed at 14 days. Neutrophil-depleted mice showed a 29.8% increase in microvascular length compared with the control IgG-treated mice (Fig. 2h, i). Multiphoton microscopy revealed that neutrophil depletion caused a significant increase in the length of perfused cortical microvessels (Fig. 2j, k). There was no significant difference in infarct volume between anti-Ly6G-treated and control IgG-treated mice at 14 days (Supplementary Fig. 2g, h). Together, these data indicate that neutrophils are important regulators of neovascularization and vessel function during stroke recovery.

Stroke induces NET formation. To test whether NETs are present in the circulation of ischemic mice, blood cells from mice at 3 days after stroke were stained for citrullinated histone H3 (H3Cit) and Ly6G. The results revealed a significantly higher number of neutrophils and H3Cit+ neutrophils in ischemic mice compared with sham-operated mice (Fig. 3a-c). In line with this, elevated levels of circulating DNA was found in the plasma from these mice (Fig. 3d). We then isolated neutrophils from the peripheral blood of these mice and incubated them with or without lipopolysaccharide (LPS) stimulation. We found that either unstimulated or LPS-stimulated neutrophils from ischemic mice showed a significant increase in H3Cit+ neutrophils and NET formation (Fig. 3e-g), indicating that neutrophils from mice subjected to stroke are primed to undergo NETosis.

As NETs can injure host tissue, we next asked whether NETs were produced in the ischemic brain and affect stroke outcomes. Western blot analysis of the ischemic cortex showed an increased amount of H3Cit that was most robust from 3–5 days (Fig. 3h, i), suggesting that NETs may play an important role during the delayed phases after stroke. Immunostaining revealed that the peri-infarct cortex was extensively labeled with H3Cit+ cells at 3 days (Fig. 3i). To identify which type of cells expressed H3cit after stroke, double immunofluorescence with confocal microscopy was performed on brain sections. This analysis revealed that H3Cit was colocalized with Ly6G-positive neutrophils, F4/80-positive macrophages/microglial cells, Iba1-positive microglial cells, NeuN-positive neurons, and glial fibrillary acidic protein (GFAP)-positive astrocytes (Fig. 3k). Importantly, 78.7% of the H3Cit-positive cells were Ly6G-positive neutrophils. H3Cit+ neutrophils were observed inside the blood vessels and cerebral parenchyma (Fig. 3l). Hematoxylin and eosin staining clearly indicated that DNA fibers were present in these areas.
Disruption of NETs enhances vascular remodeling after stroke.

We next investigated whether degradation of NETs with DNase 1 could improve vascular remodeling after stroke in mice. Treatment with DNase 1 did not affect the amount of neutrophils in the peri-infarct cortical areas (Supplementary Fig. 4a, b) but reduced H3Cit levels (Fig. 4a, b). Compared with the vehicle controls, administration of DNase 1 significantly reduced BBB permeability (Fig. 4c, d) and extravascular IgG deposits (Fig. 4e, f), and enhanced both Pdgfr-β+ and CD13+ pericyte coverage on brain microvessels (Fig. 4g, h and Supplementary Fig. 4c, d). Vascular branches (Supplementary Fig. 4e, f), microvascular length (Fig. 4i), perfused capillary length (Fig. 4j, k), and tomato-lectin perfused vessels (Fig. 4l, m) were also increased in the brains of mice treated with DNase 1. Next, we investigated the importance of neutrophil NETs in vascular remodeling. Our results showed that treatment with DNase 1 in combination with anti-Ly6G antibody did not further improve neovascularization and BBB leakage compared with mice treated with anti-Ly6G antibody alone (Fig. 4n-p). These data indicate that DNase 1 primarily digests NETs generated by neutrophils, and that neutrophil NETs play a crucial role in vascular remodeling after stroke. However, in addition to neutrophil NETs, H3Cit in other cells may also contribute to the impaired vascular remodeling.

Next, we detected whether NETosis regulates vascular remodeling during repair processes. We found that injection of anti-Ly6G antibody beginning 7 days after stroke reduced extravascular IgG deposits at 14 days (Fig. 5a, b). Furthermore, we observed...
significant increases in microvascular length (Fig. 5e, f) and perfused cortical vessels (Fig. 5i, j) in anti-Ly6G antibody-treated mice compared with control IgG-treated mice. Treatment with DNase 1 starting at 7 days after stroke also attenuated BBB disruption (Fig. 5c, d), increased microvessels (Fig. 5g, h), and improved capillary perfusion (Fig. 5k, l) at 14 days.

**PAD4 regulates BBB permeability and neovascularization.** Peptidylarginine deiminase 4 (PAD4) is a histone-modifying enzyme that is critical for NET formation. Quantitative PCR revealed a sustained 29.3-fold increase in PAD4 mRNA expression in the ischemic cortex at 3 days after stroke compared with sham-operated brains (Fig. 6a). Immunoblotting found a marked increase in PAD4 protein expression at 3 days after stroke (Fig. 6b).

**Fig. 2 Neutrophil depletion reduces BBB breakdown and increases neovascularization after stroke.** a, b Neutrophil and white blood cell counts in peripheral blood at 14 days after stroke in mice treated with control antibody or anti-Ly6G antibody (n = 3), unpaired two-tailed Student’s t-test was applied with *P = 0.0003 (a), *P = 0.012 (b). WBC, white blood cell. c Quantification of the number of neutrophils in the ischemic cortex at 14 days in mice treated with control antibody or anti-Ly6G antibody (n = 6 biologically independent experiments), Mann-Whitney test was applied with *P = 0.0022. d, e Representative in-vivo multiphoton microscopic images of intravenously injected FITC-dextran (MW = 40,000 Da; green) leakage in cortical vessels (d) at 14 days in sham-operated mice and ischemic mice treated with control antibody or anti-Ly6G antibody, and quantification of the permeability (P) product of FITC-dextran for each group (e) (n = 6). One-way ANOVA test was applied with *P = 0.0001 (Sham vs. Isotype), *P = 0.0354 (Isotype vs. Anti-Ly6G). Bar = 100 μm. f, g Representative confocal images (f) and quantitative analysis of IgG extravascular deposits (g) in the peri-infarct cortex at 14 days in mice treated with control antibody or anti-Ly6G antibody (n = 6). One-way ANOVA test was applied with *P < 0.0001 (Sham vs. Isotype), *P = 0.0041 (Isotype vs. Anti-Ly6G). Bar = 15 μm. h, j Representative confocal images (h) of CD31-positive microvessels and in-vivo multiphoton microscopy images of perfused cortical capillaries with intravenously injected FITC-dextran (MW = 2000,000 Da) (j) in the peri-infarct cortex at 14 days in mice treated with control antibody or anti-Ly6G antibody, compared with sham-operated mice. Bar = 50 μm (e) and 100 μm (g). i, k Quantification of microvascular density (i) and perfused capillary length (k) for each group (n = 6). One-way ANOVA test was applied with *P = 0.0003 (Sham vs. Isotype (i)), *P = 0.0004 (Isotype vs. Anti-Ly6G (i)), *P = 0.0002 (Isotype vs. Anti-Ly6G (k)). Data are presented as mean ± SD. Source data underlying graph a–c, e, g, i, and k are provided as a Source Data file.
3.8-fold upregulation of PAD4 protein expression in these areas (Fig. 6b, c). To test the hypothesis that increased release of NETs, orchestrated by PAD4, participates in stroke recovery, we first studied the role of overexpression of PAD4 on vascular remodeling. Immunohistochemical analysis indicated extensive expression of recombinant adeno-PAD4-flag-infected cells in the cortex at 4 days after injection (Fig. 6d). Overexpression of PAD4 in the cortex was also confirmed by immunoblotting (Supplementary Fig. 5a, b). PAD4-flag was present in the neurons, neutrophils, macrophages/microglial cells, and microglial cells, but was rarely detected in astrocytes (Supplementary Fig. 5d-h). Injection of PAD4 adenovirus in ischemic mice produced ~2.6-fold more NETs than control virus (Fig. 6e and Supplementary Fig. 5c). We then observed a significant increase in the number of H3Cit+ neutrophils (Fig. 6f), whereas the number of H3Cit+ macrophages/microglial cells, H3Cit+ microglial cells, H3Cit+ neurons, and H3Cit+ astrocytes were not affected by PAD4 adenoviruses treatment (Supplementary Fig. 5i-l). At 14 days after stroke, multiphoton microscopy analysis of i.v.-injected FITC-dextran showed a significant 2.5-fold increase in BBB permeability in the peri-infarct cortex in mice treated with PAD4 adenovirus (Fig. 6g, h). Administration of PAD4 adenovirus into ischemic mice also caused a significant reduction in CD13+ pericyte coverage on brain microvessels (Supplementary Fig. 5m, n). Moreover, vascular branches (Fig. 6i), microvascular length (Fig. 6j, k), and perfused cortical microvascular length (Fig. 6l, m) were significantly reduced in mice subjected to PAD4 adenovirus injection. The more severe BBB damage and poor neovascularization were functionally relevant. Compared with the control mice, PAD4-overexpressing mice had more severe neurological deficits as assayed by the beam walking test and forelimb force test (Fig. 6n-p).

To further establish the role of NETs in stroke recovery, we compared wild type (WT) with PAD4-deficient (PAD4−/−) mice or treated mice with the PAD inhibitor Cl-amidine. There was no significant difference in ischemic lesion between WT and PAD4−/− mice at 14 days (Supplementary Fig. 6a, b). Thus, this approach allowed us to provide evidence that effects of PAD4
Fig. 3 Neutrophils form NETs presenting in the brain after stroke. a Representative images of H3Cit (green) and Ly6G (red) double-positive cells in cytosplins from sham-operated mice and ischemic mice at 3 days. DNA was visualized with Hoechst 33342 (blue). Bar = 30 μm. b, c Quantification of Ly6G-positive neutrophils in the total leukocyte population (b) and the percentage of H3Cit-positive neutrophils (c) in cytosplins (n = 10 biologically independent experiments). Mann–Whitney test was applied with *P < 0.0001 (b), *P < 0.0001 (c). d Levels of plasma DNA were elevated at day 3 after stroke (n = 6 biologically independent experiments). Unpaired two-tailed Student’s t-test was applied with *P = 0.0026. e Representative immunofluorescence images of isolated peripheral blood neutrophils from sham-operated mice and ischemic mice at 3 days. Neutrophils were incubated in the presence or absence of LPS for 2.5 h and stained with Hoechst 33342 (blue) and H3Cit (green). Arrows indicate NETs. US, unstimulated. Bar = 30 μm. f, g Quantification of the percentage of H3Cit-positive neutrophils (f) and NETs (g) in isolated neutrophils (n = 10 biologically independent experiments). One-way ANOVA test was applied with *P < 0.0001 (Sham vs. Stroke in US (f)), *P < 0.0001 (Sham vs. Stroke in LPS group (f)), *P = 0.0235 (Sham vs. Stroke in US (g)), *P < 0.0001 (Sham vs. Stroke in LPS group (g)). US, unstimulated. h, i Representative immunoblots of the time course of NETs appearance (h) and quantification of the H3Cit levels (i) in the peri-infarct cortex of mice subjected to stroke or sham operation (n = 5). One-way ANOVA test was applied with *P < 0.0001 (Sham vs. 3d), *P < 0.0001 (Sham vs. 5d), *P = 0.0039 (Sham vs. 7d). j Representative confocal images showing NET formation in the peri-infarct cortex of mice after stroke. Inset is magnified on the right side. Arrows indicate NETs. Bar = 40 μm (left) and 20 μm (right). Arrows indicate extracellular DNA fibers. k Graphs compare the number of H3Cit+Ly6G+ neutrophils, H3Cit+F4/80+ macrophages/microglial cells, H3Cit+Iba1+ microglial cells, H3Cit+NeuN+ neurons, and H3Cit+GFAP+ astrocytes in mice at 3 days after stroke (n = 5 biologically independent experiments). l Representative confocal image showing the formation of intravascular and intraparenchymal NETs at 3 days after stroke. Bar = 15 μm. Independent experiments are repeated at least three times. m Representative in-vivo multiphoton microscopy images of extracellular DNA (green) and neutrophils in the peri-infarct cortex of mice at 3 days. Extracellular DNA (green) were labeled with intravenous injection of Sytox green and neutrophils (red) with intravenous injection of PE-conjugated monoclonal Ly6G antibody. Arrows indicate extracellular DNA fibers. Bar = 20 μm. Independent experiments are repeated at least three times. Data are presented as mean ± SD. Source data underlying graph b–d, f–i, and k are provided as a Source Data file.

Fig. 4 DNase 1 reduces BBB breakdown and increases neovascularization after stroke. a, b Representative immunoblots (a) and quantification of H3Cit levels (b) in the peri-infarct cortex at 3 days in mice treated with vehicle or DNase 1 (n = 5), unpaired two-tailed Student’s t-test was applied with *P = 0.0228. c Representative images of multiphoton microscopy of intravenously injected FITC-dextran (MW = 40,000 Da; green) leakage in cortical vessels at 14 days after stroke in mice treated with vehicle or DNase 1. Bar = 100 μm. d Quantification of the permeability (P) product of FITC-dextran for each group (n = 6 biologically independent animals), unpaired two-tailed Student’s t-test was applied with *P = 0.0083. e, f Representative images of IgG deposits and CD31-positive microvessels (e) at 14 days after stroke in mice treated with vehicle or DNase 1, and quantification of extravascular IgG deposits (f) for each group (n = 6 biologically independent animals), unpaired two-tailed Student’s t-test was applied with *P = 0.0052. Bar = 20 μm. g, h Representative images (g) and quantitative analysis (h) of Pdgfr-β-positive pericyte coverage on CD31-positive brain capillaries at 14 days (n = 6 biologically independent animals), unpaired two-tailed Student’s t-test was applied with *P = 0.0175. Bar = 40 μm. i Quantification of microvascular length in the peri-infarct cortex at 14 days (n = 6 biologically independent animals), unpaired two-tailed Student’s t-test was applied with *P = 0.0007. j, k In-vivo multiphoton microscopic images of perfused cortical capillaries with intravenously injected FITC-dextran (MW = 2000,000 Da) (j) and quantification of perfused capillary length (k) at 14 days (n = 6 biologically independent animals), unpaired two-tailed Student’s t-test was applied with *P = 0.0003. Bar = 100 μm. l, m Representative images of tomato-lectin perfused vessels (l) and quantification of lectin perfused vessels (m) at 14 days (n = 6), Mann–Whitney test was applied with *P = 0.0152. Bar = 40 μm. n, p Quantification of extravascular IgG deposits (n), microvascular length (o), and perfused capillary length (p) in the peri-infarct cortex at 14 days (n = 6 for Anti-Ly6G, n = 4 for Anti-Ly6G + DNase 1), unpaired two-tailed Student’s t-test was applied with P = 0.6496 (n), P = 0.5222 (o), P = 0.2844 (p). Data are presented as mean ± SD. Source data underlying graph a, b, d, f, h, i, k, and m–p are provided as a Source Data file.
deficiency on long-term outcomes are not secondary to the reduced lesion size. PAD4 deficiency did not alter the number of infiltrating neutrophils in the ischemic brain tissue (Supplementary Fig. 6c, d), whereas the levels of H3Cit in the lysates from the ischemic cortex at 3 days were greatly reduced in PAD4−/− mice and mice receiving Cl-amidine (Fig. 7a and Supplementary Fig. 7a). Similarly, examination of the brain tissues revealed that PAD4 deficiency or Cl-amidine reduced Sytox green-positive extracellular DNA fibers (Fig. 7b, c) in the ischemic cortex. Previous studies have shown that PAD4 may also function in other cells32,33. We found that PAD4 deficiency or Cl-amidine substantially reduced neutrophil NETs, as seen by the decrease in H3Cit+ neutrophils (Fig. 7d, e). However, PAD4 deficiency did not significantly affect the number of H3Cit+ macrophages/microglial cells, H3Cit+ microglial cells, H3Cit+ neurons, and H3Cit+ astrocytes (Supplementary Fig. 6e-h). Mice deficient in PAD4 and mice treated with Cl-amidine exhibited a significant reduction in perivascular IgG deposits (Supplementary Fig. 7b, c) and vascular leakage of i.v.-injected FITC-dextran (Fig. 7f, g). IgG content in capillary-depleted brain tissues (Fig. 7h and Supplementary Fig. 8a) was also dramatically reduced in PAD4−/− mice and mice receiving Cl-amidine. Consistent with these findings, the tight-junction proteins ZO-1, claudin-5, and occludin, and the adherens junction protein VE-cadherin, which are required for BBB integrity34, were enhanced in isolated brain microvessels in these mice (Fig. 7i and Supplementary Fig. 8b-e). Furthermore, PAD4 deficiency or Cl-amidine treatment significantly increased vascular branches (Supplementary Fig. 7d, e), microvascular length (Fig. 7j and Supplementary Fig. 7f), the length of perfused microvessels (Fig. 7k), and tomato-lectin perfused vessels (Fig. 7l and Supplementary Fig. 7h) in the ischemic cortex. However, treatment with anti-Ly6G antibody or DNase 1 had no beneficial effect in PAD4−/− mice on BBB permeability (Fig. 7m and Supplementary Fig. 9a), microvascular length (Fig. 7n and Supplementary Fig. 9b), and perfused capillary length (Fig. 7o and Supplementary Fig. 9c). These data indicate that the effects of PAD4 deficiency on neovascularization and vascular remodeling are due to NETs. Furthermore, we found that PAD4 deficiency had no beneficial effect on BBB permeability (1.18 × 10^4 ± 2702 vs. 1.10 × 10^4 ± 2697, P = 0.66, n = 4–6), microvascular length (12.8 ± 1.68 vs. 13.82 ± 2.31, P = 0.42, n = 4–6), and perfused capillary length (1871 ± 136 vs. 1978 ± 91, P = 0.42, n = 4–6) in mice subjected to anti-Ly6G antibody treatment. These findings suggest that PAD4 may primarily mediate the formation of neutrophil NETs after stroke. In parallel with the effects on vascular function, PAD4 deficiency or Cl-amidine treatment improved behavioral deficits in mice at 14 days after stroke (Fig. 7p-r).

NETs are responsible for STING-mediated vascular remodeling. The free DNA binds with cyclic GMP-AMP synthase to promote STING-dependent type I interferon (IFN) synthesis35,36.
We found a marked tenfold increase in the levels of IFN-β in the cortical areas at 3 days after stroke (Fig. 8a), suggesting activation of the STING pathway. We then detected significantly increased levels of STING (Fig. 8b and Supplementary Fig. 10a) and robust induction of phosphorylated TANK-binding kinase 1 (pTBK1) and TBK1-dependent IFN regulatory factor 3 (IRF3) activation (Fig. 8b and Supplementary Fig. 10b, c). Treatment with the PAD inhibitor CI-amidine reduced the levels of stroke-induced STING-mediated signaling in the cortical areas compared with vehicle-treated mice (Fig. 8c, d and Supplementary Fig. 10d–f).
Neutrophil depletion also resulted in a significant reduction in the levels of STING and the STING downstream signaling molecules including pTBK1, pIRF3, and IFN-β in the ischemic brain relative to IgG-treated controls (Fig. 8e, f and Supplementary Fig. 10g-i). To test whether the signaling part mediated by STING is related to neutrophils, we isolated neutrophils from bone marrow of ischemic mice and stimulated them with LPS in the presence or absence of the PAD inhibitor Cl-amidine. We found that the STING-mediated signals were activated by LPS stimulation and this effect was prevented by the addition of Cl-amidine (Fig. 8g, h and Supplementary Fig. 11a-c). Next, we investigated whether the IFN response affected the BBB permeability and vascular remodeling. Immunofluorescent staining and multiphoton microscopy indicated increased microvascular length (Fig. 8i, j) and perfused microvascular length (Fig. 8k, l) in the peri-infarct cortex in IFN receptor (IFNAR)-neutralizing antibody-treated mice as compared with control IgG-treated mice at day 14 after stroke. When analysing the i.v.-injected FITC-dextran, we observed a significant
Fig. 7 PAD4 deficiency or pharmacologic inhibition promotes vascular remodeling after stroke. a Representative immunobots of H3Cit levels in the peri-infarct cortex at 3 days in WT and PAD4−/− mice, and WT mice treated with vehicle or the PAD inhibitor Cl-amidine. Independent experiments are repeated at least three times. b In-vivo multiphoton microscopic images of extracellular DNA (Sytox, green) in the peri-infarct cortex of mice at 3 days after stroke. Arrows indicate extracellular DNA fibers. Bar = 20 μm. Independent experiments are repeated at least three times. c Quantification of NETs for each group (n = 6). One-way ANOVA test was applied with *P < 0.0001 (Same vs. WT stroke), *P < 0.0001 (WT vs. PAD4−/−), *P < 0.0001 (Vehicle vs. Cl-amidine). d Representative confocal images of neutrophil (Ly6G, green) and H3Cit (red) immunostaining in the peri-infarct cortex at 3 days. DNA was stained with Hoechst 33342 (blue). Bar = 40 μm. e Quantification of the numbers of H3Cit-positive neutrophils in the peri-infarct cortex at 3 days in WT and PAD4−/− mice, and WT mice treated with vehicle or the PAD inhibitor Cl-amidine (n = 6), unpaired two-tailed Student’s t-test was applied with *P = 0.0002 (WT vs. PAD4−/−), *P = 0.0010 (Vehicle vs. Cl-amidine). f, g In-vivo multiphoton microscopic images (f) of intravenously injected FITC-dextran leakage in cortical vessels at 14 days and quantification of the permeability (P) product of FITC-dextran (g) for each group (n = 6), unpaired two-tailed Student’s t-test was applied with *P = 0.0197 (WT vs. PAD4−/−), *P = 0.0260 (Vehicle vs. Cl-amidine). Bar = 100 μm. h Representative immunobots of IgG levels in capillary-depletion brain tissue at 14 days in WT and PAD4−/− mice, and WT mice treated with vehicle or Cl-amidine. Independent experiments are repeated at least three times. i Representative immunobots of the tight-junction protein ZO-1, claudin-5, and occludin, and the adherens junction protein VE-cadherin in isolated brain microvessels at 14 days. Independent experiments are repeated at least three times. j Quantification of microvascular density (j), perfused capillary length (k), and tomato-lectin perfused vessels (l) in the peri-infarct cortex at 14 days (n = 6). Unpaired two-tailed Student’s t-test was applied with *P < 0.0001 (WT vs. PAD4−/− (j)), *P = 0.0093 (Vehicle vs. Cl-amidine (j)), *P = 0.0001 (WT vs. PAD4−/− (k)), *P < 0.0001 (Vehicle vs. Cl-amidine (k)) *P = 0.0015 (WT vs. PAD4−/− (l)), *P = 0.0013 (Vehicle vs. Cl-amidine (l)). m-o Quantification of extravascular IgG deposits (m), microvascular length (n), and perfused capillary length (o) in the peri-infarct cortex at 14 days after stroke (n = 6 for PAD4−/−, n = 4 for PAD4−/− + Anti-Ly6G and PAD4−/− + DNase 1). One-way ANOVA test was applied with *P = 0.7263 (m), *P = 0.9547 (n), *P = 0.7304 (o). p-r PAD4 deficiency or Cl-amidine treatment improved neurological functions in beam walking test (p, q) and forelimb force test (r) (n = 10). One-way ANOVA test was applied with *P = 0.0407 (p), *P = 0.0056 (q), *P = 0.0013 (7d (r)) and *P = 0.0172 (14d (r)) (PAD4−/− and WT). *P = 0.0439 (p) and *P = 0.0210 (q), *P = 0.0091 (7d (r)) and *P = 0.0394 (14d (r)) (Cl-amidine and vehicle). Data are presented as mean ± SD. Source data underlying graph a, c, e, and g-r are provided as a Source Data file.

Discussion

Neovascularization and perfusion of the vascular structure in the peri-ischemic brain have important roles in stroke recovery37,38. However, these newly formed vessels are permeable and not yet fully developed28. The opened BBB leads to increased extravasation of immune cells and blood-derived toxic proteins39,40. Therefore, stability of the blood vessels and restoration of the damaged BBB may be crucial to maintaining a stable brain microenvironment. However, the mechanisms underlying vascular plasticity and the potential link between BBB opening and neovascular dysfunction after stroke are not fully understood.

Our data showed the accumulation of neutrophils in the brain during all stages of stroke, suggesting that neutrophils may cause delayed vascular damage. Indeed, neutrophil depletion reduced BBB breakdown at 14 days after stroke, demonstrating a role for neutrophils in the induction of vascular impairment during the later phases. We then found neutrophil depletion increased neovascularization and vascular perfusion. These results suggest that neutrophils are critical to disrupt stroke-induced new vessel formation and stabilization. These findings are also supported by previous studies showing that chronic BBB breakdown is associated with microvascular reductions34. Interestingly, in Alzheimer’s disease models, depletion of neutrophils by 300 μg of anti-Ly6G antibody every second day for 1 month reduced Alzheimer’s disease pathogenesis and improved memory41. However, anti-integrin therapies were previously shown to induce progressive multifocal leukoencephalopathy in patients with autoimmune disorders42. Further investigation of whether this long period of neutrophil depletion can induce side effects will be essential.

NETs release many cytotoxic proteases such as histone, elastase, and MPO, which directly induce endothelial cell damage to increase vascular permeability43. We observed that neutrophils isolated from ischemic mice formed more spontaneous NETs and showed a greater tendency to make NETs after exposure to LPS. Consistently, our data showed that stroke activated neutrophils to release excessive NETs within the vasculature and the parenchyma, concordant with elevated circulating DNA. Digestion of NETs with DNase 1 significantly reduced BBB damage, which was accompanied by increased pericyte coverage on microvessels and formation of new functional vessels, supporting NET formation as a cause of vascular injury44,45. These findings are in agreement with a previous report in mice with acute ischemic stroke46 and also strongly suggest that NETs may be critical for neutrophil-dependent vascular destabilization and regression during the delayed stages after stroke.

PAD4 is a key enzyme in chromatin decondensation30,31. We found that PAD4 was markedly upregulated in the peri-ischemic cortex. Overexpression of PAD4 resulted in amplified vascular damage and reduced neovascularization by releasing more NETs. Consistently, we showed that inhibition of NET formation by PAD4 genetic knockout or pharmacological inhibitor reduced loss of cerebrovascular integrity, increased neovascularization and capillary perfusion, and improved functional recovery. These results demonstrated that, by increasing NET formation, PAD4 impaired delayed vascular remodeling after stroke.

Our data revealed that stroke led to upregulation of the DNA sensor STING, activation of TBK1 and IRF3, and induction of the IRF3-dependent IFN-β synthesis. Silencing STING or administration of blocking antibody to IFNAR in mice increased vascular regeneration and repair. Furthermore, we found that the PAD inhibitor Cl-amidine suppressed the activation of the STING pathway and the production of IFN-β. Thus, the STING-mediated type 1 IFN response may link NETs and ischemic vascular remodeling.

In summary, our findings demonstrated that stroke caused neutrophil accumulation in the brain, releasing toxic signals such as NETs, which promoted subsequent activation of STING-dependent...
type I IFN-β production. Importantly, we also showed that increased NET formation or impaired NET clearance was detrimental to revascularization and vascular repair after stroke. We suggest that NETs are key targets for promoting stroke-mediated neovascularization and the resulting functional recovery.

Methods

Animal stroke model. Animal protocols were reviewed and approved by the Animal Care and Use Committee of the Institutes of Brain Science of Fudan University and were conducted in accordance with the ethical regulations. PAD4−/− mice on a C57BL/6J background were purchased from The Jackson Laboratory. Age-matched WT C57BL/6 mice (SLAC Laboratory Animal Co. Ltd, Shanghai, China) were used as controls. All mice were housed in a temperature-controlled environment (22 ± 2 °C) on a 12 h light–dark cycle with food and water available ad libitum. Room humidity was controlled at 55 ± 5%. Focal cortical cerebral ischemia was induced by electrocoagulation of the distal portion of the right middle cerebral artery (MCA)28,47. Male mice weighing 23–26 g were anesthetized with 1–1.5% isoflurane in 30% oxygen and 70% nitrous oxide. After making a 2 cm curved skin incision between the right eye and the right ear using surgical scissors, the temporal muscle was retracted laterally. Under an operating microscope, a 1.5 mm-diameter window was opened using a high-speed micro drill (Stoelting, CellPoint Scientific, Maryland) just rostral to the foramen ovale. The dura mater was carefully opened and the distal MCA was exposed and isolated, electrocauterized using bipolar electrocoagulation forceps, and disconnected just distal to crossing the olfactory tract. Immediately following the occlusion, the right common carotid artery (CCA) was occluded with a microvascular clip (Fine Science Tools, Foster City, CA) for 15 min. After closing of the surgical wounds, mice were allowed to recover from the anesthesia. Sham-operated animals only had the MCA and the CCA exposed.

For cerebral infarction, six coronal sections (20 μm) were stained with hematoxylin and eosin. Infarct volume was measured using the NIH ImageJ 1.46r software47 and was presented as percentage of the contralateral hemisphere.
In-vivo multiphoton microscopy. Mice were anesthetized with 1–1.5% isoflurane in 30% oxygen and 70% nitrous oxide, and kept on a heating plate (37 ± 0.5 °C).

To analyze microvascular perfusion, a 0.1 mL bolus of 10 mg/mL FITC-dextran (2,000,000 Da, Sigma-Aldrich; St. Louis, MO) was injected i.v.28,48,49. FITC-dextran (FVMPE-RS, Olympus, Japan) with an Olympus XL Plan N ×25/1.05 WMP dipping objective. Two-photon excitation was accomplished with MAITA ePHDS-OL, and Spectra Physics InSight DS-OL Lasers (Mai Tai, Spectra-Physics, Santa Clara, CA). Emitted fluorescence was detected through a 495–540 nm bandpass filter. To analyze vascular permeability, a 0.1 mL bolus of 10 mg/mL FITC-dextran (2,000,000 Da, Sigma-Aldrich; St. Louis, MO) was injected i.v.28,50.

To visualize NETs, mice were injected with 5 × 10^6 NETs (Invitrogen)52 30 min before imaging. To inject neutralizing monoclonal anti-mouse Ly6G antibody (1A8 clone; specific for neutrophils, BE0075-1, BioXcell, NH) 24 h or 7 days after cerebral ischemia44. The mice were then sacrificed with dental cement. Mice were imaged through cranial windows using an upright multiphoton laser-scanning microscope (FluoView FV10-ASW software). The total length of fields were taken and Sytox Green-fluorescence of randomly chosen 20 × 20 μm^2 regions of interest within the vessel and corresponding areas within the perivascular brain parenchyma were recorded.

Neutrophil depletion. Mice received an intraperitoneal (i.p.) injection of 100 μg monoclonal anti-mouse Ly6G antibody (1A8 clone; specific for neutrophils, BE0075-1, BioXcell, NH) 24 h or 7 days after cerebral ischemia44. The mice were then injected every second day for 3, 7, or 14 days until killing. Rat IgG2a isotype control (MAB-Mouse Ly6G, 1A8 clone; specific for neutrophils, BE0075-1, BioXcell, NH) 24 h or 7 days after cerebral ischemia44. The mice were then sacrificed with dental cement. Mice were imaged through cranial windows using an upright multiphoton laser-scanning microscope (FluoView FV10-ASW software). The total length of fields were taken and Sytox Green-fluorescence of randomly chosen 20 × 20 μm^2 regions of interest within the vessel and corresponding areas within the perivascular brain parenchyma were recorded.

Flow cytometry. Peripheral blood was subjected to red blood cell lysis buffer (Millipore) including protease inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). The levels of IFN-β was measured using the VeriKine™ Mouse Interferon Beta ELISA Kit (424001, BTL, Assay Science, NJ). According to the manufacturer’s instructions.

IFN-β measurement. Ischemic cortical tissues were homogenized in RIPA lysis buffer (Millipore) including protease inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). The levels of IFN-β was measured using the VeriKine™ Mouse Interferon Beta ELISA Kit (424001, BTL, Assay Science, NJ). According to the manufacturer’s instructions.

Flow cytometry. Peripheral blood was subjected to red blood cell lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L Na₂EDTA). Cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin

Fig. 8 STING-mediated effects on vascular remodeling are due to NETs. a Levels of IFN-β were increased in the ischemic cortex at 3 days, compared with sham-operated brains (n = 6), unpaired two-tailed Student’s t-test was applied with *P < 0.0001. b Immunoblot analysis of STING, phosphorylated TBK1 (pTBK1), and p38 in the cortex of mice without stroke or at day 3 after stroke. c Levels of IFN-β in the ischemic cortex at 3 days in mice treated with control or the PAD inhibitor CI-amilidine (n = 6). Mann–Whitney test was applied with *P = 0.0052. d Immunoblot analysis of STING, pTBK1, and p38 in the ischemic cortex at day 3. e Levels of IFN-β in the ischemic cortex at 3 days in mice treated with control antibody or anti-Ly6G antibody (n = 6), unpaired two-tailed Student’s t-test was applied with *P = 0.0148. f Immunoblot analysis of STING, pTBK1, and p38 in the ischemic cortex at day 3. g Levels of IFN-β in isolated bone marrow neutrophils from ischemic mice. Neutrophils were stimulated with LPS in the presence or absence of the PAD inhibitor CI-amilidine (n = 6). One-way ANOVA test was applied with *P = 0.0230 (Stoke vs. Vehicle), *P = 0.0160 (Vehicle vs. CI-amilidine). US, unstimulated. h Immunoblot analysis of STING, pTBK1, and p38 in isolated neutrophils for each group (n = 5). i, j Confocal images of CD31-positive microvessels (i) and quantification of microvascular density (j) in the peri-infarct cortex at 14 days in mice treated with control IgG or IFNAR-neutralizing antibody, and STING shRNA or control adenovirus (n = 6), unpaired two-tailed Student’s t-test was applied with *P < 0.0001 (Vehicle vs. IFNAR), *P = 0.0007 (Ad-con vs. Ad-sh-STING). Bar = 40 μm. k In vivo multiphoton microscopic images of perfused cortical capillaries with intravenously injected FITC-dextran (k) and quantification of perfused capillary length (l) at 14 days after stroke (n = 6), unpaired two-tailed Student’s t-test was applied with *P = 0.0006 (Vehicle vs. IFNAR), *P = 0.0080 (Ad-con vs. Ad-sh-STING). Bar = 100 μm. m, n In vivo multiphoton microscopic images (m) of intravenously injected FITC-dextran leakage in cortical vessels at 14 days and quantification of the permeability (P) product of FITC-dextran (n) for each group (n = 6), unpaired two-tailed Student’s t-test was applied with *P = 0.0028 (Vehicle vs. IFNAR), *P = 0.0053 (Ad-con vs. Ad-sh-STING). Bar = 100 μm. Data are presented as mean ± SD. Source data underlying graph a–h, j, l, and n are provided as a Source Data file.
(BSA) and resuspended in rat anti-mouse CD16/32 Fc block (2.4G2 clone; 1 : 100, 553141, BD Pharmingen) in PBS. Cell suspension was incubated with Allophycocyanin-conjugated antibody (APC-Cy7) and Terminator green dye (TGGCGTGGTCCGCTAGACAGCA-3′). Neutrophil purity was determined using an Olympus BX 63 microscope and an Olympus FV 1000 confocal microscope.

Cytospin NET analysis. Whole blood collected from the retro-orbital sinus was lysed with red blood cell lysis buffer. The cells were resuspended in 7.5% BSA in PBS and plated on slides using a Shandon Cytospin 4 (Thermo Scientific). Slides were fixed in 4% paraformaldehyde at 4 °C overnight and incubated with rabbit anti-H3Cit (1 : 1000, ab5103, Abcam) and rat anti-Ly6G (1 : 200, 551459, BD Pharmingen) antibodies overnight at 4 °C, then incubated withAlexa Fluor 488-conjugated donkey anti-rabbit and Alexa Fluor 594-conjugated donkey anti-rat secondary antibodies (1 : 1000, Invitrogen). DNA was stained with Hoechst 33342 (Pharmingen) antibodies overnight at 4 °C, then incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1 : 100, A21207), Alexa Fluor 488-conjugated donkey anti-goat IgG (1 : 100, A-11058), Alexa Fluor 488-conjugated donkey anti-rat IgG (1 : 100, A-21208), Alexa Fluor 488-conjugated donkey anti-mouse IgG (1 : 100, A-21202), Alexa Fluor 647-conjugated donkey anti-goat IgG (1 : 1000, A-21447), and biotin-conjugated donkey anti-rat IgG (1 : 1000, A18743, from Invitrogen, Weiterstadt, Germany) antibodies (1 : 200). DNA was stained with Hoechst 33342 (1 : 10,000, Invitrogen). Staining was visualized by Olympus FV 1000 laser-scanning confocal microscope and an Olympus BX63 microscope. For each animal, three fields from each animal slide were imaged at x40 objective. Images were processed using FV10-ASW 4.2 Viewer software and ImageJ 1.46 software.

Preparation of brain microvessels and capillary-depleted brain homogenates. Brains were removed and the meninges and large surface vessels were discarded. Brain tissue was homogenized in 50 mM potassium phosphate buffer, centrifuged, and suspended in 60 μl 10% cetyltrimethylammonium bromide (Sigma-Aldrich) in potassium phosphate buffer. The suspensions were sonicated for 30 s with three freeze–thaw cycles in liquid nitrogen. After centrifugation, 40 μl of supernatant was incubated with 100 μl tetramethylbenezidine solution (Sigma-Aldrich) and the reaction was stopped with 100 μl 2% H2O2. The optical density was measured at 450 μm (Bio-Tek, Vermont) and 590 μm (BioTek, Vermont). MPO activity was expressed in equivalent units by comparison with a reference curve generated using purified MPO (Sigma-Aldrich).

MOB in the sciatic nerve. Spinal cord brain cortices were homogenized in 50 mM potassium phosphate buffer, centrifuged, and suspended in 60 μl 10% cetyltrimethylammonium bromide (Sigma-Aldrich) in potassium phosphate buffer. The suspensions were sonicated for 30 s with three freeze–thaw cycles in liquid nitrogen. After centrifugation, 40 μl of supernatant was incubated with 100 μl tetramethylbenzidine solution (Sigma-Aldrich) and the reaction was stopped with 100 μl 2% H2O2. The optical density was measured at 450 μm (Bio-Tek, Vermont) and 590 μm (BioTek, Vermont). MPO activity was expressed in equivalent units by comparison with a reference curve generated using purified MPO (Sigma-Aldrich).

Preparation of brain microvessels and capillary-depleted brain homogenates. Brains were removed and the meninges and large surface vessels were discarded. Brain tissue was homogenized in 16% dextran (Sigma-Aldrich) in PBS containing 2% fetal bovine serum centrifuged at 6000 × g for 15 min. The supernatant was collected and centrifuged again to obtain capillary-depleted brain homogenates. Pellets were resuspended in PBS containing 1% BSA and passed through a 100 and 45 μm cell strainer (BD Falcon, CA). Microvessels were trapped on top of the 45 μm strainer.

Western blotting. Brain tissues, microvessels, and capillary-depleted brain homogenates were lysed in RIPA lysis buffer (Millipore) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Mannheim, Germany). Equal amounts of protein were loaded on SDS-polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes. The primary antibodies used were as follows: rabbit anti-Histone H3 (anti-H3; 1 : 100, 9715), rabbit anti-p38 (1 : 1000, 512660, Cell Signaling Technology, MA), rabbit anti-phospho-p38 (1 : 1000, 524620, Cell Signaling Technology, MA), rabbit anti-p42 (1 : 1000, 512660, Cell Signaling Technology, MA). The secondary antibodies used were as follows: Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1 : 100, A21207), Alexa Fluor 488-conjugated donkey anti-goat IgG (1 : 100, A-11058), Alexa Fluor 488-conjugated donkey anti-rat IgG (1 : 100, A-21208), Alexa Fluor 488-conjugated donkey anti-mouse IgG (1 : 100, A-21202), Alexa Fluor 647-conjugated donkey anti-goat IgG (1 : 1000, A-21447), and biotin-conjugated donkey anti-rat IgG (1 : 1000, A18743, from Invitrogen, Weiterstadt, Germany). Immunohistochemistry. Frozen sections were visualized by Olympus FV 1000 laser-scanning confocal microscope and an Olympus BX63 microscope. For each animal, three fields from each animal slide were imaged at x40 objective. Images were processed using FV10-ASW 4.2 Viewer software and ImageJ 1.46 software. The length of CD31-positive vessels was measured using the ImageJ area measurement tool and were expressed as a percentage of the CD31-positive area in 0.42 mm² regions. The numbers of Ly6G-positive and H3Cit-positive cells in the traced area were determined.

Statistical analysis. The data were analyzed using GraphPad Prism 7 software. All values are presented as mean ± SD. Multiple comparisons were analyzed by one-way analysis of variance followed by the Bonferroni multiple comparison test. When comparing two groups, unpaired Student's t-test or Mann–Whitney test was performed. Differences were considered significant at P < 0.05.

Data availability
All data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. The source data underlying Fig. 1b, c, d, h, 2a, c, e, g, i, k, 3b, d, f, i, k, m, 5b, 6a, b, c, d, e, f, i, b, k, m, p, 7a, c, e, g, r, and 8a, b, c, d, e, f, g, h, j, l, n and Supplementary Figs. 2f–i, h, 4a, b, d, 5a–c, i–l, n, b, d, h, 7a, c, e, g, 8a–e, 10a–i, 11a–c and 56 are provided as a Source Data file.
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