tPA Mobilizes Immune Cells That Exacerbate Hemorrhagic Transformation in Stroke

Kaibin Shi, Ming Zou, Dong-Mei Jia, Samuel Shi, Xiaoxia Yang, Qiang Liu, Jing-fei Dong, Kevin N. Sheth©, Xiaoying Wang©, Fu-Dong Shi©

RATIONALE: Hemorrhagic complications represent a major limitation of intravenous thrombolysis using tPA (tissue-type plasminogen activator) in patients with ischemic stroke. The expression of tPA receptors on immune cells raises the question of what effects tPA exerts on these cells and whether these effects contribute to thrombolysis-related hemorrhagic transformation.

OBJECTIVE: We aim to determine the impact of tPA on immune cells and investigate the association between observed immune alteration with hemorrhagic transformation in ischemic stroke patients and in a rat model of embolic stroke.

METHODS AND RESULTS: Paired blood samples were collected before and 1 hour after tPA infusion from 71 patients with ischemic stroke. Control blood samples were collected from 27 ischemic stroke patients without tPA treatment. A rat embolic middle cerebral artery occlusion model was adopted to investigate the underlying mechanisms of hemorrhagic transformation. We report that tPA induces a swift surge of circulating neutrophils and T cells with profoundly altered molecular features in ischemic stroke patients and a rat model of focal embolic stroke. tPA exacerbates endothelial injury, increases adhesion and migration of neutrophils and T cells, which are associated with brain hemorrhage in rats subjected to embolic stroke. Genetic ablation of annexin A2 in neutrophils and T cells diminishes the effect of tPA on these cells. Decoupling the interaction between mobilized neutrophils/T cells and the neurovascular unit, achieved via a S1PR (sphingosine-1-phosphate receptor) 1 modulator RP101075 and a CCL2 (C-C motif chemokine ligand 2) synthesis inhibitor bindarit, which block lymphocyte egress and myeloid cell recruitment, respectively, attenuates hemorrhagic transformation and improves neurological function after tPA thrombolysis.

CONCLUSIONS: Our findings suggest that immune invasion of the neurovascular unit represents a previously unrecognized mechanism underlying tPA-mediated brain hemorrhage, which can be overcome by precise immune modulation during thrombolytic therapy.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: brain ischemia ■ immune system ■ inflammation ■ stroke ■ tissue-type plasminogen activator

Meet the First Author, see p 4

tPA (tissue-type plasminogen activator) and endo-
vascular thrombectomy are the 2 pillars of treatment
for patients with acute ischemic stroke. However,
only patients with large vessel occlusion have been
shown to benefit from thrombectomy, which requires
expertise and infrastructure that are not evenly distrib-
uted across centers.1,2 Within 4.5 hours of ictus, tPA
remains the gold standard treatment for eligible isch-
emic stroke patients. However, due to the narrow ther-
apeutic window and potential severe adverse events
such as hemorrhagic transformation (HT) and mali-
gnant brain edema associated with the nonthrombolytic
effect of tPA, <5% ischemic stroke patients benefit
from tPA treatment.2
HT occurs when blood products extravasate into the infarct area during reperfusion. This is caused by increased permeability of blood-brain barrier (BBB) and vascular basal lamina dysfunction. tPA-mediated thrombolysis increases the risk of HT. Recent research suggests that the nonthrombolytic effects of tPA may contribute to this devastating complication. tPA binds several receptors, including annexin A2, LRP (low-density lipoprotein receptor–related protein) 1, and the NMDAR (N-methyl-D-aspartate receptor), resulting in differential downstream biological effects. For instance, tPA compromises BBB integrity via LRP1 expressed on endothelial cells, microglia, and astrocytic endfeet. tPA also activates PDGF-CC (platelet-derived growth factor-CC) and kallikrein, which promote BBB disruption.

The presence of tPA receptors on immune cells prompts the question of what effects tPA exerts on these cells and whether these effects contribute to HT. Indeed, tPA alters the activation status of monocytes/macrophages exposed to lipopolysaccharide and increases leukocyte infiltration into lung, kidney, and cremaster muscle during ischemia/reperfusion. Among leukocyte subsets, neutrophils have been linked to intracerebral hemorrhage after thrombolysis in animal models and patients with ischemic stroke; however, several key questions remain unanswered. First, as all the published studies evaluate circulating immune response at 24 hours after tPA thrombolysis, at that time HT has already occurred, it is unknown whether the augmented circulating leukocyte response is a cause of onset promotes neurological recovery of stroke patients, in part, by opening up the clotted blood vessels. However, the narrow time frame and the risk of intracerebral hemorrhage after tPA treatment pose major drawbacks to its clinical application. Peripheral immune cells infiltrating into ischemic brain exacerbate blood-brain barrier disruption and neurovascular injury. However, it remains unknown what effects tPA has on its receptor-bearing immune cells and whether these effects contribute to tPA-related hemorrhagic transformation. In this study, we have demonstrated that tPA swiftly mobilizes circulating neutrophils and T cells in patients with acute ischemic stroke and a rat model of embolic stroke. These neutrophils and T cells home to the ischemic brain and contribute to the emergence of hemorrhagic transformation. Further, we have discovered these deleterious effects of tPA are mediated by annexin A2 expressed by neutrophils and T cells. Pharmacologically targeting annexin A2 or inhibiting immune invasion into neurovascular unit attenuates hemorrhagic transformation and improves neurological outcome after tPA thrombolysis. Thus, by precise immune modulation, it is possible that tPA's limitations can be ameliorated to benefit more patients with acute ischemic stroke.
or consequence of tPA-related HT. Second, the direct effects of tPA on leukocytes and their contribution to HT after tPA thrombolysis are still unclear. Third, in addition to neutrophils, the role of other leukocyte subsets such as lymphocytes remains elusive in tPA-related HT. To address these questions, we have performed studies on ischemic stroke patients receiving tPA thrombolysis and a rat embolic middle cerebral artery occlusion (eMCAO) model.

METHODS
A detailed methods section is provided in the Data Supplement. Please refer to the Major Resources Table in the Data Supplement.

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

**tPA Swiftly Mobilizes Neutrophils and T Cells in Ischemic Stroke Patients and in a Rat Model of Focal Embolic Stroke**

To investigate how tPA influences the immune system, we analyzed circulating myeloid cells and lymphocytes from 71 ischemic stroke patients who received tPA and 27 without tPA. Total leukocyte and lymphocyte counts were increased by ≈15% and 19%, respectively, as early as 1 hour after tPA administration compared with cell counts before tPA treatment and control patients (Figure I in the Data Supplement). Further analysis of cellular subsets via flow cytometry revealed that neutrophils increased by 31%; T cells, including cluster of differentiation (CD)4+ T cells increased by 20%, and CD8+ T cells increased by 26% (Figure 1). Neutrophils and T cells were the main cell types influenced by tPA administration, whereas counts of monocytes, B cells, and natural killer cells remained relatively stable before and after tPA treatment; these cell counts were also comparable to those of control patients (Figure II in the Data Supplement).

Next, we induced embolic stroke in rats by delivering a 4-cm fibrin-enriched clot to the origin of middle cerebral artery via a catheter and administered tPA at 3 hours post-ischemia (Figure III in the Data Supplement). This model more accurately recapitulates the key components of thromboembolic stroke and subsequent tPA-induced thrombolysis in human ischemic stroke, relative to the intraluminal filament middle cerebral artery occlusion model. In eMCAO rats, the count of circulating neutrophils was increased at 1 hour after tPA administration by ≈4-fold as compared with controls receiving saline. The increase of circulating neutrophils was sustained up to 12 hours post-thrombolysis (Figure 2A and 2B). CD4+ and CD8+ T cells were also increased by ≈2-fold in peripheral blood samples of eMCAO rats treated with tPA, with elevation of CD8+ T cells detected as early as 15 minutes after tPA administration (Figure 2B). A transient increase in B-cell count was also observed following tPA treatment (Figure IVB in the Data Supplement), this was not apparent in blood samples of stroke patients. Monocytes and natural killer cells were not significantly impacted by tPA administration (Figure IVA and IVC in the Data Supplement). In addition, no significant sex impact on the peripheral immune response of eMCAO rats after tPA thrombolysis was recorded (Figure V in the Data Supplement).

Notably, cell counts in peripheral blood samples obtained from sham rats receiving tPA did not exhibit significant changes. The reason for this disparity is unclear but could imply that brain ischemia renders immune cells more susceptible to tPA. To further characterize the newly mobilized neutrophils after tPA thrombolysis, we sorted circulating neutrophils from rats and utilized RNA sequencing to characterize the molecular features. In circulating neutrophils from eMCAO rats receiving tPA, we identified a total of 2575 altered genes (upregulated, 1449 genes; downregulated, 1126 genes) after tPA thrombolysis as compared with eMCAO group receiving PBS. Moreover, 820 altered genes were identified in eMCAO+PBS group versus eMCAO+tPA group (Figure VIA and VIB in the Data Supplement). Among the altered genes, we found a significant increase of genes related to neutrophil chemotaxis, activation, and infiltration, including CCR2 (C-C motif chemokine receptor 2) and MMP (matrix metallopeptidase) 9 (Figure 2C and 2D). This result indicates that tPA thrombolysis induces profound changes in peripheral neutrophils of eMCAO rats. Similarly, the expression of the adhesion molecule receptor CD49d and activation molecule CD69 was increased in CD4+ and CD8+ T cells following tPA administration (Figure 2E). Together, these results indicate that tPA preferentially augments peripheral neutrophils and T cells in ischemic stroke patients and in rats following eMCAO.

**Neutrophil and T-Cell Invasion of the Cerebrovascular Compartment Is Associated With HT Following tPA Thrombolysis**

We next examined the destination of migrating immune cells following tPA thrombolysis in eMCAO rats. Compared with saline-treated rats, neutrophils, CD4+ T cells, and CD8+ T cells were observed in ischemic brain 4 hours post-tPA administration, becoming more pronounced at 12 hours (Figure 3A). The counts of brain-infiltrating neutrophils and T cells were associated with
their corresponding counts in blood after tPA XCT thrombolysis (Figure VII in the Data Supplement). In addition, upregulation of CCR2, CXCR2 (C-X-C motif chemokine receptor 2), MMP9, and TLR4 (toll-like receptor 4) was observed in brain-infiltrating neutrophils (Figure VIII in the Data Supplement). Importantly, while the infiltration of these cells in the brain parenchyma of eMCAO rats was not observed at 1 hour after tPA administration, the accumulation of neutrophils and T cells in the microvessel lumen of the ipsilateral hemisphere was evident at this early time (Figure 3B and 3C).

Having determined that these activated immune cells were physiologically associated with the cerebral blood vessel endothelium, we went on to test whether this finding is related to postthrombolysis HT. To this end, we analyzed the relationship between counts of circulating cells at 1 hour after tPA infusion and cerebral hemorrhage volume at 24 hours after eMCAO. Intracerebral hemorrhage was measured by 7T rodent magnetic resonance imaging scanning using a T2* sequence, which is sensitive to brain hemorrhage.20 Linear regression analysis confirmed that brain hemorrhage volume was associated with the numbers of neutrophils, CD4+ T cells, and CD8+ T cells in peripheral blood samples at 1 hour after tPA infusion (Figure 3D). Considering that HT occurs at a median of 5 to 10 hours following tPA administration, these data suggest that tPA-mobilized neutrophils and T cells may contribute to the development of brain hemorrhage.

Direct Effects of tPA on Neutrophils and T Cells and Their Impact on Endothelial Injury Following Hypoxia and Glucose Deprivation

To determine whether tPA directly acts on neutrophils and T cells, we sorted these cells from blood samples obtained from rats at 3 hours post-eMCAO and sham controls. Following exposure to 1 to 100 µg/mL tPA, flow cytometry analysis was performed to evaluate the...
expression of adhesion molecules in these cells. A time- and dose-dependent elevation of CCR2 on neutrophils and CD49d on T cells was observed at 12 hours in cells isolated from eMCAO rats; no significant upregulation in cells from sham rats was detected (Figure 4A through 4C). Neutrophils exhibited a quicker response to tPA treatment than did T cells, as CCR2 expression level was increased as early as 1 hour after tPA treatment at 100 µg/mL (Figure 4A), while the CD49d expression of T cells was not upregulated until 12 hours after tPA treatment (Figure 4B and 4C). To compare newly released versus old neutrophils and T cells, we injected Sulfo-NHS-LC Biotin into recipient rats to label circulating neutrophils and T cells.23 Such an approach allows identification of freshly released neutrophils and T cells after 1 hour as Biotin−, whereas old neutrophils and T cells are Biotin+. In eMCAO rats receiving Sulfo-NHS-LC-Biotin injection, we found upregulation of CCR2 and MMP9...
Figure 3. tPA (tissue-type plasminogen activator) facilitates the migration of peripheral neutrophils and T cells to the neurovascular unit that was associated with hemorrhagic transformation.

A, Brain tissue of embolic middle cerebral artery occlusion (eMCAO) rats receiving saline or tPA treatment at 3 h after surgery was analyzed using flow cytometry to determine the brain infiltration of neutrophils and T cells. Dot plots at the top show the gating of neutrophils (CD45+ CD11b+ granulocyte+), CD4+ T cells (CD45+ CD3+ CD4+), and CD8+ T cells (CD45+ CD3+ CD8+) of rat brain tissue after eMCAO. Statistical graphs at the bottom show the absolute cell numbers of neutrophils, CD4+ T cells, and CD8+ T cells in the brain tissue of eMCAO rats at indicated time points after tPA administration. n=6 in each group.

B and C, Left, Immunohistochemical staining of brain slices of eMCAO rats at 1 and 12 h after tPA or saline administration for CD3 (B), neutrophil (C), and CD31 to detect the immune cells that accumulated within the microvessel lumen, as well as which infiltrated into the parenchyma that was located within the ipsilateral ischemic area. White triangles signify cells accumulated in the microvessel lumen, white stars denote the cells infiltrating into the parenchyma. Scale bar=50 µm. Right, Graphs show the absolute cell numbers of T cells (B) and neutrophils (C) within blood vessels or parenchyma and statistical analysis. n=10 in each group.

D, Brain hemorrhage volume of eMCAO rats treated by tPA was measured by magnetic resonance imaging T2* images at 24 h after ischemia.

E–G, The correlation between brain hemorrhage volume at 24 h after stroke and circulatory cell counts of neutrophils, CD4+ T cells, and CD8+ T cells at 1 h after tPA thrombolysis, n=12. Data are shown as mean±SEM; 2-way ANOVA was performed in A–C and t test in D. Linear regression analysis was performed in E–G. Linear regression line, R² (goodness of fit), and P are shown in the graphs. *P<0.05, **P<0.01. CD indicates cluster of differentiation; FSC, forward scatter; and SSC, side scatter.
in newly released Biotin− neutrophils versus old Biotin+ neutrophils (Figure IX in the Data Supplement). Similarly, we observed upregulation of CD69 and CD49d in CD4+ T cells (B) and CD8+ T cells (C) at 12 h after tPA exposure. n=6 per group. D–F, Endothelial bEND3 cells seeded in cell culture inserts (D) or cover slides (E) coated by collagen and fibronectin, or in plates (F), were exposed to 4 h of HGD. After this, cells were cocultured with neutrophils or T cells treated with or without tPA. For neutrophils, tPA was pretreated for 4 h because of its quick response. For T cells, tPA was pretreated for 12 h. Neutrophils and T cells were washed with PBS to remove tPA in the medium before coculture. The ratio of endothelial cells to neutrophils/T cells was 2:1. D, Blood-brain barrier permeability was determined by measuring the FITC (Fluorescein)-dextran diffused from the upper chamber to lower chamber over 12 h. n=6 in each group. E, Immunofluorescence staining images show the tight junction protein claudin-5 expression of endothelial cells. Scale bar=50 µm. F, Twelve hours after coculture, cell lysates were collected for quantitative measurement of claudin-5 by Western blot. n=6 per group. Data are shown as mean±SEM; 2-way ANOVA in A–D or 1-way ANOVA in F. *P<0.05, **P<0.01. CD indicates cluster of differentiation; and FITC, Fluorescein.

Figure 4. Neutrophils and T cells exposed to tPA (tissue-type plasminogen activator) exacerbate endothelial cell injury after hypoxia and glucose deprivation (HGD).

A–C, Isolated neutrophils and T cells from embolic middle cerebral artery occlusion (eMCAO) or sham rats at 3 h after surgery were cultured with tPA at indicated concentrations. Flow cytometry analysis of CCR2 (C-C motif chemokine receptor 2) expression in neutrophils (A) and CD49d in CD4+ T cells (B) and CD8+ T cells (C) at 12 h after tPA exposure. n=6 per group. D–F, Endothelial bEND3 cells seeded in cell culture inserts (D) or cover slides (E) coated by collagen and fibronectin, or in plates (F), were exposed to 4 h of HGD. After this, cells were cocultured with neutrophils or T cells treated with or without tPA. For neutrophils, tPA was pretreated for 4 h because of its quick response. For T cells, tPA was pretreated for 12 h. Neutrophils and T cells were washed with PBS to remove tPA in the medium before coculture. The ratio of endothelial cells to neutrophils/T cells was 2:1. D, Blood-brain barrier permeability was determined by measuring the FITC (Fluorescein)-dextran diffused from the upper chamber to lower chamber over 12 h. n=6 in each group. E, Immunofluorescence staining images show the tight junction protein claudin-5 expression of endothelial cells. Scale bar=50 µm. F, Twelve hours after coculture, cell lysates were collected for quantitative measurement of claudin-5 by Western blot. n=6 per group. Data are shown as mean±SEM; 2-way ANOVA in A–D or 1-way ANOVA in F. *P<0.05, **P<0.01. CD indicates cluster of differentiation; and FITC, Fluorescein.

In newly released Biotin− neutrophils versus old Biotin+ neutrophils (Figure IX in the Data Supplement). Similarly, we observed upregulation of CD69 and CD49d in newly released Biotin− T cells versus old Biotin+ T cells (Figure IX in the Data Supplement). As tPA can induce endothelial injury, we also determined whether tPA-induced endothelial injury could contribute to immune activation after tPA administration. For this purpose, we performed coculture experiments using endothelial cells exposed to tPA and immune cells isolated from eMCAO rats. We found that tPA-induced endothelial injury alone is insufficient to activate neutrophils and T cells (Figure X in the Data Supplement).

Next, we sought to determine whether tPA-activated neutrophils or T cells exacerbate endothelial injury—the key pathological mechanism underlying HT24. An in vitro BBB model was established by seeding a monolayer of endothelial bEND3 cells on collagen and fibronectin-coated inserts in a transwell culture system. This in vitro BBB model was exposed to hypoxia combined with
glucose deprivation for 4 hours, followed by coculture with tPA-treated neutrophils or T cells. FITC (Fluorescein)–dextran that leaked from the upper chamber to the lower chamber was quantified to assess the permeability. Relative to baseline, hypoxia glucose deprivation increased the diffusion of FITC–dextran as early as 1 hour after hypoxia induction. tPA-treated neutrophils or T cells exacerbated hypoxia glucose deprivation–induced FITC–dextran diffusion by ≈2-fold, whereas untreated neutrophils or T cells produced only a trend toward increasing BBB leakage without statistical significance (Figure 4D). In addition, tPA-treated neutrophils or T cells promoted the degradation of the tight junction protein claudin-5 (Figure 4E and 4F). These findings suggest that tPA directly activates neutrophils and T cells, which exacerbates BBB disruption after tPA thrombolysis.

Annexin A2 Bridges tPA and Immune Cells After Thrombolysis

To identify the tPA receptor that mediates the effects of tPA on immune cells, we screened the expression of several tPA receptors (LRP1, LRP4, annexin A2, and NMDAR) in immune cells collected from blood of eMCAO rats. Among examined receptors, annexin A2 was highly expressed by ≈40% of neutrophils. Comparison of annexin A2 expression among different immune cell types revealed that neutrophils and T cells were the predominant cell populations expressing annexin A2 (Figure XI in the Data Supplement). We then examined the potential downstream pathways that mediate the effects of tPA on immune cells. KEGG pathway enrichment analysis revealed that MAPK (mitogen-activated protein kinase) signaling pathway was the most enriched pathway in neutrophils sorted from eMCAO rats following tPA thrombolysis; genes of MAPK family members are highly expressed in neutrophils of eMCAO rats receiving tPA (Figure 5A and 5B), suggesting that MAPK pathway might mediate the activation of immune cells after tPA administration. Western blot analysis demonstrates activated p38 MAPK in neutrophils of eMCAO rats after tPA treatment (Figure 5C). In addition, a selective p38 MAPK inhibitor prevented the activation of neutrophils by tPA in eMCAO rats (Figure 5D), suggesting the contribution of tPA–annexin 2–MAPK axis to tPA-induced mobilization of immune cells.

Next, we tested whether annexin A2 is necessary for the effect of tPA on neutrophils or T cells. Neutrophils and T cells isolated from healthy rat bone marrow or spleen, respectively, were transfected with annexin A2 siRNA to knock down annexin A2 expression (Figure XII in the Data Supplement). After labeling with Molday ION Rhodamine B, 5×10^6 cells were injected via tail vein to recipient rats before eMCAO surgery and tPA administration (Figure 6A). Molday ION Rhodamine B is a fluorescence-conjugated nanoparticle that can be used to trace extrinsically transferred cells in vivo as we reported previously. Compared with cells transfected with control siRNA, annexin A2 knockdown inhibited tPA-induced activation of p38 MAPK (Figure XIII in the Data Supplement). The expression of CCR2 and CD49d on neutrophils and T cells was also reduced after annexin A2 knockdown at 12 hours post-tPA administration (Figure 6D, 6E, and 6G through 6J), together with reduced transmigration of these cells into the ischemic brain (Figure 6F and 6K). These findings suggest that the tPA receptor annexin A2 is required for the effects of tPA on peripheral immune cells.

Suppression of the Transmigration of Neutrophils and T Cells Reduced tPA-Associated HT and Improved Neurological Function Following eMCAO

We next sought to examine whether inhibition of T cell or neutrophil migration reduces the extent of HT associated with tPA thrombolysis. To inhibit tPA-induced immune activation, an anti-annexin A2 mAb was given to eMCAO rats immediately before tPA administration. We found significant reduction in brain hemorrhage volume in eMCAO rats receiving anti-annexin A2 mAb (Figure XIV in the Data Supplement). We then tested 2 immune modulating drugs that have translational potential. RP101075 is a second generation of S1PR (sphingosine-1-phosphate receptor) modulator, which selectively inhibits S1PR1-dependent lymphocytes egress from secondary lymphoid organs. Compared with eMCAO rats receiving saline, eMCAO rats receiving tPA at 3 hours after ischemia had significantly increased brain hemorrhage at 24 hours poststroke (saline versus tPA, 5.6±0.7 versus 14.7±1.4 mm³) without amelioration of infarction (Figure 7A through 7F). In eMCAO rats receiving RP101075, we found significantly reduced numbers of circulating and brain-infiltrating T cells (Figure XVA and XVB in the Data Supplement). RP101075 significantly reduced brain hemorrhage in eMCAO rats receiving tPA with a 46% reduction as compared with tPA-treated rats (tPA+RP101075 versus tPA, 7.9±1.5 versus 14.7±1.4 mm³). In addition, the infarct volume of tPA+RP101075-treated eMCAO rats was 40% smaller than that of tPA-treated rats (tPA+RP101075 versus tPA, 79±1.5 versus 147.4±14.7 mm³). Although tPA thrombolysis at 3 hours after eMCAO did not significantly improve the neurological deficit as compared with eMCAO rats receiving saline, the combination of RP101075 and tPA significantly reduced neurological deficits, neuronal death, and improved long-term sensorimotor function up to 4 weeks after eMCAO (Figure 7G through 7I; Figure XVI in the Data Supplement).

In addition, we examined whether inhibition of neutrophil migration using bindarit reduces tPA-associated
hemorrhage. Bindarit is a CCL2 (C-C motif chemokine ligand 2) inhibitor that blocks the migration of myeloid cells including neutrophils.\textsuperscript{27} Importantly, tPA administration upregulated CCR2 expression on neutrophils, suggesting the involvement of CCL2-CCR2 pathway in tPA-mediated neutrophil transmigration. The extent of neutrophil infiltration into brain parenchyma was reduced in bindarit-treated rats (Figure XVC and XVD in the Data Supplement). Combination of bindarit with tPA reduced volume of brain hemorrhage by 34% compared with tPA-treated rats at 24 hours post-ischemia (tPA+bindarit versus tPA, 9.7±1.5 versus 14.7±1.4 mm$^3$). Infarct volume was reduced by 36% in rats receiving combined treatment of tPA and bindarit relative to rats receiving tPA alone (tPA+bindarit versus tPA, 146.2±19.0 versus 231.4±14.7 mm$^3$). Bindarit also reduced acute neurological deficits at 24 hours after ischemia and improved sensorimotor function at 4 weeks (Figure 7G through 7I). These data as a whole suggest that inhibition of neutrophil and lymphocyte transmigration may reduce the hemorrhagic risk and improve the efficacy of tPA thrombolysis for ischemic stroke.

**DISCUSSION**

Previous studies suggest that tPA upregulates the expression of MMPs on brain vascular endothelium\textsuperscript{8} and accelerates the degradation of the extracellular matrix of blood vessels, thereby contributing to HT following thrombolytic therapy. In addition to directly activating...
endothelial cells, the present study demonstrates that tPA mobilizes peripheral neutrophils and T cells, which transmigrate to the brain vasculature. Neutrophils and T cells exposed to tPA subsequently exacerbate BBB disruption and promote intracerebral hemorrhage. Further, we show that the action of tPA on neutrophils and T cells requires annexin A2 and involves the downstream MAPK pathway. These results suggest that tPA-mediated neurovascular inflammation represents a new mechanism underlying HT following thrombolytic therapy in ischemic stroke (Figure XVII in the Data Supplement).

The rationale of studying the action of tPA on immune cells as early as 1 hour after tPA administration is manifold. First, tPA is quickly metabolized in human plasma with a half-life of \( \approx 5 \) minutes. Hence, any significant effects directly related to tPA administration would occur soon after infusion. Second, HT is the major adverse event related to tPA administration and mostly occurs...
within 24 hours of treatment, with a median onset from 5 to 10 hours. Consequently, any discernible effects at later than 24 hours is presumed to be secondary effects to HT because the direct effects of tPA would have subsided by this time. Therefore, 24 hours has commonly been used as the time-point to assess the effects of tPA and related adverse events. In the present study, neutrophil and T-cell populations were swiftly increased in peripheral blood samples obtained from ischemic stroke patients as early as 1 hour post-tPA infusion, implying that the action of tPA on the peripheral immune system occurred before the emergence of adverse events within the brain.

In some stroke cases, HT occurs as a pathological consequence of brain infarction. Particularly following large and embolic stroke, this rate is worsened by intravenous tPA administration. A meta-analysis of 12 trials demonstrates that intravenous tPA causes 60 additional symptomatic intracerebral hemorrhages per 1000 treated stroke patients. Postthrombolysis hemorrhage is the most feared complication of intravenous thrombolysis, with almost 50% mortality in cases with symptomatic hemorrhage following tPA. HT occurs when the integrity of the endothelial cells lining brain vasculature and BBB becomes compromised. Endogenous tPA engendered during brain ischemia also increases the permeability of BBB, which may prime microvessels for the deleterious extravascular effects of therapeutically administered exogenous tPA. Increased BBB permeability before tPA treatment, which is determined by stroke severity and time to treatment, is thought to promote leakage of

Figure 7. Pharmacological inhibition of neutrophil or lymphocyte transmigration attenuates tPA (tissue-type plasminogen activator)-associated brain hemorrhage.

Groups of embolic middle cerebral artery occlusion (eMCAO) rats received saline or tPA at 3 h after surgery. The S1PR (sphingosine-1-phosphate receptor) 1 modulator RP101075 (0.6 mg/kg) or CCL2 (C-C motif chemokine ligand 2) inhibitor bindarit (50 mg/kg) was given by oral gavage immediately before tPA administration. A and B, At 24 h after eMCAO, infarct volume and intracerebral hemorrhage volume were evaluated by magnetic resonance imaging (MRI) scanning. T2-weighted images (T2WI) of rat brains show the ischemic lesion (red dashed line; A) and hemorrhagic lesions (blue arrow; B). C and D, Gross brain images (C) and histology staining images (D) show the brain hemorrhage in the ipsilateral hemisphere of eMCAO rats of indicated groups at 24 h after ischemia. Scale bar=50 µm. E and F, Measurement of infarct volume (E) and hemorrhage volume (F) based on MRI images of eMCAO rats in indicated groups. G, Neurological deficits were measured by assessing mNSS score at day 1 after eMCAO. H and I, Sensorimotor function was measured using adhesive removal test and corner test until 28 d after eMCAO. Data are shown as mean±SEM; n=10 in saline group, 11 in tPA group, 10 in RP101075 group, 12 in tPA+RP101075 group, 10 in Bindarit group, and 10 in tPA+Bindarit group. Kruskal-Wallis test in G and H, and 2-way ANOVA in I. *P<0.05, **P<0.01. mNSS indicates modified neurological severity score.
exogenously administered tPA into the perivascular space. However, according to a recent meta-analysis of individual patient data from 6756 patients, fatal intracranial hemorrhage risk was similar irrespective of treatment delay and stroke severity, suggesting that intravascular effects of tPA may, at least in part, play a role in HT formation. This study presents the first definitive evidence that tPA thrombolysis swiftly mobilizes neutrophils and T cells to accumulate in the cerebrovascular compartment. These cells then act on endothelial cells and induce BBB disruption. The combination of augmented focal inflammation after ischemia together with the direct damage exerted by tPA-mobilized immune cells results in grave consequences for the brain vasculature, leading to hemorrhagic complications.

Understanding the inflammatory mechanisms governing the emergence of HT in ischemic stroke provides an opportunity to counter this devastating complication of tPA. In the present study, we have adopted 2 approaches to decouple the interaction between tPA and immune cells via interference of cell migration and targeting a receptor that conjugates tPA. The identification of annexin A2 as a mediator between tPA and immune cells suggests that targeting annexin A2 may block the action of tPA on immune cells. In addition to mediating adverse effects on immune cells, annexin A2 also plays an important role in accelerating the thrombolytic effects of tPA by bridging tPA and fibrin. We previously found that recombinant annexin A2 enhances tPA-mediated thrombolysis. In addition to facilitating thrombolysis, our new finding suggests that recombinant annexin A2 could simultaneously abrogate the binding of tPA to annexin A2 expressed on immune cells, thus suppressing leukocyte mobilization post-thrombolysis. In line with this postulate, our previous studies have demonstrated that recombinant annexin A2 reduces HT, preserves BBB integrity, and attenuates local inflammation.

The fact that inhibiting the transmigration of lymphocytes and neutrophils reduces tPA-related brain hemorrhage is of clinical impact. Emerging evidence indicates that interactions between lymphocytes and endothelial cells foster microvascular dysfunction and secondary infarct growth after brain ischemia. Activated CD4+ and CD8+ T cells are sources of IFN-γ (interferon-γ), perforin, IL (interleukin)-23, IL-17, and other inflammatory factors that lead to neuronal cell death and BBB disruption. Depletion of lymphocytes or inhibition of their egress from lymphoid organs attenuates ischemic injury. Here, we demonstrate that tPA administration accelerates the recruitment of T cells to the brain vasculature, contributing to subsequent BBB disruption and hemorrhage, and inhibition of lymphocyte egress mitigates tPA-associated brain hemorrhage. It is also noteworthy that neutrophils are among the first peripheral cell populations to respond to brain ischemia, and they exacerbate ischemic brain injury via release of proteases such as MMPs and formation of extracellular traps. One recent study suggests that neutrophil adhesion to brain endothelium leads to stalled blood flow in cerebral microcirculation, highlighting a detrimental role of neutrophils in microvascular dysfunction. In line with prior reports, the present study demonstrates that tPA promotes neutrophil migration via a CCL2-CCR2 pathway, and inhibition of CCL2 synthesis reduced neutrophil transmigration following tPA treatment, thus attenuating brain hemorrhage.

Recently completed phase II clinical trials have evaluated the safety and efficacy of combining the immune modulator fingolimod with tPA thrombolysis in the treatment of patients with acute ischemic stroke. The outcome of these trials indicates that the combination is safe and potentially reduces HT and improves outcome, even when administered beyond the approved 4.5-hour time window for tPA. The present study provides novel insight into the underlying mechanism of the improved efficacy observed in these trials and rationalizes large-scale, controlled clinical trials to confirm the benefits of adjuvant immune therapy in future clinical trials of thrombolysis.

In all, the present study establishes that tPA thrombolysis induces a rapid surge of neutrophils and T cells in the circulation within an hour of administration, which is an active contributor to postthrombolysis HT but not a secondary response to tPA-associated adverse events. The direct effects of tPA on immune cell are mediated by annexin A2 that is the molecular switch to turn on the rapid response of neutrophils and T cells to tPA administration. Our study suggests that precise modulation of peripheral immune components could be an attractive pathway to prevent tPA-associated HT.

Several questions remain. First, it is uncertain whether inhibiting the transmigration of neutrophils or T cells is sufficient to curb HT in a clinical setting. Second, the ideal patient population to benefit from immune modulation as an adjunct therapy to tPA, as well as the optimal timing and duration of therapy, remains unclear. Only male animals were used in the present study. Although we found a similar responsiveness of circulating immune cells to tPA treatment in male versus female patients with ischemic stroke, the sex difference in tPA-related immune activation requires future investigations. Third, in addition to tPA, an altered peripheral environment after brain ischemia and immune-endothelial cellular interactions may also contribute immune cell action in conjunction with tPA. Additionally, the question of whether inhibition of neutrophils and T cells would increase the risk of infection remains unanswered, as is whether we can disassociate the ability of annexin A2 to mediate the thrombolytic effect of tPA and its action on immune cells to prevent HT at a new level. Answers to these questions will likely pave the way to a novel pharmacological
strategy, which attenuates HT—a major limitation of ITPA-mediated thrombolysis in stroke.

**ARTICLE INFORMATION**

Received June 8, 2020; revision received October 15, 2020; accepted October 16, 2020.

**Affiliations**

Department of Neurology, Tianjin Neurological Institute, Tianjin Medical University General Hospital, China (K.S., M.Z., D.-M.J., X.Y., Q.L., F.-D.S.). China National Clini-
cal Research Center for Neurological Diseases, Jing-Jin Center for Neuroinflam-
mation, Beijing Tiantan Hospital, Capital Medical University, China (K.S., F.-D.S.).

**Supplemental Materials**

Expanded Materials and Methods

**Sources of Funding**

This work was supported, in part, by the National Science Foundation of China (91642205, 81830038, and 81701176); the Advanced Innovation Center for Human Brain Protection, Capital Medical University, Beijing, China; and National Key Research and Development Program of China (2018YFC1912200).

**Disclosures**

None.

**References**

1. Leira EC, Savitz SL. In the era of thrombectomy, let us also protect the majority of patients with stroke who only require medical treatment! Stroke. 2018;49:1538–1540. doi: 10.1161/STROKEAHA.118.021411

2. Thebaud AM, Gauberti M, Ali C, Martinez De Lizarrondo S, Vivien D, Yepes M, Roussel BD. The role of plasminogen activators in stroke treat-
ment: fibrinolysis and beyond. Lancet Neurol. 2018;17:1121–1132. doi: 10.1016/S1474-4422(18)30323-5

3. Lin L, Hu K. Tissue plasminogen activator and inflammation: from phenotype to signaling mechanisms. Am J Clin Exp Immunol. 2014;43:30–36.

4. Fan X, Yu Z, Liu J, Lu N, Hajjar KA, Furie KL, Lo EH, Wang X, Annexin A2; a tissue plasminogen activator amplifier for thrombolytic stroke therapy. Stroke. 2010;41:554–558. doi: 10.1161/STROKEAHA.110.596106

5. Yepes M, Sandvik M, Moore EG, Bugge TH, Strickland DK, Lawrence DA. Tissue-type plasminogen activator induces opening of the blood-brain bar-
rier via the LDL receptor-related protein. J Clin Invest. 2003;112:1533–1540. doi: 10.1172/JCI19212

6. Mantuano E, Azzam P, Brifault C, Banki MA, Glider AS, Campama WM, Gonas SL. Tissue-type plasminogen activator regulates macrophage activation and innate immune. Blood. 2017;130:1364–1374. doi: 10.1182/blood-2017-04-780020

7. Nicole O, Docagne F, Ali C, Margail I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A. The proteolytic activity of tissue-plasminogen activator
enhances NMDA receptor-mediated signaling. Nat Med. 2001;7:59–64. doi: 10.1038/3358

8. Wang X, Lee SR, Ariai K, Lee SR, Touji K, Rebeck GW. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasmin-
ogen activator. Nat Med. 2003;9:1313–1317. doi: 10.1097/01.nrn296

9. Siao CJ, Tiirka SE. Tissue plasminogen activator mediates microglial activa-
tion via its finger domain through annexin II. J Neurosci. 2002;22:3352–3358. doi: 10.2620/jn.102622

10. Su EJ, Friedriksson L, Geyer M, Folestad E, Cale J, Andrae J, Gao Y, Pietras K, Mann K, Yepes M, et al. Activation of PDGF-CC by tissue plasminogen activator impairs brain-barrier integrity during ischemic stroke. Nat Med. 2008;14:731–737. doi: 10.1038/nrn187

11. Simão F, Ustunkaya T, Clermont AC, Feenner EP. Plasma kalikrein medi-
ates brain hemorrhage and edema caused by tissue plasminogen activ-
ator therapy in mice after stroke. Blood. 2017;129:2280–2290. doi: 10.1182/blood-2016-09-740760

12. Zhao Y, Sharma AK, LaPar DJ, Kron IL, Iaiwadi G, Liu Y, Jones DR, Laubach VE, Lau CL. Depletion of tissue plasminogen activator attenu-
ates lung ischemia-reperfusion injury via inhibition of neutrophil extrava-
sion. Am J Physiol Lung Cell Mol Physiol. 2011;300:L718–L729. doi: 10.1152/ajlpc.00272010

13. Uhl B, Zuchtingel G, Puh-Westerheide D, Prænter M, Rehberg I, Fabrius M, Hessenauer M, Holzer E, Khandoga A, Fürst R, et al. Tissue plasminogen activator promotes postischemic neutrophil recruitment via its proteolytic and nonproteolytic properties. Arterioscler Thromb Vasc Biol. 2014;34:1495–1504. doi: 10.1161/ATVBAHA.114.303721

14. Ranjdayalay K, Umachandran V, Davies SW, Syndeercome-Court D, Guttenedge CN, Timmis AD. Thrombolytic treatment in acute myocardial infarction: neutrophil activation, peripheral leukocyte responses, and myo-
cardial injury. Br Heart J. 1991;66:10–14. doi: 10.1136/hrt.66.11.10

15. Roelofs JJ, Rouschop KM, Leemans JC, Claessen N, de Boer AM, Frederiks WM, Lijnen HR, Weening JJ, Florquin S. Tissue-type plasmino-
gen activator modulates inflammatory responses and renal function in ischemia reperfusion injury. J Am Soc Nephrol. 2006;17:131–140. doi: 10.1667/ASN2005010089

16. Gautier S, Uo T, Tagzirt M, Lefebvre C, Laprais M, Pérotoux O, Dupont A, Lesy D, Bordet R. Impact of the neutrophil response to tPA in acute ischemia- colonizing-stimulating factor on the risk of hemorrhage when used in combination with tissue plasminogen activator during the acute phase of experimental stroke. J Neuroinflammation. 2014;11:96. doi: 10.1186/1742-2094-11-96

17. Perez-de-Puig I, Miro-Mur F, Ferrer-Ferrer M, Gelpi E, Pedragosa J, Justicia C, Ura X, Chamorro A, Planas AM. Neutrophil recruitment to the brain in mice and human ischemic stroke. Acta Neuropathol. 2015;129:239–257.

18. Shi J, Peng H, You S, Liu Y, Xu J, Xu Y, Liu H, Shi R, Cao Y, Liu CF. Increase in neutrophils after recumbent tissue plasminogen activator thrombolysis predicts poor functional outcome of ischaemic stroke: a longitudinal study. Eur J Neurology. 2018;25:687–695. doi: 10.1111/ejn.13579

19. Pektzel MY, Yilmaz E, Arısava EM, Topcuoglu MA. Neutrophil-to-lympho-
cyte ratio and response to intravenous thrombolysis in patients with acute ischemic stroke. J Stroke Cerebrovas Dis. 2019;28:1853–1859. doi: 10.1016/j.jstrokecerebrovasdis.2019.04.014

20. Uo T, Poley C, Maestri I, Pérotoux M, Mendyk AM, Lesy D, Bordet R, Gautier S. Neutrophils in ITPA-induced hemorrhagic transformations: main culprit, accomplice or innocent bystander? Pharmacol Ther. 2019;194:73–83. doi: 10.1016/j.pharmthera.2018.09.005

21. Zhang L, Zhang RL, Jiang G, Ding G, Chopp M, Zhang ZG. Focal embolic cerebral ischemia in the rat. Nat Protoc. 2010;5:359–347. doi: 10.1038/nprot.2010.058

22. Zhu Z, Fu Y, Tian D, Sun N, Han W, Chang G, Dong Y, Xu X, Liu Q, Huang D, et al. Combination of the immune modulator fringolimid with alteplase in acute ischemic stroke: a pilot trial. Circulation. 2015;132:1104–1112. doi: 10.1161/CIRCULATIONAHA.115.06371

23. Casanova-Ocebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chèvre R, Cachier A, González N, Kunisaki Y, Zhang D, van Roonen N, Silberstein LE, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clear-
ance. Cell. 2013;153:1025–1035. doi: 10.1016/j.cell.2013.04.040

24. Álvarez-Sabín J, Maisterra O, Santamarina E, Kase CS. Factors influencing haemorrhagic transformation in ischaemic stroke. Lancet Neurol. 2013;12:689–705. doi: 10.1016/S1474-4422(13)70055-3

25. Jin WH, Yang X, Li Z, Li M, Shi SX, Wood K, Liu Q, Fu Y, Han W, Xu Y, et al. Noninvasive tracking of CD11b+ cells with a paramagnetic and fluorescent nanoparticle in brain ischemia. J Cereb Blood Flow Metab. 2016;36:1464–1476. doi: 10.1017/S0271794016001137
26. Sun N, Shen Y, Han W, Shi K, Wood K, Fu Y, Hao J, Liu Q, Sheth KN, Huang D, et al. Selective sphingosine-1-phosphate receptor 1 modulation attenuates experimental intracerebral hemorrhage. *Stroke*. 2016;47:1899–1906. doi: 10.1161/STROKEAHA.115.012236

27. Zoja C, Corna D, Locatelli M, Rottoli D, Pezzotta A, Morigi M, Zanchi C, Buelli S, Guglielmiotti A, Perico N, et al. Effects of MCP-1 inhibition by bindartin therapy in a rat model of polyelicytic kidney disease. *Nephron*. 2019;129:52–61. doi: 10.1159/000369149

28. Hernandez-Guillamon M, Garcia-Bonilla L, Solé M, Sosti V, Parés M, Campos M, Ortega-Aznar A, Domínguez C, Rubiera M, Ribó M, et al. Plasma VAP-1/SSAO activity predicts intracranial hemorrhages and adverse neurological outcome after tissue plasminogen activator treatment in stroke. *Stroke*. 2010;41:1528–1535. doi: 10.1161/STROKEAHA.110.584623

29. Tian DC, Shi K, Zhu Z, Yao J, Yang X, Su L, Zhang S, Zhang M, Gonzales RJ, Liu Q, et al. Fingolimod enhances the efficacy of delayed alteplase administration in acute ischemic stroke by promoting anterograde reperfusion and retrograde collateral flow. *Ann Neurol*. 2018;84:717–728. doi: 10.1002/ana.25352

30. Wardlaw JM. Overview of cochrane thrombolysis meta-analysis. *Neurology*. 2001;57:589–576. doi: 10.1212/wnl.57.suppl_2.s69

31. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluemink E, Brott T, Cohen G, Davis S, Donnan G, et al; Stroke Thrombolysis Trialists’ Collaborative Group. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase in acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet*. 2014;384:1929–1935. doi: 10.1016/S0140-6736(14)60584-5

32. Wang X, Fan X, Yu Z, Liao Z, Zhao J, Mandeville E, Guo S, Lo EH, Wang X. Effects of tissue plasminogen activator and annexin A2 combination therapy on long-term neurological outcomes of rat focal embolic stroke. *Stroke*. 2014;45:619–622. doi: 10.1161/STROKEAHA.113.003823

33. Zhu H, Fan X, Yu Z, Liu J, Murata Y, Lu J, Zhao S, Hajjar KA, Lo EH, Wang X. Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke. *J Cereb Blood Flow Metab*. 2010;30:1137–1146. doi: 10.1038/jcbfm.2009.279

34. Kleinschnitz C, Kraft P, Dreykluft A, Hagedorn I, Göbel K, Schuhmann MK, Langhauser F, Helluy X, Schwarz T, Bittner S, et al. Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood*. 2013;121:679–691. doi: 10.1182/blood-2012-04-426734

35. Liesz A, Zhou W, Marcsó E, Karcher S, Bauer H, Schwarting S, Sun L, Bruder D, Stegemann S, Cerwenka A, et al. Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. *Brain*. 2011;134:704–720. doi: 10.1093/brain/awr008

36. Shichita T, Sugiyama Y, Oboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, et al. Pivotal role of cerebral interleukin-17-producing gammadermat cells in the delayed phase of ischemic brain injury. *Nat Med*. 2009;15:946–950. doi: 10.1038/nm.1999

37. Gan Y, Liu Q, Wu W, Yin JX, Bai XF, Shen R, Wang Y, Chen J, La Cava A, Poursina-Laurent J, et al. Ischemic neurons recruit natural killer cells that accelerate brain infarction. *Proc Natl Acad Sci USA*. 2014;111:2704–2709. doi: 10.1073/pnas.1315943111

38. Ritzel RM, Lai YJ, Crapser JD, Patel AR, Schreengost A, Grenier JM, Mancini NS, Patrizii A, Jellison ER, Morales-Scheiding D, et al. Aging alters the immunological response to ischemic stroke. *Acta Neuropathol*. 2018;136:99–110. doi: 10.1007/s00401-018-1859-2

39. Cruz Hernández JC, Bracko O, Kersbergen CJ, Muge Y, Haft-Javaherian M, Berg M, Park L, Vinacscik LK, Ivasky I, Rivera DA, et al. Neutrophil adhesion in brain capillaries reduces cortical blood flow and impairs memory function in Alzheimer’s disease mouse models. *Nat Neurosci*. 2019;22:413–420. doi: 10.1038/s41593-018-0329-4

40. Liu Q, Jin WN, Liu Y, Shi K, Sun H, Zhang F, Zhang C, Gonzales RJ, Sheth KN, La Cava A, et al. Brain ischemia suppresses immunity in the periphery and brain via different neurogenic innervations. *Immunity*. 2017;46:474–487. doi: 10.1016/j.immuni.2017.02.015

41. Shi K, Wang Z, Liu Y, Gong Y, Fu Y, Li S, Wood K, Hao J, Zhang GX, Shi FD, et al. CFHR1-modified neural stem cells ameliorated brain injury in a mouse model of neuromyelitis optica spectrum disorders. *J Immunol*. 2016;197:3471–3480. doi: 10.4049/jimmunol.1600135

42. Shi E, Shi K, Ou S, Sheth KN, Lawton MT, Ducruet AF. Chronic inflammation, cognitive impairment, and distal brain region alteration following intracerebral hemorrhage. *FASEB J*. 2019;33:9616–9626. doi: 10.1096/fj.201900257R

43. Mao L, Li P, Zhu W, Cai W, Liu Z, Wang Y, Luo W, Steetler RA, Leak RK, Yu W, et al. Regulatory T cells ameliorate tissue plasminogen activator-induced brain haemorrhage after stroke. *Brain*. 2017;140:1914–1931. doi: 10.1093/brain/awx111

44. Campos F, Qin T, Castillo J, Seo JH, Arai K, Lo EH, Waeber C. Fingolimod reduces hemorrhagic transformation associated with delayed tissue plasminogen activator treatment in a mouse thromboembolic model. *Stroke*. 2013;44:505–511. doi: 10.1161/STROKEAHA.112.690943

45. Salas-Perdomo A, Miró-Mur F, Gallizioli M, Brait VH, Justicia C, Meissner A, Urra X, Chamorro A, Planas AM. Effects of MCP-1 inhibition by bindarit on experimental intracerebral hemorrhage. *Sci Rep*. 2019;9:8309. doi: 10.1038/s41598-019-44845-5