Original Contribution

# Pharmacological Modulation of Neutrophil Extracellular Traps Reverses Thrombotic Stroke tPA (Tissue-Type Plasminogen Activator) Resistance

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***Background and Purpose***—Recanalization of the occluded artery is a primary goal in stroke treatment. Unfortunately, endovascular treatment is not always available, and tPA (tissue-type plasminogen activator) therapy is limited by its narrow therapeutic window; importantly, the rate of early arterial recanalization after tPA administration is low, especially for platelet-rich thrombi. The mechanisms for this tPA resistance are not well known. Since neutrophil extracellular traps (NETs) have been implicated in this setting, our aim was to study whether NET pharmacological modulation can reverse tPA resistance and the role of TLR4 (Toll-like receptor 4), previously related to NET formation, in thrombosis.

***Methods***—To this goal, we have used a mouse photothrombotic stroke model, which produces a fibrin-free thrombus composed primarily of aggregated platelets and thrombi obtained from human stroke patients.

***Results***—Our results demonstrate that (1) administration of DNase-I, which promotes NETs lysis, but not of tPA, recanalizes the occluded vessel improving photothrombotic stroke outcome; (2) a preventive treatment with Cl-amidine, impeding NET formation, completely precludes thrombotic occlusion; (3) platelet TLR4 mediates NET formation after photothrombotic stroke; and (4) ex vivo fresh platelet-rich thrombi from ischemic stroke patients are effectively lysed by DNase-I.

***Conclusions***—Hence, our data open new avenues for recanalization of platelet-rich thrombi after stroke, especially to overcome tPA resistance.

***Visual Overview***—An online visual overview is available for this article. (***Stroke*. 2019;50:00-00. DOI: 10.1161/STROKEAHA.119.026848.)**

**Key Words:** animals ■ blood platelets ■ goals ■ inflammation ■ mice



Ischemic stroke, which is caused by a blood clot that occludes cerebral arteries,[1] is one of the leading causes of death and the most frequent cause of permanent disability worldwide. The obstruction of the blood flow by a thrombus leads to cerebral injury and the subsequent disability. Therefore, recanalization of the occluded artery is a primary goal in stroke treatment. In this vein, only 2 treatment regimens are currently approved by the US Food and Drug Administration and the European Medicines Agency: (1) pharmacological thrombolysis using tPA (tissue-type plasminogen activator), which promotes degradation of fibrin in the occluding thrombus and (2) mechanical removal of the thrombus via endovascular thrombectomy. Endovascular treatment is unfortunately not always available, and tPA therapy is limited by the narrow therapeutic time window (4.5 hours after stroke onset)[2]; in addition, the rate of early arterial recanalization after tPA administration is low (less than half of the patients receiving this treatment), especially in the case of occlusive platelet-rich thrombi (around 6%),[3] which are extremely resistant to thrombolytic drugs as they contain little or no fibrin.[4,5]

The exact reasons for this so-called tPA resistance are currently unknown, but thrombus composition is believed to play an important role.[6] The fact that this information is crucial for designing efficient and safe thrombolytic strategies is leading to an active investigation based on the analysis of thrombi responsible for intracranial occlusion and, in particular, of intravenous tPA-resistant ones.

Neutrophil extracellular traps (NETs) have been found to be implicated in thrombosis.[7,8] NETs are fibrous networks of extracellular DNA released by neutrophils under the form

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of decondensed chromatin associated with histones and neutrophil granule proteins such as myeloperoxidase and neutrophil elastase, which contribute to platelet aggregation and thrombus formation.[9,10] Recent studies showed that thrombus NET content is associated with poor outcome in myocardial infarction and stroke and may be responsible for reperfusion resistance, including mechanical or pharmacological approaches with intravenous tPA.[11–13] Moreover, it has been demonstrated that infusion of DNase-I, which degrades extracellular DNA and thereby NETs, improves outcome after ischemic stroke and myocardial infarction in mice.[12–14] These data suggest that NETs may play a significant role as a potential therapeutic target to improve the efficacy or even substitute tPA-induced thrombolysis; however, the effect of DNAse-I on occluded blood vessel recanalization was not demonstrated. Importantly, NET formation has been related to both platelet and neutrophil TLR4 (Toll-like receptor 4) activation,[15,16] but their role in the setting of thrombotic stroke is unknown.

With this background, our aim was to study the involvement of NETs on thrombus formation and outcome and the role of TLR4 in this setting. Specifically, we have used a photothrombotic stroke model in mice, to produce a tPA-resistant, fibrin-free thrombus composed primarily of aggregated platelets and fresh thrombi obtained from human stroke patients. Our results demonstrate for the first time that (1) a late administration of DNase-I, but not of tPA, successfully recanalizes the occluded blood vessel by photothrombosis and subsequently improves stroke outcome; (2) a preventive administration of Cl-amidine, which impedes NET formation, completely precluded the development of the photothrombotic occlusion; (3) platelet TLR4 is implicated in NET formation after photothrombotic stroke; and (4) ex vivo fresh platelet-rich thrombi from patients with ischemic stroke are efficiently lysed by DNase-I—a finding with important therapeutic repercussions.

## Materials and Methods

Details of materials and experimental procedures are available in the online-only Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

### Animals

All experiments were performed in C57bl/6 male mice, 8 to 10 weeks old, and weighting 20 to 25 g (Harlan, Spain). Transgenic mice that express PF4-Cre (Cre recombinase enzyme under platelet factor 4 promoter) or under the lysozyme M promoter (LysM-Cre) were kindly donated by Dr Andrés Hidalgo. Each type of transgenic mouse was crossed with TLR4$^{loxP/loxP}$ mice, kindly donated by Prof Timothy Billiar (University of Pittsburgh), to delete TLR4 in platelets and myeloid cells, respectively.

Mice were kept in ventilated cages at 22°C in a 12-hour light/dark cycle and 35% humidity with ad libitum access to food and water. All procedures were performed in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Ethics Committee on Animal Welfare of University Complutense (PROEX No. 016/18) and are reported according to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible. Mortality rate in our study was <7%.

Physiological parameters were not significantly different between groups (Table I in the online-only Data Supplement).

### Induction of Focal Brain Ischemia

All groups were performed and quantified in a randomized fashion (coin toss) by investigators blinded to each specific condition. To induce cerebral infarction, we used a modified version of the photothrombotic method originally described by Watson et al[17,18] in which the occlusion of the middle cerebral artery was induced by the photochemical reaction between rose bengal and a laser beam, which caused endothelial cell damage with subsequent platelet adhesion and aggregation and thrombi formation. Further details are provided in the online-only Data Supplement.

The ferric chloride stroke model was performed as described previously.[19] Further details are provided in the online-only Data Supplement.

For reperfusion, saline, tPA (10 mg/kg intravenously),[20] or DNase-I (50 µg in 250 µL of saline intraperitoneally and a second dose of 10 µg intravenously)[21] was administered 3 hours after occlusion. A recovery of brain perfusion beyond 50% of the basal level in the ischemic territory was considered as a successful reperfusion of the occluded vessel.

PAD (peptidylarginine deiminase) inhibitor N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-l-ornithine amide (Cl-amidine; Cayman) was used to inhibit NET formation.[22] Cl-amidine blocks NET formation by inhibiting PAD 4—an enzyme that mediates chromatin decondensation and has been identified to regulate both NETosis and pathological thrombosis.[22] Cl-amidine was dissolved in PBS, and a dosage of 10 mg/kg was administered. Cl-amidine was injected intraperitoneally 20 minutes before rose bengal and laser beam exposure. Vehicle (PBS) injection served as control.

### Assessment of Neurological Deficits

Modified neuroseverity score was used to measure functional deficits induced by middle cerebral artery occlusion (MCAO) in mice. Sensory and motor deficits were evaluated through the neuroseverity score adapted for mice.[23] Further details are provided in the online-only Data Supplement.

### Infarct Size Determination and Quantification of Hemorrhages

Infarct volume was assessed at 24 hours using magnetic resonance imaging (Icon [1T-MRI]; Bruker BioSpin GmbH, Ettlingen, Germany). Infarct size was determined as described.[24] Hemorrhages were studied by staining it with diaminobenzidine.[20] Further details are provided in the online-only Data Supplement.

### Bleeding Time

Mice were anesthetized with isofluorane, and a 3-mm segment of the tail tip was removed with a scalpel. Tails were submerged in PBS at 37°, and bleeding was monitored as described previously.[25] Bleeding time was quantified in seconds (time from initiation to cessation of blood flow).

### Blood and Brain Mouse Collection and Determinations

Details for determination of plasma cytokines, histology, and immunofluorescence analysis are provided in the online-only Data Supplement.

### Patient Thrombi Collection and Processing

Details for thrombectomy procedure, human thrombi collection, lysis, and immunofluorescence are provided in the online-only Data Supplement.

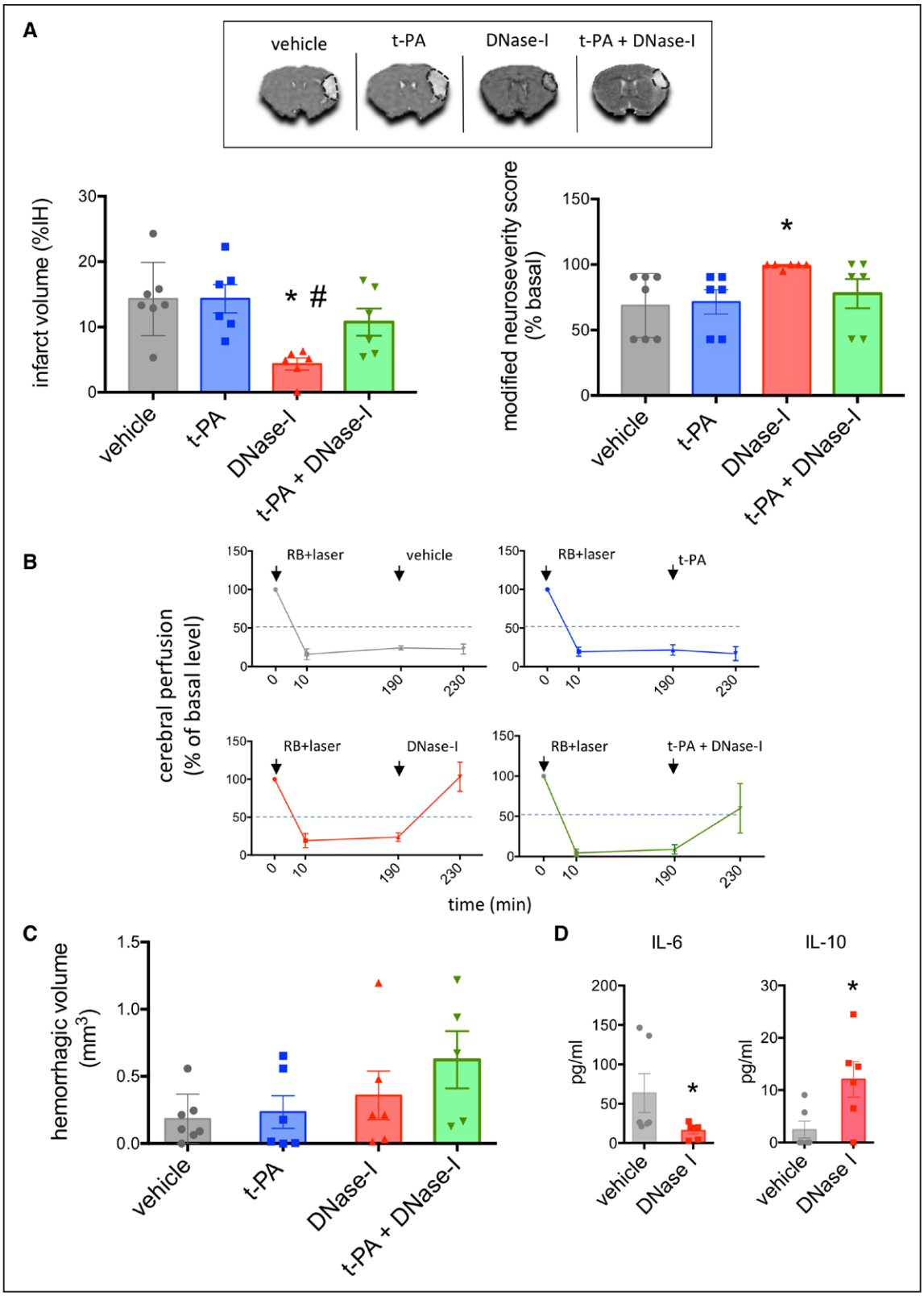


**Figure 1.** Neutrophil extracellular traps degradation by DNase-I recanalizes the occluded vessel improving stroke outcome. **A**, Effect of tPA (tissue-type plasminogen activator), DNase-I, and tPA+DNase-I (administered 3 h after ischemic insult) on infarct volume at 24 h after ischemic insult (**left**; representative images of infarct volume; **top**) and on neurological deficit at 24 h after occlusion (**right**). Sensory and motor deficits were evaluated through the neuroseverity score adapted for mice (see Methods for details). Data are expressed as mean±SEM (n=6–7) and compared by 1-way ANOVA followed by Bonferroni post hoc testing (**P*<0.05 vs vehicle group; #*P*<0.05 vs tPA-treated group). **B**, Mean of recordings of perfusion in saline, tPA, DNase-I, and tPA+DNase-I–treated groups (basal, 10 min, 190 min, and 230 min). **C**, Effect of tPA, DNAse-I, and tPA+DNase-I (administered 3 h after ischemic insult) on hemorrhagic volume ($mm^3$). **D**, Effect of DNase-I on plasma concentration of IL (interleukin)-6 and IL-10, 24 h after the occlusion. Cytokine plasma levels were measured by a BD Cytometric Bead Array. Data are expressed as mean±SEM (n=6–7) and analyzed by Mann-Whitney *U* test (**P*<0.05 vs vehicle group). IH indicates infarcted hemisphere; and RB, Rose bengal.

## Statistical Analysis

Data were expressed as mean±SEM for the indicated number of experiments. Statistical analysis was performed with Prism4 software (GraphPad Software, La Jolla, CA) using parametric or nonparametric unpaired Student *t* test or ANOVA comparisons with a $P<0.05$ was considered statistically significant. Correlation analysis was performed by use of a nonparametric Spearman correlation.

# Results

## NET Degradation by DNase-I Recanalizes the Occluded Vessel by Photothrombosis and Improves Stroke Outcome

The photothrombotic ischemic model was selected because it is known to produce a fibrin-free thrombus composed primarily of aggregated platelets,[5,26] a composition that could lead to tPA resistance. The clot was defined as stable when laser Doppler flowmetry displayed a drastic fall of brain perfusion that remained constant for 20 minutes (mean reduction of 70% to 80%). This thrombotic model produced an infarct lesion, as assessed 24 hours after the occlusion using magnetic resonance imaging, and caused functional deficits (Figure 1A and 1B). A stable thrombus was formed in 81.1% (Figure 2) of the animals, associated with a rapid and sustained reduction of brain perfusion for 20 minutes (mean reduction of 70% to 80%; Figure 1C).

Similar to the clinical situation, administration of tPA 3 hours after thrombus formation in some animals (75%) did neither lead to reperfusion (the blood flow did not return to basal levels; Figure 2) nor did affect infarct volume and functional outcome (MCAO+tPA 3 hours, n=6–7; Figure 1A and 1B; $P>0.05$), showing that the photothrombotic model is adequate to recapitulate tPA resistance. However, the administration of DNase-I (a promoter of NET degradation[21]) 3 hours after thrombus formation induced an effective vessel recanalization, as shown by the recovery of the initial cerebral blood flow, which remained stable within the first 30 minutes after DNase-I injection (Figure 1C) in 75% of the animals (Figure 2). The administration of DNase-I also caused a reduction in the infarct volume (MCAO+DNase-I 3 hours, 27.8% of reduction; n=6–7; $P<0.05$ versus MCAO+saline 3 hours; Figure 1A and 1B) and a better functional outcome 24 hours after MCAO (n=6–7; $P<0.05$ versus MCAO+saline 3 hours; Figure 1A and 1B). The administration of DNase-I did not produce any significant changes in the physiological parameters of both naive and ischemic animals (Table I in the online-only Data Supplement).

Interestingly, the combination of tPA+DNase-I, administered 3 hours after the photothrombotic occlusion, did not affect either infarct volume or neurological outcome (MCAO+[tPA+DNase-I] 3 hours, n=6; Figure 1A and 1B; $P>0.05$) when compared with vehicle or tPA groups, despite the fact that 62.5% of treated mice showed an effective reperfusion (Figure 2).

The administration of the different treatments, either alone or in combination, did not affect the hemorrhagic volume ($mm^3$) in mouse brains (Figure 1C).

To explore further the mechanisms involved in the protective effect of DNAse-I, we performed a quantitative analysis of different plasma cytokines using a customized cytometric bead array. Animals treated with DNase-I showed a significant reduction in plasma protein levels of IL (interleukin)-6 and an increase in the levels of IL-10, 24 hours after the ischemic insult, when compared with vehicle group (n=6–7; $P<0.05$; Figure 1D). No significant differences were found between groups in IL-2, IL-4, IFN (interferon)-γ, TNF (tumor necrosis factor), and IL-17A protein levels (data not shown).

To identify signs of NETosis in the ischemic brain, we used different antibodies against Cit-H3 (citrullinated histone 3), neutrophil elastase, and neutrophil cells (NIMP-R14, rat monoclonal neutrophil antibody). Our data show NETosis as demonstrated by the presence of neutrophils with Cit-H3 and neutrophil

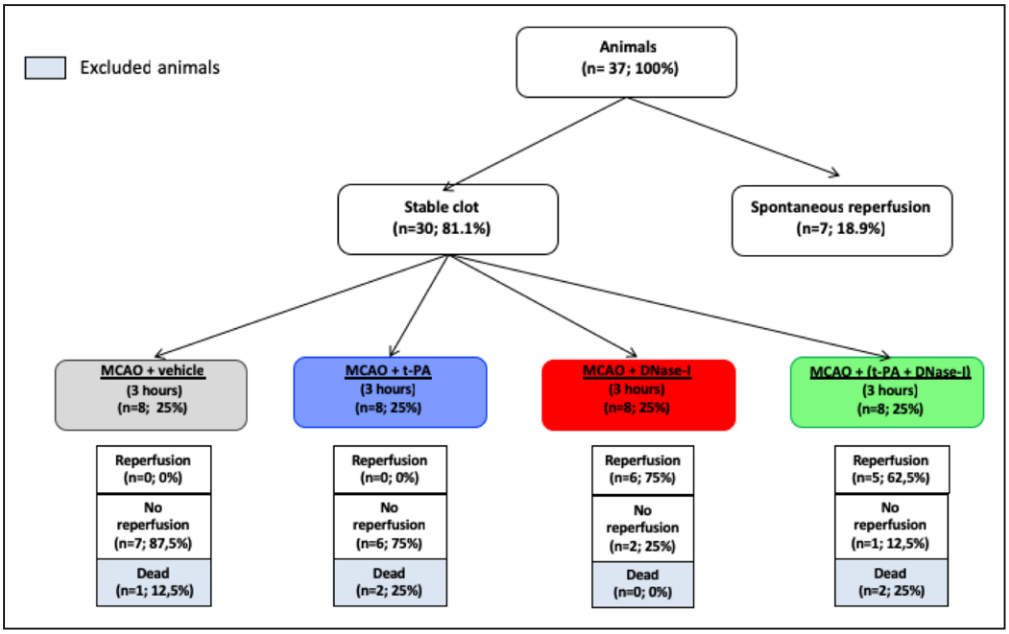


**Figure 2.** Flowchart of the study. MCAO indicates middle cerebral artery occlusion; and tPA, tissue-type plasminogen activator.

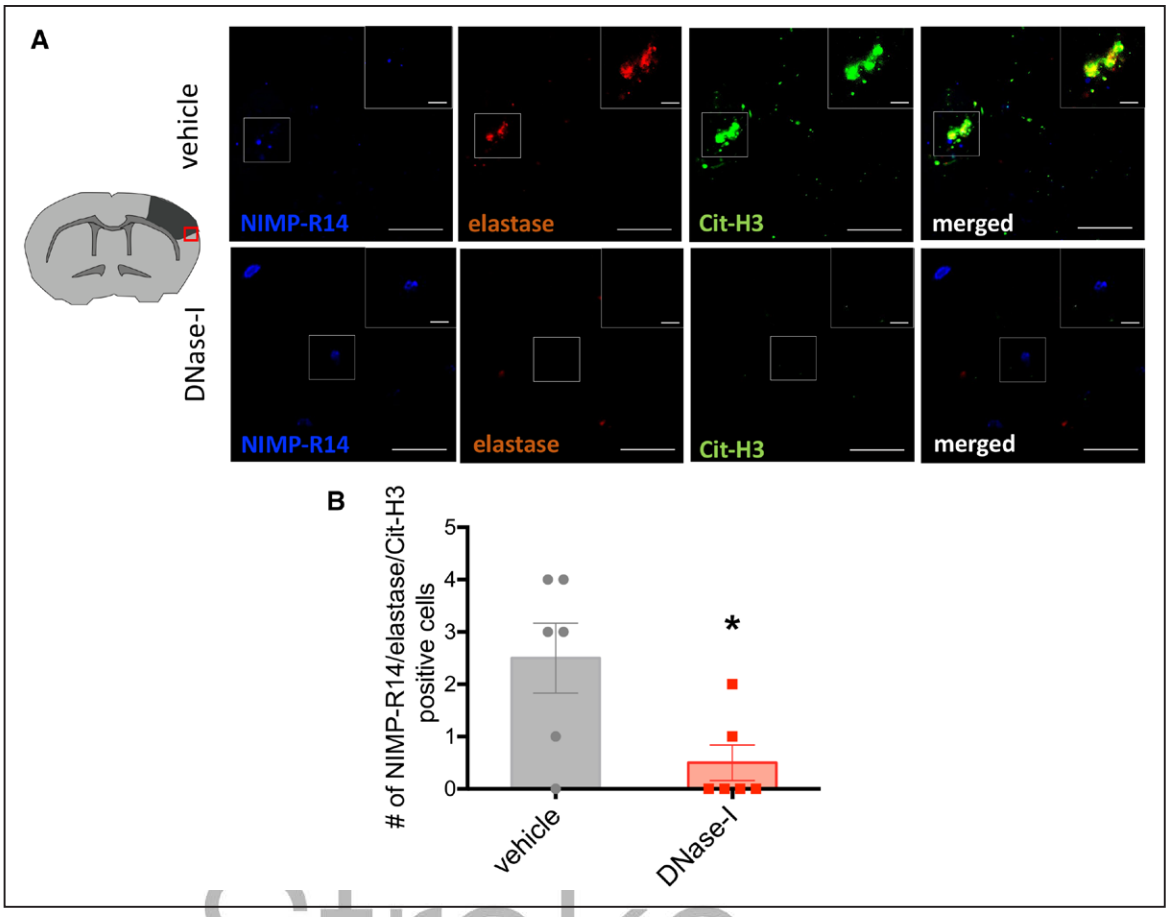


**Figure 3.** Neutrophil extracellular traps degradation by DNase-I on ischemic mouse brain. **A**, Triple immunostaining of NIMP-R14 (rat monoclonal neutrophil antibody), elastase, and Cit-H3 (citrullinated histone H3). **B**, NIMP-R14, elastase, and Cit-H3–positive cells in brain were counted, and results are represented as mean±SEM (n=6).



elastase positive staining in the ischemic cortex 24 hours after stroke (Figure 3). However, no signs of NETosis were identified in the brain of those mice treated with DNase-I (Figure 3).

## Inhibition of NET Formation by Cl-Amidine Precludes Thrombotic Occlusion

To corroborate the role of NETs in platelet-rich thrombosis, we explored the effect of Cl-amidine—a compound that blocks NETs[22]—in 2 different types of platelet-rich thrombosis (photothrombotic and ferric chloride–induced stroke models) as demonstrated by the presence of CD41 in both type of thrombi (Figure 4A). The administration of Cl-amidine before MCAO inhibited the formation of a stable thrombus in both models, as shown by the maintenance of basal blood flow levels (Figure 4D), and reduced the size of the ischemic lesion, in contrast with the control group (n=5–6; $P<0.05$; Figure 4B and 4C).

## Genetic Deletion of Platelet but Not Myeloid TLR4 Mediates Thrombosis by NET Formation After Photothrombotic Stroke

To gain a better understanding on the mechanisms leading to NET formation and subsequent thrombosis, we explored the role of neutrophil and platelet TLR4, as both have been involved in this setting.[15,16] First, to study neutrophil TLR4, we used transgenic mice that express the Cre recombinase enzyme under the LyzM (lysozyme M promoter), crossed with $TLR4^{loxP/loxP}$ mice, to obtain a deletion of TLR4 selectively in myeloid cells.[27] When thrombosis was induced in the middle cerebral artery of these mice, a stable thrombus was formed as shown by a rapid and sustained reduction of brain perfusion for 20 minutes (Figure 5C), which did not affect infarct volume and functional outcome when compared with $TLR4^{loxP/loxP}$ group (n=6–8; Figure 5A and 5B). These data suggest that TLR4 on neutrophils is not necessary for thrombus formation.

Because NETosis depends on the activation of neutrophils through platelet TLR4,[15] we then decided to analyze the role of platelet TLR4 in thrombus formation by using $TLR4^{loxP/pf4\text{-}cre}$ mice, in which platelet TLR4 is not expressed. Importantly, in this case, our data show that platelet-specific ablation of TLR4 in mice inhibited arterial thrombosis, as shown by a lack of effect on basal blood flow levels (Figure 5C). As expected, this was accompanied by a significant reduction of infarct volume 24 hours after the ischemic insult (n=6–8; $P<0.05$; Figure 5A and 5B) and a better functional outcome (n=6–8; $P<0.05$ versus $TLR4^{loxP/loxP}$; Figure 4B) when compared with $TLR4^{loxP/loxP}$ mice. When we assessed bleeding time in the tail vein on $TLR4^{loxP/pf4\text{-}cre}$ mice, no significant differences were found when compared with control mice (n=8–10; Figure 5D), discarding an impairment of platelet function in these animals.

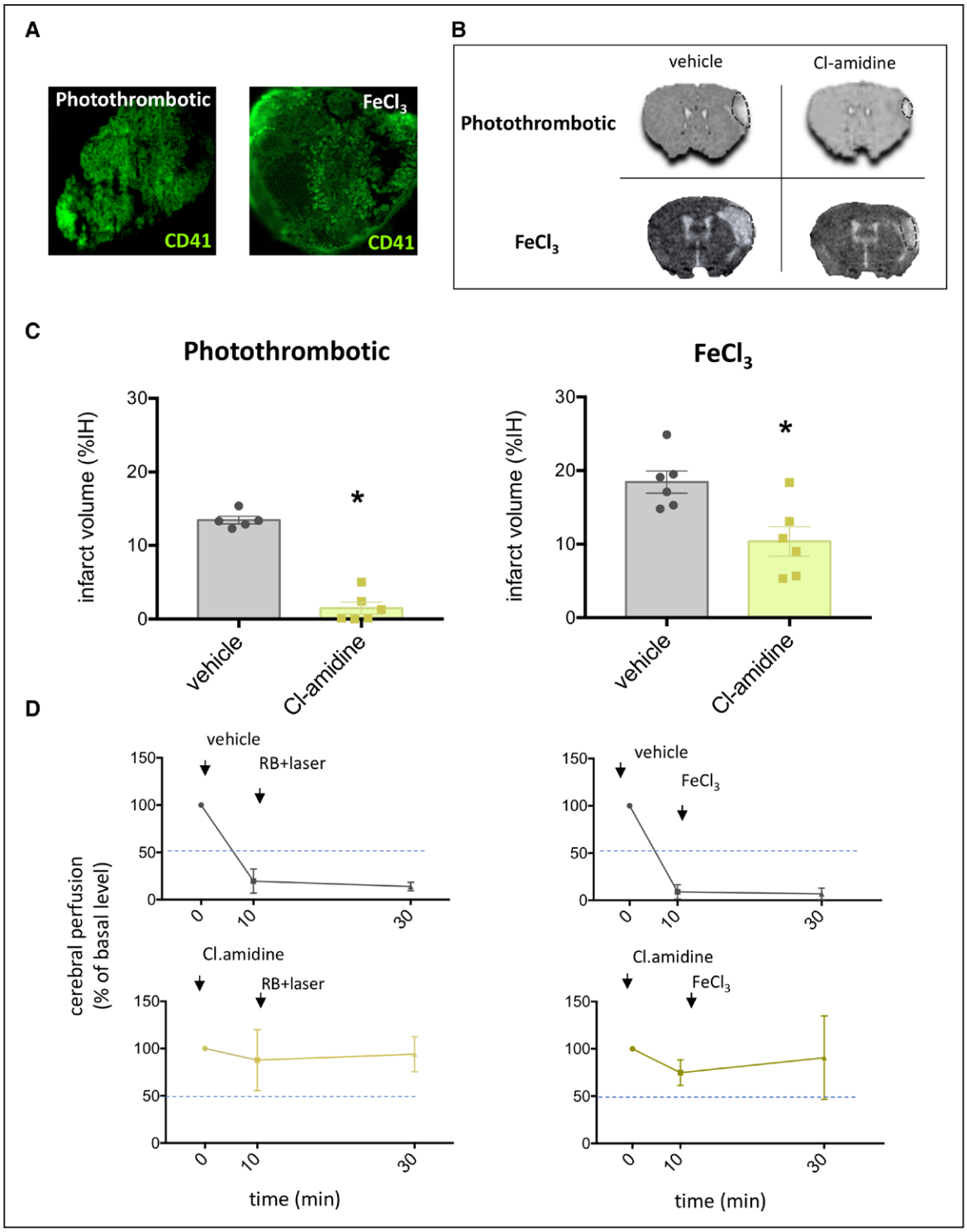


**Figure 4.** Inhibition of neutrophil extracellular trap formation by Cl-amidine precludes thrombotic occlusion. **A**, Representative immunohistological images of thrombi after photothrombotic or ferric chloride ($FeCl_3$)-induced thrombosis. **B**, Representative images of infarct volume 24 h after the occlusion induced by either photothrombosis or $FeCl_3$. **C**, Effect of pretreatment with Cl-amidine on infarct volume 24 h after the occlusion. **D**, Mean of recordings of perfusion in vehicle and Cl-amidine–treated groups (basal, 10 min, and 30 min). Data are expressed as mean±SEM (n=5–6) and analyzed by Mann-Whitney *U* test (*$P$<0.05 vs vehicle group). IH indicates infarcted hemisphere; and RB, Rose bengal.

## Promotion of NET Degradation by DNase-I Accelerates Ex Vivo Lysis of Human Ischemic Stroke Thrombi

Since our next objective is to extrapolate our results to patients, we obtained fresh thrombi from patients with stroke. Patient characteristics are listed in Figure 6A. Thrombi were divided into 4 equal parts, 3 of which were submitted to an ex vivo incubation with either vehicle, tPA (1 ug/mL), or DNase-I (0.1 mg/mL) for 4 hours (Figure 6B). Both tPA and DNase induced a partial lysis in >85% of the thrombi; however, while tPA

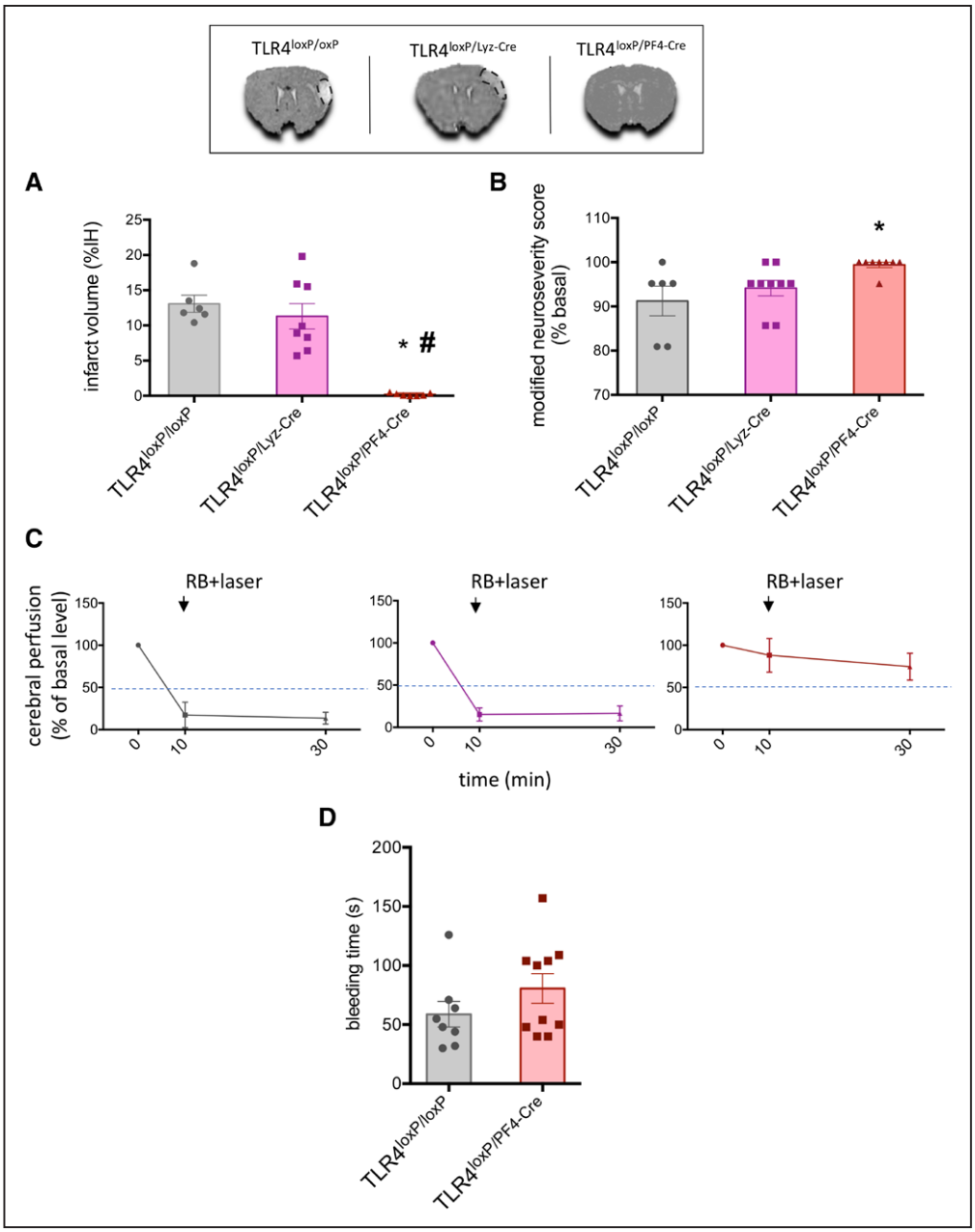


**Figure 5.** Genetic deletion of platelet but not myeloid TLR4 (Toll-like receptor 4) mediates thrombosis by neutrophil extracellular trap formation after photothrombotic stroke. **A**, Effect of TLR4 on infarct volume 24 h after the ischemic insult. Representative images of infarct volume (**top**). **B**, Effect of TLR4 deletion on neurological deficit 24 h after the ischemic insult. Sensory and motor deficits were evaluated with the neuroseverity score adapted for mice (see Methods for details). **C**, Mean of recordings of perfusion (basal, 10 min, and 30 min) in middle cerebral artery occlusion transgenic mice. Data are expressed as mean±SEM (n=6–8) and compared by 1-way ANOVA followed by Bonferroni post hoc testing (*$P$<0.05 vs TLR4$^{loxP/loxP}$; #$P$<0.05 vs TLR4$^{loxP/Lyz\text{-}Cre}$ group). **D**, Effect of deletion of TLR4 on bleeding time. Data are expressed as mean±SEM (n=8–10) and analyzed by Mann-Whitney $U$ test. IH indicates infarcted hemisphere; and RB, Rose bengal.



reduced thrombus size by about a 25% of their weight, DNase-I reduced it in >75% of their basal weight (Figure 6B), supporting that NET formation is involved in these thrombi. Careful analysis of thrombi results revealed the presence of 2 thrombi subsets depending on their response to DNase-I, that we classified as DNase-I responders versus nonresponders (Figure 6C). We hypothesized that these differences could be due to their platelet content. To confirm this hypothesis, a fourth part of the thrombi was used for histological analysis of platelet content, using CD41 as platelet marker. Immunofluorescence staining revealed that CD41 staining was more intense in DNase-I responder thrombi (n=4–6; $P$<0.05 versus nonresponder group; Figure 6C). Of note, a negative correlation was found between the mean optical density of CD41 immunoreactivity, indicative of platelet density, and thrombus weight (calculated as percentage of basal) after treatment (Figure 6D).

## Discussion

Recanalization of the occluded artery is a primary goal in acute stroke treatment, for which only 2 treatment regimens

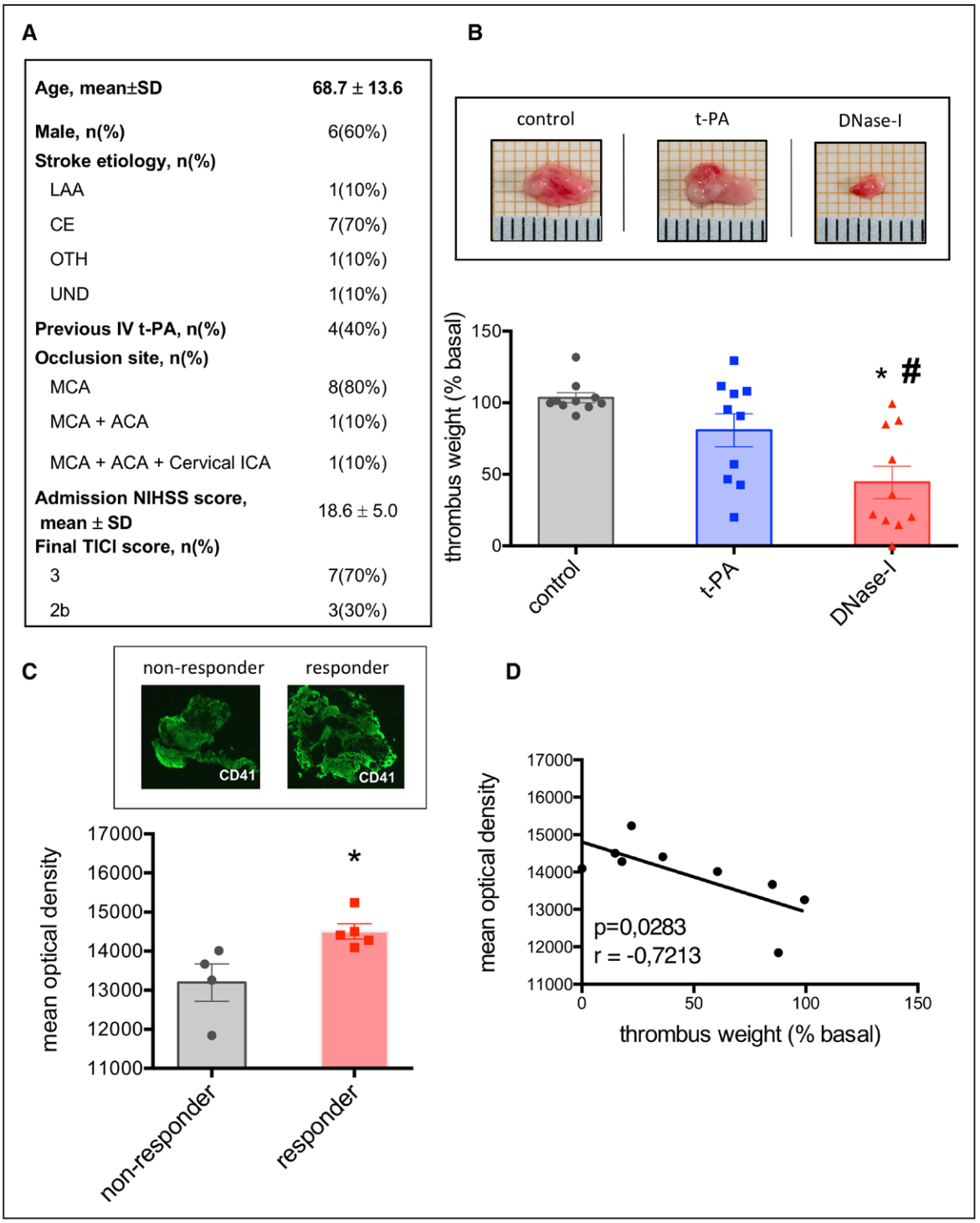


**Figure 6.** Promotion of neutrophil extracellular trap degradation by DNase-I accelerates ex vivo lysis of fresh human ischemic stroke thrombi. **A**, Clinical characteristics of acute ischemic stroke patients from whom thrombi were collected and used for this study (n=10). **B**, Thrombus weight expressed as percentage of original weight was measured 4 h after treatment. Sections of thrombi retrieved from patients with ischemic stroke (**top**). Data are mean±SEM; n=9 to 10; **P*<0.05 vs control group; #*P*<0.05 vs tPA (tissue-type plasminogen activator)-treated group. **C**, Mean optical density of CD41 (platelet marker) immunofluorescence in DNase-I responder and nonresponder thrombi detected by confocal laser scanning microscopy. Representative images of CD41 expression in human thrombi (**top**). Data are mean±SEM; n=4 to 6; **P*<0.05 vs nonresponder. **D**, Correlation analysis of mean optical density of CD41 and thrombus weight, and Spearman correlation analysis. ACA indicates anterior cerebral artery; CE, cardioembolic; ICA, internal carotid artery; IH, infarcted hemisphere; IV, intravenous; LAA, large artery atherosclerosis; MCA, middle cerebral artery; NIHSS, National Institutes of Health Stroke Scale; OTH, other cause; TICI, thrombolysis in cerebral infarction; and UND, undetermined.

are currently approved, either pharmacological thrombolysis using tPA or mechanical removal of the thrombus via endovascular thrombectomy. Endovascular thrombectomy is unfortunately not always available, and tPA therapy is limited by a narrow therapeutic window and by the so-called tPA resistance, shown by its low success rate (almost half of

the patients), especially in the case of occlusive platelet-rich thrombi. Our present results demonstrate that (1) a late administration of DNase-I, which promotes NETs lysis, but not of tPA, recanalizes the occluded vessel improving stroke outcome; (2) a preventive treatment with Cl-amidine, impeding NET formation, completely precludes thrombotic occlusion; (3) platelet but not neutrophil TLR4 mediates NET formation after stroke; and (4) ex vivo fresh platelet-rich thrombi from patients with ischemic stroke are effectively lysed by DNase-I. Hence, our data provide new therapeutic possibilities for recanalization of platelet-rich thrombi after stroke, especially to overcome tPA resistance.

To date, the precise reasons for the low success rate of tPA in ischemic stroke are not fully known, but thrombus composition may play an important role. The presence of NETs in thrombi from ischemic stroke patients,[12,14] as well as their role in thrombosis in animal models is well known.[28,29] Previous experimental studies using ischemia models and treatment with DNase-I have shown discordant results.[21,30] Therefore, to approach tPA resistance, we have used a photothrombotic stroke model in mouse producing thrombi composed primarily of aggregated platelets. First, our results show that our photothrombotic model effectively recapitulates tPA resistance as a late administration of tPA (3 hours after occlusion) failed to recanalize the occluded blood vessel.

More importantly, our data show, for the first time, that the administration of DNase-I (as late as 3 hours after thrombus formation) successfully recanalizes the occluded blood vessel without inducing intracerebral hemorrhage, reduces the levels of proinflammatory cytokines and increases those of anti-inflammatory ones, and subsequently improves outcome after photothrombotic stroke, suggesting that DNase-I has a neuroprotectant effect. We also show signs of NETosis (neutrophils with Cit-H3 and neutrophil elastase positive staining) in the ischemic cortex after stroke, which are not present in the brain of those mice treated with DNase-I. The implication of NETs is further supported by our findings that show, also for the first time, that inhibition of NET generation with Cl-amidine impedes the formation of a stable clot in vivo after photothrombotic stroke and does not cause any ischemic lesion. Our results are confirmed by the similar data obtained when using ferric chloride–induced stroke, which is another platelet-rich thrombosis model. All these data strongly suggest that the development of a tPA-resistant, platelet-rich thrombus depends on NETs formation. Interestingly, the combination of tPA+DNase-I, which induced reperfusion in most animals, did not affect either infarct volume or neurological outcome, suggesting other detrimental effects of tPA beyond its inability to induce reperfusion at late times.

To investigate the mechanisms by which the extracellular traps released by neutrophils mediate thrombosis, we explored the role of platelet and neutrophil TLR4, both known to modulate NETosis.[15,16] By using specific transgenic mice with a deletion of TLR4 selectively in myeloid cells or platelets, we demonstrate for the first time that TLR4 in platelets but not in neutrophils is determinant for the photothrombotic formation of a stable thrombus, which, in turn, depends on NETs. This is in agreement with data in the literature[15] showing that platelet TLR4 accounts for the activation of neutrophils to release their content to the extracellular space. Interestingly, the TLR4 agonist HMGB1 (high mobility group box 1) has been reported to account for NETosis induction in noninfectious diseases[31] and in a deep venous thrombosis animal model,[32] pointing to this molecule as a likely inducer of NETosis in our system. Taken together, our data show that platelet-rich thrombi, which are formed mainly by NETs in a platelet TLR4-dependent way, are resistant to lysis with tPA but not with DNase-I in mice.

In this study, we have focused on late tPA administration as relevant in the context of tPA resistance. However, although out of the scope of the present work, the effect of tPA on platelet-rich thrombosis at earlier time points is an interesting issue that deserves further studies. An additional limitation of our study is that our conclusions refer to the acute phase of stroke. Further studies are required to elucidate the impact of our findings in the recovery phase.

In the context of extrapolating our results to thrombus from patients with stroke, a recent report suggested that thrombus NET content might be responsible for reperfusion resistance.[13] Interestingly, these authors claimed that DNAse-I alone lacks ex vivo thrombolytic effect on frozen human thrombi, and, therefore, NET modulation is insufficient for an effective reperfusion in human stroke. However, in our study, ex vivo fresh human thrombi from patients with stroke are remarkably and effectively lysed by DNase-I, more efficiently than by tPA, regardless of the infarct type, a result found thanks to the use of fresh rather than frozen human thrombi, thus supporting that NETs can be independently targeted for successful thrombolysis of tPA-resistant, platelet-rich thrombi.

## Conclusions

Overall our findings provide new insights into the pathology of ischemic stroke that could be of high relevance for acute ischemic stroke management. Notably, our results provide an alternative treatment for recanalization of platelet-rich thrombi contributing, therefore, to a possible change in the therapeutic management of acute ischemic stroke.

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Drs Lizasoain and Moro equally directed the study.

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## Disclosures

None.

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