Stroke

BASIC AND TRANSLATIONAL SCIENCES

Influenza A Infection Increases Severity of Acute Ischemic Stroke Through Neutrophil Activation and Hypercoagulability

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BACKGROUND: Respiratory viruses, such as influenza viruses and SARS-CoV-2, cause severe infections of the respiratory system. Cohort studies and clinical observations indicate that patients with severe influenza A virus (IAV) infections are at an increased risk of developing an ischemic stroke event. However, the underlying mechanisms remain elusive. To this end, we investigated the consequences of IAV infection on cerebral damage in a mouse model of ischemic stroke.

METHODS: We intranasally inoculated male C57BL6/N mice with the mouse-adapted IAV strain A/Puerto Rico 8/34 or PBS as a vehicle control. At 3, 7, and 10 days post-infection, mice were subjected to transient middle cerebral artery occlusion, followed by sacrifice 24 hours after reperfusion for subsequent analysis. The anticoagulant drug acetylsalicylic acid was administered as treatment 1 day before transient middle cerebral artery occlusion.

RESULTS: Our research demonstrated a time-dependent deterioration of cerebral ischemia after transient middle cerebral artery occlusion, resulting in increased infarct volume and a worsened neurological outcome at the propagation and inflammation phases of infection. Our observations revealed an elevation in procoagulant activity and an increase in thrombosis within the microvasculature after infection and stroke. This effect was attributed to an infection-mediated inflammatory milieu and accelerated neutrophil response. Upon infection, the release of increased neutrophil extracellular traps by neutrophils had detrimental consequences for transient middle cerebral artery occlusion development. Administration of acetylsalicylic acid or control antiviral therapy prevented the IAV-induced exacerbation of stroke and reduced brain damage by reducing NETosis and coagulation.

CONCLUSIONS: These findings suggest that IAV infections enhance the systemic propensity for NETosis and foster a procoagulant state, thereby increasing the risk of cerebral damage and thrombosis following stroke. Targeting a combination of neutrophils and coagulation molecules simultaneously represents a promising treatment approach for clinical stroke.

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

Key Words: blood platelets ■ influenza A virus ■ ischemic stroke ■ neutrophils ■ thrombosis

Ischemic stroke is a significant global health concern, with neuropathological events affecting over 12.2 million patients worldwide each year.[1] Viral infections have been identified as a risk factor for acute ischemic stroke and a predictor of poor clinical outcome.[2] This effect has also been described in various studies for the recently emerged SARS-CoV-2 virus.[3] Nevertheless, the mechanisms and consequences of infection, which influence thrombo-inflammatory components, remain unclear. Systemic inflammation in respiratory diseases

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Nonstandard Abbreviations and Acronyms

ASA	acetylsalicylic acid
CD	cluster of differentiation
COX	cyclooxygenase
GPIX	glycoprotein 9
H3Cit	citrullinated histone H3
HC	healthy control
IAV	influenza A virus
IAV-NP	influenza A nucleoprotein
NET	neutrophil extracellular trap
PAI-1	plasminogen activator inhibitor-1
PSGL-1	P-selectin glycoprotein ligand-1
tMCAO	transient middle cerebral artery occlusion
tPA	tissue-type plasminogen activator

has been demonstrated to create a conducive environment for thrombotic events and a hypercoagulative state in patients.[4,5] It has been demonstrated that the seasonal incidence of stroke exhibits a seasonal variation that closely resembles the occurrence of respiratory tract and influenza virus infections.[6] Case studies have shown that infections originating from chronic bacterial and viral infections, as well as infections that require hospitalization, are risk factors for ischemic stroke.[7] Influenza A virus (IAV) infects the lung endothelial cells, causing vascular leakage and lung barrier damage, leading to proinflammatory, procoagulant responses, and enhanced oxidative stress.[8,9] Here, neutrophils are one of the most abundant immune cells in the human blood and play a fundamental role in viral condemnation and protection.[10] However, they can dramatically increase in number in the peripheral blood and contribute to excessive inflammatory processes and severe lung damage when overactivated.[11,12]

Beyond their role in thrombosis, platelets also contribute to the immune response. During IAV infection, platelets can engulf the virus, leading to the release of complement C3 and the formation of neutrophil-platelet aggregates. This process also leads to the release of neutrophil extracellular traps (NETs) from neutrophils that are composed of extracellular fibers primarily consisting of DNA and decorated with antimicrobial proteins.[13] In the context of ischemic stroke, NETs are released and increase the risk for thrombosis.[14] Interestingly, ischemic stroke pathology and respiratory infections share thrombo-inflammatory characteristics, thereby introducing inflammatory preactivation to the peripheral blood.[15,16] Nevertheless, an investigation into the risk assessment among infection phases and the consequences for thrombo-inflammatory mechanisms that may lead to an increased risk for a thrombotic event in the brain has yet to be conducted.

This study used an experimental mouse model of IAV infection to investigate its potential role as a prothrombotic driver in ischemic stroke pathology. Specifically, we examined the inflammatory alterations associated with the acute infection phase (days 1–4), the symptomatic phase (days 4–8), and the recovery phase (days 8–11) following IAV infection. Our findings revealed that IAV infection resulted in exacerbated cerebral damage, significantly elevated platelet activation state, and an increased concentration of soluble procoagulant factors in the blood. In addition, we demonstrated that neutrophils undergo preactivation during IAV infection, rendering them more susceptible to NETosis. The administration of acetylsalicylic acid (ASA) demonstrated a marked reduction in platelet and neutrophil activation, thereby lowering the risk of thrombotic events following stroke in mice.

METHODS

Data Availability

All data are included in this article and the Supplemental Material.

Experimental Animals

Animal experimentation was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) and the IMPROVE guidelines.[17,18] A total of 132 male C57BL/6 N mice were purchased from Charles River (Sulzfeld, Germany) and housed in a temperature- and humidity-controlled, specific pathogen-free animal facility with a 12-hour light-dark cycle, as well as food and water available ad libitum. Twelve experimental mice have been excluded due to (1) the course of infection, (2) hemorrhagic transformation, or (3) inadequate infarct propagation. Investigators randomly assigned mice to experimental groups, and all efforts were made to minimize animal suffering and the number of animals used. We used a score system allowing us to evaluate the symptoms arising from the experimental procedures, for example, intranasal inoculation, infection, and ischemic stroke surgery. Surgeries, treatments, and evaluation of readout parameters were performed by different investigators blinded to the group allocation with unblinding before statistical analysis. We used the online Web tool of GraphPad to assign mice randomly to treatment groups (https://www.graphpad.com/quickcalcs/randomize1/).

Infection Model

Male mice were infected with a mouse-adapted influenza virus A/Puerto Rico 8/34. Mice received an intraperitoneal injection of xylazine (0.1 mg/g IP) and ketamine (0.005 mg/g IP) followed by an intranasal application of 50-µL 300-plaque-forming units/mL A/Puerto Rico 8/34 virus or vehicle (PBS). Mice were weighed and monitored daily for symptoms of infection, that is, reduced body weight, condition of fur, and changes in respiratory behavior.

Ischemia Model and Neurological Assessment

At days 3, 7, and 10 post-infection, mice were subjected to 30-minute transient middle cerebral artery occlusion (tMCAO).[19] Mice were anesthetized with 4% isoflurane (Piramal) in 100%

oxygen for 3 to 5 minutes (World Precision Instruments, Small Animal Anesthesia System, EZ-7000). Anesthesia was maintained with ≈2% to 3% isoflurane, and body temperature was kept at 37 °C during surgery, using a feedback-controlled warming device (World Precision Instruments, Small Animal Anesthesia System, EZ-7000). Occlusion of the middle cerebral artery was induced as described.[20] In brief, the silicone filament (602112; Doccol Corporation, United States) was introduced to the middle cerebral artery via the common carotid artery. Assessment of global neurological functions following tMCAO was measured utilizing the Bederson score.[20] Mice were euthanized 24 hours after tMCAO induction. Blood samples were taken by heart puncture before perfusion with PBS and the collection of brains and lungs.

Pharmacological Interventions

In a prophylactic approach, mice were injected with 30-mg/kg IP ASA (A5376; Sigma-Aldrich), 24 hours prior, and on the day of tMCAO induction.[21] A therapeutic approach to reduce consequences of infection was therapy with 10-mg/kg IP antiviral medication (Oseltamivir).[22]

ELISA

NET quantification was performed based on the H3Cit (citrullinated histone H3) association with DNA. In brief, brain neutrophils were liberated using a tissue dissociation kit (130-110-201; Miltenyi Biotec). Neutrophils from the brain and blood were then isolated using MACS sorting (130-097-658; Miltenyi Biotec) and cultivated for 2 hours followed by stimulation with phorbol-12-myristate-13-acetate (P1585; Sigma-Aldrich) to test their response to external stimuli. Antihistone H3 antibody–coated 96-well plates were prepared to measure NET amounts using a CellDeath ELISA Kit (11774425001; Roche). Plasma-containing platelets were isolated from EDTA blood using a Ficoll-Paque gradient (400*g* for 30 minutes, RT). The upper layer of Ficoll-Paque contains cytokines, plasminogen, fibrinogen, platelets, and H3cit-DNA complexes. To measure MMP-9 (matrix metalloproteinase-9), CCL-5 (chemokine [C-C motif] ligand 5), IL-6 (interleukin 6), and MCP-1 (monocyte chemoattractant protein-1) from brain homogenate supernatant and blood plasma samples, we used multiparametric precoated plates for LUMINEX analysis (VbeVHbCg; R&D Systems). We used precoated plates to measure plasminogen (ab198511; Abcam) or fibrinogen (Abnova; KA1399) from the blood plasma. CD (cluster of differentiation) 62P platelet activation was then measured using Mouse P-Selectin/CD62P DuoSet ELISA (DY737; R&D Systems). All analyses were performed following the manufacturer's protocols.

Histological Staining

Infarct sizes were analyzed using 2,3,5-triphenyltetrazolium chloride staining, followed by volumetric infarct measurement.[23] Coronal brain sections were additionally subjected to immunofluorescence staining as described previously.[23] The following primary antibodies were used: anti-CD31 (mca2388; BioRad, 1:200), anti-GPIX (glycoprotein 9; M052-0, Emfret, 1:100), anti-Ly6G (lymphocyte antigen 6 complex, locus G; 127601; Biolegend, 1:200), anti-CD3 (ab5690; Abcam, 1:100), anti-CD-11b (mca74g; BioRad, 1:200), anti-CD45 (MA1-10231; Thermo Fisher, 1:300), and anti-IAV-nucleoprotein (ab20343; Abcam, 1:100). All secondary antibodies were diluted 1:1000. Fluorescence stainings were visualized using a Leica DMi8 microscope, Hamamatsu C11440-22 CU camera, and Leica Application Software X (LasX 3.0.2.16120). Images were processed using ImageJ (National Institutes of Health).

Flow Cytometry

Mice were euthanized, and blood was collected before perfusion with ice-cold PBS. Peripheral blood underwent ammonium-chloride-potassium lysis and multiple washing steps before staining. Immune cells were stained for anti-CD3 PacBlue (100.214; Biolegend, 1:400), anti-CD45-HorizonV500 (561.487; Biolegend, 1:400), eFluor 780 Viability dye (103.210; Thermo Fisher, 1:400), anti-CD11b-APC (101.212; Biolegend, 1:400), anti-Ly6G-PerCP-Cy5.5 (127.615; Biolegend, 1:400), and H3Cit (ab5103; Abcam, 1:200). H3Cit was additionally stained with a phycoerythrin-coupled antibody (1:500). Fluorochrome compensation was performed with BD Diva integrated compensation matrix and single-stained, mixed-tissue controls. Flow cytometric analysis was performed on the FACSAria III flow cytometer (BD Bioscience); data analyses were performed using FlowJo software. The ProCyte Dx hematology analyzer (IDEXX, United States) was used to measure platelet counts.

Statistics

Sample sizes for animal studies were calculated using power analyses to detect differences with 80% power based on an expected effect size of 0.5 and variance from previous preclinical stroke research. An α level of 5% was applied with the Bonferroni adjustment for pairwise comparisons. Data are presented as mean±SD. Statistical analyses were conducted using GraphPad Prism, and normality was assessed with the D'Agostino-Pearson test. The differences between multiple groups were evaluated using 1- or 2-way ANOVA or the nonparametric Kruskal-Wallis test. Post hoc analyses were performed using the Bonferroni tests for comparisons across all conditions, the Dunnett test for comparisons between each condition and a control group, or the Dunn tests following the Kruskal-Wallis test. Statistical significance was defined as $P \leq 0.05$.

Study Approval

The study was approved by the national government committee (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, LANUV, Germany; AZ81-02.04.2021.A073) and conducted in agreement with the German Animal Welfare Act (German Ministry of Agriculture, Health, and Economic Cooperation). Germany generally follows Directive 2010/63/EU on the protection of animals used for scientific purposes and has adapted its national regulations to incorporate key aspects of Directive 2010/63/EU.

RESULTS

IAV Infection Increases the Pathological Consequences of Ischemic Stroke

To determine the consequences of infection on ischemic stroke pathology (Figure 1A), we first determined

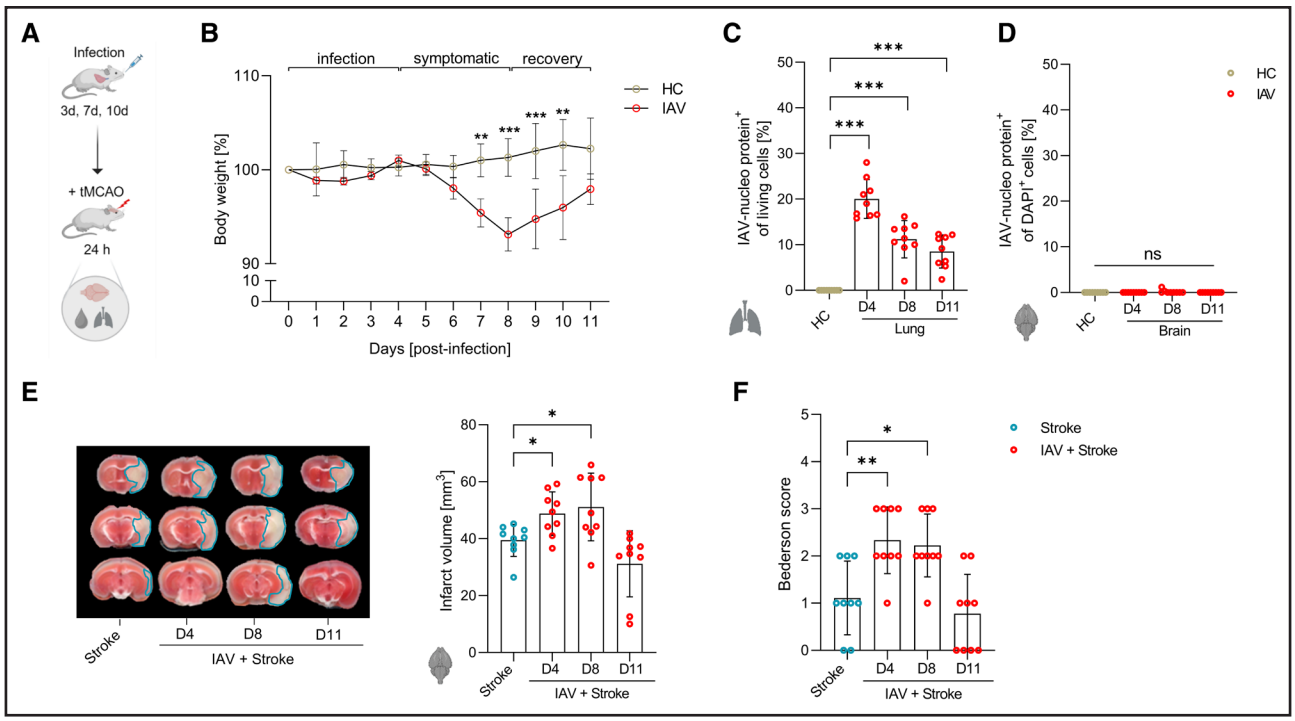

Figure 1. Influenza A virus (IAV) infection deteriorates ischemic stroke outcome and increases brain damage.
A, Mice are infected with a dose of 300-plaque-forming units/mL influenza virus A/Puerto Rico 8/34 (IAV). Cerebral occlusion of 30-minute transient middle cerebral artery occlusion (tMCAO; stroke) was induced on days 3, 7, and 10 after infection, followed by analysis of lung, brain, and blood 24 hours post-ischemia induction (days 4, 8, and 11). **B**, Body weight of IAV-infected mice compared with healthy control (HC) mice. **C**, Flow cytometric quantification of IAV-NP (influenza A nucleoprotein) in lung cells of infected and uninfected mice (n=9). **D**, Histological quantification of IAV-NP in the brain of infected and uninfected mice (n=5). **E**, Representative 2,3,5-triphenyltetrazolium chloride staining of 3 corresponding coronal brain sections on days 4, 8, and 11 with or without IAV infection 24 hours after tMCAO induction and quantification of brain infarct volumes (n=9). **F**, Evaluation of neurological deficits using the Bederson score at days 4, 8, and 11 after infection (n=9). *P<0.05, **P<0.01, and ***P<0.001; 1-way ANOVA followed by the Sidak comparison test compared with the respective HC or stroke group. DAPI indicates 4′,6-diamidino-2-phenylindole.

successful respiratory IAV infection, which resulted in a notable reduction in body weight on days 7 to 10 after induction in comparison to healthy controls (HCs; Figure 1B). The infection was further confirmed by flow cytometric analysis for IAV-NP (influenza A nucleoprotein) in lung cells, which peaked 4 days after infection. The number of infected cells demonstrated a decline with the prolongation of the infection period (Figure 1C). IAV-NP was not detected in brain sections of IAV-infected mice (Figure 1D). Subsequently, we induced ischemic stroke in mice by 30-minute tMCAO during infection (days 3, 7, and 10) and analyzed the brains 24 hours after ischemia (Figure 1E). Interestingly, we identified a marked increase in infarct volume at days 4 and 8 post-infection, in comparison to day 11 post-infection and noninfected stroke mice (Figure 1E). The increased infarct volume led to a notable neurological impairment following infection (Figure 1F). Interestingly, IAV-NP$^+$ cells correlated positively with stroke volume size on days 4 and 8 (Figure S1A and S1B), whereas no correlation was observed at day 11 (Figure S1C).

To evaluate whether the IAV infection indeed acts as a mediator for increased severity in ischemic brain damage, we administered a neuraminidase inhibitor (Oseltamivir) following inoculation. The results demonstrated that oseltamivir treatment successfully rescued the weight loss of infected mice (Figure S2A). In accordance, during the viral titer accumulation at day 4, infected mice exhibited significantly fewer IAV-NP$^+$ respiratory epithelial cells (Figure S2B). Furthermore, a reduction in the viral burden resulted in a decrease in ischemic brain damage (Figure S2C and S2D) and equalized the infarct volume to that observed in ischemic stroke without infection at days 4, 8, and 11 (Figure S2E). In addition, investigation of neurological deficits showed no aggravation during the course of infection compared with infected stroke mice (Figure S2F).

Prolonged Thrombogenic Activity After IAV Infection

We further aimed to elucidate the thrombogenic influence of an IAV infection on the organism and its impact on damage propagation in ischemic stroke. Our data showed a significant increase in occluded brain vessels on days 4 and 8 post-infection in conjunction with tMCAO compared with uninfected tMCAO mice

(Figure 2A). The occlusion of vessels on day 11 post-infection was comparable to noninfected tMCAO mice (Figure 2A). To further investigate the underlying mechanism, we analyzed the general blood composition in the respective experimental groups. We found a significant decrease in platelet counts in IAV-infected tMCAO mice at days 4 and 8 in comparison to noninfected tMCAO mice (Figure 2B). Nevertheless, platelet numbers remained unaltered in IAV-infected mice alone (Figure S3A). In addition, we observed a significant elevation in CD62P$^+$ activated platelets in the blood until day 11 post-infection, indicative of a preactivated state of platelets (Figure 2C). To further investigate the potential impact of lung infection on platelet activation, we measured the CD62P fold change of platelets in the blood on days 4, 8, and 11 with and without tMCAO. We found an increase in reactive platelets in mice suffering from IAV infection at all phases of infection. This effect was even more pronounced after tMCAO induction, indicating that platelets have an elevated activation state that may contribute to an increased risk for thrombus formation (Figure 2C; Figure S3B). We additionally found increased fibrinogen concentrations in the blood of infected mice following tMCAO (Figure 2D). Interestingly, fibrinogen levels in the peripheral blood were also increased during IAV infection on days 4 and 8 in the absence of stroke compared with HCs (Figure S3C). Plasminogen concentrations were elevated on day 11 after infection in mice with (Figure 2E) and without stroke (Figure S3D).

Circulating Neutrophils Are Preactivated Through Influenza A Infection

To investigate the potential source for increased vascular thrombosis and cerebral damage, we focused on the concept of thrombo-inflammation, which is a relevant process in ischemic stroke and IAV pathology.[24] First, we proved lung inflammation during the influenza A infection, showing elevated numbers of CD45$^+$ immune cells

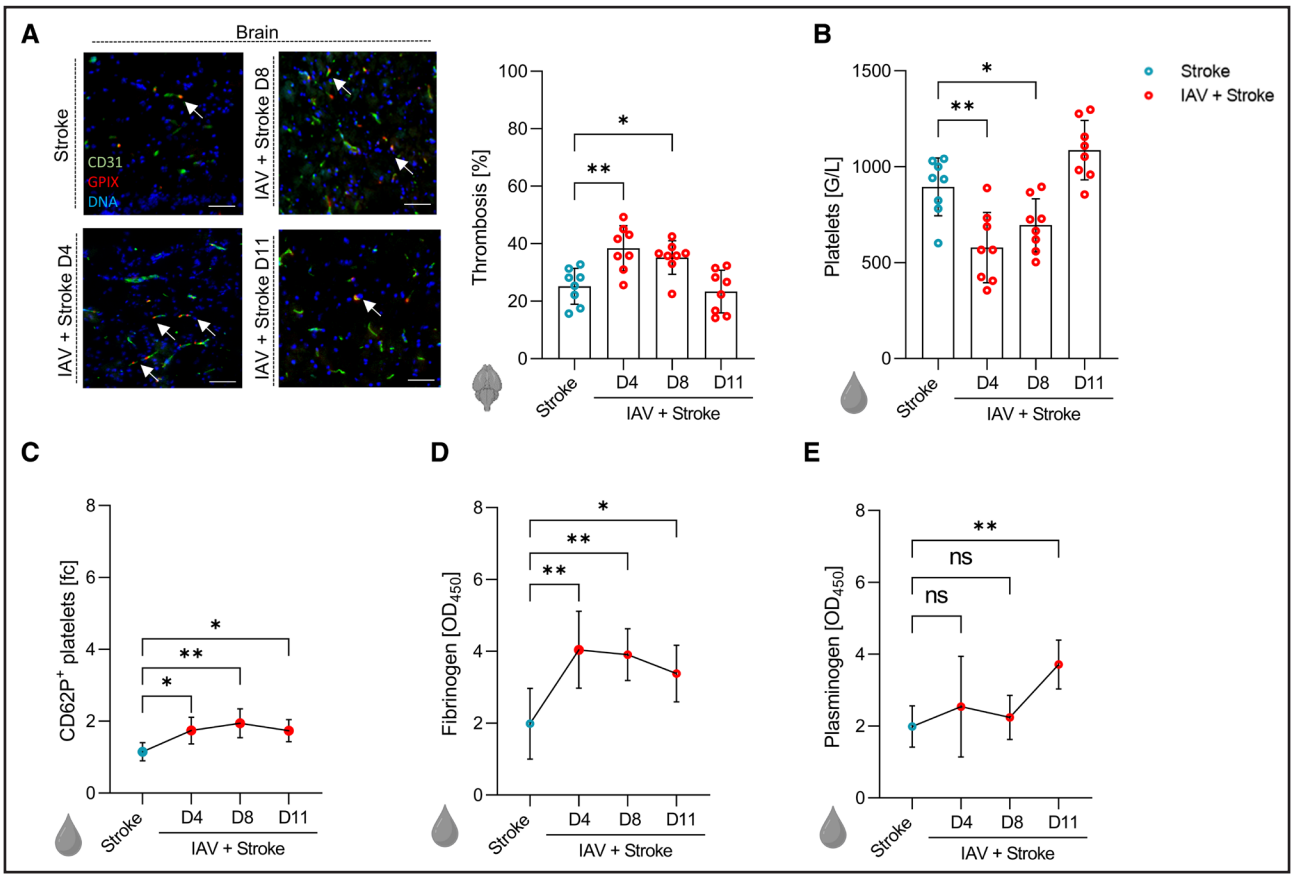

Figure 2. Prolonged thrombogenic activity after influenza A virus (IAV) infection increased vessel occlusion in the ischemic brain.

A, Percent of GPIX (glycoprotein 9)$^+$ occluded CD (cluster of differentiation) 31$^+$ vessels in the ipsilateral hemisphere of the ischemic brain of transient middle cerebral artery occlusion (tMCAO) or IAV-infected+tMCAO mice. **B**, Number of platelets in the blood of tMCAO or IAV-infected+tMCAO mice at days 4, 8, and 11 (n=8). **C**, Determination of CD62P$^+$ activated platelets in blood plasma of respective experimental groups (n=4–6). **D**, Quantitative determination of liberated fibrinogen (n=6). **E**, Plasminogen in the blood plasma in respective experimental groups (n=6). *P<0.05 and **P<0.01; 1-way ANOVA followed by the Sidak comparison test compared with the respective healthy control or stroke group. fc indicates fold change; G/L, billions/L; and OD, optical density.

at days 4, 8, and 11 compared with HC (Figure S4A). We then analyzed compositional changes in lymphocytes, macrophages, and neutrophils throughout the course of infection. The frequency of neutrophils in the peripheral blood significantly increased on day 8 after infection (Figure S4B), whereas lymphocytes dramatically decreased at days 4 and 8 after IAV infection (Figure S4C), and monocytes/macrophages did not change over the course of infection (Figure S4D). IAV infection in combination with tMCAO similarly reduced the frequency of lymphocytes in the blood (Figure S4E) and induced a significant reduction of lymphocytes in the brain after tMCAO at day 4 after infection (Figure S4F). We found a general increase in monocytes in the blood at days 8 and 11 after IAV infection combined with tMCAO compared with tMCAO alone (Figure S4G). In contrast, the number of CD11b$^+$ immune cells in the brain remained unaltered in the tMCAO infection setting in comparison to noninfected stroke mice (Figure S4H).

Neutrophils have been demonstrated to contribute to the exacerbation of ischemic stroke damage through the release of inflammatory cytokines and the release of NETs.[14] Accordingly, we investigated whether IAV infection is capable of preactivating neutrophil granulocytes. Following IAV infection in conjunction with ischemic stroke, neutrophils significantly increased in the brain of infected tMCAO mice compared with noninfected stroke mice on day 8 (Figure 3A and 3B). In the blood, neutrophils were significantly increased on days 4 and 8 compared with uninfected tMCAO mice (Figure 3C).

Although neutrophils were not elevated at each time point during infection, H3Cit-DNA complexes, a marker for NETs, increased in the blood and the brain after ischemic stroke compared with noninfected stroke mice

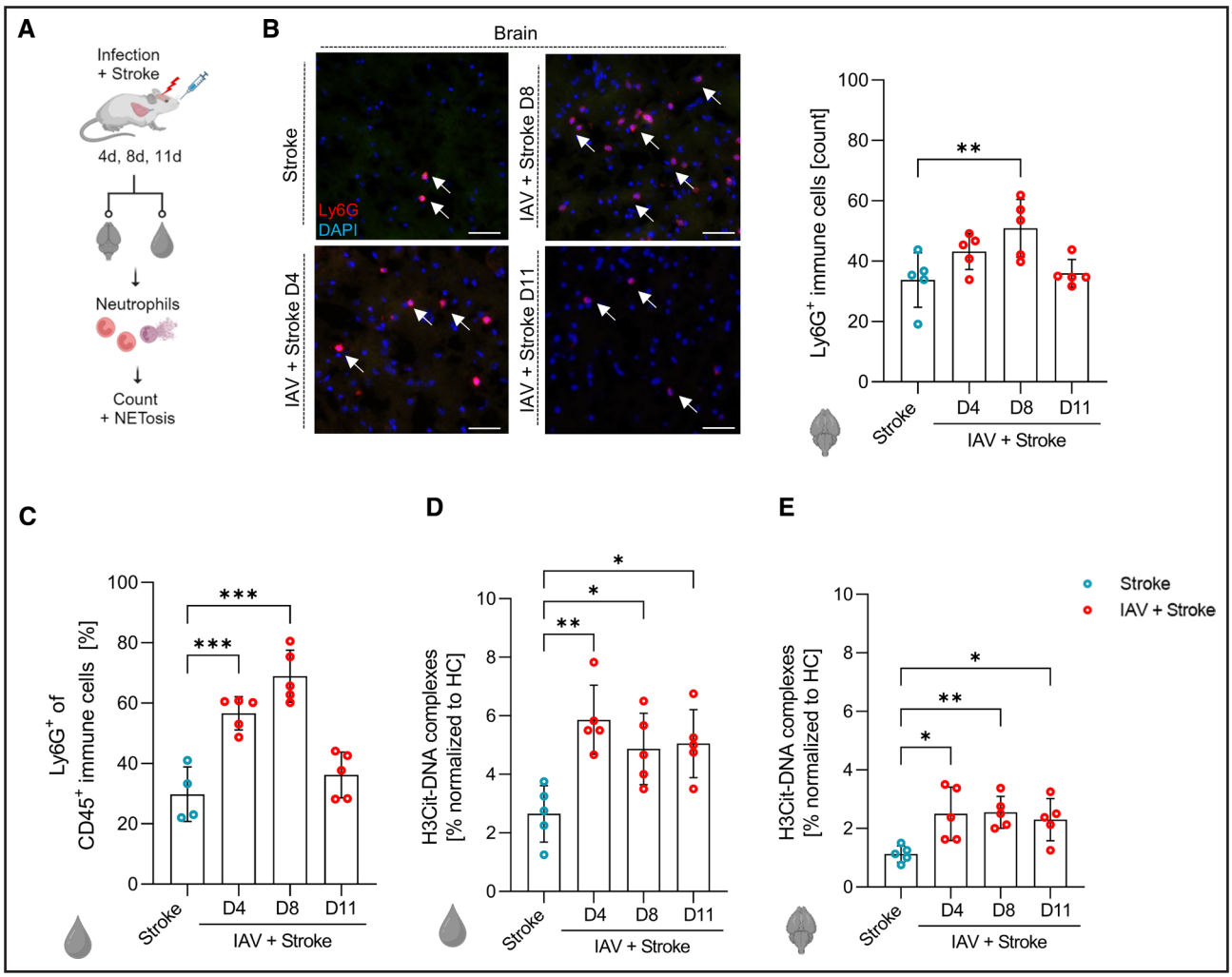

Figure 3. Influenza A virus (IAV) infection increases neutrophil count and NETosis abundance.
A, Schematic representation of the experimental paradigm. Mice were infected with IAV followed by transient middle cerebral artery occlusion (tMCAO) on day 3, 7, or 10. **B**, Total number of lymphocyte antigen 6 complex, locus G (Ly6G)$^+$ neutrophils in the ischemic brain from tMCAO and IAV-infected tMCAO mice determined with histological staining (n=5). **C**, Percentage of Ly6G$^+$ neutrophils in the blood from tMCAO and IAV-infected+tMCAO mice measured with flow cytometry (n=5). **D**, Relative levels of H3Cit (citrullinated histone H3)$^+$DNA complexes (NETosis) from the blood plasma (n=5). **E**, Brain homogenates (n=5). *P<0.05, **P<0.01, and ***P<0.001; 1-way ANOVA followed by the Sidak comparison test (infarct volumes) compared with the respective healthy control or stroke group. CD indicates cluster of differentiation.

(Figure 3D and 3E). To prove preactivation, we tested the neutrophils' threshold to release NETs upon stimulation. In vitro stimulation of neutrophils, derived from the blood of stroke mice, revealed that neutrophils induce NETosis beginning at a concentration of 1-nmol/L phorbol-12-myristate-13-acetate, independent of the prior infection (Figure 4A through 4D). Yet, we found a significant increase in H3Cit-DNA complexes on day 4 after infection at 1-nmol/L phorbol-12-myristate-13-acetate and at all stages of infection at concentrations of 10- and 50-nmol/L phorbol-12-myristate-13-acetate compared with noninfected stroke mice (Figure 4A through 4D). This phenomenon was highlighted by a significant elevation in neutrophil-dependent cytokines MMP-9, CCL-5, and IL-6 in the blood and brain tissue derived from mice with stroke combined with IAV infection compared with noninfected stroke mice at day 8 (Figure S4I through S4L). However, the concentrations of MMP-9, CCL-5, and IL-6 in infected mice had returned to poststroke levels by day 11 after infection. Although these concentrations were lower in the blood compared with the brain, their progression over the course of IAV infection followed a similar pattern (Figure S4I through S4L).

ASA Reduces Preactivation of Thrombotic Factors During IAV Infection and Ameliorates Ischemic Brain Damage

To address the identified trigger of exacerbated ischemic damage, we took advantage of ASA, which has emerged as a target to reduce NETosis, adhesion, and the activation of neutrophils.[25] In addition, ASA also impairs platelet aggregation and activation and has been shown to reduce ischemic brain damage in mice.[26] In this study, prophylactic ASA treatment was observed to significantly reduce infarct volume in noninfected mice (Figure 5A and 5B). Furthermore, it mitigated the increase in cerebral damage caused by IAV infection on days 4 and 8 (Figure 5B). This limitation of cerebral damage was also reflected in improved neuromotor function (Figure 5C). Interestingly, subjecting infected and ASA-treated mice to tMCAO led to increased platelet count in the blood, suggesting less thrombotic formation compared with untreated mice poststroke (Figure 5D). In the blood of stroke mice, comparable amounts of platelets were observed during the course of infection in comparison to HC mice treated with ASA (Figure S5A). Investigation of CD62P$^+$ platelets revealed a significantly lower level of platelet activation in IAV-infected mice after ASA treatment on days 4, 8, and 11 compared with HC without treatment (Figure S5B). This reduced platelet activation was even more pronounced when infected and noninfected mice received ASA before stroke induction compared with untreated stroke mice (Figure 5E). Consequently, we investigated microthrombosis in the brains and found that ASA not only reduced thrombosis in stroke mice but also mitigated the increased severity caused by IAV infection (Figure 5F). The concentration of liberated fibrinogen in the blood was elevated in mice that had been treated with ASA and infected with IAV (Figure S5C), whereas plasminogen levels remained unchanged during IAV infection in comparison to HC mice (Figure S5D).

ASA Decreases the Activation State of Neutrophil Granulocytes and Reduces NETosis Induction

Next, we evaluated the impact of ASA treatment on the neutrophil granulocyte response. The frequency of neutrophils in the blood of infected tMCAO mice decreased under ASA treatment compared with stroke mice on day 4 (Figure S6A). No reduction was observed at other time points of infection with or without tMCAO; in fact,

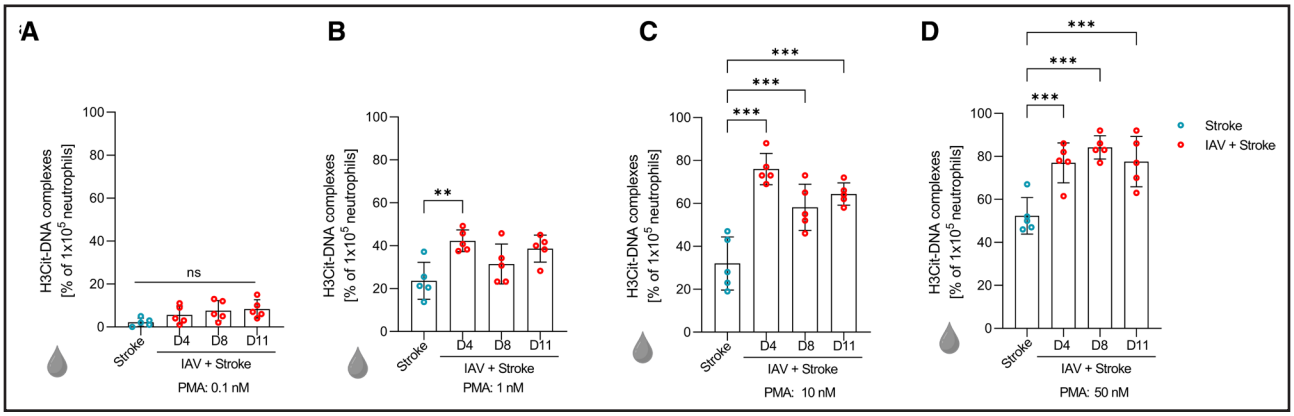

Figure 4. Circulating neutrophils from influenza A virus (IAV)–infected mice are prone to undergo NETosis during ischemic stroke.

A through **D**, Quantification of H3Cit (citrullinated histone H3)+DNA complexes of blood isolated lymphocyte antigen 6 complex, locus G (Ly6G)$^+$ neutrophils from transient middle cerebral artery occlusion (tMCAO) or IAV+tMCAO mice (n=5). Neutrophils were stimulated with 0.1-, 1-, 10-, or 50-nmol/L phorbol-12-myristate-13-acetate (PMA) to induce NETosis. **P<0.01 and ***P<0.001; 1-way ANOVA followed by the Sidak comparison test.

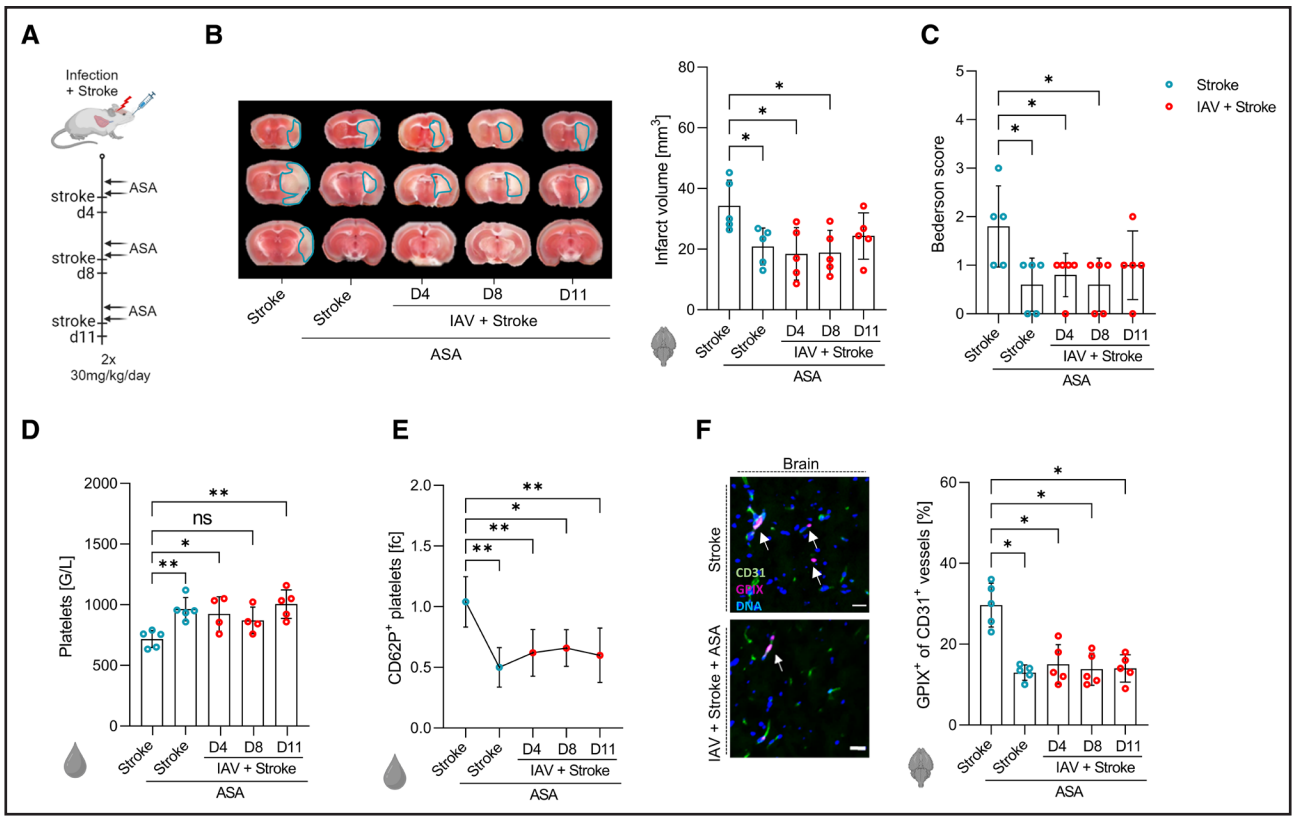

Figure 5. Acetylsalicylic acid (ASA) treatment diminishes the influenza A virus (IAV)–induced procoagulant state and reduces ischemic stroke damage.

A, Schematic representation of the experimental paradigm. Mice underwent an IAV infection and were treated with ASA 24 hours before and on the day of transient middle cerebral artery occlusion (tMCAO) induction. **B**, Representative 2,3,5-triphenyltetrazolium chloride staining of 3 corresponding coronal brain sections of days 4, 8, and 11 of IAV-infected mice 24 hours after tMCAO induction, with or without ASA treatment, and analysis of brain infarct volumes from stroke, stroke+ASA, and IAV+stroke+ASA conditions (n=5). **C**, Evaluation of corresponding neurological deficits using a Bederson score (n=5). **D**, Number of platelets in the blood of stroke, stroke+ASA, and IAV+stroke+ASA mice (n=5). **E**, Determination of CD (cluster of differentiation) 62P$^+$ activated platelets in blood plasma of respective experimental groups (n=5). **F**, Percent of GPIX (glycoprotein 9)$^+$ occluded CD31$^+$ vessels in the ipsilateral hemisphere of the ischemic brain of respective experimental groups (n=5). $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$; 1-way ANOVA followed by the Sidak comparison test (infarct volumes) compared with the respective healthy control or stroke group. fc indicates fold change; G/L, billions/L; and OD, optical density.

neutrophils were increased in the brain at day 8 in ASA-treated IAV-infected mice (Figure S6A and S6C). As previously described, an increase in H3Cit-DNA complexes was observed in the blood of infected stroke mice. Notably, ASA treatment in stroke mice showed a reduction in H3Cit-DNA complexes in comparison to untreated stroke mice. In the infected tMCAO group, significantly fewer H3Cit-DNA complexes were found under ASA treatment (Figure 6A), while no impact of ASA was observed in uninfected mice without tMCAO (Figure S6D). We found that ASA significantly reduced the number of neutrophils in the brains of infected tMCAO mice at all time points (Figure S6B). This was accompanied by a reduced activation capacity of neutrophils during tMCAO without IAV infection, but no differences were found in IAV infection following tMCAO (Figure 6B; Figure S6D). Nevertheless, ASA led to comparable amounts of neutrophil-dependent cytokines in the brain and blood, with MMP-9, CCL-5, and IL-6 levels comparable to those observed in uninfected tMCAO mice (Figure S6E through S6H).

As administration of ASA resulted in a reduced number of H3Cit-DNA complexes within the blood of stroke mice (Figure 6A), neutrophils were isolated from the blood and restimulated to assess preactivation. We observed a significant reduction in activation in ASA-treated and IAV-infected tMCAO mice compared with mice without ASA treatment (Figure 6C through 6F). We also examined the general preactivated state of neutrophils in infected mice on days 4, 8, and 11 without stroke. Here, ASA was capable of significantly reducing the capacity of neutrophils to facilitate the formation of NETs (Figure S6I through S6L). These findings highlight that IAV infection has a profound impact on the coagulation system, thereby increasing the risk of thrombotic events throughout the entire organism.

DISCUSSION

In this study, we demonstrated that IAV infection is a serious trigger for preactivation of neutrophil granulocytes

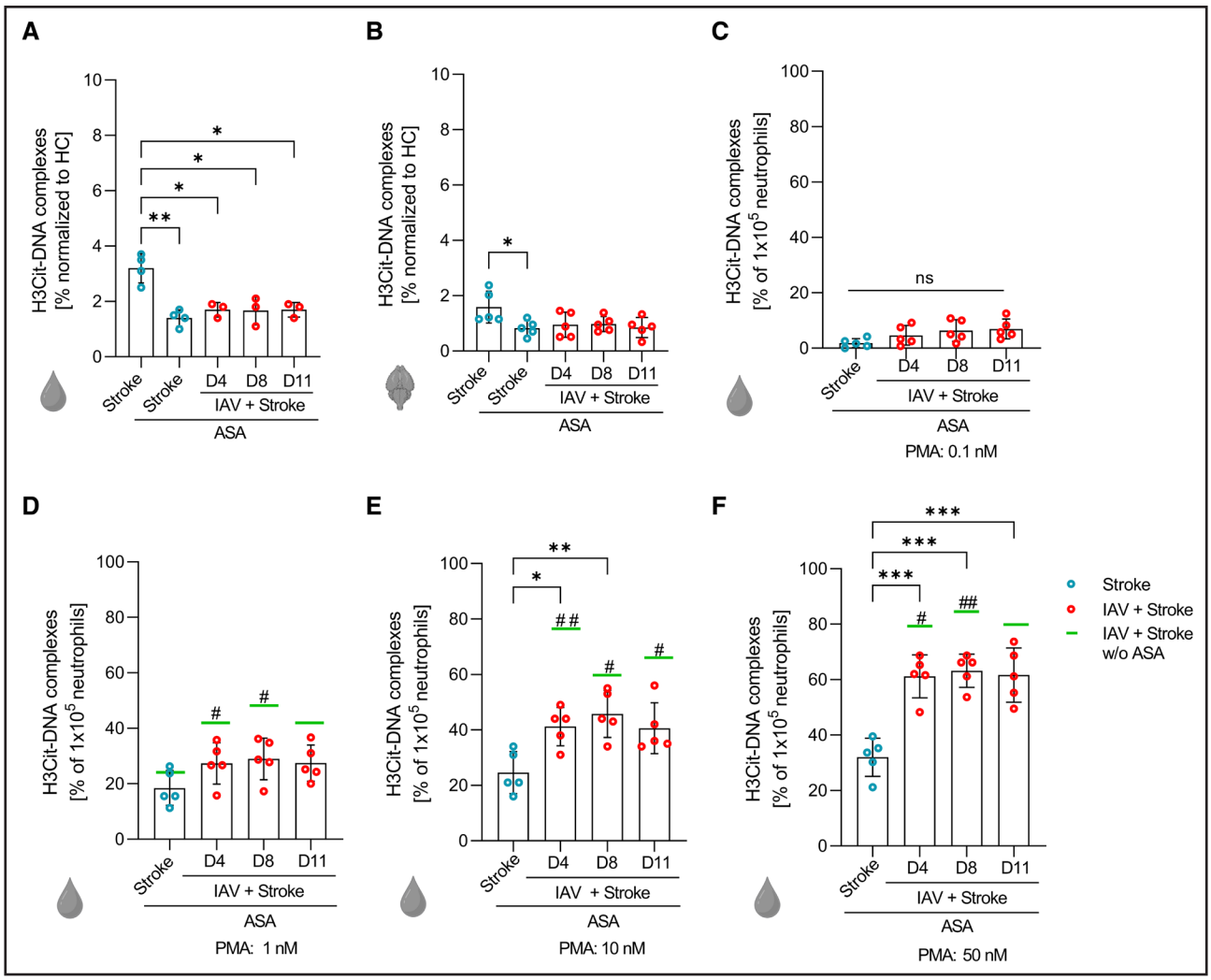

Figure 6. Acetylsalicylic acid (ASA) treatment reduces NETosis in influenza A virus (IAV)–infected stroke mice.
A, Relative levels of H3Cit (citrullinated histone H3)+DNA complexes (NETosis) from the blood plasma (n=3–4). **B**, Relative levels of H3Cit$^+$DNA complexes in ischemic brain homogenates (n=5). **C** through **F**, Quantification of H3Cit$^+$DNA complexes of blood isolated lymphocyte antigen 6 complex, locus G (Ly6G)$^+$ neutrophils from transient middle cerebral artery occlusion (tMCAO) and IAV-infected mice following tMCAO and treatment with ASA. Neutrophils were stimulated with 0.1-, 1-, 10-, or 50-nmol/L phorbol-12-myristate-13-acetate (PMA) to induce NETosis (n=5). In addition, results were compared with the corresponding experimental groups without ASA (Figure 4A–4D; indicator: green line). */#P<0.05, **/##P<0.01, and ***P<0.001; 1-way ANOVA followed by the Sidak comparison test compared with the respective healthy control (HC) or stroke group. CD indicates cluster of differentiation.

and platelets, leading to exacerbation of ischemic brain damage. Interestingly, we found that NETs from neutrophils in particular mediate damage in the brain microvasculature, leading to enhanced thrombus formation. Overall, we identified this mechanism of IAV infection as a risk factor for the development of ischemic stroke.

Our results showed increased cerebral damage in tMCAO on days 4 and 8 after IAV infection with 50 µL of 300 plaque-forming units/mL. This expands previous findings in a permanent MCAO, which showed enhanced cerebral damage 3 to 5 days post-infection, notably at an LD50 (lethal dose, 50%) dose of 1×10^5 plaque-forming units.[27] Conclusively, a sublethal dosage of IAV used in our study is already sufficient to trigger systemic effects. Increases in infarct volumes were attributed to an inflammatory cascade initiated by the virus, which disrupts the blood-brain barrier.[27] Retrospective studies indicate that the risk of myocardial infarction and ischemic stroke is significantly elevated after influenza-like illness.[2,28] Ischemic stroke and influenza-like illness were dramatically correlated within 15 days of infection, followed by a persistent risk for up to at least 60 days.[2] This has been attributed to systemic inflammation and the release of proinflammatory cytokines, which can increase the likelihood of thrombotic events.[2,29] Interestingly, vaccination against IAV or treatment with the antiviral oseltamivir may reduce inflammation in the lung.[22,30] Oseltamivir was indeed effective in our study to reduce viral burden in the lung and stabilize body weight, and had a direct influence on reducing the infarct volume.

Although the underlying mechanisms have not been investigated in detail, previous studies indicate that IAV infection significantly increases thrombogenic and coagulation activity.[31] Mice infected with A/PR/8/34 showed increased abundance of fibrinogen in bronchoalveolar lavage, peaking at day 4, followed by increased levels of plasminogen and D-dimer at day 6.[32] In the peripheral blood of our model, we found a permanent increase in fibrinogen in the blood from day 4 to day 11 after infection, while plasminogen increase was delayed until day 11. Of note, IAV infection induces disturbance in tPA (tissue-type plasminogen activator) and PAI-1 (plasminogen activator inhibitor-1), which can either lead to pathological fibrin deposition or hemorrhagic transformation.[33] During IAV infection, platelet aggregation led to pulmonary microvascular thrombosis, endothelial damage, and hyperinflammatory cytokine responses in mice.[34] These results are consistent with our findings of increased thrombosis in the ischemic brain. In our study, we found decreased numbers of platelets in the blood on day 4, while platelet activation was significantly increased until day 11 after infection, which is in line with studies describing a decrease in platelet counts and activated platelets during infection.[35] This results in an increased susceptibility to ischemic stroke events during IAV infection because preactivation of coagulation components in the blood, which comprise the capillary endothelium, particularly in small vessels, can lead to ischemic stroke.

During IAV infection, there is an increased influx of neutrophils into affected tissues, including the brain in the context of ischemic stroke.[27] These neutrophils are activated, contribute to inflammation and tissue damage, and may exacerbate ischemic injury by promoting further inflammation and disruption of the blood-brain barrier.[27] In line with an elevated neutrophil count in the blood in our experimental setup, a study performed in patients showed that the neutrophil-leukocyte ratio increases in the blood after IAV infection compared with HCs.[36] The rise in neutrophil-leukocyte ratio between days 2 and 3 post-inoculation, peaking around day 6, gradually returned to baseline by day 7, which was comparable to the disease progression in our mouse model. During thrombotic processes, neutrophils promote thrombus formation, interact with PSGL-1 (P-selectin glycoprotein ligand-1), and release NETs, facilitating the clotting processes.[14,37] Elevated levels of NET biomarkers, for example, H3Cit, have been correlated with worse outcomes and poor functional recovery in patients with ischemic stroke.[14,38] In our study, we found a close correlation between occurring NETs in the brain and the blood with cerebral damage after IAV infection. In patients with severe influenza infection, isolated neutrophils showed a greater capacity to release NETs compared with HCs, even without additional stimulation in vitro.[39] In this study, we confirmed that the extent of NETosis induction is diminished in IAV-infected mice, thereby indicating an increased risk of thrombotic and inflammatory events.

ASA is known to suppress the production of prostaglandins and thromboxane A2 by irreversibly inactivating COXs (cyclooxygenases).[40] This effect inhibits platelet aggregation and clot formation, thus supporting the reduction of vascular inflammation.[41] This is achieved by inhibiting platelet activation, which is a critical factor in the hyperinflammatory response seen in severe influenza pneumonia.[15] Controversially, studies also suggest that ASA exacerbates certain influenza virus infections in mice and need to be carefully interpreted.[15] Previous studies showed that administration of ≥30-mg/kg ASA significantly reduced infarct volume and improved neurological deficits in tMCAO mouse models.[42] In rats, administration of ASA after tMCAO decreased inflammatory and thrombotic processes.[43] Similarly, in our study, ASA treatment reduced ischemic brain damage and improved functional outcome. We found stabilization of platelet numbers and their activation status, as well as reduced thrombosis in the brain. Neutrophils were reported not to decrease under ASA treatment on days 3 and 6 in the lung post-infection.[44] However, we found that ASA treatment was able to partially prevent neutrophil accumulation in the blood of infected mice and fully prevented the increase in neutrophils in infected stroke mice. It has been demonstrated that the inhibition of NET formation by neutrophils is associated with a reduction in the phosphorylation of the NF-κB p65 (nuclear factor kappa-light-chain-enhancer of activated B cells, subunit 65) subunit, a key signaling pathway involved in the generation of NETs.[45] We also found that ASA pretreatment can stabilize and reduce the response in neutrophils for NET formation. Altogether, ASA has been shown to reduce neutrophil and platelet accumulation at sites of inflammation, thereby limiting the formation of microvascular thrombosis already described in sepsis.[46,47]

In routine clinical practice, the optimal risk management of viral infections remains poorly understood, making it challenging to determine the appropriate approach in such scenarios.[48,49] In addition, it is important to consider the intensity of infection and relevant risk factors in the patient population when evaluating treatment strategies.[50] Therefore, our study aims to investigate the potential benefits of antiviral therapy in combination with carefully calibrated antiplatelet therapy.

Our study comprises several limitations and is an exploratory study that provides a foundation for future confirmatory follow-up work. This study investigates only male mice, not considering sex differences or age. It is worth noticing that female mice mount a stronger innate and adaptive immune response, with higher antibody titers leading to faster viral clearance during influenza A infection.[51] Also, estrogen was shown to have protective effects on infarct volumes and is beneficial for recovery in female mice,[52] which should be addressed in further experiments. Moreover, we did not check for the longitudinal outcome of influenza A-infected and stroke mice, which is interesting regarding the risk assessment and

impact on functional recovery. As influenza A infection itself represents a comorbidity that can significantly influence the outcome of stroke in mice, the detailed assessment of neurological deficits should be investigated with more sensitive scoring methods. In addition, NET formation contributes to lung tissue damage during infection and aggravates tissue injury after ischemic stroke.[12,14,32] Given the pleiotropic effects of ASA targeting NETs and platelets, single-target treatments with NETosis inhibitors or DNAse should, therefore, be considered to unveil the precise role of NETs in future experiments.

In summary, our findings demonstrate that IAV infection leads to substantial activation of platelets and neutrophils, thereby increasing the risk of thrombotic events in the tMCAO mouse model. These results demonstrate that coagulation factors remain activated for an extended period following an IAV infection. This study opens up an attractive treatment option and a window of opportunity for intensified monitoring of patients with influenza infection who are at increased risk of ischemic stroke.

ARTICLE INFORMATION

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Author Contributions

Drs Haupeltshofer, Kleinschnitz, and Langhauser were involved in conceptualization. Drs Haupeltshofer, Steinbach, and Szepanowski were involved in data curation. Drs Haupeltshofer, Hansmann, Mausberg, and Steinbach were involved in formal analysis. Drs Casas and Kleinschnitz were involved in funding acquisition. Drs Haupeltshofer, Blusch, Hansmann, Szepanowski, and Wenzek were involved in investigation. Drs Haupeltshofer, Blusch, Hansen, Knuschke, Langhauser, Steinbach, Wenzek, and Westendorf were involved in methodology. Drs Haupeltshofer and Szepanowski were involved in project administration. Drs Haupeltshofer, Blusch, Casas, Hansen, Kleinschnitz, Knuschke, Mausberg, and Westendorf were involved in resources. Drs Haupeltshofer and Hansen were involved in software. Drs Kleinschnitz, Knuschke, and Langhauser were involved in supervision. Drs Haupeltshofer, Hansmann, Langhauser, and Mausberg were involved in validation. Drs Haupeltshofer, Blusch, Hansen, and Steinbach were involved in visualization. Drs Haupeltshofer and Casas were involved in writing-original draft. Drs Haupeltshofer, Blusch, Casas, Hansen, Hansmann, Kleinschnitz, Knuschke, Langhauser, Mausberg, Steinbach, and Szepanowski were involved in writing-review and editing.

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Disclosures

None.

Supplemental Material

ARRIVE Checklist
References 53, 54

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