T Cells Prevent Hemorrhagic Transformation in Ischemic Stroke by P-Selectin Binding

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***Objective*—**Hemorrhagic transformation is a serious complication of ischemic stroke after recanalization therapies. This study aims to identify mechanisms underlying hemorrhagic transformation after cerebral ischemia/reperfusion.

***Approach and Results*—**We used wild-type mice and $Selplg^{-/-}$ and $Fut7^{-/-}$ mice defective in P-selectin binding and lymphopenic $Rag2^{-/-}$ mice. We induced 30-minute or 45-minute ischemia by intraluminal occlusion of the middle cerebral artery and assessed hemorrhagic transformation at 48 hours with a hemorrhage grading score, histological means, brain hemoglobin content, or magnetic resonance imaging. We depleted platelets and adoptively transferred T cells of the different genotypes to lymphopenic mice. Interactions of T cells with platelets in blood were studied by flow cytometry and image stream technology. We show that platelet depletion increased the bleeding risk only after large infarcts. Lymphopenia predisposed to hemorrhagic transformation after severe stroke, and adoptive transfer of T cells prevented hemorrhagic transformation in lymphopenic mice. $CD4^+$ memory T cells were the subset of T cells binding P-selectin and platelets through functional P-selectin glycoprotein ligand-1. Mice defective in P-selectin binding had a higher hemorrhagic score than wild-type mice. Adoptive transfer of T cells defective in P-selectin binding into lymphopenic mice did not prevent hemorrhagic transformation.

***Conclusions*—**The study identifies lymphopenia as a previously unrecognized risk factor for secondary hemorrhagic transformation in mice after severe ischemic stroke. T cells prevent hemorrhagic transformation by their capacity to bind platelets through P-selectin. The results highlight the role of T cells in bridging immunity and hemostasis in ischemic stroke.

***Visual Overview*—**An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:1761-1771. DOI: 10.1161/ATVBAHA.118.311284.)**

Key Words: blood platelets ■ brain ■ hemorrhage ■ ischemia ■ lymphocytes ■ mice

Remarkable progress has been made in recent years in the treatment of acute ischemic stroke with the wider implementation of systemic and endovascular reperfusion therapies.[1] However, some patients may remain functionally dependent or die despite these interventions as the result of complications. Hemorrhagic transformation (HT), or bleeding into an area of ischemic brain, ranges from small petechiae to large parenchymatal hematomas.[2] HT occurs in ≈12% of patients with ischemic stroke,[3] but the risk increases to 26% after revascularization therapies.[4] HT is considered clinically relevant when it becomes symptomatic (symptomatic intracerebral hemorrhage [sICH]), which occurs in ≈3% of patients receiving systemic thrombolysis[5] and 5% of patients treated with combined intravenous and intra-arterial therapy.[4] The overall rate of sICH after mechanical thrombectomy is 4.4%,[6] but it may increase up to 11.3% when multiple device passes are required to achieve recanalization.[7] Several clinical features, blood markers, genetic factors, and neuroimaging findings have been associated with HT in patients with ischemic stroke.[8,9] HT is more frequent after large infarctions,[3,5,8–10] and critical determinants are severe damage of the vascular endothelium and the blood-brain barrier.[8,11,12] Yet, better understanding of the mechanisms underlying HT is of utmost clinical relevance because it may lead to identifying more sensitive predictors and new therapeutic strategies to minimize the burden of this serious complication.

Recent studies suggested that the neutrophil to lymphocyte ratio (NLR) predicted an increased risk of sICH after thrombolytic therapy in patients with stroke.[13] However, it is currently unknown whether there is a causal association between neutrophils, NLR, and the hemorrhagic risk or, alternatively, whether changes in white blood cells follow the severity of stroke, or concurrent complications. Acute stroke induces lymphopenia by

Received on: March 4, 2018; final version accepted on: May 29, 2018.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/journal/atvb/doi/suppl/10.1161/atvbaha.118.311284.

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Nonstandard Abbreviations and Acronyms	
Fut 7	fucosyltransferase 7
HT	hemorrhagic transformation
MCAo	middle cerebral artery occlusion
NLR	neutrophil to lymphocyte ratio
PSGL-1	P-selectin glycoprotein ligand-1
tPA	tissue-type plasminogen activator

mechanisms involving complex neurohormonal responses.[14–17] However, there is no information on whether stroke-induced lymphopenia increases the hemorrhagic risk.

Leukocytes interact with platelets[18] though several mechanisms, such as platelet P-selectin binding to PSGL-1 (P-selectin glycoprotein ligand-1 or CD162), which is expressed in neutrophils and T cells, but not B cells.[19–21] Functional P-selectin binding epitopes in the PSGL-1 molecule require post-translational modifications involving protein glycosylation mediated by various glycosyltransferases, including Fut7 (fucosyltransferase 7).[22] Expression of Fut7 in T lymphocytes is inducible on activation and polarization of naive T cells, and it is required for T-cell migration into inflamed tissues.[22–24] T cells exacerbate brain damage after cerebral ischemia/reperfusion because they facilitate platelet and leukocyte adhesion to the vessel wall and promote thromboinflammation.[25,26]

In our study, postischemic lymphopenia facilitated HT. We used Rag2$^{-/-}$ mice, which show severe lymphopenia because of lack of αβ and γδ T cells, B cells, and NKT (natural killer T cells) cells. These mice were prone to HT after severe ischemic stroke unless they were reconstituted with T cells. This study identified a new mechanism mediated by the PSGL-1/P-selectin axis by which T cells and platelets prevent secondary HT after severe brain ischemia/reperfusion in mice.

Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. An extended version of this section is available in the online-only Data Supplement.

Animals

We used adult (10–16 weeks) male mice on the C57BL/6J background (n=584). Mice included Rag2$^{-/-}$ mice (B6(Cg)-*Rag2$^{tm1.1Cgn}$*/J, no. SN 008449, The Jackson Laboratory) wild-type mice (no. SN 000664, Jaxmice), PSGL-1–deficient (*Selplg*$^{-/-}$) mice, and Fut7$^{-/-}$ mice.[27,28] Animal work was conducted following the local and the European regulations. The ethical committee of the University of Barcelona (CEEA [Comité Ètic d'Experimentació Animal]) approved the experimental procedures. Experimental animal work is reported in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Brain Ischemia

Occlusion of the right middle cerebral artery (MCAo) was performed under isoflurane anesthesia using a monofilament (no. 701912PK5Re, Doccol Corporation, Sharon, MA), as described.[29] Two days postischemia, we assessed the neurological dysfunction with a neurological score modified from a previously reported neuroscore.[30]

Drug Treatments

Treatment was randomly allocated and was administered in a blinded fashion. Ten minutes after reperfusion, mice received an intraperitoneal injection of rabbit anti-mouse platelet serum (15 µL diluted in saline to 200 µL; no. CLA31440, Cederlane) or the vehicle (saline).

Evaluation of HT

At 48 hours of reperfusion, mice were anesthetized with isoflurane and perfused through the heart with heparinized saline. The severity of brain hemorrhage was assessed in coronal brain sections (1-mm thick) of fresh tissue by giving scores (0–5, where 0 means absence of hemorrhage and 5 means large hematomas)[31] and with histological measures.

Magnetic Resonance Imaging

The mouse brain was studied with magnetic resonance imaging (7T, BioSpec, Bruker) using ParaVision 6.0 software. Infarct volume was measured on images obtained with T2*-weighted turboRARE fast spin-echo.[29] Cerebral blood flow was measured with a pulsed arterial spin labeling method. For assessment of brain bleeds, mice received a T2*-weighted magnetic resonance imaging scan using a gradient-echo pulse sequence.

Immunofluorescence

Mice were perfused through the heart with heparinized saline followed by 4% paraformaldehyde, and the brain was processed to obtain cryostat sections for immunofluorescence. Primary antibodies were rabbit polyclonal antibodies against pan-laminin or Glut-1 (facilitated glucose transporter, member 1) and rat monoclonal antibodies against CD41. Please see the Major Resources Table in the online-only Data Supplement. Images were obtained on a confocal microscope (TCS SPE-II, Leica Microsystems) with LAS software (Leica).

Isolation and Administration of T Cells for Adoptive Transfer

T cells were isolated from the spleen and the cervical and inguinal lymph nodes of donor mice through negative selection (Pan T-cell isolation kit-II mouse, no. 130-095-130, Miltenyi Biotech). Dead cells were removed (Dead cell removal kit, no. 130-090-101, Miltenyi Biotech), and viable T cells (15–20×10^{6} cells) were administered intravenously to Rag2$^{-/-}$ mice the day before MCAo. Rag2$^{-/-}$ mice receiving adoptive transfer of T cells were compared with littermate Rag2$^{-/-}$ mice that did not receive cell administration.

Quantification of Brain Hemoglobin Content

Brain hemoglobin content was determined with a colorimetric assay (no. 700540, Cayman Chemicals).

Blood Cell Counts

Blood was collected from the vena cava in EDTA and was analyzed in a hematology system (ADVIA2120i, Siemens).

Flow Cytometry

Cell isolation from brain tissue was performed as described.[32] Cells were incubated at 4°C for 10 minutes with FcBlock followed by primary antibodies. See the Major Resources Table in the online-only Data Supplement. Aqua (live/death, Molecular Probes) was used to gate on live cells. Brain cells and blood were studied in a BD FacsLSRII cytometer using the FacsDiva software (BD Biosciences). Data analysis was performed with FlowJo software (v10 FlowJo, LLC). P-selectin binding assay was performed using chimeric P-selectin/hIgM (human immunoglobulin M). Dynamic interactions of T cells with platelets were studied in diluted fresh whole blood, and cell conjugates were visualized with ImageStream MARKII technology (Amnis, EMD Millipore).

Platelet and T-Cell Conjugate Analysis

Diluted whole blood was prepared as described.[33] Briefly, 50 µL of blood was diluted in 200 µL of Tris-buffered saline containing 20 U/mL

of heparin. One milliliter of Tyrode's buffer supplemented with 1.25 mmol/L of $CaCl_2$ was added. Fifty microliter were incubated for 20 minutes with a mixture of antibodies. See the Major Resources Table in the online-only Data Supplement. Samples were immediately analyzed in a BD-FACS LSRII flow cytometer. For P-selectin binding assay, 10^5 leukocytes were incubated with a mix of antibodies for 15 minutes at 6°C and then with anti-hIgM fluorescein isothiocyanate for 15 minutes at 6°C. Cells were analyzed as above. For Amnis ImageStream analysis, whole diluted blood was stained with antibodies for 20 minutes at room temperature, red blood cells were lysed for 10 minutes at room temperature, and washed with Tyrode's buffer. Data were collected with INSPIRE software (EMD-Milipore) and was analyzed with IDEAS software (EMD-Milipore) using the wizard composite for CD3 and CD41 to generate images of platelet-T cell conjugates.

Statistics

The statistical tests used in each experiment and n values are reported in the Figure legends.

Results

Platelet Depletion Increases the Hemorrhagic Risk Only in Large Infarcts

Reperfusion therapies are not recommended in ischemic stroke patients with low platelet counts because of a high risk of HT.[34] Antiplatelet drugs also increase the risk of HT after reperfusion therapies, particularly in patients with large infarctions.[9] To find out the contribution of platelets in preventing hemorrhage secondary to brain ischemia, we treated mice with an antiplatelet serum that markedly reduced platelet numbers (Figure 1A). We induced different degrees of ischemic brain damage by MCAo for either 30 or 45 minutes. Forty-five–minute MCAo induced larger infarct volumes, as expected, and caused HT in some mice while HT was infrequent after 30-minute MCAo (Figure I in the online-only Data Supplement). Platelet depletion after induction of ischemia (Figure 1B) did not significantly increase brain bleeding after 30-minute MCAo while it produced large hematomas after 45-minute MCAo (Figure 1C and 1D). After 45-minute ischemia, we detected the presence of platelets in the ischemic brain tissue in different locations (Figure 1E), that is, intravascular platelets blocking the lumen of some capillaries, platelets within parenchymal hemorrhages, or platelet clusters surrounding the abluminal side of the endothelium. Platelets surrounded blood vessels in zones with extravasated fibrin(ogen; Figure 1E), suggesting the formation of extravascular hemostatic clots sealing the ruptured vessels.

Involvement of NLR, Neutrophils, and Lymphocytes in HT

A high NLR is associated with HT in patients with ischemic stroke treated with thrombolysis.[13] We measured blood cell counts 48 hours after 45-minute ischemia to assess for the contribution of blood cells in HT in our stroke mouse models. Neutrophils tended to increase after ischemia, whereas lymphocyte numbers markedly decreased, in agreement with the reported stroke-induced lymphopenia,[14–17] and the NLR as well as platelet numbers increased (Figure 2A). Infarct size was positively correlated to the hemorrhagic score (Figure 2B). Accordingly, examining infarct size in each category of HT score (Figure 2C) showed that mice with hemorrhages had larger infarctions and worse neurological deficits than mice devoid of bleeding (HT score=0) or with a few small petechiae (HT score=1; Figure 2D and 2E). Nonetheless, the mice with the highest hemorrhagic scores did not show the largest infarctions (Figure 2D), suggesting that the magnitude of bleeding might be influenced by other factors in addition to infarct size. We then examined blood cell counts in relation to the degree of hemorrhage. We did not find apparent relationships between the degree of hemorrhage and neutrophil numbers or the NLR (Figure 2F and 2G). However, the most noticeable finding was that mice with the highest hemorrhagic scores showed lower lymphocyte counts (Figure 2H), and lymphocyte counts were inversely correlated with the HT score (Figure 2I). To account for infarct size and lymphocyte counts, we calculated the volume-to-lymphocyte ratio by dividing infarct size (% of hemispheric volume) by blood lymphocyte number (cells/nL). The volume-to-lymphocyte ratio progressively increased with increasing hemorrhagic score (Figure 2J). These results suggest that the highest risk of bleeding occurs in large infarcts with the lowest lymphocyte counts.

Low T-Cell Numbers Increase HT After Severe Ischemia

Lymphopenic $Rag^{-/-}$ mice offer an experimental model to investigate whether low lymphocyte counts might be involved in HT. However, evidence shows that $Rag^{-/-}$ mice develop smaller lesions than the wild-type mice after transient ischemia,[25,26] stressing that it is necessary to account for the extent of the infarct to address the relationship between lymphocytes and the risk of HT. $Rag2^{-/-}$ mice showed smaller infarct volumes than the wild-type mice after 30-minute ischemia (Figure II in the online-only Data Supplement). However, infarct volumes were similar in wild-type mice and $Rag2^{-/-}$ mice after 45-minute ischemia, and we did not detect differences in cerebral blood flow recovery after MCAo between genotypes (Figure II in the online-only Data Supplement). Nonetheless, after 45-minute ischemia, the $Rag2^{-/-}$ mice seemed to bleed more, as assessed macroscopically in the tissue sections (Figure 3A) and microscopically in paraffin sections (Figure 3B). To find out whether this effect was attributable to the lack of T cells, we performed adoptive transfer of T cells to $Rag2^{-/-}$ mice the day before ischemia (see Figure III in the online-only Data Supplement for viability and purity of injected T cells). Forty-eight hours after 45-minute MCAo, the lymphocyte counts×10^3 (mean±SD, n) were 0.830±0.386 (ranging from 0.170 to 1.670; n=26) in wild-type mice; 0.132±0.094 (ranging from 0.030 to 0.420; n=23) in $Rag2^{-/-}$ mice; and 0.279±0.204 (ranging from 0.070 to 0.880; n=13) in $Rag2^{-/-}$ mice that had received adoptive transfer of T cells. The increase of T cells after adoptive transfer was statistically significant (Mann-Whitney test, P=0.003). Notably, adoptive transfer of T cells lowered the HT score in $Rag2^{-/-}$ mice (Figure 3C; Figure IV in the online-only Data Supplement). We also quantified the brain hemoglobin content and performed T2*-weighted magnetic resonance imaging (Figure 3D and 3E). In all these measurements, adoptive transfer of T cells to the $Rag2^{-/-}$ mice consistently reduced the signs of secondary hemorrhage. In independent

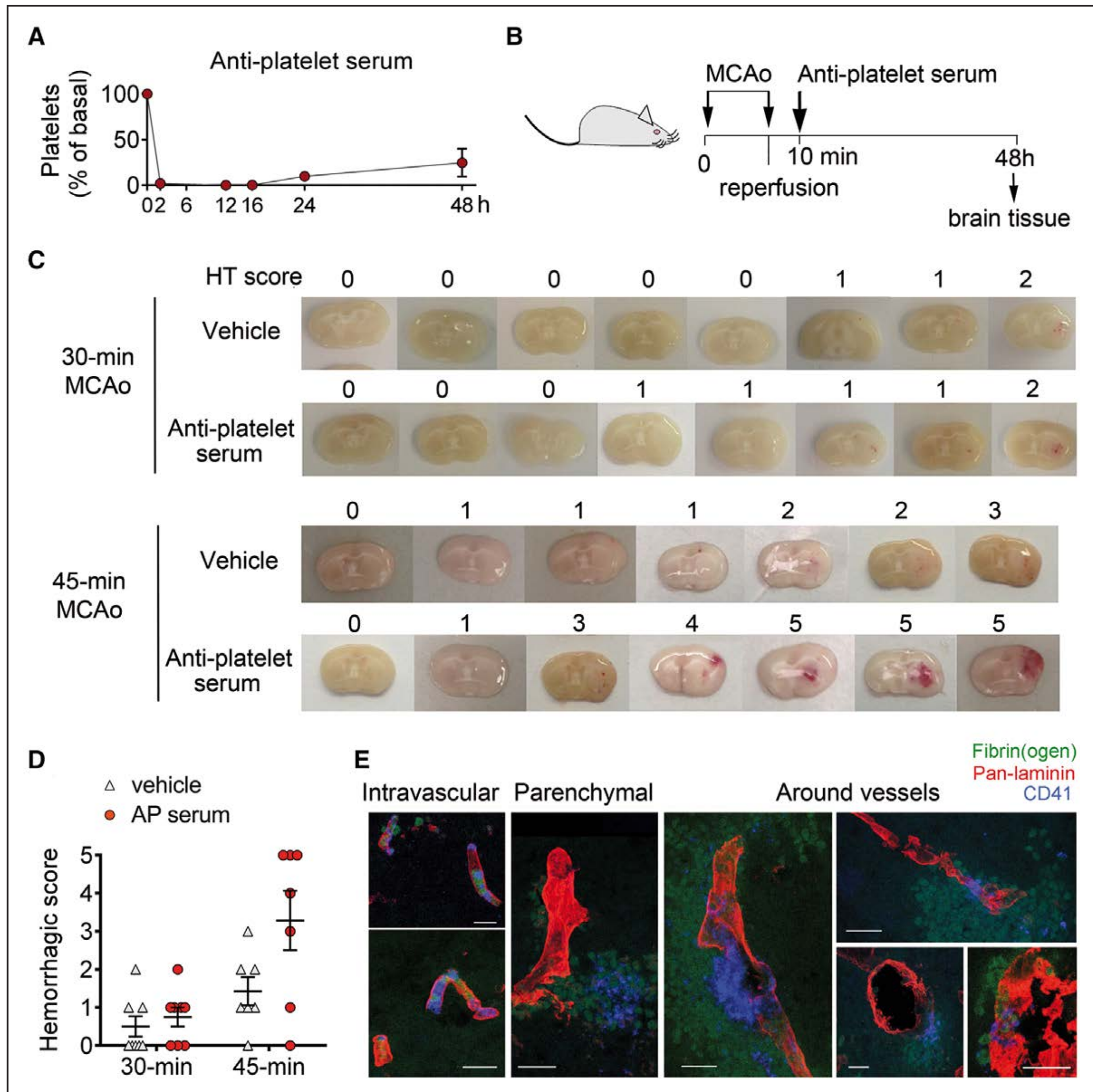

Figure 1. Platelet depletion increases brain bleeding after severe ischemia but not mild ischemia. **A**, Time course of circulating platelets after antiplatelet (AP) serum administration to control mice (n=3) to verify thrombocytopenia. **B**, Mice received antiplatelet serum or the vehicle (saline) intraperitoneally 10 min after reperfusion (reperf.) after 30-min (n=8 mice per group) or 45-min (n=7 mice per group) middle cerebral artery occlusion (MCAo). **C** and **D**, Fresh brain tissue sections were obtained at 48 h to assess for the presence of blood in the infarcted tissue. One section of each mouse is shown for illustrative purposes. Hemorrhagic transformation (HT) was assessed assigning a hemorrhagic score ranging from 0 (no hemorrhage) to 5 (large parenchymal hematoma). Platelet depletion did not significantly increase bleeding after 30-min MCAo (Mann-Whitney test, P=0.567). After 45-min MCAo, the hemorrhagic score tended to be higher in the platelet-depleted group (Mann-Whitney, P=0.092), but 1 mouse of each group did not bleed at all. However, within the mice showing some degree of blood in the brain tissue (n=6 per group), the hemorrhagic score was significantly higher in the platelet-depleted group (Mann-Whitney test, *P=0.035). **E**, After 45-min ischemia, platelets (CD41$^+$, blue) are seen in the brain tissue in the vessel lumen (Intravascular), free in the brain parenchyma (parenchymal), and as clusters surrounding the vasculature (around vessels). Images are representative of 5 mice. Pan-laminin (red) shows the vessel basal lamina, and fibrin(ogen; green) shows vascular leakage. Scale bar, 15 µm.

groups of mice, we tested whether administration of T cells to Rag2$^{-/-}$ mice affected the size of the brain lesion and the neurological deficit. T cells increased infarct volume after 30-minute MCAo but not after 45-minute MCAo (Figure 4). However, in the latter severe ischemia model, adoptive transfer of T cells reduced the hemorrhagic score and attenuated the neurological deficits (Figure 4). Altogether, these results showed that T cells prevented HT after ischemic stroke in mice with marked lymphopenia.

Crosstalk of T Cells and Platelets

Platelets support interaction of platelet-binding T cells to the vessel wall,[18] and T cells facilitate adhesion of leukocytes and platelets to the vascular endothelium after cerebral ischemia/reperfusion.[25] We found fewer platelet clusters around blood vessels of the ischemic tissue of Rag2$^{-/-}$ mice compared with wild-type mice but not after adoptive transfer of T cells (Figure 5A). The effect was not because of low platelet counts in these mice (mean±SD platelet number×10^3, wild-type

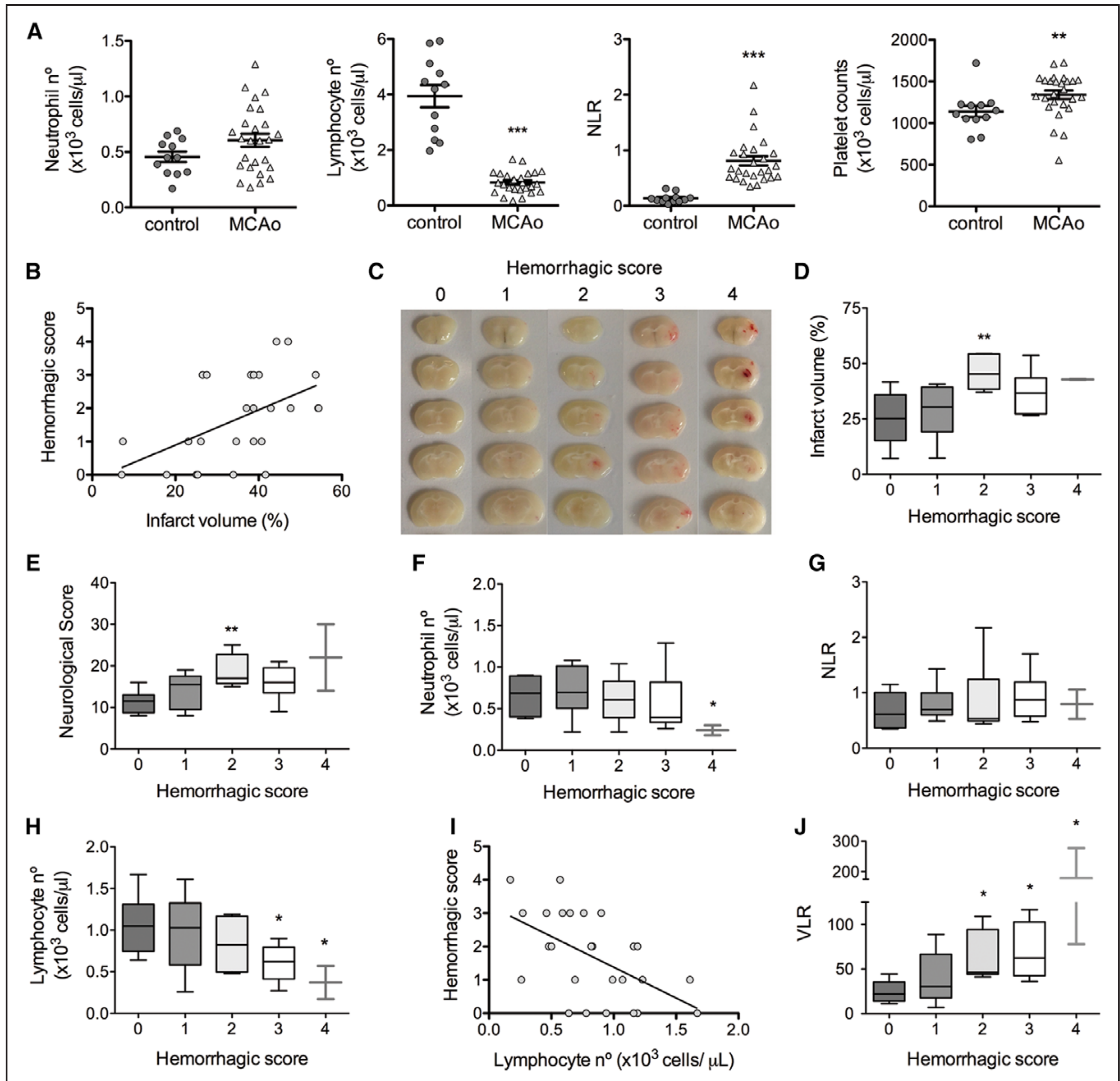

Figure 2. Low lymphocyte counts are associated with hemorrhagic transformation. Results of blood counts for control mice (n=12) and ischemic mice 48 h after 45-min middle cerebral artery occlusion (MCAo; n=26). **A**, After ischemia, neutrophils tended to increase (unpaired *t* test with Welch correction, $P=0.053$), lymphocyte numbers strongly decreased (***$P<0.001$), the neutrophil to lymphocyte ratio (NLR) increased (***$P<0.001$), and platelet numbers increased (**$P=0.006$; Mann-Whitney tests). **B**, The hemorrhagic score was positively correlated with infarct volume (Spearman $r=0.541$; $P=0.004$; n=26). **C**, We grouped ischemic mice (n=26) according to their hemorrhagic score, as illustrated with representative brain tissue sections. There were n=6 mice in each score category, excepting for score=4 with n=2 mice. **D**, Infarct volume was larger for mice showing bleeds (Kruskal-Wallis test $P=0.017$; Dunn test **$P<0.01$ vs score=0). **E**, Similar results were found for the neurological score (Kruskal-Wallis test $P=0.078$; Dunn test **$P<0.01$ vs score=0). **F** and **G**, Neutrophils and the NLR showed no apparent changes between hemorrhagic score categories (Kruskal-Wallis test $P=0.238$), but mice with hemorrhagic score=4 had low neutrophils (Dunn test, $P<0.05$ vs score=0). **H**, Lymphocytes tended to decrease progressively for higher hemorrhagic scores (Kruskal-Wallis test $P=0.080$; Dunn text *$P<0.05$ vs score=0). **I**, The hemorrhagic score was inversely correlated with lymphocyte number (Spearman $r=-0.554$; $P=0.003$; n=26). **J**, To account for infarct size and lymphocyte number, we calculated for each mouse the volume-to-lymphocyte ratio (VLR) as the ratio between infarct volume and the number of blood lymphocytes (cell counts/nL). VLR increased with increasing hemorrhagic score (Kruskal-Wallis test $P=0.011$; Dunn test *$P<0.05$ vs score=0. **A**, **B**, and **I**, Values for each mouse, and (**D**–**H**, **J**) represent group values as box and whiskers from min to max.

mice: 1240±205, n=8; Rag2$^{-/-}$ mice: 1430±296, n=7). We detected T cells within zones of platelet accumulations in the infarcted brain tissue of wild-type mice (Figure 5B) and hypothesized that an interaction of T cells with platelets might be involved in preventing HT after ischemic stroke. Specific subsets of T cells form conjugates with platelets in blood.[21] Accordingly, flow cytometry of blood showed ≈5% to 10% of T cells positive for CD41 (Figure VA in the online-only

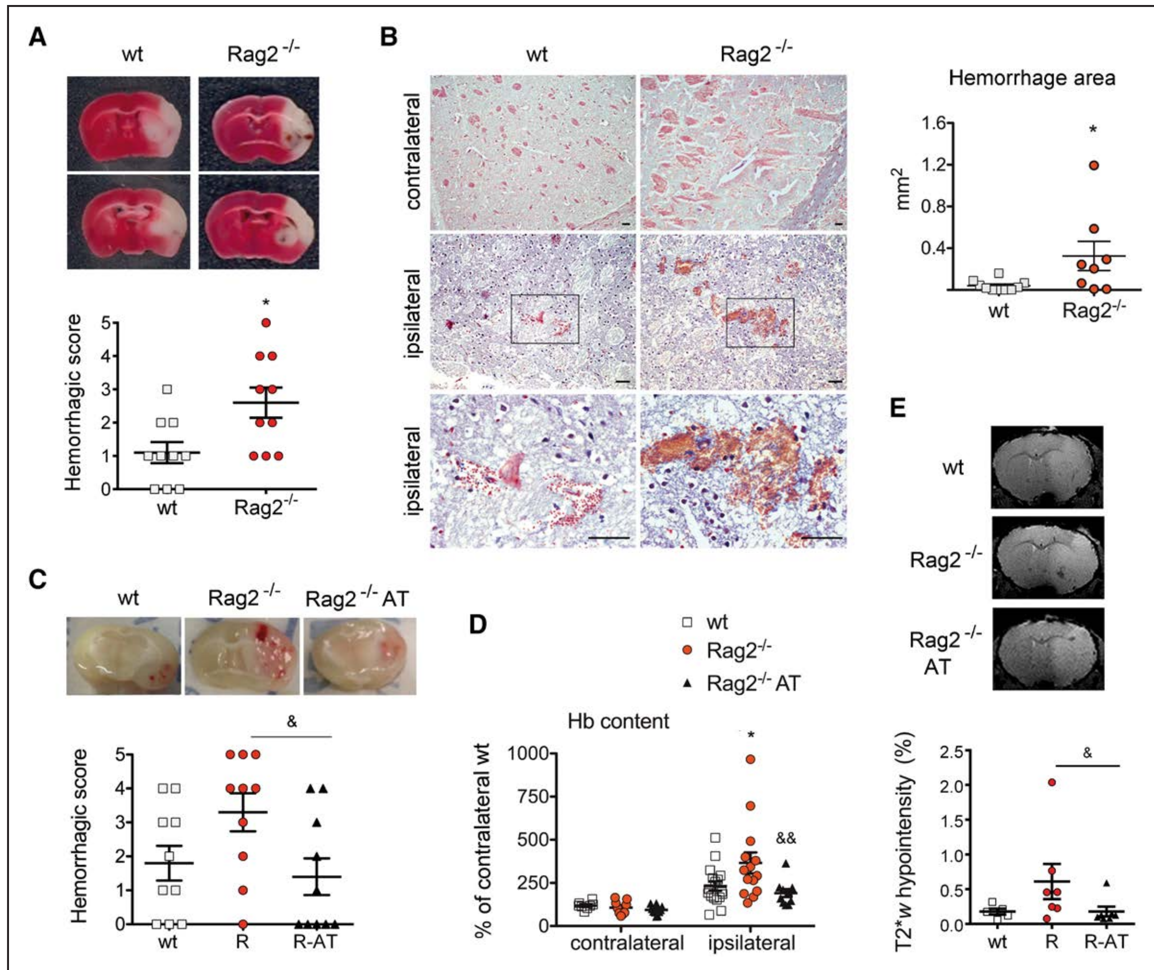

Figure 3. Adoptive transfer of T cells prevents hemorrhagic transformation after severe ischemia in lymphopenic mice. Results were obtained 48 h after 45-min middle cerebral artery occlusion (MCAo). **A**, Representative 2,3,5-triphenyltetrazolium chloride brain sections illustrate the higher hemorrhagic scores in lymphopenic Rag2$^{-/-}$ mice than wild-type (wt) mice (n=10 per group) (Mann-Whitney test, *P=0.021). **B**, Carstair histological brain sections. **Bottom**, Magnifications of (**middle**). Rag2$^{-/-}$ mice (n=8) show larger hemorrhagic areas than wt mice (n=9; Mann-Whitney test, *P=0.026). **C**, Hemorrhage in fresh brain sections of independent groups of wt mice, Rag2$^{-/-}$ mice (R), and littermate Rag2$^{-/-}$ mice receiving adoptive transfer (AT) of T cells (R-AT) (n=10 per group). AT of T cells reduced the hemorrhagic score in Rag2$^{-/-}$ mice (Kruskal-Wallis test, P=0.045; Dunn test, &P<0.05; extended data and validation are shown in Figure IV in the online-only Data Supplement). **D**, Brain hemoglobin (Hb) content (ELISA assay) was higher in the ipsilateral hemisphere of Rag2$^{-/-}$ mice (n=14) than wt mice (n=17) but not after AT of T cells (n=10; 2-way ANOVA by group, P=0.047; and hemisphere, P<0.001; and Bonferroni test *P<0.05 vs wt mice; &&P<0.01 vs Rag2$^{-/-}$ mice). **E**, T2*-weighted magnetic resonance imaging (MRI) in wt mice (n=5), Rag2$^{-/-}$ mice (R) (n=7), and Rag2$^{-/-}$ mice receiving T cells (R-AT) (n=7) shows reduced hypointensity volumes (% of hemispheric volume) in the ischemic hemisphere of Rag2$^{-/-}$ mice after AT of T cells (Kruskal-Wallis test P=0.094; Dunn test, &P<0.05 vs R). The corresponding MRI infarct volume (T2 maps) did not differ between wt mice (35.7%±9.4%), Rag2$^{-/-}$ mice (38.1%±7.6%), and Rag2$^{-/-}$ mice with T-cell AT (36.4%±12.4%). Scale bar, 50 µm.

Data Supplement), suggesting binding of activated platelets to a subset of T cells. Image stream technology combining high-resolution microscopy with flow cytometry confirmed the presence of circulating T cell-platelet conjugates (Figure 5C).

Although the majority of blood T cells are CD162$^+$, only a subset of them showed binding of a soluble P-selectin chimera (Figure VB in the online-only Data Supplement) and formed platelet conjugates (Figure VC in the online-only Data Supplement). Accordingly, CD4$^+$ memory T cells of wild-type mice bound soluble P-selectin, whereas naïve T cells did not, and memory T cells deficient in Fut7 were unable to bind soluble P-selectin (Figure VIA in the online-only Data Supplement). Likewise, platelets mainly bound CD4$^+$ memory T cells (Figure VIB in the online-only Data Supplement), whereas Fut7$^{-/-}$ or PSGL-1–deficient (Selplg$^{-/-}$) memory T cells unable to bind P-selectin showed less platelet-T cell conjugates than the wild-type T cells (Figure 5D).

Active P-Selectin Binding Sites in T Cells Are Required to Prevent HT After Severe Ischemic Stroke

In vivo, PSGL-1–deficient mice (Selplg$^{-/-}$ mice) showed a higher hemorrhagic score than wild-type mice after 45-minute ischemia (Figure 6A), suggesting that P-selectin binding was necessary to protect large infarcts from HT. To find out whether active P-selectin binding sites in T cells were involved in this effect, Rag2$^{-/-}$ mice received adoptive transfer of T cells obtained from either wild-type mice, Selplg$^{-/-}$ mice, or Fut7$^{-/-}$ mice. Selplg$^{-/-}$ and Fut7$^{-/-}$ T cells did not prevent

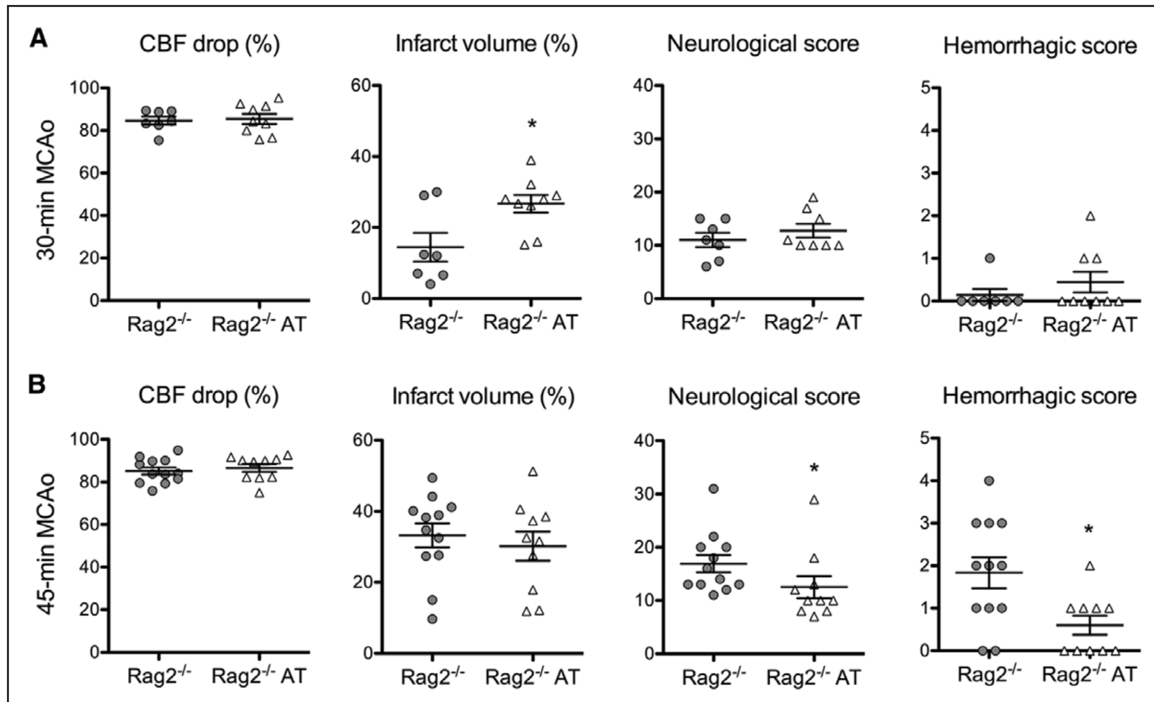

Figure 4. Effect of adoptive transfer of T cells on the outcome of brain ischemia. **A** and **B**, Rag2$^{-/-}$ mice received adoptive transfer (AT) of T cells the day before ischemia (Rag2$^{-/-}$ AT) or were untreated (Rag2$^{-/-}$). Mice were subjected to either 30-min or 45-min middle cerebral artery occlusion (MCAo) and were studied at 48 h. The drop in cerebral blood flow (CBF) induced by MCAo was similar in all groups. **A**, After 30-min ischemia, the mice that received T cells (n=9) showed a larger infarct volume (%) than the mice that did not (n=7; *t* test, **P*=0.017), but the differences in the neurological score were not statistically significant (Mann-Whitney test, *P*=0.186), and the hemorrhagic score did not change (Mann-Whitney test, *P*=0.402). **B**, After 45-min ischemia, the Rag2$^{-/-}$ mice with AT of T cells (n=10) showed an infarct volume similar to that of untreated Rag2$^{-/-}$ mice (n=12; *t* test, *P*=0.564). However, treatment with T cells improved the neurological score (Mann-Whitney test, **P*=0.017) and reduced the hemorrhagic score (Mann-Whitney test, **P*=0.020).

HT after severe ischemia, in contrast to the effect of wild-type T cells (Figure 6B; Figure VII in the online-only Data Supplement). These results demonstrate that P-selectin binding through functional PSGL-1 confers T cells the property to prevent HT after severe ischemia. Given that the subset of T cells with P-selectin–binding capacity are the memory T cells, our result suggests that memory T cells induce prohemostatic effects in large cerebral infarctions at risk of bleeding.

Discussion

The current best treatment for ischemic stroke is reperfusion therapy with thrombolysis or mechanical thrombectomy in suitable patients. However, the benefits of these therapies may be hindered by complications, such as HT. HT occurs more frequently in patients with large infarctions,[3,5,9] but little is known about the underlying mechanisms. This study demonstrates that T cells reduce the risk of HT. It also confirms the relevance of infarct size as a critical factor that increases the hemorrhagic risk. Altogether, these results highlight the crosstalk between T cells and platelets bridging immunity and hemostasis to prevent HT after acute ischemic stroke.

Growing evidence supports the fact that platelets may contribute to the endothelial barrier function by providing physical coverage to the damaged endothelium and thus prevent vascular leakage at sites of severe endothelial injury.[35–37] Hence, our results support that platelets sealed the damaged vessels and reduced the leakage of blood to the ischemic brain parenchyma. This phenomenon was in part mediated by lymphocytes, given that lymphopenia favored HT in large infarctions but did not influence this risk of HT after small infarctions. Functional binding sites on PSGL-1 in T cells were necessary to prevent HT after severe ischemia. PSGL-1 interacts with selectins and is involved in leukocyte adhesion to the activated endothelium and platelets.[38,39] Lymphocytes and platelets interact through a complex crosstalk with functional consequences for both cell types.[18] Memory CD4^{+} T cells are the circulating T cells that form conjugates with platelets. Platelet conjugation selectively enhances the adhesion of memory T cells to the endothelium of inflamed venules.[39] This phenomenon might underlie the recognized role of T cells in promoting thromboinflammation and worsening brain damage after ischemia/reperfusion.[25,26] However, platelets also support P-selectin–mediated adhesion of lymphocytes to the arterial wall when blood flow is disturbed[40] and after exposure of subendothelial matrix proteins that can occur at arterial sites of injury.[41] Our study suggests that memory T cells facilitate the formation of platelet plugs in the abluminal side of severely injured vessels. We found that the mechanism involves the PSGL-1/P-selectin axis, but identification of the precise molecular and cellular determinants of T cell–mediated platelet aggregation around the injured vessels will require more investigation.

The results highlight the intimate crosstalk between platelets and lymphocytes and their double role in hemostasis and inflammation, which in the context of cerebral ischemia can bear predominantly negative (thrombo-inflammatory) or positive (hemostatic) effects, likely depending on the vascular bed,

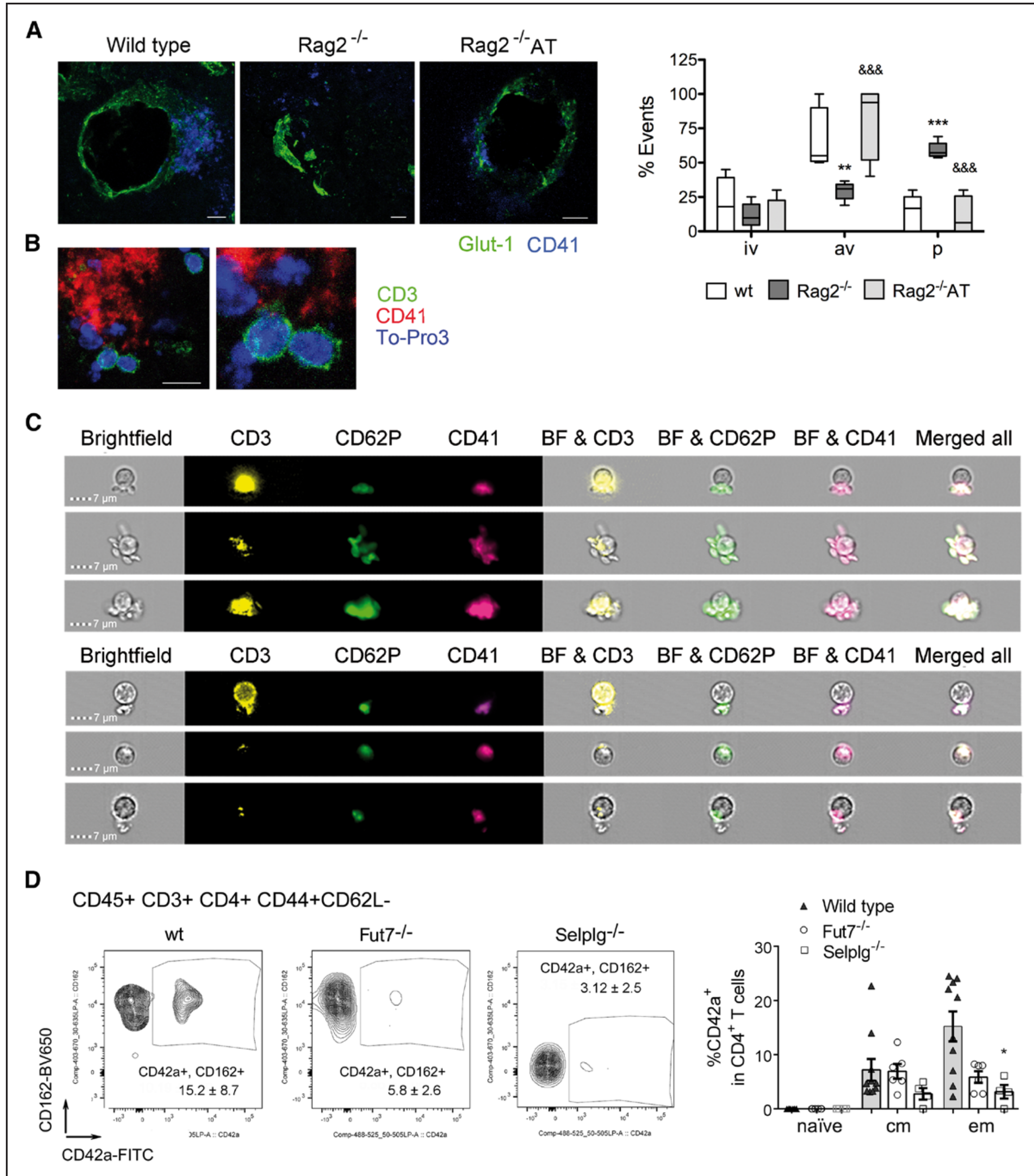

Figure 5. Platelets in the ischemic brain tissue and platelet-T-cell conjugates in blood. **A**, Platelets (CD41+, blue) and vascular endothelial cells (Glut-1+ [facilitated glucose transporter, member 1], green) in the ischemic brain tissue 48 h after 45-min middle cerebral artery occlusion representative of wild-type mice (n=5), Rag2−/− mice (n=5), and Rag2−/− receiving adoptive transfer of T cells (Rag2−/−AT; n=4). Assessment of platelet distribution in the tissue shows less platelet clusters around blood vessels and more platelet groups in the parenchyma of Rag2−/− mice. Adoptive transfer of T cells to Rag2−/− mice prevents these effects. Values are the % of events in each location are the average of measures obtained by 2 independent observers. Two-way ANOVA by animal group and platelet location followed by the Bonferroni test (***P*<0.01 and ****P*<0.01 vs wild-type [wt] mice, &&&*P*<0.001 vs Rag2−/− mice). **B**, CD3+ T cells (green) are seen in the ischemic tissue near zones of CD41+ platelet aggregation (red). Nuclei are stained with To-Pro3 (blue). Scale bar, 10 µm. **C**, ImageStream analysis (Amnis) in whole fresh blood (n=4 control wild-type mice) allows direct visualization of platelets (CD41+, CD42a+) and activated platelets (CD62P+) bound to T cells. **D**, Plots show CD45+CD3+CD4+CD44+CD62L− effector memory T cells and illustrate reduced conjugates of platelets and CD4+ effector memory T cells in blood obtained from mice deficient in Fut7−/− (n=6) or PSGL-1 (P-selectin glycoprotein ligand 1; Selplg−/−; n=4) vs the blood of wt mice (n=10). The graph shows the results of platelet binding to naïve, central memory (cm), and effector memory (em) CD4+ T cells. For effector memory T cells, Kruskal-Wallis test *P*=0.024, Dunn test **P*<0.05 vs wt cells. For central memory T cells, Kruskal-Wallis test *P*=0.09. Values in the plots and the bar graphs are the mean±SEM of the number of mice indicated above, and the value for each individual mouse is shown in the graphs. av indicates around the abluminal side of vessels; iv, intravascular; and p, parenchyma.

the integrity of the vessels, and the risk of HT. T-cell prohemostatic effects prevail in large infarctions at risk of experiencing HT. In contrast, detrimental effects of T cells become apparent in infarctions of mild or moderate size bearing a low risk of HT. This study identifies lymphopenia as a risk factor for HT of large infarctions, raising the question of whether T-cell

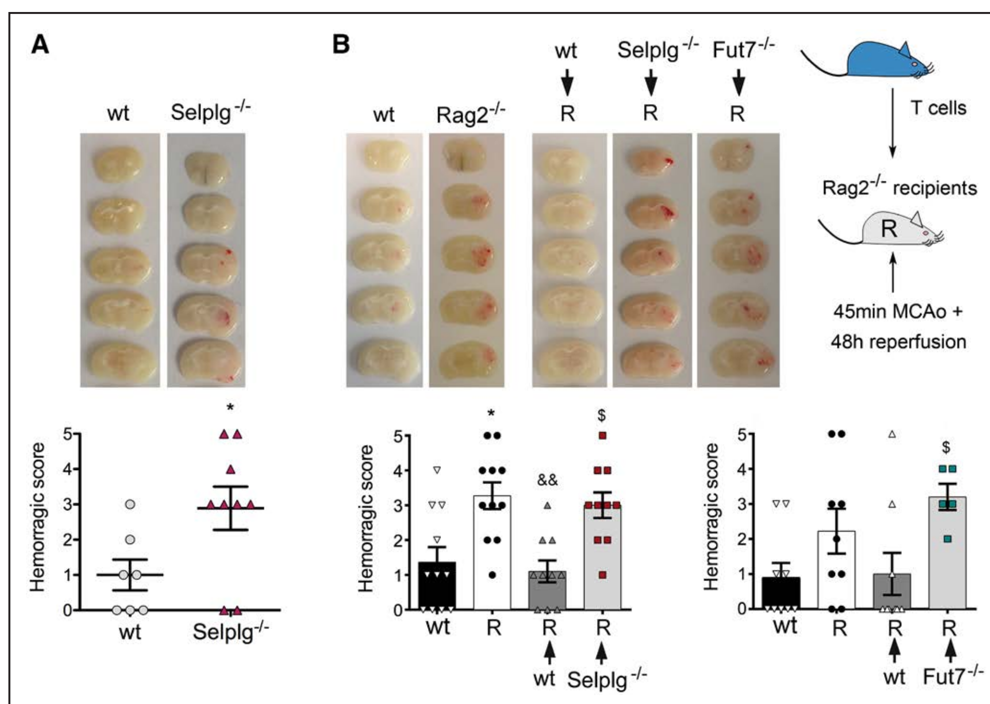

Figure 6. T cells unable to bind P-selectin do not prevent hemorrhagic transformation after severe ischemia. **A**, Images show fresh tissue sections representative of wild-type (wt) mice (n=7) and PSGL-1 (P-selectin glycoprotein ligand-1)–deficient (Selplg$^{-/-}$) mice (n=9) 48 h after 45-min middle cerebral artery occlusion (MCAo). Each column corresponds to a different mouse. Selplg$^{-/-}$ mice showed a higher hemorrhagic score than the wt mice (Mann-Whitney test, **P*=0.049). **B**, The 45-min MCAo was induced in wt mice and Rag2$^{-/-}$ mice (R) that were untreated or received adoptive transfer (AT) of wt T cells, Selplg$^{-/-}$ T cells, or Fut7$^{-/-}$ T cells. Hemorrhage was assessed at 48 h of reperfusion, and representative images of fresh tissue sections per group are shown (see extended data in Figure VII in the online-only Data Supplement). We first compared wt mice (n=11), untreated Rag2$^{-/-}$ mice (n=11) and Rag2$^{-/-}$ mice receiving wt T cells (n=10) or Selplg$^{-/-}$ T cells (n=10). Wt T cells reduced the hemorrhagic score, but Selplg$^{-/-}$ T cells did not (Kruskal-Wallis test, *P*=0.001; and Dunn test **P*<0.05 vs wt; &&*P*<0.01 vs untreated Rag2$^{-/-}$mice; $*P*<0.05 vs Rag2$^{-/-}$ mice treated with wt T cells). In independent groups of wt mice (n=9), untreated Rag2$^{-/-}$ mice (n=9), and Rag2$^{-/-}$ mice receiving either wt T cells (n=9) or Fut7$^{-/-}$ T cells (n=5), Rag2$^{-/-}$ mice receiving Fut7$^{-/-}$ T cells showed a higher hemorrhagic score than those receiving wt T cells (Kruskal-Wallis test, *P*=0.0628; Dunn test, $*P*<0.05 vs Rag2$^{-/-}$ mice receiving wt T cells).

administration after severe stroke could potentially reduce HT without exacerbating brain damage. Interestingly, a recent study reported that administration of regulatory T cells reduced HT induced by delayed treatment with tissue-type plasminogen activator (tPA) in suture and embolic stroke mouse models, and the effect was related to attenuation of tPA-induced endothelial damage.[42] PSGL-1 is involved in the suppressive activity of regulatory T cells, at least under certain experimental conditions.[43] Further studies are needed to find out whether PSGL-1 mediates the effects of regulatory T cells in stroke.

Previous clinical studies have stressed the contribution of neutrophils and the NLR to the risk of HT after thrombolysis.[13,44] In contrast, we did not find a clear relationship between neutrophil counts and the NLR with HT. A main difference between our study and those previous studies is that we did not use tPA. tPA promotes neutrophil transmigration to reperfused tissue via proteolytic activation of plasmin and gelatinases,[45] and it induces neutrophil degranulation.[46] Furthermore, neutrophil extracellular traps impair tPA-induced thrombolysis.[47] Therefore, the potentially damaging effect of neutrophils at the blood-brain barrier could be exaggerated by tPA.

Animals with large infarctions develop lymphopenia but the extent of the response shows some variation potentially attributable to the magnitude of neurohormonal responses.[17] Several lines of evidence suggest the possibility of neuroanatomical correlates of stroke-induced immunodepression[48] that could explain why poststroke lymphopenia is more severe in some individuals than others despite similarly large lesion sizes. A limitation of our study is that it was performed only in male mice. The results will require validation in female mice because of sex-dependent differences in the inflammatory response and contribution of immune cells to stroke outcome.[49]

Collectively, our results highlight the role of platelets and memory T lymphocytes attenuating HT of ischemic stroke by binding platelets through functional PSGL-1, thus identifying a new cellular and molecular target for secondary HT prevention after ischemic stroke.

Acknowledgments

We thank Dr J.M. Jou (Core Facility, Hospital Clinic) for help in blood analyses and Dr B. Hurtado for advice on platelet studies. We thank A. Hernández, C. Castellví, and F. Ruiz for excellent technical assistance. We acknowledge the Cytomics and Image Platforms of Institut d'Investigacions Biomèdiques August Pi i Sunyer for technical help.

Sources of Funding

This study was supported by grants of Spanish Ministerio de Economía y Competitividad (SAF2014-56279R, SAF2017-87459-R to A.M. Planas) and the ISCIII-Subdirección General de Evaluación (FIS PI15/00430 to A. Chamorro) cofinanced by the Fondo Europeo

de Desarrollo Regional (FEDER). M. Gallizioli had a fellowship of the European Innovative Training Networks program (FP7-PEOPLE-2013-ITN-n°07962), F. Miró-Mur had a PERIS award by the Health Department of Generalitat de Catalunya, and Y. Zhao had a scholarship of China Scholarship Council. Part of this work was performed at the Centre de Recerca Biomèdica Cellex, Barcelona. The CERCA (Centres de Recerca de Catalunya) Programme of Generalitat de Catalunya supports Institut d'Investigacions Biomèdiques August Pi i Sunyer. The Centro Nacional de Investigaciones Cardiovasculares (CNIC) is supported by the MECI and the Pro CNIC Foundation and is a Severo Ochoa Center of Excellence (MECI award SEV-2015-0505).

Disclosures

None.

References

1. Fisher M, Saver JL. Future directions of acute ischaemic stroke therapy. *Lancet Neurol*. 2015;14:758–767. doi: 10.1016/S1474-4422(15)00054-X.
2. Fiorelli M, Bastianello S, von Kummer R, del Zoppo GJ, Larrue V, Lesaffre E, Ringleb AP, Lorenzano S, Manelfe C, Bozzao L. Hemorrhagic transformation within 36 hours of a cerebral infarct: relationships with early clinical deterioration and 3-month outcome in the European Cooperative Acute Stroke Study I (ECASS I) cohort. *Stroke*. 1999;30:2280–2284.
3. Terruso V, D'Amelio M, Di Benedetto N, Lupo I, Saia V, Famoso G, Mazzola MA, Aridon P, Sarno C, Ragonese P, Savettieri G. Frequency and determinants for hemorrhagic transformation of cerebral infarction. *Neuroepidemiology*. 2009;33:261–265. doi: 10.1159/000229781.
4. Mosimann PJ, Sirimarco G, Meseguer E, et al. Is intracerebral hemorrhage a time-dependent phenomenon after successful combined intravenous and intra-arterial therapy? *Stroke*. 2013;44:806–808. doi: 10.1161/STROKEAHA.112.675678.
5. Paciaroni M, Agnelli G, Corea F, Ageno W, Alberti A, Lanari A, Caso V, Micheli S, Bertolani L, Venti M, Palmerini F, Biagini S, Comi G, Previdi P, Silvestrelli G. Early hemorrhagic transformation of brain infarction: rate, predictive factors, and influence on clinical outcome: results of a prospective multicenter study. *Stroke*. 2008;39:2249–2256. doi: 10.1161/STROKEAHA.107.510321.
6. Goyal M, Menon BK, van Zwam WH, et al; HERMES Collaborators. Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *Lancet*. 2016;387:1723–1731. doi: 10.1016/S0140-6736(16)00163-X.
7. Zaidat OO, Castonguay AC, Linfante I, et al. First pass effect: a new measure for stroke thrombectomy devices. *Stroke*. 2018;49:660–666. doi: 10.1161/STROKEAHA.117.020315.
8. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, Sharp FR. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab*. 2014;34:185–199. doi: 10.1038/jcbfm.2013.203.
9. Whiteley WN, Slot KB, Fernandes P, Sandercock P, Wardlaw J. Risk factors for intracranial hemorrhage in acute ischemic stroke patients treated with recombinant tissue plasminogen activator: a systematic review and meta-analysis of 55 studies. *Stroke*. 2012;43:2904–2909. doi: 10.1161/STROKEAHA.112.665331.
10. Fagan SC, Nagaraja TN, Fenstermacher JD, Zheng J, Johnson M, Knight RA. Hemorrhagic transformation is related to the duration of occlusion and treatment with tissue plasminogen activator in a nonembolic stroke model. *Neurol Res*. 2003;25:377–382. doi: 10.1179/016164103101201526.
11. Khatri R, McKinney AM, Swenson B, Janardhan V. Blood-brain barrier, reperfusion injury, and hemorrhagic transformation in acute ischemic stroke. *Neurology*. 2012;79(13 suppl 1):S52–S57. doi: 10.1212/WNL.0b013e3182697e70.
12. Krueger M, Bechmann I, Immig K, Reichenbach A, Härtig W, Michalski D. Blood-brain barrier breakdown involves four distinct stages of vascular damage in various models of experimental focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2015;35:292–303. doi: 10.1038/jcbfm.2014.199.
13. Maestrini I, Strbian D, Gautier S, Haapaniemi E, Moulin S, Sairanen T, Dequatre-Ponchelle N, Sibolt G, Cordonnier C, Melkas S, Leys D, Tatlisumak T, Bordet R. Higher neutrophil counts before thrombolysis for cerebral ischemia predict worse outcomes. *Neurology*. 2015;85:1408–1416. doi: 10.1212/WNL.0000000000002029.
14. Prass K, Meisel C, Höflich C, Braun J, Halle E, Wolf T, Ruscher K, Victorov IV, Priller J, Dirnagl U, Volk HD, Meisel A. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med*. 2003;198:725–736. doi: 10.1084/jem.20021098.
15. Urra X, Cervera A, Villamor N, Planas AM, Chamorro A. Harms and benefits of lymphocyte subpopulations in patients with acute stroke. *Neuroscience*. 2009;158:1174–1183. doi: 10.1016/j.neuroscience.2008.06.014.
16. Mracsko E, Liesz A, Karcher S, Zorn M, Bari F, Veltkamp R. Differential effects of sympathetic nervous system and hypothalamic-pituitary-adrenal axis on systemic immune cells after severe experimental stroke. *Brain Behav Immun*. 2014;41:200–209. doi: 10.1016/j.bbi.2014.05.015.
17. Miró-Mur F, Laredo C, Renú A, Rudilosso S, Zhao Y, Amaro S, Llull L, Urra X, Planas AM, Chamorro Á. Adrenal hormones and circulating leukocyte subtypes in stroke patients treated with reperfusion therapy. *Brain Behav Immun*. 2018;53:18–33.
18. Li N. Platelet-lymphocyte cross-talk. *J Leukoc Biol*. 2008;83:1069–1078. doi: 10.1189/jlb.0907615.
19. Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflammation. *Curr Opin Cell Biol*. 2003;15:531–538.
20. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689. doi: 10.1038/nri2156.
21. Li N, Ji Q, Hjemdahl P. Platelet-lymphocyte conjugation differs between lymphocyte subpopulations. *J Thromb Haemost*. 2006;4:874–881. doi: 10.1111/j.1538-7836.2006.01817.x.
22. Blander JM, Visintin I, Janeway CA Jr, Medzhitov R. Alpha(1,3)-fucosyltransferase VII and alpha(2,3)-sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 and migration into inflammatory sites. *J Immunol*. 1999;163:3746–3752.
23. Syrbe U, Jennrich S, Schottelius A, Richter A, Radbruch A, Hamann A. Differential regulation of P-selectin ligand expression in naive versus memory CD4+ T cells: evidence for epigenetic regulation of involved glycosyltransferase genes. *Blood*. 2004;104:3243–3248. doi: 10.1182/blood-2003-09-3047.
24. Weninger W, Ulfman LH, Cheng G, Souchkova N, Quackenbush EJ, Lowe JB, von Andrian UH. Specialized contributions by alpha(1,3)-fucosyltransferase-IV and FucT-VII during leukocyte rolling in dermal microvessels. *Immunity*. 2000;12:665–676.
25. Yilmaz G, Arumugam TV, Stokes KY, Granger DN. Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation*. 2006;113:2105–2112. doi: 10.1161/CIRCULATIONAHA.105.593046.
26. Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G. Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood*. 2010;115:3835–3842. doi: 10.1182/blood-2009-10-249078.
27. Sreeramkumar V, Adrover JM, Ballesteros I, et al. Neutrophils scan for activated platelets to initiate inflammation. *Science*. 2014;346:1234–1238. doi: 10.1126/science.1256478.
28. Nácher M, Blázquez AB, Shao B, Matesanz A, Prophete C, Berin MC, Frenette PS, Hidalgo A. Physiological contribution of CD44 as a ligand for E-selectin during inflammatory T-cell recruitment. *Am J Pathol*. 2011;178:2437–2446. doi: 10.1016/j.ajpath.2011.01.039.
29. de la Rosa X, Cervera A, Kristoffersen AK, Valdés CP, Varma HM, Justicia C, Durduran T, Chamorro Á, Planas AM. Mannose-binding lectin promotes local microvascular thrombosis after transient brain ischemia in mice. *Stroke*. 2014;45:1453–1459.
30. Orsini F, Villa P, Parrella S, et al. Targeting mannose-binding lectin confers long-lasting protection with a surprisingly wide therapeutic window in cerebral ischemia. *Circulation*. 2012;126:1484–1494. doi: 10.1161/CIRCULATIONAHA.112.103051.
31. Sun L, Zhou W, Mueller C, Sommer C, Heiland S, Bauer AT, Marti HH, Veltkamp R. Oxygen therapy reduces secondary hemorrhage after thrombolysis in thromboembolic cerebral ischemia. *J Cereb Blood Flow Metab*. 2010;30:1651–1660. doi: 10.1038/jcbfm.2010.50.
32. Miró-Mur F, Pérez-de-Puig I, Ferrer-Ferrer M, Urra X, Justicia C, Chamorro A, Planas AM. Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation. *Brain Behav Immun*. 2016;53:18–33. doi: 10.1016/j.bbi.2015.08.010.
33. Nieswandt B, Schulte V, Bergmeier W. Flow-cytometric analysis of mouse platelet function. *Methods Mol Biol*. 2004;272:255–268. doi: 10.1385/1-59259-782-3:255.
34. Powers WJ, Rabinstein AA, Ackerson T, et al; American Heart Association Stroke Council. 2018 guidelines for the early management of patients

with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2018;49:e46–e110. doi: 10.1161/STR.0000000000000158.
35. Rodrigues SF, Granger DN. Role of blood cells in ischaemia-reperfusion induced endothelial barrier failure. *Cardiovasc Res*. 2010;87:291–299. doi: 10.1093/cvr/cvq090.
36. Ho-Tin-Noé B, Demers M, Wagner DD. How platelets safeguard vascular integrity. *J Thromb Haemost*. 2011;9(suppl 1):56–65. doi: 10.1111/j.1538-7836.2011.04317.x.
37. Rodrigues SF, Granger DN. Blood cells and endothelial barrier function. *Tissue Barriers*. 2015;3:e978720. doi: 10.4161/21688370.2014.978720.
38. Lalor P, Nash GB. Adhesion of flowing leucocytes to immobilized platelets. *Br J Haematol*. 1995;89:725–732.
39. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol*. 2004;4:325–335. doi: 10.1038/nri1351.
40. Skilbeck C, Westwood SM, Walker PG, David T, Nash GB. Population of the vessel wall by leukocytes binding to P-selectin in a model of disturbed arterial flow. *Arterioscler Thromb Vasc Biol*. 2001;21:1294–1300.
41. Spectre G, Zhu L, Ersoy M, Hjemdahl P, Savion N, Varon D, Li N. Platelets selectively enhance lymphocyte adhesion on subendothelial matrix under arterial flow conditions. *Thromb Haemost*. 2012;108:328–337. doi: 10.1160/TH12-02-0064.
42. Mao L, Li P, Zhu W, Cai W, Liu Z, Wang Y, Luo W, Stetler RA, Leak RK, Yu W, Gao Y, Chen J, Chen G, Hu X. Regulatory T cells ameliorate tissue plasminogen activator-induced brain haemorrhage after stroke. *Brain*. 2017;140:1914–1931. doi: 10.1093/brain/awx111.
43. Angiari S, Rossi B, Piccio L, et al. Regulatory T cells suppress the late phase of the immune response in lymph nodes through P-selectin glycoprotein ligand-1. *J Immunol*. 2013;191:5489–5500. doi: 10.4049/jimmunol.1301235.
44. Gautier S, Ouk T, Petrault O, Caron J, Bordet R. Neutrophils contribute to intracerebral haemorrhages after treatment with recombinant tissue plasminogen activator following cerebral ischaemia. *Br J Pharmacol*. 2009;156:673–679. doi: 10.1111/j.1476-5381.2009.00068.x.
45. Carbone F, Vuilleumier N, Bertolotto M, et al. Treatment with recombinant tissue plasminogen activator (r-TPA) induces neutrophil degranulation *in vitro* via defined pathways. *Vascul Pharmacol*. 2015;64:16–27. doi: 10.1016/j.vph.2014.11.007.
46. Uhl B, Zuchtriegel G, Puhr-Westerheide D, Praetner M, Rehberg M, Fabritius M, Hessenauer M, Holzer M, Khandoga A, Fürst R, Zahler S, Krombach F, Reichel CA. 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.
47. Ducroux C, Di Meglio L, Loyau S, et al. Thrombus neutrophil extracellular traps content impair tPA-induced thrombolysis in acute ischemic stroke. *Stroke*. 2018;49:754–757. doi: 10.1161/STROKEAHA.117.019896.
48. Urra X, Laredo C, Zhao Y, Amaro S, Rudilosso S, Renú A, Prats-Galino A, Planas AM, Oleaga L, Chamorro Á. Neuroanatomical correlates of stroke-associated infection and stroke-induced immunodepression. *Brain Behav Immun*. 2017;60:142–150. doi: 10.1016/j.bbi.2016.10.004.
49. Dotson AL, Wang J, Saugstad J, Murphy SJ, Offner H. Splenectomy reduces infarct volume and neuroinflammation in male but not female mice in experimental stroke. *J Neuroimmunol*. 2015;278:289–298. doi: 10.1016/j.jneuroim.2014.11.020.

Highlights

- Large ischemic brain lesions facilitate hemorrhagic transformation at reperfusion after ischemic stroke.
- Platelets seal leaky blood vessels and prevent hemorrhages secondary to severe brain ischemia/reperfusion.
- Lymphopenia increases the risk of hemorrhagic transformation after severe ischemic stroke.
- Transfer of T cells to lymphopenic mice prevents hemorrhagic transformation after brain ischemia/reperfusion.
- CD4 memory T cells bind platelets through the P-selectin glycoprotein ligand-1/P-selectin axis, and T cells unable to bind P-selectin do not prevent hemorrhagic transformation in lymphopenic mice.