Thromboinflammation in Stroke Brain Damage

Simon F. De Meyer, PhD; Frederik Denorme, MS; Friederike Langhauser, PhD; Eva Geuss, PhD; Felix Fluri, MD; Christoph Kleinschnitz, MD

The main goal of ischemic stroke treatment is rapid recanalization of the occluded blood vessel to limit brain injury and to salvage threatened cerebral tissue. To achieve early vessel recanalization, thrombolysis using recombinant tissue-type plasminogen activator is currently the only approved pharmacological intervention. Only recently, endovascular therapy has made its way into the clinic extending the therapeutic time-window and increasing reperfusion rates. However, despite fast restoration of blood vessel patency, progressive stroke still develops in many patients, which has led to the concept of reperfusion injury. During the past decades, many studies have been addressing the mechanisms underlying ischemic stroke damage and cerebral reperfusion injury, but the picture remains far from complete. It has become clear that both thrombotic and inflammatory pathways are important pathophysiologic contributors to ischemic brain damage. At ischemic vascular lesions, blood platelets adhere and become activated, increasing the risk of secondary thrombotic events. At the same time, cerebral ischemia elicits a strong inflammatory response involving upregulation of cell adhesion molecules and cytokines as well as adhesion, activation, and transmigration of several subsets of leukocytes. Interestingly, emerging insights indicate an important link between these thrombotic and inflammatory pathways in stroke, which led to the concept of thromboinflammation in stroke pathology. In this review, we focus on recently discovered thromboinflammatory pathways of ischemic stroke and discuss the clinical potential of targeting thromboinflammation as a novel treatment strategy in stroke management. An overview of the key components is given in Tables I to III in the online-only Data Supplement.

Collagen–von Willebrand Factor–Glycoprotein Ib Axis

Collagen, von Willebrand factor (vWF), and platelet glycoprotein (GP) Ib together form an important axis that is crucial for initial platelet adhesion at sites of vascular injury. On exposure of the subendothelial matrix, platelets are able to adhere to exposed collagen via their collagen receptors GP VI and integrin αvβ3. However, especially under conditions of high shear rates, platelet adhesion is also dependent on the interaction between the platelet receptor GP Ib and vWF (Figure). vWF is a large, multimeric plasma glycoprotein that is synthesized by endothelial cells and megakaryocytes. Via its A3 domain, vWF is able to bind to exposed fibrillar collagen after which high shear blood forces expose the binding site for platelet GP Ibα in the vWF A1 domain. The vWF–GP Ib binding is reversible and does not produce stable platelet adhesion but rather decelerates platelets under high shear forces, necessary for the definitive arrest to collagen. Importantly, vWF activity is regulated by its size. Ultralarge and therefore highly active vWF multimers are released from storage granules of endothelial cells (Weibel–Palade bodies) and platelets (α-granules) after stimulatory signals. To prevent accumulation of thrombogenic ultralarge vWF, the metalloprotease ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type I repeats-13) rapidly cleaves newly released ultralarge vWF into smaller less thrombogenic multimers. Because of its pivotal role in platelet recruitment, the collagen–vWF–GP Ib axis has become a promising anti-thrombotic target.

Upon platelet adhesion, binding of collagen to GP VI induces platelet activation (Figure). GP VI is a platelet-specific immunoglobulin superfamily receptor that exerts strong signal-transduction on ligation. This results in full platelet activation with release of soluble platelet agonists including adenosine 5-diphosphate, adenosine 5-triphosphate, and thromboxane A2. Platelet activation signaling induces functional upregulation of GP Ibβ/Illa, the most abundant receptor on the platelet surface. Upon activation of the platelet, the GP Ibβ/Illa receptor shifts from an inactive state to an active ligand-binding conformation. Activated GP Ibβ/Illa mediates further platelet aggregation by binding to its primary ligands fibrinogen and the C1 domain of vWF, thereby cross-linking activated platelets and stabilizing the growing platelet plug (Figure).

In recent years, several mouse studies using the transient middle cerebral artery occlusion model have clearly shown the pathogenic involvement of the collagen–vWF–GP Ib axis in stroke.
ischemic stroke progression. Stroke injury was significantly reduced on blocking the interaction between platelet GP VI and collagen. Similarly, when binding of vWF to collagen was prevented, the same protective effect was observed, showing that binding of platelets to collagen, either directly via GP VI or indirectly via vWF, is an important step in ischemic stroke injury. The crucial role of vWF in stroke development was further demonstrated using animals deficient for this adhesion molecule. Indeed, cerebral infarctions were significantly reduced in animals that are completely deficient in vWF. Both endothelial cell–derived and platelet-derived vWF mediate ischemic neurodegeneration. Correspondingly, mice lacking the vWF cleaving enzyme ADAMTS13 presented with worse stroke outcomes, whereas infusion of recombinant ADAMTS13 reduced stroke injury via a vWF-dependent mechanism. Further experimental evidence for the involvement of the collagen–vWF–GP Ib axis in ischemic stroke is provided by studies showing a protective effect on blocking the vWF–GP Ib interaction either by inhibitory antibodies, or by using transgenic mice lacking the extracellular GP Ib domain on platelets, or by a transgenic approach using vWF mutants unable to bind GP Ib. In addition, Elvers et al. showed that mice lacking phospholipase D1, necessary for transduction of activation signals downstream of vWF-occupied GP Ib, are also significantly protected from stroke injury after transient middle cerebral artery occlusion.
In parallel with experimental studies, an increasing amount of clinical data also supports the crucial involvement of the collagen–vWF–GP Ib axis in stroke. The association between high levels of vWF or low levels of ADAMTS13 and ischemic stroke has been well established. Most convincingly, several prospective studies have identified high levels of vWF as a strong predictor of both the risk and severity of ischemic stroke. 

On a genetic level, patients with von Willebrand disease have been reported to have a lower risk for stroke. Likewise, vWF polymorphisms that significantly raise the risk of ischemic stroke have also been found. Intriguingly, not all of these are associated with higher vWF levels, implying that other mechanisms such as increased vWF activity could contribute to the risk of stroke as well. In line with this, polymorphisms in the GPIBA gene that enhance the interaction of GP Ib with vWF have been found to be associated with an increased risk of stroke. Similarly, also single nucleotide polymorphisms in the ADAMTS13 gene are associated with the incidence of ischemic stroke in a Swedish population. 

The thromboinflammatory nature of the collagen–vWF–GP Ib axis in ischemic stroke is an intriguing concept that grew from the observations that initial steps of platelet adhesion, but not later steps of platelet aggregation determine infarct growth after transient middle cerebral artery occlusion. Indeed, targeting platelet aggregation by inhibition of GP IIb/IIIa failed both in murine stroke models and in patients. Thus, processes other than thrombus growth that are mediated by this important platelet adhesion axis may underlie ischemic stroke injury. Interestingly, recent studies showed that vWF and GP Ib are potent mediators of inflammatory processes, in particular, by promoting leukocyte adhesion, rolling, and extravasation. 

vWF-mediated acute cerebral inflammation in ischemic stroke was clearly demonstrated by Khan et al and also recent studies in mouse models of myocardial infarction showed a thromboinflammatory role of the vWF axis in the pathophysiology of ischemia/reperfusion. Likewise, increasing evidence shows that GP VI also contributes to inflammatory processes by inducing pro-inflammatory platelet microparticle formation, by enhancing neutrophil damaging activities, or by inducing platelet activation and thus secretion of platelet-derived inflammatory mediators. Indeed, activated platelets are increasingly recognized to participate in acute inflammatory reactions by secreting potent immune mediators including interleukin-1α, interleukin-1β, transforming growth factor-β, histamine, serotonin, and soluble CD40 ligand (CD40L), some of which were already shown to mediate cerebral ischemia injury in animal models. CD40 and CD40L are expressed by not only platelets but also endothelial cells, and several leukocyte subtypes and CD40/CD40L interactions consequently mediate complex inflammatory cell–cell interactions. In addition, platelet CD40L triggers inflammatory activation of endothelial cells, thereby upregulating E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1. In addition, platelet components such as polyphosphates and GP Ib also interact with coagulation factor XII (FXII), thereby initiating not only the intrinsic coagulation pathway but also inflammation through activation of the kallikrein–kinin system. Hence, primary platelet adhesion and subsequent platelet activation promote both thrombosis and inflammation that together aggravate ischemic stroke injury.

**Contact-Kinin Pathway**

The contact-kinin pathway plays an important role in the thromboinflammatory pathology of ischemic stroke not only by fostering vascular permeability and inflammation via kinins such as bradykinin but also by promoting thrombus formation through activation of the intrinsic pathway (also known as the contact pathway; Figure). The contact-kinin pathway is initiated on activation of FXII (generating activated FXII [FXIIa]) via contact with a negatively charged surface or negatively charged inorganic polyphosphates released from activated platelets. Apart from triggering the intrinsic coagulation cascade via activation of FXI, FXIIa also cleaves plasmakallikrein to form plasmakallikrein, which in turn cleaves high molecular weight kininogen, inducing the release of the proinflammatory hormone bradykinin from high molecular weight kininogen. Plasmakallikrein can also activate FXII, generating a positive feedback loop.

Because deficiency of FXII in patients is not associated with an overt bleeding phenotype, the contact pathway was originally thought to be dispensable for physiological hemostasis. The generation of FXII-deficient mice, however, challenged this dogma as these mice have severely impaired thrombus formation by generating unstable thrombi. Intriguingly, these mice were also significantly protected from ischemic stroke without increasing intracranial hemorrhages. Analogous observations were obtained upon pharmacological inhibition of FXII by a selective inhibitor rHA-Infestin4. The absence of downstream factors of FXII, such as FXI or FIX, similarly protected mice from stroke. These studies clearly pointed out the central pathophysiological role of the intrinsic coagulation pathway in brain ischemia/reperfusion injury. From a mechanistic point of view, reduced thrombus formation and fibrin deposits in the cerebral microvasculature of FXII-deficient mice led to better reperfusion rates, most probably attributable to enhanced clearance of unstable thrombi from the cerebral microvasculature after reopening of large proximal vessels.

As described above, FXIIa not only initiates coagulation but also triggers inflammation through the activation of the kallikrein–kinin system. The involvement of this system in experimental stroke was recently demonstrated as both genetic and pharmacological inhibition of plasmakallikrein protected mice from ischemic stroke without an increase in infarct-associated hemorrhage. Plasmakallikrein deficiency led to reduced intracerebral thrombosis, enhanced cerebral blood flow, maintenance of the blood–brain barrier, and reduced local inflammation. Thus, by targeting both coagulation and inflammation, plasmakallikrein inhibition may offer a safe strategy for stroke. Also downstream of plasmakallikrein, absence of high molecular weight kininogen has been shown to be protective after transient middle cerebral artery occlusion. Indeed, Langhauser et al recently identified high molecular weight kininogen as key mediator of experimental
ischemic stroke by enhancing microvascular thrombosis, blood–brain barrier leakage, and inflammation.

Further downstream, the cellular effects of kinins are mediated by 2 different bradykinin receptors B1R and B2R, which on activation trigger inflammatory processes in the target organ. Although B2R is constitutively expressed, B1R is expressed at low baseline levels but induced selectively during injury. Inhibition of B1R but not of B2R protects against ischemic brain damage and is associated with less edema formation and attenuation of the local posts ischemic inflammatory response.47

An interesting plasma glycoprotein that could be of therapeutic interest is the serpin, C1 inhibitor. C1 inhibitor tackles both sides of the contact-kinin system by inhibiting FXIIa and plasmaskallikrein and was shown to protect rodents from reversible brain ischemia in several clinically relevant scenarios by its combined anti-inflammatory and antithrombotic mode of action.48

In support of the experimental data, the contact-kinin pathway has been shown to be activated in a small cohort of patients with stroke.49 Results on FXII levels are less clear; however, FXI levels are higher in patients with stroke and are associated with poor prognosis.50–53 In addition, inhibitors of the plasma kallikrein–kinin system and FXII specifically are negatively associated with stroke risk.54 Contact system deficiency is rare in humans and to date no controlled clinical trial has correlated contact system deficiency with thrombotic disease. For FXII, relationships of polymorphisms with thrombotic outcomes have been conflicting.55 However, the deficiency of FXI, the prime substrate of FXIIa, protected from stroke in a Jewish population.56 Also tree polymorphisms are associated with poor prognosis.50–53 In addition, inhibitors FXI levels are higher in patients with stroke and thrombotic outcomes have been conflicting.55 However, the deficiency of FXI, the prime substrate of FXIIa, protects against ischemic brain damage and is associated with less edema formation and attenuation of the local posts ischemic inflammatory response.47

Inflammatory Cells

Brain ischemia is followed by the activation of the immune system in a sterile inflammatory reaction, resulting in the upregulation of cell adhesion molecules, cytokines, and chemotactants, as well as infiltration of leukocytes in the ischemic tissue (Figure). In the early phase of cerebral ischemia, leukocytes are recruited by cell adhesion molecules expressed on endothelial cells and infiltration into the parenchyma occurs at later stages. Recruitment of leukocytes, including neutrophils, monocytes, and lymphocytes, occurs in successive waves, which significantly influence the pathogenesis of ischemic brain injury.

Neutrophils are among the first cells that respond to a cerebral ischemic insult, but their exact role in stroke pathology is still not totally understood. Although neutrophil phagocytic activity may help in removing necrotic cell debris and tissue healing, the destructive nature of neutrophils is causing collateral tissue destruction, disruption of the blood–brain barrier, and cerebral edema.58 Neutrophils contribute to cerebral injury by releasing proteolytic enzymes and free radicals that have direct neurotoxic activity. In addition, intravascular accumulation of neutrophils impairs local blood flow leading to the no-reflow phenomenon of the affected microcirculation.59

Interestingly, evidence is accumulating that neutrophils, apart from their well-known inflammatory function, also promote intravascular thrombus formation. Neutrophils have been shown to be an essential source of tissue factor in the early phase of thrombus formation (Figure).60,61 Interaction of neutrophils with injured endothelial cells, through lymphocyte function–associated antigen-1/intercellular adhesion molecule-1 interactions, leads to fibrin and thrombus formation by providing tissue factor before platelet accumulation.62 Tissue factor, together with coagulation factor VIIa, initiates the extrinsic coagulation pathway. Neutrophils also release proteases such as cathepsin G and elastase that act on coagulation factors, promoting clot formation.63,64 Inhibition of cathepsin G decreases thrombus formation, thereby reducing brain injury and improving neurobehavioral outcome in a mouse model of ischemic stroke.64 Also platelets interact with neutrophils, and platelet–neutrophil complexes were shown to be increased in patients with ischemic stroke.52 Ligands of the neutrophil integrin Mac-1 include fibrinogen, vWF, and GP Ib, and the contribution of these interactions in stroke still needs to be investigated. Furthermore, platelet P-selectin binds to P-selectin glycoprotein ligand-1 on neutrophils, which leads to activation of neutrophils, inducing the release of neutrophil proteases. In an elegant study, Sreeramkumar et al65 recently demonstrated that this interaction aggravates ischemic brain damage via P-selectin glycoprotein ligand-1 clusters that scan for the presence of activated platelets before neutrophils trigger inflammation and potentially cause thromboinflammatory injury. Additional evidence for the contribution of neutrophils to thrombus formation comes from the prothrombotic activity of neutrophil extracellular DNA traps.66–68 These neutrophil extracellular DNA traps have been shown to bind platelets, red blood cells, and provide a matrix for the activation of the coagulation contact pathway.69,70 Signs of neutrophil extracellular DNA trap formation were recently described in experimental stroke,68 and the DNA-degrading enzyme DNase-I was shown to reduce ischemic brain injury.69 Additional studies are needed to assess the potentially adverse prothrombotic effects of neutrophils in cerebral ischemia and reperfusion injury to better understand their thromboinflammatory role in stroke.

Recently T cells, which belong to the adaptive immune system, have received a lot of attention in ischemic stroke as novel evidence suggests that T cells contribute critically to stroke development. Rag knockout mice lacking functional T cells are less susceptible to ischemic neurodegeneration.70,71 In particular, regulatory T cells (T reg) play a detrimental role in stroke although also protective effects have been attributed to this intriguing cell population.72 We showed that T reg are prone to interact with endothelial cells via intercellular adhesion molecule-1 (expressed on endothelial cells) and lymphocyte function–associated antigen-1 (expressed on T cells) causing increased thrombus formation, impaired cerebral reperfusion and microvascular dysfunction, which finally triggers secondary infarct growth in the acute phase after experimental ischemic stroke (Figure).73 Depletion of platelets prevented T reg–driven infarct progression, underlining the thromboinflammatory role of T reg in the acute phase of ischemic brain injury.73 Correspondingly, pharmacological expansion of T reg enhanced thromboinflammation and ischemic neurodegeneration.74 However, it has been reported that T reg...
induce cerebroprotection in the later phases of brain ischemia in mice, and further studies are needed to fully elucidate the role $T_{reg}$ in the different stages of ischemic stroke.75

**Thromboinflammation in Clinical Stroke**

Albeit the concept of thromboinflammation is meanwhile well established on a preclinical basis, its relevance in patients with stroke is less clear. Lukasik et al76 investigated the efficacy of acetylsalicylic acid (ASA; aspirin, 150 mg daily) on platelet-related inflammatory factors, namely P-selectin and CD40L in both patients with acute ischemic stroke and healthy volunteers. Although the study yielded evidence for a hyperactivation of platelets in the acute stage of cerebral ischemia, the platelet α-granule–derived inflammatory mediators and monocyte–platelet aggregates were only reduced in healthy individuals but not in stroke subjects on ASA.76 Similarly, Chronos et al77 found that ASA does not inhibit ADP- or thrombin-induced platelet α-granule secretion, and another study revealed that ASA treatment is unable to attenuate either resting P-selectin expression or leukocyte–platelet aggregate formation.78

Clopidogrel is another platelet inhibitor commonly used in secondary stroke prevention. This second-generation thienopyridine binds to the platelet P2Y12 receptor, which results in a reduced release of proinflammatory mediators from platelet α-granules such as soluble P-selectin and CD40L and, therefore, in attenuated platelet–leukocyte interactions.79 Clopidogrel, in combination with ASA, significantly decreased serum levels of tumor necrosis factor-α and C-reactive protein when compared with ASA alone in patients with acute coronary syndrome,80 but whether this holds true also in ischemic stroke remains unclear.

Another receptor possibly involved in platelet-mediated inflammatory processes is GP IIb/IIIa. There is some evidence that GP IIb/IIIa receptors can be acquired by neutrophils via platelet-derived microparticles.81 Acquired GP IIb/IIIa receptors colocalize with β2-integrins and cooperate with nuclear factor-κB activation, implicating that GP IIb/IIIa receptors mediate neutrophil-induced inflammation. Interestingly, GP IIb/IIIa receptor inhibitors, such as abciximab, epifibatide, and tirofiban, have been shown to prevent nuclear factor-κB activation.81 However, a recent Cochrane meta-analysis including the Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II) study and the Study of Efficacy of Tirofiban in acute Ischemic Stroke (SETIS) showed that GP IIb/IIIa inhibitors are associated with a significant risk of intracerebral hemorrhage with no evidence of any reduction in death or neurological symptoms in patients with acute stroke.82 Although the exact reasons for this unfavorable risk-to-benefit profile are unclear, it might be attributed to the release of prothrombotic and proinflammatory platelet CD40 ligand from subthreshold GP IIb/IIIa inhibition.83

Apart from novel compounds that in the first place counteract thrombus formation, there are several interesting studies on novel immunomodulatory agents in acute stroke therapy. Recently, Fu et al84 reported that oral fingolimod, a compound that sequesters circulating lymphocytes within their lymphoid organs and that is approved for the treatment of relapsing-remitting multiple sclerosis, administered within 72 hours of stroke onset was safe, limited secondary tissue injury from baseline to 7 days, decreased microvascular permeability, and attenuated neurological deficits. In addition, combination of fingolimod together with alteplase attenuated reperfusion injury and improved clinical outcomes in patients with stroke in another pilot study.85 These clinical findings are in good accordance with the results from stroke models. Here, fingolimod lowered the amount of circulating T cells within the brain vasculature, thereby reducing the frequency of detrimental T-cell–platelet and T-cell–endothelium interactions.86 Blocking of T-cell transmigration might be another promising approach to counteract brain damage in stroke. Although blocking of very late antigen-4 expressed on T cells produced conflicting results in preclinical stroke models,87–91 a phase II clinical trial testing a monoclonal antibody directed against very late antigen-4, that is, natalizumab in the acute phase of stroke has been completed in April 2015 and publication of the results is expected soon (Effect of natalizumab on infarct volume in acute ischemic stroke (ACTION); https://clinicaltrials.gov/ct2/show/NCT01955707?term=natalizumab&rank=36).

Given the central role of the collagen–vWF–GP Ib axis in ischemic stroke models, pharmacological inhibition of vWF-mediated platelet adhesion could become a promising strategy in stroke treatment.5 A promising class of novel inhibitors targets vWF function by blocking the binding of platelet GP Ib to vWF, such as the aptamer ARC1779 and the nanobody caplacizumab. Both compounds significantly improved disease progression in patients with thrombotic thrombocytopenic purpura.92,93 Interestingly, in patients undergoing carotid endarterectomy, administration of ARC1779 reduced cerebral emboli signals.94 Recombinant ADAMTS13 (BAX930) limits vWF function by cleaving the thrombogenic ultralarge multimers in smaller, less reactive ones. Currently, participants are being recruited for a phase 1 study to evaluate the safety of recombinant ADAMTS13 in thrombotic thrombocytopenic purpura (https://clinicaltrials.gov/ct2/show/NCT02216084). No clinical data on the use of these vWF inhibitors in ischemic stroke are available yet, but it will be interesting to follow their further clinical development and in particular their potential antithromboinflammatory effects in ischemic stroke injury.

**Conclusions**

There is now ample evidence that both thrombotic and inflammatory processes are highly intertwined in the complex pathophysiology of brain ischemia. Common pathways of thrombus formation and inflammation contribute to cerebral injury in stroke and therefore form an attractive target for the development of novel antithromboinflammatory therapeutics. The currently emerging insights on thromboinflammation in stroke are only the tip of the iceberg but form a solid basis for further exploration of the critical interface between inflammation and thrombosis in the ischemic and reperfused brain. These intriguing data call for further evaluation, especially in humans, of the potential benefits of reducing thromboinflammation in stroke and other cardiovascular diseases.
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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Thrombo-inflammation in stroke brain damage
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### Supplemental table I: Evidence for the involvement of the collagen-vWF-GPIb axis in thrombo-inflammation in experimental stroke

<table>
<thead>
<tr>
<th>Target</th>
<th>Challenge</th>
<th>Stroke model (stroke assessment)</th>
<th>Species</th>
<th>Stroke phenotype</th>
<th>Mechanism</th>
<th>ICH risk</th>
<th>References</th>
</tr>
</thead>
</table>
| vWF              | vWF−−                                            | 60 or 120 min tMCAO (day 1 to day 7) | Mouse   | Beneficial       | ▪ Prevention of platelet adhesion  
▪ Blockade of collagen or GPIbα binding to vWF  
▪ Reduction of post-ischemic inflammation | No       | 1-4        |
| GPIb             | GPIbα-deficiency, anti-GPIbα Fab, Anfibatide      | 60 or 90 min tMCAO (day 1 to day 7) | Mouse   | Beneficial       | ▪ Prevention of Platelet adhesion/tethering  
▪ Increase of cortical perfusion | No       | 5-8        |
| GPIIb/IIIa       | anti-GPIIb/IIIa (Fab)2                            | 60 min tMCAO (day 1)              | Mouse   | Detrimental/Neutral | ▪ Inhibition of platelet aggregation | Yes      | 3,6        |
| GPVI             | anti-GPVI mAb, Revacept (GPVI-Fc fusion protein)  | 60 min tMCAO (day 1 to day 7)     | Mouse   | Beneficial       | ▪ Prevention of platelet adhesion  
▪ Prevention of integrin activation/secretion  
▪ Blockade of vWF-collagen interaction | No       | 3,6,9      |
| ADAMTS13         | Adams13−−                                         | 30, 60 or 120 min tMCAO (day 1)   | Mouse   | Detrimental      | ▪ Accumulation of UL-vWF  
▪ Increase in post-ischemic inflammation  
▪ Post-ischemic hypoperfusion | No       | 2,4,10     |
| ADAMTS13         | recombinant ADAMTS13                             | 120 min tMCAO (day 1)             | Mouse   | Beneficial       | ▪ Prevention of platelet adhesion | No       | 2          |
| PLD1/2           | Pld1−−, Pld2−−, small molecule PLD1 inhibitor    | 60 min tMCAO (day 1)              | Mouse   | Beneficial       | ▪ Prevention of platelet activation/signaling  
▪ Prevention of platelet α-granule release  
▪ Reduction of thrombus formation | No       | 11-13      |

Fab, Fab fragment; GP, glycoprotein; ICH, intracranial hemorrhage; mAb, monoclonal antibody; tMCAO, transient middle cerebral artery occlusion; PLD, phospholipase D1, (UL-) vWF, (ultralarge) von Willebrand factor.
### Supplemental table II: Evidence for the involvement of the contact-kinin pathway in thrombo-inflammation in experimental stroke

<table>
<thead>
<tr>
<th>Target</th>
<th>Challenge (genetic modification, pharmacological intervention)</th>
<th>Stroke model (stroke assessment)</th>
<th>Species</th>
<th>Stroke phenotype</th>
<th>Mechanism</th>
<th>ICH risk</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXII/FXIIa</td>
<td><strong>F12</strong>&lt;sup&gt;-/-&lt;/sup&gt;, inhibition by rHA-infestin-4, PCK or C1-INH</td>
<td>60 or 90 min tMCAO (day 1 to 7)</td>
<td>Mouse and rat</td>
<td>Beneficial</td>
<td>▪ Prevention of thrombus formation ▪ Enhancement of cortical reperfusion ▪ Attenuation of inflammation</td>
<td>No</td>
<td>14-17</td>
</tr>
<tr>
<td>FXI/FXIa</td>
<td><strong>F11</strong>&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>60 min tMCAO (day 1)</td>
<td>Mouse</td>
<td>Beneficial</td>
<td>▪ Prevention of thrombus formation</td>
<td>NA</td>
<td>14</td>
</tr>
<tr>
<td>KNG</td>
<td><strong>Kng</strong>&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>60 min tMCAO (day 1 to 7), pMCAO</td>
<td>Mouse</td>
<td>Beneficial</td>
<td>▪ Decrease in microvascular thrombosis ▪ Reduction in edema formation ▪ Attenuation of inflammation</td>
<td>No</td>
<td>18</td>
</tr>
<tr>
<td>PK</td>
<td><strong>Pk</strong>&lt;sup&gt;-/-&lt;/sup&gt;; inhibition by C1-INH, anti-PK pAb, DX-88</td>
<td>30/60 min tMCAO (day 1 to 7), pMCAO (day 1)</td>
<td>Mouse and rat</td>
<td>Beneficial</td>
<td>▪ Prevention of intracerebral thrombosis ▪ Enhancement of cerebral blood flow ▪ Reduction of edema formation ▪ Decrease in inflammation</td>
<td>No</td>
<td>17,19,20</td>
</tr>
<tr>
<td>B1R</td>
<td><strong>B1r</strong>&lt;sup&gt;-/-&lt;/sup&gt;, inhibition by R-715</td>
<td>60 min tMCAO (day 1 to day 5)</td>
<td>Mouse</td>
<td>Beneficial</td>
<td>▪ Reduction in edema formation ▪ Attenuation of inflammation</td>
<td>No</td>
<td>21</td>
</tr>
<tr>
<td>B2R</td>
<td><strong>B2r</strong>&lt;sup&gt;-/-&lt;/sup&gt;, inhibition by Hoe-140</td>
<td>60 min tMCAO (day to day 5)</td>
<td>Mouse</td>
<td>Neutral</td>
<td>/</td>
<td>No</td>
<td>21</td>
</tr>
</tbody>
</table>

B1R, bradykinin receptor B1; B2R, bradykinin receptor B2; C1-INH, C1-inhibitor; FXII/XI(a), (activated) factor XII/XI; ICH, intracranial hemorrhage; KNG, kininogen; NA, not assessed; pAb, polyclonal antibody; pMCAO, permanent middle cerebral artery occlusion; PCK, H-D-Pro-Phe-Arg-chloromethylketone; PK, plasmakallikrein;; rHA-infestin-4, recombinant human albumin-infestin-4; tMCAO, transient middle cerebral artery occlusion;
<table>
<thead>
<tr>
<th>Target</th>
<th>Challenge</th>
<th>Stroke model</th>
<th>Species</th>
<th>Stroke phenotype</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
</table>
| Neutrophils (general aspects) | ▪ Targeting neutrophil activation/recruitment (e.g. inhibition of CXCR-1/2; inhibition of IL1β/TNFα)  
▪ Targeting neutrophil endothelial adhesion/ transmigration/neurovascular interactions (e.g. ICAM-1/MAC-1/PECAM-1/PSGL-1)  
▪ Targeting neutrophil mediated blood–brain barrier damage (e.g. MMP-9/CEACAM-1) |              |         |                 | ▪ Reduction in edema formation  
▪ Attenuation of brain injury  
▪ Improvement of local cerebral blood flow (prevention of “no-reflow” phenomenon)                                                                 | Summarized in 22 |
| Neutrophils (thrombo-inflammatory aspects) | Cathepsin G deficiency (CatG−/−), inhibition of Cathepsin G  
PSLG-1 (anti-PSGL-1)  
Mac-1 (Mac-1−/−)  
DNase-1 (recombinant human DNase-1) | 60 min tMCAO (day 2)  
cMCAO (day 1 and 2)  
60 min tMCAO (day 1)  
60 min tMCAO (day 1) | Mouse  
Mouse  
Mouse  
Mouse | Beneficial  
Beneficial  
Beneficial  
Beneficial | ▪ Improvement of cerebral blood flow  
▪ Reduction of thrombus formation  
▪ Inhibition of platelet neutrophil interaction  
▪ Reduced platelet adhesion  
▪ Reduced leucocyte adhesion  
▪ Prevention of NET formation | 23  
24  
25  
26 |
| T cells  
Inhibition of VLA4-VCAM-1 interaction (anti-CD49d mAb) | T cell deficient mice (Rag1−/−)  
FTY-720 | 60 min tMCAO (day 1 to 7)  
60 and 90 min tMCAO (day 1 and 3)  
30 min tMCAO (day 1 to day 7)  
60 min tMCAO (day 1 to 4) | Mouse  
Mouse  
Mouse  
Mouse | Beneficial  
Beneficial  
Neutral  
Neutral/Beneficial | ▪ Antigen-independent effect  
▪ Sequestration of circulating T lymphocytes  
▪ Reduction of microvascular thrombosis  
▪ Improvement of cerebral blood flow  
▪ No impact on immune cell infiltration, platelet function, edema formation  
▪ Reduction of immune cell infiltration  
▪ Upregulation of VCAM-1  
▪ Reduction of leukocyte invasion (only in cMCAO) | 27,28  
29  
30  
31 |
<table>
<thead>
<tr>
<th>Regulatory T cells</th>
<th>cMCAO (day 1 to 7)</th>
<th>cMCAO)</th>
<th></th>
<th></th>
</tr>
</thead>
</table>
| LFA-1 (Lfa-1<sup>-/-</sup>) | 60 min tMCAO (day 1) | Mouse | Beneficial | ▪ Reduction of platelet adhesion  
▪ Reduction of leukocyte adhesion |
| Treg depletion (Foxp3 deficiency or anti-CD25) | 60 min tMCAO (day 1 to 7) | Mouse | Beneficial | ▪ Preservation of the cerebral vasculature  
▪ No immunomodulatory function |
| | 30 to 90 min tMCAO (day 1 to 7)  
cMCAO (day 1 to 7) | Mouse and rat | Neutral (only in tMCAO)  
/Detrimental (only in cMCAO) | ▪ Increase in neuroinflammatory biomarkers  
▪ Increase in immuno-inflammatory biomarkers (only in detrimental stroke phenotype) |
| | 30 and 60 min tMCAO (day 1 to 3) | Mouse | Detrimental | ▪ Increase in immune cell accumulation  
▪ Increased thrombus formation |
| Treg expansion (adoptive transfer,  
HDACi or CD28SA) | 30 to 90 min tMCAO (day 1 to 28)  
cMCAO (day 1 to 7) | Mouse and rat | Beneficial | ▪ Reduction of inflammatory biomarkers (only in cMCAO) |

CD28SA, CD28 super agonist; CEACAM-1, Carcinoembryonic antigen-related cell adhesion molecule-1; cMCAO, coagulation middle cerebral artery occlusion; CXCR-1, CXC-Motiv-Chemokinrezeptor-1; CXCR-2, CXC-Motiv-Chemokinrezeptor-2; FoxP3, Forkhead-Box-Protein P3; LFA-1, Lymphocyte function-associated antigen-1; HDACi, histone deacetylase inhibition; ICAM-1, Intercellular adhesion molecule-1; MAC-1, Macrophage-1 antigen; MMP-9, mAb, monoclonal antibody; MMP-9, matrix metalloproteinase-9; PECAM-1, Platelet endothelial cell adhesion molecule; PSLG-1, P-selectin glycoprotein ligand-1; Rag1, Recombination activating gene-1; tMCAO, transient middle cerebral artery occlusion; SA, super agonist; VCAM-1, Vascular cell adhesion molecule-1; VLA-4, very late antigen-4
References


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