Stroke

FOCUSED UPDATES: STROKE NEUROIMMUNOLOGY

Thromboinflammation in Brain Ischemia: Recent Updates and Future Perspectives

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ABSTRACT: Despite decades of promising preclinical validation and clinical translation, ischemic stroke still remains as one of the leading causes of death and disability worldwide. Within its complex pathophysiological signatures, thrombosis and inflammation, that is, thromboinflammation, are highly interconnected processes leading to cerebral vessel occlusion, inflammatory responses, and severe neuronal damage following the ischemic event. Hence, we here review the most recent updates on thromboinflammatory-dependent mediators relevant after stroke focusing on recent discoveries on platelet modulation, a potential regulation of the innate and adaptive immune system in thromboinflammation, utterly providing a thorough up-to-date overview of all therapeutic approaches currently undergoing clinical trial.

Key Words: inflammation ■ ischemic stroke ■ microglia ■ thromboinflammation ■ thrombosis

Current therapeutic options for patients with stroke primarily focus on vessel recanalization through pharmacological lysis or mechanical removal of the occlusion. However, despite continuous improvements in stroke care, ≈80% of all patients reaching the clinics are not eligible to undergo pharmacological or mechanical recanalization procedures, emphasizing the current need to improve stroke outcomes.[1] Thromboinflammation, a process referring to the complex interplay between both thrombotic and inflammatory pathways, has become increasingly recognized as an important contributor to poststroke damage. In this review, we provide an update on 3 key thromboinflammatory pathways described previously.[2] First, the thromboinflammatory activity of platelets and their key receptors and ligands in stroke brain damage will be discussed. Second, the contact kinin system, together with platelets, also promotes both coagulation and inflammation and thus forms an interesting therapeutic as well. Third, the thromboinflammatory micro-environment leads to recruitment of peripheral leukocytes, which can further exacerbate the pathology of cerebral ischemia via both thrombotic and inflammatory mechanisms. Hence, we here highlight the function of the innate immune system upon brain ischemia also providing a broad overview of most relevant rodent transgenic lines, thus encouraging novel research perspectives and further preclinical investigation. Additionally, we present a complete, descriptive, and up-to-date overview of all therapies currently under clinical assessment. Thus, this article thoroughly revises the latest discoveries in the thromboinflammation field and its current potential to reach clinical translation and ultimately patient benefit.

See related article, p 1432, 1438, 1449, 1460, 1473, 1500

PLATELETS

Current insights suggest that initial platelet adhesion and early platelet activation rather than platelet aggregation regulate thromboinflammatory damage in ischemic stroke. The main receptors mediating platelet adhesion are the GP (glycoprotein) VI and integrin $\alpha_2\beta_1$, both binding to collagen and the GPIbα subunit of the GPIb-IX-V complex, which interacts with the VWF (von Willebrand Factor). VWF is synthesized in endothelial cells or megakaryocytes being either constitutively secreted into the blood or stored in endothelial Weibel-Palade bodies and platelet α-granules. Upon immobilization at sites of endothelial damage or activation, VWF unfolds due to high shear forces, leading to

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Nonstandard Abbreviations and Acronyms

B1R	bradykinin receptor 1
B2R	bradykinin receptor 2
BBB	blood-brain barrier
FXI	factor XI
FXII	factor XII
GP	glycoprotein
IFNγ	interferon-γ
IL	interleukin
MC	mast cells
MDM	monocyte-derived macrophages
MMP	matrix metalloprotease
MS	multiple sclerosis
NE	neutrophil elastase
NET	neutrophil extracellular traps
NK	natural killer
PK	plasmakallikrein
PSGL	P-selectin-glycoprotein ligands
TF	tissue factor
tMCAO	transient occlusion of the middle cerebral artery
tPA	tissue-type plasminogen activator
VWF	von Willebrand Factor

exposure of the GPIbα binding site in the VWF A1 domain. The VWF A1-GPIbα interaction is reversible, permitting platelets to roll and decelerate on immobilized VWF, therefore, facilitating contact of platelets with the exposed subendothelial matrix and engagement of their collagen receptors. The GPIbα-VWF and GPVI/α2β1-collagen interactions induce downstream intracellular platelet signaling, resulting in platelet activation and the release of secondary mediators such as adenosine 5-diphosphate, adenosine 5-triphosphate, and thromboxane A_2. Additionally, platelet activation also leads to a conformational change of GPIIb/IIIa (glycoprotein IIb/IIa), the most abundant receptor on the platelet surface. This change from an inactive to a high-affinity state allows binding of GPIIb/IIIa to its ligands, mainly fibrinogen and VWF, resulting in platelet aggregation. Importantly, besides immobilized VWF, GPIbα interacts with multiple other ligands such as P-selectin and macrophage antigen-1 on leukocytes. These platelet-leukocyte interactions, potentially together with direct VWF-leukocyte interactions, could also become particularly relevant in orchestrating the thromboinflammatory process in stroke.

Mouse studies showed that absence of VWF leads to a significant reduction in ischemic stroke brain injury and improved functional outcome after transient occlusion of the middle cerebral artery (tMCAO) without signs of intracranial hemorrhage.[3,4] Restoration of VWF in either plasma[3,5] or the platelet compartment[6] is sufficient to reverse the protection observed in VWF-deficient animals, illustrating the pathophysiological activity of VWF in ischemic stroke irrespective of its cellular origin. Similarly, mice lacking CD69, a negative regulator of endothelial VWF release, developed worse brain damage after ischemic stroke.[7] Additionally, deficiency of the VWF-cleaving enzyme ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), an important metalloprotease that limits VWF activity, leads to worse stroke outcomes after tMCAO, whereas infusion of recombinant ADAMTS13 limits infarct growth.[4,8–10] Reconstitution of VWF-deficient animals with different mutants of VWF via hydrodynamic gene transfer revealed a pathophysiological role for the VWF A1 domain (binding to GPIbα), and the VWF A3 domain (binding to collagen) but not the VWF C4 domain (binding to GPIIb/IIIa).[5]

The VWF A1-GPIbα axis is considered a novel pharmacological target to limit thromboinflammation in the stroke brain.[11] The protective effect of blocking this interaction in experimental stroke has been extensively described using several strategies, including (1) the usage of Fab fragments of the p0p/B monoclonal antibody against GPIbα,[6,12–15] (2) the snake venom–derived GPIbα antagonist anfibatide,[16–18] (3) an anti-VWF nanobody that blocks GPIbα binding to the A1 domain,[19] or (4) the use of transgenic GPIbα/IL4Rα (interleukin-4 receptor alpha) mice.[20] In fact, the protective effect of blocking GPIbα was maintained in aged and comorbid, that is, atherosclerotic, diabetic, and hypertensive animals, without increasing bleeding complications.[15] These protective benefits have been directly associated with a reduction in both thrombotic and inflammatory pathways, leading to improved vessel patency and tissue perfusion.[14,21] Indeed, VWF-deficient mice showed less thrombosis in the cerebral microvasculature[5,6,22] while ADAMTS13 absence resulted in an increased number of thrombi in the brain lesions after stroke.[9] Anti-GPIbα treatment reduced the number of fibrin(ogen)-positive blood vessels and microthrombi and significantly reduced thrombus burden in the affected cerebral microvasculature.[12,16,18] Furthermore, VWF deficiency is associated with reduced neutrophil infiltration and reduced expression levels of the proinflammatory cytokines IL-6, IL-1β, and tumor necrosis factor-α in the ischemic hemisphere,[8,22] whereas an increase of these inflammatory responses is observed in of ADAMTS13-deficient mice.[8,9,23] Similarly, inhibition of the VWF A1 domain also significantly reduced the recruitment of monocytes, neutrophils, and T cells in the ischemic brain.[19] Blockade of GPIbα equally led to decreased expression of IL-6, IL-1β, and tumor necrosis factor-α.[12,16,17]

Besides the VWF-GPIbα axis, platelet GPVI has also become a promising platelet target to limit thromboinflammation in ischemic stroke. Engagement of GPVI induces potent platelet activation. Thus, GPVI depletion on mouse platelets led to a significant reduction of infarct volumes without increasing the risk of intracranial

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hemorrhage, even in combination with r-tPA (recombinant tissue-type plasminogen activator).[13,15,24] Mice suffering from defective signaling downstream of GPVI showed direct protection against severe stroke brain injury.[25–28] Conversely, augmentation of GPVI signaling increased infarct volumes and worsened neurological deficits.[29] GPVI thus mediates reperfusion injury, most likely by promoting platelet adhesion and platelet activation.

Remarkably, whereas thrombus formation requires both platelet adhesion via GPIbα and GPVI, and platelet aggregation via GPIIb/IIIa, the latter seems to play a minor role in acute stroke injury. Indeed, targeting platelet aggregation by inhibition of GPIIb/IIIa failed both in murine models[13,15] and patients[30] being associated with a significant increase of bleeding risk. Interestingly, procoagulant platelets are a specific subset of platelets with a particular phenotype that includes a high degree of externalized phosphatidylserine, inactivation of GPIIb/IIIa, and an ability to generate microparticles, being elevated in patients with stroke.[31] In fact, mice with platelets lacking the ability to become procoagulant were protected from brain injury upon stroke, an effect that was partly attributed to detrimental platelet-neutrophil interactions.[32] Thus, lowering the levels of procoagulant platelets with recombinant AnnexinA1 reduced platelet-neutrophil interactions and improved ischemic stroke outcomes in mice without increasing the bleeding risk.[33] Hence, specific targeting of procoagulant platelets could become a novel way to interfere with platelet-mediated thromboinflammation (Figure 1).

CONTACT-KININ PATHWAY IN THROMBOINFLAMMATION

The contact-kinin pathway consists of serially connected serine proteases which play a major role in thromboinflammation after ischemic stroke. Due to their dual activity, the contact-kinin pathway not only triggers thrombus formation by activating intrinsic coagulation via FXI (factor XI) but is also involved in the regulation of inflammatory processes, vascular permeability, and blood pressure via kinins, such as bradykinin.

The contact-kinin pathway is initiated by the activation of FXII (factor XII). Classically, FXII is known to interact with negatively charged surfaces released from activated platelets to be further stimulated[34] finally leading to FXI activation which results in thrombin generation and the subsequent formation of a fibrin clot.

Individuals with congenital deficiency of FXII do not have bleeding diathesis or abnormal hemostasis suggesting that the intrinsic coagulation pathway is not associated with physiological hemostasis.[35] In fact, FXII-deficient rats and mice also display a normal hemostatic capacity even under surgical interventions.[36,37] Interestingly, these animals are protected from experimentally induced arterial thrombosis[36,37] as well as ischemic stroke without increasing the risk of intracerebral hemorrhage.[38,39] Similar results were obtained when FXIIa (activated FXII) was selectively inhibited with recombinant human albumin-infestin in mice[40] and rats.[41] Thus, the FXII-induced intrinsic coagulation pathway remains essential for hemostasis but crucial for thrombosis.

FXIIa not only initiates coagulation but also triggers the kallikrein-kinin system. PK (plasma kallikrein) is synthesized in the liver as an inactive precursor, plasmaprekallikrein, which must undergo proteolytic processing by FXIIa to become activated. Upon activation, PK cleaves high molecular weight kininogen inducing the release of the proinflammatory nonapeptide bradykinin from kininogen. Inhibition of distinct members of the kallikrein-kinin system significantly reduced stroke volume and improved functional outcome 24 hours after transient focal cerebral ischemia induced by tMCAO. Indeed, both the genetic blockade of PK,[42] kininogen[43] and B1R (bradykinin receptor 1),[44] together with the pharmacological blockade of PK[42,45] resulted in reduced intracerebral thrombosis, maintenance of the blood-brain barrier (BBB), and reduced local inflammation. In addition to cleaving kininogen, PK also cleaves and activates FXII, creating a strong positive feedback loop that modulates platelet aggregation.

As previously mentioned, activated PK also induces the release of bradykinin from kininogen. Bradykinin is a potent inflammatory mediator binding to 2 different receptors (B1R [bradykinin receptor 1] or B2R [bradykinin receptor 2]) being involved in the regulation of vascular permeability and inflammatory processes. Binding to its receptors promotes intracellular calcium release, leading to downregulation of claudin-5 and subsequent BBB leakage.[46] This associated BBB damage following ischemic stroke allows macromolecules to reach the brain parenchyma thereby promoting brain edema, weakening the BBB, and subsequently increasing the risk of hemorrhagic transformation.[47]

Recent studies described how tPA induces bradykinin generation by a plasmin-dependent mechanism leading to B2R activation and increased BBB permeability in mice. Indeed, elevated bradykinin levels and an increase in cleaved kininogen could also be observed in patients with stroke after tPA infusion, although the precise underlying mechanism is still controversial.[48] Specifically, intravenous administration of tPA increased PK activity and cleavage of kininogen in mice. However, in mice being deficient for FXII or PK, kininogen was not cleaved after tPA administration, even if plasminogen was generated. Therefore, cleavage of kininogen by tPA requires PK and FXII, suggesting that tPA-induced cleavage of kininogen is mediated by a cascade of plasmin, FXII, and PK. From the pathophysiological perspective, pharmacological inhibition or genetic deletion of either PK or FXII reduced intracerebral hemorrhage, edema, and infarct volume caused by intravenous administration of tPA after thrombotic MCAO, therefore,

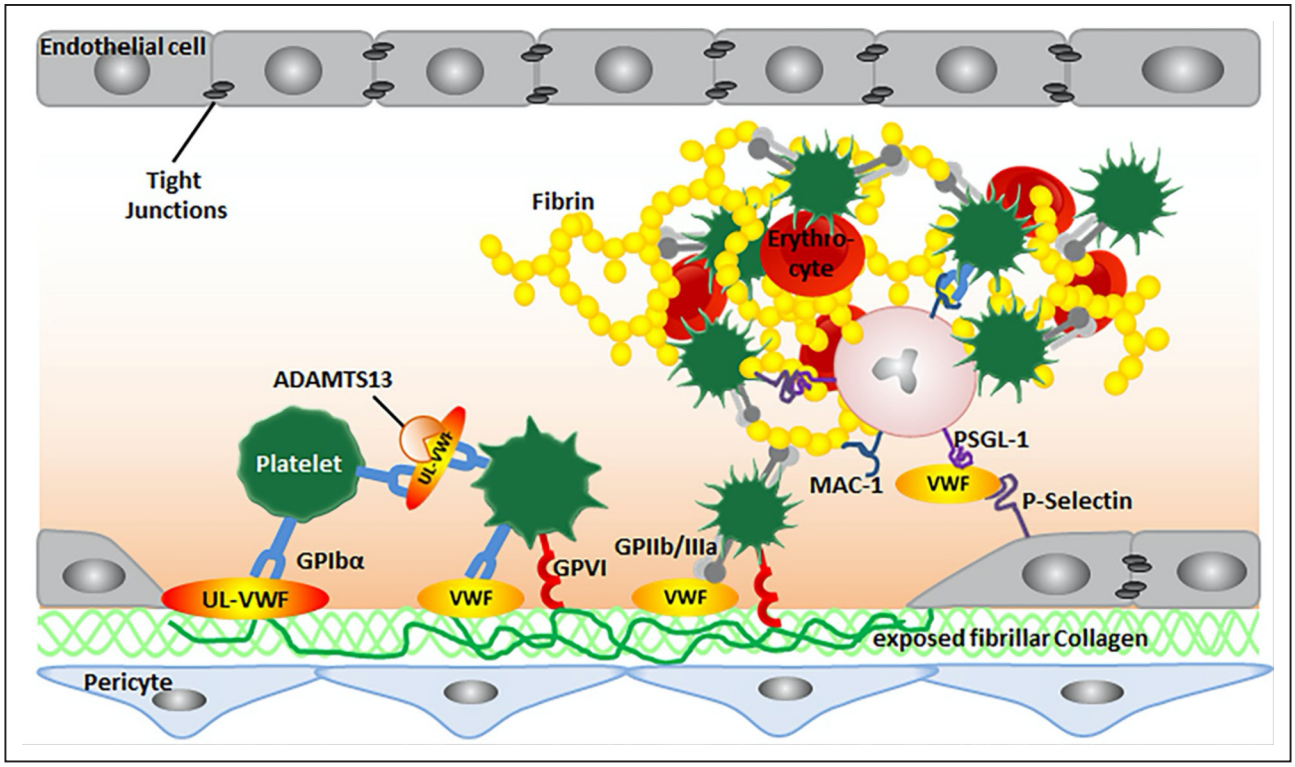

Figure 1. Platelet adhesion and thrombus formation.
Initial tethering of blood platelets is mediated by binding of GP (glycoprotein) Ibα to exposed VWF (von Willebrand Factor)/UL-VWF (ultralarge VWF). Firm adhesion of platelets to the exposed subendothelial matrix via GPVI initiates platelet activation with upregulation of GP IIb/IIIa, which mediates further stable adhesion and aggregation via binding to fibrinogen and VWF. Via PSGL (P-selectin-glycoprotein ligand)-1, neutrophils can bind to VWF, that is anchored on endothelial cells through binding with P-selectin. MAC-1 indicates macrophage-1 antigen.

supporting the concept that FXII activates PK during tPA treatment in stroked mice. On the other side, expression of bradykinin receptors B1R and B2R is upregulated approximately at 4 hours after stroke onset in mice.[44] This upregulation in combination with bradykinin generation by tPA could explain the increasing risk for brain edema and hemorrhagic transformation associated with late administration of tPA after stroke (Figure 2).

ROLE OF THE ADAPTIVE IMMUNE SYSTEM IN THOMBOINFLAMMATION

Thrombosis and inflammation are highly interconnected processes leading to cerebral vessel occlusion, inflammatory responses, and severe neuronal damage. Specifically, upregulation of adhesion molecules, platelet activation, and secretion of cytokines and chemokines, directly contribute to leukocytes recruitment which remarkably influences the pathomechanism of cerebral ischemia.

T cells have recently become increasingly important in the pathogenesis of ischemic stroke over the last years. In particular, RAG1$^{-/-}$ (recombination activation gene 1) mice (lack of mature B and T cells) develop smaller infarct volumes and improved functional outcome after tMCAO[49] independently of the platelet aggregation activity.[50] However, adoptively transferred CD4$^+$, CD8$^+$, or γδ T cells into immune-deficient RAG1$^{-/-}$ mice restored reperfusion injury in an antigen-independent manner,[50] suggesting that T cells are crucial contributors to ischemic brain damage.

The role of classically inflammatory Forkhead box P3 regulatory T cells (Tregs) is currently under discussion. Indeed, the absence of Tregs leads to exacerbated brain damage and increased inflammation after tMCAO.[51] Contrary, adoptive transfer of Tregs into RAG1$^{-/-}$ mice or, selective expansion of Tregs using an anti-CD28 superagonist in wild-type mice, results in aggravated cerebral damage.[52,53] Similarly, genetically induced deletion of Tregs (DEREG mice) results in amelioration of ischemic damage.[53] From the clinical perspective, reduced numbers of Tregs in hemorrhagic transformation thrombi were observed in patients with ischemic stroke compared with nonhemorrhagic patients after thrombectomy.

While modulating platelet function, blocking GP Ibα directly on platelets resulted in decreased T-cell infiltration and improved reperfusion.[54] However, the same experimental approach in RAG1$^{-/-}$ mice results in protection from ischemic brain damage, clearly indicating T-cell dependency.[54] Additionally, T-cell infiltration and retention in the microcirculation after tMACO were attenuated in VWF-deficient mice, highlighting the initial coagulation mechanism important for thromboinflammatory immune cell recruitment.[19] Moreover, platelet-derived soluble CD84 increases CD4$^+$ T-cell mobility, thus fostering thrombotic

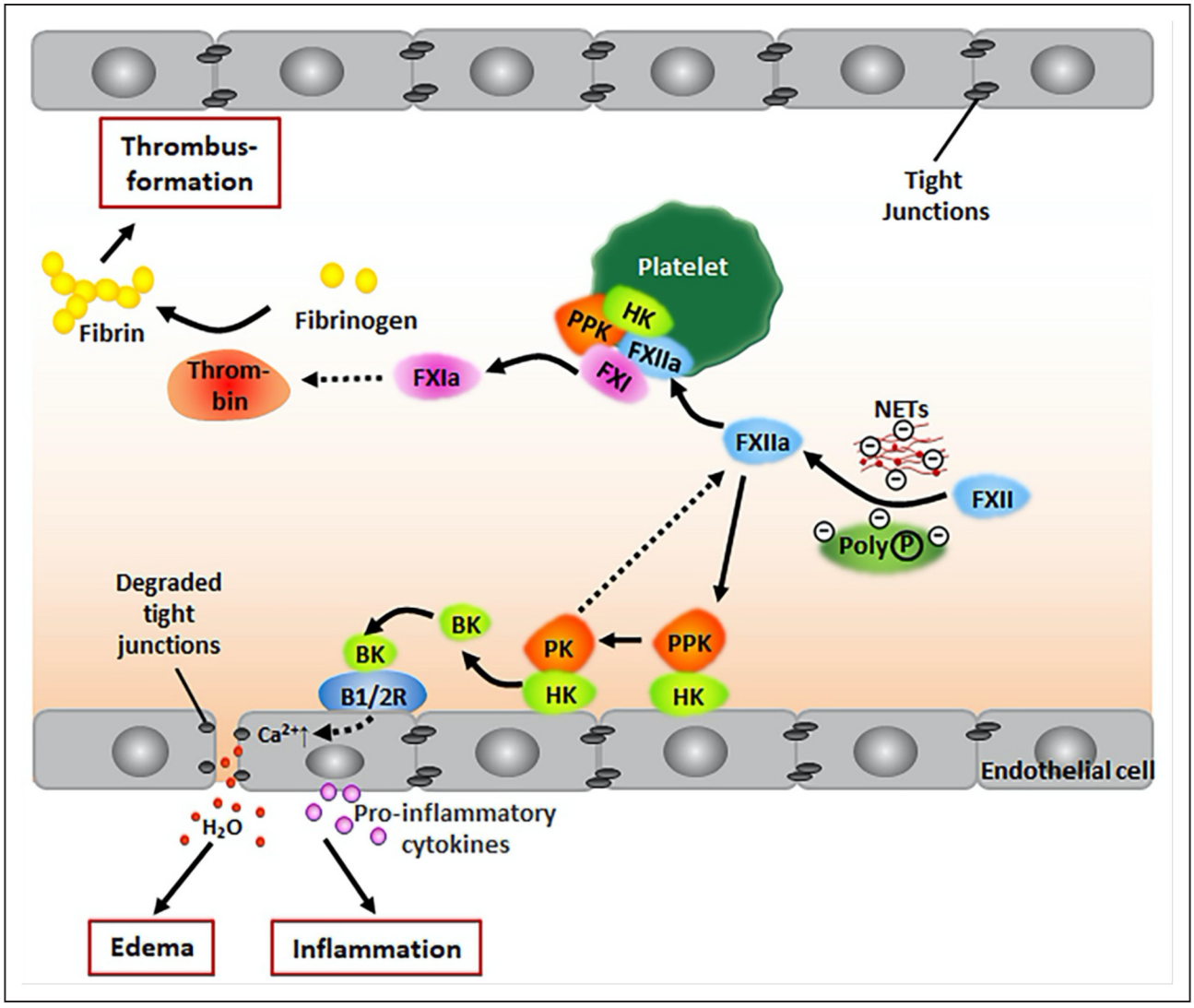

Figure 2. The kallikrein-kinin system.
Release of negatively charged polyphosphates from activated platelets or negatively charged NETs (neutrophil extracellular traps) can activate coagulation FXII (factor XII). Activation of FXII results in the assembly of a protein complex on platelets consisting of FXIIa (activated FXII), membrane-bound kininogen, prekallikrein, and FXI (factor XI). Kininogen thereby brings all components into reactive proximity, enabling the activation of FXI and leading to thrombin generation and formation of fibrin. FXIIa also triggers the activation of the kallikrein-kinin system: FXIIa cleaves PPK (plasmaprekallikrein) to form active PK (plasmakallikrein), which in turn cleaves HK (high molecular weight kininogen) to release the inflammatory peptide hormone BK (bradykinin), whose cellular effects are mediated by bradykinin receptor B1 and B2R [bradykinin receptor 2]. Activation of these receptors triggers edema formation and proinflammatory cytokine release that induce microglia activation, inflammation, and finally neuronal cell death. Poly P indicates poly-phosphate.

activity and transmigration into the ischemic hemisphere, whereas in patients, high platelet CD84 expression levels were associated with poor stroke outcome.[55]

Prolonged arrest of effector T cells at the endothelial barrier via upregulated integrin-pairs VLA-4 (very late antigen-4):VCAM (vascular cell adhesion molecule)-1 and LFA-1 (lymphocyte function–associated antigen-1):ICAM (intercellular adhesion molecule)-1 interactions have already been described in ischemic stroke.[56] Indeed, inhibition of α4-integrin reduced the infiltration of $CD4^+$ including Tregs and $CD8^+$ T cells into the ischemic hemisphere.[57,58] In fact, LFA-1 is known to be predominantly expressed on Tregs, exerting its deleterious effects by binding to the epithelium and leading to microvascular dysfunction, thrombus formation, and secondary infarct growth. These observations highlight the deleterious effects of T cells in presence of platelets and extensive T-cell adhesion in ischemic stroke.

The influence of B cells still remains controversial in the field. Pharmacological depletion of B cells (anti-CD20 antibodies) or genetic deletion ($JHD^{-/-}$ [Jh region deficient] mice) had no effect on stroke volume and functional outcome upon ischemic stroke. Similarly, reconstitution of B cells in $RAG1^{-/-}$ mice was negligible.[49,57] In contrast, in $\mu MT^{-/-}$ (inactivating mutation in the first M transmembrane exon of the μ heavy chain domain) mice, a different model

of B-cell depletion, increased infarct volume, mortality rates, functional deficits, and activated immune cells were identified in the stroked hemisphere.[59] Interestingly, adoptively transferred B cells and enriched anti-inflammatory IL-10^{+} B cells (Bregs [regulatory B cells]) were able to significantly reduce ischemic damage.[58,59] In fact, adoptive transfer of IL-10$^{-/-}$ B cells failed to achieve protection after tMCAO, although further studies described that Bregs significantly promoted the recruitment and expansion of Tregs in ischemic stroke.[60] Thus, further experimentation is required to indeed assess the potential neuroprotective role of B cells to ultimately understand their immunologic role poststroke (Figure 3).

ROLE OF THE INNATE IMMUNE SYSTEM IN THOMBOINFLAMMATION

Neutrophil granulocytes are among the first cells attracted to the ischemic brain. In fact, these short-lived immune cells are detected in large numbers within hours to 3 days after ischemic injury.[61,62] In patients with stroke, elevated neutrophil-platelet interactions were detected with the number of neutrophils in blood being associated with greater infarct volume.[63,64] Precisely, neutrophils are shown to promote thrombus formation, BBB leakage, and exacerbate cerebral blood flow by secreting proinflammatory IL-1β, cathepsin G, elastase, MMP (matrix metalloprotease)-9, and reactive oxygen/nitrogen species.[65–69] Additionally, as an essential source of TF (tissue factor), neutrophils can drive the intrinsic coagulation cascade via activation of FVIIa (activated FVIIa) and further promote thrombus formation.[67] Moreover, through direct binding to injured endothelial cells via LFA-1:ICAM-1, neutrophils are able to induce TF-mediated formation of fibrin and thrombus even before platelets are accumulating.[70] Neutrophils interact with platelets via binding of P-selectin:PSGL (P-selectin-glycoprotein ligand)-1 resulting in neutrophil activation. In fact, neutrophils use PSGL-1 clusters to search their environment for activated platelets even before the inflammatory process.[71] Additionally, neutrophils can produce extracellular traps (NETs [neutrophil extracellular traps]) that bind platelets, erythrocytes, and immune cells, thereby also providing matrix for the contact coagulation pathway.[72] In a recent study, aPLs (antiphospholipid antibodies) were connected to neutrophil-driven thrombus formation under toll-like receptor 4 activation and reactive oxygen species secretion. Here, TF expression on NETs was deeply connected to aPLs binding correlating with neutrophil-platelet aggregation[73] being even associated with resistance to tPA lysis. Accordingly, NETs promote a procoagulant phenotype of platelets and endothelial cells facilitating VWF and PAI (plasminogen activator inhibitor)-1 release.[74] Translationally, abundant NETs are accounted for reduced tPA-based thrombolysis and poor clinical outcome.[74–76] Specifically, inflammasome signaling proteins including caspase-1 are elevated in NETs present in thrombi of patients with stroke, therefore, leading to worsening outcomes after the ischemic event.[77]

Neutrophils also release NE (neutrophil elastase) that contributes to cathepsin G-induced platelet aggregation, cleavage of tissue factor protease inhibitor, degradation of MMP-9, increased neutrophil chemotaxis, and release of NETs. Indeed, genetic depletion[69] or NE inhibition with agaphelin[78] significantly reduced infarct volume, BBB disruption, and ameliorated inflammatory response upon stroke. Moreover, tPA application induces neutrophil transmigration via liberated plasmin and MMP worsening stroke outcome and inducing hemorrhagic transformation.[79]

Monocyte-derived macrophages (MDMs) are mononuclear leukocytes recruited from (1) the spleen and later (2) the bone marrow during an ischemic event. MDMs can be detected as early as 3 hours in the injured hemisphere and peak at 7 days after stroke,[80] being attracted by significantly increased amounts of CCL2 (chemokine [C-C motif] ligand 2) secreted from pericytes, endothelial and neural cells.[81] Proinflammatory Ly6C^{high} CCR2^{+} (C-C chemokine receptor type 2) monocytes are the first attracted to the ischemic brain,[82–84] appearing 3-fold higher numbers of LyC6^{high} monocytes in blood 24 hours after stroke.[85] In fact, splenectomy or splenic irradiation result in reduced MDM infiltration, smaller infarct volumes, less atrophy, neuronal damage, and BBB damage demonstrating stroke-specific MDMs originated from the spleen.[80] However, selective depletion of proinflammatory Ly6C^{high} monocytes, proinflammatory (M1—) or anti-inflammatory (M2—) macrophages had no influence on ischemic stroke volume neither in functional outcome.

Infiltrating macrophages are classically associated with inflammation and cerebral damage after ischemic stroke, although they undergo dynamic effector changes (M1 to M2 phenotype) during infarct development, maturation, and regeneration.[86] Conflicting findings made challenging to identify the exact role of CCR2^{+} macrophages during the acute phase of ischemic stroke. In CCR2$^{-/-}$ deficient mice or CCR2 antagonist treatment, monocyte influx into the brain is prevented and results in increased infarct volume, aggravation of neurological outcome, vascular instability,[87] and hemorrhagic transformation.[82] Recently, the CXCL12 (C-X-C motif chemokine ligand 12)/CXCR4 (C-X-C chemokine receptor type 4) axis has been described as another important chemokine gradient for monocyte recruitment to the ischemic hemisphere. CXCR4 is responsible for the initial monocyte migration and the territorial restriction of MDMs to the lesion area. Thus, deficiency of CXCR4 results in reduced infiltration of monocytes into the ischemic brain and consequently increased infarct volume and worsen neurological deficits.[88]

Natural killer (NK) cells are related to ischemic brain damage.[89,90] Temporal kinetics revealed that NK cells infiltrate the brain as early as 3 hours and peak at 3 days after an ischemic event. Specifically, NK cells localized in the peri-infarct areas or the ischemic penumbra in tMCAO mice and

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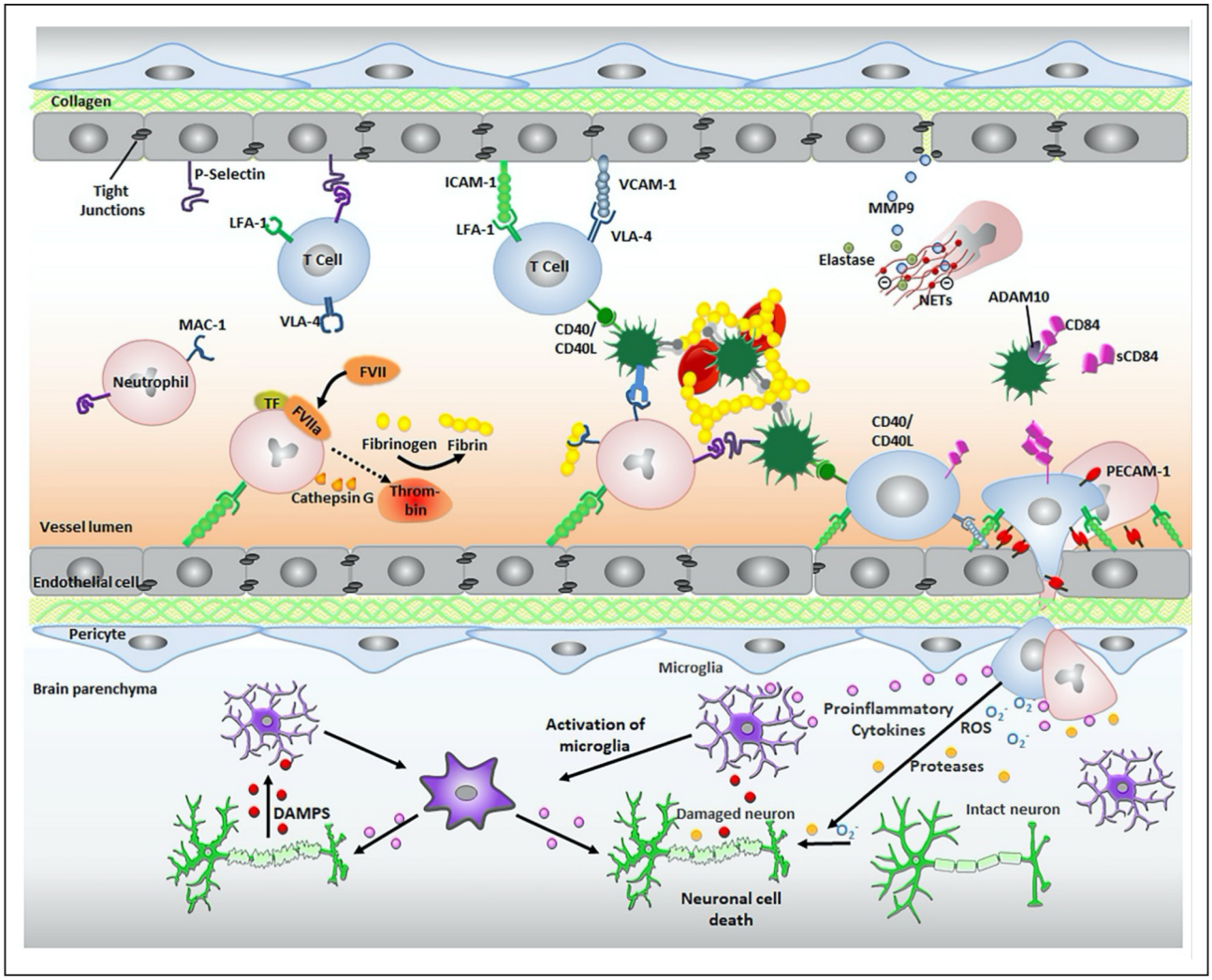

Figure 3. Recruitment of leukocytes following ischemic stroke.
The ischemic insult activates circulating leukocytes and the endothelium resulting in an upregulation of adhesion molecules. T cells are recruited to the injured endothelium via P-selectin/PSGL (P-selectin-glycoprotein ligand)-1 interaction. Stable tethering to the vessel wall is achieved by the interaction between ICAM (intercellular adhesion molecule)-1/LFA (lymphocyte function–associated antigen)-1 and VCAM (vascular cell adhesion molecule)-1/VLA (very late antigen)-4. T cells interact with activated platelets via CD40/CD40L to form a solid thrombus. Upon platelet activation, CD84 is shed from the platelet surface by ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10) resulting in sCD84 (soluble CD84) that binds to CD84 on T cells and enhances transendothelial migration of T cells. Neutrophils contribute to thrombus formation as they interact with platelets (via MAC-1 [macrophage-1 antigen]/GP Iba and P-Selectin/PSGL-1), participate in fibrin cross-linkage (via MAC-1/fibrin interaction), and trigger thrombin activation by inducing the extrinsic TF (tissue factor)/FVIIa (activated FVIIa) pathway and activation of FVIII by cathepsin G. Neutrophils also release NE (neutrophil elastase), that activates MMP (matrix metalloprotease) 9 and contribute to blood-brain barrier (BBB) damage, and NETs (neutrophil extracellular traps). Infiltration of immune cells into the brain parenchyma triggers liberation of reactive oxygen species (ROS), proinflammatory cytokines, and proteases, and induces neuronal cell damage. Furthermore, dying neurons release damage-associated molecular patterns (DAMPs) that activate microglial cells and thereby aggravate the ischemic brain damage. PECAM indicates platelet endothelial cell adhesion molecule.

postmortem tissue of patients with ischemic stroke.[90] NK cells exert their pathogenic, inflammatory, and neurotoxic capacities via secretion of IFNγ (interferon-γ) and perforin.[90] In addition, Zhang et al[91] described NK cells to participate in the BBB disruption in IP (interferon gamma induced protein)-10–dependent process. In the ischemic brain, increased amounts of IL-15 and CX3CL1 are secreted with both being necessary for NK cell recruitment. In addition, IL-15 causes maturation and enhanced cytotoxic function in NK cells.[92,93] Unfortunately, limited research is available about the interaction of NK and the thromboinflammatory process. Others suggest that IFNγ-producing NK cells critically promote deep vein thrombosis through neutrophil NET-formation.[94] However, their role during thromboinflammation is not fully understood.

The pathogenic role of mast cells (MC) in ischemic stroke was highlighted in few preclinical studies.[95] Under hypoxic conditions, MC release granules and cytokines leading to thrombosis, neurotoxicity, and immune cell recruitment in vivo and in vitro.[96,97] In the tMCAO setup, MC deficiency or

inhibition, led to decreased BBB breakdown, brain edema, and neutrophil recruitment.[98] Additionally, the absence of MCs in the infarct area was identified in postmortem tissue from patients with stroke, suggesting that the role played by MCs in stroke pathophysiology is not significantly relevant.[99]

CLINICAL ASPECTS OF THROMBOINFLAMMATION

Ischemia-reperfusion injury in hospitalized patients with stroke is tightly connected to neuroinflammatory processes including (1) immune cells infiltration into the brain parenchyma, (2) BBB damage, and (3) thrombi formation. Hence, novel strategies tackling thromboinflammation are currently under assessment in different clinical scenarios.[100]

Antiplatelet Therapy for Targeting Thomboinflammation

Antiplatelet therapy has been broadly explored in several clinical settings through different potential therapeutic targets.

GPIIb/IIIa Antagonists

Most advanced GPIIb/IIIa inhibitors including abciximab and tirofiban have been clinically assessed in patients with stroke resulting in controversial results. Indeed, the administration of abciximab led to severe intracranial hemorrhage in several treated patients irrespective of the injection time point or ischemic onset resulting in the termination of a Phase III clinical trial after the inclusion of 808 patients.[30] Similarly, patients receiving tirofiban were associated with significantly increased risk of intracranial bleeding and poor outcome poststroke,[101] despite showing safety and promising efficacy in a subset of patients. Despite these controversial results, the novel GPIIb/IIIa inhibitor tirofiban has recently entered clinical testing as a potential stroke treatment (https://www.clinicaltrials.gov; Unique identifier: NCT04491695), suggesting that this antiplatelet strategy should still be clinically considered.

$P2Y_{12}$ Receptor Antagonists

The $P2Y_{12}$ receptor plays a central role in platelet function, hemostasis, and thrombi formation also promoting platelet-leukocyte interactions.[102] Therefore, specific $P2Y_{12}$ antagonists aim to improve thromboinflammatory outcomes poststroke regulating platelet aggregation and leukocyte recruitment. Indeed, the $P2Y_{12}$ antagonist cangrelor is currently being tested in a phase III clinical trial for acute ischemic stroke in combination with the standard-of-care (REPERFUSE [Reperfusion With $P2Y_{12}$ Inhibitors in Addition to Mechanical Thrombectomy for Perfusion Imaging Elected Acute Stroke Patients], https://www.clinicaltrials.gov; Unique identifier: NCT04667078). Additionally, this strategy aims to be repurposed to other cardiovascular indications, that is, coronary artery disease and atrial fibrillation, undergoing clinical testing at the moment.

GPVI Antagonists

Direct blockage of the GPVI-collagen interaction using the dimeric GPVI-Fc fusion protein Revacept has been assessed in a recently completed phase II clinical trial suggesting promising results in patients with transient ischemic attack stroke (https://www.clinicaltrials.gov; Unique identifier: NCT01645306). As an additional pharmacological approach, the humanized Fab fragment ACT017 has already been proved as safe and well-tolerated in healthy volunteers (https://www.clinicaltrials.gov; Unique identifier: NCT03803007), later translated to a phase III efficacy trial currently ongoing in patients with acute ischemic stroke (ACTISAVE [Adaptive Efficacy and Safety Study of Glenzocimab Used as an Add-On Therapy on Top of Standard of Care in the 4.5 Hours Following an Acute Ischemic Stroke], https://www.clinicaltrials.gov; Unique identifier: NCT05070260).[103] Thus, specific GPVI inhibitors are currently considered as one of the most promising therapeutic approaches for patients with stroke within the platelet field.

FXI: Safer Anticoagulant Therapy

Humans with congenital deficiency of FXI are associated with a reduced risk of ischemic events and thromboembolism.[104] Therefore, clinical trials aiming to elucidate the potential of interfering with the FXI-kallikrein-kinin pathway are currently ongoing. Indeed, oral administration of the FXIa inhibitor BMS-986177 in combination with aspirin and clopidogrel is currently being tested to further prevent secondary ischemic events in transient ischemic attack patients (https://www.clinicaltrials.gov; Unique identifier: NCT03766581), although this strategy remains novel and partially exploratory where bleeding profile still remains uncertain (Table).

From Platelet Modulation to Neuroinflammation

Not only platelets can be proinflammatory since inflammatory mediators can also activate platelets[105] resulting in a complex thromboinflammatory crosstalk. Thus, immunomodulatory treatments are still considered as a promising therapeutic strategy for patients with ischemic stroke. Due to its role in stroke pathomechanism, inhibition of microglia activation and decreased migration of T cells is, therefore, considered as a potential therapeutic strategy to tackle an ischemic event. Minocycline, an antibiotic agent commonly used for the treatment of broad range infections is also able to modulate microglia, T-cell recruitment, and neuronal apoptosis, and thus considered as a potential stroke treatment. Indeed, a systematic review and meta-analysis including several randomized clinical trials concluded demonstrated the neuroprotective effect of minocycline specially in acute ischemic stroke subgroups.[106] However, follow-up clinical trials demonstrated safety but

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Table. Targeting Thromboinflammation in Ischemic Stroke: Current Clinical Scenario

NCT number	Disease indication	Study design	Target	Intervention	Mechanism of action	Status
NCT03766581	Transient ischemic attack	Global, randomized, double-blind, placebo-controlled, dose-ranging study	FXIa	BMS-986177 in combination with aspirin and clopidogrel	Anticoagulant therapy	Phase II (ongoing)
NCT00073372	Brain ischemia	Multinational, multicenter, randomized, double-blind, placebo-controlled trial	Glycoprotein IIb/IIIa	Abciximab	Antiplatelet therapy	Phase III (Terminated)
NCT04491695	Acute ischemic stroke	Randomized controlled trial		Tirofiban Hydrochloride		Phase II/III (ongoing)
NCT04667078	Acute ischemic stroke	Randomized, interventional trial (REPERFUSE)	$P2Y_{12}$ receptor	Cangrelor		Phase III (ongoing)
NCT05070260	Acute ischemic stroke	Randomized, double-blind, multicenter, multinational, placebo-controlled, parallel-group, single-dose, adaptive trial (ACTISAVE)	Glycoprotein VI	Glenzocimab (ACT017)		Phase II/III (ongoing)
NCT01645306	Acute ischemic stroke	Multicentre, randomized, dose-finding, double-blind, and placebo-controlled		Revacept		Phase II (completed)
NCT05032781	Acute ischemic stroke	Open label study	Not specific	Intraarterial cold saline, minocycline, and magnesium	Modulate microglia, T-cell recruitment, and neuronal apoptosis	Phase I (ongoing)
NCT04876638	Aneurysmal subarachnoid hemorrhage	Randomized, interventional study		Minocycline		Phase II (ongoing)
NCT05065216	Ischemic stroke	Randomized double-blind placebo-controlled study (ReMEDy II)	Tissue kallikrein-1	DM199	Antithombo-inflammatory therapy	Phase II/III (ongoing)
NCT01955707	Acute ischemic stroke	Multicenter, Double-Blind, Placebo-Controlled, Randomized, Parallel-Group Study (ACTION)	Cell adhesion molecule α4-integrin	Natalizumab	Anti-inflammatory therapy	Phase II (completed)
NCT04629872/ NCT04675762	Acute ischemic stroke	Nonrandomised intervention study	S1P receptor	Fingolimod in endovascular treatment	$CD4^+$ T cells trafficking, lymphocytes migration, and aggregation	Phase II (ongoing)
NCT04718064	Acute ischemic stroke	Randomized interventional study		Revascularization pretreated with fingolimod		n/a
NCT04088630	Intracerebral Hemorrhage	Randomized, double-blinded, placebo-controlled pilot trial		Fingolimod		Phase I (ongoing)

ACTION indicates Safety and Efficacy of Intravenous Natalizumab in Acute Ischemic Stroke; ACTISAVE, Adaptive Efficacy and Safety Study of Glenzocimab Used as an Add-On Therapy on Top of Standard of Care un the 4.5 Hours Following an Acute Ischemic Stroke; FXIa, activated FXI; NCT, National Clinical Trial; ReMEDy, Evaluation to Assess Safety and Tolerability of DM199 in Subjects With Acute Ischemic Stroke; REPERFUSE, Reperfusion With $P2Y_{12}$ Inhibitors in Addition to Mechanical Thrombectomy for Perfusion Imaging Elected Acute Stroke Patients; and S1P, sphingosine-1-phosphate.

not full efficacy in larger patient cohorts,[107] although its therapeutic role is still under clinical evaluation in different ongoing trials (https://www.clinicaltrials.gov; Unique identifiers: NCT05032781 and NCT04876638). In line with microglia inhibition, blocking neutrophil infiltration also reached clinical evaluation unfortunately not beneficial and, therefore, terminated due to futility when using CD18 antagonist antibodies.[107]

As previously mentioned, the kallikrein-kinin system is directly involved in the regulation of proinflammatory processes and vascular permeability leading to thromboinflammation upon stroke.[2] Towards clinical translation, the first human recombinant tissue kallikrein-1 compound, that is, DM199, is currently under evaluation in patients with acute ischemic stroke within a phase II/III trial to evaluate safety, tolerability, and efficacy of this therapeutic strategy (ReMEDy, https://www.clinicaltrials.gov; Unique identifier: NCT05065216). Specifically, up to 75 clinical centers are expected to participate including the estimated enrollment of 364 patients (Table).

Repurposing Multiple Sclerosis Towards Stroke Therapy

An impaired neuroinflammatory profile remains a common pathophysiological element in several neurovascular and neurodegenerative diseases. Indeed, leukocyte influx, microglia activation, immune cell migration, and subsequent neurodegeneration have been broadly considered as potential therapeutic targets both in ischemic stroke and multiple sclerosis.[108] In particular, the US Food and Drug Administration-approved humanized monoclonal antibody Natalizumab

able to reduce the inflammatory activity in multiple sclerosis brain has already been considered as a promising repurposing strategy for stroke therapy. Despite blocking the white blood cell influx upon brain ischemia, natalizumab treatment reported no significant differences in infarct volume and neurological deficits[109] subsequently leading to trial termination due to null effect (ACTION II trial).[110]

Following a similar therapeutic approach, the sphingosine-1-phosphate receptor agonist fingolimod has recently reached clinical evaluation as a potential stroke therapy. Precisely, fingolimod directly blocks CD4+ T cells trafficking, lymphocytes migration, and aggregation, therefore, reducing thromboinflammation. Patients receiving fingolimod treatment showed smaller lesion volumes and restricted lesion growth within the first week, while National Institutes of Health Stroke Scale scores were significantly lower already one day after starting the treatment. Moreover, fingolimod remains effective when combined with the standard-of-care (r-tPA), importantly, not being associated with hemorrhagic transformation. Indeed, this therapeutic strategy resulted in direct prevention of reperfusion injury, reduced cytokine levels,[111] and overall improved patient prognosis.[112] Thus, fingolimod aims to be a promising addition to the currently available stroke treatment, although this approach is still under clinical testing. Indeed, several clinical trials are currently under recruitment which aims to evaluate (1) the potential extension of the treatment beyond the approved therapeutic time window, that is, 4.5 hours after onset (https://www.clinicaltrials.gov; Unique identifier: NCT04629872), (2) the administration of fingolimod as a pretreatment before revascularization (https://www.clinicaltrials.gov; Unique identifier: NCT04718064), and (3) the potential therapeutic benefit after developing an intracranial hemorrhage (https://www.clinicaltrials.gov; Unique identifier: NCT04088630). Thus, fingolimod aims to be the first potential anti-inflammatory treatment with a high likelihood of reaching daily clinical routine (Table).

CONCLUSIONS

Broad experimental evidence suggests that thrombotic and neuroinflammatory processes deeply modulate the complex stroke pathophysiology. Indeed, the role of platelet activation, thrombi formation, and peripheral immune cells upon stroke has gained increasing attention over the last years resulting in a solid basis for novel pharmacological approaches. Clinical translation exploring the critical interface between neuroinflammation and thrombosis has led to last-stage human studies close to final approval demonstrating the clinical potential of targeting thromboinflammation in patients with brain ischemia.

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Disclosures

None.

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