



Review

Targeting vascular inflammation in ischemic stroke: Recent developments on novel immunomodulatory approaches

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ABSTRACT

Ischemic stroke is a devastating and debilitating medical condition with limited therapeutic options. However, accumulating evidence indicates a central role of inflammation in all aspects of stroke including its initiation, the progression of injury, and recovery or wound healing. A central target of inflammation is disruption of the blood brain barrier or neurovascular unit. Here we discuss recent developments in identifying potential molecular targets and immunomodulatory approaches to preserve or protect barrier function and limit infarct damage and functional impairment. These include blocking harmful inflammatory signaling in endothelial cells, microglia/macrophages, or Th17/γδ T cells with biologics, third generation epoxyeicosatrienoic acid (EET) analogs with extended half-life, and miRNA antagonists. Complementary beneficial pathways may be enhanced by miRNA mimetics or hyperbaric oxygenation. These immunomodulatory approaches could be used to greatly expand the therapeutic window for thrombolytic treatment with tissue plasminogen activator (t-PA). Moreover, nanoparticle technology allows for the selective targeting of endothelial cells for delivery of DNA/RNA oligonucleotides and neuroprotective drugs. In addition, although likely detrimental to the progression of ischemic stroke by inducing inflammation, oxidative stress, and neuronal cell death, 20-HETE may also reduce susceptibility of onset of ischemic stroke by maintaining autoregulation of cerebral blood flow. Although the interaction between inflammation and stroke is multifaceted, a better understanding of the mechanisms behind the pro-inflammatory state at all stages will hopefully help in developing novel immunomodulatory approaches to improve mortality and functional outcome of those inflicted with ischemic stroke.

1. Introduction

Stroke ranks first among all neurological disorders in most countries worldwide, according to the report on the global burden of disease 1990–2015 (Group, 2017), resulting in \$34 billion costs to health care in the United States alone (Benjamin et al., 2017). About 87% of all strokes are the ischemic type resulting from blockade of blood supply to the parts of the brain. Each year around 692,000 people in the United States have an ischemic stroke, putting an enormous burden on national healthcare through long-lasting disability (Benjamin et al., 2017). Stroke can occur at any age; however, almost one third occurs below the age of 65 years (Roger et al., 2012). Women are at higher risk for stroke after menopause because of the lack of protective sex hormone (Shekhar et al., 2017b; Sudlow and Warlow, 1997). African Americans have nearly twice the incidence of stroke and a higher rate of mortality as well (Benjamin et al., 2017). Various risk factors are involved in the

pathogenesis of ischemic stroke, thus requiring understanding the pathogenetic mechanisms that could help design new treatments.

Stroke management had a breakthrough after the success of thrombolytic clinical trials [NINDS (National Institute of Neurological and Stroke rt, 1995) and ECASS III (Hacke et al., 2008)] and recent extension of the therapeutic window with mechanical thrombectomy [MR CLEAN (Berkhemer et al., 2015), DAWN (Nogueira et al., 2018), and DEFUSE 3 (Albers et al., 2018)], arresting the injury during the acute stage and decreasing mortality and long-term disability. Ischemic stroke therapy can be targeted at different stages of pathogenesis: presymptomatic (preventive therapy), symptomatic (arresting progression), and recovery stages (assisting recovery).

Numerous in vivo and clinical studies (Elkind et al., 2004) have suggested the importance of inflammatory pathways in the pathogenesis of ischemic stroke. Corticosteroids, the most widely used anti-inflammatory agent, theoretically could protect from stroke (de Courten-

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Myers et al., 1994); however, apart from the vasculitis, steroids failed to demonstrate a benefit (De Reuck et al., 1988; Norris and Hachinski, 1986; Sandercock and Soane, 2011). The exact reason for steroid failure in thromboembolic infarction is not known, but we can speculate that steroids likely inhibit inflammatory pathways both during the early phase when inflammation is more damaging and later when inflammation helps clear up debris and supports wound healing. Similarly, the use of anti-inflammatory agents such as COX2 inhibitors and non-steroidal agents have failed to provide theoretical benefit and, moreover, increased the cerebrovascular risk (Andersohn et al., 2006; Bresalier et al., 2005; Solomon et al., 2005), such as stroke, bleeding, and atrial fibrillation – with the exception of non-selective cyclooxygenase (COX) inhibitors, such as aspirin, which demonstrated mainly secondary preventative benefit (1997a; 1997b). Aspirin has independent anti-inflammatory effects through modulation of NF- κ B-regulated genes (Pierce et al., 1996; Yamamoto et al., 1999).

After an infarction, the core develops non-reversible injury and later becomes a nidus for inflammation. The region immediately surrounding the dead core (penumbra) is the tissue at risk from ongoing ischemia and inflammation. Thus, an important consideration is required for these viable tissues where most of the anti-inflammatory therapy is focused on protecting by halting or slowing the inflammatory cascade. An important sequelae of infarction and thrombolytic therapy is the reperfusion injury that further enhances the progression of injury, thus requiring the development of novel therapies. Potential therapeutic targets may include microglia/macrophages, leukocytes, mast cells, cytokines, interleukins, free radicals, cell adhesion molecules, matrix metalloproteinase (MMP), signaling and transcription factors, complement system, etc. There is a need to develop efficacious pluripotent anti-inflammatory agents that withstand the rigor of well designed randomized clinical trials.

2. Background and rationale for immunomodulatory stroke therapy

The blood–brain barrier (BBB) provides a protective selective-barrier between the circulating blood and the extracellular fluid in the central nervous system and neural parenchyma. Tight junctions between endothelial cells, a defining feature of the BBB, serve to block the diffusion of hydrophilic or large molecules, as well as microscopic particles into the cerebrospinal fluid. The current understanding of the BBB views it as one aspect of the dynamic neurovascular unit (NVU), comprising endothelial cells, surrounding vascular smooth muscle cells or pericytes, basement membranes, and astrocyte end-feet processes and neuronal projections (Iadecola, 2017). The NVU functions to regulate communication between the brain and cerebrovascular network, most notably in the context of coupling neuronal activity to blood flow.

In response to ischemia, resulting typically from thrombosis or an embolism, innate immune cells in the brain and periphery are recruited to the site of injury, along with the subsequent engagement of cells of the adaptive immune system. A variety of danger signals together with pro-inflammatory cytokines and chemokines drive the initial response. Endothelial cells are activated with loss of tight junctions and integrity of the BBB, which is further weakened by MMPs (released by neutrophils, pericytes, pro-inflammatory M1 resident/infiltrating myeloid cells, and endothelial cells), as well as by reactive oxygen species, and oxidative stress (Jin et al., 2017; Petrovic-Djergovic et al., 2016; Rayasam et al., 2017; Takata et al., 2011). This creates a route for entry of immune cells into the brain parenchyma along with solutes and water that result in interstitial inflammation and edema that further damages neuronal tissue. In addition, activated endothelial cells express selectins and adhesion molecules that facilitate migration of leukocytes into the brain via paracellular or transcellular routes. The early engagement of the (alternative) complement system and platelets contributes to thrombogenesis, which along with endothelial cell sloughing and neutrophil extracellular trap (NET) formation can further

block blood flow and comprise oxygen delivery. Consequently, the extent of the initial damage can be magnified several fold over time.

The inflammatory process following an ischemic stroke occurs in three phases of increasing lengths of time: initiation (hours), propagation (days), and resolution (weeks). Each phase is associated with a distinct pattern of immune cell response and extracellular signaling molecules. As expected, adaptive immunity is engaged during the propagation phase following an initial innate immune response. A detailed description of the temporal pattern of inflammation following an ischemic stroke can be found elsewhere (Petrovic-Djergovic et al., 2016; Rayasam et al., 2017). In theory, any of the 3 phases could be targeted clinically; however, limiting the extent of inflammation or its duration during the propagation phase is more practical. Multiple preclinical studies have documented the benefit of targeting inflammation as a means of limiting tissue damage from ischemic stroke. There is one caveat, viz., some degree of inflammation is predicted to be necessary to remove injured or necrotic tissue and contribute to wound healing; for example, blocking the recruitment of infiltrating macrophages impaired functional recovery from stroke in a mouse model (Wattanani et al., 2016). Aspects of the M2 (anti-inflammatory) – M1 (pro-inflammatory) transition in microglia vs. infiltrating macrophages during the propagation phase of inflammation in ischemic stroke are still unsettled (Rayasam et al., 2017). Here we focus on recent developments to employ immunomodulatory therapy to treat ischemic stroke.

3. Recently identified therapeutic aims and therapies

3.1. Thrombin

Thrombin (factor IIa) is a blood-derived protease that participates in the final stage of coagulation pathway and converts fibrin to fibrinogen, thus helping clot production and wound healing. Thrombin enters the brain tissue after stroke or inflammation causing impairment of BBB via altering the permeability of endothelial cells and microvasculature by an unidentified pathway (Bartha et al., 2000; Kim et al., 2004). We know that thrombin is directly (Mhatre et al., 2004) and indirectly toxic to neurons via activating microglia and astrocytes (Choi et al., 2008, 2003) and its inhibition with Dabigatran results in decreased expression of inflammatory proteins and reactive oxygen species (Dittmeier et al., 2016; Tripathy et al., 2013). Most of the thrombin-mediated harmful action occurs through a protease-activated receptor (PER) 1, but additional receptors sites PER 2, 3, 4 (Bartha et al., 2000; Choi et al., 2008; Vajda et al., 2008) may be involved as well. Thrombin increases cytosolic Ca^{2+} concentration via releasing Ca^{2+} from endoplasmic reticulum through IP_3 activation (Brailoiu et al., 2017). It also increases nitric oxide (NO) production and reactive oxygen species in the mitochondria and cytosol. Furthermore, it increases fibrin stress fibers in brain microvascular endothelial cells resulting in BBB leakage (Brailoiu et al., 2017). Direct acting thrombin antagonists such as Dabigatran have been shown to decrease the size of infarction, inflammation, and BBB permeability (Dittmeier et al., 2016).

3.1.1. Inflammatory response from thrombolytic therapy

Tissue plasminogen activator (t-PA) is a serine protease innately produced in the body and the recombinant form (rt-PA), the most widely used thrombolytic medicine, works by catalyzing the conversion of plasminogen to plasmin facilitating fibrinolysis. rt-PA therapy is the standard of care for patients within 4.5 h of stroke-like symptoms, but there is 6% risk of symptomatic hemorrhagic conversion (Hacke et al., 2008; National Institute of Neurological and Stroke rt, 1995) (most feared complication of rt-PA), and it harbors a higher bleed rate as complication after delayed administration beyond 4.5 h. The real-world data suggests that due to the short therapeutic window, only 3% of patients actually receive rt-PA (Go et al., 2014; Graham, 2003) and by preventing the toxic effect of rt-PA, the therapy administration time

could be extended. Activation of MMPs (Jin et al., 2010b; Vivien et al., 2011) and PARP activity (Crome et al., 2007) have been implicated in rt-PA-mediated vascular toxicity (damaging BBB, damage to microvessels, etc.) (Jickling et al., 2014; Wang et al., 2015a, 2004). Further study showed that rt-PA through plasmin enhances endothelial micro-particle release, which has an impact on growth and morphology of cerebral endothelial cells (Garraud et al., 2016). Microparticles are membrane vesicles that are produced by cell activation or apoptosis and contribute to inflammation, thrombosis, and angiogenesis. Others have reported that rt-PA induces human brain endothelial and astrocyte morphological changes, which was linked to activation of the Rho kinase pathway in astrocytes and postulated to be responsible for increased BBB permeability (Niego et al., 2012).

3.1.2. Expanding thrombolytic time window

Several agents have been explored to extend the therapeutic window of rt-PA by counteracting the harmful effect of plasminogen activator. The adjunctive treatment can be divided based on the mechanism of action such as agents: 1) protecting blood-brain barrier, e.g., atorvastatin, batimastat, candesartan, etc.; 2) enhancing vascularization or angiogenesis, e.g., coumarin derivative IMM-H004 and granulocyte-colony stimulating factors (G-CSF); 3) with antioxidant properties, e.g., vitamin C (ascorbic acid); and 4) increasing oxygen delivery, e.g., dodecafluoropentane emulsion nanodroplets or normobaric oxygen. A summary of agents and their major inflammation-related targets that showed promise in preclinical studies as adjunct therapy with t-PA is presented in Table 1 [see also (Knecht et al., 2017; Pena et al., 2017)].

3.2. Hyperbaric oxygenation

Although hyperbaric oxygenation (HBO) therapy is not recommended by American Heart Association and Stroke Association guidelines (Jauch et al., 2013; Misir et al., 2017), HBO therapy is a widely used non-pharmacologic and non-invasive clinical treatment of ischemic stroke (Hu et al., 2014; Yang and Mu, 2017). It enhances oxygen supply, stabilizes the BBB, and decreases infarct size and cerebral edema in global or focal ischemia stroke (Neumann-Haefelin et al., 2000; Veltkamp et al., 2005). The mechanisms of HBO against ischemic stroke involve alleviating inflammation and oxidative stress (Ding et al., 2017; Misir et al., 2017; Veltkamp et al., 2005).

COX2 plays an important role in post-ischemic neuroinflammation that contributes to the aggravation of brain injury after ischemia stroke. Previous studies indicated that HBO preconditioning decreases inflammation by reducing expression of COX2 in the global cerebral ischemia rat model, and improves neurologic function (Cheng et al., 2011). Myeloperoxidase (MPO) is a pro-inflammatory enzyme that is mainly released by activated neutrophils and macrophages (Loria et al., 2008). MPO is reported to be expressed in the penumbra striatum area in the middle cerebral artery occlusion (MCAO) rat model. HBO treatment for 2 days or 3 weeks decreases expression of MPO, with the longer course treatment more effective in reducing MPO levels (Lee et al., 2013). HBO also suppresses leukocyte accumulation in the brain ischemic area and inhibits neutrophil–endothelial interactions caused by ischemia/reperfusion injury (Buras and Reenstra, 2007; Ding et al., 2014).

The role of HBO in regulating oxidative stress in ischemic stroke remains controversial. HBO may increase production of reactive oxygen species and reactive nitrogen species especially after extensive treatment (Thom, 2009). However, other studies indicated that HBO can stimulate expression of anti-oxidant enzymes, including superoxide dismutase (SOD) and catalase, which can reduce total reactive oxygen species production (Wahhabaghai et al., 2015). In addition, HBO is able to alleviate inflammation and oxidative stress by increasing NO production. But further study is needed to clarify which isoforms of NO synthase (NOS) are important for HBO treatment (Yu et al., 2016). Although it is commonly accepted that reactive oxygen species have a

detrimental role in the progression of post-ischemic stroke injury, delayed HBO treatment improved neurologic recovery in the late-chronic phase of stroke through induction of the hypoxia inducible factor-1 α (HIF-1 α) activated Wnt/ β -catenin pathway by increased reactive oxygen species production (Hu et al., 2014). The sublethal increase in reactive oxygen species was implicated in enhanced endogenous neurogenesis.

3.3. Biologics and inflammation

Limited evidence supports the conclusion that targeting the inflammatory process with biopharmaceutical drugs has utility in attenuating ischemic stroke damage (Table 2). Greater progress in the area is anticipated to be facilitated by the recent commercial development of fully human monoclonal antibodies (Van Taunay et al., 2018). Indeed, an early study targeting intercellular adhesion molecule 1 (ICAM-1), a binding protein for leukocytes on activated endothelial cells, was terminated early because of an adverse immune response. Historically, the technology behind the clinical application of monoclonal antibodies has progressed from murine to chimeric human-mouse to humanized to fully human types. The chimeric mAb, abciximab, which prevents platelet aggregation and thrombus formation, also proved ineffective in treating ischemic stroke; however, this was due to increased brain hemorrhage (Adams et al., 2008). Natalizumab, a humanized mAb against the integrin on T cells that binds the vascular cell adhesion molecule 1 (VCAM-1) on activated endothelial cells was shown to be safe in a small phase II trial of patients with acute ischemic stroke ($n = 79$ treatment/82 placebo) (Elkins et al., 2017). No reduction in infarct size was observed with natalizumab, although some benefits on functional outcomes were seen. A larger multicenter placebo-control study (NCT02730455) involving 270 participants and treatment with two doses of natalizumab at either of 2-time points after stroke was recently completed; however, the results are not yet available.

Two recent studies were designed to test whether immune tolerance towards E-selectin, via nasal delivery of the recombinant protein, was safe in patients who had suffered a stroke and was effective in inducing an anti-inflammatory T cell ($> Th2$ and $> Treg$ vs. $Th1$) profile. Unfortunately, the studies were terminated or suspended, and no results are available. During the propagation phase of inflammation, microglia and macrophages that are polarized towards the pro-inflammatory M1 phenotype secrete IL-1 β and tumor necrosis factor alpha (TNF- α), which act to compromise the BBB (Rayasam et al., 2017). A small ($n = 34$) phase II randomized controlled trial provided promising results that IV delivery of a recombinant human IL-1 receptor antagonist (rhIL-1ra), over 72 h post-stroke might reduce plasma markers of inflammation (CRP and IL-6) and improve clinical outcome in those with cortical infarcts (Emsley et al., 2005; Sobowale et al., 2016). A larger ($n = 80$) follow-up phase 2 randomized controlled trial showed that IL-1Ra was safe, well-tolerated, and reduced circulating inflammatory markers associated with worse clinical outcome (Smith et al., 2018). However, a trend towards improved functional outcome at 3 months was offset by negative effect that was postulated to represent an interaction with the thrombolytic drug, alteplase. Finally, investigators on the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), a large ($n = 10,061$) randomized, double-blind trial, recently reported that MI (myocardial infarction) patients treated with this anti-IL-1 β monoclonal antibody, had lower rate of recurrent cardiovascular events (including stroke), independent of lipid-level lowering (Ridker et al., 2017).

Ischemic stroke has been described as a thromboinflammatory disease, since thrombosis and inflammation are highly intertwined processes that interact at multiple points as contributors to brain injury and stroke progression (De Meyer et al., 2016). Moreover, downstream microvascular thrombosis likely contributes to incomplete reperfusion in $\sim 25\%$ of patients who undergo successful thrombolysis (Alexandrov

Table 1
Promising pre-clinical t-PA adjunct therapy for ischemic stroke.

Mode	Adjunct Treatment	Primary Mechanism	Major Targets	Outcomes	References
BBB protection	Atorvastatin	HMG-CoA reductase inhibitor	ICAM-1 PAR-1 Collagen type IV MMP-9	Reduced Reduced Reduced Increased	(Zhang et al., 2009)
	Batimastat	Broad spectrum MMP inhibitor	MMP inhibitor	Unreported	(Sumii and Lo, 2002)
	Bryostatins	PKC modulator	MMP-9 MMP-2 PKC ϵ PKC α PKC δ	Decreased Not changed Increased Not changed Not changed	(Tan et al., 2015)
	Candesartan	AT1R blocker	MMP-9 MMP-2 MMP-3 NF- κ B TNF- α p-eNOS	Not changed Not changed Decreased Decreased Decreased Decreased	(Ishrat et al., 2013)
	Cilostazol	PDEIII-inhibitor	MMP-9 Claudin 5	Decreased Enhanced	(Ishiguro et al., 2010)
	Cromoglycate	Mast cell inhibitor	MMP-9 MMP-2	Decreased Decreased	(Marinkovic et al., 2014)
	Fasudil	nonselective ROCK inhibitor	Rho kinase	Decreased	(Fukuta et al., 2018)
	Neural Stem Cell + minocycline	Cell replacement	MMP-9	Decreased	(Eckert et al., 2017)
	G-CSF	Cell growth promotor	Ang-1 Ang-2 CD34 eNOS VEGFR2 vWF	Not changed Increased Increased Increased Increased Increased	(dela Pena et al., 2015)
	GM6001	MMP inhibitor	MMP-9 Claudin Occludin ZO-1	Decreased Not changed Enhanced Enhanced	(Mishiro et al., 2012)
Angiogenesis	Imatinib (neutralizing antibody)	PDGFR- α antagonist	PDGF-CC pro-MMP-9 Akt (in vitro) Ang-1 CD31 CD31 + Ki67 MMP-2 Occludin Tie2	Decreased Increased Increased Increased Not co-localized Decreased Increased Decreased	(Su et al., 2008) (Zuo et al., 2014)
	IMM-H004 (Coumarin derivative)				
	Minocycline	Antibiotic	MMP-9	Decreased	(Murata et al., 2008)
	Ascorbic acid	Antioxidant	MMP-9	Decreased	(Allahtavakoli et al., 2015)
	Dodecafluoropentane emulsion nanodroplets	Oxygen transporters	(O ₂ transporter)		(Culp et al., 2015)
	Normobaric Oxygen	Slow BBB damage	MMP-9 Reactive oxygen species	Decreased Decreased	(Henninger et al., 2009; Liang et al., 2015)
Antioxidant					
Oxygen Delivery					

Ang-1, angiopoietin 1; AT1R, angiotensin II type 1 receptor; BBB, blood brain barrier; G-CSF, granulocyte-colony stimulating factor; HMG-CoA, β -Hydroxy β -methylglutaryl-CoA; ICAM-1, intercellular adhesion molecule 1; MMP, matrix metalloproteinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PAR-1, protease-activated receptor-1; PDEIII, phosphodiesterase 3; PDGFR- α , platelet-derived growth factor receptor A; p-eNOS, phospho-eNOS; PKC, protein kinase C; ROCK, rho kinase; Tie2, tyrosine kinase 2; TNF- α , tumor necrosis factor; VEGFR2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor.

et al., 2004; De Silva et al., 2009). CD147 (cluster of differentiations 147) was recently identified as a potential target for ischemic stroke injury linking microvascular inflammation and thrombosis (Jin et al., 2017). This type I transmembrane glycoprotein is expressed by key players in both processes (platelets, leukocytes, and endothelial cells) and is upregulated in the brain after cerebral ischemia. In a mouse model of ischemic stroke (transient MCAO/tMCAO), post-treatment with an anti-CD147 function-blocking antibody (α CD147) preserved BBB integrity, decreased inflammatory cell infiltrates, and reduced microvascular thrombosis, which was associated with reduced intravascular fibrin and platelet accumulation (Jin et al., 2017). At the level of the endothelial cell, treatment with α CD147 reduced activity of MMP9, a known disruptor of the BBB, as well as activation/expression of key modulators of inflammatory and thrombotic responses after ischemic stroke.

To date, the therapeutic utility of biologics for treatment of ischemic

stroke has fallen short of the promise predicted by preclinical models. Hopefully, better identification of the most appropriate targets and the relatively recent commercial development of human monoclonal antibodies will accelerate translational progress in this area.

4. Recently identified molecular/cellular targets and approaches

4.1. Cytochrome P450-derived eicosanoids on vascular inflammation in ischemic stroke

Lipid mediators play key roles in inflammation and brain homeostasis. During the ischemic period, depletion of ATP and high-energy phosphates induces massive cell death, in part via the release of neurotransmitters, such as glutamate, or by diminished activity of the sodium–potassium pump (Fan et al., 2016a). Decreased Na⁺,K⁺-ATPase activity subsequently exerts membrane depolarization resulting in

Table 2
Recent clinical trials using biologics to treat ischemic stroke.

Drug	Target/MOA	Trial	Outcomes/Notes
Abciximab (Fab fragment of the human-murine monoclonal antibody)	Glycoprotein IIb/IIIa receptor of platelets / Inhibits platelet activation and aggregation	NCT00073372	Terminated due to intracerebral hemorrhage.
Canakinumab (Fully human mAb)	IL-1 β	NCT01327846 (CANTOS)	Lower rate of recurrent cardiovascular events
Enlimomab (murine IgG2a mAb)	ICAM-1 / Reduces leukocyte adhesion to EC	Phase III	Terminated due to worse outcome and immune side effects associated with murine antibody
E-Selectin (recombinant protein)	Ligands for EC selectins on leukocytes / Mucosal tolerance	NCT00012454 NCT00069069	Terminated/suspended; results not available
Natalizumab (humanized monoclonal antibody)	Cell adhesion molecule α 4-integrin / Blocks T-cell interaction with EC VCAM-1	NCT01955707	Treatment up to 9 h after stroke did not reduce infarct size; Associated benefits on functional outcomes warrant further investigation
rhIL-1ra/Anakinra	Receptor for IL-1 α or IL-1 β / Antagonist of M1 microglia/M ϕ inflammatory signaling	(a) small (n = 34) phase II randomized controlled trial (b) ISRCTN74236229	(a) Lower blood neutrophil & WBC counts, CRP, and IL-6. Improved functional outcome (b) Lower plasma IL-6 and CRP. No improved favorable outcome on modified Rankin Scale

CANTOS, Canakinumab Anti-inflammatory Thrombosis Outcome Study; CRP, C-reactive protein; EC, endothelial cell; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; mAb, monoclonal antibody; MOA, mechanism of action; M ϕ , macrophage; VCAM-1, Vascular cell adhesion protein 1; WBC, white blood cell.

calcium influx and cell death. Glutamate stimulates receptors elevating intracellular Ca²⁺ levels and cell death, as well as activating various phospholipases including phospholipase A2 (PLA2), phospholipase C and D (PLC and PLD) to release diacylglycerol (DAG) and arachidonic acid (AA) from the cell membrane (Fan et al., 2016a; Ugidos et al., 2017). There is a positive feedback between AA, glutamate release, and depolarization-evoked Ca²⁺ accumulation (Freeman et al., 1990; Ruehr et al., 1997). Considerable evidence indicates that the principal AA metabolites 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) play important roles on vascular inflammation in ischemic stroke.

4.1.1. 20-hydroxyeicosatetraenoic acid

Formation of 20-HETE is catalyzed by the cytochrome P450 (CYP) 4A and 4F enzymes in hepatocytes, renal epithelial cells, cardiomyocytes, neurons, glial cells, leukocytes, platelets, vascular smooth muscle cells (VSMC), endothelial cells, and pancreatic islets (Fan et al., 2016a, 2015b; Tunaru et al., 2018). The effects of 20-HETE via the Gq protein-coupled receptor GPR75 in endothelium and VSMCs (Fan and Roman, 2017; Garcia et al., 2017), and GPR40 in β islet cells (Tunaru et al., 2018) have been reported in a wide variety of physiological and pathophysiological events. 20-HETE inhibits Na⁺, K⁺-ATPase and sodium transport in the kidney (Fan et al., 2015b). In the vasculature, 20-HETE increases vascular tone and impairs vasodilation via a wide range of signal pathways that have been intensively discussed in recent reviews (Fan et al., 2016a, 2015b; Imig, 2016; Wu and Schwartzman, 2011). 20-HETE is critical to maintain the myogenic response and autoregulation in both renal and cerebral circulations (Fan et al., 2015a; Ge et al., 2014). 20-HETE induces angiogenesis, vascular and cardiac hypertrophy, vascular restenosis, platelet aggregation, endothelial dysfunction, inflammation, apoptosis, and promotes glucose-mediated insulin secretion (Fan et al., 2016a; Tunaru et al., 2018). 20-HETE plays an essential role in hypertension, renal disease, ischemic/reperfusion injury in the kidney, brain, and heart, polycystic kidney disease, vascular restenosis, hepatorenal syndrome, preeclampsia, stroke, and dementia (Fan et al., 2016a).

The role of 20-HETE on the onset, development, and recovery in ischemic stroke has not been fully elucidated. After an ischemic stroke or MCAO, 20-HETE levels in plasma and brain tissues is enhanced in patients and in experimental animals (Shekhar et al., 2017a). Inhibition of 20-HETE not only reduces infarct size, but also improves neurological outcomes in MCAO models (Dunn et al., 2008; Renic et al., 2009). Blockade of 20-HETE synthesis alleviated neuronal cell death in brain slices subjected to oxygen and glucose deprivation in association with decreased superoxide production and caspase-3 activation (Renic et al.,

2012). The neuroprotective effect of 20-HETE inhibition could be explained by amelioration of inflammation, as well as oxidative stress, by diminishing recruitment of leukocytes, decreasing production of IL-1, IL-6, chemokine (C-C motif) ligand 2 (CCL2) (aka monocyte chemoattractant protein-1 (MCP-1)), interferon gamma (IFN- γ), TNF- α , and reducing expression of ICAM-1 and VCAM-1 on B-lymphocytes and endothelium (Shekhar et al., 2017a; Toth et al., 2013).

To date, it seems that almost all evidence indicates that 20-HETE has a detrimental impact in ischemic stroke determined by elevated levels after ischemic stroke, enlarging infarct size, and worsening neurological outcomes. However, many questions remain unanswered. First, what are the sources that enhance 20-HETE production after ischemic stroke? This question is especially important in humans. Indeed, the AA release during ischemia and within 24 h of reperfusion (Adibhatla et al., 2006; Ugidos et al., 2017) greatly promotes 20-HETE production. It is well established that hypercholesterolemia is one of the major risk factors for ischemic stroke and there is a good possibility that some of the stroke patients, whose plasma 20-HETE was measured, took fibrates to lower blood triglyceride levels. Notably, fibrates are strong CYP4A inducers that could increase production of 20-HETE (Fan et al., 2016a). Moreover, genetic variants in CYP450 4A and 4F enzymes are associated with higher incidence and changes in plaque stability in patients with ischemic stroke (Fan et al., 2016a). However, variants of CYP4F2 (rs2074900, rs2108622) and CYP4A11 (rs1126742) have only been reported to reduce 20-HETE production in vitro (Fan et al., 2016b; Gainer et al., 2005; Stec et al., 2007); whether other variants actively enhance formation of 20-HETE is not known. Secondly, 20-HETE is a potent vasoconstrictor and plays an important role in maintaining intact myogenic response and autoregulation in cerebral circulation (Fan et al., 2015a). In this regard, 20-HETE could be beneficial to reduce the onset of stroke, since if there is impaired cerebral autoregulation, the brain is more vulnerable to enhanced perfusion pressure in cerebral vessels that would amplify BBB leakage, infiltration of circulating inflammatory factors, glial activation, and neurodegeneration, which are all events that crucially contribute to the onset of stroke and ischemic damages. However, the published reports on the role of 20-HETE on cerebral autoregulation in ischemic stroke is still not elucidated, and further investigation is needed. After acute ischemic injury, cerebral blood flow (CBF) is decreased at 1–2 h after reperfusion, and there is a second fall at 7 h after reperfusion (Huang et al., 2016). Administration 20-HETE synthesis inhibitors in MCAO rodents reduced infarct sizes, had no effect on the fall of CBF during the ischemic period and up to 2 h after reperfusion, but delayed or ameliorated the 2nd fall (Dunn et al., 2008; Marumo et al., 2010; Poloyac et al., 2006; Renic et al., 2009). However, these data are not definitive to conclude that there is impaired

autoregulation of CBF, since the perfusion pressure in cerebral vessels is below the autoregulatory range in acute ischemic status and when the vessels remain occluded during reperfusion. Importantly, these experiments were performed in C57BL/6J mice, Sprague-Dawley, Wistar and Wistar-Kyoto (WKY) rats, spontaneously hypertensive rats (SHR) and spontaneously hypertensive stroke-prone rats (SHRSP) that have normal or elevated endogenous 20-HETE levels (Dunn et al., 2008; Marumo et al., 2010; Misir et al., 2017; Poloyac et al., 2006; Renic et al., 2009). Therefore, results from these studies can only explain the role of 20-HETE on progression of outcomes of ischemic stroke. It has to be mentioned, tissue plasminogen activator (t-PA), the only FDA-approved drug treatment for ischemic stroke patients, and COX inhibitors commonly used for acute ischemic stroke treatment, all could reduce CBF, along with other confounders such as antihypertensive drugs and vasodilators (Fan et al., 2013; Huang et al., 2016; Jordan and Powers, 2012). In fact, our recent studies using Dahl salt sensitive (SS) rats that have decreased 20-HETE production demonstrated that there is impaired autoregulation of CBF, BBB leakage, and neurodegeneration after induction of hypertension (Fan et al., 2018). These are associated with elevated infiltration of B cells and macrophages to the brain. However, whether the preexisting low level of 20-HETE increases genetic susceptibility of onset of ischemic stroke is unknown. More recently, two more CYP4F2 variants (rs771576634, rs115517770) that reduce 20-HETE activity have been reported (Kim et al., 2018), but it remains to be determined if they or the aforementioned variants are linked to stroke.

In summary, 20-HETE is detrimental to the progression of ischemic stroke by inducing inflammation and oxidative stress and neuronal cell death. Theoretically, 20-HETE may reduce the susceptibility of onset of ischemic stroke by maintaining autoregulation of CBF and reducing inflammation; however, evidence supporting this hypothesis is modest. More experimental animal studies and clinical trials are needed indeed.

4.1.2. EETs and soluble epoxide hydrolase inhibitors

Epoxyeicosatrienoic acids (EETs) are eicosanoids produced from arachidonic acid by cytochrome P450 (CYP) epoxygenases that have anti-inflammatory, anti-apoptotic, pro-angiogenic, and vasodilatory actions (Campbell et al., 2017). They are formed by a wide number of cells types relevant to cardiovascular, renal, and cerebral pathologies, most notably by arterial and venous endothelial cells. The four biologically active regioisomers, 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET exhibit variable differences in the rank order of potency in different biological functions or tissues, likely reflecting the contributions of several G protein-coupled receptor subtypes. The anti-inflammatory genomic actions of EETs involve in part inhibition of I κ B kinase and subsequent NF- κ B activation and inflammatory signaling, which includes inhibiting the expression of pro-inflammatory cytokines and adhesion molecules on endothelial cells. EETs also activate nuclear erythroid-related factor 2 (Nrf2), which induces the expression of antioxidant proteins and enzymes. At the cellular level, EETs are postulated to maintain ER-mitochondria homeostasis and limit reactive oxygen species formation by modulating the ER stress response and maintaining mitochondrial function (Inceoglu et al., 2017).

EETs are rapidly converted to less bioactive or even pro-inflammatory diols by soluble epoxide hydrolase (sEH), which is expressed by a number of cells types, most notably by endothelial cells and astrocytes. Its expression may be enhanced by different cellular stresses, including ER stress, thereby attenuating EETs' protective actions (Inceoglu et al., 2017, 2015; Mak et al., 2017). Accumulating evidence has shown that salubrinal, an agent that limits ER stress by enhancing the unfolded protein response, has protective effects on the BBB in ischemic stroke by blocking the progression of the inflammatory response that is linked to microglial reactivity and production of MMP9 (Anuncibay-Soto et al., 2018). Besides attenuating neuronal death, the primary basis for the delayed microglial reactivity, salubrinal may also attenuate loss of EETs, although this possibility needs to be investigated.

Older studies for the most part have demonstrated the neuroprotective effect of sEH inhibition in rodent models of ischemic stroke when delivered before or at the onset of ischemia (Dorrance et al., 2005; Liu et al., 2016; Shaik et al., 2013; Simpkins et al., 2009), and one study in mice documented the effectiveness of sEH inhibition given at reperfusion (Zhang et al., 2007). Recent results using a newer generation sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea (TPPU), with higher potency and longer-circulatory half-life, offers support for the clinical relevance of sEH inhibitors to treat ischemic stroke. TPPU also is more water-soluble and shows less binding to plasma proteins than older sEH inhibitors, and readily crosses the intact BBB. A study using the rat model of focal cerebral ischemia observed that TPPU given IP at the time of reperfusion, inhibited expression of pro-inflammatory cytokines IL-1 β and TNF- α , reduced infarct size, and improved functional outcome (Tu et al., 2018). TPPU also enhanced expression of anti-inflammatory TGF- β , but surprisingly not IL-10, and decreased the number of peri-infarct activated microglia. In addition, TPPU increased neuronal and astrocyte expression of VEGF, an angiogenic and endothelial protective factor. These results suggest that TPPU delivery might be useful as an adjunct therapy for neuroprotection from reperfusion in patients undergoing thrombolysis or thrombectomy. Concurrent with the development of improved sEH inhibitors, has been the synthesis of orally active EET analogs with enhanced biological potency and half-life, attributable in some cases to inhibition of sEH activity (Campbell et al., 2017). The efficaciousness of these third generation EET analogs in preventing ischemic stroke injury awaits investigation.

4.2. Microglia

Microglia are the resident immune cells of the CNS that patrol and act as sentinels of the brain (Nimmerjahn et al., 2005). Upon activation, microglia becomes polarized, adopting either a classically activated M1 phenotype that has pro-inflammatory actions or the alternatively activated M2 phenotype that has anti-inflammatory actions (Prinz and Priller, 2014; Taylor and Sansing, 2013). TREM2 (Triggering Receptor Expressed on Myeloid Cells-2) acts as a sensor for altered micro-environment in the brain enhancing the phagocytic capacity of microglial cells (Stefano et al., 2009). Depletion of TREM2 in vivo severely impairs microglial cell morphology, migration, and capacity to respond to environmental stimuli (Wang et al., 2015b). In ischemic stroke, microglia are activated within minutes and are persistently activated for days (Denes et al., 2007; Taylor and Sansing, 2013).

Failure of anti-inflammatory strategies during stroke could be partly associated with dual or multiphasic roles played by microglia (Jin et al., 2010a; Lai and Todd, 2006). Thus, targeting microglial activation after stroke may provide an effective way to limit brain injury caused by stroke. Also, the relatively long time interval (many hours) between the onset of ischemia and a fully developed microglial activation state makes targeting microglial response clinically feasible.

- Studies indicate that while ablation of proliferative microglia exacerbated ischemic damage (Lalancette-Hebert et al., 2007; Szalay et al., 2016), implantation of cultured microglia reduced damage (Kitamura et al., 2004; Szalay et al., 2016).
- Mammalian target of rapamycin (mTOR) pathway is a known regulator of immune responses, and inhibition of the mTORC1 pathway pharmacologically or genetically prevents microglial polarization toward the M1 pro-inflammatory phenotype in the context of ischemic stroke (Li et al., 2016; Xie et al., 2014). This is associated with reduced secondary damage and improved motor function. Both direct effects on macrophages and microglia, as well as enhanced anti-inflammatory activity of regulatory T cells (Tregs) have been implicated.
- Poly (ADP-ribose) polymerases (PARPs) catalyze the transfer of ADP-ribose units from NAD⁺ to target proteins including histones

and transcriptional factors. Several studies have examined the effects of PARP inhibitors after stroke and have found that they suppress microglial activation and improve neuronal survival (Hamby et al., 2007; Kauppinen et al., 2009; Takahashi et al., 1997).

- Minocycline therapy initiated one day after focal ischemia and continued for 13 days resulted in reduced microglial activation, improved scores on behavioral tests, and improved survival rate (Hayakawa et al., 2008).
- A direct beneficial effect of targeting inflammation is supported by studies showing that post-insult treatment with HDAC inhibitors valproic acid and sodium butyrate suppressed microglial activation, reduced the number of microglia, and inhibited other inflammatory markers in the ischemic brain (Kim et al., 2007).

In summary, these results suggest that inhibition of the mTORC1 pathway, PARPs, and HDACs or targeting of TREM2 or minocycline therapy may prove useful in attenuating microglial polarization toward the M1 pro-inflammatory type, which could ameliorate the ischemic brain injury.

4.3. T cells and IL-17

The first response to ischemic stroke reperfusion is the local and innate immune response that triggers activation of microglia, macrophages, and neutrophils. Microglia, which are the resident macrophages of the brain, are the first line of defense and respond quickly. They are activated by damage associated molecular patterns (DAMPs), which are released from the damaged brain cells after ischemic stroke (Kigerl et al., 2014). Activation of these microglia can be both beneficial and deleterious to the central nervous system (CNS). Activation of microglia can generate an enhanced inflammatory response, increase oxidative stress, and neuronal damage. These DAMPs will increase the infiltration of neutrophils through toll-like receptors and inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-17 (Kigerl et al., 2014). These signals and activation of the receptors will upregulate adhesion receptors (CD15 and CD11b) that allow neutrophils to bind to endothelial cells and migrate to the damaged area (Mantovani et al., 2011). The presence of neutrophils can cause more injury to the CNS by increasing inflammation, releasing lytic enzymes that destroy brain tissue, and by causing cerebral vascular blockage (del Zoppo et al., 1991; Mantovani et al., 2011; Zheng and Yenari, 2004).

Ischemic reperfusion injury also includes the adaptive immune response, in which both T and B lymphocytes play a major role and infiltrate into the CNS. Several studies have shown that ischemic stroke patients have higher serum antibody titers, derived from B cells, and circulating T cells versus normal patients (Bornstein et al., 2001; Planas et al., 2012). The exact antigen for activation of the adaptive response is not fully known or understood; however, some evidence points to the innate immunity being responsible for the presence of antigens needed to initiate the adaptive immune response (Planas et al., 2012). T cells are believed to be the most responsible for the CNS injury after stroke. Evidence to support this claim are from studies in which total lymphocyte deficient mice were protected from stroke (Hurn et al., 2007). In this study lymphocyte deficient male mice were given ischemic stroke induced by the MCAO surgery (Hurn et al., 2007). Twenty-two hours after MCAO, these mice were compared to their normal C57BL/6 mice to which they had a reduction in infarct volume and inflammatory cytokines (Hurn et al., 2007). Furthermore, B cell-deficient mice were not protected against stroke, supporting the notion that T cells are critical for CNS injury after ischemic stroke (Hurn et al., 2007).

The post-ischemic stroke brain will recruit T cells (CD4⁺) to the site of injury after stroke. These T cells can release cytotoxic molecules to cause brain injury (Arumugam et al., 2005). Both Th1 and Th17 cells are known to be present in ischemic stroke brain and play a role in stroke pathogenesis (Arumugam et al., 2005). They both produce pro-inflammatory cytokines, such as IL-2, IL-12, IFN- γ , TNF α , and IL-17

which contribute to the pathology of stroke (Arumugam et al., 2005). Activation of Th1 cells in the CNS can promote vascular permeability, which can damage the BBB and activate microglia, neutrophils, and brain endothelial cells (Arumugam et al., 2005). Th2 cells on the other hand are neuroprotective and secrete the anti-inflammatory cytokines, such as IL-4, IL-5, IL-10, and IL-13 (Arumugam et al., 2005).

An important cytokine that has recently been associated with ischemic stroke is interleukin 17 (IL-17). IL-17 is a pro-inflammatory cytokine produced primarily by Th17 and gamma delta T cells ($\gamma\delta$ T cells) (Waisman et al., 2015). These cells are also hypothesized to play a role in the pathology of other CNS diseases, such as multiple sclerosis (MS) and depression (Waisman et al., 2015). IL-17 contributes to neurological damage, infarct size, and inflammation post-ischemic stroke. Furthermore, the presence of IL-17 has been shown to impair BBB integrity in MS patients and rodent models of stroke (Waisman et al., 2015). IL-17 was first discovered in the late 1990's in both mice and humans, and shown to mobilize and activate neutrophils to the site of inflammation, activate NF- κ B signaling, IL-6 secretion (a pro-inflammatory cytokine associated with stroke), and proliferation of T-cells (Yao et al., 1995a, 1995b). IL-17, also known as IL-17A, was the first discovered among a family of 6 cytokines (IL-17 A-F) (Fossiez et al., 1998; Waisman et al., 2015). This cytokine is ~ 32 kDa and binds to a receptor family of 5 members that are ubiquitously distributed throughout all organs of body, but more concentrated in spleen and kidney (Fossiez et al., 1998).

Several human and rodent studies have shown an increase in IL-17 expressing cells in the tissue and circulation after ischemic stroke (Gelderblom et al., 2012; Kostulas et al., 1999; Li et al., 2005, 2001; Shichita et al., 2009; Waisman et al., 2015). In fact, a Swedish study by Kostulas and colleagues showed that IL-17 mRNA expressing peripheral blood mononuclear cells (PBMCs) along with other cytokines (IL-8, IL-1 β), was elevated in patients as early as 1–3 day post stroke (Kostulas et al., 1999). Additionally, there was a positive correlation between the amount of IL-17 expressing PBMCs and the severity of stroke according to the Scandinavian stroke scale (Kostulas et al., 1999). However, 20–31 days post stroke, IL-17 levels were similar to healthy patients (Kostulas et al., 1999). Thus, patients with the worst stroke outcomes had higher levels of circulating IL-17 that appear early after ischemic stroke. In another study with Chinese ischemic stroke patients, levels of IL-17 mRNA were elevated in the ischemic hemisphere of the brain versus the normal hemisphere (Li et al., 2005). These findings were also replicated in the Sprague-Dawley rat with ischemic stroke via the MCAO method of inducing ischemic stroke (Li et al., 2005, 2001). In the rat, IL-17 levels were elevated as early as 1 h post-surgery and peaked at 6 days, and then returned to basal levels around day 20 (Li et al., 2005, 2001). Therefore, it can be concluded that in both the rodent and human with ischemic stroke, IL-17 appears to be elevated early and may play a significant role in the inflammatory response, infarct size, and neurological outcomes after ischemic stroke.

To test the hypothesis that IL-17 plays a significant role in inflammation and the pathology associated with ischemic stroke, studies were conducted on IL-17 deficient mice with MCAO and MCAO treated mice with IL-17 neutralizing antibodies (Gelderblom et al., 2012; Shichita et al., 2009). Mice with MCAO injury and administration of IL-17 neutralizing antibodies at day 3 post stroke, displayed a decrease in infarct size, improved neuroglial deficient, and diminished neutrophil infiltration (Gelderblom et al., 2012). In a landmark paper, IL-17 and $\gamma\delta$ T cell knockout mice with MCAO had a smaller infarct size, decreased inflammatory response, and reduction in neurological deficits, mortality rate, and apoptosis of neuronal cells (Shichita et al., 2009). In this study, the authors concluded that depletion of $\gamma\delta$ T cells, via knockout mice or administration of $\gamma\delta$ T neutralizing antibodies, completely ameliorated the neurological damage from I/R injury (Shichita et al., 2009). Therefore, this study suggests that $\gamma\delta$ T cells are the major contributors of IL-17 and neurological damage after ischemic stroke. Hence, therapies against IL-17 or $\gamma\delta$ T cell directly after the ischemic

stroke could be promising, especially since IL-17 seems to appear early after ischemic stroke and play a role in the pathology of stroke. Thus far, targeting IL-17 has not caused any negative side effects. However, as a cautionary note, targeting cytokines and inflammatory cells can be counterproductive and deleterious, thus more studies are warranted on the blockade of IL-17 in ischemic stroke.

IL-17 plays a role in BBB breakdown, which occurs in patients and rodents after ischemic stroke. There are several mechanisms as to how IL-17 can cause BBB damage. One mechanism is by Th17 lymphocyte migration across the BBB. Migration of Th17 cells across the BBB into the brain can increase IL-17 secretion and thus generate a pro-inflammatory response in the brain to increase lymphocyte recruitment and cytokine secretion (Kebir et al., 2007). IL-17 on astrocytes in the CNS has been shown to increase the inflammatory cytokines CCL2, IL-6, TNF- α , and IL-8 (CXCL8); all of which are known to cause neuronal damage (Waisman et al., 2015). Studies by Kebire and colleagues showed that Th17 cells in the brain have cytolytic activity that can increase the production of granzyme B, which has the capacity to kill neurons (Kebir et al., 2007). Furthermore, in this study they showed that brain endothelial cells that line the BBB have receptors for IL-17, which increase permeability of the membrane. *Ex vivo* experiments with a monolayer of human BBB-ECs and *in vivo* experiments with experimental autoimmune encephalomyelitis (EAE) mice showed that IL-17 decreased expression of two important tight junction proteins, occludin and zonula occludens (ZO)-1, which keep the BBB membrane intact (Kebir et al., 2007). Additionally, by activating IL-17 receptors, IL-17 disrupts the tight junctions in the BBB and allows for uncontrolled lymphocyte and cytokine infiltration from the circulation. A third mechanism is by IL-17-mediated induction of NADPH oxidase reactive oxygen species generation, which can alter endothelial cell morphology by causing endothelial cell contraction at the BBB and downregulation of tight junction proteins (Huppert et al., 2010). Reactive oxygen species can lead to BBB destabilization via activation of mitogen-activated protein (MAP) kinase cascades, NF- κ B expression, tight junction protein turnover, apoptosis of endothelial cells, and increased matrix metalloproteinases (Abdullah and Bayraktutan, 2014; Aslam et al., 2012; Huppert et al., 2010; Voigt et al., 2013).

In summary, the role of inflammation has been shown to play a major role in the pathophysiology of ischemic stroke. IL-17 blockade appears to decrease neurological damage after ischemic stroke. Thus, therapies designed to target IL-17 or cells (Th17 or $\gamma\delta$ T-cells) that produce IL-17 are needed for ischemic stroke patients. Furthermore, other therapies that suppress Th1 pro-inflammatory cell activation and enhance Th2 cells anti-inflammatory actions should be considered.

4.4. miRNAs

miRNAs are small noncoding RNAs that control the expression levels of proteins by regulating mRNA translation or stability. A full accounting of the potential role of miRNAs as therapeutic targets or agents for ischemic stroke is beyond the scope of our review and the reader is referred to some recent articles on the topic (Chandran et al., 2017; Gaudet et al., 2017; Kaur et al., 2018; Khoshnam et al., 2017). Rather, we focus on several miRNAs that have garnered considerable interest and are illustrative of the potential benefits and challenges of this approach.

miR-130a is generally considered pro-angiogenic and is expressed by microvascular endothelial cells of the BBB, among other cell types. However, conflicting evidence is reported on whether its circulating levels are increased or decreased in acute ischemic stroke patients (Jin and Xing, 2017; Tan et al., 2009). Nonetheless, a recent animal and cell culture study reported evidence that ischemia-induced miR-130a in endothelial cells compromised BBB integrity by targeting Homeobox 5A, a transcription factor linked to the expression of the tight junction protein occludin (Wang et al., 2018b). Delivery of an antagomir miR-130a within 30 min of MCAO reduced brain edema, BBB permeability,

and infarct size, and improved neurological function. As discussed, the basic conclusion that miR-130a negatively regulates BBB integrity with ischemia is supported by the preclinical findings of others, although more than one mechanism has been implicated.

miR-124 is highly expressed in the nervous system and is considered anti-inflammatory (Gaudet et al., 2017). Early in ischemic stroke it is released by damaged neurons and is upregulated by the anti-inflammatory Th2 cytokines IL-4 and IL-13 (Rayasam et al., 2017). miR-124 plays a critical role in polarizing myeloid cells towards the M2 anti-inflammatory phenotype in part by targeting components of IL-6 and TNF- α signaling (Gaudet et al., 2017; Rayasam et al., 2017). But miR-124 also attenuates toll-like receptor signaling that likely is important for tissue repair (Gaudet et al., 2017). Evidence from several rodent studies have provided support for the idea that miR-124 is neuroprotective when delivered acutely by polarizing brain microglia/macrophages towards an anti-inflammatory M2 phenotype (Doepfner et al., 2013; Hamzei Taj et al., 2016; Sun et al., 2013). Prior knockdown, however, proved to have beneficial actions on infarct size and functional outcome (Zhu et al., 2014), suggesting that miR-124 may also have detrimental actions. Besides the identified harmful effects on neurons, miR-124 may upregulate reactive oxygen species production and cause apoptosis of brain vascular endothelial cells by attenuating protective PI3K/AKT signaling (Wang et al., 2018a).

miR-155 is a multifunctional microRNA that is specifically expressed in hematopoietic cells and cells involved in vascular remodeling, including endothelial cells, T-cells, and myeloid cells (Roitbak, 2018). Tail vein injection of a specific anti-miR-155 inhibitor was reported to promote functional recovery in a mouse model of stroke when carried out 48 h later (Caballero-Garrido et al., 2015; Pena-Philippides et al., 2016). This action was associated with improved blood flow, reduced infarct size and edema, attenuated neuronal damage and inflammatory cytokine/chemokine presence, and preserved microvascular integrity in the peri-infarct area. These two study as well as several others, have attributed the beneficial actions of miR-155 inhibition to an early (~7 days) upregulation (de-repression) of key molecules linked to signaling networks in endothelial cells important for barrier function of the microvascular tight junctions, increased vasodilation, angiogenesis, cytoprotection, and cell survival. These include SMAD5, Rictor, eNOS, Rheb, and ZO-1. Inhibiting miR-155 may also attenuate JAK/STAT inflammatory signaling and M1 microglia/macrophage polarization by upregulation of SOCS1 and SHP-1 (Roitbak, 2018). At later time points (~14 days), increased expression of the miR-155 target C/EBP- β , could further modulate the inflammatory response by upregulating expression of anti-inflammatory or modulatory cytokines (e.g., IL-10, IL-4, and IL-5), along with increased inflammatory cytokines (IL-6 and IL-17) and M1 polarization of microglia and macrophages (CD68). Thus, inhibiting miR-155 may serve to tamp down early reactive inflammation by strengthening BBB function and inhibiting M1 polarization, while at later times miR-155 inhibition would favor a controlled inflammatory, phagocytic M1 microglia/macrophage milieu to clear necrotic debris and is thus reparative (Roitbak, 2018).

Although holding much promise, miRNA-based therapeutics faces many challenges before it becomes common practice. These challenges include delivery issues, as well as untoward and unpredicted actions due to multiple targets and divergent signaling events downstream of the intended target.

5. Emerging pharmacological approaches

Drug delivery to the ischemic tissue poses a challenge to the drug development and scientific community alike. Currently, to effectively reach the tissue, the drug needs to be delivered systemically and at a higher dose. That alone comes with significant side effects such as global immunosuppression in case of anti-inflammatory drugs. To address this, different pharmacological drug delivery mechanisms, e.g., nanoparticles (NP) are currently being investigated. Micelles, inelastic

spherical shells, nanotubular particles, liposomes, golden NPs, and polymers fall into the category of NP (Panagiotou and Saha, 2015). The use of liposomal encapsulation technology (LET) is a method of generating sub-microscopic lipid bilayer vesicles called liposomes, which encapsulate numerous materials. These liposomes have both lipophilic and hydrophilic properties and act as a safe vehicle agent in drug delivery. Conjugating these liposomes results in better target penetration, e.g. in myocardial cells (Dasa et al., 2017) and CNS (Wang et al., 2015c). HAIYPRH (T7), a peptide targeted to the transferrin receptor that is highly expressed on brain capillary endothelial cells, can mediate transport of nanocarriers across the BBB. Recently, T7-conjugated PE-Gylated liposomes loaded with the neuroprotectant agent ZL006, which blocks neuronal NOS and PSD-95 interaction, reduced infarct volume and neurological deficits in the rat MCAO model when delivered IV at the time of reperfusion (Wang et al., 2015c). In addition, dexamethasone conjugated DNA nanotubes demonstrated a promising anti-inflammatory response in post-ischemic mice muscle tissue (Sellner et al., 2017).

Apelin-13 is a neuroprotective peptide originally found in bovine stomach tissue extracts. Intranasal delivery of Apelin-13 after focal ischemic stroke in mice resulted in decreased inflammation, infarct volume, and neuronal death in the penumbra, while promoting angiogenesis and long-term functional recovery (Chen et al., 2015). In another study, the role of circular RNA DLGAP4 (circDLGAP4), which acts as an endogenous sponge for miR-143, was investigated. Plasma levels of circDLGAP4 are decreased in acute ischemic stroke patients. Overexpression of circDLGAP4 by prior lentiviral delivery reduced infarct area and BBB damage in the tMCAO mouse model by attenuating the harmful consequences that miR-143 has in endothelial cells on BBB integrity (Bai et al., 2018).

Promising results were recently reported for a novel DNA complex that contains both a DNA decoy to inhibit NF- κ B activity and a DNA aptamer as ligand of the transferrin receptor to target delivery to brain endothelial cells (Hu et al., 2016). This DNA complex demonstrated anti-inflammatory actions at the level of brain endothelial cells in vivo in the mouse lipopolysaccharide (LPS)-induced model of inflammation, suggesting that it could be utilized in the inhibition of inflammation in the ischemic stroke and other neuro-inflammatory diseases affecting cerebral vasculature. Another promising pharmacological approach targeting BBB breakdown is Sac-1004, a pseudo-sugar derivative of cholesterol, which enhances endothelial barrier by stabilizing the cortical actin ring. Sac-1004 suppressed IL-1 β -induced monolayer hyperpermeability of human brain microvascular endothelial cell loss of tight junctions, and expression of adhesion molecules and activation of NF- κ B (Zhang et al., 2017). In a rat model of acute transient cerebral ischemia, Sac-1004 reduced BBB leakage and loss of tight junction proteins, inhibited glial activation, and ameliorated neurological deficits and ischemic damage (Zhang et al., 2017).

6. Conclusions and future directions

It is evident that the interaction between inflammation and stroke is multifaceted. By understanding the mechanisms behind the pro-inflammatory state during the acute to chronic stage will help in developing focused anti-inflammatory medications. By extending the thrombolytic therapy window, more patients will reap rt-PA benefit and have less long-term disability. Developing agents to block pro-oxidants and target pro-inflammatory endothelial cell events and preserve the BBB, modulating eicosanoid pathways (20-HETE), and utilizing biologics to directly counteract damaging inflammation could be used as effective adjunct therapies to exponentially extend the clinical benefit of thrombolysis and thromboectomy to many more ischemic stroke victims; utilizing the beneficial effects of controlling microglial activation, miRNA-based treatments, and pro-angiogenic novel therapies will assist neural plasticity and foster faster, more complete recovery after mild to moderate size stroke and hopefully from even devastating larger

hemispheric infarction.

Beyond targeting inflammation during acute and subacute stroke, the role of targeting inflammation during the chronic stroke is very limited. After the subacute phase, there is Wallerian degeneration, cortical reorganization, axonal sprouting, and neuronal GABA activity in both hemispheres (Brown et al., 2007; Paik and Yang, 2014). There is continued increased activities of T and B cells in the chronic stage of infarction (Doyle et al., 2015; Vindegaard et al., 2017). Nonetheless, some pro-inflammatory effects may not be all detrimental and may assist recovery and restoration of the healthy tissue (Peruzzotti-Jametti et al., 2014). *In vivo* studies on neural stem cells for neural restoration had shown some promising results (Bacigaluppi et al., 2009; Pollock et al., 2006; Smith et al., 2012) leading to clinical trials, including a single center open-label, phase III trial that is actively recruiting in South Korea (NCT01716481, clinicaltrials.gov). Stem cell restoration therapy not only has an anti-inflammatory role, but may also help in new neural cell replacement and neural plasticity (Sinden et al., 2017). It is still not clear what the optimal time for neural stem cell transplant is, and thus the research community is looking forward to a successful phase III clinical trial. The role of genetics cannot be ignored. Future treatment lies in focusing on developing drugs that are specific to patient DNA fingerprint, e.g., multiple genes have been associated with higher risk of ischemic injury throughout life. Finding specific biomarkers, e.g. biochemical, imaging, and DNA biomarkers would help in achieving these goals. Last but not the least, the development of primary preventative medications are much desired for overall risk reduction.

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