Title

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Journal

Cerebrovascular and Brain Metabolism Reviews, 35(6)

ISSN

1040-8827

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Publication Date

2015-06-01

DOI

10.1038/jcbfm.2015.45

Peer reviewed

Journal of Cerebral Blood Flow & Metabolism (2015) 35, 888–901

www.jcbfm.com

REVIEW ARTICLE

Targeting neutrophils in ischemic stroke: translational insights from experimental studies

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Neutrophils have key roles in ischemic brain injury, thrombosis, and atherosclerosis. As such, neutrophils are of great interest as targets to treat and prevent ischemic stroke. After stroke, neutrophils respond rapidly promoting blood–brain barrier disruption, cerebral edema, and brain injury. A surge of neutrophil-derived reactive oxygen species, proteases, and cytokines are released as neutrophils interact with cerebral endothelium. Neutrophils also are linked to the major processes that cause ischemic stroke, thrombosis, and atherosclerosis. Thrombosis is promoted through interactions with platelets, clotting factors, and release of prothrombotic molecules. In atherosclerosis, neutrophils promote plaque formation and rupture by generating oxidized-low density lipoprotein, enhancing monocyte infiltration, and degrading the fibrous cap. In experimental studies targeting neutrophils can improve stroke. However, early human studies have been met with challenges, and suggest that selective targeting of neutrophils may be required. Several properties of neutrophil are beneficial and thus may important to preserve in patients with stroke including antimicrobial, antiinflammatory, and neuroprotective functions.

Journal of Cerebral Blood Flow & Metabolism (2015) **35,** 888–901; doi:10.1038/jcbfm.2015.45; published online 25 March 2015

Keywords: atherosclerosis; cerebral ischemia; immune system; neutrophil; stroke; thrombus

INTRODUCTION

Ischemic stroke is a leading cause of adult disability, cognitive impairment, and mortality worldwide. Though major advances in our understanding of cerebral ischemia have been made, there remains a great need to better prevent and treat stroke. Neutrophils have important roles in acute ischemic brain injury, atherosclerosis, and thrombus formation. As such, neutrophils are of interest as treatment targets to decrease ischemic brain injury and prevent stroke.

Neutrophils are among the first cells in the blood to respond after ischemic stroke, contributing to disruption of the blood brain barrier (BBB), cerebral edema, and brain injury.[1] This is mediated by factors released from neutrophils including reactive oxygen species (ROS) (superoxide, hypochlorous acid), proteases (matrix metalloproteinases, elastase, cathepsin G, proteinase 3), cytokines (IL-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α)), and chemokines (CCL2, CCL3, CCL5) (Figure 1).[2] Neutrophils also are involved in the major processes that cause ischemic stroke, thrombosis and atherosclerosis.[3–5] They promote clot formation through interactions with platelets, proteolytic cleavage of clotting factors (tissue factor pathway inhibitor (TFPI) and coagulation factor X), and release of prothrombotic molecules (neutrophil extracellular traps (NETs) and tissue factor). Neutrophils promote atherosclerosis and plaque rupture by enhancing monocyte infiltration, producing oxidized low density lipoprotein (oxLDL), and releasing proteolytic enzymes that degrade the fibrous cap.

Given the important roles of neutrophils in ischemic stroke, they have emerged as treatment targets. In experimental studies, targeting neutrophils can reduce infarct size and improve stroke outcomes. However, early studies in humans have been met with challenges, and neutrophil directed treatments have as yet to translate to patients with stroke. In this review, we summarize the roles of neutrophils in ischemic stroke and discuss their potential as targets in ischemic brain injury, atherosclerosis, and thrombosis. Aspects important to the translation of neutrophil therapy to patients with ischemic stroke are discussed.

NEUTROPHIL RESPONSE IN ISCHEMIC STROKE

In patients with ischemic stroke, the number of circulating neutrophils rise within the first few hours of stroke onset (Figure 2).[6] This increase is associated with stroke severity,[7] infarct volume,[8] and worse functional outcomes.[9] In contrast to neutrophils, lymphocytes decrease after ischemic stroke. Thus, the neutrophil-to-lymphocytes ratio is increased after stroke, and is associated with mortality and infarct size.[10]

The rise in neutrophils after stroke occurs as a result of enhanced production, increased release from the bone marrow and spleen, and possibly from a reduction in neutrophil apoptosis.[11] Neutrophils express several endothelial adhesion molecules (P-selectin glycoprotein ligand-1 (PSGL-1), ESL-1, CD44, lymphocyte function-associated antigen 1 (LFA-1), and macrophage-1 antigen (MAC-1)) within 15 minutes of ischemia (Figure 3). By 2 hours, neutrophil rolling and adhesion is present in the pial vessels of the brain.[12–14] After 6 to 8 hours, neutrophils have surrounded cerebral vessels and infiltration has begun.[15,16] By 24 to 48 hours of ischemic stroke, neutrophil infiltration into brain has peaked.[14,17] The increase in neutrophils after ischemic stroke is associated with increased expression of adhesion molecules, cytokines/chemokines, proteases, and ROS (Table 1).

A preponderance of data to date suggest neutrophil pro-inflammatory activation after stroke is associated with increased

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Received 19 September 2014; revised 23 January 2015; accepted 26 January 2015; published online 25 March 2015

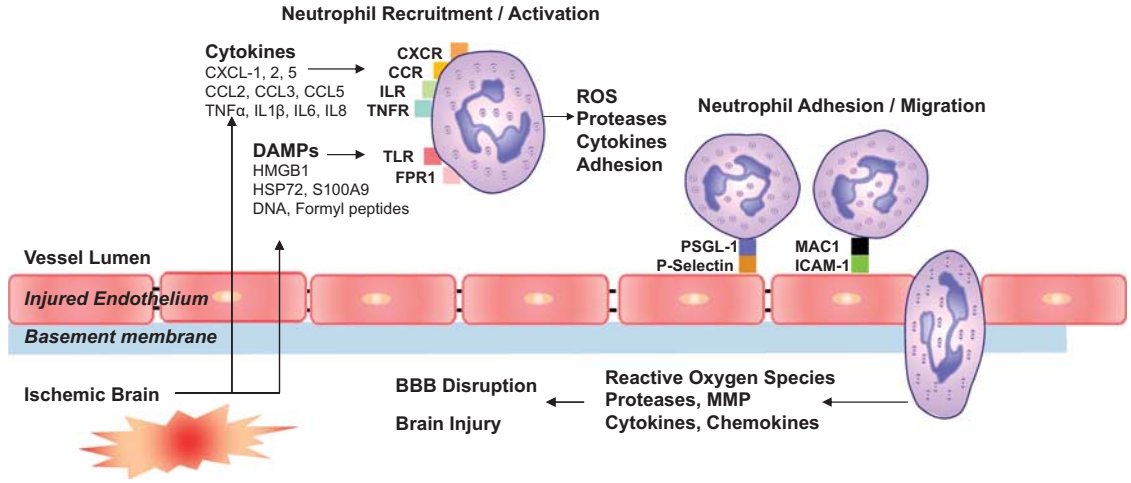

Figure 1. Neutrophils activation and adhesion in acute ischemic stroke. After ischemic brain injury, a number of cytokines and DAMPs are released. These promote neutrophil recruitment and activation, including the release of reactive oxygen species, proteases, and cytokines. Neutrophils adhere to activated endothelium through adhesion molecules that promote neutrophil–endothelial interactions and neutrophil migration with resulting effects on the blood–brain barrier and brain parenchyma. CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; CCR, CC-chemokine receptor; CXCR, CXC chemokine receptor; DAMPs, damage-associated molecular patterns; IL, interleukin; ILR, interleukin receptor; FPR, formyl peptide receptor; HMGB1, high mobility group box 1; HSP72, heat-shock protein 72; ICAM-1, intracellular adhesion molecule-1; MAC-1, macrophage 1 antigen; MMP-9, matrix metalloproteinase 9; PSGL-1, P-selectin glycoprotein ligand-1; TLR, toll-like receptor; TNF*α*, tumor necrosis factor alpha.

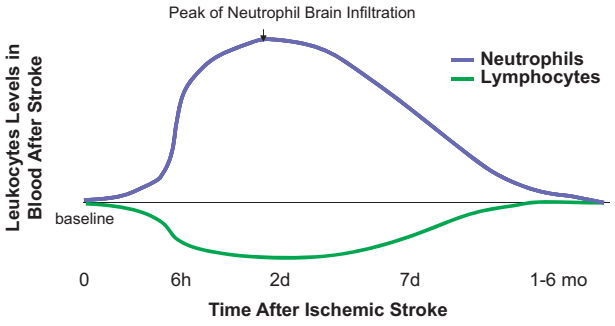

Figure 2. Change in neutrophil and lymphocyte count in blood over time after ischemic stroke.

infarct size, increased BBB disruption, hemorrhagic transformation (HT), and worse neurologic outcomes. These studies are summarized below. However, it is important to recognize that studies of neutrophils in stroke have been hampered by the lack of markers specific to neutrophils. Many of the markers associated with neutrophils can also be present on other immune cells. For example, myeloperoxidase (MPO) is often interpreted as a marker of neutrophils, however, is also expressed by monocytes/macrophages and microglia. Thus, when interpreting studies one must determine whether reported associations are truly reflective of neutrophils versus a potential contribution from other cells.

TARGETING NEUTROPHILS IN ACUTE ISCHEMIC STROKE

In patients with stroke, the degree of neutrophil accumulation in regions of cerebral ischemia correlates with stroke severity and worse stroke outcome.[18] Similar findings have also been observed in experimental studies. This accumulation occurs early, at the same time as brain injury. As a result, interest in targeting neutrophils as an avenue to reduce brain injury in ischemic stroke developed. However, uncertainty remains as to whether neutrophil accumulation is merely a response to brain ischemia versus a contributing factor to brain injury.[19]

Several studies have begun to evaluate neutrophils as treatment targets to reduce ischemic brain injury and improve stroke outcomes. After ischemic stroke, neutrophils are recruited to ischemic brain.[2,20] This entails a series of steps involving activation of neutrophil expression and release of proinflammatory factors (cytokines, proteases, and ROS), neutrophil rolling, adhesion, tethering, and transmigration across cerebral endothelium (Figure 3).[2,20] Targeting neutrophils at one or more of these steps has been assessed as treatments for acute ischemic stroke including: (1) reducing neutrophil activation and recruitment; (2) blocking neutrophil adhesion to endothelial cells; and (3) blocking neutrophil transmigration and neurovascular interactions. Targeted molecules are summarized in Table 2 and presented in detail below.

TARGETING NEUTROPHIL ACTIVATION AND RECRUITMENT IN ISCHEMIC STROKE

A number of factors released after brain ischemia act on neutrophils including cytokines, chemokines, and damage-associated molecular patterns (DAMPs) (Figure 1).[2] These factors activate neutrophil expression of proinflammatory molecules, ROS, cytokines/chemokines, and proteases (neutrophil activation). They also result in the recruitment of neutrophils from the bone marrow, spleen, and peripheral circulation to the site of injury and the expression of adhesion molecules.

Chemokine and Cytokine Activation of Neutrophils

Several chemokines and cytokines released after ischemic brain injury act on neutrophils to initiate recruitment and activation. The CXC chemokines CXCL1, CXCL2, and CXCL5 (CXCL8 in humans) and their receptors CXCR1, CXCR2, and CXCR4 are increased and contribute to neutrophil release from bone marrow, recruitment to ischemic tissue, and expression of adhesion molecules.[21–23] In human stroke, CXCL5 and CXCL1 are increased in the cerebral spinal fluid (CSF).[24,25] CC-Chemokines CCL2, CCL3, and CCL5 are also

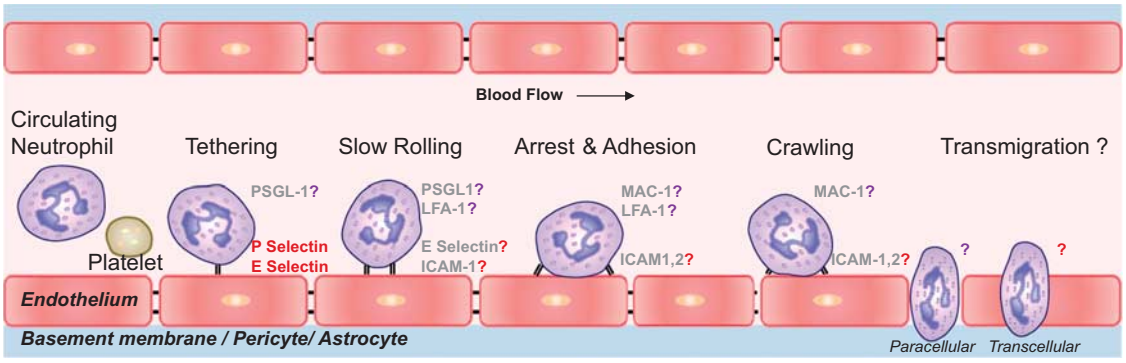

Figure 3. Neutrophil recruitment, adhesion, and transmigration. Neutrophils express specific adhesion molecules that bind activated endothelium to promote adhesion and migration. In ischemic stroke, the adhesion molecules expressed on neutrophils and endothelium involved in neutrophil tethering, rolling, arrest, adhesion, crawling, and transmigration remain poorly defined and may differ from the peripheral vasculature. Further studies are required to delineate the molecules involved in the neutrophil recruitment cascade after ischemic stroke and to determine the extent of transmigration. '?' indicates unclear role in ischemic stroke; ICAM-1, intracellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen 1; MAC-1, macrophage 1 antigen; PSGL-1, P-selectin glycoprotein ligand-1.

increased after ischemia and contribute to neutrophil recruitment and activation through receptors CCR1, CCR2, and CCR5.[26]

Inhibition of chemokines and chemokine receptors has shown variable results in ischemic stroke. In a mouse ischemic stroke model, inhibition of CXC chemokines with Evasin-3 impaired neutrophil activation but had no effect on stroke outcomes.[27] However, inhibition of CXCR1 and CXCR2 with Reparixin in a rat stroke model did reduce ischemic brain injury, improved motor outcomes, and reduced brain levels of MPO and interleukin 1β.[28] When the CXCR2 receptor alone is blocked with SB225002, no improvement in stroke outcomes occurs despite a reduction in neutrophil activation and infiltration.[29] Thus, it appears that therapies targeting CXC chemokines or CXCR1 and CXCR2 have uncertain applications in ischemic stroke, as improvement in stroke outcome has not consistently been observed.

CCR2 is another chemokine receptor that mediates neutrophil recruitment. When stroke is induced in a CCR2 knockout mouse, infarct volume is reduced as is cerebral edema and neutrophil infiltration into ischemic brain.[30] However, CCR2 knockout also reduces monocyte activation. Thus, therapies targeting CCR2 are not neutrophil specific and it remains unclear whether the beneficial effects in ischemic stroke relate to neutrophils or monocytes.

Tumor necrosis factor and IL-1β also contribute to neutrophil activation and are increased in ischemic stroke.[31,32] In animal stroke models. IL1-receptor antagonism decreases invasion of peripheral immune cells (including neutrophils) into ischemic brain, reduces infarct size, cerebral edema, glial activation, and improves behavioral outcomes.[33] In patients with acute ischemic stroke, treatment with intravenous IL1-receptor antagonism has been shown to be safe and to be associated with a reduction in circulating neutrophils and IL-6.[34] In experimental stroke, neutralizing TNF with antibodies or binding proteins reduces infarct size and improves outcome.[35] In mice deficient in TNF infarct size and behavioral outcomes are improved.[36] It is important to note that TNF and IL1β act on many cell types and have multiple effects. Thus, whether the observed effects of TNF or IL1β modulation on ischemic stroke are mediated through neutrophils or other cell types remains unclear. Another molecule involved in neutrophil chemotaxis is the GABA type B receptor 2. In ischemic stroke, it has been shown to stimulate neutrophil chemotaxis.[37]

Damage-Associated Molecular Patterns/Toll-Like Receptors

Ischemic brain injury results in the release of a number of DAMPs, including high mobility group box 1 (HMGB1), Hsp72 (heat-shock protein 72), S100A9, peroxiredoxin, mitochondrial peptides, and extracellular nucleic acids (DNA, RNA) (Figure 1).[38–40] Damage-associated molecular patterns act on neutrophils through specific receptors (toll like receptors (TLRs), co-receptors, and FPR1),[38] and result in a proinflammatory response involving the production of cytokines, proteases, and ROS.[38]

Targeting DAMPS and TLRs has shown promise as a treatment in ischemic stroke. When HMGB1 levels in plasma are reduced with cannabinoids, there is a reduction in infarct size and activated neutrophils.[41] Neutralization of peroxiredoxin with antibodies reduces inflammatory response and infarct volume growth.[40] In patients with ischemic stroke, increased neutrophil expression of TLR4 on days 3 and 7 is associated with worse stroke outcome and infarct volume.[42] When TLR4 is knocked out in mice, ischemic brain injury is reduced.[43] Despite the absence of TLR4, MPO+ cells and Iba1+ microglial cells were increased in brain, suggesting an increase of brain MPO+ cells (which could be neutrophils or macrophage/microglia) does not necessarily equate to worsening of brain injury in stroke.

TARGETING NEUTROPHIL ENDOTHELIAL ADHESION IN ISCHEMIC STROKE

Neutrophil adhesion to endothelial cells is an important step in the immune response to tissue injury (Figure 3).[2,20] In ischemic stroke, disrupting neutrophil interactions with endothelial cells has showed positive results in animals.[44] Targets that have been evaluated include blocking intracellular adhesion molecule 1 (ICAM-1), MAC-1 (CD11b/CD18), and selectins. In patients with ischemic stroke targeting neutrophil adhesion has been evaluated in three clinical trials: the Enlimomab Acute Stroke Trial, the Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN) trial, and the LeukArrest study. Unfortunately, the benefits observed in animals did not translate to the patients. Though disappointing, insight into factors that may improve the translational effectiveness of future neutrophil directed therapies in ischemic stroke were identified.

Intracellular Adhesion Molecule 1

Intracellular adhesion molecule 1 is an adhesion molecule involved in the adhesion of activated neutrophils and other leukocytes to endothelial cells. After ischemic stroke, blood levels of ICAM-1 increase in both animals and patients. In Wistar rats, blocking ICAM-1 with an antibody reduces ischemic brain injury.[45] Likewise, in a mouse MCAO stroke model genetic deletion of ICAM-1 or immunodepletion of neutrophils reduces infarct

Table 1. Factors that are expressed by or act on neutrophils in ischemic stroke

Class	*Molecule*	*Description*	*Cellular source*	*Timing post stroke*	*Ref, Species*
Adhesion Molecule	PSGL-1	P-selectin glycoprotein ligand-1 (CD162), binds P Selectin on platelets and endothelial cells	N, M, E, Ba, B, T, CD34+	Increased 24 hours Elevated at 90 days	[147] H
	MAC-1	Macrophage-1 antigen (CD11b-CD18, CR3, *a*M*β*2 integrin) binds ICAM-1	N, M, E, Ba, NK	Increased 24 hours Increased 7 days	[147] H [50] M [51] R, Rb
Protease	Elastase	Protease, cleaves elastin, CR1, tight-junctions, C3bi, immunoglobulin, cytokines	N	Increased 24 hours Increased 7 days	[148] H
	Proteinase 3	PR3, Neutrophil proteinase 4, a serine protease	N	Increased	[149] H
	Cathepsin G	Chymotrypsin-like proteinase, neutral proteinase, a protease	N	Increased	[110,111] M
	MMP-9	Protease, degrades extracellular matrix, collagen	N, M	Increased 3 hours Increased 6 hours Increased 24 hours	[75,150,151] H
Cytokine	IL-1*β*	Interleukin-1-beta, cytokine, neutrophil chemoattraction, activation	M N, NK, T, B	Increased	[32] H,R,M
	TNF-*α*	Tumor necrosis factor-alpha, cytokine, neutrophil chemoattraction, activation	M N, NK, T, B	Increased	[32,152,153] H
	IL-8	Interleukin-8 (neutrophil chemotactic factor), cytokine, chemotaxis, angiogenesis	M N,T	Increased 24 hours	[32,154] H
	CXCL1	Chemokine CXC ligand-1 (GRO1, neutrophil activating protein 3, cytokine-induced neutrophil chemoattractant 1, CINC), neutrophil chemotaxis	M	Increased 6 hours Increased 12 hours	[21–23] M
	CXCL2	Chemokine CXC ligand-2 (GRO2, macrophage inflammatory protein 2*α*), neutrophil chemotaxis	M	Increased	[21–23] M
	CXCL5	Chemokine CXC ligand 5 (ENA78), neutrophil chemotaxis	M, E	Increased	[21–23] M
Other	MPO	Myeloperoxidase, peroxidase enzyme, produces hypochlorous acid	N, M	Increased 1–5 days	[16,155,156] H,R
	NGAL	Lipocalin-2, sequesters iron siderophores, growth factor	N, kidney	Increased 1–3 days Elevated $>$1year	[149,157] H

Cell Source Legend: N = neutrophils, M = monocytes/macrophage/microglia, Ba = Basophils, E = eosinophils; B = B cells, NK = natural killer cells; T = T cells, CD34+ = CD34+ hematopoietic progenitor cells. Species Legend: H = human, M = mouse, R = rat, Rb = rabbit. Abbreviations: CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; CCR, CC-chemokine receptor; CXCR, CXC chemokine receptor; IL, interleukin; MAC-1, macrophage 1 antigen; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; PSGL-1, P-selectin glycoprotein ligand-1; TNF*α*, tumor necrosis factor alpha.

Table 2. Neutrophil therapeutic targets in acute ischemic stroke, and their effect on ischemic brain injury

Treatment	*Molecular target*	*Effect on ischemic brain*
Anti-ICAM-1 antibody (Enlimomab trial)	ICAM-1	*Humans* –no improvement in stroke outcome, no decrease in stroke severity; increase infection, increase hemorrhagic transformation[47] *Rodents (M,R), Rabbit* –decrease infarct volume, improved functional outcomes[45,158,159]
Neutrophil inhibitor factor (UK-276,279, ASTIN trial)	CD11b, CD18	*Humans* –no improvement in stroke outcome, no decrease in stroke severity, no increased infection[54] *Rodents (M,R)* –decrease infarct volume, improved functional outcomes[52,53]
Anti-CD18 antibody (Hu23F23F2G, LeukArrest)	CD18	*Humans* –no improvement in stroke outcome[48] *Rodents (M,R)* –decreased infarct volume, improved functional outcomes[158] Rabbit[51]
Anti E selectin, Anti P selectin antibody	E selectin	*Rodents (M), Primates* –decrease infarct volume, improved functional outcomes[55,56]
Anti L selectin antibody	L selectin	*Rodents (R)* –no effect[59]
Reparixin Evasin-3 SB225002	CXCR1, CXCR2, CCR2	*Rodents (M)* –variable to no effect on infarct volume and functional outcomes[27–29]
CB2 agonist	P38 MAPK, CXCL2 HMGB1–TLR4	*Rodents (M)* –decrease infarct volume, improved functional outcomes[41]
Robo1 inhibition	Robo1, Slit1 PMN	*Rodents (M)* –decrease infarct volume, improved functional outcomes, increase Slit1 PMN[63]
Apocynin	NOX inhibition	*Rodents* –decrease infarct volume, improved functional outcomes, decrease Neutrophils
NXY-059	ROS	*Humans* –no improvement stroke outcome, no reduction in hemorrhagic transformation[89] *Rodents (R) Rabbits* –decreased infarct size, decreased hemorrhagic transformation [88,160]
N-t-Butyl-Phenylnitrone	ROS	*Rodents (R)* –decreased hemorrhagic transformation[90] *Rabbits* –increased hemorrhagic transformation[91]
Edaravone	ROS	*Humans* –increased hemorrhagic transformation[93] *Rodents (R)* –decrease blood brain barrier disruption and hemorrhagic transformation[92]
PKC delta	Protein kinase C	*Rodents* –decrease infarct volume, decreased neutrophil infiltration[62]
CD47 knockout	CD47	*Rodents (M)* –decrease cerebral edema, neutrophil infiltration, MMP-9[60]
CD73 knockout	CD73, regulates leukocyte trafficking	*Rodents (M)* –increase infarct volume, increase leukocyte infiltration[61]
TLR4 knockout	TLR4	*Rodents (M)* –decrease infarct volume[43]

Abbreviations: CXCL, CXC-chemokine ligand; CCR, CC-chemokine receptor; CXCR, CXC chemokine receptor; ICAM1, intracellular adhesion molecule-1; M, mouse; MMP-9, matrix metalloproteinase 9; R, rat; ROS, reactive oxygen species; TLR, toll-like receptor; TNF*a*, tumor necrosis factor alpha.

volume, decreases mortality, and improves outcomes.[46] Of interest, this beneficial effect in ischemic stroke is mostly observed in transient MCAO models and not in permanent stroke models.

The benefits of ICAM-1 therapy in animals led to a human trial of an antibody targeted against ICAM-1 (Enlimomab).[47] The Enlimomab Acute Stroke Trial randomized 625 patients with ischemic stroke to Enlimomab or placebo within 6 hours of stroke onset.[47] Treatment lasted for 5 days. At 90 days patients in the Enlimomab-treated group had increased morbidity and mortality compared with placebo ($P = 0.004$). There were more infections and fever in the Enlimomab group, and those with fever had worse outcomes.[47] The reasons Enlimomab failed to translate to human stroke remain unclear. It has been suggested that the Enlimomab antibody used was not sufficiently humanized and thus stimulated an immune response that worsened stroke.[48] This was supported by a follow-up rat study that found injections of a mouse antibody against ICAM-1 (1A29) did not improve stroke outcome. Furthermore, the mouse 1A29 antibody induced an immune response in rats resulting in activation of circulating neutrophils, complement, and microvascular endothelium.[49] Thus, any potential benefit of blocking ICAM-1 in patients with acute ischemic stroke may have been overshadowed by the immune activation of insufficiently humanized Enlimomab. Another potential contributing factor to the failure of the Enlimomab study may be that blocking ICAM-1 is not sufficiently specific to target neutrophils. Though ICAM-1 is important for neutrophil adhesion, it also mediates the adhesion of other leukocytes that may be important to stroke recovery and host response to infection. Indeed, infection was increased in the Enlimomab group and may have contributed to worse outcomes associated with treatment. Heterogeneity of stoke in patients compared with animals may also have contributed. Inflammation and neutrophil contribution to brain injury in experimental stroke may have been more prominent and homogenous. In humans, there may be greater heterogeneity in inflammation and neutrophil contribution to barrier disruption and brain injury reducing the power to observe a beneficial outcome. Potential methods to identify patients with neutrophil proinflammatory activation likely to cause brain injury may aid in selecting patients for treatment with anti-neutrophil therapies.

Macrophage-1 Antigen and Lymphocyte Function-Associated Antigen 1

Neutrophil adhesion to endothelial cells is also mediated by the integrin MAC-1 (also called CD11b-CD18, CR3, $\alpha M\beta 2$ integrin) and LFA-1 (also called CD11a-CD18, $\alpha L\beta 2$ integrin) (Figure 3). Macrophage-1 antigen is expressed on activated neutrophils as well as monocytes and natural killer cells. Lymphocyte function-associated antigen 1 is expressed on activated neutrophils, T cells, B cells, and macrophages. In a MAC-1 knockout mouse, infarct size is reduced, mortality is decreased, and neutrophil infiltration into ischemic brain is lessened.[50] In rodent and rabbit stroke, inhibition of MAC-1 with a monoclonal antibody reduces infarct volume and improves functional outcomes.[51] When MAC-1 is blocked with recombinant neutrophil inhibitory factor (rNIF, UK-279,276) infarct size and cerebral edema in rodents are decreased, and functional outcomes are improved.[52,53] However, this benefit only occurs when rNIF is administered early after cerebral reperfusion (2 to 6 hours) and is not effective in models where no reperfusion

occurs. Thus, similar to studies of anti-ICAM-1, timing of treatment and status of reperfusion are important to effectiveness of neutrophil inhibition.

The success of MAC-1 inhibition in animal ischemic stroke led to two human studies, ASTIN and LeukArrest. In the ASTIN study, 966 acute ischemic stroke patients were randomized to the MAC-1 inhibitor UK-279,276 (neutrophil inhibitor factor) or placebo within 6 hours of onset.[54] UK-279,276 did not improve stroke outcomes at 90 days. However, the rates of infection, fever, or serious side effects were not increased in treated patients. This suggests that the failure of this neutrophil adhesion therapy to improve stroke outcomes did not relate to increased infection risk or impaired host response to pathogens. Reasons for the lack of benefit observed with UK-279,276 have included: (1) differences in effects of UK-279,276 on neutrophils in rodents compared with humans; (2) the dose or timing of administration did not produce the desired biologic effect; and (3) variable reperfusion in humans compared with rodent stroke models. Notably, the median time to treatment in the ASTIN trial was 4.1 hours (interquartile range 1.6 hours). Given neutrophils display increased expression of MAC-1 within 15 minutes of stroke, earlier treatment may have shown greater benefit.[12,13]

LeukArrest was a trial of a humanized IgG1 antibody (Hu23F23F2G) that targets the CD18 component of MAC-1 and LFA-1. In rabbits, Hu23F23F2G reduced brain injury and neutrophil infiltration.[51] This led to a phase III trial in patients with ischemic stroke. Within 12 hours of stroke onset, patients were randomized into three groups: LeukArrest antibody treatment at enrollment only, treatment at enrollment and again at 60 hours, or placebo. The trial was stopped early because it was unlikely to show benefit.[48] Details regarding safety outcomes are not known as final results were never reported. As in the ASTIN study anti-MAC-1 treatment was initiated relatively late into the course of ischemic stroke (12 hours). Whether earlier treatment within the first 1 to 3 hours of stroke onset may produce similar benefit as observed in animals remains unclear.

Selectins

Ischemic injury causes endothelial cells to express P-selectin and E-selectin (Figure 3). These selectins promote neutrophil tethering and rolling adhesion by binding PSGL-1.[20] In rodent and primate ischemic stroke, blocking E-selectin or P-selectin with a monoclonal antibody decreases infarct volume and improves functional outcomes.[55,56] When stroke is induced in a P-selectin knockout mouse, infarct size is decreased, as is BBB disruption and granulocyte infiltration.[57] L-selectin may contribute to tethering of a rolling neutrophil.[58] In ischemic stroke, blocking L-selectin did not protect against or reduce ischemic brain injury.[59] Therapies targeting selectins in patients with ischemic stroke have not yet been performed.

TARGETING NEUTROPHIL TRANSMIGRATION AND NEUROVASCULAR INTERACTIONS IN ISCHEMIC STROKE

In ischemic stroke details regarding the molecular mechanisms of neutrophil transmigration and infiltration into ischemic brain remain less well characterized. In the peripheral vasculature, molecules important to neutrophil transmigration include adhesion molecules on neutrophils (MAC-1, LFA-1, CD99, PECAM, and JAMA) and endothelial cells (ICAM-1, ICAM-2, ESAM, CD99, PECAM, and JAM) (Figure 3). In ischemic stroke, a few molecules have been found to contribute to neutrophil transmigration including CD47, CD73, PKC delta, and Slit1/Robo1. CD47 is a cell surface glycoprotein that binds endothelial integrins and facilitates vascular transmigration. Transient focal cerebral ischemia in a CD47 knockout mouse results in a reduction in neutrophil infiltration, matrix metallproteinase (MMP-9), and cerebral edema compared with controls.[60] CD73 is an ecto-5' nucleotidase that regulates neutrophil and other leukocyte trafficking into brain.[61] In chimeric mice lacking CD73, cerebral infarct volumes are larger and leukocyte infiltration is increased.[61] PKCdelta is another molecule that contributes to neutrophil adhesion and migration. In mice lacking PKCdelta, transient focal cerebral ischemia produces smaller infarcts with reduced neutrophil infiltration.[62] In mice transplanted with PKCdelta null donor bone marrow, infarct size is reduced and neurologic outcome is improved, an effect reversed by transplantation of wild-type bone marrow. Slit1 is expressed on neutrophils and prevents infiltration across cerebral endothelial cells through interactions with Robo1.[63] After ischemic stroke, there is a transient reduction in Robo1 expression that permits infiltration of Slit1 expressing neutrophils into brain.[63] Neutrophils that transmigrate into the central nervous system (CNS) via IL-1 may promote CNS injury and neurotoxicity through the release of NETs.[64] The above studies imply that blocking neutrophil transmigration across injured vessel walls after ischemic stroke may improve outcomes after stroke. Further study is required to determine whether these may be potential targets in human ischemic stroke.

There exists some uncertainty regarding the extent of neutrophil infiltration into ischemic brain. Many pathologic studies of ischemic stroke show neutrophils on the luminal and abluminal sides of vessels in infarcted brain, suggesting that they are within ischemic brain.[14] In patients with ischemic stroke, brain neutrophil accumulation is reported to occur in one of three patterns: (1) marked early neutrophil accumulation within 12 hours of stroke that persists for prolonged periods >30 days; (2) moderate neutrophil accumulation that resolves within 30 days; and (3) minimal neutrophil accumulation that resolves by 6 to 9 days of stroke.[18] However, a recent study found that Ly6G-positive neutrophils enter into ischemic brain parenchyma far less than previously thought.[65] In a mouse MCAO model, Ly6G-positive neutrophils were predominantly observed on the luminal surface or in the perivascular spaces of cerebral vessels over a period of 1 hour to 2 weeks. In addition, in post-mortem brain tissue from patients with ischemic stroke, neutrophils were predominantly perivascular with very few identified within ischemic brain tissue.[65] These findings suggest that neutrophils may affect ischemic brain not through direct activity on brain parenchyma, but rather through effects on the BBB and neurovascular unit. Future studies need to carefully determine whether neutrophils are truly in brain or are mainly perivascular.

TARGETING NEUTROPHIL MEDIATED BLOOD–BRAIN BARRIER DISRUPTION IN ISCHEMIC STROKE

The BBB is disrupted after ischemic stroke. Neutrophils contribute to this disruption through the release of proteases (matrix metalloproteinases, elastase, cathepsin G, and proteinase 3), ROS and during the process of migrating across cerebral endothelium (Figure 1).[2,65,66] The BBB disruption has an important role in poststroke cerebral edema and HT. The importance of neutrophils in BBB disruption and HT is highlighted by the fact that neutrophil proteins (MMP-9) and genes (LTF, NGAL, CEACAM8 (carcinoembryonic antigen-related cell adhesion molecule 8), and CRISP3) are predictive of HT in patients with stroke.[67] Furthermore, inhibiting or depleting neutrophils reduces BBB disruption and the rate of HT.[66,68] In contrast, promoting neutrophil activation with lipopolysaccharide enhances BBB disruption in rodent stroke.[69] Thus, targeting neutrophil-mediated BBB disruption may have utility to reduce cerebral edema and the rate of HT poststroke.

Targeting Neutrophil Matrix Metallproteinase 9

Neutrophils are an important source of MMP-9 within the first 24 hours of ischemic stroke. Matrix metallproteinase 9 is increased

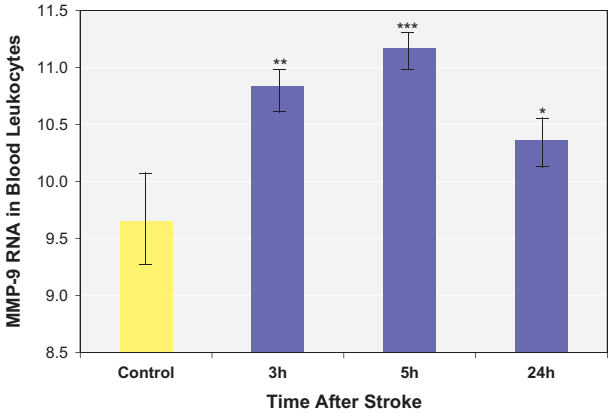

Figure 4. Change in matrix metalloproteinase 9 (MMP-9) mRNA in peripheral leukocytes over the first 24 hours in patients with ischemic stroke. (*P=0.02, **P=9.9 × 10-5, ***P=8.6 × 10-8)

in plasma within the first 2 to 6 hours of stroke in patients, primates, and rodents.[70,71] We found increased leukocyte MMP-9 mRNA within 3 hours of stroke (Figure 4).[72,73] Increased plasma MMP-9 levels correlate with BBB disruption and predict tPA-related HT.[74] In humans with ischemic stroke, MMP-9-positive neutrophil infiltration is associated with BBB breakdown, basal lamina type IV collagen degradation, and HT.[75]

Targeting neutrophil MMP-9 has been evaluated as a treatment in ischemic stroke. Mice lacking leukocyte MMP-9 have decreased BBB disruption and infarct size,[76] which appears to be mediated by leukocyte MMP-9 and not by brain MMP-9.[76,77] In neutrophils, MMP-9 is regulated by CEACAM1. Knocking down CEACAM1 promotes neutrophil MMP-9 expression and enhances BBB breakdown in ischemic stroke.[78] Pharmacological inhibition of MMP-9 poststroke also reduces BBB disruption and HT.[79] For example, the rate of HT in rats can be reduced with BB-94, a broad spectrum MMP inhibitor, and minocycline, an inhibitor of MMP-9 and microglia.[80] Ongoing studies are evaluating the role of minocycline to reduce HT in stroke patients. Though early inhibition of MMP-9 reduces HT in animals, delayed MMP-9 inhibition enhances brain injury and worsens stroke in part by impairing vascular remodeling.[81]

Granulocyte colony-stimulating factor (G-CSF) has activity to induce neutrophil mobilization. In a rat MCAO stroke model, G-CSF increases peripheral neutrophil numbers at 24 hours, and this is associated with an increase in MMP-9 and tPA-related HT.[82] It was found to potentiate neutrophil release of MMP-9 in the present of tPA. In humans, G-CSF administered after ischemic stroke has also been found to increase peripheral leukocyte count. This increase was not associated with any improvement in neurologic outcome at 90 days, nor an increased risk of HT. Though the rationale as to why G-CSF did not show clinical benefit is unclear, it may have enhanced early mobilization of inflammatory neutrophils that offset any potential beneficial effect of G-CSF.

Targeting Neutrophil Elastase

Neutrophil elastase degrades basal lamina and extracelular matrix. In experimental stroke, pharmacological inhibition of neutrophil elastase reduces BBB permeability, decreases cerebral edema, and improves neurologic outcomes.[83] In mice lacking elastase, ischemia-induced BBB disruption is reduced, as is infarct volume, cerebral edema, and leukocyte-endothelial adhesion.[84] Furthermore, pharmacological inhibition of elastase in MMP-9-null mice further decreased infarct volume and BBB disruption, indicating effects independent of MMP-9.[84] These results are important since they suggest that combined inhibition of MMP-9 and elastase may be more effective than either alone.

Targeting Neutrophil Reactive Oxygen Species

Neutrophils are an important source of ROS after stroke and reperfusion of ischemic brain.[44,45] Reactive oxygen species disrupt the neurovascular unit through damage to endothelial cells, pericytes, smooth muscle cells, and astrocytes. This results in increased BBB permeability, cerebral edema, and HT.

Superoxide radicals are mediators of reperfusion-induced BBB disruption.[85] Neutrophils generate superoxide by the enzyme nicotinamide adenine dinucleotide phosphate oxidase (NOX). Inhibition of superoxide results in reduced BBB disruption in rodents. When NOX is inhibited with apocynin or by genetic ablation, the severity of BBB disruption is reduced, as are infarct volumes and the degree of neutrophil infiltration.[86,87]

Several studies have evaluated ROS as a target to reduce BBB disruption and HT in ischemic stroke. Though animal studies of the spin-trap-agent NXY-059 showed promise,[88] it failed to improve stroke outcome or reduce HT in stroke patients.[89] Another spin trap agent, N-t-Butyl-Phenylnitrone, also reduces tPA-related HT in rodent stroke[90] but worsens HT in rabbit stroke.[91] The free radical scavenger edaravone decreases BBB disruption and HT in rodent stroke[92] but increased HT in stroke patients.[93] Benidipine, a dihydropyridine calcium channel blocker, also inhibits neutrophil ROS production and improves stroke outcomes in rats.[94] Given these variable results, evaluation of candidate compounds in multiple animal models of HT may be beneficial.[95]

IMPROVING THE TRANSLATION EFFECTIVENESS OF NEUTROPHIL THERAPY IN STROKE

Though neutrophils are frequently considered to have a negative impact on ischemic stroke, they may also have beneficial functions that need to be considered in the development of anti-neutrophil therapies. Among these functions are roles in preventing systemic infection, wound healing, monocyte recruitment, and neuroprotection.

In addition, neutrophil therapies have often been effective in stroke models with reperfusion and not in those without reperfusion. Given the variable reperfusion in human stroke, anti-neutrophil therapies in animals should be shown to be effective in stroke models with and without reperfusion. Anti-neutrophil therapies should be tested in multiple stroke models/species and there may be species-specific effects. Behavioral outcomes and infarct volumes should be assessed beyond 24 to 72 hours after stroke onset to better evaluate long-term effects of anti-neutrophil therapy.

Neutrophils, Infection, and Stroke

Neutrophils are important in preventing infection. Given stroke-associated infection is linked to worse stroke outcomes,[69,96] therapies targeting neutrophils should not impair host defense against pathogens if it is to be of benefit in ischemic stroke. In the Enlimomab study, ischemic stroke patients treated with the anti-ICAM-1 antibody had increased rates of infection, including six cases of meningitis. The presence of infection was associated with worse stroke outcomes. In future studies targeting neutrophils in ischemic stroke, the impact on host response to infections should be assessed. Of note, the increased infection rate observed in the Enlimomab study was not identified in rodent preclinical studies. Thus, it may be worthwhile to evaluate neutrophil therapies in ischemic stroke models where the immune system and vulnerability to infection are similar to that in humans (e.g., primates, cats, dogs, or rabbits).

Infectious activation of neutrophils worsens stroke. In rodents, when lipopolysaccharide (a gram-negative bacteria coat protein) is administered at time of stroke infarct size is increased and neurologic outcome worsened.[69] This effect can be abolished either by induced neutropenia or by use of an IL-1 receptor

antagonist. In a related study, administration of IL-1 in rodent stroke increased infarct size, enhanced BBB disruption, and increased neutrophil MMP-9. Inhibition of MMP-9 reduced infarct size, cerebral edema, HT, and improved neurologic outcome.[97] These results indicate that a complex relationship exists between infection, neutrophils, and ischemic stroke.

Neutrophils and Neuroprotection in Stroke

If anti-neutrophil therapy is to improve stroke outcomes, then it is important that any beneficial effects of neutrophils are not inhibited. Protective roles of neutrophils have been described in intestinal injury. When neutrophils are removed from inflamed tissue injury can be enhanced.[98] In ischemic brain neutrophils are an important source of MMP-9, which is known to have biphasic effects. Early MMP-9 inhibition improves outcomes whereas late MMP-9 inhibition worsens stroke by impairing vascular remodeling.[99] Matrix metalloproteinase 9 may also contribute to degradation of proinflammatory DAMPs including HMGB1.[100] Neutrophils release vascular endothelial growth factor and contribute to angiogenesis in the cornea, thus may be have a role in cerebral angiogenesis after stroke.[101,102] Neutrophils have been found to promote neurotoxicity through the release of NETs (decondensed DNA and proteases) when stimulated with IL-1.[64] Thus, potentially neuroprotective properties of neutrophils could be enhanced by preventing acquisition of a neurotroxic neutrophil phenotype. This might include inhibition of IL-1-dependent CNS transmigration and/or inhibition of the release of neurotoxic proteases and decondensed DNA.

Neutrophils also contribute to the resolution of inflammation and repair by promoting their own removal and releasing anti-inflammatory molecules (annexin-1, lipoxin A4, resolvins, and protectins).[2] A subset of neutrophils called N2 neutrophils have anti-inflammatory properties that may have protective effects in stroke.[103] Treatment with rosiglitazone (PPARγ agonist) increases the percentage of N2 neutrophils, neutrophil infiltration, and neutrophil clearance, suggesting a role in resolution of inflammation. This change from a proinflammatory N1 phenotype to an anti-inflammatory N2 phenotype has been suggested to occur when neutrophils accumulate in the CNS above a certain level.[104] Studies of neutrophil depletion have not consistently demonstrated improved outcomes in stroke.[105] Likewise, when neutrophils are increased with G-CSF, patient outcomes with ischemic stroke are not worsened, and in animal stroke models infarct volume is reduced.[82,106,107] This may support the notion that under certain conditions neutrophils are not deleterious and may be beneficial. Thus, in future studies of neutrophils in stroke it will be important to determine factors that promote favorable neutrophil phenotype such as level of neutrophil accumulation in the CNS, timing poststroke, reperfusion status, and neutrophil phenotype. It will also be important to not only assess stroke outcome at early time points where the harmful effects of neutrophils may be most prominent, but also days to weeks after stroke when the beneficial effects of neutrophils may become more apparent.

Neutrophils also influence the recruitment and entry of beneficial cells into brain such as B cells, T cells, monocytes, progenitor cells and mast cells.[108] For example, neutrophils express cytokines that act on B cells such as BAFF and APRIL.[5] Given depletion of regulatory B cells is associated with worse stroke outcome, the effect of neutrophils on B cells in stroke warrants study.[109] Neutrophils also release a number of proteins involved in monocyte recruitment and infiltration including azurocidin, LL37, cathepsin, CCL2 and IL-6.[110,111] Recruited monocytes contribute to resolution of ischemic inflammation through phagocytosis of apoptotic neutrophils and cellular debris. To optimize neutrophil targeted therapies, improved understanding is needed regarding the relationship between neutrophils and other cells important to stroke outcome.

STROKE RISK

Neutrophils have been associated with risk of ischemic stroke. In patients without a prior history of stroke, an increased neutrophil count is associated with an increased risk of future stroke.[112,113] Among patients with ischemic stroke or myocardial infarction, an increased neutrophil count is independently associated with an increased risk of stroke, myocardial infarction, and vascular death.[114,115]

Modulating neutrophils may reduce the risk of ischemic stroke. Colchicine inhibits neutrophil function and prevents degranulation. Colchicine reduces the risk of future cardiovascular events including stroke in patients with coronary artery disease.[116] Stroke risk may also be influenced by the neutrophil membrane protein FLAP (five-lipoxygenase-activating protein) involved in the synthesis of leukotrienes.[117] A single-nucleotide polymorphism in FLAP is associated with an increased risk of myocardial infarction and stroke.[118] A product of FLAP, leukotriene B4, is increased in neutrophils of patients with myocardial infarction.[118]

NEUTROPHILS AND THROMBUS FORMATION

Neutrophils may also increase stroke risk though their effects on thrombus formation and atherosclerosis. Embolism of thrombus formed in the vasculature or heart is central to the pathophysiology of ischemic stroke. The importance of clot is demonstrated by the benefit of thrombolysis in acute ischemic stroke and antiplatelets/anticoagulation in stroke prevention. Emerging evidence indicates neutrophils are key contributors to clot formation (Figure 5). Indeed, depleting neutrophils results in a distinct reduction in thrombus formation.[119] Neutrophils promote thrombus through several mechanisms including formation of tissue factor, interactions with platelets, release of NETs, and release of proteases that act on coagulation factors.[119,120]

Neutrophil Tissue Factor and Thrombosis

Tissue factor interacts with coagulation factor VIIa to initiate activation of the extrinsic coagulation pathway and promote thrombus formation. Activated neutrophils are an important source of tissue factor.[119,120] Whether neutrophil-derived tissue factor promotes thrombosis in ischemic stroke requires further study. Tissue factor is increased in rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease, each of which are associated with ischemic stroke. Neutrophil-derived tissue factor may contribute to this increased stroke risk.

Neutrophil Platelet Interactions

Neutrophils have a number of interactions with platelets that result in enhanced platelet aggregation and clot formation.[121] Ligands expressed by neutrophils include PSGL-1 that binds platelet P-selectin, MAC-1 (CD11b-CD18, $\alpha_M\beta_2$) that binds platelet GPIb*α*, and binds a fibrin-glycoprotein IIbIIa (gp IIbIIIa, $\alpha_{IIb}\beta_3$ integrin) complex (Figure 5). Platelets also release soluble CD40L that stimulate neutrophil expression of MAC-1. In ischemic stroke, neutrophil–platelet interactions may be important to thrombus formation and vessel occlusion. In patients with recent ischemic stroke, neutrophil–platelet complexes are increased.[122] The stroke prevention therapies both dipyridamole and candesartan inhibit neutrophil expression of adhesion molecules, which prevent thrombus formation.[123] Abciximab and eptifibatide act on the gpIIbIIa receptor, which mediates neutrophil–platelet interactions. Clopidogrel blocks platelet activation and resultant confirmation change in gpIIbIIIa, and thus also prevents neutrophil–platelet interactions.[124] In sickle cell disease, ischemic stroke may result from altered neutrophil–platelet interactions and enhanced neutrophil-dependent platelet aggregation.[125]

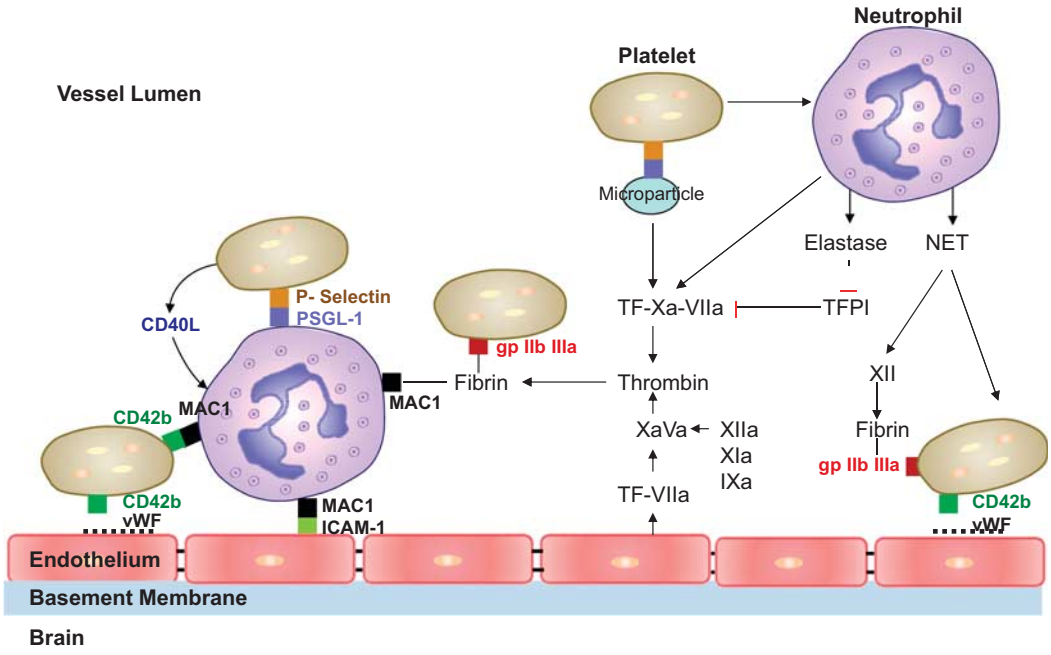

Figure 5. Role of neutrophils in thrombus formation. Neutrophils promote thrombosis through interactions with platelets, proteolytic cleavage of clotting factors (TFPI, coagulation factor X), and release of prothrombotic molecules (NETs and tissue factor). ICAM-1, intracellular adhesion molecule-1; MAC-1, macrophage 1 antigen; NET, neutrophil extracellular traps; PSGL-1, P-selectin glycoprotein ligand-1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; vWF, von Willebrand factor.

Neutrophil Proteases and Thrombosis

Neutrophil-derived proteases contribute to thrombus formation. Cathepsin G and elastase act on coagulation factor X to promote coagulation.[126] Elastase also degrades TFPI*α*, which increases levels of tissue factor that promote clot formation.[126] Neutrophil cathepsin G has a role in neutrophil–platelet interactions.[127] Inhibiting cathepsin G reduces bleeding time and has greater anti-thrombotic effect than aspirin. In rodent stroke, inhibiting cathepsin G improves cerebral blood flow, reduces brain injury, and improves behavioral outcomes.[127] Neutrophils also release the protease ADAMTS13, which cleaves hyperactive ultra-large von Willebrand factor and affects thrombosis in ischemic stroke.[128] Thus, targeting neutrophil proteases may have potential as novel anti-thrombotic therapies to prevent ischemic stroke.[127]

Neutrophil Extracellular Traps and Thrombosis

Neutrophil extracellular traps may also contribute to thrombus formation in ischemic stroke.[129] They are derived from neutrophils and composed primarily of DNA. Though they typically bind pathogens, NETs can trigger platelet activation and promote thrombus formation.[130] Blocking NET formation with DNAse reduces clot formation.[131] Neutrophil extracellular traps have been implicated in deep venous thrombosis but as yet not in ischemic stroke.[129] Neutrophils also release DNA–histone complexes that not only trap and eliminate pathogens but also promote thrombus formation. The thrombogenic potential of NETs is supported by the finding that DNase inhibits thrombus formation related to DNA–histone complexes.[132]

NEUTROPHILS IN ATHEROSCLEROSIS

Atherosclerosis is a major cause of ischemic stroke occurring in both the extracranial and intracranial vasculature that supplies the brain. Neutrophils contribute to both the formation of atherosclerosis and to the rupture of plaque that causes thrombosis and brain ischemia (Figure 6).[133]

Neutrophils in Atherosclerotic Plaque Formation

In atherosclerosis, neutrophils are recruited early to sites of endothelial injury through cytokines, chemokines, and adhesion molecules (Figure 6). In mice deficient in the adhesion molecules P-selectin (platelets),[134] CD18 (neutrophils, monocytes), or ICAM-1 (endothelial cells), formation of atherosclerotic plaque is reduced.[135] P-selectin is important for platelet deposition in plaques. Platelets secrete CCL5 that acts on neutrophil CCR5 receptor to promote neutrophil recruitment to injured endothelium.[136] Neutrophils adhere to endothelium through CD18 and ICAM-1. In turn, recruited neutrophils promote monocyte recruitment via CRAMP (cathelicidin antimicrobial peptide).[5] In mice, deletion of CRAMP or depletion of neutrophils reduces atherosclerosis.[137,138]

Chemokines and cytokines may be potential targets to reduce atherosclerosis in stroke (Figure 6). Evasin-3 is a CXC chemokine-binding protein that inhibits neutrophil activation. When administered in rodent models, carotid atherosclerosis is decreased, intraplaque neutrophil content is reduced, and matrix metalloproteinase-9 activity is diminished.[27] The beneficial effects of increased HDL (high-density lipoprotein) on atherosclerosis may be mediated in part through neutrophils. Increasing HDL decreases neutrophil activation by proinflammatory cytokines (TNF-*α*, IL-1, and IL-8).[4]

Neutrophils also contribute to atherosclerosis by promoting the formation of oxidized lipids (Figure 6). Neutrophil NADPH oxidase and MPO are a major source of ROS that promote formation of oxLDL. Oxidized low density lipoproteins are taken up by scavenger macrophages to form foam cells in atherosclerotic plaques (Figure 6). Neutrophil-derived ROS also contribute to vessel injury and endothelial dysfunction in atherosclerosis, cause vascular smooth muscle proliferation, and activate MMPs which contribute to plaque rupture.[4,139] The beneficial effect of statins in atherosclerosis may relate in part to effects on neutrophils. Statins decrease the production of ROS by inhibiting neutrophil NADPH oxidase, as well as reducing neutrophil activation, adhesion molecule expression, and platelet interactions.[4]

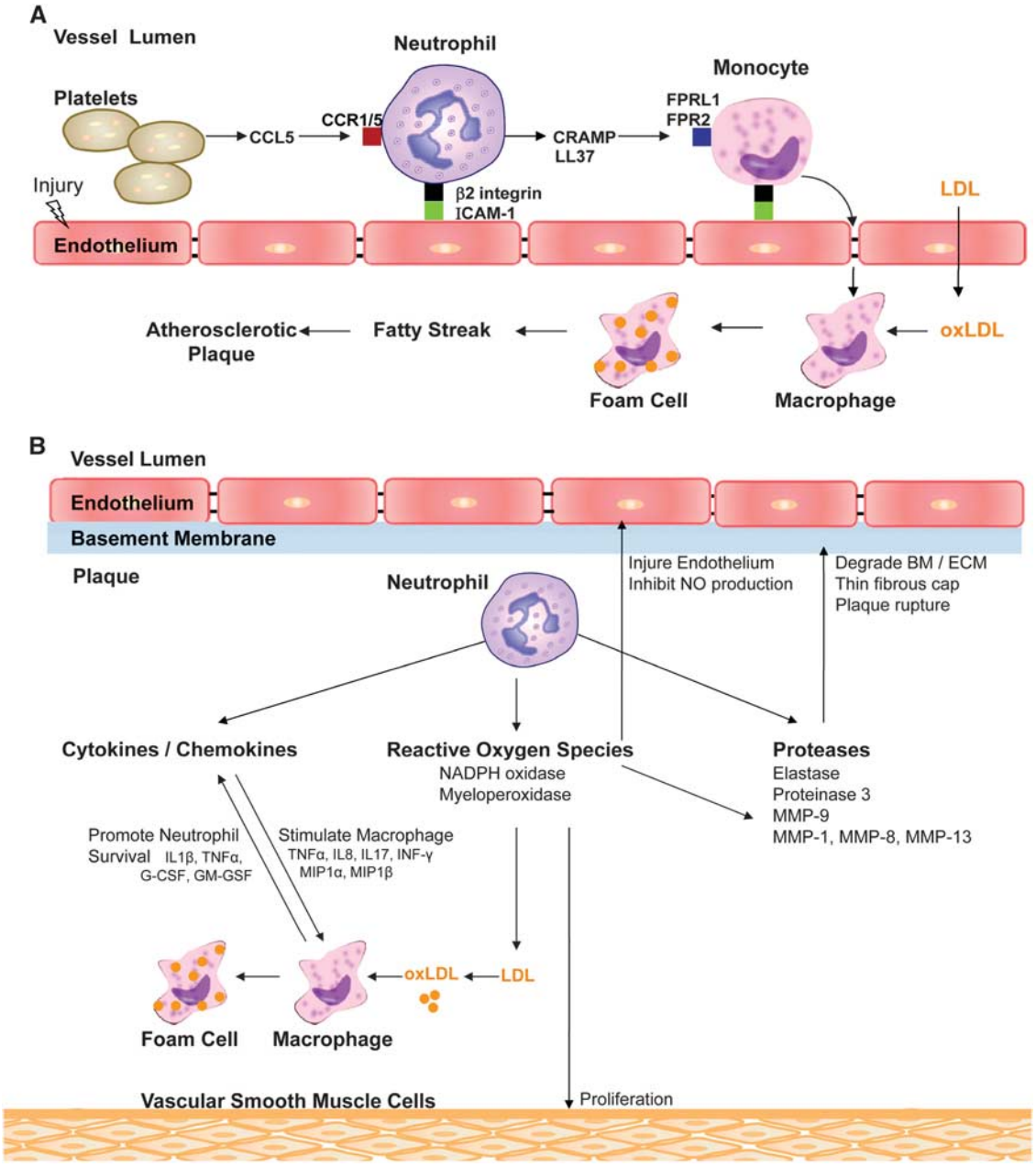

Figure 6. Role of neutrophils in atherosclerosis. (**A**) Neutrophils promote the formation of atherosclerosis though interactions with platelets and by enhancing monocyte infiltration into damaged endothelium. (**B**) Neutrophils promote atherosclerotic plaque progression and rupture via the release of cytokines, reactive oxygen species that activated macrophage foam cells, producing oxidized-LDL, and proteolytic degradation of the fibrous cap. BM, basement membrane; CCL, CC-chemokine ligand; CRAMP, cathelicidin antimicrobial peptide; ECM, extracellular matrix; FPR, formyl peptic receptor; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; INF-γ, interferon gamma; LL37, Cathelicidin; MAC-1, macrophage 1 antigen; MMP, matrix metalloproteinase; oxLDL, oxidized low density lipoprotein; PSGL-1, P-selectin glycoprotein ligand-1; TLR, toll-like receptor; TNF*α*, tumor necrosis factor alpha.

Proteases released by neutrophils also contribute to atherosclerosis (Figure 6). Neutrophil-derived extracellular matrix-degrading proteases can promote endothelial dysfunction by degrading vascular basement membrane and type IV collagen. In turn, dysfunctional endothelium promotes additional neutrophil recruitment through increased expression of adhesion molecules (*β*2 integrins, P selectin, E selectin, and ICAM-1) and IL-8. Neutrophils are an important source of MMPs, which contribute to atherosclerosis. MMP-1, MMP-8, and MMP-13 have been shown to promote atherosclerosis in mice[140,141] and to be overexpressed in human atherosclerotic plaque.[142] Matrix metalloproteinase-8 has also been associated with plaque instability,[142] and genetic polymorphism in MMP8 is related to progression of atherosclerosis and plasma levels of the adhesion molecule vascular cell adhesion molecule-1.[141] Neutrophils are also a primary source of MMP-9. MMP-9 promotes plaque instability and rupture through effects on extracellular matrix. Treatment with statins increases plaque stability in part by reducing neutrophil MMP-9 expression and neutrophil infiltration.[143]

In humans, atherosclerotic plaque instability correlates with the presence of intraplaque neutrophils.[144] Progression of aortic arch atheroma is associated with increased neutrophils in addition to

increased risk of stroke and myocardial infarction.[145] In humans, an increase in neutrophil counts is associated with hypoechoic unstable carotid plaques, symptomatic carotid artery stenosis, and cerebral microembolization.[146]

CONCLUSIONS

Neutrophils are of great interest as treatment targets to decrease ischemic brain injury and prevent stroke. After ischemic stroke, neutrophils promote BBB disruption, cerebral edema, cellular injury, and neurologic impairment. Targeting neutrophil activation, recruitment and adhesion, as well as release of proteases, ROS, and cytokines have been evaluated as stroke treatments. Neutrophils also contribute to clot formation and atherosclerosis and thus present novel targets to prevent stroke by reducing platelet aggregation, thrombosis, and plaque formation and rupture.

Several therapies used to treat stroke have known effects on neutrophils including statins, candesartan, and dipyridamole. As our understanding of neutrophils in ischemic brain injury and stroke pathogenesis advances, it is likely that novel agents targeting neutrophils will be added to our armamentarium to treat ischemic stroke.

AUTHOR CONTRIBUTIONS

All authors contributed to the work presented in this paper. All authors were involved in data acquisition, analysis, and interpretation. GCJ and FRS drafted the manuscript. All authors made critical revision of the manuscript important intellectual content.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors thank the National Institutes of Health/ NINDS and the American Heart Association for support that made this review possible.

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