The American Journal of Pathology, Vol. 189, No. 4, April 2019

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The American Journal of
PATHOLOGY

ajp.amjpathol.org

REVIEW

Ischemia-Reperfusion Injury in Sickle Cell Disease

From Basics to Therapeutics

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Accepted for publication December 7, 2018.

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Sickle cell disease (SCD) is one of the most common hereditary hemoglobinopathies worldwide, affecting almost 400,000 newborns globally each year. It is characterized by chronic hemolytic anemia and endothelial dysfunction, resulting in a constant state of disruption of the vascular system and leading to recurrent episodes of ischemia-reperfusion injury (I/RI) to multiple organ systems. I/RI is a fundamental vascular pathobiological paradigm and contributes to morbidity and mortality in a wide range of conditions, including myocardial infarction, stroke, acute kidney injury, and transplantation. I/RI is characterized by an initial restriction of blood supply to an organ, which can lead to ischemia, followed by the subsequent restoration of perfusion and concomitant reoxygenation. Recent advances in the pathophysiology of SCD have led to an understanding that many of the consequences of this disease can be explained by mechanisms associated with I/RI. The following review focuses on the evolving pathobiology of SCD, how various complications of SCD can be attributed to I/RI, and the role of timely therapeutic intervention(s) based on targeting mediators or pathways that influence I/R insult. *(Am J Pathol 2019, 189: 706–718; https://doi.org/10.1016/j.ajpath.2018.12.012)*

Sickle cell disease (SCD) includes a group of inherited disorders caused by mutations in the hemoglobin subunit β.[1] The molecular defect was discovered by Pauling and Itano and later described by Ingram almost 6 decades ago.[1] The prominence of sickle hemoglobin was seen in Africa, the Middle East, and India several thousand years ago. These areas are highly endemic to malaria-causing protist (*Plasmodium falciparum*), and sickle cell trait confers a survival advantage and relative resistance against malaria attributable to balanced polymorphism. However, patients with homozygous disease (HbSS) have severe outcomes and may develop lethal complications attributable to falciparum malaria.[2] Today, SCD has a measurable health and economic impact in the United States and worldwide. This inherited disease is found in 1 of every 365 African Americans and is responsible for approximately 113,000 hospitalizations and $488 million in costs annually.[3] The clinical hallmark of SCD is episodes of acute pain and other common manifestations, including cerebrovascular events, such as transient ischemic attacks, ischemic strokes [including silent cerebral infarcts (SCIs)], intracerebral hemorrhages, acute chest syndrome (ACS), pulmonary hypertension (PH), bacterial infections, splenic infarcts, and progressive multiorgan dysfunction syndrome.[3] This review focuses on ischemia-reperfusion injury (I/RI) in SCD with the main emphasis on the immunopathology (neutrophils and platelets) and discusses various I/RI-associated SCD clinical outcomes and therapeutic interventions.

Pathobiology of SCD

SCD was originally considered to be a disease of abnormal erythrocyte polymerization, but in recent years the trend has shifted toward a more complex pathophysiologic makeup, consisting of a polymerization defect, disrupted endothelial

Supported by the NIH National Heart, Lung, and Blood Institute grant 1R01HL134959-01A1 (F.N.E.G.).
Disclosures: None declared.

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https://doi.org/10.1016/j.ajpath.2018.12.012

and cellular state, and resultant vasculopathies and organ damage (Figure 1).[3–5]

Defective Globin Synthesis

SCD is caused by a mutation in the β-globin gene that changes the sixth amino acid from glutamic acid to valine.[1] This mutation produces a hydrophobic motif in the deoxygenated hemoglobin S (HbS) tetramer that results in binding between the β_1 and β_2 chains of two hemoglobin molecules.[5] The resultant crystallization produces a polymer nucleus that grows and fills the erythrocyte, disrupting its architecture and flexibility and promoting cellular dehydration and decreasing ATP content[6] with physical and oxidative/nitrosative stress.[7] HbSS disease (the focus of this review) is the most common type of SCD occurring through inheriting copies of the *HbS* gene homozygous for the sickle mutation.[5] Inheriting only 1 *HbS* gene results in a less severe phenotype termed HbAS (heterozygote).[5]

Red Cell Deformability and Hemolysis in SCD

HbS polymerization results in altered erythrocyte biology that significantly affects red blood cell (RBC) membrane stability, increasing RBC-dependent cellular interactions, causing hemolysis, and reducing the lifespan of sickle erythrocytes.[5,8] These effects are more pronounced under deoxygenated conditions, resulting in phosphatidylserine exposure to outer RBC surface.[5] Because of the abnormal sickle shape, sickle red blood cells (SS RBCs) are not able to traverse small capillaries and thus stick to the postcapillary endothelial surface via RBC adhesion molecules, such as CD36 and integrin $\alpha_4\beta_1$,[9] where they provoke unpredictable episodes of microvascular occlusion and premature RBC destruction (hemolytic anemia), resulting in acute painful crisis.[10]

Hemolysis is driven by abnormal HbS polymerization and promotes inflammation by scavenging nitric oxide and metabolizing its precursor arginine, leading to an oxidative/nitrosative stress state.[11] The resultant heme loaded microparticles get attached to the endothelium and increase the expression of adhesion molecules, thus promoting leukocyte recruitment and subsequent inflammation.[12] Heme-bound iron stimulates expression of placental growth factor in erythroid cells, which contributes to pulmonary vasoconstriction and right ventricular hypertrophy, resulting in PH in SCD. This ubiquitously expressed molecule promotes Toll-like receptor 4 (TLR4) signaling in endothelial cells and macrophages, activating NF-κB and triggering vaso-occlusion through Weibel-Palade body degranulation and adhesion molecule expression in SCD.[13,14] Heme also stimulates neutrophils to release their extracellular traps in SCD.[15] Although the mechanism is currently unknown, it has been suggested to be linked with reactive oxygen species (ROS) generation in neutrophils (Figure 1).[16,17] Furthermore, heme can act as a chemotactic molecule or by producing leukotriene B4 by macrophages, thereby inducing neutrophil migration.[15]

Endothelial Dysfunction and Chronic Inflammation

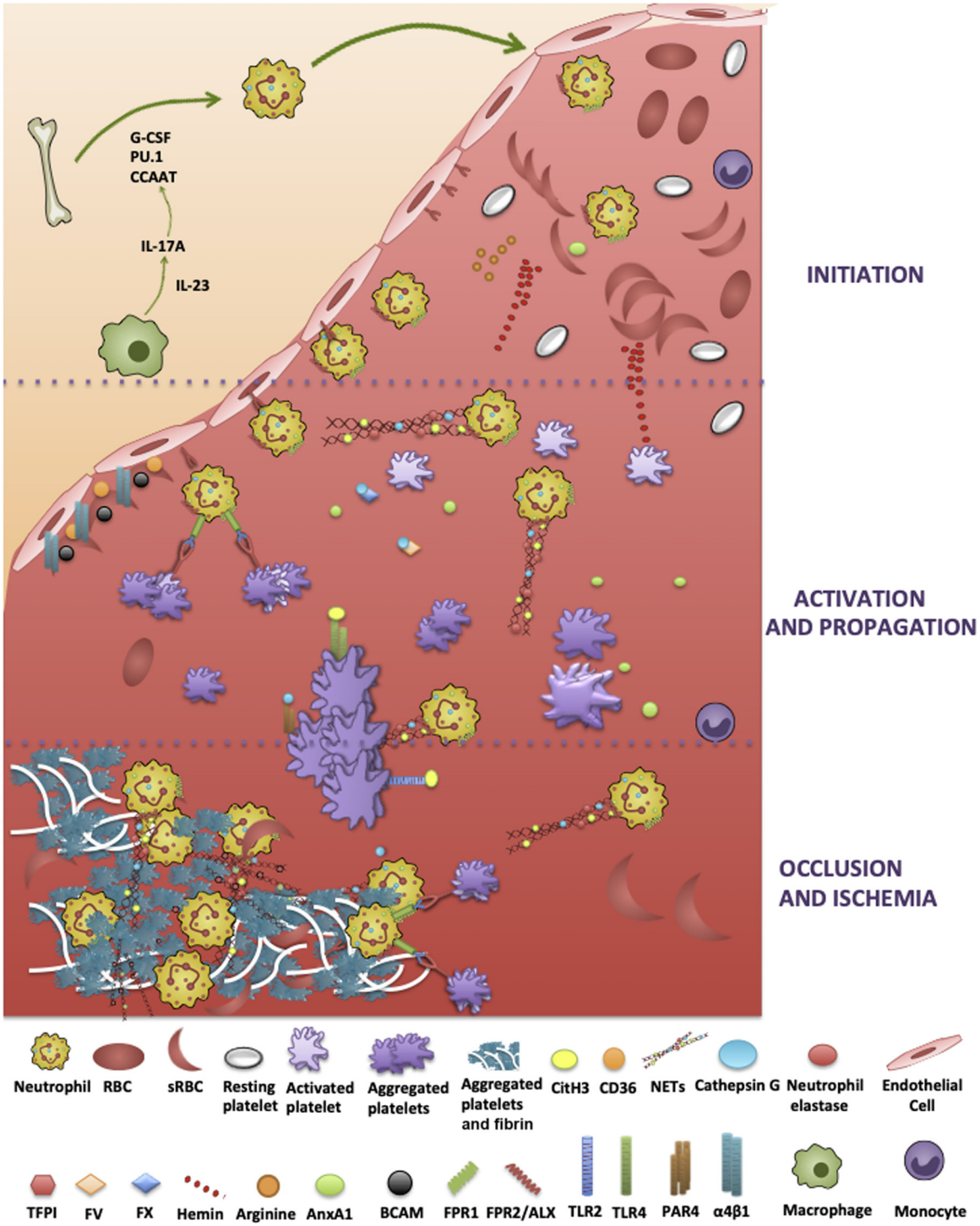
The microvasculature in SCD assumes a proinflammatory, procoagulant, and prothrombotic state,[18] with the endothelium itself playing a significant role in both initiating and maintaining the disruptive state in SCD.[10,18] The hyperactive endothelium in SCD leads to an enhanced RBC and neutrophil adhesion, resulting in slowed flow and sickling in postcapillary venules (retrograde logjamming), and subsequent vaso-occlusion and ischemia[18] (Figure 1). Evidence that SS RBCs may induce endothelial dysfunction has been obtained *in vitro* as well as *in vivo* models.[9] For example, endothelial adherent SS RBCs and decreased nitric oxide availability increase expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 and selectins (eg, P-selectin). In addition, damaged RBCs release hemoglobin, which is oxidized to methemoglobin. Methemoglobin is unstable and as such rapidly releases free heme, which can activate the underlying endothelium.[14,15] In addition, inflammatory mediators, such as interleukin (IL)-6, monocyte chemoattractant protein-1, and platelet-activating factor, are also released from an activated endothelium in SCD and other disease states.[5,18,19]

The endothelium in SCD also plays a key role in driving thromboinflammatory responses by releasing prothrombotic microparticles and tissue factor from circulating endothelial cells.[18,20] Additional factors include decreased thrombomodulin, tissue factor pathway inhibitor, and von Willebrand factor.[18] SCD microvasculature is also highly proangiogenic, which has been attributed to the hypoxic environment and increased levels of various proangiogenic factors, including vascular endothelial growth factor, placental growth factor, angiopoietin-1, angiopoietin-2, and erythropoietin in the circulation.[18,21] For further reading about the role of the endothelium in SCD, please refer to the review by Hebbel et al.[18]

I/RI in SCD

I/RI is a well-known phenomenon associated with microvascular dysfunction that occurs in a wide range of pathologic conditions, including SCD.[22] I/RI is fundamentally divided into two discrete phases composed of an initial ischemic insult attributable to vaso-occlusion followed by reperfusion, which consists of a pronounced proinflammatory response both locally and remotely (called reperfusion injury), resulting in microvascular dysfunction.[23,24] The extent of I/RI dysfunction depends on the vascular system involved, underlying comorbidities, and most importantly the magnitude and the duration of ischemia.[24] Hence, prompt treatment for ischemic events is paramount in I/RI-based complications.[24]

At a cellular level, prolonged ischemia and vaso-occlusion trigger anerobic metabolism and lactic acid production, resulting in ATP depletion and intracellular acidosis. As a consequence, ATPases (eg, sodium-potassium ATPase) are inactivated, contributing to an increase in intracellular and mitochondrial calcium (calcium overload) attributable to dysfunctional calcium ion efflux and reduced reuptake of calcium ion by the endoplasmic reticulum.[24] Concomitantly, excessive production of ROS and calcium overload results in the opening of the mitochondrial permeability transition pore, further reducing ATP levels.[24] Xanthine oxidase, which has a significant impact on I/RI, is formed from xanthine dehydrogenase under hypoxic conditions and ATP catabolism. Release of xanthine oxidase allows superoxide and hydrogen peroxide production, initiating the overall I/RI.[25] The univalent reduction of molecular oxygen in I/RI results in the



production of primary ROS superoxide anion radical and can directly oxidize various biomolecules and inactivate enzymes, initiate apoptosis, enhance proinflammatory stimuli, modify the expression of adhesion molecules on the surface of leukocytes and endothelial cells, and promote nitric oxide biodeficiency.[22,24] Tissue hypoxia and ROS production during ischemia activate and recruit cells of innate and adaptive immune system to the affected site, mainly via involvement of signaling events through pattern recognition molecules (especially TLRs, such as TLR4)[22] and production of IL-17, respectively.[22] Moreover, the release of proinflammatory mediators in the ischemic area, such as ROS and cytokines/chemokines from activated endothelium and tissue-resident macrophages and mast cells, activates and recruits leukocytes (eg, neutrophils, monocytes, and lymphocytes).[26] In addition, there is abundant evidence that now suggests a role of platelets in I/RI. On tissue injury, platelets acquire an activated phenotype via glycoprotein Iba interaction with endothelial P-selectin as well as von Willebrand factor and aggregate and release platelet-derived mediators.[27] Recent evidence mainly from indirect data that target T cells (eg, immunosuppressive agents) has suggested a modulatory role of antigen-specific T cells and independent responses in I/RI by mechanisms that are not yet well elucidated.[28] Finally, the role of the complement system in I/RI is known to involve both classic and alternative pathways.[26]

The mechanisms and widespread cellular and tissue responses known in I/RI described above may explain SCD-associated microvascular complications. I/RI in SCD can lead to endothelial dysfunction[29] through a deposition of xanthine oxidase, cyclooxygenase, uncoupled endothelial nitric oxide, NADPH oxidase, cytochrome p450, and the mitochondrial electron transport chain, all of which result in an increased vascular production of superoxide.[10,30] These events help in part to explain the risk of episodic pulmonary arterial vasoconstriction attributable to exaggerated response to unrelated potential vasoconstrictors (eg, hypoxia, platelet activating factor, and incremental hemolytic rate).

Neutrophil Activation and Cellular Cross-Talk in SCD

Leukocytes (most notably neutrophils) have been implicated in the pathogenesis of SCD and in the genesis of the proinflammatory and prothrombotic phenotype associated with this disease.[4,16] These immune cells have more complex roles beyond that of just immune surveillance and being first responders in innate immunity. Neutrophils also play key roles in chronic inflammatory conditions, adaptive immune responses, and cancer biology.[31]

Neutrophilia has long been considered a risk factor in SCD and positively correlates with early death, SCIs, hemorrhagic strokes, and ACS.[3] Reduction of neutrophil count with the use of hydroxyurea markedly decreases the frequency of painful crisis and ACS in moderate and severe SCD.[4] Neutrophils in SCD also have an activated phenotype with lower levels of L-selectin (CD62L) and higher levels of CD64.[32] Various *in vivo* studies have found that SS RBCs bind to neutrophils via up-regulation of αMB2 (Mac-1) and E-selectin.[4] Neutrophil heterogeneity in patients with SCD is also explained by microbiota regulation via danger-associated molecular patterns, ATP, and pattern recognition receptors.[32] Broad-spectrum antibiotics promote better blood rheology and prolonged survival by depleting microbiota, normalizing the number of aged neutrophils, and reducing the production of neutrophil extracellular traps (NETs).[32] Moreover, antibiotic use is known to cause a significant reduction in neutrophil adhesion and Mac-1 activation.[32] For example, historical data with penicillin prophylaxis, which is commonly given to functionally asplenic patients, to mitigate vaso-occlusive crises (VOCs) in SCD.[33]

Figure 1 Proposed overview of the vascular immunopathology of sickle cell disease (SCD). The A6T mutation in the β-globin gene causes a G6V mutation in the globin polypeptide, leading to the deoxygenated hemoglobin S (HbS) variant. When red blood cells (RBCs) are deoxygenated, HbS polymerizes and the cells take on a sickle phenotype and express adhesion molecules, such as CD36, which mediate attachment with the endothelium. Hemolysis of the sickled cells produces heme microparticles and arginase into the plasma and scavenge nitric oxide and stimulates the release of adhesion molecules, such as intracellular adhesion molecule, vascular cellular adhesion molecule, P-selectin, and E-selectin. Tissue resident macrophages and dendritic cells secrete interleukin (IL)-23, causing T cells to release IL-17A, which stimulates the secretion of granulocyte colony-stimulating factor (G-CSF) to activate neutrophil production. The process of neutrophil maturation is under the control of mainly two transcription factors (PU.1 and CCAAT). Neutrophils adhere and roll along the endothelium through selectin and integrin interactions in the direction of blood flow and are activated by chemokines along the endothelial layer. DAMPs (eg, ATP, high mobility group 1) trigger Toll-like receptor (TLR)-4 and induce an inflammatory response with the secretion of cytokines and chemokines. They also send activation signals to neutrophils and modulate their phenotype (aged neutrophils) that promote sickle cell vaso-occlusion. Activated platelets cause neutrophils to release chromatin and granule proteins to form neutrophil extracellular traps (NETs) and capture sickle RBCs (sRBCs). After activation from glycolipids derived from the tissue damage, invariant natural killer T (iNKT) cells cause further neutrophil recruitment and inflammation through production of interferon-γ and CXCR3 chemokines. sRBCs have phosphatidylserine externalization and express β_2-adrenergic receptors, which activate their procoagulant nature and adhesion properties, respectively. The subsequent responses consist of continuous accumulation of leukocytes, platelets, and RBCs with the activation of the coagulation cascade. These interactions may be mediated by the production of various proinflammatory and prothrombotic mediators, such as cathepsin G, neutrophil elastase, NETs, histones (citH3), and coagulant factors (FV and FX). Neutrophil elastase can co-localize with tissue factor pathway inhibitor (TFPI) on NETs and facilitates TFPI degradation, resulting in an activated coagulation system. The presence of citH3 on NETs is known to induce platelet aggregation via involvement of TLR2 and TLR4. Cathepsin G can activate protease activated receptor (PAR)-4, resulting in further platelet activation. As more blood cells become further incorporated and recruited into growing thrombi, fibrin scaffolds are formed. Under homeostatic conditions, neutrophils generally produce annexin A1 (AnxA1), which counteracts proinflammatory responses and enables resolution. However, in SCD, AnxA1 levels are low. **Dotted lines** separate the different phases of SCD vascular immunopathology.

Neutrophils form an important link bridging inflammation and thrombosis, a phenomenon referred to as thromboinflammation, by their ability to produce proinflammatory/prothrombotic mediators, such as neutrophil serine proteases (eg, cathepsin G and neutrophil elastase)[34] and chromatin structures called NETs[35] on activation. Neutrophil serine proteases can regulate proinflammatory/prothrombotic responses by interacting with platelets, coagulation factors,[34] and binding with formyl peptide receptors on neutrophils and monocytes.[36] In addition, NETs are stimulated by pathogens as well as other stimuli (eg, activated platelets[37] and heme),[16] especially under chronic inflammatory phenotypes, such as SCD. Furthermore, a variety of studies have found that NETs can capture platelets and RBCs, forming a complex network, which can lead to VOCs.[35] Activated endothelial cells induce NET release and kill endothelial cells.[38] Our preclinical results indicate that NETs act as prothrombotic scaffolds and play a major role in cerebral thrombus formation (J.A., F.N.E.G., unpublished data)[39] (Figure 1).

Formyl peptide receptors are G-protein−coupled receptors expressed mainly by myeloid cells with proinflammatory as well as anti-inflammatory responses, depending on the disease state and ligand interaction.[40] Annexin A1 is an endogenous anti-inflammatory/proresolving mediator secreted mainly by neutrophils whose levels are known to be low in patients with SCD.[41] Annexin A1 is known to be protective in I/RI-based vascular inflammation and may have a role in the management of SCD.[42]

Platelets

Platelets have an essential role in the pathogenesis of SCD by interacting constantly with RBCs, neutrophils, and monocytes.[4] Activated platelets piggyback on neutrophils, inducing neutrophil polarization and triggering direct migration of neutrophils to initiate inflammation in a P-selectin−dependent manner.[43] Platelet function is significantly altered in SCD and is associated with an abnormal phenotype marked by surface-mobilized, activation-dependent antigens and microparticle formation.[44] Increased circulating activated platelets have been observed in patients with SCD[45] along with increased circulating levels of platelet products, such as thrombospondin and increased expression of P-selectin (which mediates the adhesion of erythrocytes and leukocytes in venules of sickle cell (β^S) transgenic mice).[43,46] In addition, evidence exists in patients with SCD of ongoing generation of thrombin,[47] which may mediate the platelet activation observed in this disease.

Cerebral I/RI

Clinical hallmarks of SCD, which include hemolytic anemia, VOC, and vascular endothelial dysfunction, all contribute to the increased risk of cerebral ischemic injury in SCD,[3] which constitutes 54% of all cerebrovascular accidents[48] and is one of the most devastating complications of SCD. The risk of overt stroke for children with SCD is >333 times higher than that for the general population, and this burden continues well into adulthood, with approximately 11% of patients with SCD having clinically apparent strokes before the age of 20 years, increasing to 24% by the age of 45 years.[48,49] These figures are likely to be far greater because of the increased incidence of SCIs, which, although quantifiable by magnetic resonance imaging (MRI), produce no or subtle transient focal neurologic signs.[50] As a consequence of overt stroke or SCIs, patients with SCD can have a global neurocognitive impairment as well as sensory and motor deficits.[51] Thus, the combined frequency and overall burden of these ischemic events in the brain of patients with SCD not only are likely to be underestimated but also still remain poorly understood.

The pathophysiology of stroke induction is similar to most other complications of SCD. The deformed and sticky RBCs adhere to the surrounding endothelium in cerebral vessels,[52] resulting in endothelial cell damage and I/RI. These events are followed by von Willebrand factor release, platelet aggregation, and innate and adaptive immune system activation as described previously.[53,54] The whole process causes further hypoxia and acidosis and consequently further sickling. Prolonged exposure to ischemia also causes irreversible and fatal consequences to resident neurons, including cellular changes, such as nuclear fragmentation, chromatin condensation, and cell body shrinkage.[55] Bloodborne cells, such as T lymphocytes, which are known to accumulate in the postischemic brain within 24 hours, also play an important role in the detrimental effects associated with cerebral I/RI in SCD by modulating the recruitment of adherent leukocytes and platelets.[56] Furthermore, increases in cerebral blood flow, as measured by high transcranial Doppler velocity, and platelet-derived growth factor−mediated cerebral arterial remodeling also contribute to the overall increased risk of cerebrovascular diseases in SCD.[57]

Remote Organ Injury in SCD

SCD mounts a widespread systemic inflammatory response attributable to chronic leukocyte and endothelial cell activation, multiple elevated inflammatory mediators (eg, IL-6, tumor necrosis factor-α, IL-1β),[10] and acute-phase reactants (eg, C-reactive peptide, secretory phospholipase A_2, and granulocyte colony-stimulating factor). This persistent, unresolved inflammation may culminate in remote organ injury or multiorgan dysfunction syndrome[58] by promoting neutrophil activation, inducing generalized leukocyte and endothelial adhesion molecule expression, and enhancing the opportunities for leukocyte-endothelial cell interactions.[10,59] The same widespread inflammatory

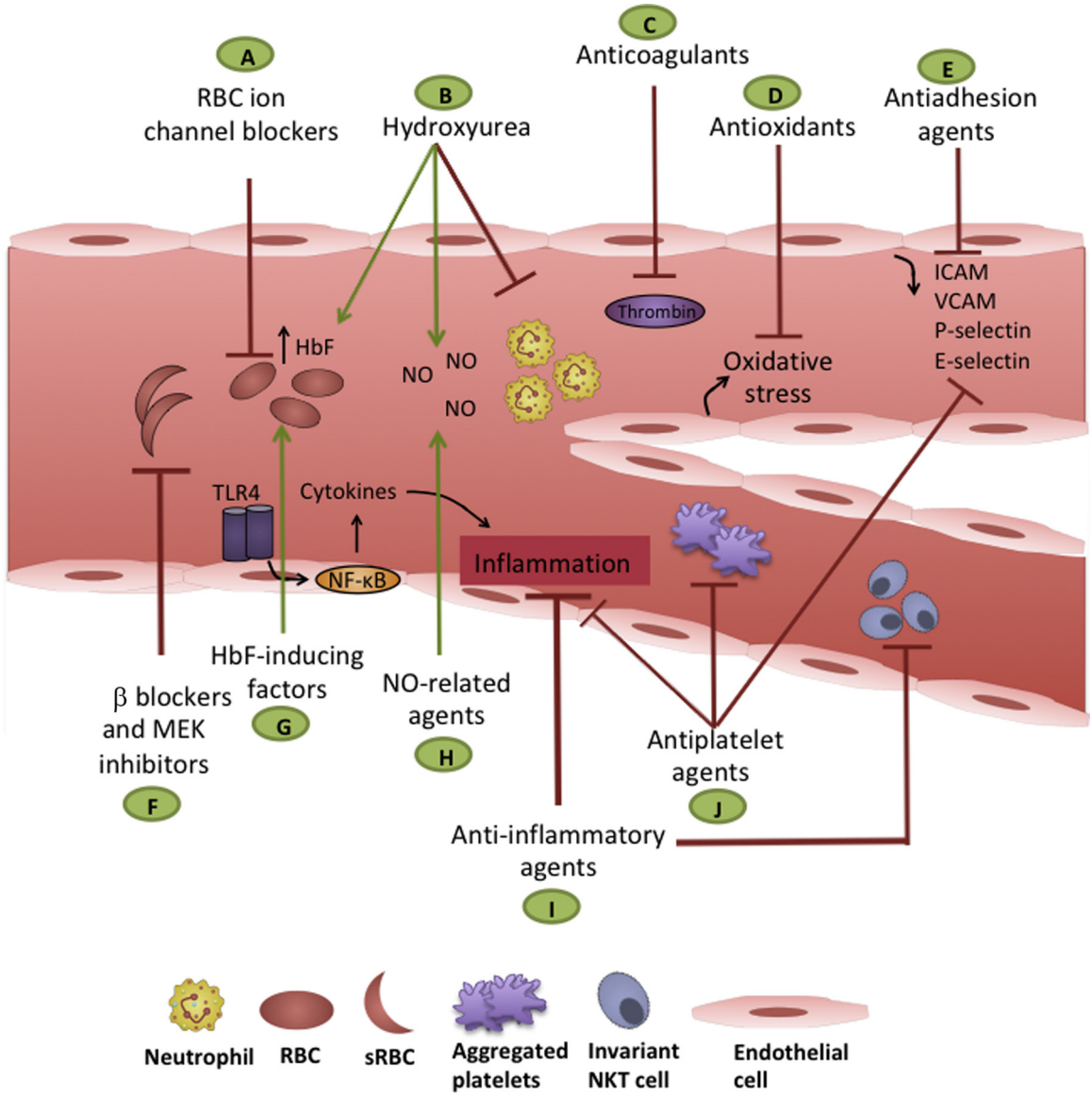


Figure 2 Schematic depiction of potential therapeutic targets in sickle cell disease (SCD). The depicted targets have been tested as potential therapeutics for ischemia reperfusion injury (I/RI) and its associated symptoms in SCD. **A:** Red blood cell (RBC) ion channel blockers (Gardos channel blockers, eg, senicapoc) can preserve sickle RBC (sRBC) hydration and improve survival. **B:** Hydroxyurea acts by inducing fetal hemoglobin (HbF), donating nitric oxide (NO), and decreasing circulating leukocytes and reticulocytes. **C:** Anticoagulants, such as direct thrombin inhibitors (eg, dabigatran), will reduce thromboinflammation. **D:** Antioxidants, such as arginine and L-glutamine, reduce the oxidant stress by improving NAD redox potential. **E:** Antiadhesion agents prevent cellular rolling and adhesion to the endothelium by decreasing the expression of intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), E-selectin, and P-selectin (eg, rivipansel and crizanlizumab). **F:** β-blockers and mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitors reduce sRBC adhesion to the vascular endothelium and can be used for management of acute vaso-occlusive episodes. **G:** HbF-inducing factors, such as hydroxyurea, and other agents (eg, decitabine, pomalidomide, butyrates) can increase HbF, lessen the HbS load, and decrease the rate of hemolysis. **H:** Nitric oxide—related agents may act by vasodilation and inhibition of vascular remodeling. **I:** Anti-inflammatory agents include antiadhesion agents, adenosine A2A receptors, and β_2-adrenergic pathways, which will help manage SCD by reducing overall inflammation and ischemia-reperfusion—related injury. **J:** Antiplatelet agents may reduce inflammatory tone, decrease platelet activation and aggregation, and reduce expression of adhesion molecules and selectins. **Green arrows** indicate activation/stimulation; **brown lines**, inhibition.

response has also been seen in sickle transgenic mice with leukocytosis, increased oxidant generation,[60] and heightened interactions between leukocytes and endothelial cells.[30]

I/RI is a central pathophysiologic process that drives remote organ injury in SCD, with the lungs being the most vulnerable organs.[24] Numerous studies have established that remote organ injury can also occur in other organs (eg, gut, liver, skeletal muscle, and heart).[24] Xanthine oxidase, which accumulates throughout the initial ischemic period, results in the generation of massive amounts of ROS, endothelial dysfunction, and xanthine oxidase—derived oxidant-induced release of chemotactic factors, which promote leukocyte recruitment. The postischemic blood also causes priming

Table 1 Emerging Ischemia and Reperfusion Therapeutics in SCD

Intervention	Target	Action and advantages	Disadvantages and adverse reactions	References
Antioxidants				
Nitric oxide (inhalation)	Nitric oxide homeostasis	Repletion of nitric oxide, treats pulmonary vasodilation, antioxidant, antiadhesive, and antithrombotic effects	Methemoglobinemia, pulmonary cytotoxicity, immunosuppression	63–65 (NCT00023296[I], NCT00142051[II],* and NCT00748423[II/III])
Sildenafil (oral)	PDE5 inhibition	Treats pulmonary arterial hypertension and erectile dysfunction	Flushing, headache, dyspepsia, and visual disturbance	66 (NCT00492531*)
L-Arginine (IV)	Nitric oxide substrate	Reduction in VOCs, reduction in total opioid use, significant decrease in length of stay in the hospital	Nothing significant	67–69 (NCT01142219[III], NCT00513617[II], and NCT01142219[III])
L-glutamine[†] (oral)	NAD precursor	Improves altered erythrocyte NAD redox potential, significant reduction in pain crisis and hospitalization	Constipation, nausea, headache, abdominal pain, cough, pain in extremity, and chest pain	70 (NCT01179217[III])
Anti-inflammatory and antineutrophil agents				
Rivipansel (IV)	Pan-selectin inhibition	Faster VOC resolution, reduction in hospital stay and use of opioids for pain management	Nothing significant	71,72 (GMI-1070 and NCT00911495[I/II])
Crizanlizumab (SelG)	P-selectin inhibition	Significantly reduces SCD-related pain crises	Headache, back pain, nausea, and arthralgia	73 (NCT01895361[II])
Sevuparin (DFO2)[‡]	Antiadhesive (inhibits P-selectin, L-selectin, thrombospondin, von Willebrand factor, and fibronectin)	*In vivo* murine model suggests decrease in erythrocyte adhesion, normal blood flow, and decrease in VOCs	Bleeding risk, HIT	74–76 (NCT0251838[II])
Regadenoson	$A_{2A}R$ agonist, iNKT inhibition	No reduction in iNKT cell activation and no clinical improvement	NR	77 (NCT01085201[I])
Sulfasalazine	NF-κB inhibition	Management of arthritis and inflammatory bowel diseases	Hepatic dysfunction, leukopenia, agranulocytosis, megaloblastic anemia	78,79 (preclinical)
Statins	Up-regulating eNOS levels, smooth muscle migration, and proliferation	Management of dyslipidemia and coronary artery disease	Hepatic dysfunction, myopathy, renal dysfunction	80 (preclinical)
Propranolol	BCAM/Lu and ICAM-4 (LW)	Management of atrial fibrillation, hypertension, coronary artery disease, and cardiomyopathy	Cardiac dysfunction, bronchoconstriction, PAD exacerbation, hypoglycemia	81 (NCT02012777[I]*)
Vepoloxamer (Mst-188)	Multiple mechanisms and improves vascular rheology	Reduction in the duration and severity of pain crisis	Phase 3 showed no effect	82 (NCT00004408[III] and NCT01737814[III])
IVIG	Antibody binding via the Fc domain of the IgG molecules to the common IgG receptors; modulate neutrophil function via FcγRIII receptors; Mac-1 stabilization	Management of common immunodeficiency, autoimmune neuropathies, and vascular diseases	Concurrent infections, anaphylaxis, thromboembolic events, CNS and renal complications, hemolysis, and neutropenia	83–85 (NCT01757418[I/II])

(*table continues*)

Table 1 (*continued*)

Intervention	Target	Action and advantages	Disadvantages and adverse reactions	References
Antiplatelet and anticoagulant therapy				
Dabigatran	Direct thrombin inhibitor	Reduced neutrophil infiltrates in the pulmonary tissue	Bruising and minor bleeding	86
Rivaroxaban	Factor X inhibitor	Reduce inflammation, coagulation, and endothelial cell activation, oral drug	Bleeding abnormalities	87 (NCT02072668[II])
Prasugrel	P2Y12 inhibition	Reduce platelet activation and vaso-occlusive pain, oral drug; well tolerated and safe	Phase 3 showed nonsignificant reduction in vaso-occlusive crises among children with SCD, increased bleeding risk	88,89 (NCT01794000)

*Terminated.
[†]US Food and Drug Administration approved.
[‡]US Food and Drug Administration approved for a rare pediatric disease designation.
$A_{2A}R$, adenosine A2A receptor; BCAM/Lu, basal cell adhesion molecule and its isoform Lutheran; CNS, central nervous system; eNOS, endothelial nitric oxide synthase; HIT, heparin induced thrombocytopenia; ICAM-4 (LW), I]intercellular adhesion molecule-4 (Landsteiner-Wiener blood); iNKT, invariant natural killer T; IV, intravenous; IVIG, intravenous immunoglobulin; NR, not recorded; PAD, peripheral artery disease; PDE5, phosphodiesterase type 5 inhibitor; SCD, sickle cell disease; VOC, vaso-occlusive crisis.

and recruitment of neutrophils and complement system to remote tissues.[23]

Therapeutic Strategies for Treating I/RI in SCD

Despite the discovered molecular nature of SCD, huge disparities exist in developing therapies compared with other diseases, mainly because of the limited investment from the pharmaceutical industry and marginal clinical trials. Hydroxyurea, exchange blood transfusions, and the recently approved L-glutamine are currently the only available disease-modifying therapies.[3] Most of the focus of the treatment to date has been on the symptomatic and preventive aspects of the disease, including antibiotic prophylaxis, routine immunizations, and management of acute painful crisis with appropriate analgesia and hydration.[3] Successful stem cell transplantation can cure sickle cell anemia, but it is only performed in a few patients (with variable outcomes) because of its high cost, lack of suitable donors, widespread complications, and lack of availability in every center.[61] Other recent strategies lie in the field of gene editing. Using CRISPR-Cas9 gene editing, scientists have been able to repair the defective genes in cells taken from patients with SCD by up to 25%, although less success was observed when the edited cells were tested *in vivo*.[62] However, this idea of correcting the gene is far from being a readily available and acceptable clinical strategy for the treatment (and cure) of SCD.

The main focus of SCD management has been on the associated pain, which has given good short-term benefits without a substantial long-term therapeutic impact. Understanding the complex pathophysiologic mechanisms of SCD has led to multiple potential targets in the I/RI cascade, which may have long-term beneficial outcomes (Figure 2 and Table 1[63–89]). Some of these targets and strategies are discussed here.

AntiSickling Agents

Antisickling is the most common disease-modifying strategy in SCD. These agents increase fetal hemoglobin (HbF) levels, lessen the HbS load, and decrease the rate of hemolysis.[3] The most common antiswitching agent used is hydroxyurea; other agents that result in HbF induction have not proven to be as efficacious. Hydroxyurea was approved by the US Food and Drug Administration (FDA) for use in SCD in February 1998 and is believed to be acting via multiple pathways to induce HbF,[90] although the actual mechanism of action is still under debate. However, it is known that hydroxyurea acts as a nitric oxide donor,[91] stabilizing erythrocyte structure and decreasing circulating leukocytes and reticulocytes, all of which mitigate I/RI in SCD.[92]

Multiple trials have evaluated the role of hydroxyurea in SCD in both adults and children, demonstrating reductions in severity and frequency of VOC.[93,94] Furthermore, hydroxyurea significantly decreases adhesion molecules, such as VCAM-1, and reduces the adhesion of RBCs to the endothelium.[95] There are many trials in progress with different end points [eg, HbF response, acute complications, maximum tolerated dose, and percentage of dense RBCs

(NCT01960413, NCT02225132, NCT02042222)]. Of interest, other HbF-inducing agents (eg, decitabine, pomalidomide, butyrates ACY-957, Pracinostat, KLF1-ASOs, valproic acid, and trichostatin) are in preclinical or clinical testing or in development.[3]

Anti-Inflammatory Agents and Antineutrophil Agents

Many aspects of SCD, including multifocal microvascular occlusions and chronic inflammation, are consistent with I/RI. Targeting mechanisms of the inflammatory cascade, such as adhesive interactions between cellular components (eg, leukocytes, platelets, RBCs, and the endothelium), are of great interest as therapeutic targets.[10] Table 1 provides a comprehensive list of anti-inflammatory and antioxidant agents that have been tried in various studies. Some drugs and receptors or pathways of particular interest are as follows.

Selectin Inhibitors

Selectins mediate cellular rolling and adhesion to the endothelium. In particular, endothelial cells, as well as platelet-derived P-selectin, have a key role in the pathogenesis of VOCs, and P-selectin mediates adhesion of SS RBCs to vessels and assists in the formation of neutrophil-platelet aggregates.[73] *In vivo* studies have found that inhibition of adhesion molecules reduces VOCs significantly,[30,73,96] with the most commonly studied antiadhesive agent, the pan-selectin inhibitor GMI-1070 (rivipansel), reducing VOCs and disease severity *in vivo*.[96] A recently completed phase 1/2 study found high tolerability of GMI-1070 and suggested increased blood flow in a subset of patients (NCT00911495).[71] Telen et al[72] completed a phase 2 study, which revealed clinically relevant reductions in time of resolution of VOCs, length of stay in the hospital, and opioid dose needed after GMI 1070 administration (NCT01119833).

Several studies have found positive effects in specifically blocking P-selectin. In a recent double-blind, randomized, placebo-controlled phase 2 trial, patients receiving a high dose of P-selectin inhibitor crizanlizumab (SelG1) had a significantly lower rate of sickle cell—related pain crises than placebo.[73] Sevuparin (low-molecular-weight heparin) has low anticoagulant activity but is a potent P-selectin blocker, inhibiting HbSS RBC adhesion to the endothelium and reducing tumor necrosis factor—induced VOCs.[76] Sevuparin is currently undergoing a phase II trial (NCT0251838) for the management of acute VOCs.

Adenosine A2A Receptors and β_2-Adrenergic Pathway

The β_2-adrenergic system also plays a significant role in adhesion pathways via activation of basal cell adhesion molecule and its isoform Lutheran and intercellular adhesion molecule-4 (Landsteiner-Wiener blood).[81] Epinephrine elevates cAMP and increases adhesion in a B-adrenergic and protein kinase A—dependent manner (NCT02012777).[81] Invariant natural killer T (iNKT) cells become activated and increase in number during painful VOCs in SCD. Concomitantly, there is an increase in NF-κB phosphorylation, an increase in adenosine A2A receptor expression, and an increase in interferon-γ levels, all instigating inflammation during VOCs. Adenosine A2A receptor agonists can decrease the iNKT cell activation and may limit painful crisis in SCD. In a recent phase 1 study, regadenoson, an adenosine A2A receptor agonist, reduced the activation of iNKT cells without causing any toxic effects.[77] However, the phase 2 trial did not find any improvement in the clinical outcomes and nonsignificant reduction of iNKT cell activation between regadenoson treatment and placebo groups in SCD.[97] Sulfasalazine is a potent and specific inhibitor of NF-κB activation[78] and modulates endothelial function and significantly ameliorates secondary inflammatory damage (by reducing the expression of intercellular adhesion molecule, E-selectin, and VCAM, thus decreasing leukocyte recruitment) caused by SCD in multiple animal and human studies.[79,98,99]

Targeting Neutrophils by IVIGs

The working mechanism of immunomodulation by intravenous immunoglobulins (IVIGs) is still not completely understood, but research in the last decade has primarily focused on classic antibody binding via the Fc-domain of the IgG molecules to the common IgG receptors. Multiple studies have found a role of IVIG in modulating neutrophil activation, decreasing RBC-neutrophil interactions, inhibiting adhesion, and increasing their flow and survival.[83] These effects are mainly mediated via neutrophil specific FcγRIII receptors[84] and Mac-1.[85] An ongoing phase 1/2 trial (NCT01757418) is currently recruiting patients to assess the effects of IVIG on sickle cell pain crisis. In contrast to GMI-1070, low-dose IVIG at 200 to 400 mg/kg may have a better overall efficacy because of less of an increase in white blood cell and neutrophil counts, resulting in an increased effect on RBC capture.[85]

Antiplatelet and Anticoagulants

Although drugs that target the generation (eg, warfarin) or activity (eg, heparin) of thrombin have been tested for prevention of sickle cell crises, high oral doses proved not to be beneficial because of major bleeding complications.[100] However, lower doses, which may have greater benefit, have not yet been evaluated in randomized trials in patients with SCD. Nonetheless, thrombin remains a viable therapeutic target for prevention of the enhanced thrombosis in SCD.[86] In a preclinical study, a 10-day treatment of sickle mice with dabigatran (a direct thrombin inhibitor) resulted in reduced neutrophil infiltrates in the pulmonary tissue.[86] Prasugrel, an oral P2Y12 receptor inhibitor that prevents ADP-dependent platelet activation, has been also tested in various preclinical and clinical

trials for determining effects on vaso-occlusive events; so far, the results have failed to prove any substantial reduction.[88,89]

Oxidative and Nitrosative Targets

Oxidative and nitrosative stress and nitric oxide depletion play a pivotal role in the pathophysiologic mechanisms of SCD. Nitric oxide inhibits leukocyte adhesion and platelet adhesion and activation, thus blunting hemolysis and thromboinflammation associated with SCD.[10,101] Several agents that target different aspects of oxidant and nitric oxide biology have been used to modify SCD and reduce VOC events (Table 1). Some of the particular interest are as follows.

Nitrite Therapy

Initial studies in animals, as well as humans, had some promise with inhaled nitric oxide therapy. de Franceschi et al[63] subjected transgenic mice with SCD to hypoxia and reoxygenation, coupled with inhalation of nitric oxide, and found that this prevented histopathologic lung damage, attenuated inflammatory responses, modulated genes involved in I/RI, modulated vascular rheology, and attenuated RBC dehydration. However, in adults with SCD and mild to moderate ACS, nitric oxide therapy failed to show any reduction in treatment, suggesting that nitric oxide therapy is complex and requires further understanding.[64] The failure of inhaled nitric oxide may be because it mainly targets the lungs and was used under acute care settings, which may have been insufficient for I/RI affected tissues[101] with less systemic action.

Phosphodiesterase and Endothelin Receptor Inhibition

SCD is associated with a significant oxidant stress, which is attributable to lower redox potential attributed mainly to arginase release from intravascular hemolysis and endothelial production under a proinflammatory state.[67] The resulting deficit in arginine is known to contribute to VOC and early mortality.[67] Preliminary clinical studies with arginine therapy, including a phase 2 trial, found efficacy in managing acute VOCs[67] and other complications, such as leg ulcers[68] and PH.[69]

L-glutamine is a precursor of NAD and prevents oxidant damage to RBCs by improving the NAD redox potential.[70] A recent phase 3 trial (NCT01179217) found that it provides clinical benefit over placebo by reducing the frequency of painful crisis and hospitalization.[102] L-glutamine was recently approved by the FDA to reduce acute complications of SCD in adult and pediatric patients ($\geq$5 years old).[103] In addition, a multicenter randomized clinical trial of sildenafil for PH in patients with SCD (termed the walk-PHaSST) was stopped because of serious adverse effects in the treatment group.[66] Results were also inconclusive when endothelin receptor blockade was used (Asset-1 and -2, with bosentan) as a potential therapy for PH.[104]

Conclusions

The role of I/RI in the pathophysiology of SCD is complex and needs further understanding. Reperfusion injury attributable to ROS production and cellular activation plays a major role in SCD and should set the stage for more robust and durable preclinical and clinical research, ultimately paving the way for new disease-modifying agents. In addition, SCD is a thromboinflammatory state that contributes to chronic inflammation and I/RI. Targeting various thrombosis and coagulation mechanisms and pathways may also be another viable therapeutic strategy for drug discovery programs. The recent FDA approval of L-glutamine, promising results with crizanlizumab, and the continued success of hydroxyurea in both the pediatric and adult populations have given more impetus to the study of the various checkpoints in the I/RI-based pathophysiology in SCD. In summary, because SCD is a systemic and widespread disease that involves a panoply of cellular mediators and pathways, a more individualized multitargeted therapeutic approach might be the future for SCD medicine.

Acknowledgment

We thank Youmna Moufarrej (Louisiana State University Medical School) for her help with the illustrations.

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