Neutrophils, Platelets, and Inflammatory Pathways, at the Nexus of Sickle Cell Disease Pathophysiology

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Sickle cell disease (SCD) is a severe genetic blood disorder characterized by hemolytic anemia, episodic vaso-occlusion and progressive organ damage. Current management of the disease remains symptomatic or preventative. Specific treatment targeting major complications such as vaso-occlusion is still lacking. Recent studies have identified various cellular and molecular factors that contribute to the pathophysiology of SCD. Here we review the role of these elements and discuss the opportunities for therapeutic intervention.

Introduction

Sickle cell disease originates from a single nucleotide mutation of β-globin, which leads to polymerization of the mutated hemoglobin upon deoxygenation, and dramatic alteration in the shape and surface properties of red blood cells (RBCs). Through interactions with multiple blood and immune cell populations, sickle RBCs promote inflammation, obstruct the vasculature, and injure the endothelium, leading to broad manifestations that affect most vital organs. Although the molecular origin of the disease is clear, the mechanisms that contribute to the complex manifestation and severe outcome of the disease have not been fully elucidated. Recent studies have revealed that many cell types that are not affected by the β-globin mutation play important roles in the pathophysiology of SCD, leading to an evolving multicellular paradigm that has triggered enthusiastic investigations into novel therapeutics for the disease.
Neutrophils in Sickle Cell Disease

Neutrophils are a critical component of innate immunity. As the most abundant immune cells in the circulation, they provide immune protection against invading pathogens but can also promote certain inflammatory diseases. Neutrophils are initially suggested to promote disease progression in SCD by clinical epidemiological studies. SCD patients were found to exhibit marked variation in disease severity. For example, in patients with painful crises, the most common disease manifestation, the rates of crises vary from 0 to >10 episodes per year. Notably, patients with more severe clinical manifestations tend to have higher neutrophil counts compared to racially matched controls. High leukocyte counts also positively correlate with early death, silent brain infarcts, hemorrhagic strokes and acute chest syndrome (ACS) in SCD patients, implicating leukocyte count (neutrophil in particular) as a major risk factor for SCD.

Further evidence supporting a role for neutrophils in SCD pathophysiology comes from the identification of myeloid growth factors, granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), as absolute contraindications in SCD individuals. In early reports, severe or fatal crises have occurred in SCD patients administered with either GM-CSF or G-CSF to treat leg ulcer, mobilize hematopoietic stem cells (HSCs) or correct neutropenia. More recently, a patient was reported to have a rare co-existence of SCD and severe congenital neutropenia, exhibiting significantly alleviated disease manifestations compared to his siblings. However, when the
patient received G-CSF to treat neutropenia, the course of the disease dramatically worsened.\textsuperscript{19}

By contrast, reduction in neutrophil count can benefit SCD. In a multi-center study of hydroxyurea (MSH), hydroxyurea treatment – the most commonly used therapeutics for SCD patients – markedly decreased the frequency of painful crises and ACS in patients with moderate to severe SCD.\textsuperscript{20} Hydroxyurea has been shown to effectively induce fetal hemoglobin (HbF) expression in RBC, but it has also many other effects that benefit SCD.\textsuperscript{21-24} For example, hydroxyurea treatment significantly decreases soluble VCAM-1 levels in patient plasma, and reduces adhesion of sickle RBCs to the endothelium.\textsuperscript{22,23} In addition, recent studies suggest that hydroxyurea treatment increases nitric oxide (NO) species, which may or may not be associated with induction of HbF.\textsuperscript{21,25-27} Interestingly, hydroxyurea treatment shows beneficial effects even in patients with no detectable rise of HbF, whereas all patients that respond well clinically to hydroxyurea treatment exhibit decreased numbers of neutrophils.\textsuperscript{22,28,29}

Neutrophils from patients with SCD also exhibit an activation phenotype characterized by lower expression level of L-selectin (CD62L) and higher level of CD64.\textsuperscript{30} In addition, CD11b/CD18 membrane expression is also approximately 70\% higher on neutrophils from SCD patients compared to controls.\textsuperscript{31} These neutrophils show increased adhesive properties, which could be reduced by stimulation of the NO/cGMP-dependent pathways.\textsuperscript{32} Hydroxyurea treatment is
found to suppress neutrophil activation as demonstrated by correction of neutrophil activation markers.\textsuperscript{33} Further studies suggest that hydroxyurea treatment has immediate benefits on sickle cell vaso-occlusion by inhibiting neutrophil recruitment and activation, with a mechanism that involves the amplification of the NO-cGMP pathway.\textsuperscript{25,26} These findings suggest an important role of neutrophils in the pathophysiology of SCD.

**Neutrophil-RBC Interactions Promote Vaso-occlusion**

The first evidence that neutrophils may directly participate in the pathogenesis of SCD comes from the observation that sickle RBCs bind to neutrophils \textit{in vitro}.\textsuperscript{3} This observation is supported by \textit{in vivo} studies in SCD mice (Berkeley mice\textsuperscript{34}), where the dynamics of circulating blood cells are analyzed in the cremasteric microcirculation using intravital microscopy.\textsuperscript{35} In this model, sickle RBCs are found to predominantly interact with adherent leukocytes in post-capillary venules. These interactions are triggered by surgical trauma, enhanced and sustained by TNF-\textit{α} administration, leading to a lethal vaso-occlusive crisis (VOC). Mice lacking both P- and E-selectins, in which leukocytes are prevented from recruitment to the endothelium, are protected from VOC in this model.\textsuperscript{35}

Further studies using multichannel fluorescence intravital microscopy (MFIM) identify Gr-1\textsuperscript{+} neutrophils as the major leukocyte population that is recruited to post-capillary venules and interacts with circulating RBCs in TNF-\textit{α} stimulated mice.\textsuperscript{36} Sickle RBCs are captured by activated \(\alpha_4\beta_2\) integrin (Mac-1) clustered on
the leading edge of adherent neutrophils, leading to acute lethal VOC. The activation of Mac-1 on adherent neutrophils requires a secondary wave of signals transduced by E-selectin ligand-1 (ESL-1) upon engagement of neutrophils by endothelial E-selectin. Inactivation of E-selectin and Mac-1 by either genetic manipulation or antibody blocking prevents neutrophil-RBC interactions, leading to improved blood flow and prolonged survival of SCD mice.

These studies suggest that vaso-occlusion in SCD may arise from a multistep and multicellular process as detailed in Fig. 1. First, endothelial cells are activated by internal and exogenous inflammatory stimuli, enabling the recruitment of circulating neutrophils to post-capillary venules. Then adherent neutrophils interact with circulating sickle RBCs through activated Mac-1, leading to transient or prolonged obstruction of venular blood flow. The obstruction of blood flow increases the transit time of RBCs, produces ischemia, enhances RBC sickling and further promotes neutrophil recruitment and heterotypic interactions, all of which culminate in vascular occlusions that may cause life-threatening crises.

**Neutrophil Heterogeneity and Microbiota Regulation**

Circulating neutrophils have long been considered a homogenous population. However, heterogeneity may arise from their aging in the circulation and replenishment by newly released neutrophils from the bone marrow. Recent studies show that aged neutrophils, marked by CD62L$^{lo}$ CXCR4$^{hi}$, represent an overly active neutrophils subset that exhibits enhanced Mac-1 activation and...
neutrophil extracellular traps (NETs) formation under inflammatory conditions. Interestingly, neutrophil aging is driven by microbiota-derived signals through neutrophil toll-like receptors (TLRs) and Myd88-mediated signaling (Fig. 1). Microbiota depletion or genetic deficiency of these signaling pathways leads to reduced aged neutrophil numbers and diminished Mac-1 activation in the vasculature. In SCD mice, the aged neutrophil population is dramatically expanded, and the increased aged neutrophil counts correlate positively with neutrophil adhesion, Mac-1 activation and neutrophil-RBC interactions. Microbiota depletion using broad spectrum antibiotics largely normalizes the numbers of aged neutrophils in SCD mice and markedly reduces neutrophil adhesion, Mac-1 activation and neutrophil-RBC interactions, leading to significantly improved blood flow and prolonged survival. In addition, chronic depletion of the microbiota protects SCD mice from tissue damage. For example, splenomegaly and liver damage induced by SCD are significantly alleviated in antibiotics-treated SCD mice.

Patients with SCD have also been analyzed for aged neutrophil numbers. Consistent with the rodent data, the numbers of aged neutrophils are dramatically increased in SCD patients compared to healthy controls. Strikingly, in the patient population taking penicillin prophylaxis, the numbers of aged neutrophils are significantly reduced. Although penicillin V can clearly prevent morbidity and mortality from Streptococcus pneumoniae infection in young children with SCD, whether antibiotics can mitigate vaso-occlusive episodes
remains unknown. These results thus raise the possibility that antibiotics therapy may have a broader impact in the management of the disease, which should be evaluated in clinical trials.

**Platelets in Sickle Cell Disease**

Platelets are essential for hemostasis but can also promote inflammation.\(^{42}\) Platelet activation is elevated in SCD patients under steady-state condition, which is further enhanced during VOC.\(^{43-45}\) Activated platelets may promote the adhesion of sickle RBCs to human vascular endothelium by secreting thrombospondin,\(^{46}\) and may contribute to thrombosis and pulmonary hypertension in SCD.\(^{43}\) Platelets can also bind to erythrocytes, monocytes and neutrophils to form aggregates.\(^{45,47}\) Significantly more platelet-monocyte aggregates and platelet-neutrophil aggregates are observed in the circulation of both SCD patients and transgenic SCD mice compared to controls.\(^{48}\) Activated platelets bind to neutrophils to form aggregates in a P-selectin-dependent manner.\(^{49}\) Pulmonary dysfunction is significantly ameliorated in SCD mice when platelet-neutrophil aggregate formation is blocked by P-selectin neutralizing antibody or the platelet inhibitor Clopidogrel.\(^{49}\) At the molecular level, the serine/threonine kinase isoform AKT2 in neutrophils appears essential for neutrophil crawling and neutrophil-platelet interactions on activated endothelial cells during vascular inflammation.\(^{50}\) Inhibition of AKT2 diminishes neutrophil adhesion and neutrophil-platelet interactions, leading to improved blood flow in SCD mice.\(^{50}\)
Platelets can also be captured by activated Mac-1 integrin clustered on the leading edge of adherent neutrophils, promoting vascular damage and lung injury by generating oxidative species in a mouse model of transfusion-related acute lung injury (TRALI). In addition, platelet interaction can also induce neutrophil polarization through P-selectin glycoprotein ligand-1 (PSGL-1) transduced signals, triggering directed migration of neutrophils to initiate inflammation. Although these studies indicate a role for platelets in the pathogenesis of SCD, direct evidence that platelets participate in major complications such as vaso-occlusion in SCD is still lacking. Our own studies have found surprisingly little platelet accumulation at sites of active VOC (P.S.F., unpublished data).

**Hemolysis, NETs, and Acute Lung Injury**

Hemolysis in SCD arises from damaged sickle RBC membranes, causing chronic anemia and the release of hemoglobin into the circulation, which promotes inflammation by depleting NO, generating oxidative stress, and releasing heme, the prosthetic moiety of hemoglobin. A large fraction of extracellular heme is carried by the erythrocyte-derived microparticles in circulation. These heme-loaded microparticles transfer heme to endothelial cells, leading to vascular dysfunction and vaso-occlusion in SCD. Activation by extracellular heme increases expression of adhesion molecules on endothelial cells, thus promoting leukocyte recruitment. Heme can also contribute to the pathogenesis of SCD by
activating placenta growth factor (PlGF) production from erythroid cells via erythroid Krüppel-like factor.\textsuperscript{55}

Recent studies have found that plasma heme levels are elevated during VOC in both human patients and SCD mice, leading to neutrophil activation and the formation of NETs in pulmonary vasculature.\textsuperscript{56} NETs in pulmonary vasculature contribute to acute lung injury in SCD mice challenged with TNF-\(\alpha\), which is significantly alleviated by administration of DNAse I or hemopexin, the reagent that dismantles NETs or scavenges heme, respectively.\textsuperscript{56} Administration of exogenous heme or hemin, the oxidized form of heme, can trigger VOC or ACS in SCD mice through activation of the endothelium, which is largely prevented by TLR4 inhibition, suggesting that extracellular heme/hemin may signal through TLR4 to trigger an inflammatory response.\textsuperscript{57,58} In addition, hemolytic transfusion reactions (HTRs) induce lethal acute vaso-occlusion in a model of alloimmune, IgG-mediated HTRs in SCD mice.\textsuperscript{59} These studies suggest that VOC and acute lung injury may be interconnected by neutrophils, NETs, and hemolysis.

Platelets can also activate neutrophils to produce NETs. During bacterial infection, platelets are activated by TLR4 ligand in the circulation, and then bind to the adherent neutrophils, leading to the formation of NETs in liver sinusoids and pulmonary capillaries.\textsuperscript{60} Platelets also promote NET formation in TRALI.\textsuperscript{61} Targeting platelets activation with aspirin or a glycoprotein IIb/IIIa inhibitor reduces NET formation and alleviates lung injury.\textsuperscript{61} Similarly, aspirin inhibits
neutrophil-platelet aggregate formation and thereby protects the lungs in an acute lung injury model.\textsuperscript{62} These studies raise a possibility that platelets may contribute to SCD pathophysiology by promoting NET formation, although direct evidence is still needed.

**Inflammation in Sickle Cell Disease**

SCD has long been recognized as a chronic inflammatory disease. Accumulating evidence reveals that cell types not affected by the mutation, such as leukocytes and endothelial cells, play important roles in SCD pathophysiology. In addition, the list of molecules that promote inflammation in SCD is also expanding (Fig.2).

**Cells that promote inflammation in SCD:**

**Monocytes** exhibit an activated phenotype and promote inflammation in SCD.\textsuperscript{63-65} Monocytes from SCD patients can activate NF-κB pathway in endothelial cells. The activation of NF-κB pathway results in higher expression of adhesion molecules, including E-selectin, ICAM-1, VCAM-1, thus promoting mononuclear leukocytes adhesion to endothelial cells. The activation of endothelial cells by monocytes does not require cell-cell contact, but is mediated pro-inflammatory cytokines produced by monocytes. When TNF-α and IL-1β from monocytes are blocked with neutralizing antibodies, the upregulation of E-selectin expression by endothelial cells is abrogated.\textsuperscript{63} Importantly, monocytes isolated from SCD patients have higher TNF-α and IL-1β expression levels compared to healthy controls.\textsuperscript{64} Although the mechanisms that contribute to monocyte activation in
SCD remain largely unclear, recent studies have identified several molecular and cellular factors important for this phenomenon. The first clue has emerged from the studies showing that platelet binding to monocytes leads to monocyte activation. The percentage of platelet-monocyte aggregates is significantly higher in SCD patients compared to healthy controls, suggesting an increased activation level of monocytes. The elevated levels of PIGF in SCD patients also contribute to monocytes activation. PIGF, an angiogenic growth factor belonging to the vascular endothelial growth factor family, is produced by erythroid cells. Clinical studies found that PIGF levels positively correlate with the severity of disease manifestation in patients with SCD. PIGF significantly increases the expression of pro-inflammatory cytokines including IL-1β, TNF-α, IL-8, monocyte chemoattractant protein-1 and VEGF via the activation of Flt-1 and the PI3K/AKT and ERK-1/2 pathways in peripheral blood monocytes.

Invariant natural killer (iNKT) cells have been shown to play a key role in promoting pulmonary inflammation and dysfunction in SCD. iNKT cells are a subset of T cells that have limited T cell receptor (TCR) repertoire and recognize lipid antigens presented by CD1d on antigen presenting cells. iNKT cells are thought to bridge innate and adaptive immunity by producing large amounts of cytokines shortly after stimulation. In NY1DD transgenic SCD mice, more numerous and activated iNKT cells (CD69+ IFN-γ+) that are hypersensitive to hypoxia/reoxygenation are found in the spleen, liver, and lung compared to controls. iNKT cells cause baseline pulmonary dysfunction by producing IFN-
γ and IFN-γ induced CXCR3 chemokines in SCD mice, which can be prevented by genetic deficiency or pharmacological inactivation of iNKT cells. Further studies have found that iNKT cells in SCD mice also exhibit higher expression levels of the adenosine 2A receptor (A2AR), a receptor that triggers anti-inflammatory response upon activation. Treatment of SCD mice with A2AR agonist regadenoson could effectively prevent baseline pulmonary dysfunction in SCD mice. Importantly, SCD patients also exhibit more numerous and activated iNKT cells in the circulation. During painful VOC, iNKT cells become more activated (CD69+ IFN-γ+ IL-4+), and express higher levels of A2AR in an NF-κB dependent manner in SCD patients. These results suggest that targeting iNKT cells by either humanized monoclonal antibodies or A2AR agonist may benefit SCD.

Endothelial cells have been described to have an activated phenotype in SCD. The activation of endothelial cells may be induced by adhesion of sickle RBCs, which produces oxidative stress, activates NF-κB pathway and promotes the transmigration of monocytes. Treatment with a NF-κB pathway inhibitor reverses the activation phenotype of circulating endothelial cells in SCD patients and of both circulating and tissue endothelial cells in SCD transgenic mice in vivo. Furthermore, monocytes-derived TNF-α and IL-1β also activate endothelial cells via the NF-κB pathway. In addition, platelets can activate endothelial cells in patients with SCD. More recently, endothelial cells have been shown to be activated by hemolysis. In particular, heme can be transported
to endothelial cells by circulating microparticles, activating endothelial cells through TLR4-mediated signaling.\textsuperscript{53,57} The activation of endothelial cells is critical in sickle cell vaso-occlusion.\textsuperscript{78} In a hypoxia/reoxygenation-induced vaso-occlusion model, the anti-inflammatory reagent dexamethasone significantly inhibits leukocyte-endothelium interaction and prevents vaso-occlusion by reducing the expression of adhesion molecules on endothelial cells.\textsuperscript{78} Similar beneficial effects are observed when VCAM-1 or ICAM-1 is blocked with monoclonal antibodies.\textsuperscript{78} In another vaso-occlusion model induced by TNF-\(\alpha\) and surgical trauma, a pan-selectin antagonist, GMI-1070, has been found to reverse acute vascular occlusion.\textsuperscript{79}

**Molecules that promote inflammation in SCD:**

**Pro-inflammatory cytokines** play critical roles in the pathophysiology of SCD. SCD patients exhibit higher levels of several pro-inflammatory cytokines, including TNF-\(\alpha\), IL-6, IL-1\(\beta\), GM-CSF, IL-3, endothelin-1 and prostaglandin E\(2\).\textsuperscript{80-84} Pro-inflammatory cytokines activate endothelial cells and leukocytes through the NF-\(\kappa\)B pathway.\textsuperscript{66} The activation of NF-\(\kappa\)B pathway results in increased expression of endothelial adhesion molecules, such as ICAM-1, VCAM-1, P-selectin and E-selectin, promoting leukocyte adhesion and triggering the ensuing vaso-occlusive events.\textsuperscript{82} For example, TNF-\(\alpha\) alone with surgical trauma is enough to trigger lethal acute vaso-occlusion in transgenic SCD mice.\textsuperscript{35}
**Chemokines** are a family of small molecules that can induce chemotaxis.\(^8^5\) Chemokines contribute to SCD pathogenesis by acting on leukocytes.\(^5^9\) In NY1DD SCD mice, higher levels of CXCL9 and CXCL10 are observed in the lung.\(^7^0\) These chemokines recruit CXCR3-expressing leukocytes, promoting inflammation and causing pulmonary dysfunction. Neutralization of CXCR3 with a monoclonal antibody significantly alleviates pulmonary dysfunction in these animals.\(^7^0\) CXCL1, a neutrophil chemoattractant, is also a key inflammatory mediator of acute vaso-occlusion in Berkeley SCD mice.\(^5^9\) CXCL1 is elevated during hemolytic transfusion reaction (HTA) induced VOC. Administration of recombinant CXCL1 alone triggers lethal VOC in SCD mice, and blockade of CXCL1 receptor, CXCR2, inhibits VOC in this model.\(^5^9\) Importantly, elevated levels of IL-8 or CXCL8 are observed in SCD patients during crises and have been proposed to be a VOC predictor.\(^8^6,^8^7\) IL-8 may play a role in SCD pathophysiology by promoting neutrophil adhesion to endothelial cells.\(^8^8\)

The **coagulation cascade** also plays an important role in promoting vascular inflammation in SCD.\(^8^9\) Increased tissue factor (TF) expression, exposure of phosphatidylserine (PS) on sickle RBCs, circulating microparticles and activated platelets together can lead to a well-documented hypercoagulable state and thrombotic complications.\(^9^0,^9^1\) Although direct evidence that coagulation pathways contribute to major disease manifestation such as vaso-occlusion is still lacking, emerging evidence suggests that hypercoagulation and chronic inflammation in SCD are correlated. For example, a recent study shows that
induction of endothelial TF expression depends on the NFκB pathway in peripheral mononuclear cells.\textsuperscript{92} In addition, the histone deacetylase inhibitor trichostatin A (TSA) and the TSA analog suberoylanilide hydroxamic acid (SAHA), drugs with multimodal activities, reduces both TF and VCAM-1 expression in SCD pulmonary vascular endothelial cells with or without a hypoxia/reoxygenation challenge.\textsuperscript{93} Hydroxyurea treatment is also found to reduce several markers of coagulation activation.\textsuperscript{94,95} Furthermore, inhibition of TF has been shown to attenuate inflammation-associated vascular injury, as measured by plasma levels of IL-6, serum amyloid P, and soluble VCAM-1.\textsuperscript{96} Inhibition of TF also reduces pulmonary expression of MCP-1, KC and MPO in two models of SCD (BERK and Townes).\textsuperscript{96} Similarly, inhibition of FXa and deficiency of thrombin receptor, protease activated receptor-2 (PAR-2), in non-hematopoietic cells attenuate systemic plasma levels of IL-6.\textsuperscript{97} More recently, it has been reported that genetic deletion of circulating prothrombin alleviates multi-organ pathologies in SCD mice.\textsuperscript{98} In addition, thrombospondin-1 (TSP1) can stimulate microparticle release from RBCs. These microparticles trigger reactive oxygen species (ROS) production by endothelial cells, promote leukocyte adhesion, and induce endothelial apoptosis in a PS-dependent manner, leading to acute vaso-occlusive events in SCD mice.\textsuperscript{99}

**High mobility group box 1 (HMGB1)** has been recently identified as a key player in promoting inflammation in SCD. HMGB1 is a danger-associated molecular pattern (DAMP) molecule released from activated immune cells and
necrotic cells, which can activate the TLR4 signaling pathway.\textsuperscript{100} The levels of HMGB1 are elevated and further increase during VOC in both SCD patients and transgenic SCD mice. HMGB1 accounts for the majority of sickle cell plasma-induced TLR4 activity both \textit{in vitro} and \textit{in vivo}.\textsuperscript{101}

**Inflammation, Neuropathy and Pain**

Most patients with SCD experience moderate to severe pain throughout their lifetime. The refractory nature of pain and the development of steady, persistent chronic pain in some patients suggest that neuropathy may contribute to pain in SCD. In a recent analysis of descriptors of pain reported by patients, pain in SCD turns out to be nociceptive and neuropathic, contrary to common expectations that SCD pain is only nociceptive.\textsuperscript{102} This finding has triggered investigations to ascertain whether an adjuvant analgesic treatment can improve pain management. Interestingly, trifluoperazine, a potent Ca\textsuperscript{2+}/calmodulin protein kinase II\textalpha{} (CaMKII\textalpha{}) inhibitor commonly used to treat neuropathic pain, shows significant beneficial effects on SCD patients, suggesting a role for neuropathy in the pathogenesis of SCD pain.\textsuperscript{103}

Studies in SCD animal models have revealed neurochemical alterations in SCD. Similar to human patients, SCD mice exhibit altered sensitivity to pain as demonstrated by musculoskeletal and cutaneous hyperalgesia. Peripheral nerves and blood vessels are structurally altered in the skin, with decreased expression of \upsilon{}-opioid receptors and increased calcitonin gene-related peptide
and substance P immunoreactivity.\textsuperscript{104} Interestingly, activators of neuropathic and inflammatory pain, including p38, STAT3, and MAPK, exhibit increased phosphorylation, correlating with increases in COX-2, IL-6, and TLR4 levels in the spinal cord.\textsuperscript{104} Further studies identify a direct role for inflammation in pain pathophysiology in SCD, where mast cells promote neurogenic inflammation and nociceptor activation through the release of substance P in the skin and dorsal root ganglion. Targeting mast cells by genetic manipulation or pharmacologic inhibition effectively alleviates pain in SCD mice.\textsuperscript{105} These studies therefore suggest that neurogenic inflammation or neuropathy may be novel targets for pain management in SCD.

**Emerging Therapies targeting Neutrophils, Platelets and Inflammatory Pathways in Sickle Cell Disease**

Since polymerization of the abnormal hemoglobin represents a key step in the pathophysiology of SCD, it has been an important therapeutic target for over five decades. Any agents directly inhibiting this step, by HbF induction or by other mechanisms, will have numerous downstream effects on adhesion and inflammation. Our current understanding of the critical role of other cell types besides RBCs and key inflammatory processes, have led to the development of novel targeted therapies that are summarized below.

**Targeting neutrophils:**
Hydroxyurea, the only FDA approved therapy for SCD, is a potent inducer of HbF.\textsuperscript{106} However, hydroxyurea has multiple mechanisms by which it produces its clinical response. The Evidence-Based Management of Sickle Cell Disease: Expert Panel Report 2014 (available at http://www.nhlbi.nih.gov/health-pro/guidelines/sickle-cell-disease-guidelines/) recommends dose escalation of hydroxyurea to achieve mild myelosuppression (absolute neutrophil count 2,000/uL to 4,000/uL) based on aggregate clinical and laboratory parameters. This is often accompanied by reduction in baseline thrombocytosis. While both of these effects may independently contribute to its clinical efficacy, they also affect the maximum tolerated dose, a clear predictor of HbF response.\textsuperscript{107} It is also interesting to note that short-term administration of either hydroxyurea or the phosphodiesterase 9 (PDE9) inhibitor ameliorates VOC in a SCD murine model via NO-mediated cGMP elevating effects that lead to reduced neutrophil adhesion and heterotypic RBC-neutrophil interactions, suggesting that hydroxyurea may have immediate benefit in the setting of VOC.\textsuperscript{26} PDE9 is highly expressed in hematopoietic cells and is thus a promising therapeutic target. PF04447943, a PDE9 inhibitor is currently being evaluated in a phase I study (NCT02114203).

Rivipansel (GMI-1070), a synthetic pan-selectin inhibitor, has been shown to inhibit predominantly E-selectin-mediated leukocyte adhesion and dramatically reduce RBC-leukocyte interactions, leading to improved blood flow and prolonged survival in SCD mice during VOC.\textsuperscript{79} In a phase I clinical trial, GMI-
1070 has been well tolerated without significant adverse effects. SCD patients receiving GMI-1070 exhibited a modest increase in total peripheral white blood cell count without clinical symptoms. Results of a prospective multicenter, randomized, placebo-controlled, double-blind, phase II study of 76 SCD patients with acute VOC reveals a positive, but non-significant trend, in the primary endpoint of time to VOC resolution in GMI1070 compared to placebo and a clinically significant decline of 63 hours (48%) in median values. In addition, an 83% reduction of opioid use (p=0.01) in the active arm has been noted. Currently, a phase III clinical trial of rivipansel in SCD is registered (NCT02187003).

**Intravenous immunoglobulin (IVIG)** can reverse acute VOC in preclinical models by rapidly inhibiting neutrophil adhesion to the endothelium and abrogating RBC-neutrophil interactions. IVIG beneficial signaling is mediated by FcγRIII receptors, the only Fc receptor expressed on murine neutrophils, resulting in the recruitment of Src homology 2-containing tyrosine phosphatase-1 (SHP-1) and the inhibition of adhesion and αMβ2 integrin activation. The protective effects of IVIG are abrogated in SHP-1-deficient mice, suggesting an important role of SHP-1 signaling in regulating neutrophil adhesion and activation. IVIG administration in SCD patients in a phase I study demonstrates no significant adverse events with doses up to 800mg/kg, indicating safety and tolerability in acute VOC. Since lower doses show better
biomarker and clinical efficacy profiles, the 400mg/kg dose is selected for further study in an ongoing phase II trial (NCT01757418).

Numerous preclinical and early phase clinical studies have demonstrated the benefit of targeting P-selectin. The efficacy of SelG1, a humanized anti-P-selectin monoclonal antibody, in reduction of pain crises rates is currently being tested in an ongoing phase II trial (NCT01895361). Heparins also act via the inhibition of P-selectin-mediated adhesion in addition to inhibition of ligand binding to leukocyte integrin Mac-1 and other anti-inflammatory effects. This is in addition to their expected anticoagulant effect. Tinzaparin, a low molecular weight heparin, has been studied in a randomized double-blind clinical trial. 253 subjects were enrolled, ages 12 years and older, in a study in which reduced duration of VOC and no severe bleeding complications were reported. A feasibility study of unfractionated heparin in acute chest syndrome is currently recruiting (NCT02098993).

**Targeting platelets:**

**Prasugrel** is a thienopyridine P2Y12 adenosine diphosphate (ADP) receptor antagonist that inhibits ADP-mediated platelet activation and aggregation. Randomized double-blind placebo-controlled phase II studies to examine safety have been completed in adults. Mean pain rates (percentage of days with pain) and intensity in the prasugrel arm are decreased compared with placebo. However, these reductions do not reach statistical significance. Platelet surface
P-selectin and plasma soluble P-selectin, biomarkers of platelet and endothelial activation, are significantly reduced in SCD patients receiving prasugrel compared with placebo. Prasugrel is generally well tolerated and a phase III trial in children is ongoing (NCT01794000). Ticagrelor is a novel antiplatelet agent that also inhibits ADP-mediated platelet activation, but unlike Prasugrel, does not require metabolic activation. A phase II trial is currently registered (NCT02482298).

**Targeting inflammation:**

Regadenoson is an A$_{2A}$ receptor (A$_{2A}$R) agonist reported in a phase I study in SCD patients to reduce iNKT cell activation to levels noted in healthy controls and SCD patients at baseline.$^{75}$ Notably, iNKT cell activation is associated with increased phosphorylation of NFkB p65, increased expression of A$_{2A}$R and higher levels of IFN-$\gamma$. Although NFkB p65 phosphorylation was reduced to baseline levels, the reduction in A$_{2A}$R expression and IFN- $\gamma$ levels did not reach statistical significance.$^{75}$ No toxicity has been noted. Based on these positive findings in the biological endpoints, a phase II randomized placebo-controlled trial is currently recruiting (NCT01788631).

Several additional agents targeting inflammation via modulation of oxidant stress, nitric oxide bioavailability, coagulation and inflammatory cytokines are listed in **Table 1**. Of note, a randomized placebo-controlled double-blind trial of omega-3 fatty acids conducted at a single center in Sudan shows promising results.
Omega-3 treatment reduces VOC events and transfusions in enrolled patients (n=140) monitored for a year.\textsuperscript{120} This well tolerated therapy warrants further study. **Glutamine** enhances nicotinamide adenine dinucleotide (NAD) in sickle RBCs\textsuperscript{121}. A recently completed phase III trial of Glutamine in SCD (NCT01179217) conducted at 31 sites in the United States and enrolled 230 patients. A statistically significant reduction in pain crises and hospitalization is apparent in the treatment arm, with improvements in several secondary outcomes including the rates of acute chest syndrome.

**Conclusion**

In summary, advances in the understanding of the pathophysiology of sickle VOC have led to several new exciting agents that are currently being evaluated. Given the complexity and multiplicity of events leading to the vaso-occlusive event, it is likely that a multi-targeted multi-modal approach will be required to achieve the best outcome.
Conflict of interest:

P.S.F. has been a consultant and his laboratory has received funding from Glaxo Smith Kline and NKT Therapeutics.

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Author Contributions

D.Z., C.X., D.M. and P.S.F. wrote the manuscript.
Table 1. Agents targeting Neutrophils, Platelets and Inflammatory Pathways

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<th>Mechanism of Action</th>
<th>Phase (Study ID)</th>
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<td>Pan-selectin inhibitor</td>
<td>3 (NCT02187003)</td>
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<td>Unfractionated Heparin</td>
<td>Inhibits P-selectin and Mac-1 ligand binding</td>
<td>2 (NCT02098993)</td>
</tr>
<tr>
<td>Platelets</td>
<td>Prasugrel</td>
<td>ADP receptor blockade</td>
<td>3 (NCT01794000)</td>
</tr>
<tr>
<td></td>
<td>Ticagrelor</td>
<td>ADP receptor blockade</td>
<td>2 (NCT02482298)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Regadenoson</td>
<td>A2AR agonist, blocks iNKT activation</td>
<td>2 (NCT01788631)</td>
</tr>
<tr>
<td></td>
<td>NKTT120</td>
<td>Humanized monoclonal antibody, depletes iNKT cells</td>
<td>1 (NCT01783691)</td>
</tr>
<tr>
<td></td>
<td>Montelukast</td>
<td>Inhibits leukotriene receptors and mast cell degranulation</td>
<td>2 (NCT01960413)</td>
</tr>
<tr>
<td></td>
<td>Mometasone</td>
<td>Inhaled corticosteroids</td>
<td>2 (NCT02061202)</td>
</tr>
<tr>
<td></td>
<td>Rivaroxiban</td>
<td>Anti-Xa, inhibits IL-6 and VCAM-1</td>
<td>2 (NCT02072668)</td>
</tr>
<tr>
<td></td>
<td>Omega-3 fatty acids</td>
<td>Reduces endothelial activation</td>
<td>2 (ISRCTN80844630)</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>Increases NADPH, reduced oxidative stress</td>
<td>3 (NCT01179217)</td>
</tr>
<tr>
<td></td>
<td>N-acetylcysteine</td>
<td>Increases glutathione, reduces oxidative stress</td>
<td>3 (NCT01849016)</td>
</tr>
<tr>
<td></td>
<td>Simvastatin</td>
<td>Activates endothelial NO synthase</td>
<td>2 (NCT01702246)</td>
</tr>
<tr>
<td></td>
<td>Atorvastatin</td>
<td>Activates endothelial NO synthase</td>
<td>2 (NCT01732718)</td>
</tr>
<tr>
<td></td>
<td>L-arginine</td>
<td>Substrate for NO</td>
<td>2 (NCT02447874)</td>
</tr>
<tr>
<td></td>
<td>Vaporized Cannabis</td>
<td>Targets cannabinoid receptors to reduce chronic pain and</td>
<td>1, 2 (NCT01771731)</td>
</tr>
</tbody>
</table>

*Several therapies have multiple mechanisms of action


33. Benkerrou M, Delarche C, Brahimi L, et al. Hydroxyurea corrects the dysregulated L-selectin expression and increased H(2)O(2) production of


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Figure Legend

Figure 1: Multicellular and Multistep Model of Sickle Cell Vaso-occlusion. Sickle cell vaso-occlusion arises from a cascade of interactions among red blood cells (RBCs), neutrophils, and endothelial cells. Activation of endothelial cells leads to the recruitment of neutrophils, which is initiated by rolling of neutrophils on endothelial selectins, followed by adhesion mediated by integrins. Adherent neutrophils receive a secondary wave of signals transduced through E-selectin, leading to the activation of $\alpha_M \beta_2$ (Mac-1) integrin on the leading edge. Activated Mac-1 on adherent neutrophils mediates the capture of circulating sickle RBCs, producing temporary or prolonged obstruction of venular blood flow. Circulating neutrophils exhibit considerable heterogeneity in their pro-inflammatory properties. Signals derived from the microbiota drive the neutrophil aging in the circulation, generating an overly active aged subset that exhibits enhanced Mac-1 activation and neutrophil extracellular traps (NETs) formation. Aged neutrophils play an important role in promoting sickle cell vaso-occlusion. Currently, whether NET formation plays a role the vaso-occlusive process remains unclear.

Figure 2: Inflammation in Sickle Cell Disease. SCD has been recognized as a chronic inflammatory disease. Multiple cell types and molecules are involved in its inflammatory pathways. Hemolysis of red blood cells (RBCs) leads to the release of heme into the circulation, which can activate endothelial cells through the TLR4 pathway and induce placenta growth factor (PIGF) release from RBCs. PIGF also activates monocytes, leading to the production of pro-inflammatory
cytokines TNF-α and IL-1β. The adhesion of platelets or sickle RBCs can also activate endothelial cells, producing increased expression of adhesion molecules including ICAM-1, VCAM-1, P-selectin, and E-selectin, and thus promoting the recruitment of neutrophils. Invariant natural killer (iNKT) cells exhibit an activated phenotype and contribute to pulmonary dysfunction in SCD by producing IFN-γ and inducing CXCR3 chemokines. Platelets can form aggregates with circulating leukocytes, including neutrophils and monocytes. Activated neutrophils roll and adhere to the endothelium and initiate VOC by capturing sickle RBCs. Heme can also induce NET formation, which promotes acute pulmonary injury in SCD.

**Abbreviations:** NETs, neutrophil extracellular traps; HMGB1, high mobility group box 1; PIGF, placenta growth factor.
Figure 1

Microbiota-derived signals

“Nascent”

TLRs / Myd88

“Aged”

Rolling

Adhesion

Heterotypic interaction

Blood flow

NETs

Neutrophils

RBCs

Endothelial cells

Vaso-occlusion

TLR4  TLR2  P-selectin  E-selectin

Mac-1  VLA-4  α4β7 integrin  PSGL-1

CD44/ESL-1  ICAM-1  VCAM-1  MADCAM-1
Activated endothelial cells: Increased ICAM-1, VCAM-1, P-selectin and E-selectin

TNF-α, IL-1β activate NETs

sickled RBC

neutrophil

TNF-α

iNKT cell

IFN-γ

CXCL9, CXCL10

chemotaxis

CXCR3-expressing lymphocytes

Pulmonary dysfunction

endothelial cells

sickled RBC

neutrophil

monocyte

iNKT cell

platelet

Blood flow

platelet-neutrophil aggregate

P-selectin

CD69

Adenosine A2a Receptor

iNKT cell

PIGF

deoxygenation

sickled RBC

hemolysis

heme

HMGB1

monocyte

PIGF

TLR-4

heme

Figure 2
Neutrophils, platelets, and inflammatory pathways, at the nexus of sickle cell disease pathophysiology

Dachuan Zhang, Chunliang Xu, Deepa Manwani and Paul S. Frenette