Published in final edited form as:

Clin Hemorheol Microcirc. 2018 ; 68(2-3): 263–299. doi:10.3233/CH-189012.

Inflammation in Sickle Cell Disease

Nicola Conran¹ and John D. Belcher²

¹Hematology Center, University of Campinas – UNICAMP, Cidade Universitária, Campinas-SP, Brazil.

²Division of Hematology, Oncology and Transplantation, Vascular Biology Center, Department of Medicine, University of Minnesota, Minneapolis, Minnesota, USA

Abstract

The primary β -globin gene mutation that causes sickle cell disease (SCD) has significant pathophysiological consequences that result in hemolytic events and the induction of the inflammatory processes that ultimately lead to vaso-occlusion. In addition to their role in the initiation of the acute painful vaso-occlusive episodes that are characteristic of SCD, inflammatory processes are also key components of many of the complications of the disease including autosplenectomy, acute chest syndrome, pulmonary hypertension, leg ulcers, nephropathy and stroke. We, herein, discuss the events that trigger inflammation in the disease, as well as the mechanisms, inflammatory molecules and cells that propagate these inflammatory processes. Given the central role that inflammation plays in SCD pathophysiology, many of the therapeutic approaches currently under pre-clinical and clinical development for the treatment of SCD endeavor to counter aspects or specific molecules of these inflammatory processes and it is possible that, in the future, we will see anti-inflammatory drugs being used either together with, or in place of, hydroxyurea in those SCD patients for whom hematopoietic stem cell transplants and evolving gene therapies are not a viable option.

Keywords

Cytokine; Endothelium; Hemolysis; Hydroxyurea; Vaso-occlusion

1. Chronic inflammatory mechanisms in sickle cell disease

Sickle cell disease (SCD) is caused by a mutation in the β -globin gene, resulting in the production of hemoglobin S (HbS) [1], which polymerizes upon deoxygenation of the red blood cell (RBC). This primary pathophysiological alteration has countless consequences, including the production of inflammatory molecules and responses that ultimately lead to vaso-occlusion of microvessels, the principal manifestation of SCD. In addition to driving

Address correspondence to: Nicola Conran: Hemocentro, Rua Carlos Chagas 480, Cidade Universitaria, Barao Geraldo, Campinas 13083-878 SP. Tel: +55 19 3521 8533. conran@unicamp.br; John D. Belcher: Vascular Biology Center, Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN. Tel: + 1 612-624-2611, belcher@umn.edu.

⁹Disclosures of conflicts of interest

Nicola Conran receives research funding from Bayer AG. Dr. Belcher receives research funding from CSL Behring.

the vaso-occlusive processes that culminate in the acute painful episodes that are the major cause of hospitalization in SCD patients, inflammatory responses are also key components of numerous complications of the disease including autosplenectomy, acute chest syndrome, pulmonary hypertension, leg ulcers, nephropathy and stroke [2–8]. While it is difficult to identify the exact events that trigger the chronic inflammatory state that defines SCD, we herein present some of the pathophysiological mechanisms that may contribute to and drive inflammation in SCD.

1.1 Sources of inflammation in sickle cell disease

Red cell alterations—HbS, and its polymerization under deoxygenated conditions, causes RBCs to adopt their characteristic sickled shape, predisposing the cells to a number of intracellular and membrane alterations. These cellular changes alter the RBC redox balance, impair the cells' deformability, make them more adhesive, and leave them susceptible to microparticle release, as well as complement and coagulation system activation, all of which can contribute to inflammatory processes in SCD [9].

Red cell dehydration is a major factor in erythrocyte sickling; K/Cl cotransport activity is augmented in sickle RBCs [10, 11] and repeated RBC sickling can increase intracellular Ca^{2+} concentrations [12], resulting in the activation of the erythrocyte Ca^{2+} -dependent K^{+} Gardos channels [13]. Ensuing intracellular K^{+} and water losses result in the generation of dense dehydrated RBCs, whose numbers have been associated with increased RBC sickling and the incidence of complications of the disease [14–16]. Continuous oxygenation and deoxygenation cycles can culminate in the generation of reactive oxygen species (ROS) in the RBC due to the autooxidation of oxyhemoglobin to methemoglobin and the generation of superoxide anion and hydrogen peroxide. In the case of sickle RBC, this ROS production is aggravated by the instability of HbS [17, 18], coupled with the oxidative effects of free iron, hemoglobin and heme deposits on the cell membrane [15, 19]. The consequent oxidation of RBC membrane proteins may exacerbate the abnormal rheological properties of sickle RBC [20], further impairing the deformability of these cells [21], and may even be a contributing factor to the adhesivity of the sickle RBC [22]. A significant part of ROS production in sickle cells is mediated enzymatically by NADPH oxidase, which is regulated by protein kinase C, Rac GTPase, and intracellular Ca^{2+} signaling within the sickle RBC [23]. Plasma from patients with SCD and isolated cytokines, such as transforming growth factor (TGF)- β 1 and endothelin-1, enhance RBC NADPH oxidase activity and increase ROS generation.

Repeated HbS polymerizations, together with RBC dehydration and membrane oxidative injury, result in alterations to the cytoskeleton and membrane components that can leave RBCs irreversibly sickled [24, 25]. These irreversibly sickled RBCs retain their sickled shape irrespective of whether they carry polymerized HbS or not, and they tend to be dense with low concentrations of fetal hemoglobin (HbF), decreased deformability, increased osmotic fragility and a short survival [25, 26]. Irreversibly sickled cells probably contribute to the inflammatory state in SCD by participating in hemolytic and occlusive mechanisms in blood vessels [9, 27, 28]. Dehydrated and dense sickle RBCs also externalize negatively-charged phosphatidylserine (PS) on their plasma membrane that, besides contributing to

sickle RBC adhesive properties [29–31], may activate the coagulation cascade by promoting the generation of thrombin [32, 33], though more recent data have suggested that whole blood thrombin generation in SCD may arise from a cellular component rather than PS exposure [34]. Thrombin generation and activation of the coagulation system will be covered in a further subsection of this review.

The contribution of sickle cell adhesiveness to endothelial activation and vaso-occlusive mechanisms has long been recognized [35, 36]. While normal RBCs present negligible adhesivity, sickle RBCs are much more adhesive due to the expression of multiple adhesion molecules on their cell surface. Sick RBCs express $\alpha 4\beta 1$, Lu/BCAM, LFA-3 and ICAM-4 [37–39], in addition to PS, on the plasma membrane, allowing the cells to adhere to and bind extracellular matrix proteins and counter receptors on the endothelium. Additionally, sickle RBCs also interact with activated neutrophils and platelets, forming heterocellular aggregates that can adhere to the vessel walls, resulting in the obstruction of microcirculatory flow [40–43].

In addition to membrane alterations, sickle RBCs produce significant quantities of microparticles (MPs) [44], usually observed as spheroid vesicles of 100–300 nm in size that present PS and phosphatidylethanolamine on their membrane surface [45]. Red cell microparticles are generated during reticulocytosis, a process that is exacerbated in SCD [46], and as a result of repeated sickling/unsickling. Continuous HbS polymerization causes the formation of long spicules, due to alterations in the spectrin-actin meshwork, which can uncouple the lipid bilayer from the cytoskeleton [44, 47, 48], allowing the subsequent release of microvesicles. Additionally, elevated intracellular calcium can activate calpain-1 and other proteases that may destabilize the membrane and allow microparticle release [49]. Other triggers of erythrocyte MP shedding in SCD may include increased thrombospondin-1 [50], RBC membrane oxidation and increased acid sphingomyelinase activity [47, 51, 52]. Red cell MP may be an important source of inflammation in SCD, as they carry large quantities of hemoglobin, heme and iron, potentially making a significant contribution to the quantity of inflammatory hemoglobin products in the SCD circulation. Sick RBC MP can induce the production of IL-6, TNF α and IL-1 β in monocytes [52] and more recent evidence suggests a role for red cell MP in transferring heme to endothelial cells in SCD, causing vascular injury and potentially triggering vaso-occlusive events [53]. The role of MPs in SCD are further discussed in detail in this special SCD issue in *Clin Hemorheol Microcirc* (see the review of Romana et al.).

Hemolysis and hemoglobin products—Hemolysis is a primary inflammatory trigger in SCD. The alterations in sickle RBCs described above leave these cells more rigid and less deformable and therefore more susceptible to rupture in the circulation. The lifespan of sickle RBCs may be as short as 7–14 days, as compared with the lifespan of approximately 120 days for normal RBC [1, 54]. The premature destruction of up to 10% of the total number of RBCs may occur every 24 h in SCD, with approximately 30% of total hemolysis occurring intravascularly [54, 55] and plasma cell-free heme concentrations of 25 μ M, or more, being reported in steady-state SCD individuals [56].

Upon the lysis of RBCs, liberation of cell-free hemoglobin into the circulation occurs. If not sequestered immediately by plasma hemoglobin-binding proteins, such as haptoglobin [57], this cell-free hemoglobin elicits the immediate consumption of nitric oxide (NO), a gaseous signaling molecule that is constitutively produced by endothelial nitric oxide synthase (NOS) [56]. In addition to its vasodilating properties, endothelial-derived NO [58] has significant anti-inflammatory effects, reducing leukocyte activation and leukocyte-endothelial interactions, and the emigration of leukocytes from the blood vessels to tissue [59–62]; indeed the installation of vascular hemolysis in mice induces an immediate systemic and vascular inflammatory response, in which NO depletion apparently triggers instantaneous and extensive leukocyte recruitment in the microcirculation [63]. NO also modulates the production of endothelin-converting enzyme-1 [64] and arrests vascular smooth muscle cell proliferation, and is therefore important for preventing vascular remodeling, a mechanism that is central to pulmonary hypertension [65, 66]. NO also significantly inhibits platelet aggregation and the release of pro-inflammatory cytokines and tissue factor from leukocytes and endothelial cells [61, 62, 67–69]. In contrast, in the presence of superoxide, NO forms the highly reactive peroxynitrite, which can oxidize lipids, proteins and lipoproteins and promote cellular apoptosis and necrosis [70–72].

Following the reaction of oxyhemoglobin with NO in the blood vessel, Hb-Fe³⁺ is formed, which readily releases heme [73], a highly inflammatory, hydrophobic, iron-containing molecule that can activate the innate immune pattern recognition receptor, toll-like receptor (TLR) 4 on monocyte/macrophages and endothelial cells [74, 75]. Heme and the TLR4 cognate ligand, lipopolysaccharide (LPS), can also activate TLR4 signaling on other cell types such as T-cells, platelets and mast cells [76–79]. Heme activation of TLR4 on lung endothelium can trigger acute chest syndrome in SCD mice [80], tissue factor expression on monocytes [81], and neutrophil NET formation [82, 83]. Heme derived from sickle RBCs can activate TLR4 independently of LPS, leading to oxidant production, inflammation, and vaso-occlusion [74]. Heme-induced TLR4 signaling requires all of the proximal components of classical TLR4 signaling, including TLR4, CD14, MD-2, and MyD88 ([75] and unpublished data). Heme-induced TNF- α secretion occurs independently of the LPS binding site on MD-2/TLR4 and is antagonized by protoporphyrin IX. The inflammatory components of heme-mediated TLR4 signaling include NF- κ B activation, inflammasome assembly, cytokine production, adhesion molecule expression, and rapid (within minutes) mobilization of Weibel-Palade bodies (WPB) to endothelial cell surfaces, which can release a large number of pro-inflammatory and hemostatic proteins. Blockade of P-selectin or von Willebrand Factor (vWF) with functional blocking IgG significantly inhibits heme/TLR4-induced vaso-occlusion. Heme-mediated TLR4 activation can also be blocked by the plasma high-affinity heme-binding protein hemopexin, which is depleted in SCD [74, 80, 84, 85].

In addition to heme, RBCs contain numerous other molecules with inflammatory potential, called damage-associated molecular patterns (DAMPs); thus, in addition to the release of hemoglobin and, consequently heme, hemolysis is also accompanied by the release of a number of DAMPs, some of which are listed in Table 1. One such red cell DAMP is adenosine 5' triphosphate (ATP) [86], which in addition to acting as a universal energy source, also has vasoactive properties [87]. Extracellular ATP acts as a signaling molecule via the activation of purinergic P2 receptors [88], where binding of ATP to the P2X₇

receptor is known to lead to K^+ efflux via ATP-gated cation channel opening and trigger assembly of the inflammasome platform, with subsequent IL-1 β and IL-18 processing, in certain inflammatory cells [89]. Extracellular ATP can also be rapidly converted to adenosine by ectonucleotidases; when in the extracellular environment, the adenosine purine molecule can exert both anti-inflammatory and pro-inflammatory effects, depending on the receptor with which it interacts. As such, while the interaction of adenosine with the Adora2a adenosine receptor selectively inhibits the iNKT cells, adenosine signaling through the Adora2b adenosine receptor on the RBC membrane appears to induce erythrocyte sickling in SCD [90, 91].

Vaso-occlusive processes and ischemia-reperfusion injury—Vaso-occlusive processes occurring primarily in the microcirculation reduce tissue oxygenation and can culminate in tissue damage and ischemia-reperfusion physiology, which in themselves are highly inflammatory mechanisms. Vaso-occlusion comprises a multi-step and multi-component process consisting of the initiation, propagation and resolution of vascular obstruction [92], in which numerous cell types and molecular interactions play roles. *In vitro* techniques and *in vivo* models have suggested that vaso-occlusion is initiated by the adhesion of RBCs [35, 36] and activated leukocytes (which then mediate the secondary adhesion of red cells and platelets) [93, 94] to the endothelium, with the positing of “erythrocentric” and “leukocentric” theories for the precipitation of vaso-occlusion [1] that are not necessarily mutually exclusive. More recent data have also suggested a major role for platelets in vaso-occlusive processes, the adhesion of platelets to endothelial cells leads to their activation and expression of endothelial ICAM-1 and E-selectin and IL-8 secretion via an NF κ B-dependent pathway [95], probably due to the release of potent platelet-derived inflammatory mediators such as IL-1 β , CD40 ligand, TNFSF14 (tumor necrosis factor superfamily member 14; LIGHT) and IL-6 [95–98]. Furthermore, neutrophil-platelet microemboli reportedly trigger lung arteriole vaso-occlusion [40], and it is possible that the adhesion of platelets to damaged endothelium may in fact precede and mediate the adhesion of larger neutrophils and red cells to the vessel wall under some circumstances (Chweih et al, *unpublished observations*).

Vaso-occlusive processes in SCD cause ischemia-reperfusion injury, defined as the tissue damage that occurs as the result of the interruption of the blood supply causing hypoxia, followed by resolution and consequent reperfusion of the tissue [99]. Cells undergoing cell death mechanisms present cytosolic calcium accumulation, mitochondrial dysfunction and cell swelling [5, 99] and release major inflammatory DAMPs, such as ATP, heme, high-mobility group box 1 (HMGB1) and heat shock proteins (Table 1), which are reportedly increased in SCD [100, 101]. These DAMPs can promote multiple inflammatory pathways, including NET formation and inflammasome assembly, as will be later described [89, 102]. When blood flow is restored following disruption of vaso-occlusion, the tissue is then reperfused and the damaged tissues are reoxygenated, paradoxically causing further damage, due to the production of ROS and calcium overload [99]. A major effect of ischemia-reperfusion injury may be the activation of iNKT cells, which can induce pulmonary inflammation by triggering IFN- γ and INF- γ -inducible chemokines [103].

Infections—Individuals with SCD, particularly children, are very susceptible to bacterial infections due to functional asplenia [104]. The use of penicillin prophylaxis and vaccines, in most children with SCD, has drastically reduced the incidence of severe infections in these patients [105], but nevertheless it is probable that bacterial infections are important triggers of inflammatory responses in the SCD population due to further neutrophil activation and the release of pathogen-associated molecular patterns (PAMPs) that can prime inflammatory cells, aggravate inflammatory processes and trigger innate immunity pathways [106, 107].

Histamine—Fasting plasma histamine is elevated in steady state SCD and further augmented during painful vaso-occlusive episodes [108]. It is probable that the major source of plasma histamine in SCD derives from mast cell activation and consequent degranulation, although small amounts of histamine may also be released from intracellular stores during platelet activation [109, 110]. Mast cell activation has been demonstrated in SCD and contributes to sickle pain pathobiology [111], furthermore, morphine administration (often used during painful vaso-occlusive episodes) may exacerbate cutaneous mast cell degranulation [112], with systemic morphine exposure being suggested to increase the risk for acute chest syndrome in SCD [113]. Histamine, in addition to its roles in vasoregulation, smooth muscle contraction, neurotransmission, and vascular permeability [114], induces vWF release and thrombospondin-1 and P-selectin exposure on endothelial cells [30, 115, 116], in turn mediating the adhesion of RBCs [30, 117, 118]. Histamine may also elevate circulating leukocyte numbers [119], promote inflammatory angiogenesis [120] and modulate invariant natural killer T cell activation [121, 122].

Oxidative stress—Oxidative stress and inflammation are intrinsic to SCD and inextricably linked to its pathophysiology [23, 96, 123–161]. SCD patients have an imbalance between the production of oxidants and antioxidant capacity, which is a critical factor in endothelial cell dysfunction, inflammation, vaso-occlusion, and organ pathology [162–172]. There are multiple potential sources of oxidants in SCD, including accelerated hemoglobin S (HbS) autooxidation [173], released heme/iron catalyzed Fenton reactions [174], increased expression and activity of various isoforms of NADPH oxidase (NOX) [23, 135, 175, 176], xanthine oxidase [177], cytochrome P450, cyclo-oxygenase [178], mitochondria [179], and uncoupled NOS [180]. Although anti-oxidants act to scavenge ROS produced by these enzymes, their effectiveness has been limited in clinical trials likely because of their non-specificity and the important role oxidative stress plays in the induction of countervailing cytoprotective pathways such as the Nrf2/HO-1 axis. Nevertheless, the US FDA's recent approval of the use of supplementation with L-glutamine in SCD (see Section 6), highlights the fact that anti-oxidant approaches may be a useful adjuvant therapy in SCD, and targeting of oxidant production by specific enzyme systems has the potential to target the formation of oxidants that contribute directly to SCD pathophysiology.

Thrombin generation and activation of complement—SCD is a hypercoagulable state with an increased incidence of prothrombotic events. Whole blood thrombin generation is significantly elevated in whole blood, but reduced in plasma [34] and this pro-thrombotic state, involving the activation of platelets and the coagulation system, may play a

role in some of the complications of the disease, including ischemic stroke and venous thromboembolism (VTE) [181]. Abnormal PS exposure on the sickle RBC surface and circulating erythrocyte-derived microparticles have been suggested to induce excessive thrombin generation in SCD [33]. Evidence suggests that activation of the coagulation system and thrombin generation occur in SCD [182–185], as well as increased tissue factor expression [146, 186–189].

In 1967, Francis and Womack first reported abnormal complement activity in SCD [190]. This was revisited in 1973 by Johnston et al. who reported activation of the alternative pathway (AP) in the disease [191]. The AP can be constitutively activated by spontaneous modification of C3 yielding iC3 (tick-over mechanism), which can bind factor B [192]. The complex can then bind to activating surfaces, with amplification of C3 convertase, generation of C5 convertase, and finally C5 cleavage. In addition, thrombin can bypass classical and AP complement activation and directly cleave C3 and C5 into biologically active fragments [193, 194]. Activation of the AP and thrombin pathways is accelerated on PS-rich membrane surfaces. Importantly, C5a can activate all of the cells involved in vaso-occlusion (leukocytes, platelets, and endothelial cells), initiate P-selectin expression, elicit the release of pain mediators (histamine, tryptase and substance P) from mast cells and enhance vascular permeability.

In 1994, Chudwin et al. reported that SS-RBCs activated complement to a greater extent than did RBCs from controls [195]. They also reported that this activation was via the AP and not the classical pathway (CP). In 1995, Mold et al. demonstrated that AP activation is initiated by membrane phospholipid changes that occur in SS-RBC [196]. The alternative complement pathway is abnormally activated in SCD [191, 197] and is amplified by PS on the outer leaflet of SS-RBCs and MPs [194, 195, 198, 199]. PS on the surface of SS-RBCs and activated platelets accelerates the assembly of prothrombinase complexes, leading to thrombin generation, which can generate biologically active C3/C5 fragments, including the anaphylatoxins C3a/C5a [193, 200]. C3b opsonizes SS-RBC and C5b initiates the formation of membrane attack complexes on SS-RBC that respectively increase their susceptibility to extravascular clearance and intravascular lysis [201, 202]. Arumugam et al demonstrated that genetically reducing prothrombin levels limits inflammation, endothelial cell activation, and end-organ damage in SCD mice [154]. Sparkenbaugh et al showed that factor Xa (FXa) and thrombin contribute to inflammation in SCD mice, and inhibitors of FXa (rivaroxaban) and thrombin (dabigatran) reduced this inflammation [150].

1.2 Propagation of Chronic Inflammation in SCD

Pro-inflammatory Mediator Production—The activation of the inflammatory cells and their signaling pathways leads to the production and secretion of numerous molecules that propagate the inflammatory state in SCD, including cytokines and chemokines, growth factors, eicosanoids and peptides that can further activate other cells. Numerous type I cytokines, produced from multiple cell types, are elevated in steady-state SCD. A major leukocyte-derived cytokine is tumor necrosis factor (TNF)- α ; circulating levels of this molecule are consistently reported as elevated in SCD [142, 203–205] and TNF- α is even frequently used to trigger vaso-occlusive processes in sickle mice models [93, 206]. This

cytokine is suggested to be generated as an early consequence of ischemia-reperfusion [207] and has potent effects on both leukocytes and endothelial cells. In neutrophils, TNF- α stimulates the surface expression of β -2 integrins [208], in turn augmenting their adhesion to the blood vessel wall [208] and interactions with other cells via NF- κ B and MAPK signaling [209]. Furthermore, TNF- α degrades the endothelial glycocalyx (shown to be reduced in SCD patients) [210, 211], alters endothelium-derived NO bioavailability [212] and upregulates adhesion molecule expression on the endothelium [213], with a monocyte-dependent TNF-endothelial activation axis being recently described in sickle mice [207].

Interleukin (IL)-1 α elevation is reported in SCD and represents a primary inflammatory trigger that induces leukocyte recruitment, endothelial cell activation and the production of other inflammatory mediators [214, 215]. Increased IL-6 in SCD [203, 205, 216, 217] may play a role in acute phase protein synthesis, although this cytokine may also have beneficial effects due to its ability to inhibit TNF- α activity and up-regulate anti-inflammatory IL-10 [218]. The IL-17 cytokine is also elevated in SCD, probably reflecting increased lymphocyte activity and may stimulate the production of chemokines and cytokines involved in neutrophil recruitment to the blood vessel wall [203]. Plasma interferon (IFN)- γ , a type-2 interferon produced by multiple cell types, is apparently augmented in steady-state SCD and could modulate macrophage function and increase T helper cell expansion [216]. Platelet-derived cytokines, such as CD40L and TNSF14, are also elevated in SCD and probably participate in leukocyte and endothelial activation, with evidence for the association of increased levels of these cytokines with acute chest syndrome and elevated tricuspid regurgitant velocity, respectively [96, 97, 219]. Elevated circulating levels of both IL-1 β and IL-18 have been observed [205, 220, 221] in SCD and most likely reflect inflammasome formation in inflammatory cells; the IL-1 β cytokine may in turn go on to stimulate leukocyte and endothelial cell activation, while IL-18 could be important for stimulating vascular smooth muscle cell proliferation and migration as well as the productions of IFN- γ , IL-2 and IL-12.

Chemokine upregulation in SCD may facilitate the recruitment of leukocytes, such as neutrophils, eosinophils and monocytes, to blood vessel walls. Chemokines reported to be augmented in SCD include IL-8 (CXCL8), monocyte chemoattractant protein (MCP)-1 (CCL2), RANTES (CCL5), platelet factor (PF) 4 (CXCL4), macrophage inflammatory protein (MIP)-1 α , Eotaxin-1 (CCL11) and fractalkine [203, 205, 222–224]. Augmented growth factors in SCD stimulate leukocyte proliferation and differentiation and may regulate some of the angiogenic processes that contribute to some SCD manifestations. Such growth factors include granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-CSF (M-CSF), TGF- β , and pro-angiogenic molecules [143, 203, 205, 223, 225–230]. Other inflammatory mediators that are upregulated in SCD include substance P, and the acute phase proteins, C-reactive protein and pentraxin-3 [231–233].

The severity and symptoms of sickle cell anemia (SCA) and the other SCD genotypes are surprisingly variable [234], and genetic modulation of the production of cytokines, other inflammatory molecules, and their receptors may constitute one of the mechanisms that contribute to this variation. Polymorphisms in the genes encoding IFN γ and TNF- α have been shown to present associations with infectious complications and stroke [235–237],

while several genes in the TGF-beta/BMP signaling pathway have been associated with the incidence of leg ulcers [238], and the IL1A rs1800587 SNP may influence chronic pain in SCD [239].

Inflammatory Cell Activation

Endothelial cell activation: Endothelial cell activation plays a key role in the vaso-occlusive process. Intact, non-activated endothelium generally averts the adhesion of inflammatory cells to the vessel wall via mechanisms such as NO generation and prostacyclin release [58]. Endothelial dysfunction occurs as a consequence of endothelium-derived NO depletion by cell-free hemoglobin [56, 240], endothelial NOS uncoupling and consumption of L-arginine (the substrate for NOS) by arginase released during hemolysis [241]. The endothelium is further activated by interactions/adhesion of sickle RBCs and leukocytes with the vessel wall [242–244]. Cell-free heme also induces microvascular endothelial barrier dysfunction in the lung by inducing necroptotic death [245].

Once activated, the endothelium produces and releases a number of potent inflammatory molecules, including IL-1 β , IL-8, IL-6, IL-1 α , GM-CSF, plasminogen activator inhibitor (PAI)-1, MCP-1 and RANTES [95, 216, 217, 246–249] that contribute to the inflammatory milieu in SCD. Activated endothelium also expresses adhesion molecules such as VCAM-1, ICAM-1, E-selectin and P-selectin [250–253], which are important for red cell, leukocyte and platelet tethering and adhesion. More recently, a role for endothelin-B receptor-mediated signaling in leukocyte adhesion to the endothelium has been shown in SCD [254].

SCD appears to be associated with an imbalance in angiogenic processes, which govern the formation of new capillaries from preexisting vessels and are essential for the repair processes [255] that are necessary after tissue damage. The angiogenic process involves the interactions of several cell types for capillary formation [256], and endothelial cell proliferation and invasion in response to angiogenic mediators and alterations in oxygen tension are crucial to these mechanisms [257, 258]. SCD patients, display a number of manifestations that are suggestive of alterations in the angiogenic processes, such as proliferative retinopathy, osteonecrosis and pulmonary hypertension and moyamoya syndrome amongst others [259–266], where retinopathy and osteonecrosis are often even more frequent in HbSC disease (generally viewed as a less severe disease than homozygous HbS disease) [267, 268]. Numerous angiogenic mediators are elevated in SCD, including vascular endothelial growth factor (VEGF), VEGF D, placental growth factor (PlGF), angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), basic fibroblast growth factor [bFGF] and erythropoietin (EPO) [143, 226–228, 269, 270], furthermore, plasma from SCD individuals induces endothelial cell proliferation and capillary formation *in vitro* [270], highlighting the important role that endothelial cells play in these mechanisms.

Leukocyte activation: Leukocytes are key players in the inflammatory processes that trigger vaso-occlusion and other complications of SCD, participating in the generation of inflammatory molecules as well as physically contributing to the vaso-occlusive process. SCD is often associated with leukocytosis and a clue to the prominent role of these inflammatory cells to SCD pathophysiology was provided some time ago by the

demonstration that increased leukocyte counts are associated with increased mortality, acute chest syndrome and stroke in the disease [271]. Intravital microscopy techniques in murine models of SCD later showed that, under certain circumstances, the recruitment and adhesion of leukocytes, particularly neutrophils, to the microvenule walls may be the trigger for the onset of vaso-occlusive processes. *In vitro* and *in vivo* techniques further indicate that, following their recruitment to the vessel walls of the SCD microcirculation, β 2-integrin expression is increased on the surface of SCD neutrophils and intermediates the recruitment of red blood cells to the vessel wall, in turn promoting vaso-occlusion [93, 124, 272, 273].

In addition to their important role in cellular and molecular inflammatory responses, neutrophils, in particular, but also monocytes, eosinophils and mast cells, can also respond to the presence of microorganisms and other stimuli including alterations in ROS balance by releasing extracellular traps (ETs) [274]. ET release consists of the ejection of decondensed chromatin through the ruptured cell membrane; this extruded DNA contains histones and granular enzymes, such as neutrophil elastase [274, 275]. While these ETs have a recognized importance as a defense mechanism against microorganisms, increasing evidence indicates a role for these structures in inflammatory and autoimmune diseases [276, 277]. Neutrophil ET (NET) formation has been reported in SCD [83, 278], and may play some role in SCD pathogenesis, with a crucial role for cell-free heme and TLR4 in this formation [83, 279].

Monocyte activation has also been reported in SCD and a role for these cells in endothelial activation in the disease has also been demonstrated [141, 280]. Monocytes are important producers of pro-inflammatory cytokines [281], including TNF- α and IL-1 β [280], and can also form heterocellular aggregates with RBCs and platelets [41, 282, 283], potentially contributing to vaso-occlusive processes. Two reports suggest that monocytes may be crucial to the production of TNF- α and IL-1 β in SCD, which in turn have a critical function in endothelial activation [207, 280]. Furthermore, the exposure of murine macrophages to hemolytic RBCs or heme causes their functional phenotypic change toward a proinflammatory state [159] and the formation of the NLRP3 inflammasome [284] via activation of the TLR4 signaling pathway. Given the elevation in levels of the inflammasome-processing dependent cytokines, IL-1 β and IL-18, in SCD [205, 221], it seems reasonable to presume that, in addition to a participation of activated neutrophils [285] in the processing of these cytokines, tissue macrophages and monocytes may also make some contribution.

Invariant natural killer T (iNKT) cells are a specialized subset of T cells that recognize both self and foreign lipids presented by CD1d and can play both harmful and protective pathological roles [286]. Patients with SCD present activation of circulating iNKT during painful vaso-occlusive episodes [287] and NY1DD sickle mice reportedly have more numerous and more activated iNKT cells in the lungs, liver and spleen than wild-type mice; furthermore, these cells participate in pulmonary inflammation by producing IFN- γ and CXCR3 chemokines [103]. Moreover, pulmonary function in these mice is improved by depleting or neutralizing iNKT cells and their activity and the activation of adenosine A(2A) receptors (A(2A)Rs) on iNKT and NK cells in SCD mice can ameliorate baseline pulmonary function and prevent hypoxia-reoxygenation-induced exacerbation of pulmonary injury [288].

Platelet activation: Platelet activation [289–291] and alterations in platelet aggregation [292, 293] are characteristics of SCD; moreover, platelet activation appears to be further elevated during acute vaso-occlusive episodes [294], with augmented circulating levels of platelet microparticles and platelet-derived proteins, such as thrombospondin-1 and PF4, reflecting this activation in SCD [290, 295–297]. As well as their role in hemostasis, platelets are also major inflammatory cells [94]. The adhesion molecules, $\alpha_{IIb}\beta_3$ and P-selectin, are augmented on the surface of SCD platelets [96, 291, 298] making them more adhesive to components of the vascular wall [95, 291], with evidence that the adhesion of SCD platelets to endothelial cells induces their activation [95]. Additionally, platelets from SCD individuals produce high levels of inflammatory cytokines, such as LIGHT (TNFSF14) and CD40L [96, 97], with levels of CD40L being associated with the incidence of acute chest syndrome [219].

2. Anti-inflammatory mechanisms in SCD

The resolution of inflammation involves active innate biochemical mechanisms and anti-inflammatory molecules that enable inflamed tissues to return to homeostasis [299]. In an attempt to counter the chronic inflammation in SCD, a number of anti-inflammatory molecules and pathways are up-regulated and these and others may potentially be exploited for therapeutic purposes. The IL-10 cytokine, produced principally by macrophages, T and B lymphocytes, natural killer cells and monocytes, is a class 2 cytokine that can limit the production of pro-inflammatory cytokines including TNF- α and IL-1 β [300, 301]. Levels of IL-10 are augmented in steady-state SCD [142, 143], but reduced (compared to steady-state) in patients in vaso-occlusive crisis [302].

The heme oxygenase-1 (HO-1) enzyme is an important antioxidant and anti-inflammatory molecule that catalytically cleaves heme groups to carbon monoxide, free iron, and biliverdin following its upregulation in response to heme and oxidative signals. HO-1 gene expression is up-regulated in SCD, probably as a result of hemolytic events [142, 303]. Gene delivery of HO-1 to the liver of SCD mice, inhibits local hypoxia-induced stasis [304], while the repeated administration of SCD mice with haptoglobin for three months increased HO-1 and H-ferritin expression and decreased iron deposition in the kidney, although the changes during the period studied were unable to improve kidney function [305]. Importantly, HO-1-catalyzed heme degradation can result in the generation of carbon monoxide (CO), which also has anti-inflammatory and anti-sickling properties [306–308]. CO can increase RBC survival, as well as decrease leukocytosis and NF- κ B activation and upregulate anti-inflammatory signaling pathways in SCD [149, 306, 309].

The Nuclear factor erythroid-2-related factor-2 (Nrf2) pathway also represents an anti-inflammatory pathway whose activation may be beneficial in SCD. Nrf2 activation occurs in response to both oxidative and inflammatory stresses and orchestrates the recruitment of inflammatory cells and regulates gene expression, including HO-1, through the antioxidant response element (ARE) [310]. Nonhematopoietic Nrf2 displays a dominant protective role against tissue damage in mice with SCD and its prophylactic activation stabilizes intravascular hemolysis, reverses vascular inflammation and attenuates lung edema in these mice [311]. Furthermore, dimethyl fumarate (DMF), an activator of Nrf2-responsive genes

and a drug approved for the treatment of multiple sclerosis, enhances antioxidant defenses and inhibits inflammation and vaso-occlusive processes in SCD mice, via an HO-1 dependent mechanism [312], while the administration of CDDO-Im (2-cyano-3, 12dioxoooleana-1, 9diene-28-imidazolide) also relieves inflammation and organ failure in SCD mice [156]. *Sleeping Beauty* transposase plasmid delivery of either the human wild-type ferritin heavy chain (wt-hFHC) or hemopexin into SCD mice was able to enhance Nrf2-regulated protein activity, including HO-1, resulting in decreased NF- κ B activation and vaso-occlusive processes in these mice [152, 158]. Importantly, Nrf2 activation also upregulates γ -globin gene transcription in erythroid cells, inducing HbF production, therefore representing a therapeutic target whose activation may have multiple benefits in SCD [313–315].

Polymerization of HbS in the deoxy conformation shortens the lifespan of SS-RBCs and promotes intravascular and extravascular hemolysis [55]. When SS-RBCs are lysed intravascularly, HbS is released into the vascular space where it can consume NO and be oxidized to higher oxidative forms [56, 316, 317]. During these reactions, ferric (Fe³⁺) hemoglobin (metHb) is formed, which readily releases heme [74, 318–320]. Haptoglobin and hemopexin are plasma proteins with the highest binding affinities for hemoglobin (Hb) ($K_d = \sim 10^{-12}$ M) and heme ($K_d < 10^{-13}$ M), respectively [321]. Haptoglobin and hemopexin render Hb and heme relatively nonreactive [322–330] and deliver Hb and heme safely to CD163 receptors on macrophages [331, 332] and CD91 receptors on hepatocytes [333, 334], respectively, for endocytosis and degradation of their heme moieties by HO-1 [331, 334–338]. Haptoglobin blocks Hb translocation into interstitial tissue spaces [339] and preserves vascular NO signaling during hemolysis and both haptoglobin and hemopexin can inhibit Hb- and heme-mediated microvascular stasis in SCD mice.

Albumin can complex with heme *in vivo*, especially in haptoglobin-depleted states such as SCD [318, 340]. However, endogenous circulating serum albumin in mice, which is ~ 4 g/dL [341] or ~ 600 μ M, does not prevent heme-mediated TLR4 activation and stasis in SS-mice in response to heme infusions as low as 0.32 μ mol/kg or ~ 5 μ M plasma heme [74]. This probably reflects albumin's much lower binding-affinity for heme ($K_d = \sim 4 \times 10^{-5}$ M) versus hemopexin ($K_d < 10^{-13}$ M). Heme/hemopexin complexes predominate in sera when there is sufficient hemopexin [321, 342].

Plasma haptoglobin and hemopexin levels are often depleted in SCD patients and mice due to chronic intravascular hemolysis [84, 85, 158, 343, 344]. In animal models, increasing plasma haptoglobin or hemopexin can prevent organ toxicity caused by the Hb and heme released during hemolysis [158, 159, 324, 327, 343, 345–347]. Conversely, haptoglobin and hemopexin gene-null mice are especially prone to oxidative stress and inflammation [320, 345, 346, 348–353].

3. Inflammation and acute painful vaso-occlusive episodes

While subclinical vaso-occlusive processes occur constantly in the microcirculation of individuals with SCD, acute vaso-occlusive episodes (VOE) constitute episodic events that cause extreme pain, usually localized in the bones and joints, often leading to hospitalization

for pain management [268]. Leukocyte counts have been consistently reported as elevated above baseline upon hospital admission of patients for acute pain events [354–356], and *in vitro* studies have demonstrated that neutrophils from patients hospitalized during pain events present indications of greater activation and are more adherent [357, 358]. Additionally elevated myeloperoxidase activity and circulating neutrophil MP levels are associated with acute VOE [359], indicating that an intensification of inflammatory processes occurs at the time of VOE onset. Of the inflammatory molecules known to be elevated during steady-state in SCD, there is some evidence that some of these are further elevated during acute VOE, including the cytokines CD40L, IL-6, and IL-18 [96, 216, 302] and chemokine IL-8 (CXCL8) [203, 360]. The acute phase proteins, substance P, [231] C-reactive protein and pentraxin-3 [232] and the lipid chemoattractant, leukotriene B4 (LTB4) [361], are reported to also be further increased during VOE. In contrast, alterations in anti-inflammatory molecules may occur in VOE, with IL-10 observed to be significantly decreased in VOE compared to levels in steady-state SCD individuals [302], while IL-4 has been reported as increased in SCD VOE, possibly reflecting shifts in CD4⁺:CD8⁺ T cell ratios [362].

Lactate dehydrogenase has been suggested as a predictor of acute VOE severity [363] and cell-free heme is also increased during VOE [359], indicating that exacerbated hemolytic processes may be associated with these events [364]. Thus, given the critical role that inflammation has in driving the initiation and propagation of vaso-occlusive processes, it seems reasonable to assume that inflammatory processes may be the trigger for these acute painful VOEs, indeed in animal models, the administration of TNF- α , the neutrophil chemoattractant CXCL1, lipopolysaccharide (LPS), or exposure to hypoxia and reoxygenation induce severe inflammatory responses that culminate in experimental vaso-occlusion [74, 83, 93, 125]. However, despite the substantial data available that demonstrate the further generation of a plethora of inflammatory molecules in the circulation of SCD patients experiencing an acute painful episode, researchers have yet to convincingly describe a major inflammatory marker that may be indicative of an imminent acute event or provide a specific target for pharmacological reversal of the vaso-occlusive state in patients experiencing severe pain.

4. Role for the gut and microbiome in sickle cell pathogenesis

A huge diversity of microorganisms coexist with mammalian organisms, particularly in the intestine, and reports increasingly speculate that the microbiota may play a role in modulating inflammatory responses [365]. While the intestine has developed mechanisms to optimize protection to hosts against pathogens, this organ constitutes a primary site for foreign antigen encounter and microbial colonization [366]. Intestinal epithelial cells can detect bacterial antigens (or pathogen-associated molecular patterns, PAMPs), and initiate innate immune responses [367]. Immune cells in the vicinity, such as macrophages, dendritic cells and lymphocytes, can detect these PAMPs, which include LPS, peptidoglycan, and microbial nucleic acids, via receptors expressed on the epithelial cell surface, such as major histocompatibility complex I and II molecules and TLRs [368], in turn activating inflammatory pathways. Additionally, impaired intestinal barrier function may result in bacterial translocation, where the presence of bacterial products in the systemic circulation

can potentially exacerbate the organism's inflammatory state [369]; indeed changes in the composition of intestinal microbiota have been associated with diseases such as atherosclerosis, hypertension, heart failure, chronic kidney disease, obesity, and type 2 diabetes mellitus [370].

In addition to the role of the intestinal microbiome in providing inflammatory stimuli, recent data suggest that food intake itself may induce inflammatory mechanisms, increasing the number of peritoneal macrophages and macrophage-derived IL-1 β secretion, in a glucose-dependent manner [371]. Furthermore lipids consumed in the diet (fatty acids, cholesterol, or fat-soluble vitamins) and oligoelements (such as zinc, copper, and iron) also have an effect on the immune system, where high fat-diets reduce both innate and adaptive immune responses by affecting the activity of macrophages, dendritic cells, and T lymphocytes [372], and zinc-deficiency can lead to immune dysfunction [373].

A recent demonstration of the influence of the microbiome on the immune system in SCD was provided by a study from Zhang et al [374]. Authors reported a role for the microbiota in driving neutrophil aging (and consequently increased activity) via TLR-mediated signaling. Data suggested that neutrophils constantly receive priming signals, derived from microbiota-derived molecules that cross the intestinal barrier, and become more active and more prone to release NETs as they age in the circulation. Importantly, the microbiota apparently also influence neutrophil aging and activity in SCD; depletion of the microbiota with antibiotics in both mice and humans with SCD significantly reduced the number of total neutrophils and circulating aged neutrophils and dramatically improved inflammation-related organ damage in SCD mice [374]. Whether abnormalities in the integrity of the intestinal barriers or disequilibrium of the intestinal microbiota also occur in SCD, to amplify the effects of gut microbiota, is not known at present. However, while SCD is characterized by sterile inflammatory processes, PAMPs derived from microbiota and pathogens in the intestine may conceivably contribute to inflammatory processes in these individuals. For example, microbial-derived LPS in the gut may act as a priming signal for inflammasome formation in epithelial cells [365], which in the presence of sterile DAMPs may lead to IL-1 β and IL-18 processing.

5. Exercise and inflammation in sickle cell trait and disease

In healthy individuals, exercise significantly increases oxygen consumption and oxygen flux in active muscles [375], occasioning ROS generation and ischemia-reperfusion stress, as well as modulating inflammatory responses [376]. Exercise can induce significant metabolic alterations, and acute bouts of exercise cause temporary increases in inflammatory markers and circulating adhesion molecule concentrations, due to the requirement for muscle healing and repair [377, 378]. In contrast, chronic exercise (repeated exercise for a prolonged period) provides healthy benefits in the general population with anti-inflammatory and anti-oxidant effects [379]. In the context of SCD, such acute exercise-induced changes may cause problematic physiological disturbances for patients, potentially increasing HbS polymerization and vaso-occlusive processes [380].

Sickle cell trait (SCT) is generally considered to be a benign disorder, but there are some reports of an increased risk of exercise-related sudden death in these individuals [381, 382], possibly due to hemorheological alterations, vascular cell adhesion mechanisms and the modulation of vascular function that may cause ischemia and muscle rhabdomyolysis [383]. Indeed, decreased red blood cell deformability and increased blood viscosity occur in SCT individuals during the recovery from exercise [384] and repeated heavy exercise bouts are reported to elevate circulating soluble(s) L-selectin and P-selectin kinetics and levels in SCT individuals, when compared to control individuals, possibly indicative of leukocyte and platelet activation [385]. In contrast, physically-active SCT individuals demonstrate reduced elevations in circulating sVCAM-1, compared to untrained SCT individuals, following an acute bout of exercise, indicating that a physically active lifestyle may decrease endothelial activation in SCT carriers and reduce the risk of vascular adhesive events in the microcirculation [386].

In SCD, it is still uncertain as to whether physical activity is safe, and many individuals with SCD may present limitations in their ability to exercise due to anemia or chronic complications such as pulmonary vascular disease, congestive heart failure and lung disease [387]. Hemorheological and hematological changes in SCD patients after mild-moderate exercise have been reported to be mild, except for the formation of dense cells, with authors suggesting that low-intensity exercise may not be harmful in SCD individuals that are able to carry out this activity [388]. In terms of the inflammatory response to exercise, there is some evidence that exercise may not be significantly more inflammatory in SCD than it is in healthy individuals. In children and young adults with SCA, who were subjected to maximal exercise testing, the acute phase response appeared to be elevated following exercise, as demonstrated by elevated C-reactive protein and D-dimer, compared to controls. However, alterations in markers of endothelial activation and inflammation were not significantly changed in response to exercise, when compared to matched controls [389]. In another study, NO levels, anti-oxidant capacity, sE-selectin and sP-selectin did not change in 11 patients subjected to mild-moderate exercise, sVCAM-1 levels were increased after exercise in both the SCA individuals and in the healthy control group, while sL-selectin decreased and sICAM-1 increased with exercise only in the SCA group [390]. Indeed, there is some evidence in mice with SCD that chronic exercise could be beneficial, both for reducing rheological alterations and for the inflammatory state. Mice with SCD subjected to 8 weeks of physical activity displayed an increased hematocrit-to-viscosity ratio and a lower blood viscosity, suggesting improvements in tissue perfusion and decreased vascular resistance [391]. Furthermore, sickle cell mice subjected to moderate training (treadmill training for 1 hour a day, 5 days a week) for 8 weeks demonstrated elevations in venous oxyhemoglobin, in association with reductions in the white cell count and the plasma Th1/Th2 cytokine ratio, as well as a reduced renal expression of the genes encoding IL-1 β and endothelin-1 [392].

As such, further studies to understand why some carriers of SCT may be at greater risk from exertional death than others are needed [383], as well as to determine whether mild-to-moderate physical activity could be of benefit in SCD [379].

6. Anti-inflammatory therapeutic approaches for SCD

At present, therapeutic options for SCD are limited to hematopoietic stem cell transplantation (HSCT)[393], which is a curative option but of limited availability, chronic transfusion and hydroxyurea therapy, although the USA Food and Drug Administration have recently approved oral L-glutamine for use in SCD (<https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm566097.htm>). L-glutamine improves the redox potential of sickle RBC and, therefore, counters oxidative stress [394, 395] and the FDA has approved the use of this supplement on the basis that it reduced the frequency of sickle cell crises and hospitalization in a group of SCA patients in a randomized, double-blind, placebo-controlled, multi-center clinical trial (NCT01179217).

Anti-inflammatory effects of hydroxyurea:

Hydroxyurea (or hydroxycarbamide) is a cytostatic agent that significantly reduces the incidence of hospitalization, acute pain, acute chest syndrome and transfusion frequency in patients with SCD, and may provide an alternative to chronic transfusion therapy in a subset of patients with abnormal transcranial Doppler (TCD) velocities and at risk of stroke [396–399]. The compound acts principally by inducing the production of HbF, consequently reducing sickle hemoglobin polymerization [400]. Additionally, hydroxyurea has major anti-inflammatory properties, which may be due to both the downstream effects of the inhibition of HbS polymerization and due to direct anti-inflammatory effects. Hydroxyurea therapy significantly reduces leukocyte counts in patients with SCD, even before HbF elevation is observed [397], potentially reducing the amplitude of inflammatory responses in the disease. In addition, hydroxyurea lowers the expression and activity of adhesion molecules on the surface of RBCs, leukocytes and the endothelium in SCD [291, 401–405] and has been associated with decreased circulating concentrations of a number of inflammatory molecules, including endothelin-1, TNF- α , IL-1 β , IL-17, and GM-CSF [142, 203, 225, 406–408], in addition to increasing anti-inflammatory protein expressions [142].

Furthermore, recent data suggest that hydroxyurea may have direct effects that are independent of its ability to elevate HbF production. Hydroxyurea can act as a NO donor, *in vivo* [409], with potentially significant anti-inflammatory effects. The administration of a single dose of hydroxyurea decreased leukocyte recruitment to the microvasculature in SCD mice following an inflammatory stimulus, resulting in the inhibition of vaso-occlusive processes and prolonged animal survival following inflammatory stimulation, when used together with an amplifier of NO-cyclic guanosine monophosphate (cGMP) intracellular signaling [410]. Similarly, hydroxyurea, when given in a single dose to C57BL/6 mice, abolishes the effects of hemolysis on systemic inflammation and leukocyte recruitment in the microcirculation [249], indicating that hydroxyurea has HbF-independent and NO/cGMP-dependent effects that may involve the neutralization of hemolytic insults and inflammatory events.

Other anti-inflammatory approaches under pre-clinical and clinical development for use in SCD:

Therapeutic approaches for SCD include those that target HbS polymerization and those that target events downstream of HbS polymerization that aim to reduce the inflammatory processes that trigger vaso-occlusion [411]. Gene therapies under development for SCD intend to correct the genetic defect of the disease, or induce HbF production [412, 413]. To inhibit the polymerization of HbS, HbF inducers such as thalidomide derivatives/hybrids, histone deacetylase (HDAC) inhibitors and metformin have been studied [414–417], while anti-sickling agents under study include 5HF (Aes-103) and GBT440, which modulate the oxygen affinity of HbS [418].

A huge number of anti-inflammatory drugs are currently under investigation as potential therapeutic approaches for SCA and some of these drugs are described here and are summarized in Table 2. With the aim of reducing leukocyte adhesion to the blood vessel wall and, therefore, halt the initiation of vaso-occlusive processes, the pan-selectin antagonist, rivipansel (GMI-1070), was developed with a view to decreasing leukocyte/endothelial interactions, as well as the formation of heterocellular leukocyte aggregates, and for use at the onset of VOE. Phase II studies have shown that GMI-1070 improves clinical outcomes such as time to resolution of crisis, time to discharge, and opioid use [419–421]. Other anti-cell adhesion approaches include single dose intravenous immunoglobulin, modified heparins and poloxamer 188 [419, 422–424]. Crizanlizumab, a monoclonal antibody that neutralizes the activity of P-selectin (expressed on endothelial cells and platelets), in a phase 2 trial, reduced the incidence of SCD vaso-occlusive complications (pain, priapism, acute chest syndrome, sequestration) when used at higher doses [425]. In contrast, the anti-platelet agent, prasugrel hydrochloride, which irreversibly inhibits the P2Y₁₂ receptors, while being apparently well tolerated and safe in SCD, failed to significantly reduce vaso-occlusive complications in a large group of children and adolescents when administered for up to 24 months [426].

Statins and TNF- α antagonists may be useful for reducing endothelial activation. In a recent pilot trial, the treatment of SCD patients with simvastatin resulted in a significant reduction in the frequency of pain, oral analgesic use and circulating inflammatory markers [427], especially in individuals on hydroxyurea therapy. Given the probable major role of TNF- α in initiating and propagating SCD vaso-occlusion, TNF- α blockers, such as etanercept or infliximab, could be of some benefit. In SCD mice, etanercept ameliorated blood biomarkers of inflammation, vaso-occlusive processes triggered by hypoxia/reoxygenation, endothelial activation and histopathologic liver injury, amongst other parameters [207]. iNKT cell depletants and A2AR agonists under development for SCD could also decrease inflammation by decreasing iNKT cell numbers and inflammatory activation [90, 428].

Hemoglobin and heme scavengers, such as plasma-purified haptoglobin or recombinant hemopexin, have successfully prevented vaso-occlusion and acute chest syndrome onset in SCD mouse models [74, 80]. Sustained treatment of SCD mice with haptoglobin has been found to increase HO-1 and H-ferritin expression and decrease iron deposition in the kidney, although these effects were not associated with improved kidney function [305]; in contrast hepatic overexpression of hemopexin also inhibits inflammation and vascular stasis in SCD

mice [158]. Supplementation of SCD mice with purified plasma haptoglobin or hemopexin was shown to rapidly activate the Nrf2/HO-1 axis and inhibit inflammation and vaso-occlusion in an HO-1-dependent manner [429]. Activation of Nrf2 with dimethylfumarate (DMF), enhances antioxidant defenses, and inhibits inflammation and vaso-occlusion in SCD mice, also in an HO-1-dependent manner [312]. Triterpenoids have been suggested as a potential approach for SCD and synthetic oleanane (derived from oleanolic acid) triterpenoids (SOTs) are known to exert anti-inflammatory effects through Nrf2 activation, leading to transcription of enzymes, including HO-1 [430].

Pegylated hemoglobin carbon monoxide (CO) carriers have also been investigated in pre-clinical and clinical investigations for use in SCD. Sanguinate™ releases CO while also delivering oxygen to hypoxic tissues and a Phase Ib trial to evaluate this molecule's safety, efficacy in SCD has been completed (NCT01848925), demonstrating an acceptable safety profile in SCD patients [431]. A Phase 2 study of the safety and effectiveness of Sanguinate™ in the treatment of vaso-occlusive crises is currently in the recruitment stage (NCT02411708). Release of CO by another pegylated-Hb, MP4CO, has demonstrated significant anti-inflammatory and cytoprotective effects in mice with SCD, inducing hepatic HO-1 activity and inhibiting NF- κ B activation and hypoxia-reoxygenation-induced microvascular stasis [149]. A Phase Ib single dose escalation study demonstrated MP4CO to be well tolerated in steady-state SCD [307, 432], although a planned phase 2 study to evaluate the use of this molecule in vaso-occlusive crisis has not gone ahead due to the loss of corporate funding [NCT01925001].

Preclinical studies in SCD show that amplification of NO-cGMP dependent signaling, in combination with hydroxyurea, or not, may be of therapeutic benefit. An inhibitor of phosphodiesterase 9 (PDE9), which is highly expressed in hematopoietic cells [433] and decreases the degradation of intracellular cGMP, reduces vaso-occlusive processes in a SCD-mouse model [410]. A phase I study to investigate the safety and tolerability of PF-04447943, a PDE9 inhibitor, in steady-state SCD individuals, with and without co-administration of HU, has been completed (NCT 02114203), while a phase 1a study is currently being initiated to evaluate the safety and tolerability of another PDE9 inhibitor, IMR-687, in healthy individuals (NCT02998450). Alternatively, guanylate cyclase stimulators or activators may also represent an approach for amplifying NO signaling in SCD [434, 435].

7. Conclusion

Given the huge range of agents currently under pre-clinical and clinical investigation for use in SCD, it seems likely that the use of further anti-inflammatory approaches in addition to (or possibly even in place of) hydroxyurea will eventually be common practice for the treatment of SCD in those individuals for whom HSCT or gene therapy are not a viable option. The exact inflammatory pathway that may be the most promising therapeutic target has still not been defined, but it seems probable that anti-inflammatory drugs that ameliorate a broad range of inflammatory mechanisms, or neutralize important players in the SCD inflammatory scenario or specific inflammatory cell types may hold the key to providing benefits in these patients. However, while some of the causes and inflammatory pathways

that instigate the chronic inflammatory state in SCD are now better understood, the inflammatory mechanisms that trigger acute vaso-occlusive episodes remain extremely obscure, probably due to the constraints of studying this particular event in patients and the limitations of animal models of study. More studies that focus on defining specific triggers for these episodes are needed, in order to better define those approaches that may best prevent these triggers and to support the development of drugs for treating hospitalized patients during vaso-occlusive crisis.

Acknowledgments

Nicola Conran is supported by research grants from Fapesp, Brazil (2014/19173–5 and 2014/009843). John Belcher is supported by NIH grant R01 HL114567–05 and a grant from CSL Behring.

10. References

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Table 1.

Known and putative DAMPs that may play a role in SCD inflammatory mechanisms

DAMP	Source	Cellular function/DAMP activity	Associated Receptor(s)
Heme	Hemolysis	Released from cell-free hemoglobin following oxidation	TLR4, NLRP3
Hsp70	Hemolysis/necrotic cells	Molecular chaperone	TLR2, TLR4, CD14
ATP	Hemolysis/necrotic cells	Energy source, released from RBC and other cells following damage, hypoxia, reduced oxygen tension and oxidative stress	P ₂ Y ₂ and P ₂ X ₇
Cyclophilin A	Hemolysis/necrotic cells	Chaperone found in cell cytosolic fraction	CD147
mt DNA	Necrotic cells	Can activate leukocytes	TLR9, NLRP3
HMGB1	Necrotic cells	Nuclear protein bound to chromatin in almost all eukaryotic cells	TLR2, TLR4, TLR9, CD44, RAGE
Extracellular DNA	Necrotic cells and activated neutrophils	Released during cell death and NETosis	TLR9
S100A8	Activated leukocytes	Ca ²⁺ -binding protein that is abundant in macrophages and neutrophils	TLR4, RAGE

Compiled and adapted from [285, 436–438]

NLRP3, NLR family, pyrin domain-containing 3; RAGE, receptor for advanced glycation end products; RBC, red blood cell; TLR, toll-like receptor.

Table 2.
Selected anti-inflammatory therapies in pre-clinical and clinical investigation for sickle cell disease

Anti-inflammatory approach	Drug Class(es)	Agent(s)	Mechanism of action	Reference(s)/ ClinicalTrials.gov identifier
Hemoglobin/heme scavenging	Hemoglobin/heme scavengers	Haptoglobin hemopexin hE-Hb-B10 AIM	Binding and clearance of plasma hemoglobin or its products.	[74, 80, 439]
Intracellular inflammatory pathway modulation	Statins	Simvastatin atorvastatin	Reduction in endothelial activation and vaso-occlusive pain, possibly via HO-1 induction and inhibition of NF- κ B activity	[427, 440] NCT01702246
	Nrf2/HO-1 activators	dimethylfumarate (DMF)	DMF activates Nrf2 and HO-1, enhances antioxidant defenses, and inhibits inflammation and vaso-occlusion in SCD mice.	[312]
Immunomodulation	TNF- α blockers	etanercept infliximab	Reduce endothelial activation and have anti-inflammatory effects.	[207, 441]
	Anti-IL-1 β	canakinumab	IL-1 β neutralization reduces reperfusion injury and endothelial activation in SCD mice. Canakinumab is currently in phase 2 to evaluate efficacy, safety and tolerability in pediatric and young adult SCA patients.	[442] NCT02961218
	IL-1 receptor antagonists	anakinra	Anakinra reduces VCAM-1 expression in SCD mice.	[207]
Invariant Natural Killer T (iNKT) cell inactivation	A _{2A} R Agonists	Regadenoson	Decrease iNKT-cell activation and reduce inflammation. In phase 2 studies for SCD.	[90] NCT01085201 NCT01788631
	iNKT cell depleting agents	NKTT120	MAb that depletes iNKT cells, reducing inflammatory molecule release.	[428, 443] NCT01783691
Inhibition of cell adhesion	Selectin inhibitors	Rivipansel (GMI-1070)	Inhibition of leukocyte/endothelial adhesion molecules resulting in reduced blood cell adhesion to the blood vessel wall. Currently at Phase 3 to evaluate efficacy and safety for the treatment of vaso-occlusive crisis	[419, 421] NCT01119833 NCT02187003 NCT02433158
		Crizanlizumab (SeIG1)	Inhibition of P-selectin. In a double-blind, randomized, placebo-controlled, phase 2 trial, crizanlizumab significantly lowered the rate of sickle cell-related pain crises.	[425] NCT01895361
	Modified heparins	Tinzaparin Sevuparin	L-selectin/P-selectin inhibitor. Inhibition of leukocyte/endothelial/platelet adhesion molecules resulting in reduced blood cell adhesion to the vessel wall.	[424, 444] NCT02515838
	Non-ionic surfactants	Poloxamer 188	Significantly reduces blood viscosity, RBC aggregation and RBC adhesion to endothelial cells	[423, 445] NCT01737814 NCT02449616
CO delivery	Pegylated Hb-based CO carriers, CO-releasing molecules	MP4CO Sanguinate™	Six potential mechanisms: Anti-polymerization; anti-inflammatory; anti-	[307, 308, 432] NCT01356485 NCT01374165

Anti-inflammatory approach	Drug Class(es)	Agent(s)	Mechanism of action	Reference(s)/ClinicalTrials.gov identifier
Inhibition of platelet activity	(CORMs), inhaled CO, and oral CO. Adenosine diphosphate (ADP) receptor antagonists	Prasugrel	oxidant; anti-apoptotic; vasodilation; and red cell hydration	[426, 446] NCT01167023 NCT01476696
	$\alpha_{IIb}\beta_3$ -integrin inhibitors	Eptifibatide	Inhibit sADP-mediated platelet activation and platelet-leukocyte aggregate formation.	[447] NCT00834899
Augmentation of NO bioavailability/cGMP signaling	Arginine therapy	L-arginine	Inhibition of $\alpha_{IIb}\beta_3$ integrin-dependent platelet activation and aggregation.	[448] NCT02536170
	Nitrite therapy	Sodium nitrite	Increases availability of the substrate for NO synthesis.	[449, 450] NCT01033227 NCT01316796
	sGC stimulators and activators	IRV-1701 BAY 41-2272 Riociguat (BAY 63-2521) Cinaciguat (BAY 58-2667)	Nitrite exerts anti-platelet and anti-adhesion activities, is activated by red blood cells, and has enhanced potency under physiological hypoxia. Topical sodium nitrite also reduces leg ulcer size.	[451–453] NCT02792998 NCT03285178 NCT02633397
	Phosphodiesterase 9 inhibitors (PDE9i)	PF04447943 IMR-687 BAY 73-6691	Amplification of sGC/cGMP signaling has been shown to augment γ -globin gene expression in vitro and reduces SCD neutrophil adhesive mechanisms. Riociguat is currently approved for use in pulmonary hypertension.	[410, 433, 452, 454] NCT02114203
Oxidative stress	Anti-oxidants	L-glutamine	Amplification of cGMP signaling in hematopoietic cells. <i>In vitro</i> : PDE9 inhibition reduces SCD leukocyte adhesion and elevates γ -globin gene expression. <i>In vivo</i> : PDE9 inhibition reduces vaso-occlusive processes in SCD mice	[394, 433] NCT01179217
Red cell dehydration	Gardos channel Inhibitors	Senicapoc (ICA-17043)	Enhancement of nicotinamide adenine dinucleotide in sickle RBC. FDA approved as a supplement in SCD	[455]
Microbiota depletion	Antibiotics	Penicillin V broad-spectrum antibiotics	Reduction of potassium leakage, decreasing cell dehydration.	[374]
			Reduction in total neutrophil count and circulating aged neutrophils in SCD patients. Dramatically improves inflammation-related organ damage in SCD mice	

cGMP; cyclic guanosine monophosphate; CO, carbon monoxide; mAB, monoclonal antibody; sGC, soluble guanylyl cyclase. Table adapted from [456]