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Review

Haematopoietic stem cell health in sickle cell disease and its implications for stem cell therapies and secondary haematological disorders

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ABSTRACT

Gene modification of haematopoietic stem cells (HSCs) is a potentially curative approach to sickle cell disease (SCD) and offers hope for patients who are not eligible for allogeneic HSC transplantation. Current approaches require in vitro manipulation of healthy autologous HSC prior to their transplantation. However, the health and integrity of HSCs may be compromised by a variety of disease processes in SCD, and challenges have emerged in the clinical trials of gene therapy. There is also concern about increased susceptibility to haematological malignancies during long-term follow up of patients, and this raises questions about genomic stability in the stem cell compartment. In this review, we evaluate the evidence for HSC deficits in SCD and then discuss their potential causation. Finally, we suggest several questions which need to be addressed in order to progress with successful HSC manipulation for gene therapy in SCD.

1. Introduction

Sickle cell disease (SCD) is one of the most common monogenic disorders with high prevalence in people originating from Sub-Saharan Africa, as well as Mediterranean, middle eastern and Indian populations. It is a disease with complex pathophysiology originating from a single-base alteration (c.20 A > T) in the gene (*HBB*) encoding β-globin. This results in a single amino acid substitution of valine for glutamic acid, which changes the properties of the haemoglobin molecule and enables polymerization of deoxygenated haemoglobin. Polymer formation leads to reduced deformability of red cells, haemolysis, and microvascular occlusion. The consequent cyclical microvascular ischaemia/reperfusion creates a chronic inflammatory milieu characterised by sterile inflammation and a cascade of pathophysiological events which manifest clinically as recurrent painful crises, increased susceptibility to infection and multi-organ involvement; ultimately leading to increased morbidity and mortality.

Although SCD was first described over 100 years ago and the molecular basis of the disease has been understood for over 70 years, the development of therapeutics has been slow compared to other haematological disorders. Until recently, the only options for disease modifying therapy were hydroxycarbamide (also known as hydroxyurea) and chronic blood transfusion. Newer therapeutic agents such as Voxelotor (a modulator of haemoglobin affinity for oxygen), L-glutamine (an antioxidant agent) and crizanlizumab (a P-selectin inhibitor) have emerged in the last decade. Haematopoietic stem cell (HSC) transplantation (HSCT) from an HLA-matched sibling donor (MSD) is curative, however a major limitation for HSCT is that only about 15% of the patients have an MSD [1]. Toxicity associated with conditioning regimens, acute and chronic graft versus host disease and graft failure are additional challenges.

Gene modifying therapy offers a promising alternative to HSCT as a disease modifying or curative treatment. The current approach to these therapies involves collection of autologous HCSs followed by purification and manipulation by techniques such as gene editing or lentiviral (LV) – mediated gene addition. An increasing body of literature on laboratory observations and clinical studies, including experience in gene therapy trials have raised concerns about the health and function of HSCs in SCD. This is an important problem as gene therapy requires collection of a large quantity of healthy HSCs to be manipulated ex vivo and then reinfused into the patient after myeloablation. In this review, we examine our current understanding of the HSC health in SCD, discuss potential underlying mechanisms of HSC defects, appraise the methods available to study the integrity of HSCs and propose future areas of

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research.

2. HSC perturbation in SCD

2.1. Differences between bone marrow (BM) and peripheral blood (PB)

In PB of SCD patients during steady state, circulating HSCs and progenitors are increased compared to controls [2–5] and further increased during vaso-occlusive crisis [6]. In BM, findings have been more variable, one study highlighting a reduction in number and purity of BM $CD34^+$ cells compared to controls [2–4,7,8].

2.2. $CD34^{dim}$ and $CD34^{bright}$

One well documented observation in both PB and BM in SCD is the presence of a diffuse population of $CD34^+$ cells between the negative and conventionally staining $CD34^+$ cells, and usually referred to as $CD34^{dim}$ cells [2,3]. In PB, the $CD34^{dim}$ population is elevated irrespective of SCD treatment and shows variable antigen expression associated with progenitor and more differentiated haematopoietic precursors. The functional significance of the $CD34^{dim}$ population is not clear but is probably induced by disturbed haematopoiesis associated with SCD.

2.3. Aberrant antigen expression

In comparison to controls, $CD34^{bright}$ cells are also increased in PB and show an aberrant increase in expression of HSC markers, including integrin α 6 (CD49f), a stem cell marker which may be involved in maintaining self-renewal of pluripotent stem cell populations including HSCs [2,9]. However, such a high expression of CD49f has been shown to be associated with reduced engrafting capability in cultured $CD34^+$ cells derived from cord blood [10].This raises the question of functional capacity of these $CD34^+$ CD49f++ cells seen in SCD. Hua, Peng, et al. studied BM cells from children with SCD and thalassaemia collected as autologous back-up harvests prior to planned allogenic stem cell transplantation and compared to adult control BM. They showed expansion of a population of $CD34^+$ cells expressing the B-lymphoid marker CD10 representing over 65% of all $CD34^+$ cells and confirmed the lymphoid characteristics of these cells by single cell RNA sequence profiling. The authors suggested that expansion of this population of cells may be related to a pro-inflammatory state in SCD [8]. In another study of BM $CD34^+$ cells [3], the $CD34^+$ cells showed increased CD235a expression suggesting differentiation into erythroid lineage-restricted progenitors.

2.4. Effect of Hydroxyurea (HU) on stem cell count

Hydroxyurea treatment suppresses BM and PB $CD34^+$ cells compared to non-treated patients with a lowering in the number of colony forming progenitors [4]. A reduction in cell proliferation was demonstrated in patients on HU which is the rationale for withholding HU temporarily prior to stem cell harvesting for gene therapy [4]. In PB, HU therapy is also associated with a normalization of $CD34^{bright}$ cell counts to levels similar to that of healthy controls. The authors of this study interpreted the finding as an indication of a normalization of haematopoiesis with HU [2,11].

2.5. Functional assays for HSC in SCD

In vitro analysis of progenitor cell number and function using colony forming unit (CFU) assays have shown lineage skewing in SCD, including high levels of erythroid progenitors and increased sensitivity to erythropoietin [5,7,12]. An inverse relationship between progenitor numbers and HbF levels [5,12] suggests that stress erythropoiesis may be driving the expansion. [4,11,12,14,15]. Studies have also shown a lag in time to maximal colony formation compared to controls [13] and altered cell cycle kinetics in peripheral blood progenitors, with earlier recruitment into cell cycle [14]. Similarly, in a murine model of SCD, a lower proportion of stem cells were found to be in G0 phase of cell cycle, indicating a reduction in the number of quiescent populations of stem cells in SCD [15].

Overall function of HSCs in promoting long-term sustained haematopoiesis has also been evaluated. Employing the Berkley SCD mouse model, Javazon et al. (2012) compared HSCs from SCD mice with those from control and mice hemizygous for β^{sickle} mutation and demonstrated a reduction in engraftment potential of HSCs harvested from homozygotes [15]. In contrast, peripheral blood $CD34^+$ HSCs from SCD patients during acute crisis were able to reconstitute haematopoiesis in irradiated mice [6]. In summary, these results suggest that long term engrafting capability of non-mobilized $CD34^+$ cells from PB or BM in SCD may be limited by a relative deficiency of true HSCs [8].

Further clarification of the impact of SCD on $CD34^+$ cells and other progenitor cells is required. However, these human and murine studies indicate a pattern of disturbance in the phenotype, number and functioning of HSC in SCD.

3. Pre-clinical and early phase clinical studies of gene therapy in SCD

Low $CD34^+$ yields and relatively high proportion of $CD34^{dim}$: $CD34^{bright}$ cells have been confirmed when BM has been used as a source of HSCs for gene therapy protocols [16,17]. These problems have led to development of protocols for peripheral mobilization of $CD34^+$ cells as an alternative source of HSCs. One immediate problem which needed to be addressed was the moratorium on use of G-CSF, which, although long-established as the agent of choice for HSC mobilization, has been associated with severe adverse events in SCD patients due to vaso-occlusion, probably related to G-CSF induced hyperleukocytosis and neutrophil activation [18,19].

Plerixafor is an alternative mobilizing agent which directly inhibits the binding of stromal-cell-derived factor-1a (SDF1, also known as CXCL12) to its CXC chemokine receptor (CXCR4) on HSPC, releasing stem cells from the BM niches. There is now a considerable body of data demonstrating that Plerixafor is suitable for mobilization of HSC for genetic therapy in SCD [16,20–24]. Mobilization protocols in SCD result in increased peripheral $CD34^+$ cells peaking within 4–6 h and returning to baseline after 24 h without inducing severe hyperleukocytosis, neutrophil activation, or severe adverse clinical events [25]. Harvested $CD34^+$ cells are enriched for $CD34^{bright}$ cells with the expected phenotypic markers of HSCs [20,24]. Plerixafor-mobilized $CD34^+$ cells have immunophenotype and RNA sequencing profile consistent with quiescent HSCs and are able to repopulate haematopoiesis in conditioned immune-deficient mouse model as effectively as G-CSF mobilized stem cells from non-SCD donors [21]. However, not all SCD patients mobilize adequate $CD34^+$ cell numbers and some require repeat cycles of mobilization [20,23,25]. Although there is significant inter-patient variability in mobilization efficacy, low pre-mobilization $CD34^+$ count, increased age, markers of increased disease history and recent hydroxyurea use appear to correlate with poorer efficacy [23,25]. Current updates on gene therapy studies in SCD provide reassurance about the long-term engraftment of gene modified plerixafor-mobilized $CD34^+$ cells [22,26].

4. Genomic stability in HSCs and inherent risk of haematological malignancies in SCD

Another concern related to HSC function in SCD is the potential of diminished genomic integrity over time leading to increased risk of haematological malignancies. This is supported by recent population studies indicating a 2-to-10-fold increase in the risk of haematological malignancies in SCD compared to the general population [27–31].

Chronic perturbation of HSC function through disease is one

causative factor in the development of clonal haematopoiesis (CH) and haematological malignancies. Whole genome and whole exome sequencing have been applied by two groups to evaluate the incidence of clonal haematopoiesis in SCD, with contradictory results. One study [32] concluded that CH was more prevalent and emerges at an earlier age compared with non-SCD controls, with clones characterised by mutations associated with AML (predominantly in *DNMT3A*) [32]. However, another study failed to detect a significant increase in CH [33].

An increased rate of haematological malignancies has also been observed over prolonged follow-up of SCD patients undergoing allogeneic stem cell transplantation. The risks for leukaemia/MDS were higher with low-intensity (non-myeloablative) regimens compared with more intense regimens. These regimens rely on tolerance induction and establishment of mixed-donor chimerism; and persistence of host cells exposed to low-dose radiation triggering myeloid malignancy is one plausible aetiology, however, pre-existing myeloid mutations and prior inflammation may also have contributed [34].

A particularly concerning observation has been the development of acute myeloid leukaemia (AML) in two SCD patients enrolled in Part A of the Bluebird Bio Lentiviral gene addition therapy in SCD *(ClinicalTrials.gov: NCT02140554)* using autologous $CD34^{+}$ HSCs collected by BM harvest and transduced with BB305 lentiviral vector encoding the human beta-A-T87Q globin gene. These cases were both treated with a product manufactured from BM-derived, rather than Plerixafor-mobilized HSCs. As aforementioned BM cells are now considered less suitable for manipulation. A detailed analysis demonstrated that HSC malignancy was unlikely to be due to insertional mutagenesis [35]. In both, mutations associated with AML (*RUNX1, KRAS*, and *PTPN11*) were detected in leukaemic blasts suggesting HSC damage as a result of the underlying susceptibility of HSCs in SCD. These cases have raised questions about the long-term health of HSCs in SCD and susceptibility to malignant transformation with potential challenges to the safety and efficacy of stem cell therapies involving genetic manipulation of autologous HSCs and led to temporary suspension of clinical trials involving LV-based gene therapy (*ClinicalTrials.gov: NCT02140554, NCT04293185, NCT02140554*) [36].

Hydroxyurea (HU) is a ribonucleotide reductase inhibitor which depletes intracellular deoxynucleotide triphosphates and thereby acts as an S-phase–specific agent with inhibition of DNA synthesis and eventual cellular cytotoxicity. Its mechanism of action in SCD is multimodal, through induction of HbF by various mechanisms including bone marrow cytotoxicity-induced stress erythropoiesis, repression of ineffective erythropoiesis, translational, epigenetic and post-translational modifications [37]. It also induces mild myelosuppression resulting in reduction of absolute neutrophil, platelet, and reticulocyte counts [38,39]. Although, there is significant evidence that it improves morbidity and mortality in SCD [40–42], there is a concern, based on its mechanism of action, that it might increase the risk of haematological malignancies. One study showed increased DNA damage index and chromosomal damage in leukocytes of adult patients with sickle cell anaemia (SCA) compared with control population [43] and the deleterious DNA effects were shown to be accentuated in SCA patients on HU suggesting that HU may promote genomic instability [43]. However, another group, assessing HU genotoxicity by DNA damage biomarkers, micronuclei, nucleoplasmic bridges and nuclear buds, concluded that there was no difference in SCD patients on HU and the controls [44]. In the BABY-HUG trial, there was also no increased acquired genotoxicity in young children on HU for a period of 2 years using three laboratory assays- chromosomal karyotype, illegitimate VDJ recombination events, and micronucleated reticulocyte formation [45]. Similar negative result has been obtained in multiple mutagenicity assays in patients on HU [46]. A health insurance claims database analysis and study on clonal haematopoiesis which included patients treated with HU were similarly reassuring [33,47].

In summary, there does appear to be an increased risk of haematological malignancies in SCD, but there is uncertainty concerning the degree of risk. It seems unlikely that chronic hydroxyurea therapy is a major predisposing factor, however, further work is also needed to understand causation. New studies of single cell profiling to investigate trajectories of DNA mutational evolution during long-term follow-up and HSC interventions such as gene therapy will help to clarify these issues.

5. Potential pathogenetic mechanisms for perturbation of HSC health in SCD

HSCs are dormant in steady state but respond quickly to acute cues such as blood loss or infection by proliferation and differentiation in order to replenish lost lineage specific- blood cells or to mount an appropriate immune response. Subsequently, HSCs have the potential to revert to their pre-insult quiescence [48]. However, endogenous and exogenous stimuli can disrupt HSCs self-regeneration capacity, differentiation fate and genomic integrity; and perturb several niche components [49]. Some of the pathophysiological features in SCD that could cause HSC dysfunction are illustrated in Fig. 1 and reviewed in the following sections.

5.1. Chronic inflammation

Sterile inflammation is one of the key pathophysiological features of SCD [50], characterised by an increase in adhesion markers on damaged sickled erythrocyte membranes, as well as activated and dysfunctional endothelium and an expanded reticulocyte population. Enhanced adhesion of sickled erythrocytes to endothelial cells, platelets and neutrophils results in microvascular occlusion. Subsequent cyclical ischaemia/reperfusion injury, combined with release of microparticles from damaged erythrocytes are amongst the mechanisms triggering a thrombo-inflammatory cascade, which involves the activation of neutrophils and monocytes, production of inflammatory cytokines such as (Interleukin)IL-1α, IL6, tumour necrosis factor (TNF)-α and IL-1β and upregulation of growth factors such as GM-CSF, M-CSF, TGF-β and pro-angiogenic molecules [51,52].

An Inflammatory environment such as this exposes HSCs to cytokines and growth factors, including IL-1, IL-6, TNF α, and interferon (IFN)-γ. HSCs exit quiescence and proliferate to enhance myeloid and megakaryocytic output on exposure to these cytokines [48], and apoptosis is enhanced [53]. HSCs are directly activated through Toll-like receptors by damage- or pathogen-associated molecular patterns (DAMPs or PAMPs), driving emergency granulopoiesis and producing further inflammatory cytokines [54,55]. In the setting of an acute infection, this response is required to provide an appropriate response against the microbial invasion. However, an ongoing exposure to inflammation leads to egress of HSC and progenitors from the BM through activation of NOD1, TLR2, and TLR4, causes biased lineage output towards myeloid rather than lymphoid through exposure to growth factors such as G-CSF and GM-CSF produced by activated niche cells, and depletes long-term repopulating capacity [56]. There is a metabolic switch from anaerobic glycolysis to oxidative respiration in HSCs which results in increased reactive oxygen species (ROS) accumulation and DNA damage [55]. Chronic inflammation also promotes clonal haematopoiesis by favouring clones with mutations such as DNMT3a and TET2 that provide HSCs a survival advantage [57,58]. Additionally, chronic inflammation perturbs the non-haematopoietic BM cells such as endothelial cells and mesenchymal stromal cells produces DAMPs, pro-inflammatory cytokines and growth factors which induce cell cycling and apoptosis in HSCs, as well as damaging the nice function [55,59].

Supporting the importance of inflammation on HSC health in SCD, there is direct evidence on the effect of enhanced inflammation in SCD with increased inflammatory markers in the mononuclear compartment of BM [3] and in enhanced expression of genes involved in the inflammatory response in transcriptomic profiles of BM HSCs [21]. It seems

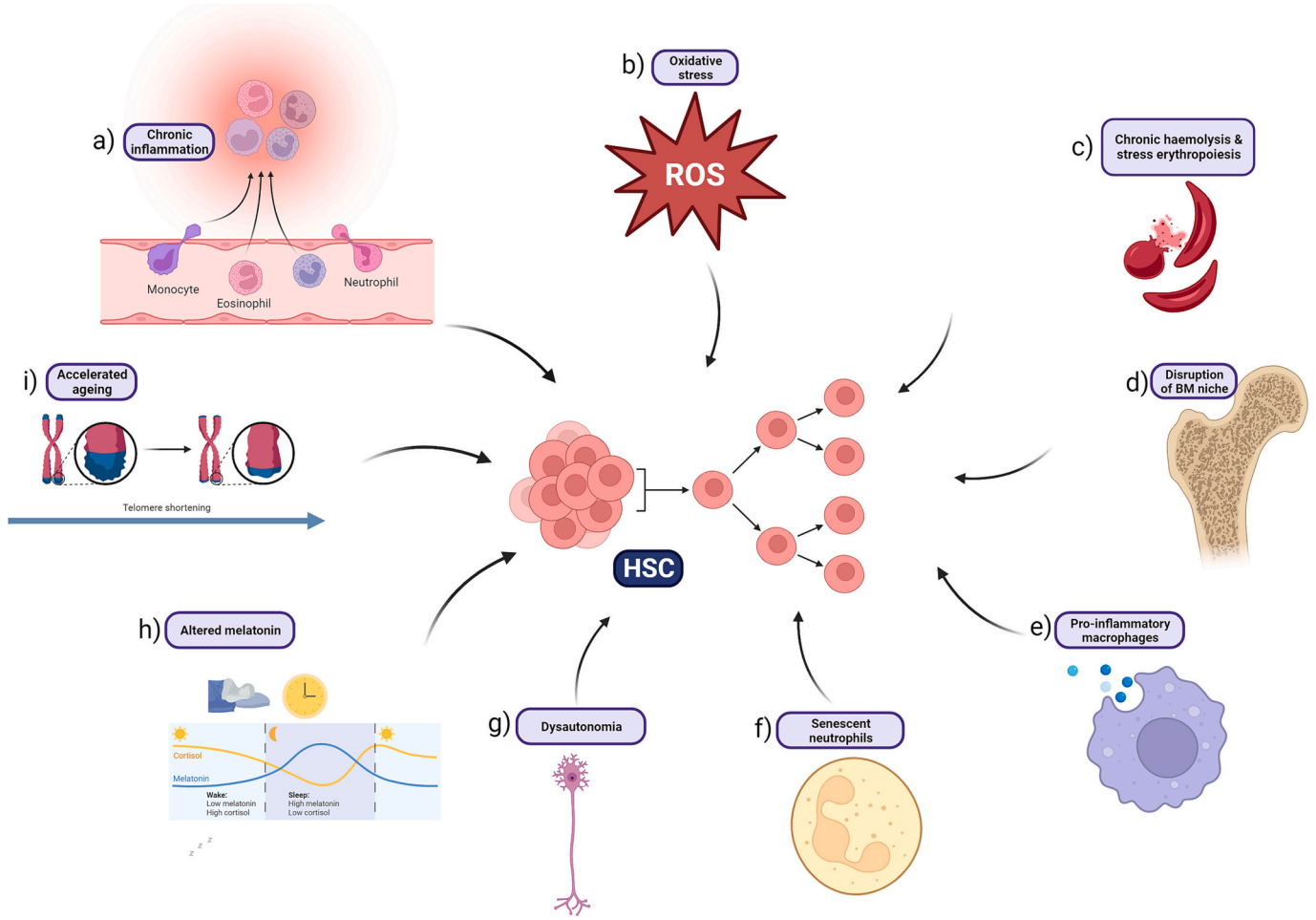

Fig. 1. Potential pathological factors affecting HSC health in SCD (Created with BioRender.com).

likely that chronic inflammation is a major driver of HSC dysfunction, but further studies are required to differentiate from the competing effects of other insults, and to determine whether pharmacological and biological approaches to suppress inflammation reverse the observed BM HSC defects.

5.2. Oxidative stress

SCD is associated with heightened oxidative stress because of imbalance between excess reactive oxygen species such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical and reduced antioxidants such as nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase, catalase, and heme oxygenase-1 [60]. Sickle haemoglobin has increased tendency to undergo auto-oxidation effecting enhanced accumulation of ROS in erythrocytes [61].

The half-life of sickled erythrocytes is much shortened by enhanced red cell breakdown. Intravascular haemolysis in SCD causes enhanced release of cell-free heme into the circulation which results in reduction of the vasodilator and an anti-inflammatory nitric oxide and production of oxygen free radicals causing endothelial dysfunction. Free heme, iron and arginase released by the breakdown of red cells mop up NO and catalyse production of ROS. A vicious cycle is perpetuated with chronic haemolysis, cyclical ischaemia reperfusion and local tissue hypoxia causing reduction of ROS scavengers leading to dysfunction of endothelial cells, platelets, neutrophils and erythrocytes which further aggregate and produce vaso-occlusion [62,63]. ROS are detrimental to DNA, lipids, proteins, and carbohydrates and cause cell dysfunction or death [62]. Javazon, Elisabeth H., et al. (2012) showed increased lipid peroxidation and ROS in HSCs in murine models of SCD. This was demonstrated to affect engraftment capability with a partial improvement seen upon the treatment with the antioxidant *N*-acetyl cysteine [15].

5.3. Chronic haemolysis and 'stress erythropoiesis'

In addition to induction of oxidative stress, free heme also has been shown to act as a damage-associated molecular pattern (DAMP), which causes epigenetic modulation of HSCs in mice resulting in an expansion of short-term (ST)-HSC and myeloid priming. Additionally, free heme exposure in mice resulted in a delayed and modified innate immune response with HSC differentiating to pro-inflammatory macrophages in mice [64].

The increased erythropoietic drive in SCD is a result of compensatory expansion of erythrocyte production with chronic haemolysis, together with induction of stress erythropoiesis pathways as a response to inflammatory stimuli. These mechanisms of enhanced erythropoiesis have been studied in detail in mouse models [65], but it is not certain how they function at a molecular and cellular level in humans. In SCD patients, populations of $CD34^{+}$ cells expressing erythroid markers have been identified in some studies [3] which may represent HSCs responding to stress erythropoietic signals. In the small number of studies looking at transcriptomics in SCD HSCs, gene expression associated with erythropoietic drive was not notably increased [8,21].

Further studies are needed in SCD to study sub-populations of HSCs with erythropoietic gene expression profiles, to determine whether chronic erythropoietic stress in SCD is a primary cause of HSC

dysfunction and depletion, and whether hydroxyurea and other therapies impact on HSC health through modifying chronic haemolysis and stress erythropoietic responses.

5.4. Disruption of BM niche

The cellular components of BM niches supporting haematopoiesis include perivascular stromal cells, vascular endothelial cells, osteolineage cells and megakaryocytes [66,67].These cells regulate quiescence, circadian rhythm of circulation, self-renewal and differentiation fates of HSC and MPP population, via production of various growth factors, chemo-attractants and cytokines such as stem cell factor, CXCL12, notch ligand, TGF-β, TPO, pleiotrophin and osteopontin, and through various direct cell-cell interactions [66].

There are surprisingly few studies on BM morphology in SCD using routine histopathological techniques, and very little information on BM trabecular and vascular disturbance and distribution of cellular niche components. Bony trabecular thinning, erythroid hyperplasia, sinusoidal dilatation, increased perivascular fibrosis and varying stages of infarct resolution are some of the histopathological findings reported in SCD, mostly from post-mortem samples [68]. Magnetic resonance imaging study of the bone marrow has shown an abnormal patchy distribution of BM signal, which increases with age. This patchiness is observed in steady-state and deteriorates during vaso-occlusive crisis [3]. Mechanisms of BM damage have been studied in mouse models of SCD, and findings include vaso-occlusion triggering HIF-1α–induced neovascularisation [69], and defective mesenchymal stromal cells induced by free heme in SCD, which was reversible by targeting oxidative stress using N-Acetyl cysteine or the TLR4 pathway [70].

The BM niches are likely to be severely disrupted as a result of a panoply of insults including erythroid hyperplasia, sinusoidal vaso-occlusion, ischaemia/reperfusion, chronic inflammation, bone remodelling, neovascularization, and fibrotic change. Further research is needed to study this aspect of SCD pathophysiology in greater detail.

5.5. Pro-inflammatory macrophages

Macrophages help maintain HSC in the protective niche environment. Various subsets of macrophages in the BM niche play key roles in HSC homeostasis and haematopoiesis during inflammatory stress [71]. Their depletion in mice has shown to cause peripheral mobilization of HSC [72,73]. HSC self-renewal and maintenance of numbers in stress-response has shown to be partly reliant on a rare subpopulation of macrophages expressing alpha-smooth muscle actin (αSMA) which supplies PGE2 for this purpose [74].

Lysosomal processing of polymerised sickle haemoglobin is impaired in macrophages in SCD. This in turn leads to inflammasome activation in the macrophages resulting in production of pro-inflammatory cytokines propagating the chronic inflammatory milieu [75]. Pro-inflammatory macrophages, which are also called classical ‘M1’ macrophages, inhibit in vitro self-renewal and expansion of HSCs upon co-culturing [76].

5.6. Senescent neutrophils

Neutrophils also have been shown to have a role in HSC regulation in mice models. Aged neutrophils, characterised by markers $CD62^{low}$ and $CXCR4^{high}$, are efferocytosed by resident macrophages in the BM to maintain homeostasis. This results in activation of macrophage Liver X receptors -LXRα and LXRβ, and egress of HSC from the BM [77]. Interleukin (IL)-23 transcription is downregulated upon efferocytosis of aged neutrophils by macrophages and dendritic cells. IL-23 causes stromal cells to produce G-CSF, mediated by IL-17 and thus aged neutrophils via the IL23-IL17-GCSF feedback circuit cause functional regulation of myelopoiesis [78,79]. Patients with SCD have an expanded population of aged neutrophils which are characterised by $CD62L^{low}$ $CXCR4^{high}$ [80]. Intestinal dysbiosis has been proposed to drive neutrophil senescence through the cross talk between neutrophil toll-like receptors (TLRs) and Myd88-mediated signalling [80,81]. These senescent neutrophils through their interaction with bone marrow macrophages may contribute to the increased HSC in circulation in SCD.

5.7. Dysautonomia

Sympathetic nervous system (SNS) and Schwann cells are known to be involved in the regulation of HSC and its niche components [67].

G-CSF induces HSC mobilization partly through activation of SNS which has a suppressive effect on osteolineage cells, thus disrupting the niche and allowing HSCs to escape the microenvironment. The SNS also has a key role in circadian circulation of HSCs. Where SNS direct the effects on HSC through the niche, nociceptive nerves are shown to maintain HSC in the BM by their direct action via the secretion of calcitonin gene-related peptide (CGRP) [82]. Non-myelinating Schwann cells also are important for quiescence of HSC through TGF-β and SMAD signalling activation [67].

Dysautonomia with a predominant sympathetic activity shown in SCD patients may modify HSC mobilization and the circadian circulation of HSC [83]. Enhanced nociceptor nerve stimulation with inflammatory stimuli in SCD [63] may also affect HSC mobilization.

5.8. Altered melatonin levels in SCD

There is in vitro evidence that melatonin has an anti-senescence and antioxidant effects on HSCs and a regulatory role on the other cellular components of the niche in addition to the circadian pattern of circulation of HSC [84]. SCD is associated with reduced melatonin levels, perhaps related to sleep disturbance, which is a very common issue in patients with SCD [85–88]. Reduced melatonin levels and altered circadian rhythm may affect stem cell health and cause HSC mobilization.

5.9. Senescence and ageing

Finally, ageing in SCD is a probable risk factor for accelerated HSC dysfunction. Ageing in the haematopoietic compartment is characterised by expansion of functionally defective HSC with reduced regenerative potential, skewed myeloid differentiation, cytopaenias, thrombopoiesis, impaired adaptive immune response and increased incidence of haematological malignancies. There are similarities in the phenotype in chronic inflammation and ageing, characterised by anaemia, immunosenescence, and thrombocytosis with a release of proinflammatory cytokines such as IL-1, TNF, and IL-6, which are collectively called senescence-associated secretory phenotype (SASP) [48]. Hence, these two are often considered together under one terminology as inflamm-ageing [89].

In the BM microenvironment, mesenchymal stem cell (MSC) transition into an adipogenic state while losing osteogenic potential, macroscopically noted as conversion of red marrow to fatty marrow [90]. Telomere shortening in stromal cells has also been observed to negatively impact on HSC function, including engraftment capability. Ageing, additionally, results in increased adipo-CAR cell numbers, loss of $EMCN^{+}CD31^{high}$ vessels and β3-adrenergic innervation. Ageing associated reduction in the number of sinusoidal endothelial cells and increased vessel diameter also result in changes to the vascular BM affecting HSC function [91].

Intrinsic mechanisms implicated with HSC ageing can be summarised as increased small RHO GTPase cell division control protein 42 (CDC42) activity causing loss of polarity of HSC cells, increased ROS production, and telomere shortening. This leads to increased expression of cyclin-dependent kinase inhibitor 2 A (CDKN2A; also known as p16INK4A) and probably of B cell lymphoma 2 (BCL-2), B cell-activating transcription factor (BATF) and p53 and further epigenetic changes

which underlie the predisposition to clonal haematopoiesis [92]. Somatic mutations in certain genes such as *DNMT3A*, *TET2*, and *ASXL1*, which accumulate with ageing, confer survival advantage to HSCs, and upon exposure to a pro-inflammatory environment, can predispose to clonal haematopoiesis and haematological malignancies [90,93].

Features of ageing, such as telomere shortening, dysfunction of the ubiquitin–proteasome system, activation of acid sphingomyelinase are seen in SCD; and organ damage in SCD mirrors what is seen in aged general population [94,95]. With these findings established in multiple other tissues, it is not unreasonable to extrapolate effects of ageing to bone marrow and HSC. Although progressive pancytopaenia is not generally observed in patients with SCD, other ageing phenotypic characteristics of the haematopoietic system such as myeloid bias and increased risk of haematological malignancies are noted in SCD and thus inflamm-ageing is likely to be important.

6. Conclusions and future considerations

In this review we have described the spectrum of HSC deficits in SCD and highlighted some of the gaps in understanding of mechanisms underlying damage. Several broad conclusions can be made: [1] Chronic inflammation is likely to be an important mediator of damage; [2] HU, a cytotoxic drug, is not clearly implicated in enhancing HSC damage, and [3] plerixafor-mobilized $CD34^+$ HSCs appear to be less perturbed than BM $CD34^+$ cells and are a suitable source of HSC for manipulation in gene therapies.

Future work is required to further characterise the defects in HSC number and function and relate to different stages of disease natural history. This will require larger studies including patients of all ages, variable disease severity and treatment history, including those on long term treatment with HU and those who are HU naïve.

In addition, there is further scope for different approaches to explore mechanisms of HSC damage, including more in-depth RNA studies of gene expression profiling to establish predominant up-regulated cellular regulatory pathways, and single cell 'omics' combined with barcoding to reveal clonal expansion and evolution, mutational burden, and development of the premalignant state.

In vivo approaches could be explored to further define the effects on HSC function in SCD, such as clonal analysis using single cell transplantation assays, analysis of somatic mutations in humans as barcodes in transplantation setting and whole genome sequencing in the non-transplantation setting [96,97].

There is an undeniable need for more therapies in SCD. Prospective investigations should aim to search for pharmacological agents which might improve HSC health in vitro and in vivo.

Finally, further long-term natural history studies of patients undergoing standard therapy and under longer-term follow up after HSC transplantation and gene therapy would be essential to better evaluate the long-term risks of clonal haematopoiesis and malignant transformation.

Practice points

- There is accumulating evidence for abnormalities in HSC number and function in SCD.
- The clinical implications of HSC dysfunction during extended follow-up of patients with SCD are not yet clear.
- Chronic inflammation is likely to be an important mediator of HSC perturbation.
- Plerixafor-mobilized $CD34^+$ HSCs appear to be less damaged than BM $CD34^+$ cells and are a suitable source of HSC for manipulation in gene therapies.

Research agenda

- Further characterisation of HSC health in patients with variable disease severity and treatment history.
- Long-term follow-up studies on outcomes related to haematopoiesis and development of clonal haematopoiesis in patients undergoing standard care, allogeneic stem cell transplantation with long-term mixed chimerism, and following autologous HSC manipulation for gene therapy.
- Evaluation of pharmacological approaches for optimising HSC health as a goal of SCD therapy and as an in-vitro treatment during HSC manipulation for stem cell therapy.

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Declaration of Competing Interest

The authors declare no competing financial interests.

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