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RESEARCH ARTICLE

Clearance of pathogenic erythrocytes is maintained despite spleen dysfunction in children with sickle cell disease

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Abstract

In children with sickle cell disease (SCD), splenectomy is immediately beneficial for acute sequestration crises and hypersplenism (ASSC/HyS) but portends a long-term risk of asplenia-related complications. We retrieved peripheral and splenic red blood cells (RBCs) from 17 SCD children/teenagers undergoing partial splenectomy for ASSC/HyS, 12 adult subjects without RBC-related disease undergoing splenectomy (controls), five human spleens perfused ex vivo with Hb_{SS}- and Hb_{AA}-RBC, and quantified abnormal RBC by microscopy, spleen-mimetic RBC filtration, and adhesion assays. Spleens were analyzed by immunohistochemistry and transmission electron microscopy (TEM). In circulating blood of SCD and control subjects, dysmorphic

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(elongated/spherocytic) RBCs were <2%, while proportions of pocked-RBC were 4.3-fold higher in SCD children than in controls. Compared to controls, splenic RBCs were more frequently dysmorphic (29.3% vs. 0.4%), stiffer (42.2% vs. 12.4%), and adherent (206 vs. 22 adherent RBC/area) in SCD subjects. By TEM, both polymer-containing and homogenous RBC contributed to spleen congestion, resulting in 3.8-fold higher RBC population density in SCD spleens than in control spleens, predominantly in the cords. Perfused spleens with normal function displayed similar congestion and retention of dysmorphic RBC as SCD spleens. The population density of active macrophages was similar in SCD and control spleens, with a relative deficit in phagocytosis of polymer-containing RBC. Despite the existence of hyposplenism, splenectomy in SCD children removes an organ that still efficiently filters out potentially pathogenic altered RBC. Innovative treatments allowing fine-tuned reduction of RBC retention would alleviate spleen congestion, the major pathogenic process in ASSC/HyS, while preserving spleen protective functions for the future.

1 | INTRODUCTION

Splenic dysfunction (hyposplenism), assessed by the presence of circulating red blood cells (RBCs) containing Howell-Jolly bodies (HJB-RBC)¹⁻⁴ or vacuoles (pocked-RBC),⁵⁻⁹ is a major feature of sickle cell disease (SCD). It appears progressively in infants, and generally worsens during childhood.^{4,5,7-9} In the general population, hyposplenism portends a risk of infectious, thromboembolic, and neoplastic complications.¹⁰ Acute splenic sequestration crisis (ASSC), the predominant spleen-related complication in SCD, is a life-threatening event defined by an abrupt drop in hemoglobin (≥ 2 g/dL) combined with acute splenomegaly.^{11,12} Subacute splenomegaly with cytopenias defines hypersplenism (HyS) which may impact the growth and development of affected children.¹³ The current management of ASSC/HyS, broadly similar in Europe and in the United States, is based on optimized care, including hydroxyurea, blood transfusion,¹⁴ and splenectomy in case of relapse (for ASSC) or impact on growth (for HyS).¹⁵⁻²¹ Partial splenectomy in patients with SCD²² has been proposed to prevent the recurrence of ASSC or HyS,^{17,18,23} while maintaining residual spleen function, although this second objective might be met only transiently.²⁴

The interconnected mechanisms underlying ASSC, HyS, and hyposplenism in SCD are not entirely deconvoluted. Direct histological exploration of spleens has been relatively limited, although progressive alterations in SCD spleens were described almost a century ago.²⁵ Splenic fibrosis and iron deposition likely resulting from macro or micro infarcts, related or not to in situ sickling of RBC, are the major pathophysiological features. Immunohistochemistry (IHC) has shown a decreased density of CD8-positive sinusoid and lymphoid follicles, the presence of Gamna-Gandy bodies, and an increased number of microvessels, compared to control spleens.²⁶ Whether congestion, the hallmark of ASSC, results predominantly from hyposplenism, or from defects in filtering structures, or conversely from hyperactive filtration is not

known. A recent modeling study based on an extensive microfluidic analysis suggested that the central pathogenic process in ASSC/HyS is the imbalance between the rate of RBC retention (V^R) and the rate of post-retention elimination (V^E) by the spleen.²⁷ However, whether RBC retention and elimination are defective, normal, or excessive was not addressed in this report, although a slow intra-macrophagic processing of sickled hemoglobin S-containing-RBC (Hb_{SS}-RBC) was observed experimentally under hypoxia.²⁷ In a recent study in a malaria-endemic area, direct comparison of circulating and splenic RBC from subjects splenectomized for trauma has generated paradigm-shifting observations on the parasite-sheltering role of the spleen in this major infection.²⁸⁻³⁰ To clarify the pathogenic processes leading to ASSC/HyS, we adopted this powerful comparative approach, completed by spleen-mimetic RBC filtration.³¹⁻³³ The unique pathophysiological relevance of this filtration method has been validated in human spleens perfused ex vivo.³⁴ We complemented these explorations by dynamic RBC adhesion assays in vitro on endothelial cell layer, and detailed morphometric observations at the tissular, multicellular, cellular, and subcellular level⁹ on spleen fragments from SCD children partially splenectomized for ASSC/HyS. This multifaceted approach uncovers the puzzling co-occurrence of hyposplenism and hypersplenism, respectively revealed by the high levels of circulating pocked-RBC and preserved ability of SCD spleens to retain stiff RBC, similarly to those observed in control spleens.

2 | METHODS

2.1 | Patients

From May 2021 through October 2023, 12 adult subjects without RBC-related disease splenectomized for pancreas tumor, 16 SCD children splenectomized for ASSC/HyS, and one SCD adult

(19 year) splenectomized for recurrent episodes of ASSC/HyS were enrolled from four hospitals of the Assistance Publique-Hôpitaux de Paris network (AP-HP) in a study titled “Pathophysiological Explorations of Red Blood Cells” ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03541525) reference NCT03541525), approved by the French Institutional Review Board and sponsored by Imagine Institute. Demographic and clinical data were collected at the time of sampling and reported in supplemental materials (Table S1).

2.2 | Spleen and peripheral blood samples

On the day of splenectomy, spleens were collected within 2 h of surgery. Peripheral blood samples were collected just before the pre-operative transfusion and/or during surgery. The spleen biopsies were aseptically processed for histological and transmission electron microscopy (TEM) studies as described.^{30,35} The proportion of pocked-RBC was quantified as described by imaging flow cytometry (IFC)⁹ and by differential interference contrast (DIC) microscopy with minor modification.^{8,9,36,37} The final concentration of glutaraldehyde used for RBC fixation was 0.1% and at least 300 RBCs were counted. Based on the kinetics of reappearance of pocked-RBC following transfusion, obtained in a previous cohort of 116 SCD patients (unpublished data), the proportion of pocked-RBC was corrected if transfusions were performed within 21 days before the splenectomy, following the formula:

$$\begin{aligned} \text{Corrected \% of pocked-RBC in case of one transfusion } (P_{\text{corr}}) &= P_{T1} + (D_{T1} \times 0.46); \text{ in case of two transfusions } (P_{\text{corr}}) \\ &= P_{T1} + (D_{T1} \times 0.46) + (D_{T2} \times 0.46), \end{aligned} \quad (1)$$

P_{T1} : proportion of pocked-RBC at the sampling, just prior transfusion; D_{T1} : number of days (between transfusion and splenectomy); D_{T2} : number of days between the second transfusion and splenectomy; NB: pocked-RBC proportion is stabilized following 21 days after transfusion in patients with SCD.⁹

2.3 | Retrieval of splenic red blood cell

Splenic RBCs were retrieved as described³⁰ with minor modifications. Briefly, spleen tissue sections (about 0.5–1 cm³ in size, 5–10 pieces) were randomly sampled from slices, put in 50 mL falcon tubes containing 30 mL of Krebs/Albumax composed of Krebs-Henseleit buffer (Sigma-Aldrich, France) with calcium chloride dihydrate (0.175 g/L), sodium carbonate (2.1 g/L), and Albumax-II (5 g/L, Gibco, France) buffered at pH 7.35–7.45. This mixture was slowly rotated for 30 min–1 h and the suspension was collected by gravity in a new 50 mL falcon tube through 40 μm SmartStrainers filters (Sigma-Aldrich, France), to be centrifuged at 1237g for 5 min. Supernatant was discarded and the pellet was washed twice in 30 mL of Krebs/Albumax. This RBC pellet is named here splenic RBC.

2.4 | Quantification of pocked-RBC and abnormal RBC

Morphometric analysis of both peripheral and splenic RBC was carried out by DIC optical microscopy on glutaraldehyde-fixed RBC to quantify the proportion of pocked-RBC and abnormally shaped cell subpopulations (i.e., elongated RBC, spherocytes)⁸ from at least 300 RBCs counted.

2.5 | Assessment of retention/enrichment rate of RBC by filtration

Spleen-mimetic microspheres was performed as described,^{32,38} with minor modifications on both peripheral and splenic RBCs from eight SCD subjects and 10 controls within 4 h of surgery (except for one control, 18 h post-surgery, spleen preserved at 4°C). The detailed method is reported in supplemental methods and Figure S1. Data were analyzed using FlowJo 10.6.2 software.

2.6 | CD71 Immunohistochemistry (IHC) analysis of spleen fragments

Spleen sections from controls and SCD children were analyzed by IHC using a rabbit polyclonal anti-CD71 transferrin receptor primary antibody (Abcam, Cambridge, United Kingdom), followed by secondary detection with N-Histofine simple stain MAX PO (Nichirei Biosciences, Tokyo, Japan) and a hematoxylin counterstain.³⁰ The number and perimeter of red pulp sinuses were quantified on 10 randomly selected areas from the red pulp using ZEN software 3.1 (blue edition, Carl Zeiss, Germany) according to the standard operation procedure, SOP (Supplemental methods, Figure S2). The following formulas were used to calculate sinuses number per spleen.

$$\begin{aligned} \text{Spleen weight(g)} &= [\text{removed portion of the spleen(g)}] \\ &+ [(1/3) \text{ of the removed portion(g)}]. \end{aligned} \quad (2)$$

Of note, 3/4 of the spleen were systematically removed for each SCD child (according to the surgeon).

$$\text{Spleen volume (mm}^3\text{)} = [\text{Spleen weight(g)} \times 1000]. \quad (3)$$

$$\begin{aligned} \text{Number of sinuses per spleen} &= \text{Spleen volume (mm}^3\text{)} \\ &\times \text{sinus number per mm}^3 \text{ (IHC)}. \end{aligned} \quad (4)$$

2.7 | Transmission electron microscopy (TEM)

Spleen fragments were observed by TEM³⁵ to assess the morphometric parameters of spleen and containing cells such as RBC subpopulation (i.e., both RBC with polymerized sickle hemoglobin/polymer-containing-RBC “Pc-RBC,” presumably Hb_{SS}-RBC or homogeneous hemoglobin/homogeneous cytoplasm-RBC “Hc-RBC,”

presumably Hb_{AA}-RBC) and macrophage, and analysis was carried out according to the following SOP (Supplemental methods, Figure S3). Forty to 85 pictures were analyzed for each of 13 spleens (five controls and eight SCD spleens). Images were analyzed using ImageJ software and statistical analysis by GraphPad Prism 8.0 software. The parameters were assessed using the following formulas:

$$\begin{aligned} &\text{Macrophage density} \\ &= \text{number of macrophage population/number of images.} \end{aligned} \quad (5)$$

$$\begin{aligned} &\text{Active macrophage density} \\ &= \left[\frac{\text{number of macrophage containing one or more RBC element}}{\text{total number of macrophages}} \right] \times 100. \end{aligned} \quad (6)$$

$$\text{RBC subpopulation density} = \text{number of RBC/number of images.} \quad (7)$$

$$\begin{aligned} &\text{Proportion of phagocytized Hc – RBC} \\ &= \left[\frac{\text{number of macrophage containing Hc – RBC}}{\text{number of macrophages containing Hc – RBC} + \text{total number of free Hc – RBC}} \right] \times 100. \end{aligned} \quad (8)$$

$$\begin{aligned} &\text{Proportion of phagocytized Pc – RBC} \\ &= \left[\frac{\text{number of macrophage containing Pc – RBC}}{\text{number of macrophages containing Pc – RBC} + \text{total number of free Pc – RBC}} \right] \times 100. \end{aligned} \quad (9)$$

$$\begin{aligned} &\text{Proportion of RBC subpopulation} \\ &= \text{number of RBC/number of total cell subtype.} \end{aligned} \quad (10)$$

2.8 | In vitro RBC dynamic adhesion assay

Circulating and splenic RBCs (three SCD children and two controls) were used to perform adhesion experiments on TNF α -activated human microvascular endothelial cell line 1 (HMEC-1), as described,^{39,40} with minor modifications (Supplemental methods). Images were analyzed using ImageJ software.

2.9 | Ex vivo perfusion of normal human spleens

Normal human spleens were retrieved from adult subjects without RBC-related disease who were splenectomized for pancreas tumors, catheterized, and perfused ex vivo as described,^{34,35,41,42} with minor modifications with mixture of hemoglobin A containing-RBC Hb_{AA}-RBC and hemoglobin S containing-RBC Hb_{SS}-RBC from adults SCD patients in transfusion program,⁹ (Supplemental methods). Spleen effluents were sampled at different time points during perfusion. Samples were used to quantify the proportion of pocked-RBC, hematocrit, elongated RBC,⁴ and hemoglobin S (mutated hemoglobin, Hb_S) using high performance liquid chromatography.

2.10 | Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0 software. Descriptive statistics, Mann–Whitney test, simple linear regression relationship, and nonparametric Spearman correlation were performed.

3 | RESULTS

3.1 | Sick cell disease children with acute sequestration crises and hypersplenism are hyposplenic

The proportion of circulating pocked-RBC was evaluated shortly before and/or upon splenectomy, using DIC microscopy and IFC in 12 controls, and 16 SCD patients with ASSC/HyS (Figure 1A,B). The median [interquartile, IQR] proportion of pocked-RBC was higher in SCD patients than in controls either by DIC (12.34% [7.04%–15.19%] vs. 3.41% [1.61%–6.59%], $p = 0.0014$, Figure 1A) than by IFC (9.99% [5.77%–13.56%] vs. 2.83% [1.64%–4.38%], $p = 0.0004$, Figure 1A). Unlike controls, SCD children with ASSC/HyS had received RBC transfusions before partial splenectomy. We thus estimated the proportion of pocked-RBC corrected for dilution by transfusion, based on the transfusion date and kinetics of reappearance of pocked-RBC following transfusion (methods, Formula (1)). After correction, the median [IQR] proportion of pocked-RBC was markedly higher in SCD children with ASSC/HyS than in controls both by DIC (14.61% [9.73%–22.13%] vs. 3.41% [1.61%–6.59%], $p = 0.0004$, Figure 1B) and by IFC (12.41% [7.01%–20.88%] vs. 2.83% [1.64%–4.38%], $p = 0.0001$, Figure 1B). These results showed that 14/16 children had more than 10% pocked-RBC in circulation by DIC, our threshold for hyposplenism.

3.2 | Spleens from SCD children with ASSC/HyS accumulate mechanically and morphologically altered RBC

We performed spleen-mimetic microfiltration on peripheral and splenic RBC (Figure 1C) retrieved upon splenectomy from 10 adult subjects without preexisting RBC-related disease (controls), nine SCD children with ASSC/HyS, and one SCD adult with relapsing ASSC to determine a retention/enrichment rate (RER).^{32,38} Peripheral RBC from controls and SCD patients had similar RER (respectively, -14.65% [-20.17% – -9.01%] vs. -15.29% [-17.68% – -9.21%], Figure 1D–Figure S1, light-red dots) unlike SCD splenic RBC that had a significantly higher RER than control splenic RBC (Figure 1D–Figure S1, deep-red dots), the mechanical retention rates being 30% higher for splenic RBC from SCD patients than splenic RBC from controls (Figure 1D). The median [IQR] spleen-to-circulation RER was 3.4 times greater in SCD patients than in controls (42.15% [40.42%–47.55%] vs. 12.43% [10.49%–15.67%], $p < 0.0001$), indicating that splenic RBCs were significantly stiffer in SCD subjects than in controls (Figure 1E). Morphometric quantification of altered

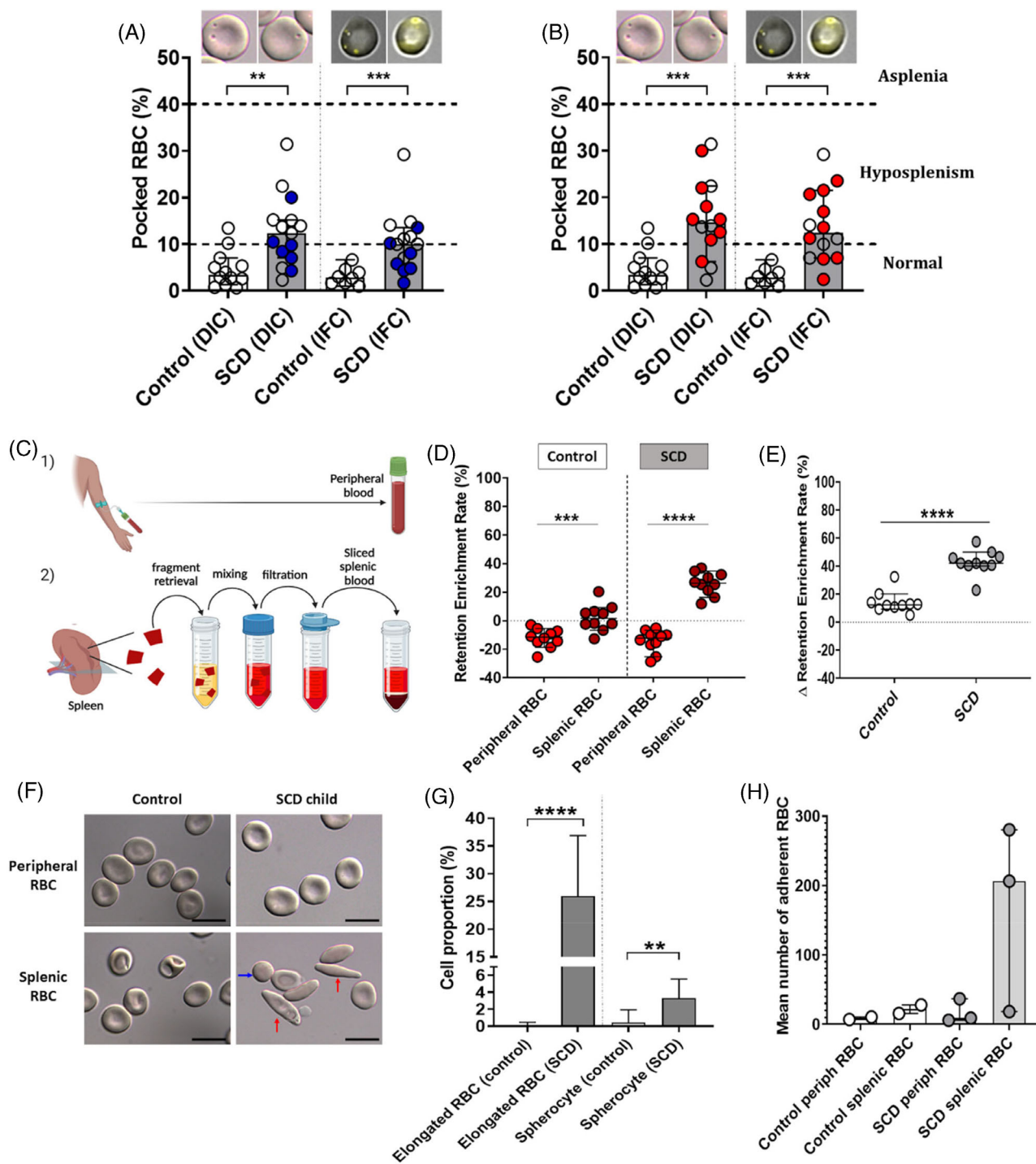


FIGURE 1 Sickle cell disease (SCD) children with acute sequestration crises and hypersplenism are hyposplenic and their spleens retain altered, elongated red blood cell (RBC) and spherocytes. From peripheral blood samples, native proportion of pocked-RBC (A), or corrected proportions based on transfusion date before partial splenectomy (B) are assessed by differential interference contrast and imaging flow cytometry⁹ in SCD children (SCD) and healthy subjects (control). Blue dots represent the native pocked-RBC proportion of transfused SCD patients requiring a correction, red dots present the corrected pocked-RBC proportion for these SCD patients. White dots are either non-transfused subjects or the ones transfused more than 21 days prior partial splenectomy. (C) Schematic representation of the peripheral (C1) and sliced splenic blood (C2) retrieval procedure. The retention/enrichment rate (RER) of splenic and peripheral RBC was assessed using spleen-mimetic microsfiltration^{32,38} from control and SCD subjects. (D) RER of peripheral (light-red dots) and splenic RBC (deep-red dots) for control and SCD subjects. (E) Difference (Δ = splenic RER - circulation RER) between splenic and peripheral RBC RER for controls ($n = 10$, white circle), and SCD children ($n = 9$) and adult ($n = 1$), (dark-gray dots). (F) Typical aspect of “spherocytic RBC” indicated by blue arrow while red arrows show elongated RBC, scale bar 10 μ m. (G) Quantification of abnormal cell subpopulations accumulated in the splenic blood from control ($n = 9$) and SCD subjects ($n = 11$). (H) Number of adherent RBC (mean) on endothelial cell layer from control ($n = 1$) and SCD subjects ($n = 3$). Mann-Whitney test was used (ns: $p > 0.1234$; *: $p < 0.0332$; **: $p < 0.0021$; ***: $p < 0.0002$). [Color figure can be viewed at wileyonlinelibrary.com]

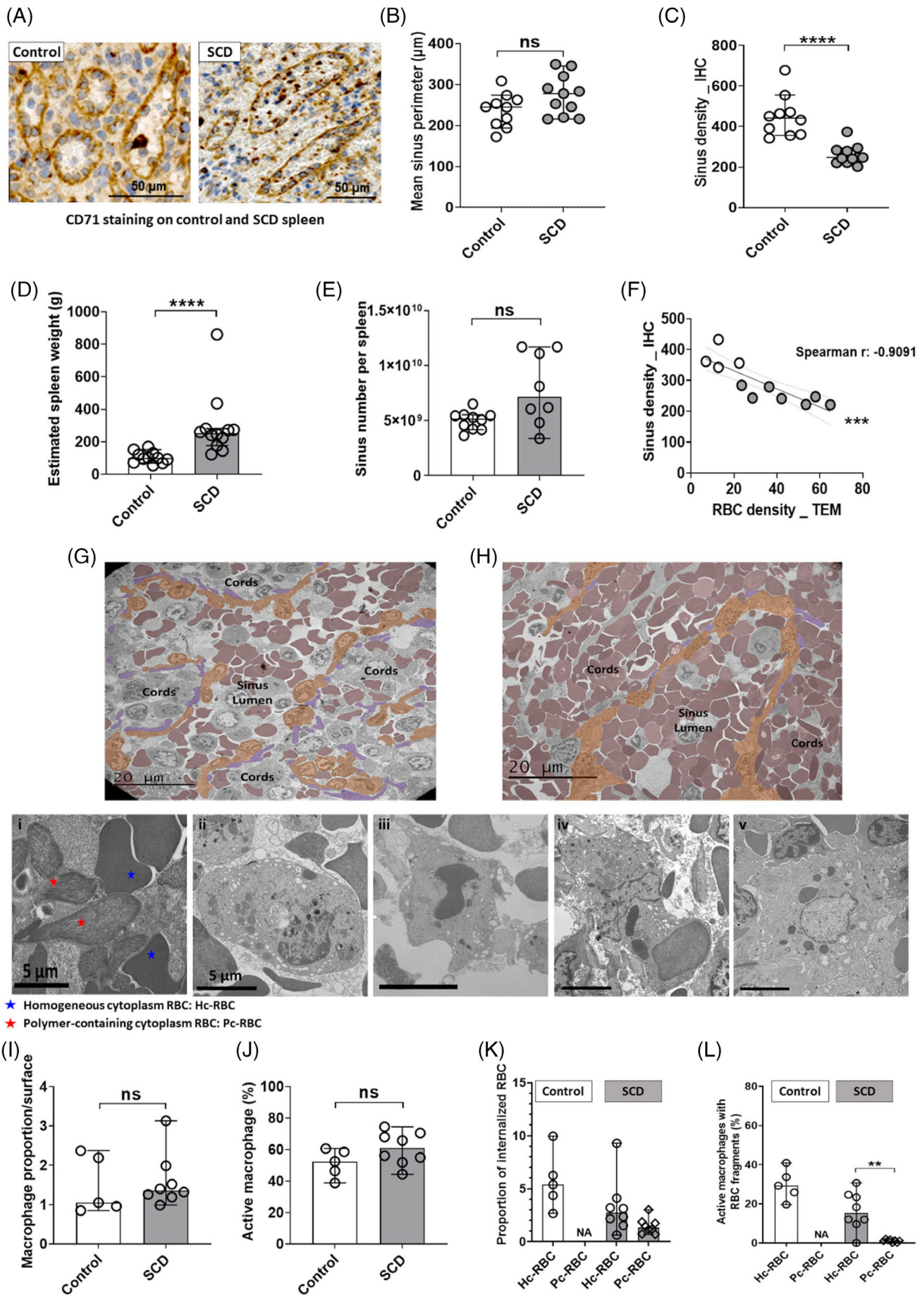


FIGURE 2 Legend on next page.

RBC (Figure 1F) using DIC microscopy showed that SCD splenic blood (patient with ASSC/HyS) was enriched in elongated RBC (median proportion 25.97% vs. 0% for controls, $p < 0.0001$) and spherocytes (3.29% vs. 0.44% for controls, $p = 0.0016$), (Figure 1G).

3.3 | Compared to splenic RBC from controls, splenic RBC from SCD children are more adherent to human microvascular endothelial cells

We performed dynamic adhesion experiments on monolayers of human microvascular endothelial cell line (HMEC-1) activated by TNF- α using peripheral and splenic RBC from controls and SCD patient. Following perfusion and washing, a higher median proportion of adherent RBC was observed in splenic compared to circulating samples in both control and SCD subjects. The median [IQR] number of adherent RBC per area of biochip under flow was 8.88 [7.08–10.68] versus 21.82 [15.67–27.97] respectively for peripheral and splenic RBC from control subjects; and 9 [5.92–36.79] versus 206 [18.42–280.5] for peripheral and splenic RBC from SCD children, respectively (Figure 1H).

3.4 | Spleens from SCD children are not depleted in sinuses, despite a congestion-related moderate reduction in sinus density

Analysis of spleens from 10 controls and 10 SCD patients using IHC with anti-CD71 antibody (Figure 2A, Figure S2) showed that both groups had similar median [IQR] sinus perimeters (244.9 μm [201.6–265.5] vs. 265.4 μm [218.7–297.8], $p = 0.1655$, Figure 2B). The number of sinuses per area was 44% lower in SCD than in control spleens (Figure 2C). Of note, the estimated spleen weights were higher in SCD children than controls (252 g [188.8–278.5] vs. 100 g [72–130], Figure 2D). As a result, the total number of sinuses per spleen (median [IQR]) was similar in SCD patients than in controls (7.15E+09 [5.12E+09–1.16E+10] vs. 5.18E+09 [4.23E+09–5.48E+09], $p = 0.0528$, Figure 2E), for 10 control and nine SCD subjects, with an insignificant trend toward higher numbers of sinuses in SCD spleens than in control spleens. Sinus population density (assessed by IHC) showed a strong

inverse correlation with the number of RBC per surface, counted on TEM pictures (Figure 2F, Spearman r : -0.9091 , white and gray dots respectively for controls and SCD subjects), as a surrogate for splenic congestion. Congestion was observed in a majority of SCD spleens fragment (Figure 2G,H).

3.5 | Splenic macrophages from SCD children internalize and digest Hb_{AA}-RBC at a higher rate than Hb_{SS}-RBC

We determined the population density of macrophages (Supplemental methods, Figure S3) in five control and eight SCD spleens by TEM acquisitions. SCD and control spleens showed no difference in population density of splenic macrophages (1.36 [1.21–1.87] vs. 1.05 [0.91–2.28], $p = 0.5237$, Figure 2I) and in the proportion of active macrophages (i.e., containing one or more RBC or RBC fragments, respectively 60.9% [52.59%–69.84%] vs. 52.41% [42.61%–59.58%], $p = 0.2222$, Figure 2J). The estimated median proportion of RBC engulfed by splenic macrophage was 5.39% [3.52%–8.1%] for RBC with homogeneous cytoplasm (Hc-RBC, presumably Hb_{AA}-RBC) in control spleens, whereas it was 2.7% [1.69%–3.88%] for Hc-RBC and 1.31% [0.84%–1.84%] for polymer-containing RBC (Pc-RBC, presumably Hb_{SS}-RBC) in SCD spleens (Figure 2K). In SCD spleens, the median percentage of macrophages processing internalized RBC (defined by the presence of RBC items smaller than 1 μm in the macrophage cytoplasm) was lower for Pc-RBC (Hb_{SS}-RBC) than for Hc-RBC (Hb_{AA}-RBC) (1.29% [0.25%–1.78%] vs. 15.2% [10.23%–24.34%], $p = 0.007$, Figure 2L).

3.6 | Both autochthonous Hb_{SS}-RBC and transfused Hb_{AA}-RBC contribute to splenic congestion, predominantly in the cords

On TEM pictures, the RBC population density was 3.8 times higher in SCD compared to control spleens (Figure 3A). The population density of Hb_{AA}-RBC (homogeneous cytoplasm, Figure 2 insert i) was 1.8 times higher in SCD spleens than in control spleens (Figure 3B), while in SCD spleens, population densities of Hb_{AA}-RBC and Hb_{SS}-RBC (polymer-containing, Figure 2 insert i) were similar (Figure 3B). These

FIGURE 2 Spleens from sickle cell disease (SCD) children with hypersplenism and acute sequestration crises showed no absolute deficit in sinus density by their macrophages internalizing and digesting Hb_{AA}-RBC at a higher rate than Hb_{SS}-RBC. Immunohistochemistry (IHC) analysis using CD71 antibody³⁰ of spleen sections from control and SCD subject (A). IHC analysis enabled to quantify the perimeter (B), and number (C) of sinuses. (D) Spleen weights were estimated according to the weight of removed part of the spleen and portion that is systematically removed by the surgeon and enabled to estimate an absolute sinus number per spleen (E). (F) Sinus density (assessed by histology) was plotted against RBC density observed in transmission electron microscopy (TEM) (G). TEM sections showing normal (G) and congested SCD (H) spleens. Sinus lumens were delineated from cords by endothelial cells (colored in brown) and basal fibers (colored in purple), RBC are represented (colored in red). Inserts show RBC with a homogeneous cytoplasm or Hc-RBC (presumably Hb_{AA}-RBC) (I), polymer-containing RBC or Pc-RBC (presumably Hb_{SS}-RBC) (i), an empty macrophage (ii), a macrophage containing a RBC with a homogeneous cytoplasm (iii), a macrophage containing a polymer-containing RBC (iv), a macrophage containing one or multiple RBC elements smaller than 1 μm (v). Proportion of macrophages per surface (I), proportion of active macrophages (J), proportion of RBC internalized (K), and macrophages that digest internalized RBC on total active macrophages (L). Scale bars = 5 μm . Mann-Whitney test (ns: $p > 0.1234$; *: $p < 0.0332$; **: $p < 0.0021$; ***: $p < 0.0002$). [Color figure can be viewed at wileyonlinelibrary.com]

observations reflected the congestion, which was qualitatively evident on histological sections in control spleens compared to SCD spleens (Figure 3C,D, respectively). Similar spleen congestion was observed in

normal human spleens perfused ex vivo^{34,35,41-43} with a mixture of Hb_{AA}-RBC and Hb_{SS}-RBC for 60 to 80 min from adults SCD patients in transfusion program⁹ (Figure 3E). We then analyzed the distribution

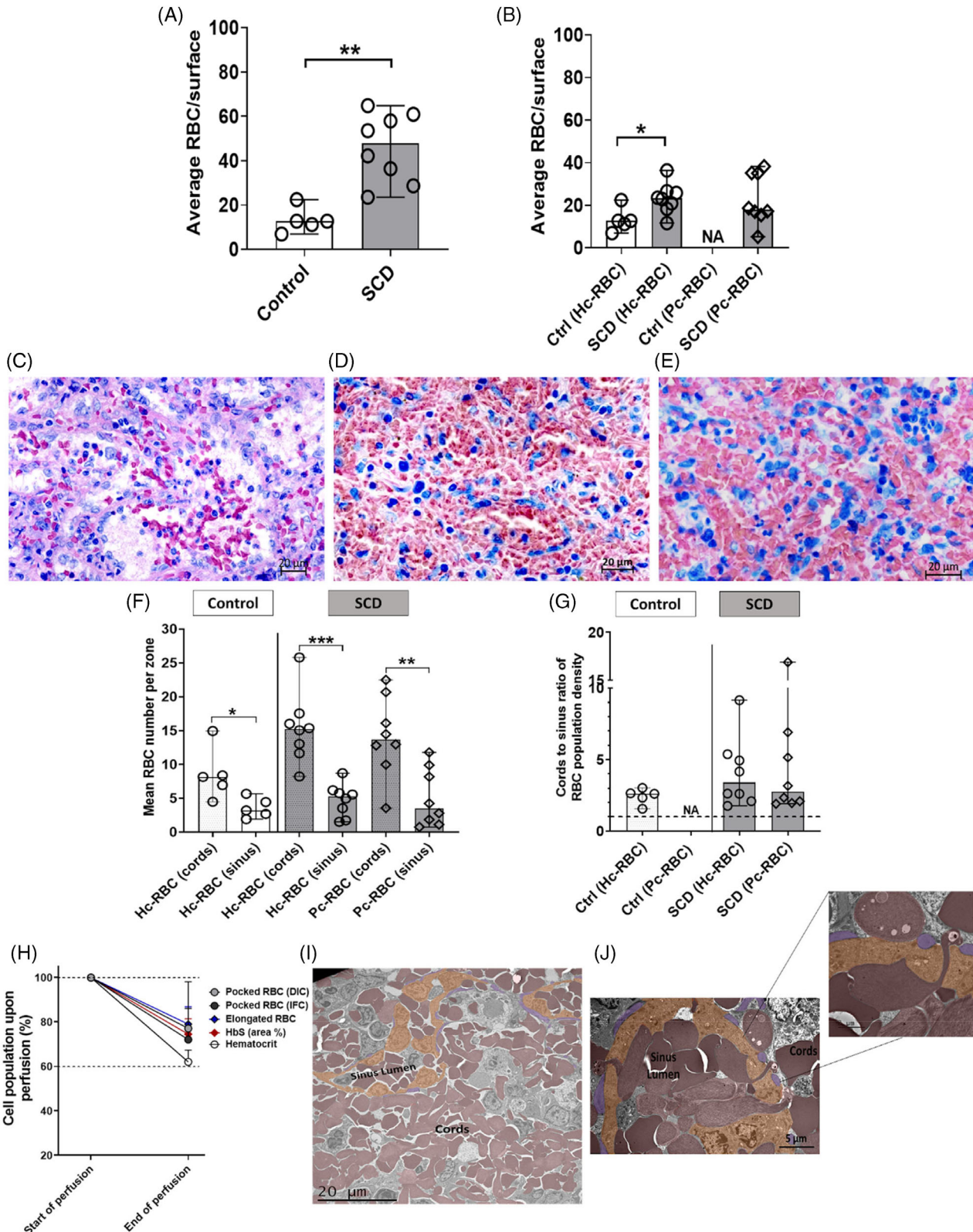


FIGURE 3 Legend on next page.

of RBC in the different compartments of the red pulp, namely the cords and sinus lumens, located respectively upstream and downstream from filtering slits. Both Hc-RBC/Hb_{AA}-RBC and Pc-RBC/Hb_{SS}-RBC were concentrated in the cords. The cord-to-sinus accumulation ratio was 2.61 [1.95–2.83] for Hb_{AA}-RBC in control spleens, while in SCD spleens, it was 3.39 [2.22–5.27] and 2.75 [1.99–6.47] for Hb_{AA}-RBC and Hb_{SS}-RBC, respectively (Figure 3F,G).

3.7 | Human spleens perfused ex vivo with mixture of Hb_{AA} and Hb_{SS}-RBC were congested and retained dysmorphic and pocked-RBC at similar rates

Human spleens from subjects without preexisting RBC or splenic disease, presumably with normal function, were perfused ex vivo. They retained 20–40% of item-containing or altered RBC subpopulations (i.e., pocked-RBC and elongated RBC, Figure 3H). By TEM, as observed in SCD spleens (Figure 1H), normal spleens perfused ex vivo showed intense RBC accumulation (Figure 3I) with a cord-to-sinus accumulation ratio of 2.72 [1.58–3.3] and 1.5 [0.8–2.61] for Hb_{AA}-RBC and Hb_{SS}-RBC, respectively (not shown). These normal spleens also retained pocked-RBC and elongated RBC at similar rates (88.54 [77.07–100] and 86.06 [72.11–100] for pocked-RBC by DIC and IFC, respectively versus 89.52 [79.04–100]). By TEM, the squeezed morphology of some pocked-RBC engaged across of the sinus wall, with a vacuole lying behind on the cordal side, was highly suggestive of ongoing pitting (Figure 3J and insert). Taken together, these observations indicate that these spleens with fully preserved filtering function (as previously demonstrated)⁴¹ retained stiff RBC just like SCD spleens.

4 | DISCUSSION

Through detailed phenotypic characterization of unique pairs of peripheral and splenic blood samples, we show here that, like normal spleens perfused ex vivo, the spleen of SCD children with ASSC/HyS clears the peripheral blood from stiff, adherent, and potentially pathogenic RBC. While congestion with RBC had been observed previously,^{2,9,25,27} the persistent ability of these spleens to very efficiently discriminate normal RBC from altered ones was unexpected. Spleen function in subjects

with SCD deteriorates at an early age,^{4,7,8} as confirmed by abnormally high pocked-RBC counts in 14 of 16 SCD children in the reported cohort. Nevertheless, we provide direct evidence that altered RBC such as elongated RBC and spherocytes accumulate in the spleen of SCD children with a spleen-to-periphery ratio greater than 10, a very strong indication of a persistent spleen filtering function. These results are in line with previous data reporting the accumulation of elongated RBC in splenic blood from young SCD patients.⁴⁴ These results are also in line with recent in vitro microfluidic studies on Hb_{SS}-RBC and Hb_{AA}-RBC showing that a large fraction of microfluidic channel slits were blocked when perfused with Hb_{SS}-RBC.²⁷ Also consistent with the preserved ability of these spleens to sense and retain rigid and dysmorphic RBC was their structural preservation. There was indeed no absolute deficit in the two main components of the splenic filtering unit, the splenon, namely sinus walls and macrophages. Like in a previous study,²⁶ we observed a moderate deficit in sinus density in the spleen tissue but this deficit was not absolute but related to spleen swelling due to congestion. The total number of sinuses per spleen was indeed at least normal compared to controls, and their mean perimeter was preserved. Unlike a previous report stating that the phagocytic function of the spleen is lost before its mechanical filtering function in SCD children,⁴⁵ we observed normal red pulp macrophage densities, both in terms of total macrophage numbers as well as numbers of active macrophages.

RBCs retained by the SCD spleens in this study were more adherent in vitro to endothelial cells compared to peripheral RBC. Increased adhesiveness and stiffness of circulating RBC is thought to trigger or contribute to vaso-occlusive crises and/or acute chest syndromes, associated with organ injury and early mortality in SCD.^{46–48} Accordingly, clearing the peripheral blood from such potentially pathogenic RBC is likely protective. The enrichment in altered and potentially pathogenic RBC in SCD spleens in this study is the first direct indication that they still efficiently discriminate altered RBC from normal ones. Even more strikingly, elongated RBCs were similarly rare in the peripheral blood of SCD patients and controls, indicating that spleens in these SCD children were still playing their role as a protective filter at the time of splenectomy, with a vast majority of circulating RBC being normally shaped and deformable.

Microscopic examinations of spleens from sickle cell anemia (SCA) patients showed entangled masses of elongated, pointed, curved, and abnormally shaped RBC in the splenic cords.²⁵ Moreover, accumulation of elongated/sickled RBC in the splenic

FIGURE 3 Hb_{SS}-RBC and transfused Hb_{AA}-RBC contribute to the congestion in sickle cell disease children with acute sequestration crises/hypersplenism. Transmission electron microscopy (TEM) was performed on spleen fragments³⁵ allowing to assess the density of red blood cell (A). Enumeration of RBC with homogeneous cytoplasm Hc-RBC (presumably Hb_{AA}-RBC) and polymer-containing RBC Pc-RBC (presumably Hb_{SS}-RBC) on TEM images (B). Histological analysis on spleen fragment from control spleen (C), SCD child (D), and human normal spleen perfused ex vivo with mixture of Hb_{AA}-RBC and Hb_{SS}-RBC (E). Red blood cells (red dots) and white cells (blue dots) are visible in the red pulp zone from histological images, highlighting congestion with red cells (D–E). Higher accumulation of RBC (assessed on TEM images) in the cords compared to sinus lumens density is highlighted (D, E). Higher accumulation of RBC (assessed on TEM images) in the cords compared to sinus lumens density is highlighted (F, G). Upon ex vivo perfusion of human spleens, proportions of pocked-RBC, elongated RBC, HbS, and hematocrit were assessed (H). TEM images from perfused spleen fragments showing spleen congestion (I) and pocked-RBC crossing inter-endothelial slits (J). Of note, RBC circulation is from cords to sinus lumen. Sinus lumens were delineated from cords by endothelial cell (colored in brown) and basal fibers (colored in purple), RBC are represented (colored in red). Mann–Whitney test was used (ns: $p > 0.1234$; *: $p < 0.0332$; **: $p < 0.0021$; ***: $p < 0.0002$). [Color figure can be viewed at wileyonlinelibrary.com]

blood (as compared to peripheral blood) from young SCD patients splenectomized for hypersplenism had been described with peripheral-to-splenic ratios varying from 1:2 to 1:14.⁴⁴ Whether these sickled RBC had been cleared from the peripheral blood or had undergone sickling in situ was not definitely determined by these observations.^{25,44} Deconvoluting the relative contributions of RBC stiffness and adhesion and RBC sickling in the pathogenesis of ASSC/HyS is not easy. Previous studies stipulated that in vivo sickling of Hb_{SS}-RBC and subsequent elongated RBC formation results from intermittent or continuous stagnation of these cells in various organs such as spleen but they did not elucidate the triggering factor of this stasis.^{44,49} Our data point to mechanical retention, a permanent physiological process, as a major player. We observed indeed that stiff Hb_{AA}-RBC accumulate in the cords as much as Hb_{SS}-RBC in SCD spleens, and that the cords-to-sinus lumens accumulation is almost identical in control spleens perfused ex vivo and SCD spleens retrieved following splenectomy. Because Hb_{AA}-RBC cannot sickle, their accumulation in SCD spleens stems from retention. In addition, microspiltration and morphological analysis of RBC, both performed at normoxia revealed the accumulation of stiff RBC in the spleens. When analyzed by TEM at normoxia, Hb_{SS}-RBC display no polymers, indicating that their retention in microsphere layers is related to their baseline stiffness and not to sickling. Taken together, these observations strongly suggest that mechanical retention is the predominant, triggering contributor to spleen congestion in ASSC/HyS, experimentally supporting previous speculations.^{44,49} In line with this interpretation of our findings, reduced deformability of circulating RBC correlated with loss of splenic function in a recent study in children living with sickle cell anemia.⁵⁰

A recent in vitro study using labeled THP-1 macrophages and RBC (Hb_{AA}- and Hb_{SS}-RBC) under normoxia and hypoxia showed that Hb_{SS}-RBC adhere to macrophages. Under hypoxia, sickled Hb_{SS}-RBC were digested slowly.²⁷ The present study displaying a very low proportion of macrophage containing Hb_{SS}-RBC fragment(s) (<1.5% of splenic red pulp macrophages), compared to Hb_{AA}-RBC (mean: 16% of splenic red pulp macrophages) is consistent with these previous findings.²⁷ We therefore suggest that hypoxia exacerbates congestion not only by in situ sickling of Hb_{SS}-RBC but also by slower intramacrophagic processing of sickled Hb_{SS}-RBC. A deficit in post-filtering of RBC is indeed a potential contributing mechanism of ASSC/HyS.²⁷

This study has at least four limitations. First, our splenectomy cohorts are unique but are not age-matched because splenectomy in children without underlying RBC disease is very infrequent. Reassuringly though, we found no published indication suggesting that RBC deformability or splenic filtration are different in adults and children, with the notable exception of premature infants. Second, we shall confirm that polymer-containing RBC and those that have a homogeneous cytoplasm correspond indeed to Hb_{SS}-RBC and Hb_{AA}-RBC, respectively. We did not observe hemoglobin polymers in either peripheral or splenic RBC in suspension from SCD children likely because these RBCs were processed at normoxia, causing mutated hemoglobin (hemoglobin S) to rapidly depolymerize before fixation. By contrast, the size of spleen fragments (about 5 mm thick) likely maintains a certain level of hypoxia until fixation, enabling the observation of Hb polymers in Hb_{SS}-

RBC.^{51,52} In addition, in human spleens perfused ex vivo, the proportion of polymer-containing RBC was broadly similar to the proportion of Hb_{SS}-RBC introduced in the perfusion RBC mixture, with adjustment for retention during the perfusion (not shown). Third, spleen size in SCD children was determined based on the proportion of spleen removed indicated by the surgeon, a somewhat subjective estimate, while only the removed part could be objectively weighed. Finally, for the demanding human spleen perfusion experiments, the quantification of elongated RBC, hematocrit, and HbS were not systematic, limiting the number of experimental data. Additional adhesion experiments are also needed to consolidate the observed trends. While technical optimizations and complementary experiments with new samples are underway, we believe that these limitations do not alter the main conclusions drawn from our results regarding the persistent ability of SCD spleen to efficiently filter out altered RBC.

The three major splenic filtering functions, namely mechanical retention of stiff RBC, their phagocytosis by macrophages, and pitting of vacuole-containing RBC, deteriorate at a different pace in SCD children. We show here that SCD spleens still efficiently filter RBC, while their ability to pit (i.e., remove particulate items from RBC), a major marker of hyposplenism, is already altered. The co-occurrence of hyposplenism and hypersplenism in children with ASSC/HyS sheds new light on the meaning of these words. In normal human spleens perfused ex vivo^{34,35,41–43} with mixtures of Hb_{AA}-RBC and Hb_{SS}-RBC, stiff and pocked-RBC were cleared at a similar pace, as expected from organs retrieved from subjects with a fully preserved spleen function. Interestingly, both ex vivo and SCD spleens leave no stiff RBC in circulation, an indication of their similarly preserved ability to retain them, which matches their almost identical levels of congestion. By contrast, SCD spleens filter (hypersplenism) but do not efficiently pit (hyposplenism). We found their sinus walls intact enough to retain 7 μ m-RBC, leading to congestion, but we suspect that they carry alterations that prevent them from sensing and expelling small (approximately 1 μ m) particulate items from RBC, a more subtle cellular function. Of note, the specific process by which the spleen operates pitting is still unknown, although observations in malaria,³⁴ here in SCD (Figure 3J), and by in silico modeling^{53,54} suggest that it takes place when RBCs cross narrow inter-endothelial slits.

Whatever the fine cellular alterations underlying the asynchronous deterioration of splenic function, splenectomy in SCD children with ASSC/HyS removes an organ that still efficiently filters out abnormal, potentially pathogenic RBC, despite simultaneous presence of markers of hyposplenism. Partial splenic embolization has recently been reported as a safe procedure, improving hematological parameters and reducing the need of blood transfusion in patients with SCD and HyS.⁵⁵ Not least, medical approaches (drug- or surgically mediated) controlling the stringency of splenic retention would alleviate the major pathogenic process in ASSC/HyS “spleen congestion” while preserving an organ potentially protective against vaso-occlusion.

AUTHOR CONTRIBUTIONS

A.S. designed the project, performed experiments, analyzed the results, and wrote the paper. A.C. and C.D. performed experiments (pocked-

RBC and sinus counting) and analyzed the results. M.M. performed adhesion experiments and revised the manuscript. L.D., A.F.M., and M.D. performed experiments and revised the manuscript. S.M. enrolled patients, collected data, and revised the manuscript. B.M. and A.E. carried out electronic microscopy experiments, analyzed the data, and revised the manuscript. C.R. carried out hemoglobin S quantification and revised the manuscript. P.A.N performed ex vivo spleen experiments and revised the manuscript. M.T., D.H., J.C., and D.W. carried out histology experiments, analyzed the data, and revised the manuscript. S.D., B.A., J.D., A.S., F.P., and M.L. included, performed the splenopancreatectomy of control subjects, and revised the manuscript. C.C. performed all the partial splenectomy of SCD children and revised the manuscript. S.A. designed the project, enrolled SCD children, and revised the manuscript. P.B. designed the project, analyzed results, and wrote the paper.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

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