



Complement Activation in Sickle Cell Disease: Dependence on Cell Density, Hemolysis and Modulation by Hydroxyurea Therapy

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Complement activation in sickle cell disease: dependence on cell density, hemolysis and modulation by hydroxyurea therapy

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Complement activation in sickle cell disease: dependence on cell density, hemolysis and modulation by hydroxyurea therapy

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Abstract

The complement system is an innate immune defense cascade that can cause tissue damage when inappropriately activated. Evidence for complement overactivation has been reported in small cohorts of patients with sickle cell disease (SCD). However, the mechanism governing complement activation in SCD has not been elucidated. Here, we observe that the plasma concentration of sC5b-9, a reliable marker for terminal complement activation, is increased at steady state in 61% of untreated SCD patients. We show that greater complement activation *in vitro* is promoted by SCD erythrocytes compared to normal ones, although no significant differences were observed in the regulatory proteins CD35, CD55, and CD59 in whole blood. Complement activation is positively correlated with the percentage of dense sickle cells (DRBCs). The expression levels of CD35, CD55, and CD59 are reduced in DRBCs, suggesting inefficient regulation when cell density increases. Moreover, the surface expression of the complement regulator CD46 on granulocytes was inversely correlated with the plasma sC5b-9. We also show increased complement deposition in cultured human endothelial cells incubated with SCD serum, which is diminished by the addition of the heme scavenger hemopexin. Treatment of SCD patients with hydroxyurea produces substantial reductions in complement activation, measured by sC5b-9 concentration and upregulation of CD46, as well as decreased complement activation on RBCs *in vitro*. In conclusion, complement overactivation is a common pathogenic event in SCD that is associated with formation of DRBCs and hemolysis and affects red cells, leukocytes and endothelial cells. This complement overactivation is partly alleviated by hydroxyurea therapy.

Introduction

Sickle cell disease (SCD) is a widespread inherited disorder with a mutation in the hemoglobin (Hb) gene (HbS), affecting over 6 million people worldwide¹. The altered characteristics of the sickle red blood cells (RBCs) leading to hemolysis, adhesion and abnormal hemorheological properties are responsible for vaso-occlusion and organ damage, in association with endothelial, neutrophil and platelet inflammatory activation. One of the hallmarks of the inflammatory process is the activation of the innate immune complement cascade². This cascade is a first line of defense against pathogens, but it induces host tissue damage when inappropriately overactivated³. Clinical observations have shown markers of complement activation in the circulation of SCD patients and increased levels of surface-bound C3 fragments on RBCs⁴⁻¹⁰. Mouse models have demonstrated that complement plays a key role in vaso-occlusion and tissue injury since complement blockade at the level of C5 or C5aR1 had a protective effect¹¹. The mechanism of this complement activation is not yet fully elucidated. *In vitro* studies and mouse models have provided evidence that the activation of the cascade in SCD could proceed by different mechanisms: on ischemic tissue, via cell-free heme/heme-carrying RBC microvesicles or directly on the surface of the SCD RBCs^{9,11-13}. The clinical relevance of the progression of the complement cascade to its terminal steps has not been investigated in detail in a large cohort of SCD patients. Homozygous severe SCD patients are treated by hydroxyurea (HU), the main effects of which are to stimulate fetal Hb (HbF) production, to decrease the dense RBC percentage (%DRBC) and to decrease cell adhesion, resulting in decreases in RBC sickling, hemolysis and vaso-occlusion^{14,15}. The impact of HU on the control of the complement-mediated inflammation is unknown.

In this study, we demonstrate that complement activation is common in SCD. This activation could be explained in part by the complement-activating surface properties of SCD RBCs and the presence of cell-free heme in the serum, rendering endothelial cells susceptible to complement attack. Complement-mediated inflammation was partially controlled in patients by HU treatment.

Patients and Methods

Cohort description

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3 Blood samples were obtained from 106 homozygous adult SCD patients (SS) at steady state,
4 treated with hydroxyurea (SS-HU) at a stable dose for more than 6 months and not treated with
5 HU (SS-NT), followed in our sickle cell referral center in Henri-Mondor Hospital. All patients
6 gave their written consent. This study was approved by the local Institutional Review Board
7 (CPP–Creteil). The exclusion criteria were secondary causes of complement activation, chronic
8 blood transfusion program or pregnancy. Steady state was defined as a visit to the clinic for
9 consultation ≥ 1 month after a VOC episode or an acute event and more than three months after
10 the most recent blood transfusion. Serum was collected from 19 additional consecutive patients
11 (11 SS-NT and 8 SS-HU).
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23 **Complement assessment**

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25 Complement assessment was performed on EDTA plasma samples at the immunology
26 laboratory of the Georges Pompidou European Hospital using standard diagnostic procedures.
27 Plasma C3, C4 (by nephelometry) and Factor H and Factor I protein concentrations (ELISA),
28 sC5b-9 (MicroVue sC5b-9 Plus EIA, Quidel, San Diego, CA, USA) and presence of anti-Factor
29 H autoantibodies (ELISA) were screened in patients' plasma. sC5b-9 was considered high >
30 450 ng/ml. Membrane expression levels of CD55 and CD59 on erythrocytes and CD46 on
31 granulocytes were measured by flow cytometry.
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38 Three samples were weakly positive for anti-Factor H antibodies, which may influence the
39 sC5b-9 levels. These 3, and 18 additional samples lacking sC5b-9 assessment, were excluded.
40 The remaining 85 samples were analyzed, with a median patient age of 33.7 years [18-58];
41 44/85 were HU patients, and 41/85 were NT patients, with similar ages (median ages of 34.4
42 and 33 years, respectively).
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48 The normal range of the studied parameters was considered that of the Caucasian controls (AA
49 c), used as a standard in the diagnostic laboratory, n=46 for sC5b-9 and n=130 for CD46, CD55
50 and CD59. Twelve ethnically matched healthy donors (AA a) were also recruited to evaluate
51 the normal ranges.
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59 ***In vitro* complement activation by RBCs**
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RBCs were purified from healthy donors (AA) and SCD patients and incubated with 33% normal human serum (30 min/37°C). AB- blood group normal human serum was used to avoid classical complement pathway activation due to the presence of anti-RBC antibodies. Released sC5b-9 was measured by ELISA (Quidel).

RBC density measurements

Density was assessed with the phthalate density-distribution technique, as previously described^{16,17}. Two phthalate-oil esters, n-butyl phthalate and dimethylphthalate, were mixed to obtain the following precise densities: 1.080, 1.084, 1.088, 1.092, 1.096, 1.100, 1.104, 1.108, 1.112, 1.116, 1.120, and 1.124 g/ml at +20°C. RBCs were washed 3 times at 4°C with isotonic saline (osmolality, 290-300 mOsm). A 50% RBC suspension was prepared in isotonic saline; then, 0.5–1 cm of each phthalate oil mixture was placed in separate glass capillary hematocrit tubes. By capillarity, each tube was filled with the patient's washed RBC suspension (~2 cm), and each tube was sealed at one end and spun in a temperature-controlled centrifuge (20°C) at 10,000×g for 10 min. The height of the RBC column below the oil in each tube was measured using either graph paper or a ruler under a magnifying glass. The percentage of dense RBCs (DRBCs), defined as cells below the phthalate oil layer, was calculated as follows: $100\% \times (\text{cells below}) / (\text{cells below} + \text{cells above})$. The results were plotted against density to generate an RBC-density-distribution curve for each patient. For each of these curves, the percentage of DRBCs was calculated as the percentage of RBCs with $d > 1.11$ mg/ml and the nondense RBCs (non-RBC) as the percentage of RBC with $d \leq 1.11$ mg/ml.

Expression of complement regulators on nondense and dense HbS erythrocytes

RBCs from each patient were separated into nondense RBCs and DRBCs, and the expression levels of CD35/CR1, CD55/DAF, CD59/MAC-IP and CD47/IAP as well as the phosphatidylserine expression were compared by flow cytometry. The arbitrary mean fluorescence intensity units for the diagnostic and *in vitro* measurements are different due to the use of different equipment and calibration procedures in two laboratories.

***In vitro* complement activation on endothelial cells (ECs)**

By flow cytometry: Human umbilical vein endothelial cells (HUVECs) from 3 different donors were cultured and exposed at resting state to serum from SCD patients (n=19, 11 SS-CT and 8 SS-HU), treated or not with HU or healthy controls (n=22) as described^{18,19}. Briefly, ECs were cultured at approximately 80% confluence, washed with PBS containing Ca²⁺ and Mg²⁺ (Gibco) and exposed to patient or healthy donor sera for 30 min at 37°C, 5% CO₂. Subsequently, the cells were washed with PBS (without ions) and detached nonenzymatically after exposure for 30 min to PBS-1%BSA-10 mM EDTA-5 mg/ml lidocaine. Deposition of C3 activation fragments (C3(H₂O)/C3b/iC3b) and C5b-9 on the EC surface was measured by flow cytometry using anti-C3c mouse monoclonal antibody (Quidel, recognizing C3b, iC3b and C3(H₂O)) and mouse monoclonal antibody against C5b-9 neoepitope, clone B7, kind gift by Prof. Paul Morgan, Cardiff, UK. Goat anti-mouse IgG Alexa Fluor 700 (Thermo Fisher) was used as a secondary antibody. Normal human serum from one donor was repeated in each experiment and served as an internal control to assure the comparability of the results.

For 8 patient sera, the contribution of heme was evaluated by addition of the heme scavenger hemopexin at 10 µM final concentration. The relative fluorescence intensity (RFI) was calculated after division of the mean fluorescence intensity of each sample to the one of the control sample, where only secondary antibody was used for the staining. Data were acquired using a BD LSRFortessa flow cytometer and analyzed in FlowJo (LLC).

By immunofluorescence: HUVECs were cultured on glass slides, washed as above, and exposed at resting state for 30 min to serum from SCD patients SS-NT (n=2) or SS-HU (n=2) and compared to healthy controls AA (n=3). The sera were diluted 1/3 with M199 medium, added to each well before the addition of the serum. The same sera were added on cells, for which hemopexin (10 µM final concentration) was added to the M199 (Gibco) medium. Deposition of C3 activation fragments C3(H₂O)/C3b/iC3b was revealed with polyclonal anti-C3c antibody coupled to FITC (Dako, Agilent group, Santa Clara, CA, USA). Anti-C5b-9 (clone B7, as above) staining was followed by a secondary anti-mouse-IgG-Alexa Fluor 488, (Thermo Fisher). After fixation and staining of the nuclei with DAPI, the immunofluorescence was assessed on whole slides by an Axioscan Slide Scanner (Zeiss) and the image analysis software Zen (Zeiss).

Statistical analyses

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3 Statistical analyses were computed with Stata v12.1 (StataCorp LP, College Station, TX, USA)
4 and Prism 5.0 (GraphPad Inc, La Jolla, CA, USA) software. Detailed descriptions of the
5 statistical tests are given in the figure legends.
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12 **Results**

13 **Complement activation proceeds to the terminal pathway in SCD patients**

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18 sC5b-9 is a biomarker for full-blown terminal complement pathway activation and was elevated
19 in the plasma of 42% (36/85) of the patients (Figure 1A, $p < 0.001$). The sC5b-9 concentration
20 was significantly higher in the SS-NT group compared to healthy controls ($p < 0.005$) and
21 compared to the patients in the SS-HU group ($p < 0.05$; Kruskal Wallis test, Dunn's multiple
22 comparisons correction) (Figure 1B). Indeed, 61% of the SS-NT patients (25/41) had increased
23 sC5b-9, contrary to only 25% (11/44) of HU patients ($p = 0.001$, Fisher's exact test). These
24 results were obtained with the diagnostic cut-off, obtained with AA c controls $n = 46$ for sC5b-
25 9 and $n = 130$ for CD46, CD55 and CD59. For sC5b-9, they appeared to be lower compared to
26 the Caucasian donors and in the same range for CD46 and CD59 (Figure 1A). Despite this
27 difference, the cut off of the Caucasian normal was retained due to the larger number of tested
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44 **RBCs from SS-NT patients activate complement**

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47 To gain a mechanistic insight on the factors that could promote the elevated sC5b-9 in the
48 plasma of SCD patients, we tested whether the RBCs could represent a complement-activating
49 surface. Indeed, after incubation of SCD RBCs with normal human serum, sC5b-9 was
50 generated at higher concentrations with SS-NT RBCs compared to AA RBCs ($p = 0.006$) and
51 SS-HU RBCs ($p = 0.038$, Figure 2A).
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57 Such increased complement activation could be related to an alteration of the expression of
58 complement regulators on HbS RBCs. Measurement of CD35 and CD55 on the surface of total
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HbS RBCs from SS-NT and SS-HU patients revealed that they were within normal ranges (Supplementary Figure 1A,B). Interestingly, CD59 of the SCD patients was in the upper normal range for the Caucasian French healthy population (AA c) and showed significantly higher expression in the SS-NT group compared to the 139 tested control subjects (Supplementary Figure 1C). To determine if this could be due to an ethnic difference in the CD59 expression level, we tested 12 healthy subjects of sub-Saharan/Antilles origin, corresponding to the ethnicity of the SCD patients (AA a). The surface expression of CD59 on RBCs was not different between the two healthy donor groups and confirmed the higher CD59 level on HbS RBCs (Supplementary Figure 1C).

Therefore, the expression level of the complement regulators on the total HbS RBC population cannot explain either their complement-activating capacity or the elevated sC5b-9 expression in the plasma of the SCD patients.

Elevated %DRBCs in patients with high plasma concentrations of sC5b-9

To determine whether the characteristics of HbS RBCs contribute to complement activation in the circulation of SCD patients, we measured the %DRBCs. As previously described, the %DRBCs was higher in the SS-NT group than in the SS-HU group ($p=0.0147$, Figure 2B). In the SS-NT group, conversely to SS-HU, %DRBCs was higher in patients with high levels of sC5b-9 ($p=0.0407$, Figure 2C). Concordantly, the sC5b-9 levels correlated with the %DRBCs in NT ($p=0.02$, $r^2=0.1424$, not shown), but not in HU patients.

Dense RBCs express less complement regulators compared to nondense RBCs

To evaluate the capacity of dense vs nondense RBCs to resist to complement activation, we measured the expression of complement regulators on their surface. CD35 and CD55 were expressed at significantly lower levels on DRBCs compared to nondense RBCs, irrespective of the HU treatment (CD35: SS-NT $p<0.0001$; SS-HU $p=0.0469$, Figure 2D; D55: SS-NT $p=0.0011$; SS-HU $p=0.0313$, Figure E). CD59 expression was also lower on DRBCs in SS-NT patients ($p=0.0131$), but no difference was seen between dense and nondense RBCs from SS-HU patients. The lower expression of the regulators, especially on the SS-NT HbS RBCs, was

not due to a general reduction in all proteins since the expression of CD47 remained unaltered between nondense and DRBCs. In addition to the reduced expression of regulators, DRBCs also had higher expression levels of phosphatidylserine, also known to promote complement activation⁹.

Overexpression of CD46 correlates with lower plasma sC5b-9

Leukocytes, and especially neutrophils, express high amounts of CD46 (membrane cofactor protein, MCP) on their surface. Interestingly, the CD46 expression levels in the SS-NT and SS-HU groups were higher compared to healthy controls of both Caucasian ($p < 0.01$ and 0.0001 respectively) and ethnically matched origin ($p < 0.05$ and $p < 0.0001$, respectively, Figure 3A). Moreover, SS-HU patients expressed significantly more CD46 on their neutrophils compared to SS-NT patients ($p < 0.05$). In the context of SS-NT, the level of CD46 inversely correlated with the plasma sC5b-9 concentration; the highest CD46 expression was associated with normal sC5b-9 ($r^2 = 0.18$, $p = 0.0056$, Figure 3B). No correlation was observed for the SS-HU because most of the patients had high CD46 and normal sC5b-9 (Figure 3C).

Complement overactivation on the surface of ECs exposed to SCD patient sera

The elevated sC5b-9 in the plasma of the SCD patients could also originate from complement activation on vascular endothelium. Therefore, we investigated the complement activation on resting ECs in presence of sera from patients with SCD or healthy controls (Figure 4A). A significant increase by flow cytometry in the deposition of C3 activation fragments ($C3(H_2O)/C3b/iC3b$) was detected in the presence of sera from SCD patients compared to healthy donors ($p < 0.0001$) (Figure 4B), and 13/19 (68%) SCD samples exceeded the established threshold of physiological deposits (average $\pm 2SD$ of the relative fluorescence intensity of the staining from healthy donor sera). Positive deposits were found in 8/11 (73%) SS-NT and 5/8 SS-HU patients (62.5%) (Figure 4C). Apart from one patient, the RFI values obtained for the patients with SCD did not exceed the positive control of maximal alternative pathway activation achieved by blockade of the regulatory activity of Factor H. The elevated C3 activation fragment deposits were confirmed by immunofluorescence (Figure 4D) when resting ECs were exposed to sera from patients with SCD or healthy controls.

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The complement activation from SCD sera proceeded to the terminal pathway since we detected elevated C5b-9 deposits by flow cytometry ($p<0.01$ for both SS-NT and SS-HU, Figure 4E) and immunofluorescence (Figure 4F).

Complement overactivation on ECs is partially heme-dependent

SCD is a prototypical disease, associated with intravascular hemolysis. Since heme activates endothelium and renders it a complement-activating surface, we tested whether it can be the factor that promotes complement deposits in our EC assay. The C3 deposits induced by incubation of the ECs with SCD patient sera were significantly decreased upon addition of heme scavenger hemopexin ($p=0.0117$), while no effect was observed in the presence of normal serum (Figure 4G). This result was confirmed by immunofluorescence (Figure 4H). Moreover, the C5b-9 deposits were also attenuated by pretreatment with hemopexin (Figure 4I).

Discussion

We provide evidence for activation of the terminal pathway of the complement cascade in SCD patients at steady state, which is controlled by treatment with HU. We describe a multimodal mechanism of complement activation, mediated by SCD RBCs and hemolysis.

Complement overactivation contributes to the pathological process in an increasing number of diseases due to its pro-inflammatory and cell damaging capacities³. This is mediated predominantly by the activation of the terminal pathway of the cascade, generating C5a anaphylatoxin and membrane-bound C5b-9. Elevated plasma sC5b-9 is a hallmark of ongoing complement activation and is measured in clinical practice. The concentration of sC5b-9 in SCD found here was similar to diseases where the contribution of complement is clearly established, such as C3 glomerulopathies and anti-neutrophil cytoplasmic antibody-associated vasculitis.^{20,21,22} Over 60% of the untreated patients presented with elevated sC5b-9. This percentage may be even higher, depending on the selected normal range for the healthy controls. Indeed, the normal range of sC5b-9 of the ethnically matched healthy donors has to be determined with more individuals. If lower sC5b-9 expression in the African population is confirmed, the number of SCD patients with elevated sC5b-9 may be even higher than

estimated here. A bias, though, could come from the fact that the ethnically matched samples were taken from the blood bank.

The elevated concentration of sC5b-9 in the SS-NT patients could be related to multiple factors, including the SCD RBCs, the process of intravascular hemolysis as well as the activation of the cascade on other blood cells or on endothelium. Indeed, SS-NT RBCs activated complement in normal serum *in vitro*. This could be explained at least in part by the higher percentage of DRBCs in the SS-NT samples. The DRBCs expressed higher levels of phosphatidylserine compared to non-DRBCs, which could contribute to complement activation as shown before⁹. Moreover, the DRBCs presented with lower surface expression of the complement regulators CD35 and CD55 compared to non-DRBCs. The differences in the expression of the regulators in the NT group were not due to reduced cell volume since another membrane marker, CD47, showed no difference in its expression between non-DRBCs and DRBCs. CD59 was expressed at lower levels on DRBCs in the SS-NT group. This result suggests a key role of CD59 in the control of sC5b-9 generation. Nevertheless, the DRBCs are present in the range of only a few percent in the circulation of an SCD patient and may not account for the totality of the complement activation and generated sC5b-9.

Neutrophils are highly abundant in the circulation, especially in SCD²⁵. Contrary to RBCs, they express the complement regulator CD46. We discovered that CD46 was elevated on neutrophils of SCD patients compared to controls. CD46 correlated inversely with the plasma sC5b-9 in the SS-NT, suggesting a defense mechanism against complement activation. Therefore, CD46 on SCD neutrophils may regulate the complement activation in plasma, reducing sC5b-9.

Further, we demonstrated that SCD patient sera induced enhanced C3 fragment deposition and C5b-9 anchoring on the membranes of resting ECs. Hemolysis-derived products or anti-endothelial antibodies present in the serum could explain this activation. This complement overactivation is concordant with the complement-mediated cytotoxicity from SCD patients' sera (modified Ham's test)⁷, the enhanced C3 deposits on ECs exposed to SCD RBC microvesicles¹² and the endothelial C3 deposits in tissues of SCD mice and biopsies of patients¹⁰⁻¹². This overactivation is indeed at least in part heme-dependent since it was inhibited by the heme scavenger hemopexin. In similar experimental settings, complement overactivation on ECs was detected in the sera of patients with atypical hemolytic uremic syndrome^{18,19,26-29}, as well as from other diseases with intravascular hemolysis, such as HELLP syndrome (hemolysis, elevated liver enzymes, and a low platelet count) and preeclampsia³⁰. It is tempting

to speculate that in these diseases, heme could be the trigger of EC activation, rendering them susceptible to complement activation^{12,13,31,32}. The capacity to activate complement on ECs disappeared in HELLP and preeclampsia 6 months after recovery. Longitudinal studies are needed to show whether this complement activation would vary throughout the disease course of individual SCD patients.

Taken together, our results show a complex, multifactorial mechanism, which could explain the complement overactivation detected in SCD patients. We discovered that most of the SS-HU patients lack elevation of sC5b-9. This novel beneficial effect of HU on the level of complement activation could be explained by the decrease in DRBCs and by the improved complement regulatory capacity of SCD patients' RBCs and neutrophils. Although CD59 was expressed at lower levels on the DRBCs in SS-NT patients, its expression remained unaltered in SS-HU patients. Moreover, treatment with HU correlated with even higher surface expression of CD46 on neutrophils. These results, together with the data from the literature, show that the beneficial effect of HU should be related to the %DRBCs, RBC microvesicles, the reduction of the activation state of the neutrophils and endothelial cells and posttranslational modifications of Hb and, hence, heme release, rather than direct effect of HU as a complement inhibitor^{17,23}. Indeed, HU-treated patients showed reduced hemolysis and higher fetal Hb and heme-scavenger hemopexin compared to NT patient²⁴. Moreover, mass spectrometry analyses of the plasma of HU-treated patients showed lower complement C9 production compared to untreated patients. Since the methods cannot distinguish between native C9 and C9 in sC5b-9, it is difficult to conclude whether C9 production was decreased or just if there was less sC5b-9. Whichever the case, this study, in line with ours, shows the decreased capacity of action of the terminal pathway under HU. Longitudinal studies are needed to show whether HU treatment could control complement activation throughout the disease course of individual patients and how it will affect complement biomarkers over time.

Taken together, recent mouse models and data from patients suggest that complement plays a key role in the SCD disease process and is a potential therapeutic target^{10-12,33}. HU treatment resulted in prevention of complement activation on blood cells. Better understanding the link between complement activation and SCD pathophysiology could help in treating many complications of SCD.

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Authors' contributions

Designed the research: LTR, VFB and PB; performed the research: LTR, PC, GB, PVM, AG, IB, VP, SP, LK, AJ; analyzed the data: LTR, PB, PC, AG, EA; PVM, VFB; assured access to patients' samples and/or care for the patients: PB, FP, FG, SP; wrote the manuscript: LTR and PB.

All authors discussed the data. None of the authors has a relevant conflict of interest.

References

1. Sundd P, Gladwin MT, Novelli EM. Pathophysiology of Sickle Cell Disease. *Annu Rev Pathol*. 2018.
2. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol*. 2015;6:262.
3. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Front Immunol*. 2015;6:257.
4. Chaplin H, Jr. The implication of red cell-bound complement in delayed hemolytic transfusion reaction. *Transfusion*. 1984;24(3):185-187.
5. Chudwin DS, Korenblit AD, Kingzette M, Artrip S, Rao S. Increased activation of the alternative complement pathway in sickle cell disease. *Clin Immunol Immunopathol*. 1985;37(1):93-97.
6. Chudwin DS, Papierniak C, Lint TF, Korenblit AD. Activation of the alternative complement pathway by red blood cells from patients with sickle cell disease. *Clin Immunol Immunopathol*. 1994;71(2):199-202.
7. Gavrilaki E, Mainou M, Christodoulou I, et al. In vitro evidence of complement activation in patients with sickle cell disease. *Haematologica*. Vol. 102. Italy; 2017:e481-e482.
8. Mold C, Tamerius JD, Phillips G, Jr. Complement activation during painful crisis in sickle cell anemia. *Clin Immunol Immunopathol*. 1995;76(3 Pt 1):314-320.
9. Wang RH, Phillips G, Jr., Medof ME, Mold C. Activation of the alternative complement pathway by exposure of phosphatidylethanolamine and phosphatidylserine on erythrocytes from sickle cell disease patients. *J Clin Invest*. 1993;92(3):1326-1335.
10. Lombardi E, Matte A, Risitano AM, et al. Factor H interferes with the adhesion of sickle red cells to vascular endothelium: a novel disease modulating molecule. *Haematologica*. 2019.
11. Vercellotti GM, Dalmaso AP, Schaid TR, Jr., et al. Critical role of C5a in sickle cell disease. *Am J Hematol*. 2018.

12. Merle NS, Grunenwald A, Rajaratnam H, et al. Intravascular hemolysis activates complement via cell-free heme and heme-loaded microvesicles. *JCI Insight*. 2018;3(12).
13. Merle NS, Paule R, Leon J, et al. P-selectin drives complement attack on endothelium during intravascular hemolysis in TLR-4/heme-dependent manner. *Proc Natl Acad Sci U S A*. 2019.
14. Bartolucci P, Habibi A, Stehle T, et al. Six Months of Hydroxyurea Reduces Albuminuria in Patients with Sickle Cell Disease. *J Am Soc Nephrol*. 2016;27(6):1847-1853.
15. Rakotoson MG, Di Liberto G, Audureau E, et al. Biological parameters predictive of percent dense red blood cell decrease under hydroxyurea. *Orphanet J Rare Dis*. 2015;10:57.
16. Bridges KR, Barabino GD, Brugnara C, et al. A multiparameter analysis of sickle erythrocytes in patients undergoing hydroxyurea therapy. *Blood*. 1996;88(12):4701-4710.
17. Di Liberto G, Kiger L, Marden MC, et al. Dense red blood cell and oxygen desaturation in sickle-cell disease. *Am J Hematol*. 2016;91(10):1008-1013.
18. Frimat M, Tabarin F, Dimitrov JD, et al. Complement activation by heme as a secondary hit for atypical hemolytic uremic syndrome. *Blood*. 2013;122(2):282-292.
19. Marinozzi MC, Vergoz L, Rybkine T, et al. Complement factor B mutations in atypical hemolytic uremic syndrome-disease-relevant or benign? *J Am Soc Nephrol*. 2014;25(9):2053-2065.
20. Chauvet S, Roumenina LT, Aucouturier P, et al. Both Monoclonal and Polyclonal Immunoglobulin Contingents Mediate Complement Activation in Monoclonal Gammopathy Associated-C3 Glomerulopathy. *Front Immunol*. 2018;9:2260.
21. Fremeaux-Bacchi V, Sellier-Leclerc AL, Vieira-Martins P, et al. Complement Gene Variants and Shiga Toxin-Producing Escherichia coli-Associated Hemolytic Uremic Syndrome: Retrospective Genetic and Clinical Study. *Clin J Am Soc Nephrol*. 2019.
22. Gou SJ, Yuan J, Chen M, Yu F, Zhao MH. Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. *Kidney Int*. 2013;83(1):129-137.
23. Jana S, Strader MB, Meng F, et al. Hemoglobin oxidation-dependent reactions promote interactions with band 3 and oxidative changes in sickle cell-derived microparticles. *JCI Insight*. 2018;3(21).
24. Brewin J, Tewari S, Menzel S, et al. The effects of hydroxycarbamide on the plasma proteome of children with sickle cell anaemia. *Br J Haematol*. 2019;186(6):879-886.
25. Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood*. 2016;127(7):801-809.
26. Galbusera M, Noris M, Gastoldi S, et al. An Ex Vivo Test of Complement Activation on Endothelium for Individualized Eculizumab Therapy in Hemolytic Uremic Syndrome. *Am J Kidney Dis*. 2019;74(1):56-72.
27. Noris M, Galbusera M, Gastoldi S, et al. Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. *Blood*. 2014;124(11):1715-1726.
28. Roumenina LT, Frimat M, Miller EC, et al. A prevalent C3 mutation in aHUS patients causes a direct C3 convertase gain of function. *Blood*. 2012;119(18):4182-4191.
29. Frimat M, Boudhabhay I, Roumenina LT. Hemolysis Derived Products Toxicity and Endothelium: Model of the Second Hit. *Toxins (Basel)*. 2019;11(11).
30. Palomo M, Blasco M, Molina P, et al. Complement Activation and Thrombotic Microangiopathies. *Clin J Am Soc Nephrol*. 2019.
31. May O, Merle NS, Grunenwald A, et al. Heme Drives Susceptibility of Glomerular Endothelium to Complement Overactivation Due to Inefficient Upregulation of Heme Oxygenase-1. *Frontiers in Immunology*. 2018;9(3008).
32. Roumenina LT, Rayes J, Lacroix-Desmazes S, Dimitrov JD. Heme: Modulator of Plasma Systems in Hemolytic Diseases. *Trends Mol Med*. 2016;22(3):200-213.
33. Merle NS, Boudhabhay I, Leon J, Fremeaux-Bacchi V, Roumenina LT. Complement activation during intravascular hemolysis: Implication for sickle cell disease and hemolytic transfusion reactions. *Transfus Clin Biol*. 2019.

Figure legends

Figure 1. Plasma sC5b-9 is elevated in SCD patients, especially in absence of treatment:

A) Quantification of the sC5b-9 in plasma of SCD patients (n=85), compared to healthy controls of Caucasian origin (AA c, n=46) or African origin (AA a, n=9). B) Stratification of the SCD patients depending on the treatment: SS-HU (treated with hydroxyurea, HU) and SS-NT (not treated with HU). The dotted line represents the cut-off of positivity for the Caucasian controls, equal to the average of the control values +2SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; Kruskal-Wallis with Dunn's test for multiple comparisons.

Figure 2. Contribution of the HbS RBC to the complement activation in SCD. A) *In vitro*

complement activation by RBCs incubated with normal AB- human serum, measured by sC5b-9 release. RBCs derived from SS-NT and SS-HU patients were compared. Levels of sC5b9 generated *in vitro* were compared across conditions (AA, SS-HU, SS-NT) using mixed-effects linear regression models, entering the 'lot' as a random effect to account for the correlation between measurements from the same 'lots'. Global comparisons were performed as well as pairwise comparisons between the three subgroups, applying the Benjamini-Hochberg false discovery rate procedure to correct for test multiplicity. B-C) RBCs were separated into nondense RBCs (non-DRBCs, density \leq 1.11 mg/ml) and dense RBCs (DRBCs, density $>$ 1.11 mg/ml). B) Comparison of the %DRBCs in SS-NT vs SS-HU patients. C) %DRBCs within the samples of patients with normal (<440 ng/ml) or high (>440 ng/ml) plasma sC5b-9 in SS-NT or SS-HU groups. B,C) * $p<0.05$, Unpaired t test with Welch's correction, data pass the D'Agostino-Pearson normality test. D-H) Phenotypic characterization of the non-DRBCs and DRBCs from SS-NT and SS-HU treated patients. Expression of: D) CD35; E) CD55, F) CD59; G) CD47, H) phosphatidylserine (PS)+ RBC% in the total RBC sample (left) and in DRBCs or non-DRBCs. P values are calculated using Wilcoxon matched-pairs signed-rank test.

Figure 3. Increased surface level of CD46 on granulocytes in SCD patients, especially those treated with HU. A) CD46 in SCD SS-NT and SS-HU patients compared to Caucasian

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Figure 4. Complement deposition on resting endothelial cells exposed to sera from SCD patients and its dependence on heme: A) Examples of deposits of C3 activation fragments on

HUVECs, measured by flow cytometry. Light gray: isotype control; dark gray: staining of cells incubated with sera from 13 different healthy donors (negative control); black: normal human serum, spiked with Factor H blocking antibody Ox24 (positive control). In color: individual sera from SCD patients (n=5). B) Quantification of the C3 activation (act) fragment deposits on HUVECs exposed to sera from healthy controls (AA, no information about ethical origin) and SCD patients (SS). C) Stratification of the patients depending on the treatment (SS-NT vs

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SS-HU). D) C3 deposits (in green) on endothelial cells, revealed by immunofluorescence. Nuclei are stained with DAPI in blue. E) Quantification by flow cytometry of the C5b-9 deposits on HUVECs exposed to sera from healthy controls (AA) and SCD patients (SS). F) Visualization of the deposits of C5b-9 (green) by immunofluorescence. Nuclei are stained with DAPI in blue. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, Mann-Whitney test in A) and Kruskal-Wallis with Dunn's test for multiple comparisons in C) and E). G-I) Hemopexin reduces complement activation on ECs exposed to SCD patient sera: G) Quantification of the C3 activation (act) fragment deposits on HUVECs exposed to sera from healthy controls (AA) and SCD patients (SS) in the presence or absence of 10 μ M hemopexin (Hx). * $p<0.05$, Wilcoxon matched-pairs signed-rank test. Visualization of the deposits of C3 activation fragments (H) and C5b-9 (I) in the presence and absence of 10 μ M hemopexin. The respective deposits are in green. Nuclei are stained with DAPI in blue.

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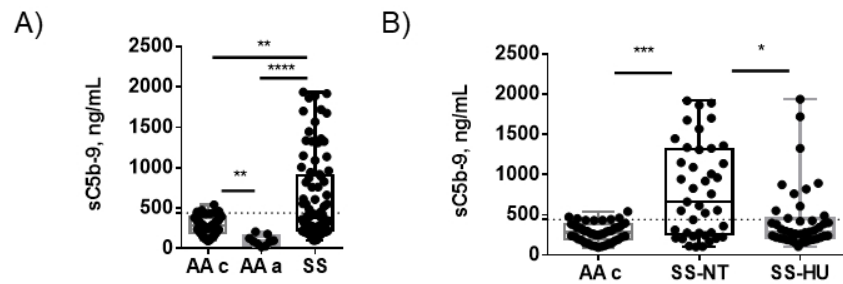


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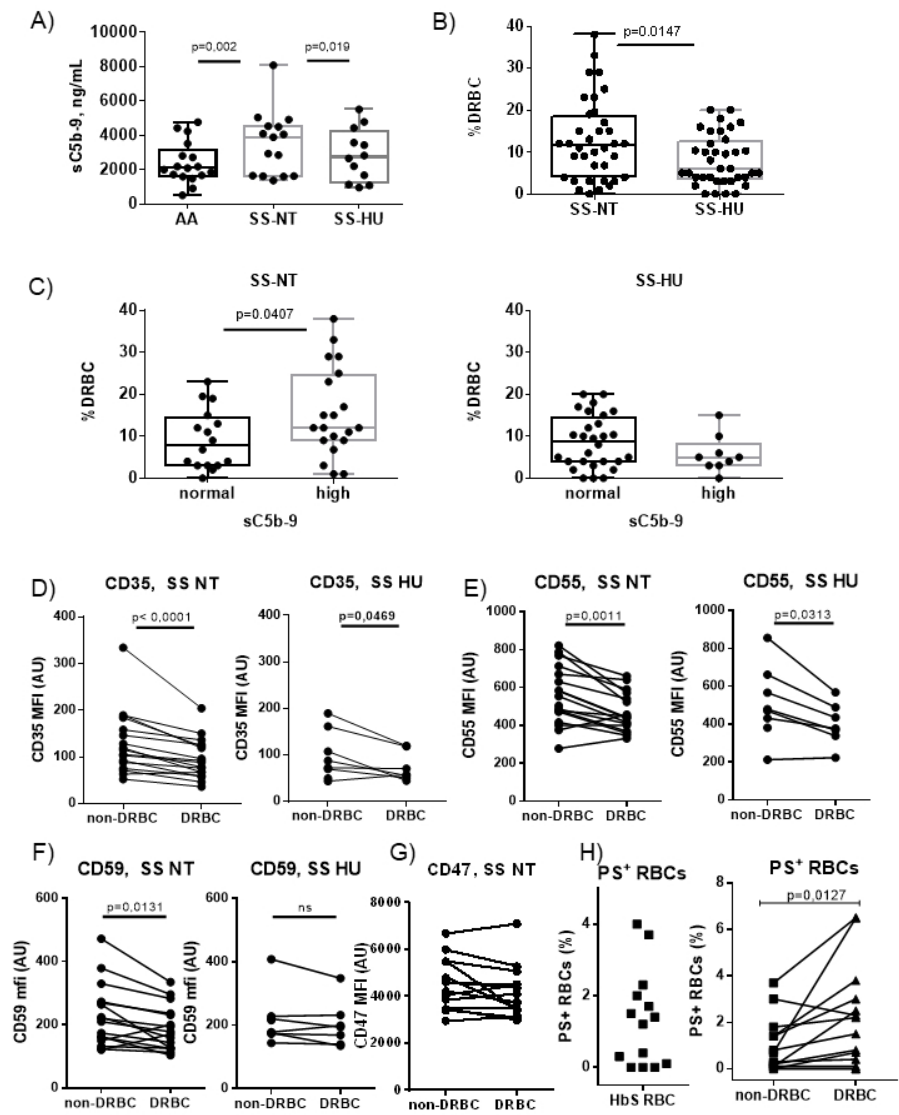


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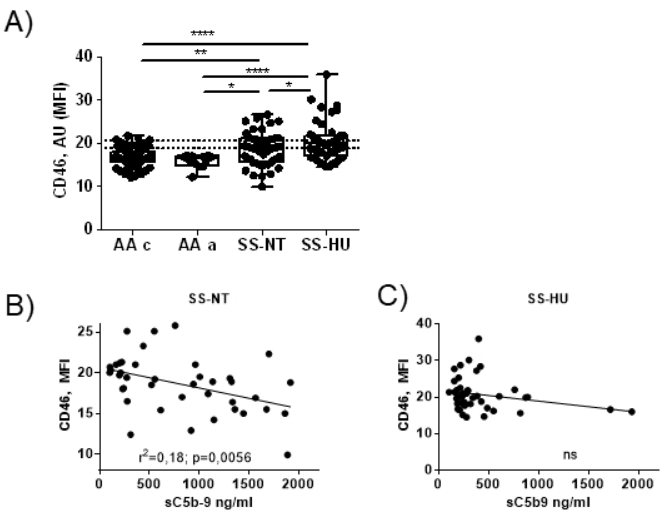


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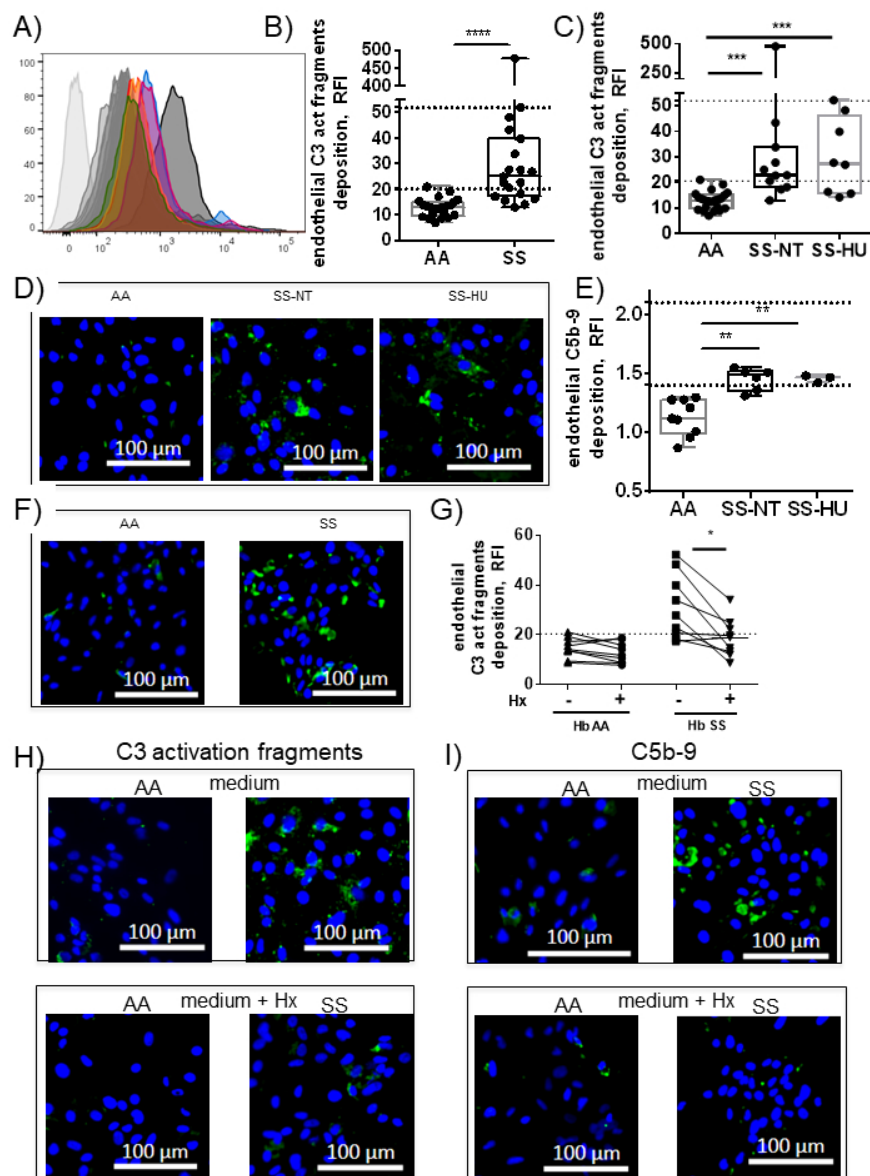
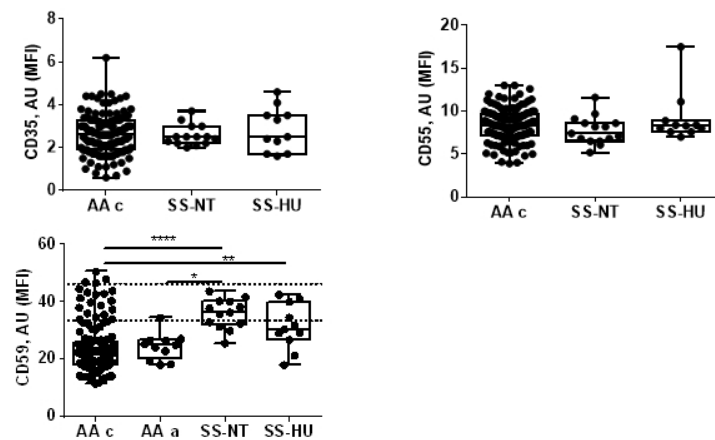


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Supplementary Figure 1. Phenotypic characterization of Hb SS RBC from SCD patients, treated or not with HU. A) CD35 (CR1); B) CD55 (DAF); C) CD59. For CD59, for which the SCD patients values were within the normal range of the Caucasian healthy controls (AA c), but significantly higher as an overall population, we determined a normal range, using also African controls (AA a).

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