Aggregation of mononuclear and red blood cells through α4β1-Lu/BCAM interaction in sickle cell disease

by Vicky Chaar, Julien Picot, Olivier Renaud, Pablo Bartolucci, Ruben Nzouakou, Dora Bachir, Frédéric Galactéros, Yves Colin, Caroline Le Van Kim, and Wassim El Nemer

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Aggregation of mononuclear and red blood cells through $\alpha_4\beta_1$-Lu/BCAM interaction in sickle cell disease

Running title: Mononuclear and sickle red blood cell interactions

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**Background and objectives.** Abnormal interactions between red blood cells, leukocytes and endothelial cells play a critical role in the occurrence of sickle cell disease associated painful vaso-occlusive crises. We investigated the interaction between circulating leukocytes and red blood cells which could lead to aggregates formation enhancing the incidence of vaso-occlusive crises.

**Design and methods.** Blood samples from sickle cell disease patients (n=25) and healthy subjects (n=5) were analyzed by imaging and classical flow cytometry after density gradient separation. The identity of the cells in the peripheral blood mononuclear cell layer was determined using antibodies directed specifically against white (anti-CD45) or red (anti-Glycophorin A) blood cells.

**Results.** Aggregates between red blood cells and peripheral blood mononuclear cells were visualized in sickle cell disease patient’s whole blood. The aggregation rate was 10-fold higher in these patients than in control subjects. Both mature red blood cells and reticulocytes were involved in these aggregates through their interaction with mononuclear cells, mainly with monocytes. Aggregates size was variable, with one mononuclear cell binding to one, two or several red blood cells. Erythroid Lu/BCAM and \(\alpha_4\beta_1\) integrin were involved in aggregates formation. The aggregation rate was lower in patients treated with hydroxycarbamide as compared to untreated patients.

**Conclusions.** Our study gives visual evidence for the existence of circulating red blood cell–peripheral blood mononuclear cell aggregates in sickle cell disease patients and shows that they are decreased during hydroxycarbamide treatment. Our results strongly suggest that erythroid Lu/BCAM proteins are implicated in these aggregates through their interaction with \(\alpha_4\beta_1\) integrin on peripheral blood mononuclear cells.
Introduction

Sickle cell disease (SCD) is a monogenic red blood cell disorder resulting from a single amino acid substitution in the hemoglobin (Hb) β-chain. This abnormal Hb, named HbS, polymerizes under deoxygenated conditions leading to less deformable sickle red blood cell (SS RBC) formation. Vaso-occlusive crises (VOC) are the main acute complication of SCD and are the consequence of blood microvessels obstruction by SS RBCs. Indeed, in addition to their propensity to sickle, SS RBCs can also adhere to vascular endothelium contributing to these VOC. Several studies, using in vitro and ex vivo models, have identified multiple adhesion proteins involved in SS RBC adhesion to endothelium. One of the most characterized RBC adhesion molecules is α4β1 integrin or Very Late Antigen-4 (VLA-4), expressed on reticulocytes, which binds to vascular cell adhesion molecule-1 (VCAM-1), thrombospondin and fibronectin. Lutheran/basal cell adhesion molecule (Lu/BCAM) proteins, the unique receptors for laminin in normal (AA) and SS RBCs, could be involved in VOC. Unlike AA RBCs, SS RBCs adhere to laminin and resist to high shear stress forces. Lu/BCAM-mediated SS RBC adhesion to laminin is stimulated by the physiologic stress mediator epinephrine through the β2-adrenergic receptor and protein kinase A (PKA) signaling pathway. Lu/BCAM proteins are also constitutively expressed on the endothelial cell surface and interact with α4β1 integrin expressed on young SS RBCs which may contribute to the abnormal adhesion of SS RBCs to resting endothelium.

In addition to SS RBCs, clinical observations suggested a role for leukocytes in the pathophysiological scheme of SCD. High leukocyte count is associated with SCD-related morbidity and mortality and experimental studies suggested a contribution of leukocytes in the vaso-occlusive process. Leukocytes from SCD patients adhere abnormally to vascular endothelium in vitro and play a critical role in the vaso-occlusive phase of sickle cell retinopathy. Infusion of epinephrine-activated human SS RBCs into nude mice induces vaso-occlusion associated with adhesion of murine leukocytes to vascular endothelium.
Furthermore, SCD mice deficient in endothelial P and E-selectins, important mediators of leukocyte recruitment to the vessel wall, are protected from vaso-occlusion. Beside their adhesion to the vessel wall through interactions with endothelial and extracellular matrix proteins, leukocytes can also interact with circulating SS RBCs leading to aggregates formation and enhancing the incidence of VOC. SS RBCs adhere to polymorphonuclear neutrophils in vitro and are captured by adherent leukocytes as shown by intravital microscopy in mice expressing human HbS as well as in a flow model of vaso-occlusion in vitro. More recently, Brittain et al showed interactions between reticulocytes and monocytes in whole blood samples and in adhesion assays in vitro. These interactions are mediated by αβ integrin, expressed on both cell types, via a bridge of soluble fibronectin. Furthermore, in vitro experiments suggested that SS RBCs bind to peripheral blood mononuclear cells (PBMCs) via erythroid LW/ICAM-4 and CD44 receptors, and induce their adhesion to endothelium.

In this study, enrichment of PBMCs by density gradient separation of SS whole blood was accompanied by an abundant and abnormal presence of RBCs in the PBMC layer fraction. Using the innovative imaging flow cytometry technique we revealed, by direct visualization, the presence of SS RBC-PBMC aggregates in this layer. We showed that the red cell population involved in the aggregates was a mixture of mature RBCs and reticulocytes, whereas monocytes represented the main PBMCs aggregating with RBCs. Our results indicated that erythroid Lu/BCAM proteins were implicated in these aggregates through its interaction with αβ integrin on PBMCs. Finally, we observed lower rates of aggregates in patients treated with hydroxycarbamide (HC) as compared to untreated patients. Considering the potential of such aggregates in promoting vaso-occlusion in SCD patients, our data could explain, at least in part, the clinical benefit of HC in reducing VOC frequency.
Design and Methods

Patients

Homozygous SCD patients (SS) at least 18 years old, able to give their informed consent and consulting at our Adult Sickle-Cell Referral Center were eligible for inclusion in this study which was approved by the local ethics committee (Comité de Protection des Personnes) and was conducted in accordance with the provisions of the Declaration of Helsinki, and local laws and regulations. Experiments were performed with freshly drawn heparin-anticoagulated venous blood from healthy adult donors or adult SS patients at steady-state, treated with HC or not. SCD steady-state was defined as a visit at least ≥ 1 months after an acute clinical event and ≥ 3 months after blood transfusion. We selected 17 SS patients untreated with HC (mean age: 34 years, SD: 8 years; sex ratio M/F: 12/5) and 8 patients on a stable HC therapy (20 mg/kg/d for > 6 months) (mean age: 37 years, SD: 10 years; sex ratio M/F: 4/4) and these 2 groups were studied independently. Blood samples from 5 healthy adult donors (AA) were used as control.

PBMC layer preparation

Venous blood was collected from the antecubital vein into lithium heparin (17 U./ml) tubes (Becton Dickinson Vacutainer, Plymounth, UK). PBMCs were separated from whole blood using Ficoll-Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA) density gradient separation and the isolated cells were washed once with phosphate-buffered saline (PBS) containing calcium (1 mM) and magnesium (1 mM).

Labeling and blocking experiments

Cells were incubated with mouse monoclonal anti-human CD45-FITC and Glycophorin A (GPA)-PE or GPA-APC conjugated antibodies (Becton Dickinson Biosciences, San Jose, CA,
USA) for 60 min and washed with PBS before flow cytometry or imaging flow cytometry analysis. For triple staining experiments, monoclonal anti-human CD71-APC conjugated antibody (Becton Dickinson Biosciences) was added. An indirect labeling was used for Lu/BCAM. Cells were incubated with a goat polyclonal antibody anti-human Lu/BCAM (R&D systems, Minneapolis, MN, USA) for 90 min, washed with PBS, then incubated with a swine anti-goat-PE secondary antibody (Beckman Coulter, Fullerton, CA, USA) together with anti-CD45-FITC and anti-GPA-APC antibodies.

For blocking experiments, the reagents were added prior to labeling. Cells from the mononuclear enriched layer were incubated for 30 min with 40 µg/mL recombinant human VCAM-1-Fc chimera (R&D systems, Minneapolis, MN, USA) or 40 µg/mL mouse monoclonal anti-human CD29 antibody (Becton Dickinson Biosciences).

**Flow cytometry analysis**

Acquisition and analysis were performed using a BD FACS Canto II flow cytometer (Becton Dickinson Biosciences) via BD FACSDiva software (v6.1.2). A total of $5 \times 10^5$ events were collected for each experiment. Monocytes and lymphocytes of the PBMC layer were identified and gated by forward and side scatter. PBMCs, RBCs and aggregates were quantified as the percentage of events that stained positive for CD45 ($% \text{CD45}^+$), GPA ($% \text{GPA}^+$) and both antigens ($% \text{CD45}^+ \text{GPA}^+$), respectively. The percentage of PBMCs involved in aggregates among total CD45$^+$ events ($% \text{PBMCs in aggregates}$) was determined for each subject according to the following formula:

$$% \text{PBMCs in aggregates} = \{(% \text{CD45}^+ \text{GPA}^+) / ( % \text{CD45}^+ + % \text{CD45}^+ \text{GPA}^+)\} \times 100$$
**Imaging flow cytometry analysis**

Acquisition and analysis were performed using the ImageStream system (Amnis Corporation, Seattle, WA, USA) and the ImageStream Data Exploration and Analysis Software (IDEAS; Amnis). This allows quantitative characterization of single cells or aggregates within a population by assessing a combination of morphology and fluorescence patterns. ImageStream system is equipped with three laser lines 405, 488 and 658 nm. Samples were prepared as described above. Spectral compensation was performed as described in Ortyn et al.. Cell populations were identified by gating on cells expressing surface markers and confirmed by visual inspection of the fluorescence pattern.

**Statistical analyses**

The Mann-Whitney test was used to determine: i) differences in RBC and PBMC proportions between untreated SS patients and AA subjects, ii) differences in the percentage of PBMCs in aggregates between SS untreated patients and AA subjects and between untreated and HC treated SS patients. Blocking experiments were analyzed using Wilcoxon's matched-pair test. $P < 0.05$ was considered significant.
Results

Abnormal co-selection of RBCs with PBMCs in SS patients

PBMCs were isolated from whole blood samples by ficoll-histopaque gradient separation. Cells from the PBMC layer were analyzed by flow cytometry using FITC-conjugated anti-CD45 and PE-conjugated anti-GPA antibodies specific of white blood cells (WBCs) and RBCs, respectively. As illustrated in Figure 1A, an abnormal high percentage of RBCs, determined by the percentage of GPA-PE events, was observed in the PBMC layer of SS patients (typical result, n=17). This percentage was highly variable among SS patients and the percentage median was significantly higher for those patients than for AA controls (26.4 vs 3.7, \(P=0.0013\)) (Figure 1B).

High rate of RBC and PBMC aggregates in SS patients

A high number of double-stained events, positive for both CD45-FITC and GPA-PE, were detected in all SS patients in contrast to AA subjects (Figure 2A, typical result, n=17). We postulated that these events could represent aggregates between RBCs and PBMCs. To test this hypothesis, we used the imaging flow cytometry approach. We analyzed the PBMC layer of one SS patient and one AA subject after CD45-FITC and GPA-PE staining. Images obtained from the SS patient showed that double stained events corresponded to PBMC-RBC aggregates (Figure 2B). Three populations of PBMC-RBC aggregates could be distinguished according to the GPA-PE intensity, all of which involving one PBMC interacting respectively with either, 1, 2 or >2 RBCs. A fourth population involving PBMCs and small RBC-derived microparticles was also observed. The rare double stained events observed in AA subjects were mainly PBMCs interacting with RBC-derived microparticles (Figure 2B, AA subject).

Based on the imaging flow cytometry approach, gating of double stained events was improved in classical flow cytometry experiments, excluding the aggregates between PBMCs
and RBC-derived microparticles. As the RBC percentage in the PBMC layer was variable among SS patients, the aggregation rate was determined by calculating the percentage of PBMCs involved in aggregates. The median of this percentage was 10-fold higher in SS patients than in healthy controls (2.11 vs 0.2, \( P=0.0048 \)) (Figure 2C).

**Aggregates include reticulocytes, mature RBCs and monocytes**

To identify the SS RBC population, young vs mature, involved in aggregation, cells were double stained with anti-GPA and anti-CD71 antibody which recognizes the transferrin receptor, specific of reticulocytes among the RBC population. First, the RBC population of the PBMC layer (Figure 3A, typical result, left panel) was analyzed and showed the presence of both mature RBCs and reticulocytes (Figure 3A, middle panel). The percentage of reticulocytes in this layer was variable among the 7 tested patients but was always higher than in whole blood, indicating a preferential selection of reticulocytes during the density separation. When aggregates were gated, they showed CD71-positive and CD71-negative populations, indicating that both young and mature RBCs were involved in aggregation with PBMCs (Figure 3A, right panel). The median of CD71-positive RBCs in aggregates was 58.4% (n=7).

The percentage of lymphocytes and monocytes was determined in the PBMC population and in the aggregates of 9 patients using the forward and side scatters (Figure 3B, typical result). In the PBMC population, the majority of the cells were lymphocytes (median: 97.8%). Despite the small percentage of monocytes in the PBMC population (median: 2.2%), they represented the majority of PBMCs involved in aggregation (median: 60.7%), indicating that RBCs interacted preferentially with monocytes within the aggregates (Figure 3C).
**αβ₁ integrin and erythroid Lu/BCAM are involved in aggregates formation**

To identify the adhesion proteins involved in aggregates formation, inhibition assays were performed with specific ligands of αβ₁ integrin, which is expressed on all PBMCs and a population of reticulocytes. When soluble VCAM-1-Fc was added, the percentage of aggregates was significantly reduced (median: 2.4% vs 3.7%, n=6, \( P=0.03 \)) (Figure 4A). Incubation with anti-β₁ blocking antibody also inhibited aggregation for 6 other samples (median: 0.98% vs 1.45%, \( P=0.03 \)) (Figure 4B), indicating that αβ₁ integrin was involved in aggregates formation.

As Lu/BCAM glycoproteins are known to interact with αβ₁ integrin, their implication in aggregation was investigated. Lu/BCAM antigens are very variable in strength among individuals and exhibit heterogeneity between individual RBCs within a person. This account for the mixed field agglutination patterns showing clumps of agglutinated cells in the presence of free cells. Lu/BCAM antigen distribution among the PBMC layer cells was analyzed. As expected, Lu/BCAM were not expressed on PBMCs (Figure 4C, middle panel). Lu/BCAM erythroid heterogeneous expression within the same individual was confirmed for all SCD patients analyzed in this experiment (Figure 4C, middle panel, typical result). These patients showed different proportions of Lu/BCAM-negative and positive (median: 56.1%, n =5) RBCs in the PBMC layer (Figure 4D). Lu/BCAM heterogeneous distribution among total RBCs was not conserved in the aggregates as all RBCs involved in aggregates were stained positive for Lu/BCAM (median: 95.1%) (Figure 4C, right panel, 4D). The absence of Lu/BCAM-negative RBCs and the exclusive presence of Lu/BCAM-positive RBCs in aggregates strongly suggested that Lu/BCAM were the erythroid proteins involved in PBMC-RBC interaction.
Aggregation rate is lower in patients treated with hydroxycarbamide

The effect of HC treatment on RBC-PBMC aggregation was investigated. Eight HC-treated patients were analyzed and compared to 17 untreated patients. The median of the aggregation percentage was significantly lower for the treated patients than for the untreated (0.68% vs 2.11%, \( P = 0.013 \)) (Figure 5), indicating that HC diminished the occurrence of aggregation events. However, aggregates were still detected in blood samples from the HC-treated patients as compared to healthy controls (median: 0.68% vs 0.29%, \( P = 0.0186 \)) (Figure 5).
Discussion

Leukocytes are believed to play a critical role in SCD by aggregating with RBCs and platelets and by adhering to and stimulating the vascular endothelium. This stimulation leads to increased expression of RBC adhesion molecules ligands, thus contributing to vaso-occlusion.\textsuperscript{15} In the present study, we revealed the presence of aggregates involving SS RBCs and PBMCs in SCD patients’ whole blood after enrichment of PBMCs by density gradient separation. Previous studies suggested interactions between the two cell types in SCD patients, using \textit{in vitro} binding assays,\textsuperscript{28,30} a SCD mouse model\textsuperscript{27} and an \textit{in vitro} flow model of vaso-occlusion.\textsuperscript{29}

Because of their size and their multiple potential interactions with the vascular wall, WBC-RBC aggregates could initiate or aggravate vaso-occlusion by disrupting the microcirculatory blood flow. Intravital microscopy studies in a SCD mouse model revealed that adherent leukocytes in inflamed venules played a direct role in vaso-occlusion by trapping circulating SS RBCs.\textsuperscript{27} Inhibition of WBC-RBC interactions with a high dose of intravenous immune globulin prevented venular vaso-occlusion in these mice.\textsuperscript{36} A novel mechanism that may contribute to vaso-occlusion was described, in which incubating leukocytes with SS RBCs, in particular with epinephrine-activated SS RBCs, stimulated their adhesion to endothelial cells \textit{in vitro}.\textsuperscript{31} Our results with the imaging flow cytometry analysis gave a strong evidence of the abnormal RBC-PBMC interactions that could occur in SCD patients’ whole blood as they visualized for the first time circulating WBC-RBC aggregates. Based on the study of Zennadi \textit{et al.},\textsuperscript{31} the presence of such interactions in whole blood could lead to activation of PBMCs and contribute to abnormal cell adhesion to the vascular wall. The imaging flow cytometry revealed that a PBMC could interact with more than one SS RBC in cell aggregates. It also allowed the imaging of aggregates between PBMCs and RBC-derived microparticles, in both SCD patients and AA controls. The imaging flow cytometry
assay was essential to determine the composition of the aggregates and to optimize the gating in the classical flow cytometry assays that followed, taking into account aggregates comprising whole cells and excluding those with RBC-derived microparticles.

Our study indicated that both SS reticulocytes and mature RBCs were involved in aggregation with PBMCs. In contrast, Brittain et al. reported that reticulocytes represented the primary cells interacting with leukocytes.\textsuperscript{30} Our results were obtained after a specific cell surface labeling of reticulocytes and showed the presence of both cell types (reticulocytes and mature RBCs) in cell aggregates, whereas Brittain et al. showed a positive correlation between the number of GPA\textsuperscript{+}/CD45\textsuperscript{+} events and reticulocyte count, which does not exclude the presence of mature RBCs in aggregates. Our finding is supported by Zennadi et al. study which showed that both SS reticulocytes and mature RBCs could interact with PBMCs and induce their adhesion to endothelial cells \textit{in vitro}.\textsuperscript{31} Moreover, in prior studies, dense (mostly mature) SS RBCs were more adherent to polymorphonuclear neutrophil monolayers than light (mostly young) SS RBCs\textsuperscript{28} reinforcing our finding concerning mature SS RBCs ability to interact with leukocytes.

In our study, SS RBCs interacted preferentially with monocytes within the aggregates, as previously described in whole blood.\textsuperscript{30} These interactions could result from the presence of activated monocytes, as SCD is characterized by an inflammatory state reflected by monocyte\textsuperscript{37-39} and polymorphonuclear neutrophil\textsuperscript{40} activation. Although the majority of the PBMCs involved in aggregates were monocytes, 39.33\% (median) were lymphocytes, indicating their ability to interact with SS RBCs. Zennadi et al. reported that SS RBCs induced adhesion of both lymphocytes and monocytes to endothelial cells,\textsuperscript{31} suggesting an interaction between SS RBCs and both PBMC types. Our results strongly support the presence of direct interactions between lymphocytes and SS RBCs \textit{in vivo}, suggesting that, in
addition to neutrophils and monocytes, lymphocytes may also contribute to the pathophysiology of SCD.

We identified \( \alpha_{4} \beta_{1} \) integrin and Lu/BCAM proteins as cell surface actors involved in RBC-PBMC aggregation. \( \alpha_{4} \beta_{1} \) integrin is expressed on leukocytes and reticulocytes whereas Lu/BCAM expression is specific of the erythroid lineage among circulating blood cells. The implication of \( \alpha_{4} \beta_{1} \) in RBC-PBMC aggregation was brought by the inhibition assays using VCAM-1-Fc and anti-\( \beta_{1} \) blocking antibody. Inhibition was variable but statistically significant in two sets of six patients, even though the aggregation was not totally abolished. This was probably due to the difficulty for VCAM-1-Fc and anti-\( \beta_{1} \) antibody to compete with the established interaction between \( \alpha_{4} \beta_{1} \) and its erythroid ligand. An interaction between reticulocyte \( \alpha_{4} \beta_{1} \) and endothelial Lu/BCAM has already been described in SCD patients.\(^{14}\) Our current study suggested that \( \alpha_{4} \beta_{1} \) on PBMC surface could interact with erythroid Lu/BCAM in the RBC-PBMC aggregates. Plasma fibronectin (Fn) was found to mediate interactions between monocytes and SS reticulocytes by bridging \( \alpha_{4} \beta_{1} \) molecules on both sides in \textit{in vitro} adhesion assays and in SCD patients blood.\(^{30}\) Our experiments showed that both SS reticulocytes and mature RBCs, which do not express \( \alpha_{4} \beta_{1} \), were involved in aggregates, suggesting that cell-cell interactions were not dependent on Fn. It was also shown that epinephrine-treated SS RBCs induced PBMC adhesion to endothelial cells via at least two erythroid receptors, LW and CD44.\(^{31}\) Nevertheless, the Lu/BCAM-\( \alpha_{4} \beta_{1} \) interaction seems to be the primary interaction responsible for the RBC-PBMC aggregates that we identified as no Lu/BCAM-negative RBC was found within these aggregates.

The effect of HC treatment on RBC-PBMC aggregation was also investigated in our study. HC therapy is associated with clinical benefit in SCD by reducing painful VOC and hospitalization frequencies\(^{32}\) but its mechanism of action is still poorly understood. We observed lower rates of aggregates in HC-treated patients as compared to untreated patients.
A similar observation has been reported in a flow model of vaso-occlusion in which HC inhibited the interactions between adherent leukocytes and flowing SS RBCs \textit{in vitro}.\textsuperscript{29} The inhibition that we detected was probably not due to a lower expression of Lu/BCAM on the surface of SS RBCs as HC-treatment increases the percentage of Lu/BCAM-positive SS RBCs and Lu/BCAM expression level/RBC.\textsuperscript{41} It could be due to the inhibition of Lu/BCAM and/or \( \alpha_4\beta_1 \) activities as both molecules are known to be activated by phosphorylation of their cytoplasmic domain.\textsuperscript{6,13} HC therapy in SCD also targets leukocytes through a reduction of their number, a diminution of their activation and a modulation of their adhesion molecules expression.\textsuperscript{42-45} Although HC decreases \( \alpha_4\beta_1 \) expression on SS reticulocytes,\textsuperscript{41,46} to our knowledge, the effect of HC-treatment on \( \alpha_4\beta_1 \) in SCD leukocytes has not been investigated yet. A recent study showed that HC treatment diminishes the gene expression of inflammatory mediators, such as TNF\( \alpha \), in SCD mononuclear cells.\textsuperscript{44} Such decrease in TNF\( \alpha \) could have a direct inhibitory effect on SCD mononuclear cell activation and could thus modulate \( \alpha_4\beta_1 \) binding activity.\textsuperscript{47} The decrease of RBC-PBMC aggregation associated with HC treatment could reflect, in part, the clinical benefit of HC therapy and more investigations are necessary to confirm this hypothesis.

In conclusion, our study gives visual evidence for the existence of RBC-PBMC aggregates in SCD patients and shows that the aggregation rate is decreased during HC treatment. Our results strongly suggest that erythroid Lu/BCAM are implicated in these aggregates through their interaction with \( \alpha_4\beta_1 \) integrin on PBMCs. Future investigations should help characterize the mechanisms leading to this interaction and evaluate the impact of these aggregates in VOC occurrence, which could generate new therapeutic perspectives.
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Authorship and Disclosures

VC designed and performed research, analyzed data and wrote the paper.

JP designed and performed cytometry experiments and figures, and commented on the manuscript.

OR designed and performed imaging cytometry experiments.

PB, RN, DB and FG collected blood samples and provided patients’ clinical data.

YC and CLVK discussed the results, gave advice and commented on the manuscript at all stages.

WEN supervised the project, designed research and wrote the paper.

The authors report no potential conflicts of interest.
References

Figure Legends

Figure 1. Abnormal SS RBC co-selection during PBMC density gradient separation. (A) Typical histograms representing flow cytometry analysis of the PBMC layer in one AA subject (left panels) and one SS patient (right panels). Upper and lower panels represent CD45-FITC (PBMCs) and GPA-PE (RBCs) staining, respectively. Bold horizontal line represents positive events’ area; percentages indicate the proportions of positive events for each marker. (B) RBC percentage in the PBMC layer in AA subjects (■) (n = 5) and SS patients (▲) (n = 17). Horizontal lines indicate medians. RBC percentage in the PBMC layer is significantly higher in SS patients than in AA subjects. *P = 0.0013, Mann-Whitney test.

Figure 2. SS patients have abnormal high rates of RBC-PBMC aggregates. (A) Typical dot plot representation of the PBMC layer flow cytometry analysis in one AA subject (left panel) and one SS patient (right panel). CD45-FITC and GPA-PE antibodies stain PBMCs and RBCs, respectively. CD45-FITC and GPA-PE double stained events represent potential RBC-PBMC aggregates. (B) Imaging flow cytometry showing dot plots of one SS patient (upper left panel) and one AA subject (lower left panel), as in (A). Right panels show typical examples of brightfield and fluorescent images from the double stained events area. Fluorescent images show, from left to right, CD45-FITC-labeled PBMCs, GPA-PE-labeled RBCs or RBC-derived microparticles and CD45-FITC/GPA-PE double stained event. The double stained area was divided into 4 parts according to the RBC:PBMC ratio in aggregates: >2:1 (P1), 2:1 (P2), 1:1 (P3). The fourth part shows the presence of PBMCs interacting with RBC-derived microparticles (P4). Double stained events from the AA subject were essentially restricted to PBMCs interacting with RBC-derived microparticles (P5). (C) Percentage of PBMCs involved in aggregates in AA subjects (■) (n = 5) and SS patients (▲) (n = 17). Horizontal lines indicate medians. *P = 0.0048, Mann-Whitney test.
Figure 3. SS RBCs and PBMCs populations involved in aggregation. (A) Aggregates include both mature SS RBCs and reticulocytes. Typical results showing cells from the PBMC layer labeled with FITC-conjugated anti-CD45, PE-conjugated anti-GPA and APC-conjugated anti-CD71 antibodies. Dot plot representation in the left panel shows gated events corresponding to SS RBCs, PBMCs and aggregates. Each of these 3 populations was gated and analyzed for CD71 expression, as shown in the middle panel. CD71 expression in aggregates is magnified on the right panel. Bold horizontal lines represent CD71-positive events. (B) Monocytes have a higher ability to aggregate than lymphocytes. Dot plot representation in the left panel is as for (A). Lymphocytes (L) and monocytes (M) were distinguished among PBMCs and aggregates using morphological parameters (FSC vs SSC, right panels). (C) Percentage of lymphocytes (○) and monocytes (▲) in the PBMC population and in aggregates. Horizontal lines indicate medians.

Figure 4. αβ₃ integrin and Lu/BCAM are involved in RBC-PBMC aggregation. Inhibition assays using VCAM-1-Fc (A) or anti-β₃ antibody (B). *P = 0.03, Wilcoxon's matched-pairs test. (C) Dot plot representation in the left panel shows gated events corresponding to SS RBCs, PBMCs and aggregates. Each of these 3 populations was gated and analyzed for Lu/BCAM expression, as shown in the middle panel. Lu/BCAM expression in aggregates is magnified on the right panel. Bold horizontal lines represent Lu/BCAM-positive events. (D) Percentage of Lu/BCAM-positive events in the RBC population (■) and in aggregates (▲) of 5 SS patients. Horizontal lines indicate medians.
Figure 5. Hydroxycarbamide decreases RBC-PBMC aggregation rate in SS patients.

Percentage of PBMCs in aggregates in SS untreated patients (■, n = 17), HC-treated patients (▲, n = 8) and AA subjects (▼, n = 5). *P = 0.013, **P = 0.0186, Mann-Whitney test.
Figure 1

A

AA subject

SS patient

CD45-FITC

PBMCs 94%

RBCs 6%

CD45-FITC

PBMCs 27%

RBCs 73%

GPA-PE

GPA-PE

B

% RBCs in PBMC layer

*  

0 25 50 75 100

AA

SS

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Figure 2

A

AA subject

SS patient

Double stained events

GPA-PE

CD45-FITC

PBMCs

RBCs

Double stained events

GPA-PE

CD45-FITC

PBMCs

RBCs

B

SS patient

Brightfield

CD45+ GPA+ GPA+

RBCs

RBC:PBMC

P1 >2:1

P2 2:1

P3 1:1

P4

RBC-derived microparticles

PBMCs

AA subject

Brightfield

CD45+ GPA+ GPA+

RBCs

RBC-derived microparticles

PBMCs

C

% PBMCs in aggregates

AA

SS

*
Figure 3

A

GPA-PE

CD45-FITC

PBMCs

RBCs

Aggregates

Events (x1,000)

CD71-APC

PBMCs

RBCs

Aggregates

B

CD45-FITC

GPA-PE

RBCs

Aggregates

PBMCs

SSC (x1,000)

FSC (x1,000)

L: 27%

M: 73%

L: 97%

M: 3%

C

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Lymphocytes

Monocytes

%
Figure 4

A

% PBMCs in aggregates

Human IgG VCAM-1-Fc

Human IgG

VCAM-1-Fc

B

% PBMCs in aggregates

Isotype control Anti-β1

Isotype control

Anti-β1

C

Aggregates

PBMCs

RBCs

CD45-APC GPA-PE Lu/BCAM-FITC

PBMCs

RBCs Aggregates

Lu/BCAM-FITC

D

% Lu/BCAM-positive RBCs

% Lu/BCAM-positive RBCs

RBCs

Aggregates
Figure 5

% PBMCs in aggregates

- Untreated SS
- HC-treated SS
- AA

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