

Decreased erythrocyte binding of Siglec-9 increases neutrophil activation in sickle cell disease

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ABSTRACT

Oxidative stress and inflammation promote vaso-occlusion in sickle cell disease (SCD). CD33-related Sialic acid-binding immunoglobulin-type lectins (CD33rSiglecs) are cell surface proteins that recognize sialic acids inhibit innate immune cell functions. We have shown that Siglec-9 on human neutrophils interact with erythrocyte sialic acids (prominently glycoporphin-A (GYPA) to suppress neutrophil reactive oxygen species (ROS). We hypothesized that altered sickle erythrocyte membrane sialic acid leads to decreased Siglec-9 binding capability, and thus a decreased neutrophil oxidative burst. SS erythrocytes express significantly more sialic acid than AA erythrocytes ($p = 0.02$). SS erythrocytes displayed significantly less Siglec-9-Fc binding $39\% \pm 11$ (mean \pm SEM) compared to AA erythrocytes $78\% \pm 5$ ($p = 0.009$). Treatment of AA erythrocytes with sialidase to remove sialic acid decreased binding to $3\% \pm 7.9$ ($p \leq 0.001$). When freshly isolated neutrophils were incubated with AA erythrocytes, neutrophils achieved $16\% \pm 6$ of the oxidative burst exhibited by a stimulated neutrophil without erythrocytes. In contrast, neutrophils incubated with SS erythrocytes achieved $47\% \pm 6$ of the oxidative burst (AA versus SS, $p = 0.03$). Stimulated neutrophils incubated with AA erythrocytes showed minimal NET formation while with SS erythrocytes NETs increased. SS erythrocytes are deficient in binding to neutrophil Siglec-9 which may contribute to the increased oxidative stress in SCD.

1. Introduction

Sickle cell disease (SCD) pathophysiology involves increased oxidative stress, hemolysis and inflammation. These effects promote downstream complications such as vaso-occlusion, ischemia-reperfusion physiology and organ damage [1–3]. Erythrocytes exert both pro-oxidative and anti-oxidative effects on disease-associated inflammation through iron-driven free radical production and endogenous anti-oxidants, respectively. In SCD, erythrocyte dysfunction caused by accelerated auto-oxidation and iron de-compartmentalization drive this oxidative stress [4–6]. Pro-inflammatory molecules and oxidants derived from immune cells, such as neutrophils, also contribute to elevated oxidative stress and inflammation.

Neutrophils are the most abundant immune cells in circulation [7,8]. In normal physiology, intravascular neutrophils remain quiescent until challenged by a pathogen or other injury. However, in SCD circulating neutrophils struggle to retain quiescence, and once activated,

are a powerful promoter of inflammation and disease progression in SCD [8,9]. This is accomplished through release of reactive oxygen species (ROS) and proteases, neutrophil extracellular trap (NET) production, and cell adhesion to the vascular wall and other blood cells participating in vaso-occlusion [1,9–11]. Past studies have sought to elucidate the mechanisms by which neutrophils remain quiescent in the bloodstream until they leave the circulation either to travel to a site of infection or for invader surveillance to eventually undergo apoptosis [8,10,12,13].

One recently discovered mechanism involves CD33-related sialic acid-binding immunoglobulin-type lectins (CD33rSiglecs) present on immune cells, which can recognize host cell-associated sialic acids in the form of “self-associated molecular patterns” (SAMPs) to down-regulate immune responses or suppress immune functions [14,15]. Siglec-9, the most abundant CD33rSiglec found on neutrophils, has been shown to interact with sialylated SAMPs on the erythrocyte membrane (prominently glycoporphin-A (GYPA)) leading to neutrophil quiescence

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and activation. This action can be disrupted through removal or modification of sialic acids on the erythrocyte membrane [16–18]. Given the 1000-fold excess of erythrocytes over neutrophils in whole blood, this finding can also explain the well-known technical problem of spontaneous activation of freshly isolated neutrophils *in vitro*.

Erythrocyte aging leads to a natural alteration of membrane topography, including a loss of membrane bound sialic acid [19–21]. In SCD, erythrocyte aging and accumulation of damage is accelerated [22,23]. However, previous studies are in disagreement in regard to comparative concentrations of SS erythrocyte membrane sialic acid [24,25]. We endeavored to explore the possible interaction of sickle erythrocyte associated sialic acid, neutrophil siglec-9 and how that interaction may contribute to the systemic oxidative stress and inflammation present in sickle cell disease.

2. Methods

2.1. Human samples

Human blood samples were obtained from healthy nonpregnant adult volunteers and steady-state and crisis SCD patients by protocols approved by the Institutional Review Board of the University of Minnesota. The study was conducted in accordance with the Declaration of Helsinki.

2.2. Erythrocyte collection and ghost production

Blood was collected directly into BD Vacutainer tubes containing EDTA. The collected blood was centrifuged at $2000 \times g$ for 10 min at 4°C to separate the cells and plasma. After centrifugation the plasma and buffy coat layers were removed. The collected erythrocytes were placed into 15 ml centrifuge tubes and washed with ice-cold PBS (pH 7.4) twice. After removing the PBS supernatant, 10 ml of ice-cold 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA was added to the washed erythrocytes. After inverting the tube several times to mix, the mixture was transferred to 50 ml tubes and placed on ice for 10 min. After incubation the cells were centrifuged at $10,000 \times g$ for 30 min with the brake off. After 30 min the supernatant was aspirated off. This process was repeated until the membrane pellet became a pale white color. At that time the supernatant was removed, and the membrane pellet was washed with ice-cold de-ionized water.

2.3. HPLC Sialic acid quantification

The DMB reagent, as per the conventional method was made with the following recipe and hydrolyzing conditions: 14 mM DMB (1,2-Diamino-4,5-methylenedioxybenzene), 18 mM sodium hydrosulfite, 0.75 M 2-mercaptoethanol, and 1.6 M acetic acid, and incubated at 50 °C for 2.5 h. The DMB-derivatized samples were analyzed on a Dionex Ultra3000 HPLC System using a Phenomenex Gemini 5 μ C18 250 \times 4.6-mm HPLC column at room temperature. The fluorescence was detected at 448 nm using excitation at 373 nm. The data collection time was expanded to 90 min. Standard samples were run with each experiment to account for slight variations in elution time. Data are expressed as picomoles of sialic acid (Neu5Ac)/microgram (μ g) RBC ghost protein or RBC.

2.4. Erythrocyte density centrifugation

Fractionation of erythrocytes was accomplished using Optiprep Density Media (Sigma Aldrich). Four gradient solutions of 1.077, 1.087, 1.097 and 1.107 g/ml were formulated according to the supplier's instructions and used to create a discontinuous gradient. 1 ml of whole blood was layered on top of the density gradient and centrifuged at 1000 g for 30 min at room temperature. Following centrifugation, each layer was collected separately, washed with 1X PBS and prepared for

future use.

2.5. Siglec-9-Fc binding assay

Approximately 3 ml of venous blood (AA and SS) was collected in EDTA, centrifuged, and erythrocytes isolated. Approximately 2 million erythrocytes were incubated with 10 μ g of rhSiglec-9 protein (R&D Systems) for 30 min on ice. Erythrocytes were then stained with either anti-CD235a (Biolegend) or anti-IgG (Biolegend) on ice for 30 min and analyzed by flow cytometry. One set of AA erythrocytes were pretreated with 20 mU of *Arthrobacter ureafaciens* sialidase (AUS) (Sigma-Aldrich) for 15 min at RT followed by protein incubation and staining [16].

2.6. Neutrophil oxidative burst assay

Neutrophils were isolated from AA volunteers using a combination of Histopaque 1077 (Sigma-Aldrich, USA) and Hesperan 0.09% (Supplier) according to instructions from the manufacturers. Healthy neutrophils were incubated with AA or SS erythrocytes (50:1 ratio) or plasma, followed by measurement of stimulated neutrophil oxidative burst (100 nM phorbol myristate acetate, PMA) as previously described [16].

2.7. *In vitro* NET assay

Isolated neutrophils from AA volunteers as stated previously and plated on Poly-L-Lysine coated 24 well plate (Greiner). Cells attached for 30 min, followed by incubation with phorbol myristate acetate, PMA (100 nM) (Sigma-Aldrich), AA/SS plasma or AA/SS erythrocytes. Neutrophils were then stained with DNA dye SYTOX orange (Life Technologies) and cell permeable dye SYTO13 (Life Technologies) and observed using confocal microscopy as previously described [10].

2.8. Statistics

Analyses were performed with Prism 8 for Mac OSX (Prism). Comparison of multiple treatment groups was made using 1-way analysis of variance (Tukey's Multiple Comparisons Test). An unpaired *t*-test was used for 2 groups.

3. Results and discussion

3.1. Erythrocyte membrane Sialic acid content

Using modern HPLC techniques AA and SS erythrocytes were analyzed to determine their respective membrane sialic acid (Neu5Ac) concentrations. SS ($n = 10$) erythrocytes express significantly more sialic acid than AA ($n = 8$) erythrocytes per μ g/membrane protein ($p = 0.02$) and per individual erythrocyte ($p = 0.03$) (Fig. 1). These results are surprising given that erythrocytes are believed to lose sialic acid as they age or undergo damage.

3.2. Erythrocyte Siglec-9Fc binding

Based on our observations above, we explored whether the altered SS erythrocyte sialome would impact the sialic acid/Siglec-9 mediated interaction with neutrophils. Initial efforts involved determining whether the altered SS erythrocyte sialome would bind recombinant soluble Siglec-9-Fc protein. When measured by flow cytometry (Fig. 2), SS ($n = 15$) erythrocytes displayed significantly less Siglec-9-Fc binding $39\% \pm 11.9$ (mean \pm SEM) compared to AA ($n = 14$) erythrocytes $78\% \pm 5.4$ ($p = 0.009$). Treatment of AA erythrocytes with neuraminidase to remove sialic acid decreased binding to $3\% \pm 7.9$ ($p \leq 0.001$). Given the significant difference in Siglec-9Fc binding percentage of AA vs. SS erythrocytes, we used a density centrifugation method to separate the erythrocytes into light ($x < 1.100$ g/ml) and

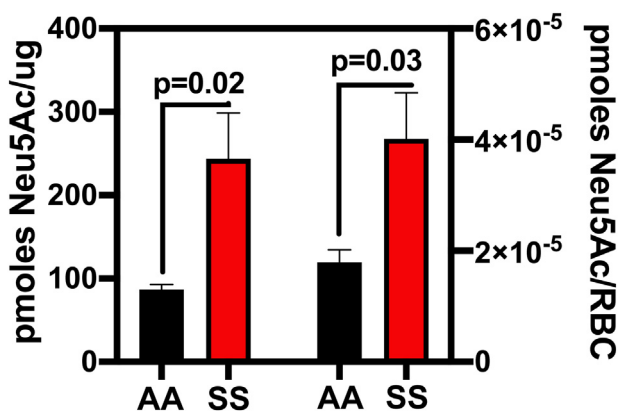


Fig. 1. Sickle erythrocytes contain more sialic acid than AA erythrocytes. Expressed as pmoles Neu5Ac/μg SS or AA erythrocyte membrane protein or red blood cells (RBC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

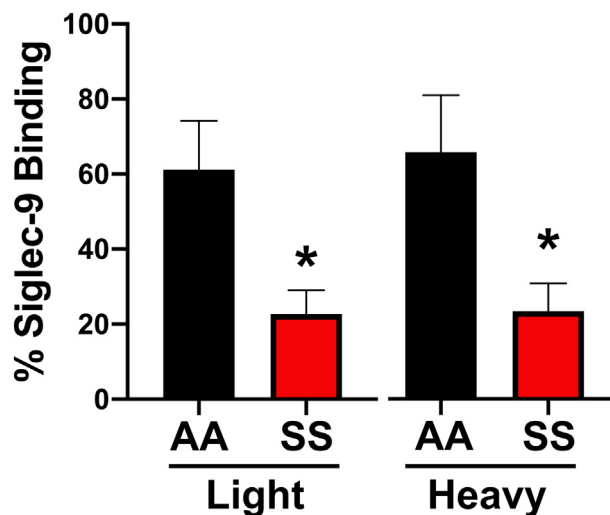


Fig. 3. Light or heavy fractions of SS erythrocytes bound less Siglec-9 Fc than AA erythrocytes. Density gradient erythrocytes from light and dense fraction of SS and AA erythrocytes were assessed for binding Siglec-9Fc by flow cytometry.

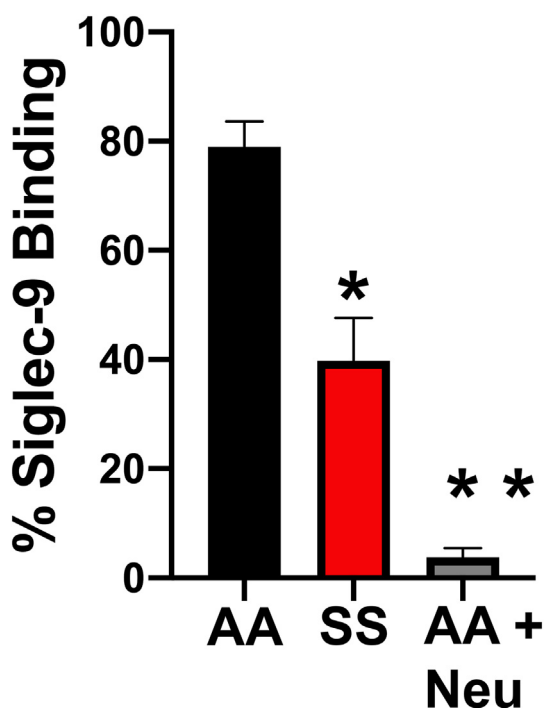


Fig. 2. Sickle erythrocytes bind less Siglec-9Fc than AA erythrocytes. AA erythrocytes, SS erythrocytes or AA erythrocytes treated with neuraminidase (AA NEU Treated) were assessed by flow cytometry as in Methods for Siglec-9Fc binding.

heavy ($x > 1.100$ g/ml). When measured by flow cytometry heavy SS erythrocytes ($n = 7$) displayed significantly less Siglec-9Fc binding ($23.43\% \pm 7.42$) compared to AA heavy erythrocytes ($n = 4$) ($65.80\% \pm 15.17$) ($p = 0.04$). The light SS erythrocytes ($n = 7$) also displayed significantly less Siglec-9Fc binding ($22.70\% \pm 6.33$) compared to light AA erythrocytes ($n = 4$) ($61.20\% \pm 13$) ($p = 0.012$) (Fig. 3).

3.3. Neutrophil oxidative burst suppression

Neutrophil ROS production was measured by flow cytometry using dihydrorhodamine 123 (DHR123) (Fig. 4). When freshly isolated neutrophils were incubated with AA ($n = 4$) erythrocytes, they were only able to achieve $16.8\% \pm 6.7$ of the oxidative burst exhibited by a PMA (100 nM) stimulated neutrophil without erythrocyte addition. In

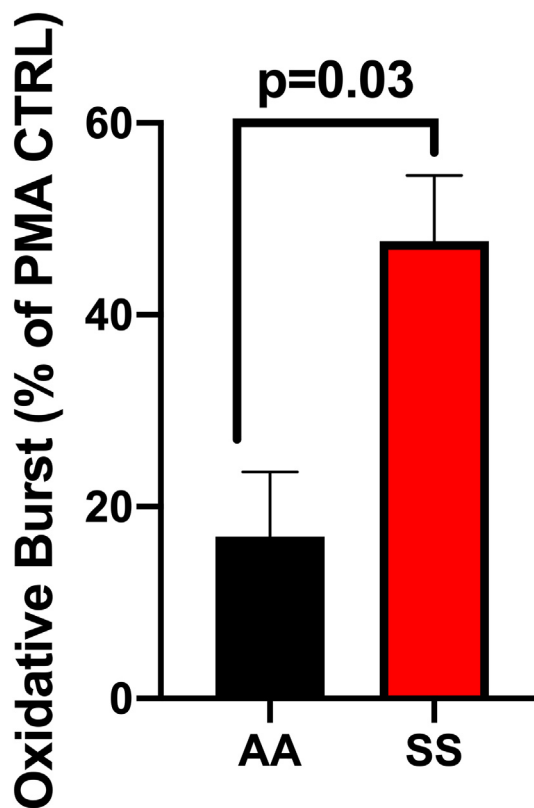


Fig. 4. AA RBCs inhibit neutrophil oxidative burst more than SS RBCs. AA and SS RBC added to neutrophils stimulated with PMA and oxidative burst measured presented a percent of control stimulated neutrophils (% of PMA CTRL).

contrast, neutrophils incubated with SS erythrocytes ($n = 12$) achieved $47.6\% \pm 6.8$ of the oxidative burst (AA versus SS, $p = 0.03$). Thus, SS erythrocytes showed significantly reduced ability to suppress neutrophil activation when compared with AA erythrocytes.

Neutrophil oxidative burst can lead to NETosis, which further contributes to the heightened inflammatory state. We show a drastic visible reduction of NETosis of neutrophils when incubated with AA erythrocytes compared with only slight visible reduction when incubated

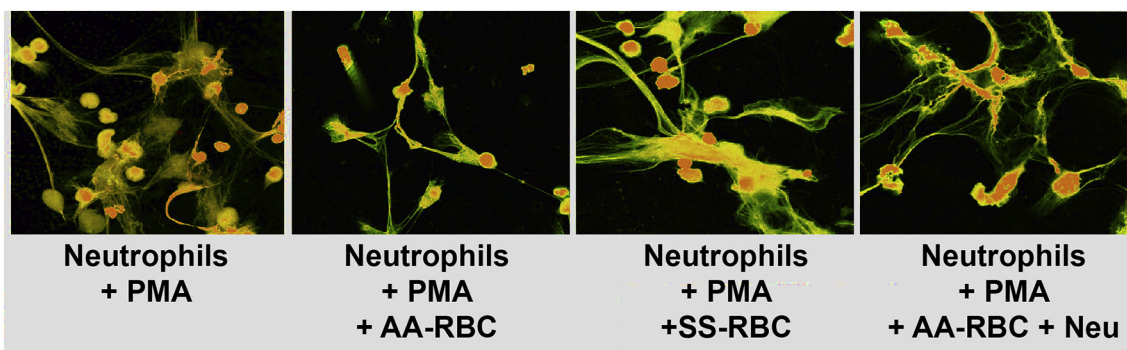


Fig. 5. AA RBC inhibit neutrophil NETosis more than SS RBCs.

Neutrophils stimulated with PMA in presence of AA or SS or AA RBC treated with neuraminidase and NETosis assessed with SYTOX orange. Intracellular DNA stains green and extracellular and membrane-compromised PMN DNA stains yellow-orange. AA erythrocytes demonstrated significantly increased ability to suppress NETosis when compared with SS erythrocytes. Neuraminidase treatment reduces the ability of the AA erythrocytes to suppress NETosis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with SS erythrocytes or sialidase treated AA erythrocytes (Fig. 5). Removal of sialic acid with neuraminidase SS RBC also inhibit neutrophil NETosis. These data are consistent with a decreased ability of SS erythrocytes to suppress neutrophil oxidative burst and activation.

4. Conclusion

A constant disease state of oxidative stress and inflammation underlies SCD pathophysiology. These results suggest that improper erythrocyte binding of neutrophil Siglec-9 may contribute to SCD pathophysiology. Previous literature has shown that GYPA, the primary Siglec-9 ligand on erythrocytes, demonstrates significant clumping on the SS erythrocyte [23]. We hypothesize that CYPA clumping might inhibit Siglec-9 binding to erythrocytes; however this hypothesis requires further testing. As noted, previous studies have shown both decreased and increased levels of sialic acid on sickle erythrocytes [26–31]. Maturation of reticulocytes has been shown to decrease GYPA on erythrocyte membranes [32], however our studies with light and heavy erythrocytes showed similar Siglec-9 binding to light and heavy sickle erythrocytes, suggesting other mechanisms might be involved.

The plasma sialome (the sum total of plasma glycoproteins) might have a separate inhibitory effect via Siglec-9. Sialic acid levels in plasma can be elevated in inflammatory states such as rheumatoid arthritis [33] and could possibly play a role in modulating inflammation. However, plasma from SS and AA individuals equally inhibited ROS formation. This is a complex issue that requires further investigation, but so far there does not appear to be a difference between AA and SS plasma.

In conclusion, this work demonstrates that despite increased sialic acid content, SS erythrocytes display a decreased ability to bind Siglec-9 and modulate neutrophil activation. This defective interaction with Siglec-9 may be a novel source of inflammation and oxidative stress in SCD. The development of neutrophil Siglec-9 agonists may prove a promising target for drugs seeking to decrease oxidation and inflammation in SCD.

Authors' contributions

Dr. Kiser designed the study, performed the assays and drafted the manuscript. Dr. Liczcano performed the HPLC studies and reviewed the manuscript, Ms. Nguyen and Ms. Becker performed assays, Drs Belcher, Varki and Vercellotti designed and conceived studies, analyzed data, supervised laboratory assays, provided resources, and reviewed and edited manuscript.

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Declaration of competing interest

Drs Belcher and Vercellotti receive research funding to their laboratory from CSL Behring (King of Prussia, PA, USA), Hillhurst (Montrose, CA, USA) and Astellas (Northbrook, IL, USA). Other authors have no conflicts of interest.

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