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CORRESPONDENCE

Impaired pro-resolving mechanisms promote abnormal NETosis, fueling autoimmunity in sickle cell disease

To the Editor:

Sickle cell disease (SCD) is a worldwide distributed hereditary red cell disorders with still high mortality and morbidity and limited therapeutic options. SCD is characterized by anemia, chronic hemolysis, and acute vaso-occlusive painful crises. The biocomplexity of SCD goes beyond red cells, involving neutrophils and soluble factors such as cytokines or alternative complement pathway intensively cross-talking with vascular endothelial cells. In addition, in SCD, the overactivation of neutrophils contributes to the production of neutrophil extracellular traps (NETs).[1,2] This might trigger endothelial vascular injury, promoting acute sickle cell related events and increasing the risk of infections in patients with SCD.[2–4] Recently, Vats et al reported a connection between NETs of liver origin and acute sickle cell related events. Interestingly they found increased NETs followed by oxy-Hb infusion.[5]

NETs are a meshwork of extracellular DNA decorated with histones and neutrophil granule proteins (e.g., myeloperoxidase, proteases), participating in host defenses against pathogens. However, balance between NETs generation/NETs clearance is crucial to limit the propagation and persistence of inflammation. Recently, the incorrect elimination of NETs has been linked to different diseases such as Systemic Lupus Erythematosus (SLE), acute-respiratory-distress-syndrome (ARDS).[6] Resolution of inflammation is an active process that is initiated by the synthesis from fatty acids of endogenous specialized pro-resolving lipid mediators (SPMs) namely resolvins (Rv), lipoxins (LX), and maresins (Mar). SPMs have pivotal roles in dampening unrelenting neutrophil-inflammatory responses, enhancing the clearance of infections, and controlling blood coagulation.[7(p1230),8,9] Chiang et al. have recently shown that Rvs reduce NETs and improve NETs clearance in a model of sepsis by *Staphylococcus aureus*, a known trigger of NETosis.[7(p1230)] Previously, we reported a reduction of RVD1 in plasma from patients with SCD and we demonstrated an impairment of pro-resolution mechanism in a humanized mouse model for SCD. We also showed that exogenous administration of RvD1 protects humanized SCD mice against acute VOCs and secondary sickle cell related organ damage.[10]

Here, we studied 23 adult patients with SCD on steady state referring to the Comprehensive center for hemoglobinopathies and rare anemias, University of Verona, and AOUI Verona from January 2019 to December 2021. Eight healthy volunteers matched by age and ethnicity were used as controls (Table S1). The study was approved by the Ethics Committee of Verona and Rovigo (#FGFR23). Written consent was obtained from each subject. SCD patients were naïve for chronic sickle cell related treatments (e.g., hydroxyurea or chronic transfusion) due to either physician-related barriers or to patients refusal. SCD patients underwent complete clinical examination and rheumatological assessment to rule out the presence of concomitant rheumatological comorbidity. For each patient the presence/absence of red flags associated with the concomitant rheumatological condition as joint inflammation, skin manifestations, sicca syndrome and Raynaud's phenomenon, were assessed. Blood was collected for hematological, biochemical assays and ANA, ENA determination as part of the standard clinical care for these patients. Additional blood was collected for research studies on whole blood and on plasma cytokines. Demographic, hematologic, and biochemical data are shown in Table S1. As expected, this unique cohort of patients with SCD displayed chronic hemolytic anemia, with increased reticulocyte count and lactate dehydrogenase (LDH). This was accompanied by increased total white-blood-cell (WBC), neutrophil, and monocyte counts (Table S1). Markers of systemic inflammation such as C-reactive-protein (CRP), erythrocyte-sedimentation-rate (ESR), and interferon (IFN)-γ were 2–3 folds higher albeit not statistically significant when compared with healthy controls (Table S1). Among the studied cytokines, SCD patients displayed a significant increase in tumor-necrosis-factor (TNF)-α ($p = .011$) and interleukin (IL)-10, suggesting both active inflammatory state and a counterregulatory mechanism to limit the sustained inflammation (Table S1). Activation of complement pathway was observed as reduction of C3 and C4 in SCD subjects compared to healthy controls (Table S1). Furthermore, we found that antinuclear antibodies (ANA), but not extractable nuclear antibodies (ENA), were significantly increased in SCD patients compared with healthy subjects. This agrees with previous reports, which detected ANA positivity in SCD patients in the absence of any autoimmune clinical manifestations.[11,12] Plasma of SCD patients was found to be enriched of nucleosomes already in the steady state compared to healthy controls and their concentration was found to be significantly increased during the painful crisis.[4] Noteworthy, these studies exclude any correlation between autoantibodies and either SCD genotype or transfusion history of the patients.[11–13] All ANA^+ SCD subjects included in our study showed a cell nuclei homogenous pattern and, although within a low titer range among 1:80 to 1: 160, they had a higher inflammatory and hemolytic status, as demonstrated by WBC and neutrophil counts and LDH values compared to ANA^- subjects (Table S2). Granzyme B (GRANB), which acts as a promoter of autoimmunity,[14] was significantly higher in ANA^+ than in ANA-

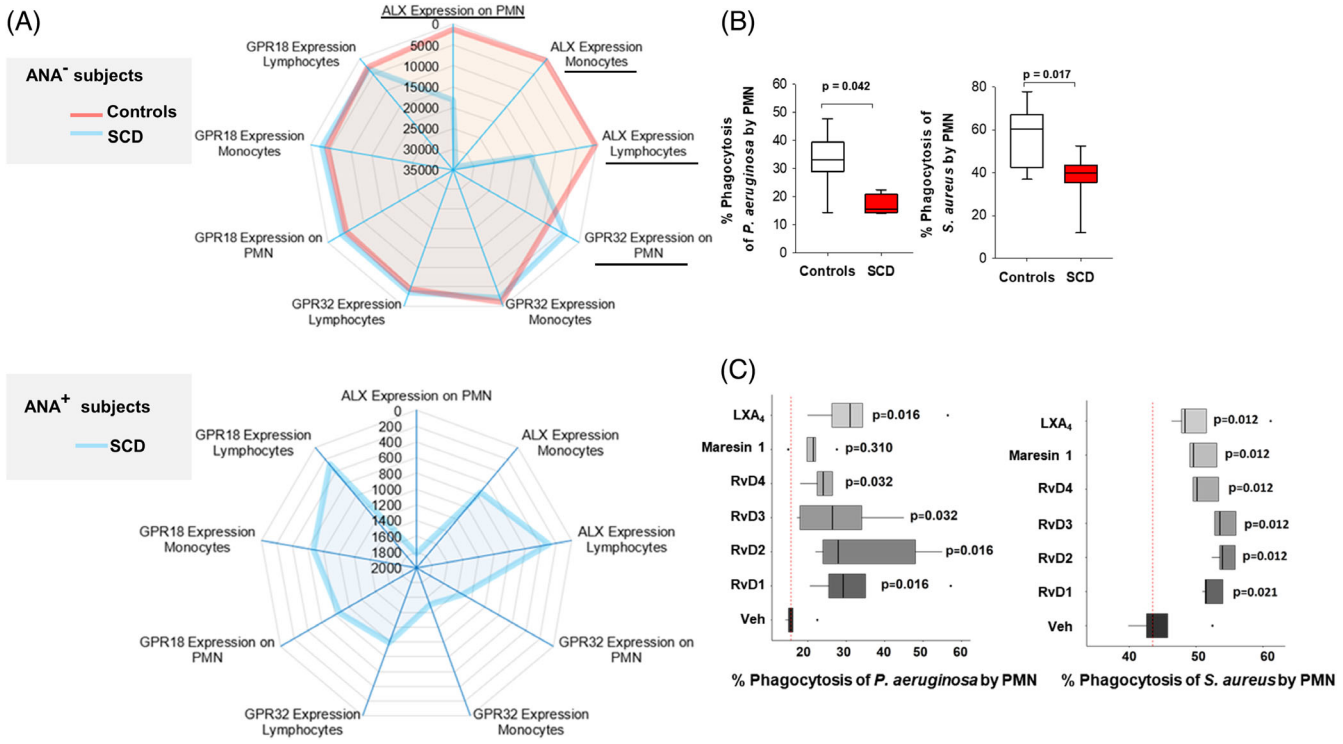

FIGURE 1 Radar plot for median values of RvD1 and D2 receptors ALX, GPR32 and GPR 18 expression on leukocytes from ANA negative (*upper plot*) and ANA positive (*lower plot*) SCD study participants. Freshly drawn blood (50 µL) from SCD ($n = 8$) or healthy volunteers ($n = 8$) were stained (30 min, 4°C, in the dark) with and APC-labeled anti-ALX (12.5 µL/test, FAB 154A RnD Systems), or anti-GPR32 (0.5 µg/test, TA340615, OriGene), or anti-GPR18 (0.5 µg/test, TA372644, OriGene) antibody. For GPR32 and GPR18 staining, cells were subsequently washed (see below) and incubated with an anti-rabbit FITC-conjugated secondary antibody (0.5 µg/test, Cat# 11-096-144, JaksonImmunoResearch). Receptor expression was determined with flow cytometry. Radar plots display multivariate data in the form of a two-dimensional chart of three or more quantitative variables represented on axes starting from the same point. (B) Differences in phagocytosis of *P. aeruginosa* and *S. aureus* by neutrophils from volunteers with SCD and healthy controls and (C) Rescue by SPM treatment of SCD neutrophils. *P. aeruginosa* (strain PA01) and *S. aureus* (strain ATCC 43300) were grown to mid-log phase ($OD_{600} = 0.45$) as and labeled with the pH-sensitive probe pHRodo (ThermoFisher) as previously reported 10 µL of a 0.45 suspension of pH-Rodo-labeled bacteria were added to 1×10^5 PMN isolated as above that were kept at 37°C/5% CO_2 on a tube rotator. Phagocytosis was determined with flow cytometry by measuring the percentage of PMN with ingested fluorescent bacteria. Data are reported as median (line), 25th/75th percentile range (box) and 95% confidence intervals (whiskers) from independent experiments with cells from $n = 9$ SCD volunteers

subgroup. This association appears to be ANA-specific, since when SCD patients were stratified based on ENA status, we found no significant differences in the same parameters (LDH ENA^- 312 [247;344] vs. ENA^+ 431 [347;498] U/L, $p = .071$; WBC ENA^- 7455 [6628;9810] vs. ENA^+ 7060 [6040, 7740] cells/µL, $p = .457$; Neutrophils ENA^- 4260 [3730; 5888] vs. ENA^+ 3340 [3050; 3745] cells/µL, $p = .111$); GRANB ENA^- 24.3 [14.8; 32.3] vs. ENA^+ 26.9 [12.2; 44.9] pg/mL, $p = .903$. Collectively, these findings were a distinct feature of ANA^+ SCD patients and were independent to the ANA dilution as no significant differences in terms of neutrophils and GRANB were found.

Since NETosis plays a key role in the modification and externalization of autoantigens involved in production of autoantibodies such as ANA,[6] we investigated NETosis in SCD patients and healthy volunteers. As shown in Figure S1A, SCD patients had a significantly higher percentage of NETs-forming PMN in whole blood at baseline compared to healthy controls. This was further increased upon stimulation, ex vivo with Ca^{2+} −ionophore as quantitatively assessed with flow cytometry using a gating strategy illustrated in Figure S1B and confirmed with immunofluorescence microscopy (Figure S1B, lower panel). Ca^{2+} was used as a stimulus to induce a maximal NETosis of neutrophils from volunteers with SCD, proving further responsiveness of these leukocytes despite persistent basal activation in the vasculature of these patients. Noteworthy, basal NETosis was higher, though not significant, in ANA^+ SCD subjects compared to ANA^-SCD patients.

Since we previously described defective pro-resolution mechanisms in humanized SCD mice,[10] we assessed the expression of cognate receptors for these SPMs on blood leukocytes from patients with SCD as a measure of their pro-resolution capability. To highlight differences in the expression of multiple GPCR across the two groups, mean fluorescence expression (MFI) values for ALX, GPR32/DRV1, and GPR18/DRV2 on PMN, monocytes, and lymphocytes were plotted as a radar plot, which is a common representation of continuous quantitative variables plotted as a point on a line. As shown (Figures 1A and S2) the overall expression of ALX, GPR32/DRV1, and GPR18/DRV2 on PMN, monocytes, and lymphocytes from ANA^+ SCD patients was significantly lower compared with ANA^- SCD

subjects, who were also characterized by relatively low LDH compared to ANA$^+$ SCD patients. However, GPR32/DRV1 expression on PMN and lymphocytes, as well as GPR18/DRV2 expression on monocytes and PMN were higher in ANA$^-$ compared with healthy controls. This could indicate selective changes in the cell surface expression of these GPCR linked to activation/triggering of leukocytes. Taken together, our data suggest a possible unbalance between NETosis and the clearance of NETs, contributing to the generation of new epitopes for autoantibody production as reported in patients with SLE.[6] This might sustain a self-feeding cycle in which overactive neutrophils, hemolysis, and autoantibodies promote and stimulate a severe NETosis in patients with SCD. Recently, Chiang et al. reported that resolvins reduce PMN NETosis in healthy individuals.[7] Thus, we determined whether they might also dampen NETs in whole blood from SCD patients. As shown (Figure S3A), both RvD1 and RvD4 significantly diminished maximally-induced NETosis in SCD, conveying a 14–10 difference in the median percentage of NETs-release up to baseline. Noteworthy, this effect was specific for RvD1 and RvD4 since RvD2 and RvE1, which also counter inflammatory response on blood cells (e.g., leukocyte L-selectin shedding, aggregation, and adhesion to vascular endothelial cells)[9,15] did not affect NETs production in SCD neutrophils (A23187 + Veh Median % NETS: 32.5 [31.2; 34.5]; A23187 + RvD2 Median % NETS: 30.2 [20.9; 45.5], $p = 1.000$; A23187 + RvE1 Median % NETS: 31.5 [29.7; 39.8], $p = 1.000$). RvD4, but not RvD1, significantly enhanced NETs clearance by SCD monocyte-derived macrophages (Figure S3B) an active, pro-resolutive, phagocytic process that does not trigger inflammatory cytokine secretion. Hence, RvD1 and RvD4 each reduce pro-inflammatory NETosis and selectively stimulate pro-resolutive mechanisms in SCD. This is extremely important in considering exogenous resolvin to limit disease progression in SCD but also to modulate the host response to infections and sepsis. Indeed, sepsis is one of the major causes of death of patients with SCD.[3] In addition, growing evidence indicates an important role of NETosis in defensive mechanisms against severe infection and sepsis.[16] Here, we first assessed that PMN from SCD patients had a deficiency in the phagocytic capacity against *Pseudomonas aeruginosa* and *S. aureus* (Figure 1B). Next, since control of infection is a defining function of resolvins and SPM, we determined their actions on phagocytosis by SCD neutrophils. As shown (Figure 1C), RvD2, RvD3, RvD4, and LXA_4 significant enhanced engulfment by PMN of *P. aeruginosa*, while Mar-1 did not and RvD1 yield an increase that was ultimately not significant. In contrast, all tested SPM but RvD2 significantly stimulated phagocytosis of *S. aureus*. Hence, each SPM gave a selective enhancement of phagocytosis by SCD neutrophils that can protect from infections.

In conclusion, our data suggest that in SCD an impaired pro-resolvin capability might detrimentally affect the balance between NETosis and NETs active clearance promoting the generation of autoantibodies such as ANA. Exogenous resolvin reduced NETs, stimulated their active clearance in SCD, and promoted neutrophil antimicrobial functions. This is consistent with the histological definition of active resolution, that is, cessation of host-detrimental excessive neutrophil activation and potentiation of host-protective neutrophil activities. Therefore, results presented here further support the importance of SPMs in reprogramming host responses in SCD to limit disease progression and infections and sepsis.

AUTHOR CONTRIBUTIONS

Antonio Recchiuti, Lucia De Franceschi, designed and carried out research and wrote the paper; Stefano Alivernini, critically revised data and wrote the paper; Jacopo Ceolan, Filippo Mazzi, Sofia Menotti characterized patients and collected biological materials, analyzed data; Alessandro Matte and Enrica Federti carried out cytokine ELISA analysis and revised the paper. Annamaria Porreca and Marta Di Nicola, supervised and carried out statistical analysis.

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CONFLICT OF INTEREST

The authors declare no competing conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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