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ORIGINAL RESEARCH PAPER

Effect of lysed and non-lysed sickle red cells on the activation of NLRP3 inflammasome and LTB4 production by mononuclear cells

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Abstract

Objective and design This study tested the hypothesis that sickle red blood cell (SS-RBC) can induce inflammasome NLRP3 components gene expression in peripheral blood mononuclear cells (PBMCs) as well as interleukin-1β (IL-1β) and leukotriene B4 (LTB4) production. Additionally, we investigated the effect of hydroxyurea (HU) treatment in these inflammatory markers.

Methods PBMCs from healthy donors (AA-PBMC) were challenged with intact and lysed RBCs from SCA patients (SS-RBC) and from healthy volunteers (AA-RBC). *NLRP3, IL-1β, IL-18* and *Caspase-1* gene expression levels were assessed by quantitative PCR (qPCR). IL-1β protein levels and LTB4 were measured by ELISA.

Results We observed that lysed SS-RBC induced the expression of inflammasome NLRP3 components, but this increase was more prominent for *CASP1* and *IL18* expression levels. Moreover, we observed that intact SS-RBC induced higher production of IL-1β and LTB4 than lysed SS-RBC. Although SCA patients treated with HU have a reduction in *NLRP3* gene expression and LTB4 production, this treatment did not modulate the expression of other inflammasome components or IL-1β production.

Conclusions Thus, our data suggest that caspase-1, IL-1β and IL-18 may contribute to the inflammatory status observed in SCA and that HU treatment may not interfere in this inflammatory pathway.

Keywords Sickle cell anemia · NLRP3-inflammasome · Hemolysis · Hydroxyurea · heme

Abbreviations

AA-PBMC	Peripheral blood mononuclear cells from healthy donors
AA-RBC	Red blood cell from healthy individuals
ASC	Apoptosis-associated speck-Like protein containing card
BEN	Benin haplotype
CAR	Central African Republic haplotype
CASP1	Caspase-1
DAMPs	Damage-associated molecular pattern molecules (DAMPs)
H_2O_2	Hydrogen peroxide
HbF	Fetal hemoglobin
HMGB1	High-mobility group protein B1
HU	Hydroxyurea
HV	Healthy volunteers
IL	Interleukin
LT	Leukotriene
LTB4	Leukotriene B4
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptors
NLRP3	Nod-like receptor family, pyrin domain containing 3
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells

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RBC	Red blood cell
SCA	Sickle cell anemia
SCD	Sickle cell disease
SS-RBC	Red blood cells from sickle cell anemia patients
TNF	Tumor necrosis factor
β^S	Beta(S)-globin

Introduction

Sickle cell anemia (SCA) is a molecular disorder characterized by systemic inflammation with frequent vaso-occlusive phenomena and ongoing hemolytic anemia. Patients present a wide range of clinical manifestations related to the pathophysiological mechanism, such as vaso-occlusive and painful episodes, increased susceptibility to infections and intravascular hemolysis which contribute to the chronic inflammatory status [1–3].

Free hemoglobin and heme released during intravascular hemolysis are able to activate several cells, including endothelial cells and leukocytes, leading to systemic inflammation, painful crises and vascular injury [4–6]. Cellular residues, endogenous molecules, such as leukotriene B4 (LTB4) [7, 8] and inflammatory cytokines [9], released during intravascular hemolysis, are able to act as signals for tissue injury, and are recognized by NOD-like receptors (NLRs) [10]. High-mobility group protein B1 (HMGB1) and heme are danger-associated molecular patterns (DAMPs) considered to be NLRs agonists [10–13].

The LTB4 synthesis from arachidonic acid is catalyzed by 5-lipoxygenase and leukotriene-A4hydrolase (LTA4H) that is mainly produced by leukocytes during the inflammatory response. LTB4 is able to recruit and activate neutrophils, monocytes and eosinophils [7].

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is expressed in a variety of cell types, including neutrophils, dendritic cells, epithelial cells, monocytes and T lymphocytes. Upon activation, NLRP3 recruits the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC) adaptor protein and pro-caspase-1 in a process that requires the participation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as a first-step of NLRP3 activation, and NLRP3 oligomerization itself as a second-step of activation, to convert pro-caspase-1 into its active form. Then, active caspase-1 converts the inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) into active forms, which consist of cytokines associated with the innate immune response in both infection and aseptic inflammation, contributing to the pathology severity [14–20].

Levels of IL-1β and IL-18 or polymorphisms of *IL1β* and *IL18* have been associated with clinical inflammatory conditions, such as multiple sclerosis, cancer, Alzheimer's disease, arthritis and SCA [8, 9, 21–27], contributing to the disease severity, and to the therapeutic failure [9, 26–28].

Hydroxyurea (HU) therapy appears to be the best treatment for patients with AF, as it increases fetal hemoglobin (HbF), increases nitric oxide (NO) production and reduces leukocyte count. HU exhibits its pharmacological effect by inhibiting ribonucleotide reductase enzyme. The treatment with HU leads to fewer symptoms, less severe hemolytic anemia and lower mortality [29–31]. In a previous study, the HU therapy was associated with high tumor necrosis factor (TNF) levels and increased plasma levels of the anti-inflammatory IL-10 [32].

This study is a sequence of a previous article from our group in this subject, from which the main difference is the addition of lysed RBCs, as an attempt to compare the effects of the release of internal RBC content with the effects of intact RBCs on NLRP3 inflammasome. Other difference is that now we work with whole blood from SCA patients treated and untreated with HU, to evaluate whether inflammasome components gene expression are modulated by this pharmacotherapy. A previous study from our group has shown that intact sickle red blood cells (SS-RBC) induce the production of inflammatory mediators and gene expression of NLRP3 inflammasome components in peripheral blood mononuclear cells (PBMC) [8].

Based on these findings, our group decided to evaluate the gene expression of *NLRP3*, *CASP1*, *IL-1β* and *IL18* and production of IL-1β and LTB4 in AA-PBMC challenged with intact or lysed RBC isolated from both SCA patients and healthy individuals. In addition, we investigated whether SCA patients treated with HU have a different profile of gene expression and inflammatory mediators' production in comparison to untreated patients.

Methods

Patients and healthy volunteers

Patients were recruited from the Bahia Hematology and Hemotherapy Foundation (HEMOBA), while healthy volunteers (individuals without hematological disorders or inflammatory conditions) were enlisted from the Faculty of Pharmaceutical Sciences (FacFAR) of the Federal University of Bahia (UFBA). A cross-sectional study included 137 children with SCA in steady state and 30 healthy volunteers. None of the included patients required blood transfusion four months prior to blood draw nor presented any evidence of infection, vaso-occlusive events or hospitalizations. Although all patients were being treated with folic acid,

none reported taking antibiotics, lipid-lowering therapy, steroidal or nonsteroidal anti-inflammatory drugs. A total of 29 patients were on HU therapy with an initial single dose of 17 mg/kg/day. The demographic, hematological and biochemical parameters of both groups are shown in Table 1. This study was conducted in accordance with the Helsinki Declaration of 1975, and its revisions, and received approval from the Institutional Review Board of the Gonçalo Moniz Institute–FIOCRUZ (protocol number CAAE: 04733612.7.0000.0040), Bahia-Brazil. All study subjects or their legal guardians were properly advised about the research, agreed with the biological sample collection and signed a term of informed consent.

Blood sampling, isolation of human peripheral mononuclear and red blood cells

Venous blood collected from SCA patients in steady state and from healthy volunteers (HV) were subjected to hemoglobin profiling by high-performance liquid chromatography, as well as to obtain RBC for experimentation and PBMCs for gene expression analysis.

PBMCs were obtained from the peripheral blood of a healthy donors by gradient centrifugation using Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) as previously described [8]. PBMC cultures were resuspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin and 100 µg/mL of streptomycin (Gibco, Grand Island, NY, USA). PBMCs were then counted, distributed in a 24-well plate (Costar, Corning, NY, USA) at a concentration of 3×10^6 mononuclear cells/mL and challenged with 3% of lysed or intact RBCs for overnight incubation at 37 °C with 5% CO_2.

Lysed RBC were obtained using a previously described technique [8, 33] with minor modifications. Briefly, whole blood was collected and centrifuged at 500 g for 10 min at 4 °C. Plasma was removed by aspiration and discarded. The RBC pellet was washed 3 times in sterile phosphate-buffered saline (PBS) (pH 7.3) for 5 min at 170 g. The RBC concentrate was evaluated for leukocyte and platelet contamination by staining with trypan blue and RBCs under 97% of purity were not considered. A phase-contrast microscope (Olympus CK2, Center Valley, PA, USA) was used for examination and images were obtained via Image Pro Plus 6.1 software (Media Cybernetics, Rockville, MD, USA). RBCs were lysed by freezing concentrate in liquid nitrogen for 10 min, followed by three thawing cycles consisting of 5 min at 37 °C.

Heme and H_2O_2 solutions

The experimental heme solution was prepared by diluting bovine hemin (Sigma, St. Louis, MO, USA), according to a previously described technique [34]. A previous study performed in our laboratory found an average heme plasma concentration of 28.5 µM in healthy subjects, and 68.6 µM in HbSS patients [34, 35], which led us to use 70 µM of heme in the experiments herein. Hydrogen peroxide (H_2O_2) (Proquimios, RJ, Brazil) was used at a concentration of 20 µM, identical to what has been described in inflammatory conditions [36].

Leukotriene-B4 and IL-1β production

Supernatants were collected from in vitro cultured PBMCs challenged with lysed or intact RBCs. EIA was used to detect LTB4 using Leukotriene B4 EIA kit in accordance with manufacturer instructions (Cayman Chemical Company, MI, USA). IL-1β production was assessed using IL-1β ELISA kits (R&D Systems, Minneapolis, USA) in accordance with manufacturer instructions. Serum and supernatant LTB4 and IL-1β dosages were similarly conducted for both HU-treated and untreated SCA patients.

Determination of β^S haplotypes

The Beta(S)-globin gene haplotypes CAR (Central African Republic) and BEN (Benin) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) techniques according to a previous study [37].

Gene expression quantification

RNAs were obtained with the use of Trizol reagent (Invitrogen, Life Technologies, CA, USA), according to the manufacturer's recommendations, and quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). RNAs were used in the reverse transcription reactions using High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, Foster City, CA) in accordance with the manufacturer's instructions. Primer sequences were: *NLRP3* (Fw: *TGC CCC GAC CCA AAC C*; Rev: *GAA GCC GTC CAT GAG GAA GA*), *IL-1β* (Fw: *AGC TAC GAA TCT CCG ACC AC*; Rev: *CGT TAT CCC ATG TGT CGA AGA A*), *IL-18* (Fw: *ATC GCT TCC TCT CGC AAC A*; Rev: *TCT ACT GGT TCA GCA GCC ATC TT*), *Caspase-1* (Fw: *AAA AAA TCT CAC TGC TTC GGA CAT* ; Rev: *TCT GGG CGG TGT GCA AA*), *GAPDH* (Fw: *CAC ATG GCC TCC AAG GAG TAA*; Rev: *TGA GGG TCT CTC TCT TCC TCT TGT*) and *β-ACTIN* (Fw: *CCT GGC ACC CAG CAC AAT*; Rev: *GCC GAT CCA CAC GGA GTA CT*).

Table 1 Hematological and biochemical markers of steady SCA patients, either treated with HU or untreated, and healthy volunteers

	SCA		HV (Mean ± SD) ($n = 30$)	*P* value[§]	*P* value[†]	*P* value[‡]
	Untreated (Mean ± SD) ($n = 108$)	Treated with HU (Mean ± SD) ($n = 29$)				
Age (years)	10.80 ± 3.11	10.24 ± 2.91	9.62 ± 3.12	0.3860	0.0576	0.3374
Hemolysis markers						
Red blood cell ($\times 10^6$/mL)	2.67 ± 0.49	2.64 ± 0.38	4.67 ± 0.42	0.7045	**< 0.0001**	**< 0.0001**
Hemoglobin (g/dL)	8.07 ± 1.06	8.13 ± 0.67	12.84 ± 1.27	0.3788	**< 0.0001**	**< 0.0001**
Hematocrit (%)	23.11 ± 3.09	23.72 ± 2.92	38.40 ± 3.39	0.2154	**< 0.0001**	**< 0.0001**
Mean corpuscular volume (fL)	87.94 ± 7.81	90.20 ± 7.24	82.18 ± 4.38	0.2253	**< 0.0001**	**< 0.0001**
Mean corpuscular hemoglobin (pg)	30.75 ± 3.33	31.73 ± 3.10	27.48 ± 1.65	0.2304	**< 0.0001**	**< 0.0001**
Reticulocytes (%)	6.94 ± 2.08	6.95 ± 2.18	0.77 ± 0.18	0.9649	**< 0.0001**	**< 0.0001**
Lactate dehydrogenase (U/L)	1153.00 ± 468.90	1007.00 ± 435.70	417.90 ± 94.61	0.1084	**< 0.0001**	**< 0.0001**
Heme (µg/mL)	4.95 ± 1.01	3.06 ± 0.58	1.64 ± 0.65	**0.0247**	**0.0018**	**0.0112**
Platelets						
Platelets ($\times 10^9$/mL)	459.50 ± 129.90	432.70 ± 160.20	301.30 ± 61.06	0.4476	**< 0.0001**	**0.0030**
Leukocytes						
Leukocytes ($\times 10^9$/mL)	13,326.00 ± 3238.00	12,941.00 ± 4901.00	6988.00 ± 1864.00	0.3126	**< 0.0001**	**< 0.0001**
Monocytes ($\times 10^9$/mL)	936.10 ± 390.60	909.60 ± 443.10	438.40 ± 186.90	0.7462	**< 0.0001**	**< 0.0001**
Neutrophils ($\times 10^9$/mL)	6337.00 ± 2738.00	5935.00 ± 2988.00	3263.00 ± 1444.00	0.2852	**< 0.0001**	**< 0.0001**
Lymphocytes ($\times 10^9$/mL)	4881.00 ± 1648.00	5358.00 ± 2517.00	4881.00 ± 1648.00	0.5964	**< 0.0001**	**< 0.0001**
Iron metabolism						
Ferritin (ng/mL)	262.40 ± 246.10	317.50 ± 162.60	32.13 ± 16.67	**0.0103**	**< 0.0001**	**< 0.0001**
Iron	92.34 ± 43.81	89.93 ± 43.09	72.52 ± 34.55	0.6861	**0.0310**	0.1374
Inflammation						
C-reactive protein (mg/L)	5.07 ± 4.05	8.52 ± 6.56	1.29 ± 0.57	**0.0010**	**< 0.0001**	**< 0.0001**
Hemoglobin pattern						
Fetal hemoglobin (%)	9.07 ± 5.75	9.42 ± 5.29	0.30 ± 0.12	0.5901	**< 0.0001**	**< 0.0001**
Fetal hemoglobin levels based on haplotype (%)						
CAR/CAR[a]	4.47 ± 2.80	10.50 ± 4.95	–	**0.0200**	–	–
CAR/BEN[b]	9.05 ± 6.00	8.24 ± 4.89	–	0.9640	–	–
BEN/BEN[c]	7.68 ± 5.98	10.65 ± 7.22	–	0.3930	–	–

Genotype frequency in SCA patients: [a]CAR/CAR: 16.2%; [b]CAR/BEN: 55.0%; [c]BEN/BEN: 20.0%

Significant *p* values are shown in bold

SCA sickle cell anemia, *HU* hydroxyurea, *HV* healthy volunteers, *BEN* Benin haplotype, *CAR* Central African Republic haplotype, *SD* standard deviation

[§]*p* values < 0.05 were considered significant among untreated SCA patients and those treated with HU (Mann–Whitney test)

[†]*p* values < 0.05 were considered significant among untreated SCA patients and HV individuals (Mann–Whitney test)

[‡]*p* values < 0.05 were considered significant among treated SCA patients and HV individuals (Mann–Whitney test)

Gene expression was analyzed by quantitative PCR (qPCR) using an ABI 7500 FAST Real-Time PCR system (Applied Biosystems TM, Foster City, CA, USA). Amplification reactions were performed using Power SYBR® Green (Applied Biosystems TM, Foster City, CA, USA), also in accordance with the manufacturer's instructions. Cycle threshold (Ct) values were obtained using the Operational Program 7500™ System (Applied Biosystems, TM, Foster City, CA, USA). Intra-assay precision and normalization of expression levels were performed according to a previous study [8]. Relative expression folds were calculated based on 2^-ddCt method [8, 38].

Statistical analysis

Comparisons of quantitative variables between groups were made using the Mann–Whitney test for non-normally distributed data. The non-parametric Kruskal–Wallis test was used to compare among three or more groups. Values of $p < 0.05$

were considered significant. All data were analyzed using Prism 5.1 software (GraphPad, San Diego, USA).

Results

Hematological and biochemical markers in SCA patients under hydroxyurea treatment

Data regarding the hematological and biochemical markers of SCA patients either in steady state, i.e., untreated ($n=108$), or treated ($n=29$) with HU, and healthy volunteers (HV, $n=30$) are represented in Table 1. HU treatment was able to reduce levels of heme ($p=0.0247$) and increase ferritin ($p=0.0103$) and C-reactive protein ($p=0.0010$) levels. There was no statistically significant difference in HbF between the groups treated and not treated with HU. However, patients with Central African Republic (CAR) haplotype and treated with HU presented HbF levels increased suggesting that this haplotype influences the improvement of this marker production in response to HU.

Lysed SS-RBC induce the expression of NLRP3 inflammasome components

To compare the impact of intact and lysed RBC on the induction of NLRP3 inflammasome component gene expression, PBMCs were challenged with intact or lysed SS-RBC or with AA-RBCs. Our results show that the PBMCs stimulated with intact AA-RBC did not exhibit any significant expression of *NLRP3*, *CASP1*, *IL1B* or *IL18*, in comparison to the basal expression of these receptors (Fig. 1a–d). Lysed AA-RBCs induced significantly higher expression of *NLRP3* (Fig. 1a) (4.57 ± 1.45; $p=0.022$) and *CASP1* (Fig. 1b) (3.94 ± 0.67; $p=0.0179$), but lower *IL1B* (Fig. 1c) (3.69 ± 1.91; $p=0.0119$) and *IL18* (Fig. 1d) (0.26 ± 0.06; $p=0.0238$) gene expression when compared with unstimulated PBMC cultures (0.38 ± 0.06).

To further investigate any discrepancies between the hemolytic and nonhemolytic state in vitro, PBMCs were challenged with intact or lysed SS-RBC under the same conditions described above. As shown in Fig. 1, stimulation with both intact and lysed SS-RBC induced significant expression of all the NLRP3 inflammasome components. Furthermore, we found substantially increased *CASP1* (Fig. 1b) and *IL18* (Fig. 1d) gene expression in the PBMCs challenged with lysed SS-RBC populations, as compared to intact SS-RBC.

In general, the challenge of PBMCs with heme and H_2O_2 induced the gene expression *NLRP3* inflammasome components. However, no significant changes in *CASP1* gene expression were observed upon stimulation with H_2O_2 (0.85 ± 0.43; $p=0.1000$), or in *IL18* expression under heme (0.57 ± 0.56; $p=0.6286$).

Lysed SS-RBC induce the production of IL-1β and LTB4

Supernants from cultured PBMCs stimulated with both intact (265.20 ± 95.17; $p=0.0179$) and lysed (92.97 ± 22.37; $p=0.0286$) SS-RBCs showed increased production of IL-1β (Fig. 2a) when compared with unstimulated PBMC cultures (11.62 ± 2.98). No statistically significant differences were seen in the levels of IL-1β in response to intact (21.54 ± 5.93; $p=0.1000$) or lysed (71.65 ± 10.46; $p=0.1000$) AA-RBCs when compared to unstimulated cell cultures.

Both intact and lysed SS-RBC and AA-RBC were found to induce LTB4 production in PBMCs. Significant statistical differences were observed when comparing LTB4 production upon stimulation with intact (3.19 ± 0.27; $p=0.0275$) and lysed (4.02 ± 0.63; $p=0.0238$) AA-RBC, as well as between intact (6.08 ± 2.29; $p=0.0286$) and lysed (2.33 ± 0.34; $p=0.0290$) SS-RBC. Moreover, enhanced LTB4 production was detected in PBMCs stimulated with intact SS-RBC in comparison to those stimulated with lysed SS-RBC ($p=0.0286$).

In addition, stimulation of cultured PBMCs with heme and H_2O_2 induced the production of IL-1β and LTB4 by these cells.

HU treatment affects NLRP3 gene expression and LTB4 production

A comparison of gene expression between untreated and treated SCA patients (Fig. 3) revealed that *CASP1* (Fig. 3b) (0.19 ± 0.02; 0.24 ± 0.14, $p=1.0000$), *IL1B* (Fig. 3c) (4.51 ± 0.96; 5.07 ± 2.58; $p=0.9451$) and *IL18* (Fig. 3d) (1.54 ± 0.27; 1.23 ± 0.86, $p=0.2788$) were unaffected by HU treatment. However, the patients treated with HU exhibited decreased *NLRP3* gene expression (Fig. 3a) (6.25 ± 1.36; 3.30 ± 1.49, $p=0.0245$).

Fig. 4 illustrates that SCA patients treated with HU showed no changes in IL-1β (Fig. 4a) (6.43 ± 1.53; 7.50 ± 1.51, $p=0.2632$); however, HU treatment did induce a decrease in LTB4 (Fig. 4b) (921.80 ± 454.20; 540.00 ± 129.80, $p=0.0245$), reaching levels similar to those seen in healthy volunteers.

Discussion

The NLRP3 inflammasome is the most common and fully characterized inflammasome and has been shown to be activated by several endogenous danger signals, such as heme

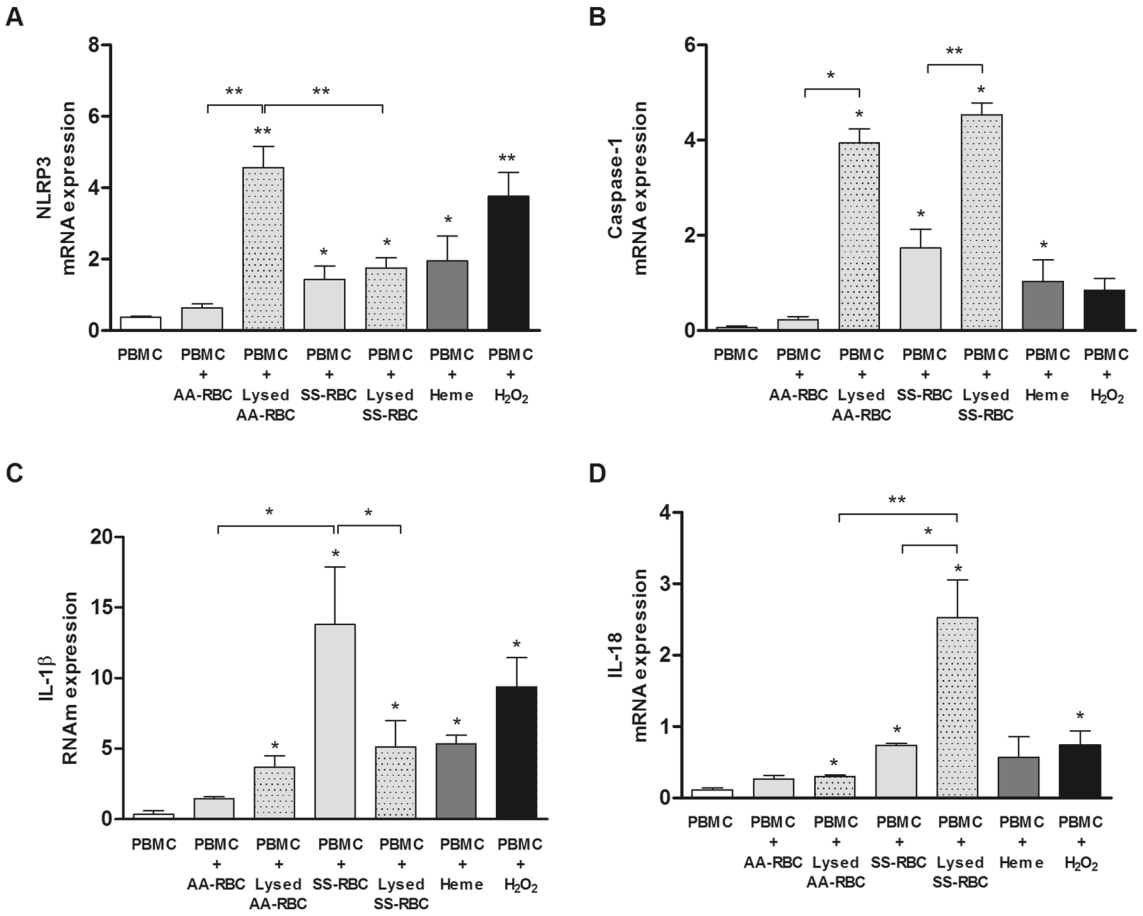

Fig. 1 Intact and lysed sickle red cells induce NLRP3 inflammasome components expression in PBMC culture in vitro. *NLRP3* (**a**), *Caspase-1* (**b**), *IL-1β* (**c**), *IL-18* (**d**) mRNA expression was evaluated in PBMC from healthy donors (AA-PBMC, $n=4$), challenged in triplicates for each stimulus, with RBC from healthy volunteers (AA-RBC, $n=10$) or from SCA patients (SS-RBC, $n=8$), for 24 h at 37 °C and 5% CO_2. For control of cell activation, heme 70 µM and H_2O_2 20 µM were used. Asterisk marker above each bar represents statistic significant difference between this and the negative control bar (untreated PBMC). Asterisk marker above each bar represents statistic significant difference between this and the negative control bar (PBMC). Asterisk marker above horizontal lines represents statistic significant difference between evaluated groups (*$p<0.05$; **$p<0.01$), Mann Whitney test. Relative expression folds were calculated based on 2^-ddCt method using non-stimulated PBMCs for calibration. *NLRP3* Nod-like receptor family, pyrin domain containing 3, *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *RBC* red blood cells, *SCA* sickle cell anemia, H_2O_2 hydrogen peroxide

and hemolysis products [10, 39]. This study aimed to investigate comparatively the role of lysed and intact RBCs in the activation of NLRP3 inflammasome in the context of SCA.

Our results showed that SS-RBCs can act as DAMPs, as indicated by the induction of the expression of NLRP3 inflammasome components by the stimulated PBMCs. When comparing the effects of lysed and intact SS-RBC, we observed a higher induction of *CASP1* and *IL18* gene expressions with lysed SS-RBC than with intact SS-RBC. On the other hand, the comparison between intact and lysed AA-RBC showed that only lysed AA-RBC were able to induce the gene expression of these NLRP3 inflammasome components. Although further studies are needed, our results suggest that erythrocyte composition differs between AA-RBCs and SS-RBCs regarding inflammasome pathway activation.

In compare to healthy donors, sickle RBCs present loss of lipid symmetry with increased expression of phosphatidylserine and adhesion molecules such as ICAM-4, CD44 and CD47, loss of the red cell membrane fluidity, and increased susceptibility to oxidative damage [40]. Ren et al. previously demonstrated that RBCs from SCA patients show an increase in arachidonic acid (AA), and a decrease in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

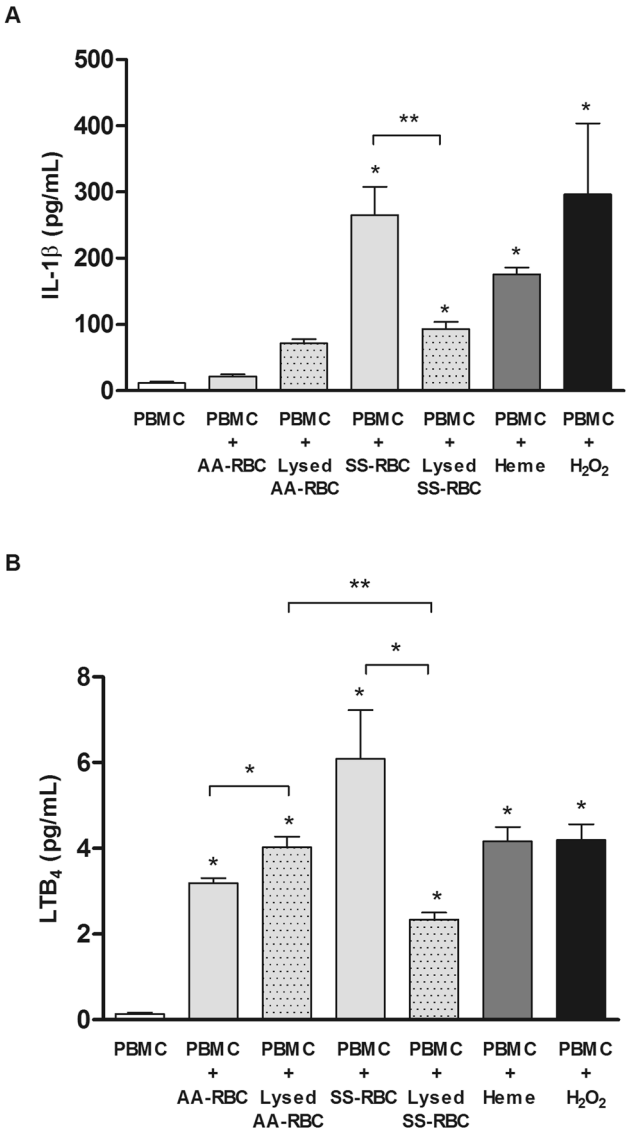

Fig. 2 Intact and lysed sickle red blood cells induce IL-1β and LTB4 production in PBMC culture in vitro. After PBMC from healthy donors (AA-PBMC, $n=4$) have been challenged with AA- or SS-RBC, levels of IL-1b (**a**) and LTB4 (**b**) were measured in cells supernatant cultured in triplicates with RBC from healthy volunteers (AA-RBC, $n=10$) or from SCA patients (SS-RBC, $n=8$), for 24 h at 37 °C and 5% CO_2. For control of cell activation, heme 70 µM and H_2O_2 20 µM were used. Asterisk marker above each bar represents a significant statistical difference between this and the negative control bar (untreated PBMC). Asterisk marker above horizontal lines represents significant statistic difference between designated groups (*$p<0.05$; **$p<0.01$), Mann–Whitney test. *LTB4* leukotriene B4, *IL* interleukin, *PBMC* peripheral blood mononuclear cells, *SCA* sickle cell anemia, *RBC* red blood cells

in comparison to RBCs from healthy volunteers suggesting that RBC alterations on membrane could justify the contrast observed between healthy individuals and SCA patients [41]. These previous findings may reinforce the differences between AA-RBCs and SS-RBC, and intact and lysed RBCs.

A different behavior was observed for *IL1β,* since PBMCs challenged with intact SS-RBC showed higher *IL1β* gene expression levels than those challenged with lysed SS-RBC. Despite the observed effects of lysed AA-RBCs on *IL1β*, *CASP1* and *NLRP3* expression, it is important to consider that significant intravascular hemolysis does not occur physiologically in healthy volunteers, whereas a large proportion of SCA patients exhibit this condition [1, 42]. Hence, the inflammation observed in SCA patients could be a consequence of constant exposure to DAMPs originated from intact or lysed RBCs [10] during intravascular hemolysis, which leads to the increased expression of NOD-like receptors and can promote leukocyte recruitment in a mechanism dependent of inflammasome components [10].

In addition to assessing gene expression, we also evaluated the protein levels of IL-1β and lipid mediator LTB4 in the supernatant from PBMC challenged with SS-RBC. We observed that both intact and lysed SS-RBC were able to induce IL-1β production, while neither lysed AA-RBC nor intact showed this effect. This finding suggests that IL-1β induction may be related to DAMPs found only in SS-RBCs.

LTB4 is an inflammatory mediator that has previously shown to be increased in SCA patients [7, 8]. LTB4 acts as a chemoattractant that enhances leukocyte migration and adhesion to vascular endothelium, promoting the inflammatory scenario in these patients. Our results show that both AA-RBC and SS-RBC were able to induce LTB4 production. However, intact SS-RBC were able to induce a higher LTB4 production than lysed SS-RBC did, reflecting differences between these conditions. Despite initial concerns over hemolysis products, such as heme, increase LTB4 levels [43], our results suggest that intact SS-RBC membrane components may show an increased ability to induce LTB4 production.

Since hematological and biochemical markers are known to differ substantially between SCA individuals and healthy volunteers, we evaluated the effect of HU treatment with respect to these parameters. In SCA, the main therapeutic target consists of attempting to shift hemoglobin production from sickle hemoglobin to fetal hemoglobin. HU yields therapeutic benefits by increasing HbF levels, improving red cell rheology and reducing red cell adhesion [30, 44].

It is known that the main effect of HU is attributable to increases in HbF, which aids the inhibition of HbS polymerization. However, in the present study, there was no significant difference in HbF between the groups treated and not treated. It is known that β^S gene haplotypes have an important role in fetal Hb synthesis. Some studies have shown that this HbF has the highest concentration in patients with Benin

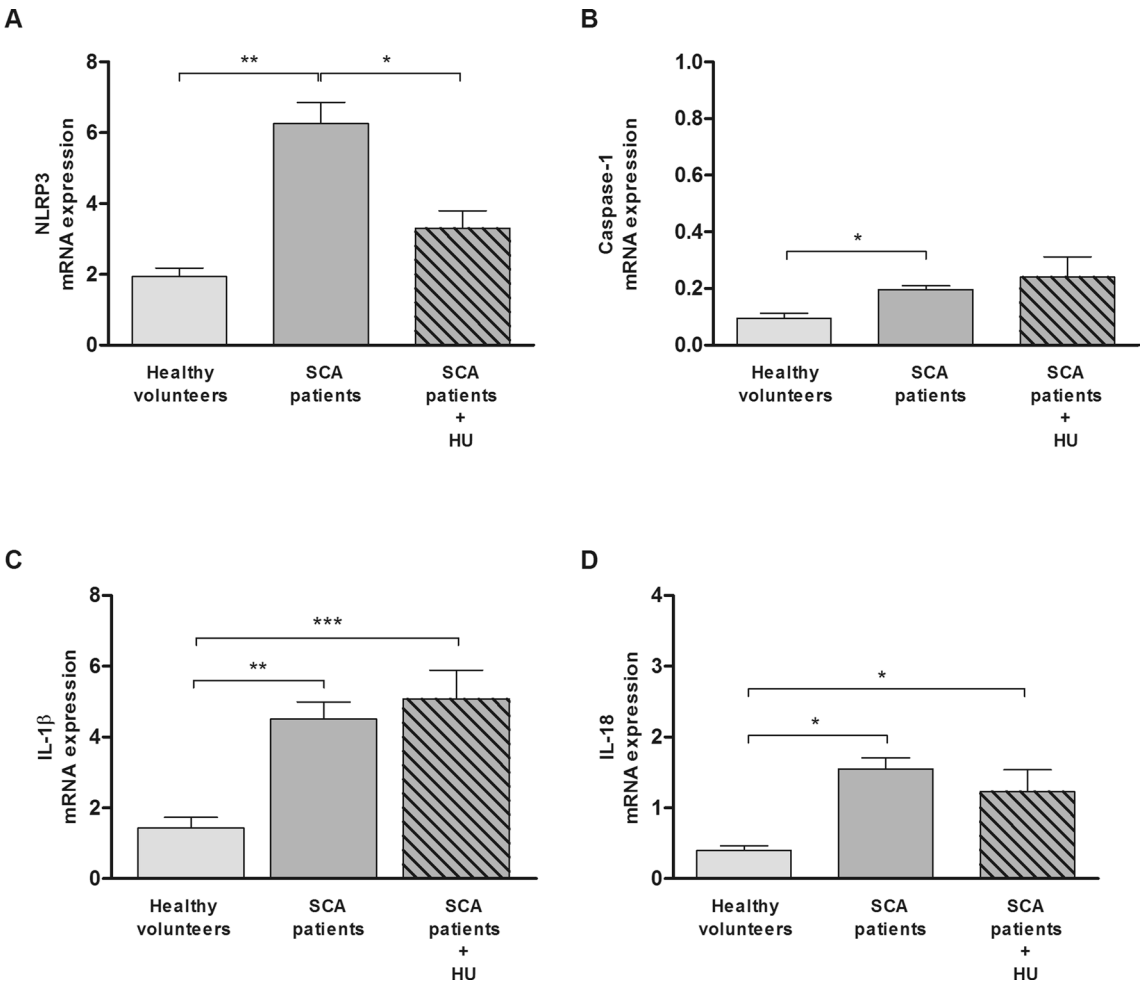

Fig. 3 Whole blood derived-mRNA presented decreased NLRP3 gene expression in SCA patients treated with HU. *NLRP3* (**a**), *caspase-1* (**b**), *IL-1β* (**c**), *IL-18* (**d**) mRNA expression were evaluated in whole blood from SCA patients treated with HU ($n=13$) comparing to untreated patients ($n=15$) and healthy volunteers ($n=20$). Asterisk marker above each bar represents a significant statistical difference between this and the control group (healthy volunteers). Asterisk marker above horizontal lines represents significant statistic difference between evaluated groups ($*p<0.05$; $**p<0.01$; $***p<0.001$), Mann Whitney test. Relative expression folds were calculated based on 2^-ddCt method using non-stimulated PBMCs for calibration. *NLRP3* Nod-like receptor family, pyrin domain containing 3, *IL* interleukin, *SCA* sickle cell anemia, *HU* hydroxyurea

(BEN) haplotype in compared to the Central African Republic (CAR) haplotype [37, 45]. This could justify the greater responsiveness to treatment with HU of patients with only CAR haplotype (CAR/CAR) in comparison to CAR/BEN or BEN/BEN. Taken together, these results demonstrate the ability of HU treatment to improve HbF production in SCA patients with CAR haplotype.

As expected, levels of heme and C-reactive protein were higher in SCA patients compared to healthy volunteers [46–48]. Moreover, we showed that HU treatment was able to reduce levels of heme and increase C-reactive protein levels. It is well known that free heme has pro-oxidant and pro-inflammatory properties [49], and that the levels of CRP are associated with endothelial injury and the chronic inflammatory state observed in SCA patients [48]. Thus, this finding may support the hypothesis that SCA patients may have persistent inflammation even under treatment with HU since this treatment does not interfere with all inflammatory mechanisms.

Ferritin is a biomarker found to be elevated in inflammation and autoimmune diseases, such as systemic lupus erythematosus [50] and in SCA vaso-occlusive crisis and infections [51]. Since the variation seen in our data with respect to ferritin levels among HU-treated patients was

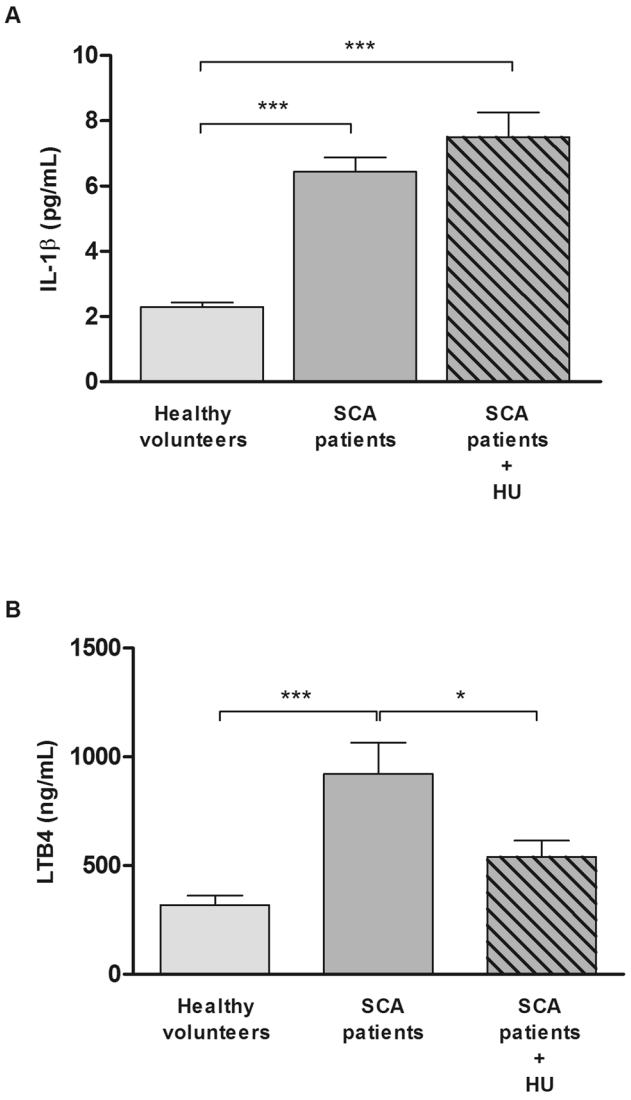

Fig. 4 HU treatment decreases LTB4 production and did not change IL-1β production in SCA patients. Levels of IL-1β (**a**) and LTB4 (**b**) were measured in serum from SCA patients untreated ($n=15$) or treated ($n=13$) with HU, and healthy volunteers ($n=20$). Asterisk marker above each bar represents a significant statistical difference between this and the control group (healthy volunteers). Asterisk marker above horizontal lines represents significant statistic difference between evaluated groups (*$p<0.05$; ***$p<0.001$), Mann–Whitney test. *LTB4* leukotriene B4, *IL* interleukin, *SCA* sickle cell anemia, *HU* hydroxyurea

very high and considering the fact that we did not perform experiments to evaluate any causal relationships between ferritin levels before and after HU treatment, we are unable to conclude that HU treatment induces an increase in ferritin.

Regarding the NLRP3 inflammasome analysis performed with whole blood-derived mRNA from SCA patients untreated in comparison with those treated with HU, we observed that HU treatment did not interfere in the expression of *CASP1*, *IL1β* and *IL18*, suggesting that HU may not affect inflammasome-dependent inflammatory conditions observed in SCA patients, as a previous work from our group has shown, that in vitro PBMCs challenged with SS-RBCs and HU did not stimulate *NLRP3* inflammasome gene expression [8]. However, herein, our results suggested that SCA patients treated with HU decrease *NLRP3* gene expression. HU treatment did not change IL-1β levels in SCA patients, yet it decreased LTB4 serum levels. LTB4 is an important chemoattractant for neutrophils [43]. Thus, reduced *NLRP3* gene expression and levels of LTB4 may suggest a better prognostic, demonstrating that HU therapy can decrease inflammation by these pathways.

Our previous studies have demonstrated that PBMCs from SCA patients exhibited high expression of *NLRP3* and *IL-1β,* and that cultured AA-PBMC challenged with intact SS-RBC exhibited increased gene expression of components of the NLRP3 inflammasome [8]. The present study observed that the NLRP3 inflammasome is modulated by lysed SS-RBC, as well as by HU treatment. Herein we show that AA-PBMC challenged with lysed SS-RBC presented increased *CASP1* and *IL18* gene expression, as well as increased IL-1β and LTB4 levels; moreover, patients treated with HU showed reduced *NLRP3* gene expression in whole blood-derived mRNA, in addition to lower levels of LTB4. The present study serves to complement a previous report by our group in that PBMCs were presently challenged with lysed SS-RBC, versus intact SS-RBC previously, and the results of each investigation are compared herein.

This study showed novel evidence for the NLRP3-inflammasome platform as an important inflammatory pathway significantly associated with the clinical inflammatory status observed in SCA patients. Interestingly, the production of key proinflammatory cytokines such as IL-1β was not affected by HU therapy. Thus, further studies are required to evaluate the biological association between the products of hemolysis and the DAMP-mediated inflammatory response in SCA. Nevertheless, in view of the findings of the present research, a potential mechanism of action by HU regarding the expression of the NLRP3 inflammasome components and the production of IL-1β and LTB4 is shown in Fig. 5. It is emphasized that the new data obtained in this work can contribute to the identification of new targets for SCA drug development with an emphasis on sickle cell DAMPs and NLRP3-inflammasome-associated molecules.

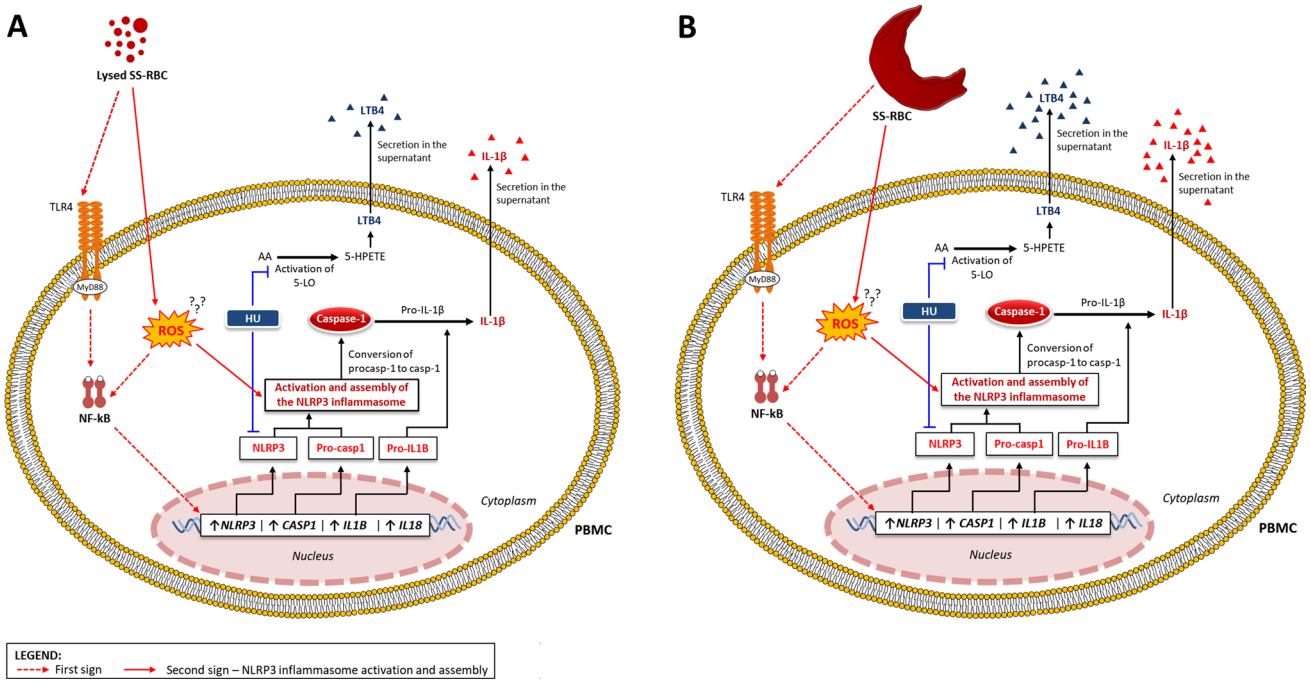

Fig. 5 Mechanistic model of inflammasome activation by SS-RBC and lysed SS-RBC and LTB4 production. Our findings suggest that lysed SS-RBC and SS-RBC may contribute to the activation of the inflammasome in PBMC, probably initiated by the recognition of sickle cell surface components and products released in hemolysis (alarms) by TLR4 (first sign). After initiation, NF-kB activation occurs that induces the expression of NLRP3, CASP1, IL1B and IL18. Then, we propose that the surface components of lysed SS-RBC and SS-RBC can also act as initiators of the second signal, responsible for the assembly and activation of the inflammasome, probably by induction of ROS (not measured) that will reflect IL-1β and IL-18 (not dosed). SS-RBC has been shown to promote the production of LTB4 more pronounced (**a**), compared to lysed SS-RBC (**b**). Leukotriene B4 (LTB4) is produced from arachidonic acid (AA) due to the activation of 5-lipoxygenase (5-LO), responsible for converting AA into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), a precursor to leukotrienes. Hydroxyurea (HU) therapy significantly decreases the production of LTB4 in PBMC but does not significantly reduce the production of IL-1β, although we have observed a significant decrease in the expression of NLRP3

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Author contributions TNP, RRO and MSG: conceived the study design. TNP and SSS: performed all the experiments. DLZ, CCG and RPS: assisted in all experiments. VMLN and IML: responsible for patients' follow-up. VVM, MMA, JRDF, JRF, JBL and GQC: discussion and accomplishment of some experiments. MOSC: assisted in the collection of samples from patients. VMB, RRO and MMA: discussion of study design, experiments and all results. TNP, SSS, RRO and MSG: wrote the paper. The manuscript has been critically reviewed and approved by all authors.

Declarations

Conflict of interest The authors declare no competing interests.

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