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Sickle red cells as danger signals on proinflammatory gene expression, leukotriene B4 and interleukin-1 beta production in peripheral blood mononuclear cell

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ABSTRACT

This study tested the hypothesis that sickle red blood cell (SS-RBC) induce Toll-like receptors (TLR) and Nod-like receptor family, pyrin domain containing 3 (NLRP3)- inflammasome expression in peripheral blood mononuclear cells (PBMC). TLR and NLRP3 inflammasome could contribute to the maintenance of the inflammatory status in sickle cell anemia (SCA) patients, since SS-RBC act as danger signals activating these pathways. In this study, first, we evaluated *TLR (2, 4, 5* and *9), NLRP3, Caspase-1, interleukin (IL)-1β* and *IL-18* expression in PBMC freshly isolated from SCA patients (SS-PBMC) in comparison with PBMC from healthy individuals (AA-PBMC). In the second moment, we investigated whether SS-RBC could interfere with the expression of these molecules in PBMC from healthy donor, in the absence or presence of hydroxyurea (HU) in vitro. TLRs and NLRP3 inflammasome expression were investigated by qPCR. IL-1β, Leukotriene-B4 (LTB_4) and nitrite production were measured in PBMC (from healthy donor) culture supernatants. *TLR2, TLR4, TLR5, NLRP3* and *IL-1β* were highly expressed in SS-PBMC when compared to AA-PBMC. Additionally, SS-RBC induced *TLR9, NLRP3, Caspase-1, IL-1β* and *IL-18* expression and induced IL-1β, LTB_4 and nitrite production in PBMC cultures. HU did not prevent *TLR* and *NLRP3* inflammasome expression, but increased *TLR2* and *IL-18* expression and reduced nitrite production. In conclusion, our data suggest that TLR and inflammasome complexes may be key inducers of inflammation in SCA patients, probably through SS-RBC; also, HU does not prevent NLRP3 inflammasome- and TLR-dependent inflammation, indicating the need to develop new therapeutic strategies to SCA patients that act with different mechanisms of those observed for HU.

1. Introduction

Sickle cell anemia (SCA) is a genetic disorder characterized by the production of abnormal red blood cells, homozygosity of hemoglobin S (HbS) and oxygen transport dysfunction. Clinically, SCA patients can be in a steady state, with subclinical manifestations, or in crisis, showing systemic inflammation, with vaso-occlusive phenomena and painful episodes, as well as susceptibility to infections and hemolysis [1–3]. SCA has been characterized as a chronic inflammatory state, with abnormal activation and elevated number of peripheral blood mononuclear cells (PBMCs),

Abbreviations: AA-PBMC, peripheral blood mononuclear cells freshly isolated from healthy individuals; AA-RBC, red blood cell from healthy individuals; ASC, apoptosis-associated speck-Like protein containing card; DAMPs, damage-associated molecular pattern molecules (DAMPs); HbF, fetal hemoglobin; HMGB1, high-mobility group protein B1; HU, hydroxyurea; LT, leukotriene; LTB4, leukotriene B4; NLR, Nod-like receptors; NLRP3, Nod-like receptor family, pyrin domain containing 3; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; RBC, red blood cell; SCA, sickle cell anemia; SCD, sickle cell disease; SS-PBMC, peripheral blood mononuclear cells freshly isolated from sickle cell anemia patients; SS-RBC, red blood cells from sickle cell anemia patients; TLR, Toll-like receptors.

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endothelial dysfunction and an increased level of multiple inflammatory mediators [4–7].

Endothelial cells, leukocytes and platelets are constantly exposed to reticulocytes, normal and sickle red blood cells. This exposure contributes to the systemic inflammation and prothrombotic phenomena [8,9]. Nitric oxide (NO) is an important molecule involved in the maintenance of vascular homeostasis. Moreover, sickle red blood cells (SS-RBC) induces leukotriene (LT) production, an inflammatory mediator derived from arachidonic acid metabolism, which induces leucocytes migration and activation [10–13]. In addition to increased levels of NO metabolites (nitrite and nitrate), SCA patients have increased levels of LTB_4, even in steady state, which are associated with the severity of clinical outcome, particularly during vaso-occlusion episodes [14–16].

Since the presence of SS-RBC is a typical feature in SCA patients, more investigation is necessary in order to identify its role in the amplification and maintenance of inflammatory immune response within these conditions. Innate immune system is able to recognize danger-associated molecular patterns (DAMPs) by TLRs (Toll-like receptors) and Nod-like receptors (NLR) [7,17]. Since TLRs and NLRs are a family of evolutionary conserved proteins that recognize pathogen (PAMPs) and DAMPs associated molecular patterns, it is expected that these receptors play a critical role in SCA pathogenesis [17–21].

In humans, at least ten TLRs (TLR1 to TLR10) are reported, each of which has specificity for a particular PAMP or DAMP [22]. Several studies have shown association between expression of these receptors and inflammatory conditions [23–25]. In SCA, for instance, high-mobility group protein B1 (HMGB1) and heme are DAMPs that act as TLR4 agonists [18,19,21,26–28]. Other TLR, such as TLR2, TLR5 and TLR9, were recently shown to recognize cellular residues and other endogenous molecules, acting as signals for tissue injury [25,29,30].

Nod-like receptors, in turn, oligomerizes under activation resulting in an inflammatory multi-protein platform, known as inflammasome. HMGB1, iron and heme are the major DAMPs considered as NLR agonists [19,21,31–34]. Classically, inflammasome consists of NLRP (usually NLRP3, Nod-like receptor family, pyrin domain containing 3), inflammatory protease caspase-1 and the apoptosis-associated speck-Like protein containing card (ASC). Then, active caspase-1 converts the inflammatory cytokines IL-1β and IL-18 into active forms [35–41]. High levels of these cytokines have been associated with different clinical inflammatory conditions, such as multiple sclerosis, cancer, Alzheimer's disease, Behcet's disease, arthritis and SCA [7,17,42–48].

Thus, TLRs and NLRP3 inflammasome may play a role in the immunopathogenesis of inflammatory diseases, such as SCA, contributing to its severity. However, these pathways can be responsible for therapeutic failure in regard of inflammation. Hydroxyurea (HU) is the drug approved for treatment of SCA patients, and is a cytotoxic agent that inhibits ribonucleotide reductase enzyme and leads to improvement in levels of fetal hemoglobin (HbF), hydration of RBC and reduction of SS-RBC-endothelial interaction [49,50]. Such effects are associated with improvement of clinical status of the patient and reduction of inflammatory markers [49,51]. However, it is not known whether HU interferes with TLR or NLR signaling pathways.

The role that inflammatory receptors, such as TLRs and NLRs, play in the immunopathogenesis of SCA, as well as the contribution of RBC from SCA patients to the induction of inflammation and the role of HU in this scenario are still not clear. In this study, we evaluated the gene expression of TLR (*TLR2, TLR4, TLR5, TLR9*) and NLRP3-inflammasome components (*NLRP3, caspase-1, IL-1β and IL-18*) in PBMC of SCA patients. Moreover, we investigated the effect of SS-RBC on the expression of these genes and production of IL-1β cytokine, LTB_4 and nitrite in PBMC from healthy controls, as well as in vitro effect of HU in these conditions.

2. Materials and methods

2.1. Study subjects

A cross sectional study was performed to include 12 SCA patients (age 9.1 ± 4.7 years), recruited at the Fundação de Hematologia e Hemoterapia do Estado da Bahia (HEMOBA). All patients were in steady state, characterized by absence of blood transfusion in a period of four months prior to blood draw. In addition, patients included in this study did not show any infection, hospitalization or vaso-occlusive event, and were not under antibiotics, corticosteroids or HU treatments, but all patients were under treatment with folic acid. The control group consisted of 9 healthy individuals (age 12.4 ± 5.6 years) recruited at the Pharmacy College (FacFAR) of Universidade Federal da Bahia (UFBA). This group was characterized by absence of hematological disorders or inflammatory conditions. All procedures followed were in accordance and approved by the Research Ethics Committee of the Fundação Oswaldo Cruz – FIOCRUZ, Brazil; and also with the Helsinki Declaration of 1975, and its revisions. Informed consent was obtained from all patients (or their parents/legal guardians).

2.2. Blood samples and preparation of red blood cells

Venous blood was collected from patients and controls using EDTA to determine hemoglobin profile and to obtain red blood cells (RBC). Briefly, each blood sample was centrifuged at 270*g* for 10 min to discard the platelet-rich plasma (PRP). Leukocytes and PRP were removed by aspiration, and RBC were washed 3 times at 170 g for 5 min with sterile phosphate buffered saline (PBS) (pH 7.3). RBC concentrate were assessed for leukocyte and platelet contamination by staining with trypan blue and then analyzed by using a phase contrast microscope (Olympus CK2, Center Valley, PA, USA).

2.3. Hematological and biochemical analysis

Hematological and biochemical parameters from patients and controls included in this studied was obtained. RBC, platelets, leukocyte, reticulocyte, hemoglobin, fetal hemoglobin, hematocrit, ferritin and C-reactive protein of the SCA patients and controls are shown in Table 1. The mean age of patients and healthy individuals was similar.

Table 1
Hematological and biochemical parameters of steady-state SCA patients and healthy volunteers.

	SCA patients (n = 12) (mean ± SD)	Healthy individuals (n = 9) (mean ± SD)	*p* value*
Age (years)	9.08 ± 4.72	12.44 ± 5.60	0.1864
RBC ($\times 10^6$/mL)	2.99 ± 0.36	4.78 ± 0.41	<0.0001
Platelets ($\times 10^9$/mL)	441.00 ± 118.10	307.10 ± 49.85	0.0056
Leukocyte ($\times 10^9$/mL)	15,178 ± 4474	6767 ± 1716	0.0002
Reticulocyte (%)	7.25 ± 2.05	0.76 ± 0.17	0.0004
Hemoglobin (g/dL)	8.68 ± 1.03	13.23 ± 1.27	0.0004
Fetal Hemoglobin (%)	7.73 ± 6.26	0.48 ± 0.42	0.0007
Hematocrit (%)	25.34 ± 3.53	39.43 ± 3.18	0.0004
Ferritin (ng/mL)	246.30 ± 213.80	36.63 ± 18.53	0.0016
C-reactive protein (mg/L)	5.17 ± 0.67	1.58 ± 1.16	0.0163

SCA sickle cell anemia, *SD* standard deviation, *RBC* red blood cells.
* Mann-Whitney test; values to $p < 0.05$ were considered a significant difference.