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Interleukin-27 and interleukin-37 are elevated in sickle cell anemia patients and inhibit *in vitro* secretion of interleukin-8 in neutrophils and monocytes

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ABSTRACT

Background and objective: Inflammation is implicated in the pathogenesis of most complications seen in sickle cell anemia (SCA) patients. We aimed to evaluate serum levels of two newly discovered anti-inflammatory cytokines (IL-27 and IL-37), and pro-inflammatory cytokines among Brazilian SCA patients that are not on hydroxyurea therapy (HbSS), compared with hydroxyurea-treated patients (HbSSHU) and healthy controls (HbAA). Furthermore, we demonstrated the effect of IL-27, IL-37, and heme on *in vitro* secretions of IL-8 in human neutrophils and monocytes.

Methods: A cross-sectional study of 82 consenting SCA (35 HbSS and 47 HbSSHU) patients in steady state and 49 HbAA consenting individuals. Clinical details were obtained from interviews and medical records. Serum levels of IL-27, IL-37, TGF-β, TNF-α, IL-1β, IL-6, and IL-8 were quantified by enzyme linked immunosorbent assay (ELISA). Neutrophils and monocytes were isolated from healthy controls, and cultured separately with or without cytokines (IL-27 and IL-37) and heme. Supernatant IL-8 concentration was determined by ELISA.

Results: Serum levels of IL-27, IL-37, IL-1β, IL-6, and IL-8 were significantly elevated in HbSS patients compared to HbAA controls. Serum IL-8 levels were significantly higher in HbSS and HbSSHU patients than in controls. IL-27 and IL-37 were positively correlated in both HbSS and HbSSHU patients. *In vitro* IL-8 production by IL-27 and IL-37 pre-treated neutrophils and monocytes was significantly inhibited even after heme addition.

Conclusions: Our findings show that IL-27 and IL-37, as well as the pro-inflammatory cytokines, are elevated in HbSS patients compared with controls, suggesting that the secretion of these anti-inflammatory cytokines is driven by the presence of pro-inflammatory cytokines. This role is probably sufficient in preventing further cellular or tissue damage but not potent enough to prevent inflammation. Therefore, IL-27 and IL-37 may be potential immuno-targets for ameliorating complications associated with elevated heme levels seen in SCA and other hemolytic anemias.

1. Introduction

Sickle cell anemia (SCA) is an inherited disease of global health burden characterized by chronic hemolysis and chronic end organ damage [1,2]. Based on the predominant pathophysiology, complications in SCA patients are classified into hemolytic endothelial dysfunction and vaso-occlusion sub-phenotypes [3]. Chronic inflammatory states in SCA patients are associated with high expression levels of inflammation markers, such as adhesion molecules and cytokines [4]. This results from complex interactions among endothelial cells, erythrocytes, leukocytes, and platelets. Placental growth factor, among other products of hemolysis, activates monocytes to secrete interleukin-8 (IL-8) and other cytokines. These cytokines activate endothelial cells and leukocytes via the NF-kB pathway, resulting in increased expression of endothelial adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin [5]. Eventually, leukocyte adhesion is stimulated and vaso-occlusion is triggered [6]. Several types of cells, such as neutrophils, endothelial cells, macrophages, and fibroblasts, produce IL-8, a CXC chemokine family member that is involved in neutrophil recruitment and function, endothelial cell proliferation, and angiogenesis [7–9].

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Tumor necrosis factor-α (TNF-α) is mainly produced by monocytes, macrophages, and other cells, such as T-cells, smooth muscle cells, adipocytes, and fibroblasts, and stimulates necrosis in tumors [8,10]. IL-8 and TNF-α induce increased adhesion of erythrocytes and leukocytes to the vascular endothelium, and this adhesion can cause vaso-occlusive crisis (VOC) and local hypoxia [11].

Several studies have shown that SCA patients display higher levels of IL-8 as well as other cytokines during VOC and steady state compared with controls, but some studies did not find statistical differences [11–18]. Results with the use of hydroxyurea (HU) are not clear-cut as some researchers found increased, decreased, or unchanged levels of IL-8 and TNF-α levels in HU-treated patients [12,15,18]. Higher plasma concentrations of IL-1β was observed during steady state and painful crises than in control subjects, and was shown to be a good predictor of stroke outcome in SCA patients [19,20]. This increase in pro-inflammatory cytokines leads to an imbalance between pro- and anti-inflammation, with the pro-inflammatory pathways being favored. Therefore, endothelial activation is worsened, and the vicious cycle continues. The pattern of alteration of plasma anti-inflammatory cytokines in SCA patients are not clear. Previous studies have shown that IL-10 and IL-4 may be higher, lower, or unchanged in SCA patients compared to controls [15,21–23].

Only a handful of studies have investigated the role of anti-inflammatory cytokines in SCA, and there are no SCA studies on two recently characterized anti-inflammatory cytokines (IL-27 and IL-37). However, recent works have described the importance of IL-27 and IL-37 in the modulation of chronic inflammatory disease [24–26]. IL-27 of the IL-12 family and IL-37 of the IL-1 family, have received special attention because of their potent anti-inflammatory properties and potential therapeutic use in patients with atherosclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis, ankylosing spondylitis, and others [24,25,27–29]. Dendritic cells and macrophages produce IL-27 after stimulation by TLR2, TLR4, and TLR9 [30,31]. Naive CD4+ T cells in the presence of IL-27 or TGF-β differentiate into IL-10 producing regulatory T cells subset 1 (Treg1) cells, potent suppressors of inflammation. In addition, IL-27 can inhibit the differentiation of naive CD4+ T cells in Th17 and/or Treg Foxp3+ [32]. IL-27 has a potential inhibitory role in some inflammatory diseases such as colitis, multiple sclerosis, atherosclerosis, autoimmune disorders, and others [33–35]. Most of these studies have focused on the role of IL-27 in regulating adaptive immune cells, but researchers have recently demonstrated that IL-27 and IL-37 can modulate the functions of neutrophils by suppressing neutrophil activation and thereby production of pro-inflammatory cytokines [36,37]. The biological role of IL-37 is still unclear, but its marked ability to inhibit inflammation is worth evaluating in SCA [38,39]. Expression of IL-37 in macrophages and epithelial cells decreases secretion of pro-inflammatory cytokines, whereas silencing of the IL-37 gene in human cells leads to a higher expression of these cytokines [38]. Based on the potential anti-inflammatory roles of IL-27 and IL-37 in chronic inflammatory disorders and the lack of studies evaluating their role in SCA, this study evaluated the serum levels of IL-27, IL-37, and other cytokines in Brazilian SCA patients and normal controls. Additionally, we demonstrated the effect of IL-27 and IL-37 on *in vitro* production of IL-8 in monocyte and neutrophil cultures stimulated with heme.

2. Materials and methods

2.1. Study participants

A cross-sectional study composed of 82 adult SCA patients on follow up at the Hematology and Hemotherapy Center of Pernambuco (HEMOPE), Recife. Control group consisted of 49 healthy adults from the same region and with the same ethnic characteristics. Patients who had been previously diagnosed hemoglobin SS (HbSS) by alkaline electrophoresis, high performance liquid chromatography (HPLC), and solubility tests were included, if in steady state. Steady state was defined as a stable clinical state for 3 months without signs or symptoms of infection or pain, other acute episodes suggestive of crisis, or blood transfusions. Individuals exhibiting any of the following were excluded: pregnancy, infections, acute or chronic inflammatory disease or the use of immunosuppressants in the last two weeks, and malignancies and SCA in acute crisis. Participants who fulfilled the above criteria were grouped as follows: HbSS, SCA patients who were not taking hydroxyurea; HbSSHU, SCA patients on hydroxyurea; hemoglobin AA control group (HbAA controls), hemoglobin AA individuals without' SCA or sickle cell trait.

All participants signed the consent form and the questionnaire was filled adequately from interviews and medical records. Some of the details obtained based on the questionnaire were bio-data and complications suffered from SCA. Venous blood (5 mL) was collected into plain sample tubes from each patient and control, in addition to routine blood samples for complete blood counts. Samples were centrifuged and the sera were stored in aliquots at −80 °C until analyzed. When collection was complete, the samples were then transported in dry ice to State University of Campinas (UNICAMP), Campinas, southeast Brazil and stored at −80 °C until cytokine assays.

The study was approved by the ethical committees of both UNICAMP, Campinas and HEMOPE, Recife (CAAE: 52941315.6.0000.5404) in accordance with Declaration of Helsinki of 1975 (revised in 2000), and all participants gave written consent.

2.2. Hematological analysis

Hematological analysis was performed using an automated cell counter (CELL-DYN Ruby analyzer, Abbott Diagnostics, abbott park, IL). Hemoglobin S, F, and A2 were determined using HPLC (BIO-RAD variant II, Hercules, CA).

2.3. Quantification of serum concentrations of pro- and anti-inflammatory cytokines

Serum levels of IL-1β, TNF-α, IL-6, IL-8, TGF-β, IL-27, and IL-37 were quantified by enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN and Biolegend, San Diego, CA) according to manufacturer's instructions.

2.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected from 5 healthy controls (80–100 mL) into sodium heparin tubes. Samples were centrifuged in Ficoll-Hypaque solution (GE Healthcare Piscataway, NJ) (density 1.077; 400 ×*g* for 30 min at 20–22 °C) and the mononuclear cells (in the interface layer of plasma and Ficoll-Hypaque solution) were transferred to new 15 mL falcon tubes. PBMCs were washed twice using RPMI 1640 medium (300 ×*g* for 10 min at 4 °C) and re-suspended in supplemented RPMI 1640 medium (Invitrogen Co, Grand Island, NY) (10% fetal bovine serum, L-glutamine [2 mM]. gentamicin [5 μg/mL], and sodium pyruvate [1 μM]). Cells were quantified and subjected to monocyte isolation. After the plasma, PBMCs and Ficoll-Hypaque solution had been removed from the peripheral blood, the buffy coat layer over the red blood cell layer was transferred to a 15 mL falcon tube for neutrophil isolation.

2.5. Isolation of monocytes

Monocytes were isolated using the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. PBMCs were re-suspended at a concentration of 1×10^7 cells in 80 μL of phosphate-bufferred saline-B-E buffer (PBS-BSA [0.5%] and EDTA [2 mM]) and incubated for 10 min at 4 °C with a mixture of biotinylated antibodies against CD16 (neutrophils and NK