Evaluation of Th17 related cytokines associated with clinical and laboratorial parameters in sickle cell anemia patients with leg ulcers

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Abstract

Leg ulcers (LUs) represent one of the main causes of morbidity in sickle cell anemia (SCA). This manifestation has been related to hemolysis, infections predisposition and inflammation that leads cytokines secretion. In this context, our study aimed to evaluate Th17 related cytokines (IL-6, IL-17A, IL-22 and IL-23) in serum and peripheral mononuclear cells culture supernatants with and without lymphoproliferative stimulation (anti-human CD3 and anti-human CD28). The cytokines levels were also correlated to clinical, hematological and biochemical parameters in SCA patients with and without LUs history (SCALU and SCAWH) as well as in healthy controls. In SCALU patients, high levels of IL-17A were associated with absence of acute chest syndrome (ACS, p = 0.0328). The other clinical parameters analyzed (osteonecrosis, stroke, splenectomy and blood transfusions history) were not significantly related with other cytokine levels. In SCALU patients may exert a preventive role in the ACS development. Furthermore, IL-6, IL-17A and IL-22 accompanied the LDH levels only in SCALU patients suggesting to serve as additional markers of hemolysis or to be related with immunity response against extracellular pathogens.

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1. Introduction

The leg ulcers (LUs) are a debilitating condition in sickle cell anemia (SCA). This manifestation represents one of the most important causes of morbidity in SCA patients [1]. The LUs usually develops in SCA between 10 and 30 years old and with more prevalent in African countries [2]. These ulcers usually appear in the ankle region and are characterized by slow healing, frequent local pain and a high number of recurrences [3]. The pathophysiology of LUs in SCA patients remains unclear. Mohan and coworkers argue that the cause is due to venous insufficiency in the regions where ulcers develop, preventing normal blood flow, causing hypoxia and tissue infarction [4].

Acute chest syndrome (ACS), osteonecrosis, priapism and stroke represent complications in SCA [5] being ACS the second most common cause of hospitalization in SCA patients [6]. The ACS is characterized by fever, leucocytosis, and respiratory symptoms leading to chronic lung damages [7–9]. These clinical manifestations are involved in two main processes in sickle cell disease: hemolysis and vascular occlusion [10]. Hemolysis leads to cardiovascular, pulmonary, gastrointestinal and renal manifestations [11]. Besides that, leg ulcers were also related to the intensity of hemolysis [12]. These events involve vascular occlusion and inflammatory process that involve many cell types, inflammatory mediators, cell adhesion molecules, and the presence of cytokines and chemokines which recruit cells to inflammatory sites, and induce immune responses [13–15]. IL-17A is the hallmark cytokine of Th17 cells and has been shown to function as a proinflammatory cytokine that upregulates...
a number of chemokines and matrix metalloproteases, leading to the recruitment of neutrophils into sites of inflammation [16]. IL-22 is a family type II cytokine that is preferentially expressed by differentiated Th17 cells [17] and thus considered as a Th17 cytokine, but it is also expressed by other cells like CD8⁺ T cells and CD11c⁺ dendritic cells as showed by Zheng and coworkers [18]. Overall, Th17 cells produce multiple effector cytokines with partially overlapping roles in immunity. IL-23 can also directly promote Th17 differentiation by increasing RORγt expression in combination with IL-1β and IL-6 [19]. IL-23 is a novel member of the IL-12 cytokine family and it is composed by a unique p19 subunit and an identical p40 subunit to IL-12 whereas IL-12 drives the classical Th1 response [20,21].

Most cytokine studies in SCA have evaluated cytokines produced by Th1 cells (IFN-γ, IL-1, IL-2, IL-12) that are involved in innate immunity [22–24] and produced by Th2 cells (IL-4, IL-5, IL-13) in humoral immunity [25,26]. On the other hand, there are few studies associating clinical profiles with other cytokines pathway such as Th17 [27,28]. Th17 cells differentiation is driven by TGF-β and IL-6 [29,30] and is reinforced by IL-23 [31,32]. These cells mainly secrete IL-17 (A and F) but also produce IL-9 [33], IL-21, IL-22, IL-26, and CCL20 [34].

These mediators are responsible for a number of effector functions in host defense and in several autoimmune diseases [35]. Due to the lack of studies in this context, our study analyzed if the cytokines IL-6, IL-17A, IL-22 and IL-23 were related to clinical parameters in SCA patients with LUS. For this reason, we evaluated hematological and biochemical markers to identify whether there is a relation between the Th17 related cytokines and the laboratory parameters in these patients.

2. Materials and methods

2.1. Ethical approval

Ethics approval was obtained from ethical committee from Health Science Center of Federal University of Pernambuco and Hematology and Hemotherapy Foundation of Pernambuco (protocols 483/10 and 57/10 respectively).

2.2. Patients

All SCA patient (HbSS) blood samples were obtained from January 2011 to June 2012 during regular clinical visits at the Hospital of Hematology and Hemotherapy Foundation of Pernambuco (HEMOPE), Brazil. The SCA patients were divided into two groups: patients with active LUs (SCALU) and without LUs history (SCAWH). Healthy individuals were used as controls and also collected in the same place totaling three groups. Informed written consent and the clinical questionnaire were obtained from all patients and controls. The inclusion criteria for all patients were at a steady-state condition and the exclusion criteria was to show no other systemic diseases that could have potentially altered their inflammatory profile functions and did not receive transfusions in less than 3 months or hydroxyurea therapy. All parameters used in the study were confirmed by clinical history contained in the medical records of the institution.

2.3. Isolation and culture conditions for peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from fresh peripheral blood collected in sodium heparin tubes, following an adapted method described previously by English and Andersen [36]. Briefly, whole blood was laid over one layer of Ficoll–Paque Plus (density gradient of 1.077 g/L, GE Healthcare, Little Chalfont, UK) and centrifuged at 400 g for 45 min, cells were washed once in PBS (pH 7.4) and washed once more in PBS. RBC lysis buffer was used for contaminating red cells for 10 min at 4 °C (Ebioscience, San Diego, CA, USA). PBMCs were incubated at 37 °C for 48 h under two conditions: unstimulated cells and stimulated cells with anti-human CD3 and anti-human CD28 (anti-humanCD3 + anti-humanCD28, Ebioscience, San Diego, CA, USA).

2.4. Cytokines measurements in serum and PBMCs culture supernatants

Serum was obtained from blood samples that were collected and centrifuged at 1258g for 15 min, and stored at −80 °C. Commercially available ELISA kits were used to determine IL-6 (BD Biosciences, NJ, USA), IL-17A, IL-22 and IL-23 (Ebioscience, San Diego, CA, USA). These cytokines were evaluated on culture supernatants of PBMCs incubated during 48 h with or without stimulation with anti-humanCD3 + anti-humanCD28 (Ebioscience, San Diego, CA, USA). The ELISA plates were read at 450 nm and 570 nm (EL808, Biotek, VT, USA). Following the detection limits of each kit: IL-6 (4.69 pg/ml), IL-17A (3.91 pg/ml), IL-22 (7.81 pg/ml), IL-23 (15.62 pg/ml).

2.5. Blood samples, hematological and biochemical evaluation

Peripheral blood samples obtained from EDTA tubes were analyzed for complete blood count (STKS, Coulter) and reticulocyte count. Sera obtained tubes were used to analyze serum levels of indirect bilirubin and lactate dehydrogenase (Cobas Integra plus 400, Roche).

2.6. Statistical analysis

Mann–Whitney test was used to correlate cytokines levels with clinical parameters using Graphpad Prism software (Version 5.0). The Spearman correlation test was used for hematological and biochemical evaluation associated with cytokines levels using Origin software (Version 8.0). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Th17 related cytokines levels in serum and PBMCs culture supernatants

First of all, we evaluated IL-6, IL-17A, IL-22 and IL-23 cytokines levels in serum patients. Only a few SCALU and SCAWH patients had cytokine levels above the kit’s detection limit (Supplementary Fig. 1). All the three analyzed groups were not significantly different regarding their cytokine levels when comparing each other (p > 0.05). Because of these results, the association of serum cytokines levels with clinical, hematological and biochemical parameters became unfeasible. In this sense, our analysis were focused on PBMC culture supernatant. The IL-17A levels of stimulated cells were significantly higher in SCAWH group than controls (0.0195). We also observed the IL-17A high levels in SCALU group compared with controls but they were not significant. The IL-6, IL-22 and IL-23 levels was no statistical significant in either group (Fig. 1).

3.2. Correlation between Th17 related cytokine levels with clinical profile

The levels of IL-6, IL-17A, IL-22 and IL-23 were correlated to: acute chest syndrome, osteonecrosis, priapism, stroke, splene-
tomy and history of transfusions. Firstly, we determined the characterization of SCA patients (Table 1). Then we compared the possibility of associations within SCALU and SCAWH groups. In SCALU patients high levels of IL17A were associated with absence of acute chest syndrome ($p = 0.0328$). However IL-17A levels were also more expressed in SCAWH than SCALU patients with ACS historic ($p = 0.0483$). The associations of IL-17A with other parameters were not significant (Table 2). The ACS was confirmed in all cases by a new pulmonary infiltrate on Chester X-ray together with SPO2. The IL-6, IL-22 and IL-23 levels were not significantly associated with any clinical parameters (Supplementary Tables 1 and 2).

3.3. Correlation between supernatant Th17 related cytokine levels with laboratorial parameters

The Th17 related cytokines were correlated with value of Hb (total hemoglobin), WBC (leukocyte count), Ret (reticulocyte count), LDH (lactate dehydrogenase) and IB (indirect bilirubin). Only in SCALU patients was observed that increased levels of LDH were positively correlated with IL-6 ($p = 0.0191$, $r^2 = 0.287$), IL-17A ($p = 0.0130$, $r^2 = 0.419$) and IL-22 ($r^2 = 0.168$) (Fig. 2). The IL-23 had no positive correlation with any laboratorial parameter (data not shown). These laboratorial markers are always significantly altered comparing sickle cell anemia patients with normal controls.

4. Discussion

The classical view of sickle cell anemia has been focused on the primary genetic defect that lead to hemoglobin polymerization causing red cells deformity, becoming rigid, obstructing blood flow, and producing acute and chronic tissue damage because of poor perfusion [37]. A more complete view that allows that the sticky, stiff, oxidizing sickle red cell is an irritant process that not only obstruct blood flow but also provokes an inflammatory response are mandatory [38]. Serum markers of inflammation have provided evidence for a state of chronic inflammation in SCA patients [39]. Inflammation promotes endothelial adherence to sickle erythrocytes, leukocytosis, in the absence of infection, common in SCA patients and predicts for stroke, acute chest syndrome, and overall mortality [61].

Our results Th17 related cytokines correlation showed no differences between detecting levels of IL-6, IL-17A IL-22 and IL23 in the serum of SCA patients and controls. In this latter case our results could be related with the nature appearing location of leg ulcers or the biological life time of these cytokines [40]. Levels of IL-17A obtained from stimulated cells could point to new hypotheses evaluation, mainly about the possible involvement of this cytokine with SCA events [27]. We associated the Th17 related cytokine levels in relation to the clinical history of each SCA group.

Vilas-Boas and coworkers [41] did not demonstrate correlation of IL-4, IL-17, IL-23 and TGF-β with clinical events in SCA patients

Table 1

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>SCALU (n = 24)</th>
<th>SCAWH (n = 42)</th>
<th>Control (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%Yes)</td>
<td>n (%No)</td>
<td>n (%Yes)</td>
</tr>
<tr>
<td>Male/female</td>
<td>13/11</td>
<td>20/22</td>
<td>10/10</td>
</tr>
<tr>
<td>Age</td>
<td>35 (21–59)</td>
<td>31 (18–50)</td>
<td>34 (21–58)</td>
</tr>
<tr>
<td>Osteonecrosis</td>
<td>8 (33)</td>
<td>6 (14.2)</td>
<td>36 (85.8)</td>
</tr>
<tr>
<td>Acute chest syndrome</td>
<td>7 (21.2)</td>
<td>18 (43)</td>
<td>–</td>
</tr>
<tr>
<td>Priapism</td>
<td>5 (4.1)</td>
<td>3 (17)</td>
<td>–</td>
</tr>
<tr>
<td>Stroke</td>
<td>1 (0.2)</td>
<td>3 (7.2)</td>
<td>39 (92.8)</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>2 (8)</td>
<td>1 (12)</td>
<td>41 (98)</td>
</tr>
</tbody>
</table>

* The priapism percentage was considered only for men. The values represents number of patients (%) except Male/Female value are means (min–max).
in steady state. Our association between IL17A with clinical parameters showed an interesting result. Only in SCALU patients, the increased level of IL-17A was related with ACS. The ACS affects SCA patients older than 2 years being considered one of the most frequent complications and major cause of morbidity and mortality in SCA [7]. Data from the Clinical Course of Sickle Cell Disease Cooperative Study indicates that the ACS occurs in 10.5% of patients per year [8] where infectious agents are responsible for more than one third of cases. Extensive antibiotic therapy is usually used in this case, even when the infection does not have a clear etiology [42]. IL-17 and IL-22 synergistically act to augment the expression of genes involved in the defense against microbial pathogens such as the β-defensin2 gene in keratinocytes [17]. For example, an analysis of mycobacteria-specific Th17 cells in exposed humans revealed that most of these cells expressed either IL-22 or IL-17 but not both cytokines [43]. Consistent with this hypothesis, mice with targeted deletion of IL-17RA have significant defects in host defense. IL-17Rs signaling is critical for G-CSF and CXC chemokine production, and IL-17R deficient mice exhibit a delay in neutrophil recruitment into the alveolar space hindering the immune response that would allow the destruction and expulsion of pathogens [44]. These data suggest that decreased levels of IL-17 could favor the risk to infection. Our associations compared to clinical parameters indicate that IL-17A may exert a preventive role in the development of ACS related to pathogens. Because the results were only found among patients with leg ulcers, possibly the action of related Th17 cytokines is antimicrobial due to predisposing factor of leg ulcers to local infections [45].

Taylor et al. hypothesized that the degree of hemolysis is a key determinant influencing a phenomic spectrum of complications that reflect the severity of sickle vasculopathy. In sickle cell disease two thirds of hemolysis occur extravascularly, the remaining one third of red cells hemolyze intravascularly. Along with reticulocyte count, indirect bilirubin level, and serum haptoglobin, LDH has been used as a marker of hemolysis. Serum LDH is also usually elevated in sickle cell anemia in the steady state. This robust hemolytic rate increases even more during vaso-occlusive pain crisis (VOC) [46]. Our results reveal that the LDH levels positively correlated with IL-6, IL-17A and IL-22 being favorable for hemolysis. This hemolysis will lead to ischemic events involving interactions between erythrocytes, leukocytes and endothelium cells [15]. Although the levels of hemoglobin, reticulocytes, leukocytes and bilirubin are abnormal when compared to healthy controls, our results indicated that there was no positive correlation related to IL-17A and IL-22 levels.

It is possible to define the presence of pathogens is strongly related to the formation of biofilms. This colonization of bacteria predominantly characterize chronic wounds, with the presence of inflammatory exudate, poor healing and chronic ulcers, present in the group with sickle cell leg ulcers [47,48]. Due to the presence of cytokines related to Th17 only in SCALU patients, there may be an association between these cytokines and the role of immunity against extracellular pathogens in these patients. Thus our data together shows that IL-17A and IL-22 cytokines act as biomarkers of clinical and hematological changes in patients with ACS points out some differences between SCALU and SCAWH patients that could be correlated with different clinical and biological behavior of these patients.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.11.012.

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