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Evaluation of immunological role of Interferon gamma, Interleukin-10 and CD4$^+$ T lymphocytes in pediatric patients with sickle cell disease

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Abstract:

Sickle cell disease (SCD) is a chronic inflammatory disease associated with increased levels of multiple cytokines as interferon gamma (IFN-γ) during vaso-occlusive (VOC) crisis and steady state. Therefore, we aimed to explore the immunological role of serum IFN-γ, interleukin-10 (IL-10) and CD4$^+$ T lymphocytes in children with SCD at steady state and VOC. This study was conducted on 30 children with SCD (15 at steady state and 15 in VOC) and 30 healthy children as controls. Detailed history, complete physical, clinical examination and laboratory investigations as: complete blood count, serum ferritin, IFN-γ, IL-10 and CD4$^+$ T lymphocytes for all patients were recorded. **Results:** Significant increase in serum IFN-γ in patients with SCD during VOC compared with patients at steady state and controls (P<0.001). Significant increase in serum IL-10 in patients with SCD at steady state compared with those during VOC and controls (P=0.008 and P=0.04 respectively). CD4 $^+$T lymphocyte decreased in patients with SCD during VOC compared with those at steady state and controls (P<0.001). There was a significant positive correlation between serum IFN-ɣ and number of crisis per year (r=0.648, P=0.0001). Serum IFN-γ was increased in patients with history of frequent crisis per year (P=0.001) and decreased in patients under hydroxyurea therapy (P<0.001). IFN-γ was increased in SCD patients. Increased IFN-γ production in SCD suggesting that functionally activated natural killer cells reflecting a host immunological mechanism leading to antigen antibody activation in SCD. Serum IL-10 increased in SCD patients at steady state. CD4 $^+$T lymphocyte decreased in patients during VOC.

Keywords: Sickle cell disease, vaso-occlusive crisis, interferon gamma, Interleukin-10, CD4 $^+$ T lymphocytes.

1. Introduction

Sickle cell disease (SCD) is an autosomal recessive disorder that associated with production of abnormal hemoglobin S [1]. SCD occurs as a result of point mutation which leads to hemogloninopathy that occurred due to changes in the structure of

amino acids sequence on the beta globin chain of the hemoglobin inducing polymerization of HbS molecules within the red cells due to substitution of glutamate by valine which causes sickling [2]. Painful VOC, recurrent infections and chronic inflammation are common in SCD. $CD4^+$ T cells differentiate into TH1 and TH2 cells in the early stages of any infection. TH1 cells induce cytotoxic $CD8^+$ T cells, leading to cytokines production as interleukin-2, interleukin-12, TNF-*γ* and interferon gamma (IFN-*γ*); and TH2 cells produce cytokines such as IL-4, IL-6 and IL-10. TH1 and TH2 cells play distinguished roles in SCD [3,4]. IFN-*γ* was produced by $CD4^+$ and $CD8^+$ T cells after activation by natural killer (NK) cells. IFN-*γ* has an immune-regulatory functions that involve mononuclear phagocytes activation, class I molecules of the major histocompatibility complex (MHC-I) up regulation, NK cell cytolytic activity stimulation and neutrophils activation [5]**.** IFN-γ has different roles in the innate and adaptive immune responses. IFN-γ function has many effects in host defense and immune regulation, including antiviral, antimicrobial and antitumor activities [6]**.** SCD is associated with higher levels of multiple cytokines during VOC and steady state [7]**.** These pro-inflammatory cytokines listed as TNF-α, IL-1β and IL-6, and extended to IFN-γ and IL-17A referred to as IL-17, that is produced by conventional Th1, Th17 $CD4^+$ T cells, and innate-like T cells that activate T cell and macrophages to stimulate cellular immunity and inflammation [8]**.** Activated CD8+ cells produce IL-10. Other types of cells can produce it, as activated TH0, TH1 and TH2 cells, B lymphocytes, and mast cells. IL-10 synthesis is inhibited by itself and IL-4 [9]. IL-10 is an anti-inflammatory cytokine leading to inhibition of various cytokines synthesis, such as IL-1, IL-6, IL-8, IL-12, and TNF-*α* to enhance the iron uptake and retention within the reticuloendothelial system and monocytes. IL-10 inhibits TH1 cells proliferation, decreasing secretion of TH1 cytokines and cytolytic function and facilitating a TH2 response development [10]**.** Therefore, we aimed to explore the immunological role of IFN-γ as a regulatory cytokine, IL-10 and $CD4^+$ T lymphocytes in children with SCD in steady state and VOC.

2. MATERIALS AND METHODS

Design:

This cross sectional study was conducted on the period from August 2019 to July 2020, and included 30 SCD (HbSS) patients (15 patients in steady state and 15 presented in VOC, they were under treatment with hydroxyurea and 30 apparently healthy controls, age and gender matched to the patients. They were recruited from pediatric hematology unit, Menoufia University hospital. The study protocol was approved by the ethical committee of the Faculty of Medicine, Menoufia University and a written informed parental consent was obtained. All patients were subjected to detailed history, complete physical, clinical examination. Laboratory investigations were done for all studied subjects including: complete blood count (CBC), serum ferritin, serum lactate dehydrogenase (LDH), IFN-γ and serum IL-10 by ELISA. $CD4^+$ T lymphocytes by flow cytometry.

Inclusion criteria:

Children with SCD diagnosed by hemoglobin electrophoresis. SCD children (HbSS) presented in VOC and steady state.

Exclusion criteria:

Patients that presented with hepatitis B or C infection were excluded.

Sample collection and assay

Five ml of whole blood were obtained under complete aseptic conditions from patients before blood transfusion and from controls, and divided into aliquots. Two ml of blood were put into EDTA containing tubes for hematological analysis of CBC and flow cytometry. Three ml of blood were left 15 minutes to clot, and then centrifuged at 3000 rpm for 10 minutes. Sera were separated and collected in 2 tubes. One for measurement of serum ferritin using automated Analyzer (Cobas e604 –Roch).

The other one was stored at -20 C for measurement of IFN-γ and IL-10 levels.

All frozen samples were thawed at room temperature (18-25^{o} C) at the time of assay.

Interferon gamma assay:

Samples were assayed for human IFN-γ in serum with double antibody sandwich (ELISA) kits ((RayBio,Catalog#:ELH-INFG,UM). In Standard wells: 50 µl of each standard were added. In test wells, 40 µl of sample were added. Fifty µl of prepared streptavidin -HRP solution were added to each standard and test wells, then 10 µl of IFN- γ –antibody added to each well microtiter ELISA plates. The plates were incubated at 37^{o} C for 1h. The plates were then washed five times with wash buffer. Then, 50 µl of chromogen solution A, and 50 µl of chromogen solution B were added to each well (color of the liquid changes into the blue) and incubated at dark for 10 min at 37^{o} C.Then 50 µl of stop solution were added to each well. (The blue changes into yellow immediately). Scanning of plates by a microplate reader set at the appropriate wave length for the color forming reaction (optical density at 450 mm). The chroma of color and the concentration of human IFN-γ of sample were positively correlated.

Interleukin-10 assay:

Samples were assayed for IL-10 in serum with double antibody (ELISA) kits (RayBio,Catalog#: ELH-IL 10, UM). All reagents, samples and standards were prepared and 100 µl of each standard and sample were added to each well microtiter ELISA plates pre-coated with monoclonal antibodies to human interleukin-10. The plate was incubated at room temperature for 2.5 hours, then washed 4 times with wash buffer (300 µl) and incubated for 1 hour at room temperature after adding 100 µl prepared biotin antibody. Then, 100 µl of prepared streptavidin solution we added to each well. The plate was incubated for 45 minutes at room temperature. Detection of bound enzyme by incubation in dark for 30 minutes after adding 100 µl tetra-methyl-benzidine (TMB) one-step substrate reagent to these wells. Finally 50 µl stop solution were added. We scanned plates by a microplate reader set at wavelength 450 mm.

The standard curve linear regression equation was calculated according to standards' concentration and the corresponding OD values (the standard density as the horizontal and the OD value for vertical), and the OD values of the samples were applied on the regression equation to calculate the corresponding sample's concentration.

Flow cytometric analysis of CD4 and CD3 expression:

Double-labelling method with monoclonal antibody to $CD4^{+}$ and $CD3^{+}$ was used.

(a)Monoclonal antibodies:

1- Fluorescein isothiocyanate (FITC) conjugated monoclonal anti-human $CD4^{+}$ (Catalog # 11-0049-42, eBioscience™, USA).

2- Phycoerythrin (PE) conjugated monoclonal anti-human $CD3^{+}$ (Catalog # 12-0037-42, eBioscience™, USA).

(b) Sample staining: first tube for detection of CD4 and CD3 expression on lymphocytes, and second tube auto control was prepared. Samples were analyzed within 24h from sampling. optimized amounts of fluorochrome-conjugated antibodies; CD4-FITC, CD3-PE were added to blood sample. Then, erythrocyte lysis was performed by lysis solution (Dako Uti-Lyse™) which consists of two reagents. Reagent A lyses RBCs to avoid their interference. Reagent B slows the lysing reaction to prevent WBCs damage. 100 µl of anticoagulated (EDTA) whole blood was added to the bottom of a 12 x 75 mm polystyrene tube along with 5µl of CD3 and 5µl of CD4 in tube, vortex and incubation at room temperature for 40 minutes in the dark was done. 100 µl of reagent A was added to sample, vortex and was incubated for 10 minutes in dark. - 1 mL of reagent B was added to sample, vortex and was incubated for 10 minutes in dark. - Unstained samples were included in second tube as controls.

(c) Flow cytometric analysis:

FCM analysis was performed using Partec CyFlow® Space Flow Cytometer, gating was done on lymphocytes (R1) on the basis of side scatter versus forward scatter (SSC/FSC). T lymphocytes subset by flow cytometry in SCD at steady sate, VOC and healthy control shown in **(Fig. 1, 2, 3).**

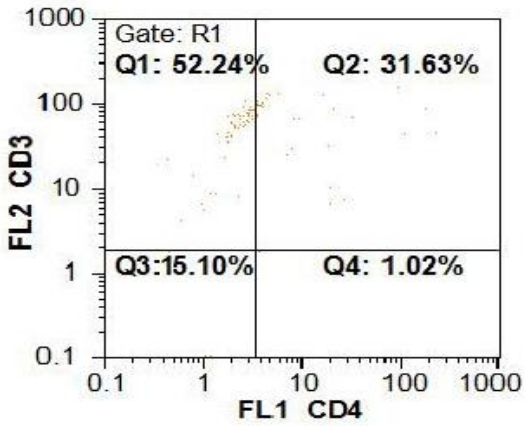

Figure 1. T-lymphocyte subsets of normal healthy control**.**

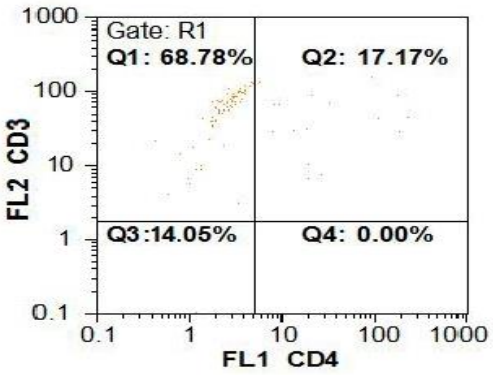

Figure 2. T lymphocyte subsets of SCD patients at steady state**.**

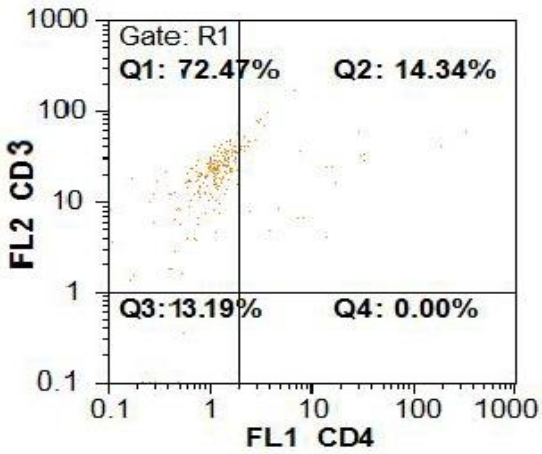

Figure 3. T lymphocyte subset of SCD Patients in VOC

Sample size calculation:

The sample size was relied upon 95% CI with 80% power, using unpaired t-test and assuming a (two-sided) α of 0.05. Based on previous study (El-Alfy et al., 2018) [11], Mean of CD4+ T lymphocytes (%) for SCD patients group was 34.41 while that of healthy control group was 30.0 and SD was 6.1 (of patients or control) with group size ratio of 1/1, the number of participants was 30 for each group.

Statistical Analysis:

Data were analyzed using statistical package for social sience (IBM SPSS) version 20 statistics (SPSS Inc., Chicago, USA). The relation between qualitative variables was done using Chi-square test. Student t-test or Mann-Whitney test used for comparison between two groups having quantitative data. Pearson's correlation coefficient or Spearman correlation was used to test correlation between numerical variables. Multiple linear regression was applied to determine relationship between dependent variable (INF, IL10 or $CD4^+$ T lymphocyte) and independent variables (other laboratory variables). A *p*-value < 0.05 was considered significant.

3. Results:

Demographic, clinical and hematological characteristics of studied groups were illustrated in **(Table 1)**. As regards hematological data, patients with SCD had lower hemoglobin, higher WBCs, neutrophil count, lymphocyte count, serum ferritin compared with controls (P<0.001). Immunological data **(Table 2)** demonstrated significant increase in IFN-γ in SCD patients in VOC compared with SCD patients at steady state and controls (P<0.001), SCD patients at steady state showed significant increase in IFN-γ compared with controls (P<0.001). Serum IL-10 showed significant increase in SCD patients at steady state compared with SCD patients in VOC and controls (P=0.008 and P=0.04 respectively) but no significant difference between IL-10 in SCD patients in VOC compared with controls. $CD4^+$T lymphocyte showed significant difference in SCD patients in VOC as it was decreased compared with SCD patients at steady state and controls (P<0.001) **(Table 2)**. There was significant correlation between serum IFN-ɣ, IL-10 and $CD4^+$T lymphocyte as regard serum ferritin (r=0.716, P=0.0001, r=-0.413, P=0.02, r=-0.821, P=0.0001 respectively), hemoglobin level and ANC. There was significant correlation between serum IFN-ɣ, and $CD4^+$T lymphocyte as regard WBC and significant correlation between serum IL-10 and absolute lymphocytes. Also, there was significant positive correlation between serum IFN-ɣ and number of crisis per year (r=0.648, P=0.0001) and frequency of blood transfusion. No significant correlation was reported between serum IFN-ɣ, IL-10 and $CD4^+$T lymphocyte as regard to age **(Table 3)**.

Multivariable linear regression analysis showed that IFN-ɣ was the significant dependent variable that contributes to increased number of crisis and blood transfusion per year **(Table 4)**. Serum IFN-γ was increased in patients with history of frequent crisis per year (P=0.001) and decreased in patients under good compliance of hydroxyurea therapy (P<0.001) **(Table 5).**

4. Discussion:

Alterations in immune phenotype and function of lymphocytes lead to increased inflammatory conditions in patients with SCD [12]. NK cells and IFN-γ levels in children with SCD evaluated through their relation to hemolysis, VOC and response to therapy [13].

Regarding laboratory data, the current study showed elevated WBCs and neutrophil counts in SCD at steady state and in VOC compared with controls. Musa et al., (2010) illustrated WBCs and neutrophil count elevation in SCD patients at steady state and in VOC compared with healthy controls and explained that by chemotactic stimuli due to infection in these patients [14]. El-Alfy et al., (2018) showed elevated WBCs, neutrophil, and lymphocytes count in patients with SCD compared to controls [11]**.** Another study showed that SCD has been associated with elevated baseline WBCs [15]. We found elevated LDH and serum ferritin in SCD patients in VOC and at steady state compared with controls. Allali et al., (2019) showed elevated LDH in SCD at steady state [16] and El-Alfy et al., (2018) demonstrated elevated serum ferritin and LDH in SCD patients at steady sate [11].

On the basis of immunological data, we demonstrated significant increase in IFN-γ in SCD patients in VOC compared with SCD patients at steady state and controls. Serum IL-10 showed significant increase in SCD patients at steady state compared with SCD patients in VOC and controls. CD4 $^{+}$T lymphocyte showed significant difference in SCD patients in VOC as it was decreased compared with SCD patients at steady state and controls.

Nnodim et al., (2015) showed a significant higher IFN-γ level in SCD patients [17]. Khalifa et al., (2018) also demonstrated a significant higher IFN-γ level in SCD during VOC and at the steady state compared to controls [18]. El-Alfy et al., (2018) showed significant increase in serum IFN-γ and $CD4^{+}$ T lymphocytes in patients with SCD at steady state compared to controls [11]. Nickel et al., (2015) found that patients with SCD, presented with VOC, have increased neutrophils and monocytes activation with higher cytokine levels [19]**.** On the other hand, Musa et al., (2010) showed no significant difference in IFN-γ in SCD patients. In addition, their study showed significant differences in $CD4^{+}$ and $CD8^{+}$ T cells percentages between patients in VOC and steady state, these differences were for $CD4^{+}$ T cells only through the expression of the cell numbers as absolute values as $CD4^{+}$ T cells were low in SCD patients in VOC and steady state. They illustrated that healthy controls had elevated serum IL-10 levels than SCD patients in VOC with no significant difference. There was a significant increase in serum IL-10 at steady-state SCD patients compared with SCD patients in VOC and controls **(14)**. Garcia et al., (2020) illustrated decreased $CD4^{+}$T lymphocytes and increased IL-10 levels in SCD compared with controls [20].

In contrast, Ojo et al., (2014) showed no significant difference in $CD4^{+}$T lymphocyte count between SCD patients and controls [21]. Koffi et al., (2003) illustrated that the helper/inducer ($CD4^{+}$) T cells proportion was not significantly elevated in SCD patients compared with the controls [22].

Pathare et al**.,** (2004) found higher IL-10 levels in SCD patients at steady state than either SCD patients in VOC or controls [7]. Graido-Gonzalez et al., (2018) demonstrated increased IL-10 levels in SCD patients in VOC than controls [23].

Serum IFN-γ was increased in patients with history of frequent crisis/ year and decreased in patients under hydroxyurea therapy. EL-Alfy et al., (2018) demonstrated significant decrease in IFN-γ in hydroxyurea treated patients [11]**.**

The mechanism of elevated NK cells with IFN-γ in SCD mainly in VOC is not understood. So, it has been demonstrated that increased reactive oxygen species, reactive nitrogen species and decreased nitric oxide levels result in RBCs, leukocytes, platelets, and endothelial cells activation. This activation results in increased pro-inflammatory and anti-inflammatory cytokines production that gives SCD the chronic inflammatory disease characteristics [24].

Immune abnormalities in SCD may participate in VOC and increase infection susceptibility and may demonstrate incidences of impaired prominence or vaccines response duration. These conditions resulting in morbidity and risk of death in SCD patients [25, 26]**.**
There is a limitation in our study was the small sample size in our governorate. In addition we recommend studying more cytokines to clarify the immune changes in SCD steady state and VOC.

5. Conclusion

IFN-γ was increased in SCD patients. Increased IFN-γ production in SCD suggesting that functionally activated natural killer cells reflecting a host immunological mechanism leading to antigen antibody activation in SCD. Serum IL-10 increased in SCD patients at steady state. $CD4^{+}T$ lymphocyte decreased in SCD patients in VOC. Cytokine expression may be changed in patients with good compliance hydroxyurea therapy; therefore hydroxyurea plays an important role in SCD patients.

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Competing interests: The authors declare that there is no conflict of interest.

Ethics approval: The Institutional Review Board (IRB) of the Menoufia Faculty of Medicine approved the study. Research work was performed in accordance with the Declaration of Helsinki. A written patient consent was taken from the parents.

Table (1): Demographic and clinical characteristics among studied groups

Demographic and clinical characteristics	SCD patients (no.=30)		Controls (no.=30)	Test of significance	P value
	Steady state patients	VOC patients			
Age (year)				Test1=1.10	P1=0.27
Mean±SD	7.8±4.4	9.3±3.9	7.3±3.7	Test2=0.11	P2=0.91
Median (Range)	6.5 (2-16.5)	9 (5-17)	7.2 (2-12)	Test3=1.02	P3=0.31
Sex				Test1=1.22	P1=0.27
Male	7 (46.7%)	10 (66.7%)	12 (40.0%)	Test2=0.18	P2=0.67
Female	8 (53.3%)	5 (33.3%)	18 (60.0%)	Test3=2.85	P3=0.09
Hb (g/dl)				Test1=4.38	P1<**0.001***
Mean±SD	7.9±0.47	8.6±0.31	12.1±0.84	Test2=21.35	P2<**0.001***
Median (Range)	7.9 (7-8.7)	8.6 (8-9)	11.8 (11-14)	Test3=20.54	P3<**0.001***
WBC ($x10^9$/l)				Test1=3.28	P1=**0.001***
Mean±SD	11.5±3.5	14.9±2.8	8.1±1.8	Test2=3.80	P2<**0.001***
Median (Range)	10.8 (8-22)	14.7 (10.9-22)	8.3 (5.5-10.7)	Test3=9.86	P3<**0.001***
Absolute lymphocytes ($x10^9$/l)				Test1=0.57	P1=0.58
Mean±SD	3.8±0.56	3.6±0.66	2.9±0.31	Test2=5.28	P2=**0.001***
Median (Range)	3.9 (2.7-4.5)	3.7 (2.7-4.7)	3 (2.4-3.4)	Test3=3.82	P3=**0.001***
ANC ($x10^9$/l)				Test1=4.15	P1=**0.0001***
Mean±SD	4.9±1.7	8.9±1.3	3.4±0.82	Test2=3.40	P2=**0.001***
Median (Range)	4.5 (2.8-10)	8.9 (7.2-11.1)	3.5 (2.3-4.5)	Test3=17.57	P3=**0.0001***
LDH (mg/L)				Test1=1.36	P1=0.18
Mean±SD	458.1±52.7	435.3±37.8	207.7±23.6	Test2=17.55	P2=**0.001***
Median (Range)	447 (389-580)	432 (379-506)	204.5 (176-267)	Test3=21.35	P3=**0.001***
Ferritin (μg/l)				Test1=3.89	P1<**0.001***
Mean±SD	488.6±234.6	936.7±151.7	44.5±13.3	Test2=5.43	P2<**0.001***

Median (Range)	389 (250-1000)	900 (700-1200)	40 (27-70)	Test3=5.43	P3<**0.001***
Hydroxyurea					
Good Compliance	13 (86.7%)	8 (53.3%)	-	Test1=3.97	P1=**0.04***
Poor Compliance	2 (13.3%)	7 (46.7%)			
Number of VOC/year					
1	12 (80.0%)	8 (53.3%)	-	Test1=8.13	P1=**0.04***
2	2 (13.3%)	0 (0.0%)			
3	0 (0.0%)	5 (33.3%)			
4	1 (6.7%)	2 (13.3%)			
Blood transfusion/year					
1	12 (80.0%)	8 (53.3%)	-	Test1=8.13	P1=**0.04***
2	2 (13.3%)	0 (0.0%)			
3	0 (0.0%)	5 (33.3%)			
4	1 (6.7%)	2 (13.3%)			

SCD: Sickle Cell Disease; VOC: Vaso-occulosive Crises; Hb: Hemoglobin; PLT: Platelet; WBC: White blood Cells; ANC: Absolute Neutrophil Count; LDH: Lactate Dehydrogenase *significant difference

P1: Steady sate patients versus vaso-occulosive patients; P2: Steady sate patients versus controls; P3: vaso-occulosive patients versus controls.

Table (2): IFN-γ, IL10, CD4 $^{+}$ T lymphocyte among studied groups

Parameters	SCD patients (no.=30)		Controls (no.=30)	Test of significance	P value
	Steady state patients	VOC Patients			
INF gamma (Pg/ml) Mean±SD				Test1=4.67	P1<**0.001***
Median (Range)	27.9±1.9	144.5±61.6	14.9±1.5	Test2=24.61	P2<**0.001***
	27.5 (25.1-32.1)	113.4 (79.7-221.8)	15.02 (12.4-17.5)	Test3=5.42	P3<**0.001***
IL-10 (Pg/ml)				Test1=2.64	P1=**0.008***
Mean±SD	78.5±34.5	45.5±24.3	53.8±32.8	Test2=2.03	P2=**0.04***
Median (Range)	75 (20-150)	40 (15-75)	50 (14-100)	Test3=0.72	P3=0.47
CD4 $^{+}$T lymphocyte (%) Mean±SD				Test1=6.07	P1<**0.001***
	22.1±1.9	17.2±2.5	29.8±2.8	Test2=9.55	P2<**0.001***
Median (Range)	22.5 (19.1-24.9)	18 (13.6-20.4)	30 (25.9-34)	Test3=14.73	P3<**0.001***

INF: interferon; IL10: interleukin *significant difference

P1: Steady sate patients versus vaso-occulosive patients; P2: Steady sate patients versus controls; P3: vaso-occulosive patients versus controls

Table (3): Correlation between either of IFN-γ, IL10 or $CD4^+$ T lymphocyte and other parameters among SCD patient

Parameters	IFN-γ	IL-10	$CD4^+$T lymphocyte
	r P value	r P value	r P value
Age (year)	0.188 0.32	-0.050 0.79	-0.129 0.49
Hb (g/dl)	0.595 **0.001***	-0.502 **0.005***	-0.479 **0.007***
WBC ($x10^9$/l)	0.543 **0.002***	-0.195 0.30	-0.517 **0.003***
Absolute lymphocytes ($x10^9$/l)	-0.046 0.81	0.435 **0.02***	0.293 0.12
ANC ($x10^9$/l)	0.685 **0.0001***	-0.397 **0.03***	-0.741 **0.0001***
Ferritin (μg/l)	0.716 **0.0001***	-0.413 **0.02***	-0.821 **0.0001***
Number of crisis/year	0.648 **0.0001***	0.331 0.07	-0.344 0.06
Blood transfusion/Year	0.648 **0.0001***	0.331 0.07	-0.344 0.06

*significant difference

Table (4): Multivariable linear regression analysis to detect predictable factors for IFN-*γ*, IL10 and CD4 $^{+}$T lymphocyte among SCD patients

Predictors	**IFN-*γ***		**IL10**		**CD4 $^{+}$T lymphocyte**	
	Beta (β)	**t and P value**	**Beta (β)**	**t and P value**	**Beta (β)**	**t and P value**
Hb (g/dl)	79.24	3.15 **0.002***	-34.72	3.26 **0.0003***	-2.79	2.48 **0.02***
WBC (x10^{9}/l)	8.86	2.55 **0.02***	-1.58	0.90 0.38	-0.369	2.27 **0.03***
Absolute lymphocytes (x10^{9}/l)	-2.58	0.11 0.91	25.17	2.67 **0.01***	1.78	1.80 0.08
ANC (x10^{9}/l)	17.67	4.06 **0.0001***	-6.12	2.71 **0.01***	-0.96	5.53 **0.0001***
Ferritin (ng/ml)	0.15	4.12 **0.0001***	-0.04	2.30 **0.03***	-0.01	8.47 **0.0001***
Number of crisis/year	46.94	5.16 **0.0001***	7.81	1.37 0.18	-1.07	1.95 0.06
Blood transfusion/year	46.94	5.16 **0.0001***	7.81	1.37 0.18	-1.07	1.95 0.06

Dependent variable was IFN-*γ*, IL-10 or CD4 $^{+}$ T lymphocytes.
*significant difference

Table (5): IFN-γ, IL-10, $CD4^+$ T lymphocyte among SCD patients regarding hydroxyurea and number of crisis

Parameters	**SCD patients (no.=30)**		**Test of significance**	**P value**
	Good Hydroxyurea compliance (No.=21)	**Poor hydroxyurea Compliance (No.=9)**		
INF gamma (Pg/ml)				
Mean±SD	52.9±34.1	163.9±82.2	3.46	0.001*
Median (Range)	29 (25.1-113.4)	212.1 (30.4-221.8)		
IL-10 (Pg/ml)				
Mean±SD	53.1±30.04	82.8±34.6	1.95	0.06
Median (Range)	55 (15-110)	75 (40-150)		
CD4T lymphocyte (%)				
Mean±SD	20.3±3.1	18.2±3.7	1.57	0.13
Median (Range)	20.2 (14-24.9)	19.1 (13.6-24.5)		
Parameters	**Crisis ≥3 (No.=8)**	**Crisis <3 (No.=22)**	**Test of significance**	**P value**
INF gamma (Pg/ml)				
Mean±SD	180.8±69.2	51.8±33.6	3.75	<0.001*
Median (Range)	212..3 (32.1-221.8)	29 (25.1-113.4)		
IL-10 (Pg/ml)				
Mean±SD	66.9±11.6	60.2±39.01	0.82	0.41
Median (Range)	70 (40-75)	57.5 (15-150)		
$CD4^+$ T lymphocyte (%)				
Mean±SD	17.4±2.9	20.5±3.1	2.38	0.02*
Median (Range)	19.1 (13.6-20.4)	20.3 (14-24.9)		

*significant difference

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