Journal of Cardiovascular Disease Research

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

IMMUNE DYSFUNCTION IN PATIENTS WITH SICKLE CELL ANEMIA

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ABSTRACT

Background: Sickle cell anemia (SCA) is a hemoglobinopathic disease associated with a chronic hemolysis and vasoocclusive events. Patients with sickle cell anemia (SCA) have increased susceptibility to impairment of immune function. The aim of the present study to evaluate immune dysfunction in patient with sickle cell anemia. **Patients and methods:** A cross-sectional study Included pediatric patients with SCD who were following up regularly, at Pediatric Hematology Outpatient Cinics, Pediatric Department in Zagazig University Hospitals. All patients were subjected to complete history taking with special emphasis. Clinical and special investigation including T-cell function evaluation, CD3, CD4, CD8, CD19 and CD56 were performed using immune phenotype analysis.

Results: There is statistically significant positive correlation between hemoglobin S and interleukin 6. There is statistically significant negative correlation between hemoglobin S and both CD 4 and, CD 56 and IgG. There is statistically non-significant correlation between hemoglobin S and either CD3, CD8, CD19, IgM, IgE, and IgA. There is statistically significant association between vasocclusive crisis and all of CD4, CD8, CD 19 and CD 56, and IgA. All were significantly lower in patients in vasocclusive crisis. There is statistically non-significant association between vasocclusive crisis and CD 3. There is statistically significant association between vasocclusive crisis and all of IgM, IgG, IgE, and IgA. There is statistically significant association between vasocclusive crisis and all of IL-6. It was significantly higher in patients in vasocclusive crisis**.**

Conclusion: It can be concluded that sickle cell anemia (SCA) patients have increased susceptibility to impairment of immune function. A significant association was reported between vaso-occlusive crisis and all of IgM, IgG, IgE, and IgA. All were significantly higher in patients in vaso-occlusive crisis. In addition; IL-6 was significantly higher in patients in vasocclusive crisis.

Keywords: Immune Dysfunction; IL-6; CD4, CD8, IgG, vasocclusive crisis

INTRODUCTION

Sickle cell anemia (SCA) is a chronic monogenic hemoglobinopathy characterized by hemolysis and vasoocclusive events. Sickle cell disease (SCD) is an inflammatory condition associated with alterations in immune phenotype and function (**1).** It is a global health issue, affecting millions of people worldwide, and its incidence is expected to increase to 400,000 neonates born per year by 2050 **(2).** SCA may leads to ischemia, infarction and ischemia-reperfusion injury with a progressive damage in multiple organs **(3).**

The genetic and molecular bases of SCD originates from mutation of the β-globin gene, leading to polymerization of the abnormal deoxygenated hemoglobin S (HbS), resulting in obstruction of small vessels by sickle-shaped red blood cells (RBC). However, the pathophysiology has been found to be much more complex than originally thought, involving many factors other than RBC **(4).**

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Individuals with sickle cell anemia (SCA) have increased susceptibility to impairment of immune function. This includes reduction in the proportion of circulating CD4+ and CD8+ T cells, reduction of CD4+ helper : CD8+ suppressor T cell ratio, aberrant activation and dysfunction of regulatory T cells (Treg), skewing of CD4+ T cells towards Th2 response and loss of IgM-secreting CD27+IgMhighIgDlow memory B cells **(5).** However, the increased neutrophils are mostly dysfunctional due to impaired chemotaxis, migration and killing ability. Impairment of the alternate pathway of complement activation through qualitative and quantitative deficiencies of factors B and C3 has also been reported **(6).**

High rates of alloimmunization, connective tissue diseases and transplant rejections **(7)**, as well as incidences of aberrant vaccine reactivity **(8)**, have bought to surface adaptive immune abnormalities in SCA. Currently, however, little has been done to characterize T and B lymphocyte phenotype, function and contribution to chronic inflammatory diseases in SCA **(9).**

Limited studies done indicate that abnormalities in both T and B cells occur in SCA. These abnormalities may be induced by SCA disease itself, or may arise as a result of complications of its treatment with repeated blood transfusions **(5).**

The aim of the present study to evaluate immune dysfunction in patient with sickle cell anemia

PATIENTS AND METHODS

A cross-sectional study Included pediatric patients with SCD who were following up regularly, at Pediatric Hematology Outpatient Cinics, Pediatric Department in Zagazig University Hospitals. This study was ethically approved from Institutional Reviewer Board (IRB) in Faculty of Medicine, Zagazig University Hospital and a written parental consent from every case or their caregivers that participates in this research was taken.

Inclusion Criteria:

Patients with sickle cell disease (Hbss-Hbsc-thalassemia, sickle cell trait) in age from 2 to 18 years of both sexes. No other conditions associated with known abnormalities of theimmune system.

Exclusion criteria:

Age below 2 and more than 18 other hemo anemias, any patient with primary or secondary immune deficiency and patients with infections, chronic inflammatory conditions other than SCD, renal or cardiac disease, rheumatoid arthritis or other autoimmune diseases, hypothyroidism, diabetes mellitus, or steroid therapy were excluded.

All patients were subjected to complete history taking with special emphasis. The frequency of vaso-occlusive crisis in the last year was divided into mild (two or less episodes requiring medical visits) or severe (three or more episodes requiring medical visits). The diagnosis of acute chest syndrome was defined as a new pulmonary infiltrate on chest X-ray and ≥ 2 of the following: chest, upper abdominal, or rib pain; dyspnea; fever, tachypnea; grunting; nasal flaring; or retractions.

Laboratory investigations:

Blood samples were collected peripheral venous blood under complete aseptic conditions and were divided into 2 portions:

- Two ml collected on potassium ethylene diamine tetra-acetic acid (K2-EDTA) (1.2 mg/ml) as an anticoagulant for complete blood count (CBC) and HB electrophoresis.
- One ml was left in plain tubes for 30-60 minutes for clotting then centrifuged at 3000 rpm for 10 minutes; serum samples were kept frozen into epindorfs at -20ºC till the time for ferritin determination.

For chemical analysis and enzyme-linked immunosorbent assay (ELISA), clotted samples were obtained and serum was separated by centrifugation for 15 minutes at 1000 xg then stored at -80°C till subsequent use in ELISA. CBC performed using Sysmex XT-1800i, examination of Leishman-stained smears for differential white blood cell (WBC) count and hemoglobin analysis by HPLC using D-10. Serum ferritin was measured at the start of the study with calculation of the mean value of the lst year prior to the study in order to know the ferritin trend.

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Special investigations:

Interleukin 6 by ELISA. For B cell function evaluation, all immunoglobulins, IgG, IgM, IgA and IgE were performed (CoBAs (Roch) chemilumenicuc). For T-cell function evaluation, CD3, CD4, CD8, CD19 and CD56 were performed using immune phenotype analysis that was performed on peripheral blood drawin into a Cytochex BCT tube. Flow cytometry data were analyzed using FloJo software (TreeStar, Ashland, OR, USA).

Flow cytometric analysis:

Evaluation of T-cell function was done by flow cytometry with the use of FloJo software (TreeStar, Ashland, OR, USA). In brief, a volume of 50 µl of blood was added to 10 µl of fluorochrome-labeled monoclonal anibody. For each set, appropriate isotypic control was done. Samples were protected from light and incubated at room temperature for 20 minutes.

Statistical analysis:

The data were analyzed using the SPSS (Statistical Package for Social Sciences) version 22 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) to calculate difference between two or more groups of qualitative variables. Quantitative data were expressed as mean ± SD. Independent samples t-test was used to compare between two independent groups of normally distributed variables. P value ≤ 0.05 was considered significant.

RESULTS

The present study showed age of onset ranged from 1 to 4 years with median 1 year. Hemoglobin S ranged from 38.1 to 92% with median 67.3%. Two patients had hepatosplenomegaly. Seven patients had vasocclusive crisis at time of study. About 73% had sickle cell anemia, 23.3% had combined sickle cell anemia and thalassemia while two patients had sickle cell trait. About 64.2% of the studied patients received blood every 3 months; about 63% of the studied patients need iron chelation therapy **(Table 1)**.

There is statistically significant difference between the studied groups regarding hemoglobin level **(Figure 1)**. There is statistically significant difference between the studied groups regarding serum ferritin level (higher in case group). There is statistically significant difference between the studied groups regarding CD4, CD8 and CD 56. All were significantly higher in control group. There is statistically non-significant difference between the studied groups regarding CD3, or CD 19.All were non-significantly higher in control group. There is statistically significant difference between the studied groups regarding IgM, IgG, IgE, and IgA. All were significantly higher in case group. There is statistically significant difference between the studied groups regarding interleukin 6 (significantly higher in case group) **(Table 2).**

There is a significant negative correlation between serum ferritin and both CD4 and CD8. There was a significant negative correlation between serum ferritin and both IgA and IgE. There is a significant positive correlation between serum ferritin and IL 6 **(Figure 2).**

There is statistically significant positive correlation between hemoglobin S and interleukin 6. There is statistically significant negative correlation between hemoglobin S and both CD 4 and, CD 56 and IgG. There is statistically non-significant correlation between hemoglobin S and either CD3, CD8, CD19, IgM, IgE, and IgA **(Table 3).**

There is statistically significant association between vasocclusive crisis and all of CD4, CD8, CD 19 and CD 56, and IgA. All were significantly lower in patients in vasocclusive crisis. There is statistically non-significant association between vasocclusive crisis and CD 3. There is statistically significant association between vasocclusive crisis and all of IgM, IgG, IgE, and IgA. All were

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significantly higher in patients in vasocclusive crisis. There is statistically significantassociation between vasocclusive crisis and all ofIL-6. It was significantly higher in patients in vasocclusive crisis **(Table 4)**.

There is statistically non-significant association between need for hydroxurea and eitherCD3, CD4, CD8, CD 19 or CD 56. There is statistically non-significant association between hydroxurea and either IgM, IgG, IgE, and IgA. There is statistically non-significantassociation between Hydroxurea and IL-6 **(Table 5)**.

Table (1): Clinico-epidemiological characteristics of studied patients:

	N=30	%
Age of onset (year) Median (range)	 1 (1 – 4)	
Hemoglobin S: Median (range)	 67.3 (38.1 – 92)	
Type: Sickle cell anemia Sickle cell anemia and thalassemia Sickle cell trait	 22 6 2	 73.3 20 6.7
Hydroyurea: Yes No	 16 14	 53.3 46.7
Vasocclusive crisis: No Yes	 23 7	 76.7 23.3
Frequency of blood transfusion Every 3 months Every 6 months	 9(64.2) 5 (35.8)	 64.2 35.8
Chelation therapy: No Yes	 11 19	 36.7 63.3

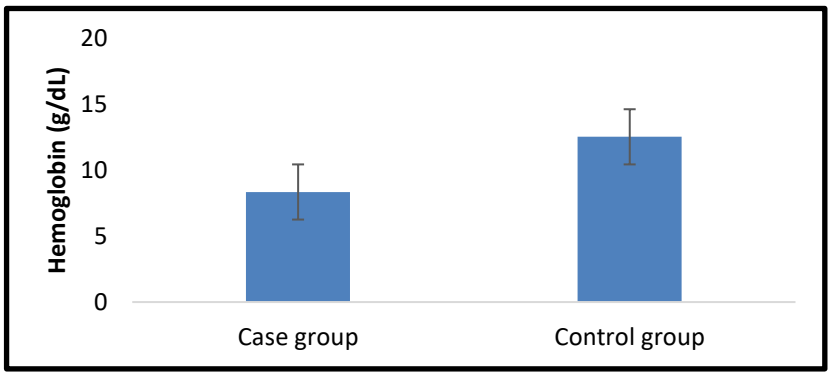

Figure (1): Simple bar chart showing comparison between the studied groups regarding hemoglobin

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Table (2): Hematological and immunological data of patients with SCD and control

Parameter	Case group	Control group	Test	
			Z	p
	N=30 (%)	N=30 (%)		
Ferritin (µg/L Median (Range)	430 (67 – 2460)	62.5 (9 – 100)	-6.175	<0.001**
CD 3	75.09 (60 – 82.8)	78 (63 – 88)	-1.096	0.273
CD 4	37.15 (18 – 79.1)	32 (23 – 54)	-2.488	0.013*
CD 8	29 (12 – 60.8)	45 (22 – 60)	-3.225	0.001*
CD 19	14 (4 – 33)	16 (4 – 36)	-1.911	0.056
CD 56	5.23 (1 – 12.5)	12 (5 – 19)	-4.993	<0.001**
IgM	66.55 (19 – 246)	190 (65 – 251)	-4.379	<0.001**
IgG	930.5 (99 – 2384)	1560 (990 – 3500)	-3.816	<0.001**
IgA	136.6 (60 – 2553)	330 (240 – 400)	-5.506	<0.001**
IgE	53.1 (17.4 – 217.8)	145 (80 – 200)	-3.58	0.001**
IL 6	111.17 (19.1 – 340)	66.3 (19.1 – 120)	-6.175	<0.001**

Z Mann Whitney test **p≤0.001 is statistically highly significant

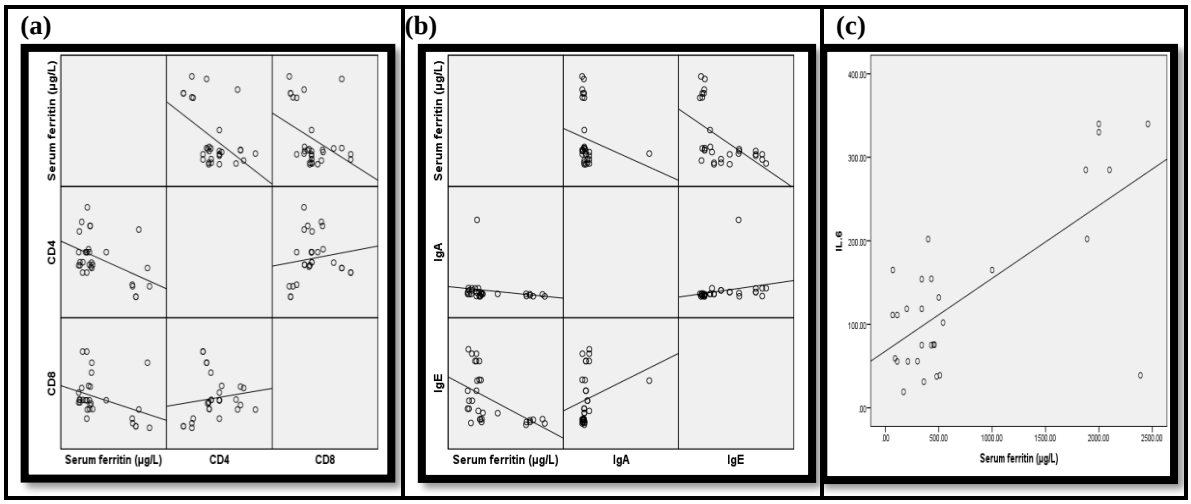

Figure (2): Scatter matrix showing: (a) significant negative correlation between serum ferritin and both CD4 and CD8; (b) significant negative correlation between serum ferritin and both IgA and IgE; (c) significant positive correlation between serum ferritin and IL 6

Table (3): Correlation between hemoglobin S and Cluster of differentiation, immunoglobins and interleukin 6 among the studied patients

	Hemoglobin S	
	r	p
CD 3	-0.216	0.252
CD 4	-0.381	0.038*
CD 8	-0.356	0.054
CD 19	-0.147	0.438
CD 56	-0.335	0.005*
IgM	-0.306	0.399

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IgG	-0.476	0.008*
IgA	-0.235	0.211
IgE	-0.321	0.083
IL-6	0.68	<0.001**

r spearman rank correlation coefficient *p<0.05 is statistically significant **p≤0.001 is statistically highly significant

Table (4): Association of Vasoocculosive crisis and cluster of differention (CD), immunoglobulins and IL 6 levels

Parameter	VOC		Test	
	Absent	Present	Z	p
	Median (range)	Median (range)		
CD 3	75.09 (60 – 82.8)	69.55 (30 – 82.8)	-0.541	0.597
CD 4	45 (29.44 – 79.1)	19.6 (11 – 62.2)	-3.204	0.001**
CD 8	29 (17 – 60)	15.5 (11 – 68.8)	-2.044	0.04*
CD 19	15 (11 – 33)	4.35 (4 – 31)	-2.167	0.031*
CD 56	8.22 (1 – 12.5)	1.63 (1 – 11.1)	-2.711	0.005*
IgM	90.3 (30 – 246)	40 (19 – 120)	-2.75	0.006*
IgG	1090.5 (110 – 2384)	105 (99 – 1682)	-2.255	0.024*
IgA	178.38 (60 – 2553)	77 (56 – 138)	-3.008	0.003*
IgE	78 (17.4 – 217.8)	22.4 (12.4 – 205.8)	-2.63	0.009*
IL 6	75.6 (19.1– 202.0)	284.9 (38.9 – 340)	-2.958	0.003*

Z Mann Whitney test *p<0.05 is statistically highly significant **p≤0.05 is statistically highly significant

Table (5): Association of hydroxyurea therapy and cluster of differntation (CD), immunoglobulins and IL6

Parameter	Hydroxurea		Test	
	Absent	Present	Z	p
	Median (range)	Median (range)		
CD 3	75.7 (41.3– 82.8)	72.79 (30 – 82.8)	-0.021	0.983
CD 4	45 (11 – 79.1)	34.9 (11 – 65)	**-1.692**	**0.091**
CD 8	29 (12 – 53.5)	28.3 (11 – 60.8)	-0.416	0.677
CD 19	13.85 (4.2 – 23)	14.5 (4 – 31)	-0.188	0.851
CD 56	3.66 (1.– 9)	9 (1 – 12.5)	-1.922	0.055
IgM	62 (19 – 181)	75.55 (30 – 246)	-0.5	0.617
IgG	930.5 (100 – 2384)	970 (99 – 1729)	-0.666	0.505
IgA	135.1 (60 – 2553)	148.48 (65 – 320)	-0.042	0.967
IgE	35.9 (19.1 – 340)	78 (37.5 – 340)	-1.20	0.227
IL 6	117.05 (19.1 – 340)	111.17 (37.5 – 340)	-0.062	0.95

Z Mann Whitney test *p<0.05 is statistically highly significant

DISCUSSION

Sickle cell disease (SCD) is a chronic, incurable condition presenting primarily as anemia (sickle cell anemia [SCA]) in people homozygous for hemoglobin S (HbS). This abnormal hemoglobin, resulting from the replacement of glutamic acid at position 6 of the β-globin chain by valine, is

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

responsible for erythrocyte distortion and fragility. The painful vaso-occlusive crisis (VOC) is the most common and is characterized by fever, leukocytosis, joint effusions, and tenderness (**10).**

The first question in our discussion: what are the etiology of immune dysfunction in SCD?

While immune abnormality in SCD have historically been attributed largely to splenic dysfunction, growing evidence exists that the immune deviation in SCD extends beyond splenic-associated abnormalities and that SCD itself is a pro-inflammatory condition with exaggerated immune activation **(11)**. SCD is associated with a chronic inflammatory state, and the hyper-inflammatory response is characterized by elevated white blood cell (WBC),increased level of inflammatory cytokines ,and abnormal activation of endothelial cell **(12)**. So there is a general consesus on the presence of abnormal immune cell counts and function in patients with SCD but the details of these abnormalities are not clear. This study focused on the immunological role of T-lymphocyte (CD3),immunological role of B lymphocyte (IgM) and the role of one of the important cytokines;IL6.

In patient with SCD, leukocytosis is observed due to the adhesion of neutrophils to the site of endothelial injury. Bacterial infection with leukocytosis is a known predisposing to VOC in patient with SCD. In addition, it has been shown that there is a statistically significant relationship between neutrophil levels and the clinical severity of SCD **(13).**

The second question in our discussion is: what are the effects of blood transfusion, serum ferritin level and chelation therapy on various immunological parameters in our study?

Patient with SCA recipients of multiple blood transfusion were found to have increase proportion of central memory CD4+T cells **(9),** reduced CD4+helper: CD8+ suppressor T cell ratios and impaired natural killer (NK) cell activity **(14)**. The reduction of CD4+helper: CD8+suppressor T cell ratio likely represents normal response to multiple blood transfusions as it has also been observed in individuals with other blood disorders necessitating repeated blood transfusion **(15).**

On the contrary our study reveals insignificant association between the need or blood transfusion and either of CD3,CD8,19 or 56,immunoglobulins orIL6. the second point: serum ferritin of interest there is a significant negative correlation between serum ferritin and all of CD4, CD8, IgM, and highly significant negative correlation to CD56 ,IgA and IgE. **Putu et al. (16)** showed that there is significant correlation between Ferritin serum and CD4 count, CD8 count and CD4/CD8 ratio. This may be explained by the need for more regular chelation jn our patient.

What are the pathogenesis of vaso-'occulosive crisis and its relation to the immune mechanism e.g: CD,Immunoglobulins and cyotokines especilly IL6?

The sequence of pathophysiological events that lead to the sickle cell VOC is not well understood. Several authors have outlined a sequence of steps occurring in the microcirculation that culminate in this painful sickle cell crisis. Polymerization of HbS, decreased blood red cell flexibility,

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

microvascular occlusion, hypoxia of tissue involved with the occluded microvascular network, and tissue damage triggering painful stimuli**.** Ischemic events produced by the occlusion of both large and small blood vessels are stressful and involve intricate interactions between red blood cells, the endothelium, and leukocytes. These interactions are known to be regulated by cytokines secreted by T cells as well as by adhesion molecules, and consequently, the immune response is implicated in the initiation and development of the sickle cell crisis **(17).**

What are the effects of hydroxyurea therapy on immunological parameters?

As regards to the CD finding in our results, there is significant correlation between patients in VOC and all of CD4,CD8,CD9,CD56, all were sig lower in patient with crisis than steady state. On the contrary **El-Alfy et al. (1)** in their study reported an elevation of CD4,CD28T-lymphocytes which is more evident among patient with vaso-occlusive crisis.

Daltro et al. (18) showed that SCD Patients Have a Lower Proportion of TCD4+ and TCD8+ Cells Compared to Healthy Control Patients. They noted a higher frequency of TCD4+ and TCD8+ in non-SCD BM compared with SCD BM (32.01% (range 19.09-46.3) vs. 24.28% (range 5.41-47.82) and 23.20% (range 13.14-31.58) vs. 16.18% (range 6.4-31.68), p = 0:05 and p = 0:01, respectively). There were no significant differences in the frequency of other T subsets between groups. Also, **Bhriye et al. (19)** reported that total T helper cells were found to be lower in patient with VOC compared to control and steady state but not statistically significant.

As regards to the immunoglobulin levels in patient with VOC, there is a significant higher levels of IgG,IgM,IgE and IgA in relation to steady state. **Change et al. (20)** demonstrate that the administration of high doses of immunoglobulin can trigger sickle cell crisis. **Nnodim et al. (21)** showed that the level of IgA was significantly higher in homozygous SCD and patient with voc when compared to control, while IgG level was not significantly decreased compared to control.

Cherif-Alami et al. (22) reported high IgG level in patient with SCD (except those under 3 years) and IgG level were increased in all age group but no consisttent difference in IgM level in all patient.

In our study there is significant higher level of IL6 in patient of SCD with VOC compared to steady state. **Tayler et al. (23)** found that increased levels of IL6 in 78 percent of their patient (45)in relation to control. He commented that the high of type2 cytokine may suppress both humoral and cell mediated immunity functions in SCD with resultant increased mortality. **Pierrot-Gallo et al. (24)** showed that the plasma level of IL6 and IL8 were significantly higher in patient compared to control with four folds increase.

What are the effects of hydroxyurea therapy on immunological parameters?

The use of hydroxyurea has been shown to significantly overturn increased lymphocyte count, particularly circulating memory T cells, in individuals with SCA. In our study there is no significant correlation between hydroxyurea therapy and all of CD, immunoglobulins and IL6. **Guarda et al. (25)** tested the effect of HU on cytokine production by monocytes. In unstimulated conditions, frequencies of monocytes expressing TNF-α, IL-1β or IL-6 were similar between the groups of patients taking or

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

not HU. Nevertheless, monocytes producing IL-8 were significantly expanded in patients not undergoing HU therapy. Upon LPS challenge *in vitro*, monocytes were able to increase the production of TNF-α, IL-1β, IL-6 and IL-8 independent of the clinical group. Importantly, HU use was associated with decreased capacity to produce TNF-α, IL-1β or IL-6 relative to that in patients who were not under HU therapy.

The first limitation of this study is that it is a cross sectional study which canot imply sausality, so further longitudinal studies are needed with more patients in steady state and during crisis. Also, we need to study more T lymphocyte cells like NK cell and other cytokines like IL 4and IL 10.

CONCLUSION

It can be concluded that sickle cell anemia (SCA) patients have increased susceptibility to impairment of immune function. A significant association was reported between vaso-occlusive crisis and all of IgM, IgG, IgE, and IgA. All were significantly higher in patients in vaso-occlusive crisis. In addition; IL-6 was significantly higher in patients in vasocclusive crisis.

No Conflict of Interest

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