LYMPHOCYTES SUBSET AND CYTOKINS ESTIMATIONON IN PATIENTS WITH SCD AT HEREDITARY BLOOD DISEASE CENTRE IN BASRAH

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ABSTRACT

A case-control study has been carried on patients with Sickle cell disease registered at the Hereditary Blood Disease Centre (HBDC) at Basrah Maternity and Children Hospital from the first of December 2016 till the end of March 2017. A total of 32 patients were included, their ages ranged from 16 to 55 years. Lymphocytes subset estimation including B.lymphocytes, T.lymphocytes, T.helper, T. cytotoxic,(CD4, CD8, and their ratio) NK natural killer, by using BD Accrui C6 flow cytometry (BD, Accuri C6)Accuricytomters. This study analyzed numerical values of selected serum cytokines Including IL1β, IL6, IL8, IL10, IL12 and TNFby using cytometric beds arraykit(CBA) on patients in VOC, and it compared these values with those obtained from the same patients in steady-state and unaffected hemoglobin AA homozygotes who served as normal healthy controls (NHC). This study show that SCD is a state of immune deregulation in which there is chronic inflammatory state with an acute exacerbations during crises, also ILs in addition to being important inflammatory markers, they play a key role in the immunological basis of crises,from other side IL-6 can be utilized as a predictor of crisis in patients during steady state or imminent crisis.

Keywords: Lymphocytes, serum cytokines ,Sickle cell disease,Basrah Maternity and Children Hospital.

No: of Figures: 1 **No: of Tables: 4** **No: of References:31**

INTRODUCTION

Sickle cell disease (SCD) is a genetic blood disease which is due to the presence of an abnormal form of hemoglobin, hemoglobin S, Hemoglobin S polymerization and denaturation results in oxidant damage to the red blood cell membrane, with subsequently disturbed homeostasis which in turn, can result in dehydrated dense cells and irreversibly sickled cells (Berlin and Elliot, 1991). SCD is associated with high morbidity and mortality among sickle cell suffers in developing countries (Ogamidi and Onwe, 2000). Red cell abnormalities lead to either hemolysis or vasoo-cclusion (VOC). VOC results from interaction between red cells, leukocytes and endothelial cell inflammation, endothelial injury, leukocytes adhesion and activation of coagulation pathways contribute to the pathophysiology of VOC crisis (Mousa *et al.*, 2010).

Pro inflammatory cytokines are believed to activate the vascular endothelium, compromise vascular integrity, and promote both erythrocyte and leukocyte adhesion in vascular beds. Multiple studies have demonstrated patients with SCD have chronically elevated levels of multiple inflammatory mediators (Black *et al.*, 2014; Salas *et al.*, 2014).

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 (Duits *et al.*, 1998). 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. Indeed, studies show that immune subsets are operative in sickle cell disease (Herna´ndez *et al.*, 1980; Graido-Gonzalez *et al.*, 1998; Koffie*t al.*, 2003; Pathare *et al*., 2004), and the susceptibility of sickle cell disease patients in crisis to infections that specifically require the help of T cells to be cleared CD4+ T cells, subdivided based on their associated cytokines, play a crucial role in inflammatory responses and the elimination of infection.(Kumar *et al.*, 2010).

This study analyzed numerical values for CD3, CD4, and CD8 T cells and levels of selected serum cytokines in patients in VOC, and it compared these values with those obtained from steady-state sickle cell disease patients and unaffected hemoglobin AA homozygotes who served as normal healthy controls (NHC). This was done in an effort to understand if any imbalance in the immune response is important in the pathogenesis of sickle cell disease.

MATERIALS & METHODS

A case-control study has been carried on patients with Sickle cell disease registered at the Hereditary Blood Disease Centre (HBDC) at Basrah Maternity and Children Hospital from the first of December 2016 till the end of March 2017. A total of 32 patients were included, their ages ranged from 16 to 55 years. A specially designed questionnaire was used which includes the date of birth, sex , Residency, details of the acute painful episode and associated

symptoms , site and frequency of Vasoocclutioncrisis per year, previous history of stroke, Acute chest syndrome,Hydroxy Urea intake, and history of splenectomy, symptoms related to infection .All patients had history of admission to Hereditary Blood Diseases Ward for the management of VOC, these patients were assessed initially (clinically and by selected laboratory data) during VOC, and then during they were in steady state (follow up).

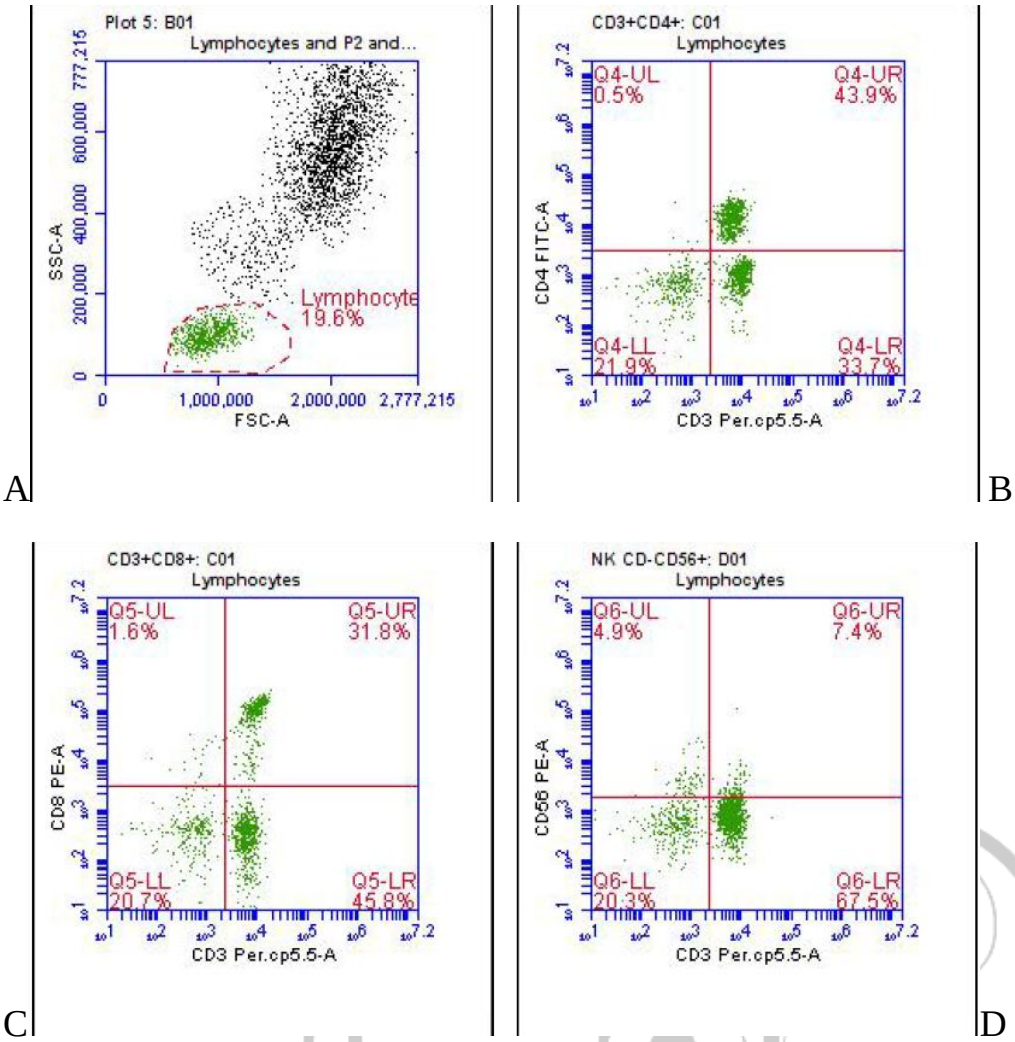
Patients recruited in study had been evaluated for the type of SCD using High Performance Liquid Chromatography (HPLC), (VARIANT™, β-ShortPrograms; Bio-Rad Laboratories, Hercules, CA, USA).This test was also done for the control group to ensure that they have normal Hb pattern.

3ml of blood were withdrawn from each patient during painful episodes and also during steady state for patients and control groups; the following investigations were done for each patient with SCD and for control group.

Lymphocytes subset estimation including B.lymphocytes, T.lymphocytes, T.helper, T. cytotoxic,(CD4, CD8, and their ratio) NK natural killer, by using BD Accrui C6 flow cytometry (BD, Accuri C6, Accuricytomters, Inc. Ann Arbor 21, MI 48103, USA) and (BD, Accuri C6 software version 1.0.264.21) are used for cell acquisition and events analysis. The machine is calibrated using 6 peaks and 8 peaks calibration beads (BD, Accuri C6, Accuri cytometers, Inc. Ann Arbor, MI48103 USA).Isotype negative control for FL1 and FL2 fluorescent detectors (IgG1 FITC and IgG2 PE respectively, eBiosciences, Bendremed system GmbH, 1030 Vienna, Austria).Panel of monoclonal anti-human IgG fluorescent-labelled antibodies was used including:

Flow Cytometry Reagents Anti-Human CD3 APC 100 tests (Lot# 17-0037-42), Flow Cytometry Reagents Anti-Human CD19 PerCP-Cyanine5.5 100 tests (Lot# 45-0199-42), Flow Cytometry Reagents Anti-Human CD4 FITC 100 tests (Lot# 11-0048-42), Flow Cytometry Reagents Anti-Human CD8 PE 100 tests (Lot# 12-0087-42) and Flow Cytometry Reagents Anti-Human CD56 (NCAM) PE 100 tests (Lot# 40 12-0567-42). All the items have been used by BD Accuri C6 flow cytometry (BD, Accuri C6, Accuricytomters, Inc. Ann Arbor 21, MI 48103, USA) and (BD, Accuri C6 software version 1.0.264.21) for cell acquisition and events analysis according to the procedure fixed in kits manuals, and some time with some modifications depending on optimal results. BD FACS Lysing Solution supplied from BectonDickinsons and company, BD Bioscience, Ireland, REF 349202).

Lymphocyte gate was made on FSC/SSC scattergram as the distinct population of events with low FSC and SSC as shown in (Fig.1) T-helper cells was determined as CD3+/CD4+ events, cytotoxic T cells as CD3+/CD8+ events, B cells as CD3-, CD19+ events and NK cells as CD3-/CD56+ events as shown in (Fig.1).



Figure(1) Lymphocyte gate and subset determination.

A- Lymphocyte subset population gate determination.

B- CD4+B cell gate determination.

C- CD8+B cell gate determination.

D- NK cell gate determination.

Biological markers evaluations by Cytometric Bead Array (CBA)

Including IL1β, IL6, IL8, IL10, IL12 and TNF.

The BD™ CBA Human Inflammatory Cytokines Kit can be used to quantitatively measure interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin- 10 (IL-10), tumor necrosis factor (TNF), and interleukin- 12 (IL-12) protein levels in a single sample. The kit performance has been optimized for analysis of specific proteins in tissue culture supernatants, EDTA treated plasma, and serum samples using one of two protocols depending on the

sample source. The kit provides sufficient reagents for 80 tests.

Principle of CBA assays:

BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead in the kit has been conjugated with a specific antibody.

The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector. Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12 proteins. The six bead populations are mixed together to form the bead array that is resolved in a red channel (FL3 or FL4) of a flow cytometer.

Statistical analysis

Statistical analysis was done using SPSS program version (18) software. Data were expressed by means ± Standard Deviation (SD).Comparisons of proportions were performed by cross tab using Chi-Square test. The Independent t- test was used for quantitative comparison and between two means of different samples. Comparisons between groups were made by using the one way analysis of variance (ANOVA) test. Correlations between variables were evaluated by the Pearson or Spearman coefficient. Mann-Whitney was used in order to overcome the underlying assumption of normality in parametric tests (the test does not assume that the difference between 2 samples is normally distributed). P value < 0.05 was considered as statistically significant.

RESULTS

The results of the current study is an output from 32 SCD patients (14 males and 18 females), their ages range from 16 – 55 years. Twenty two of them are SS, two are splenoctomized and one with splenomegaly. Ten of them are SF, two are splenoctomized and one with splenomegaly. The patients are recruited during crisis state then follow up27 of them in a steady state. There were 26 normal control subjects, matched with the patients in age and sex

Lymphocyte subset calculation

Whole blood samples were analyzed to determine whether there were any differences among the groups in Lymphocyte subset (Table 1 and 2). The mean of Lymphocyte subset for three groups are presented in Table (1).

Total lymphocyte count value as shown before that was increased significantly in crisis and steady groups compared to control the mean was (5.62 x10^9/L) (4.92 x10^9/L) (2.62 x10^9/L) respectively ,this

difference is statically significant(P value < 0.05).

B cell and T cell were significantly increased in crisis and steady as compared to control, B cell mean in crisis group (1.69 x10^9/L) , while in steady group (1.19 x10^9/L) and in control was (0.41 x10^9/L) with *P* value= .006, T cell mean in crisis ,steady and control was(3.41 x10^9/L), (3.15 x10^9/L), (1.84 x10^9/L) respectively with P value < 0.05

Although there was increased inCD4+T and CD8+T cell value in crisis and steady compared to control group, but this difference is statically insignificant (P value > 0.05). The CD4/CD8 ratio difference is statically insignificant. There was little high value in steady than crisis but this highest insignificant statically (P value > 0.05).

The value of NK cells was statically insignificant (P value > 0.05).

Table (1) Descriptive Statistical results of Total lymphocyte count, B cell, T cell, CD4+, CD8+, NK cell and CD4/CD8 ratio in all groups

Parameters		No.	Mean	P value
Total lymphocyte count	Control	26	2.6241	.005
	Crisis	32	5.62	
	Steady	27	4.92	
	Total	85	4.46	
B-lymphocytes	Control	26	0.41	.006
	Crisis	32	1.69	
	Steady	27	1.19	
	Total	85	1.13	
T-Lymphocytes	Control	26	1.84	.029
	Crisis	32	3.41	
	Steady	27	3.15	
	Total	85	2.83	

T-Lymphocytes/CD4+	Control	26	1.06	.070
	Crisis	32	1.78	
	Steady	27	1.72	
	Total	85	1.53	
T-lymphocytes/CD8+	Control	26	0.79	.056
	Crisis	32	1.63	
	Steady	27	1.42	
	Total	85	1.30	
NK cells	Control	26	0.36	.294
	Crisis	32	0.52	
	Steady	27	0.58	
	Total	85	0.49	
cd4/cd8	Control	26	1.50	.445
	Crisis	32	1.44	
	Steady	27	2.12	
	Total	85	1.67	

Table (2) Multiple Comparisons of Statistical results of Total lymphocyte count, B cell, T cell, CD4+, CD8+, NK cell and CD4/CD8 ratio in all groups

Parameters correlation			Mean	P value
Total lymphocyte count	Crisis	Control	2.99499*	.002
	Crisis	Steady	.70054	.446
	Steady	Control	2.29444*	.018
B-lymphocytes	Crisis	Control	1.28738*	.001
	Crisis	Steady	.49869	.205
	Steady	Control	.78869	.056
T-Lymphocytes	Crisis	Control	1.56490*	.011
	Crisis	Steady	.26166	.667
	Steady	Control	1.30323*	.042
T-Lymphocytes/ CD4+	Crisis	Control	.72233*	.034
	Crisis	Steady	.05279	.875
	Steady	Control	.66954	.059
T-lymphocytes/ CD8+	Crisis	Control	.84219*	.020
	Crisis	Steady	.20888	.557
	Steady	Control	.63332	.090
NK cells	Crisis	Control	.16294	.251
	Crisis	Steady	-.05981	.672

	Steady	Control	.22275	.133
cd4/cd8	Crisis	Control	-.05630	.922
	Crisis	Steady	-.67419	.241
	Steady	Control	.61789	.306

Estimation of Biological Markers (Specific cytokines)

Serum samples were analyzed to determine whether there were any differences among the groups in specific cytokine (TNF, IL β, IL-6, IL8, IL-10 and IL-12. Tables(3and4) .Although there was particular elevation in TNF, IL β and IL-10 values in patients groups especially in crisis as compare to control but this difference is statically insignificant . There was highly difference of IL-6 value among groups, The total mean was (5.75 pg/ml) and the mean in crisis group (12.96 pg/ml) , while in steady group (2.33 pg/ml) and in control was (1.02pg/ml) this difference is statically significant(*P* value= .003), with significant increase in crisis than other groups.IL-8 shown significant increase in steady group (47.0741pg/ml) than crisis group (22.71pg/ml) and all from control (7 ,85 pg/ml) ,this difference is statically significant(*P* value= .022).

Table (3) Descriptive Statistical results of specific cytokine (TNF, IL β, IL-6, IL8, IL-10 and IL-12) in all groups

Parameters		N	Mean	P value
TNF	Control	27	3.45	.255
	Crisis	32	5.68	
	Steady	29	5.22	
	Total	**88**	**4.83**	
IL-1β	Control	27	1.99	.423
	Crisis	32	2.66	
	Steady	29	2.81	
	Total	**88**	**2.50**	
IL-6	Control	27	1.02	.003
	Crisis	32	12.96	
	Steady	29	2.33	
	Total	**88**	**5.75**	
IL-8	Control	27	7.85	.002
	Crisis	32	22.71	
	Steady	29	47.07	
	Total	**88**	**25.76**	
	Control	27	1.04	.242

IL-10	Crisis	**32**	**1.78**	
	Steady	**29**	**1.27**	
	Total	**88**	**1.38**	
IL-12	Control	**27**	**2.03**	**.001**
	Crisis	**32**	**3.67**	
	Steady	**29**	**2.03**	
	Total	**88**	**2.64**	

The value of IL-12 was significantly high in crisis group (3.67 pg/ml) as compare with other, in steady group (2.03 pg/ml) and in control was (2.03 pg/ml),this difference is statically significant(*P* value= .001).

Table (4) Multiple Comparisons Statistical results of specific cytokine (TNF, IL β, IL-6, IL8, IL-10 and IL 12)) in all groups

Parameters			**Mean**	**P value**
TNF	**Crisis**	**Control**	**2.22838**	**.114**
	Crisis	**Steady**	**.46168**	**.739**
	Steady	**Control**	**1.76669**	**.220**
IL-1β	**Crisis**	**Control**	**.65729**	**.303**
	Crisis	**Steady**	**-.15308**	**.810**
	Steady	**Control**	**.81037**	**.220**

IL-6	Crisis	Control	11.94134*	.002
	Crisis	Steady	10.62882*	.006
	Steady	Control	1.31251	.737
IL-8	Crisis	Control	14.85167	.165
	Crisis	Steady	-24.36907*	.024
	Steady	Control	39.22074*	.001
IL-10	Crisis	Control	.74348	.102
	Crisis	Steady	.50475	.261
	Steady	Control	.23873	.606
IL-12	Crisis	Control	1.63766*	.004
	Crisis	Study	1.63777*	.003
	Steady	Control	-.00011	1.000

DISCUSSION

In this study absolute CD4+/CD8+ T cell are significantly increased in crisis compare to control but not to steady state patients.

T-cell show significant increase in crisis and steady compare with control group also there is significant increasing between crises and steady from side and steady with control from other side.Also this study show that CD4\CD8 ratio shows increase in steady state with little difference between control and crisis but this increase didn't reach significant value. The mean NK absolute cell count is slightly high in steady state than in crisis and control group, however, these difference are not significant.

The number of circulating T cells was found to be highly variable between individuals with SCA at steady state (Koffi*et al*., 2003 and Musa*et al*.,2010). However, most studies have reported a reduction in the proportion of circulating CD4+ and CD8+ T cells in SCA (Sanhadji*et al*.,1988 ; Kaaba and Al-Harbi 1993) with normal or increased absolute CD4+ and CD8+ T cell count (Wang *et al*.,1988).Al-Najjar (2013) found that B lymphocytes and NK cells were significantly increased in SCA patients with acute VOC, And also found that the total T lymphocytes, CD4+ T helper lymphocytes, CD8+ T suppressor lymphocytes, were not significantly increased in SCA patients with acute VOC, while in steady state there were all significantly increased.

Al-Najjar (2013) found that (CD4+:CD8+) ratio showed also significant difference between steady state patients and control . Wong *et al*., (1995) reported that during sickle cell crisis the (CD4+:CD8+) ratio was variably affected.

The cause for the observed alteration in lymphocyte phenotypes remains to be explained. With advancing age and progressive deterioration of splenic function, hyposplenism has been shown to contribute to lymphocyte abnormalities in SCA .

It is possible that the observed increase in CD4+ and CD8+ T cells likely reflect immune activity against recent or possibly ongoing vaso-occlusive events in SCA at relative steady state (Sales *et al.,* 2011; Keikhaei*et al.,* 2013).

Little has been done to characterize T and B lymphocyte, function and contribution to chronic inflammatory diseases in SCA. Limited studies done indicate that abnormalities in both T and B cells occur in SCA (Musa *et al.*,2010 and Vinger*et al.*,2014).

In this study, we found that certain cytokines in sickle cell disease were significantly increased when compared with healthy subjects namely pro-inflammatory cytokines(TNF,IL-1b, IL-6, IL-8,IL12) and anti-inflammatory cytokine (IL10) . TNF was increased in crisis group compared to steady state group as well as the control group, but the difference was not significant ,IL-1b was increased in steady more than crisis patients , However all these difference are not statistically significant. IL-1β and TNF cytokines are able to promote the production and

release of other important mediators, such as IL-8, a neutrophils-attractive chemokine. Together, these and other cytokines act in triggering the inflammatory cascade (Leal *et al*.,2013and Monaco *et al*.,2015) .Lanaro*et al*., (2009) observed an increase in the circulating levels of TNF in SCA patients at steady state, which is characteristic of a pro-inflammatory state. Moreover, Pathare*et al*., (2004) observed an increase in the circulating concentration of TNF during crisis events. Nnodim*et al*.,(2015) observed the level of IL-1b is significantly elevated in steady state CSD patients as compared with the control.

IL-6 was significantly increased in crisis group. In steady state it is also increased compared to control but the difference was not significant. In another study this increment was not statically significant (Veiga*et al*.2013). The surprising result that give by (Qari*et al*.,2012) they observed that IL-6 levels were significantly higher during the steady state than during painful crises .Other studies showed similar finding , Pathare*etal*., (2004) and Hibbert*et al* ., (2005) showed that serum concentration of IL-6 was higher in SCA patients than in normal controls, and there was also a significant increase in IL-6 levels in crisis patients when compared to steady-state patients , as shown in this study.

IL-8 in this study show significantly increase in serum level in steady group than control and even than crisis group, there is significant difference between groups but there is no significant difference between crisis and control groups, these cytokines induce increased adhesion of RBC and leukocytes to the vascular endothelium, and this adhesion can cause vaso-occlusion and local hypoxia (Pathare*et al*. 2004). Several studies have shown that patients display higher levels of IL-8 during VOC than during the stead y state (Pathare*et al*., 2004, Lanaro*et al*., 2009 and Keikhaei*et al*., 2013). In contrast to these findings, other studies showed that the levels of IL-8 were similar between patients in crises and patients in steady-state (Michaels *et al*.,1998). IL-12 show there is significant elevation in crisis group as compared with other groups and no difference in level between steady and control groups.IL-12 are proinflammatory cytokines produced by macrophages and dendritic cells in response to microbial pathogens (Hunter, 2005) .IL-12 regulates both innate and adaptive immunity. There are few reports in the literature pertaining to the role of IL-12 in SCA. Taylor *et al*. (1999) recommended to investigate IL-12 levels in SCA patients during crises. Hassan *et al*. (2009) performed a similar study of HbAS children who were infected with Plasmodium falciparum. Surprisingly, detectable levels of IL-12 were found in patients with mild malaria, but not in asymptomatic individuals. This finding could be related to the low levels of IL-10 that are typically associated with this infection, as IL-10 is a potent inhibitor of IL-12 (Hassan *et al*. 2009).

This study demonstrated that no significant difference in the level of IL-10 among groups despite of the minor elevation in crisis and steady but was not significant elevationarecent report has shown an

association between reduced IL-10 levels and the frequency, type, severity, and duration of vaso-occlusive crises in children with SCA. (Sarray*et al.*,2015) suggesting a possible influence of IL-10 on the pathophysiology of stroke. Musa *et.al.*,(2010) Observed notably low level of IL-10 in crisis as compared with steady and control. Recent work by Alsharif (20017) found number of, lymphocytes, CD3, CD4 and CD8 count , TNF, IL-6 and IL-8, were significantly elevated in steady state SCA patients when compared with control group, the results agree with this study.

Conclusion and recommendation

From this study we conclude that SCD is a state of immune deregulation in which there is chronic inflammatory state with an acute exacerbations during crises, also ILs in addition to being important inflammatory markers, they play a key role in the immunological basis of crises. from other side IL-6 can be utilized as a predictor of crisis in patients during steady state or imminent crisis.Further study recommend to highlight the details of immune deregulation and to study the possibility of clinical utilizing IL6 and IL8 as apredictors of crisis .Also to study any possible clinical value of the new generation of anti-inflammatory drugs (IL antagonists) in crisis management or prevention.

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