

CLINICAL RESEARCH ARTICLE



Impact of hydroxyurea on lymphocyte subsets in children with sickle cell anemia

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BACKGROUND: Hydroxyurea (HU) has beneficial effects in the management of sickle cell anemia (SCA), but there is a paucity of data on the effect of HU on immune cells in SCA. Herein we aimed to evaluate the effect of HU on immune profiles of Egyptian children with SCA.

METHODS: This was a controlled prospective cohort study conducted in 30 children with SCA and 30 healthy age-matched controls. Flow cytometry was used to evaluate lymphocyte profiles, including CD8+ T, CD19+ B, CD3+, CD4+, natural killer (NK), NK T, T helper 1 (Th1), Th2, T cytotoxic (Tc1), and Tc2 cells, prior to and after 1 year of treatment with HU.

RESULTS: HU treatment led to significant increases in hemoglobin (Hb), red blood cell, and hematocrit counts and a significant decrease in the percentage of sickle Hb, with subsequent improvement in SCA complications. Compared with baseline values, CD3+, CD4+, Th1, and CD8+ T cells were significantly increased, while NK, Th2, and Tc2 cells were significantly decreased, with a resulting increase in the Th1/Th2 and Tc1/Tc2 ratios.

CONCLUSIONS: HU has the beneficial effect of restoring the abnormally elevated immune parameters in children with SCA.

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IMPACT:

- Hydroxyurea treatment restores the abnormal immune parameters in children with sickle cell anemia.
- HU treatment led to significantly increased CD3+, CD4+, Th1, and CD8+ T cells, while NK, Th2, and Tc2 cells were significantly decreased, with a resulting increase in the Th1/Th2 and Tc1/Tc2 ratios.
- Our study showed the impact of HU therapy on immune parameters in children with SCA.

INTRODUCTION

Sickle cell anemia (SCA) is the most common genetic disorder worldwide and is a major cause of hemolytic anemia.¹ In Egypt, the reported carrier rate of sickle hemoglobin (HbS) ranges from 9 to 22%, with a varied distribution.² In Egypt, the phenotype of sickle cell disease (SDC) is severe, and the majority of the globin gene haplotypes were of African type.^{1,3} SCA is characterized by numerous disorders caused by HbS. When deoxygenated, HbS polymerizes into long, rigid intracellular arrays that distort the red blood cells, leading to chronic hemolytic crises, vaso-occlusive crises (VOCs), and recurrent infections.³

Children with SCA (HbSS) are reported to have a high mortality rate due to their increased susceptibility to infection. The immunocompromised state in SCA includes impaired leukocyte's function, abnormality of the alternative complement system, deficiency of circulating antibodies (Abs), and loss of natural killer (NK) cell activity.^{1,4}

There is increasing evidence that SCA causes a pro-inflammatory state with exacerbated immune activity.^{5,6} In addition, studies have

reported that the immune abnormalities in SCA have a considerable role in the pathology of the disease.^{6–8} Reports have shown that children with SCA have increased neutrophil and monocyte activation, as well as elevated cytokine levels. Moreover, the frequency and activity of NK and natural killer T (NKT) cells were observed to be elevated in SCA, which may contribute to the pulmonary ischemia–reperfusion observed in SCA.^{4,7,8}

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase that is commonly used in pediatric sickle cell centers. HU reduces the frequency of blood transfusions, pain crises, acute chest syndrome (ACS), and hospitalization in patients with SCA. Moreover, HU increases the fetal hemoglobin (HbF) level and stops the development of sickle cells, thereby preventing VOCs.^{9–11} In addition, HU has been described to reduce white blood cell (WBC) and neutrophil counts¹¹; however, its effects on other immune cells have not been fully investigated. While several studies have demonstrated immunological abnormalities in SCA, there is a paucity of data on the detailed immunophenotype of children with SCA, especially in this subregion. This

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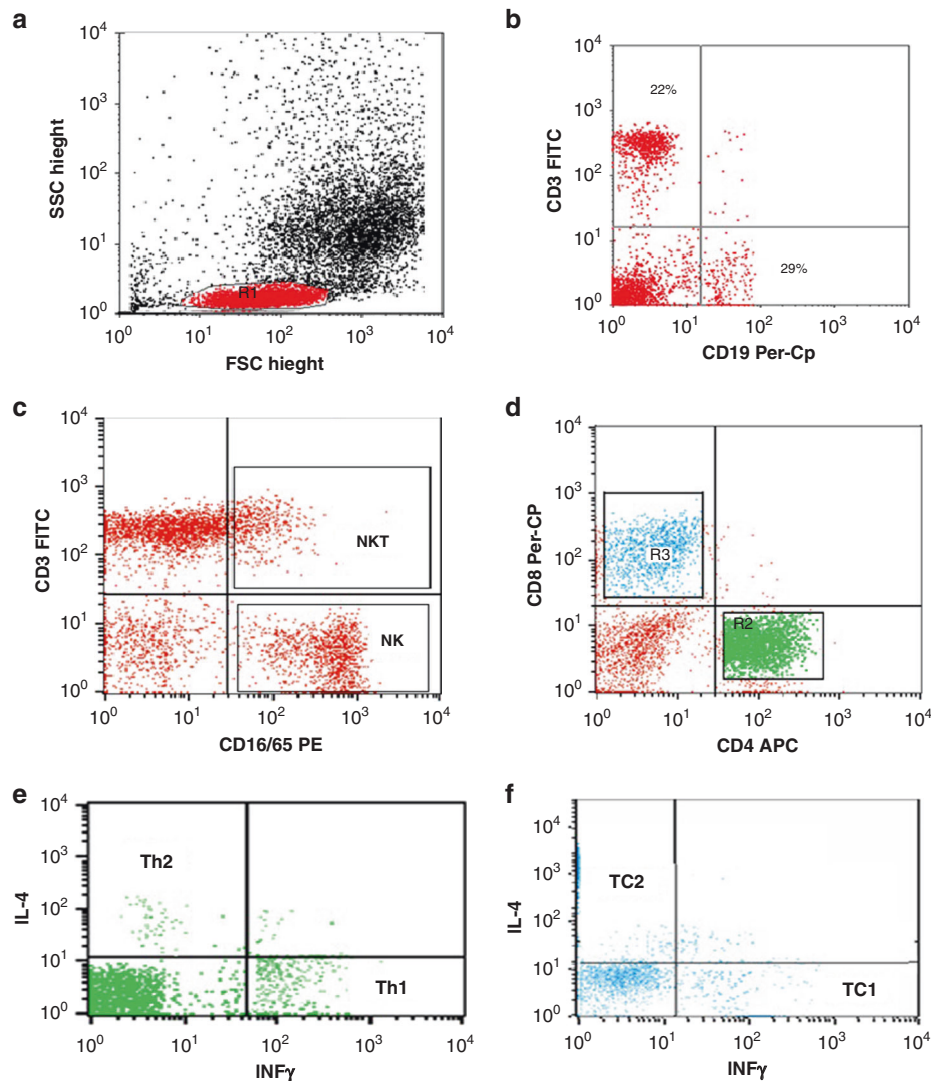


Fig. 1 Flow cytometric detection of lymphocyte subsets. **a** Scatter histogram was used to define the lymphocyte population (R1). **b, c** CD19⁺, CD3⁺, NK, and NKT cells were assessed on lymphocyte population. **d** The expression of CD4 and CD8 were assessed on lymphocyte population and then gated for further assessment of intracellular cytokines. **e** The expression of IL-4 and IFN γ on CD4⁺ lymphocytes to detect Th1 and Th2. **f** The expression of IL-4 and IFN γ on CD8⁺ lymphocytes TC1 and TC2.

study was structured to analyze the effect of HU on the immune profile of our SCA patients to obtain a better understanding of their immune phenotypes and how these are related to responses to therapy.

PATIENTS AND METHODS

All protocols of our study followed the regulations of the Ethical Committee of Assiut University (IRB No. 17300663). Informed written consent was obtained from all caregivers of children included in the study.

In our study, children with SCA (HbSS) were enrolled from the pediatric hematology outpatient clinic at Assiut University hospitals and followed up for 1 year. The subjects were 30 Egyptian children with SCA selected from a sample of 39 patients. Nine patients were excluded; six failed to provide consent, and three met exclusion criteria. We excluded any SCA patient with evidence of infection in the previous 4 weeks and/or received a blood transfusion in the 6 weeks before enrollment. Thirty age- and sex-matched healthy children were enrolled as controls. All participants of our study had the same socioeconomic background and ethnic heritage. All controls were healthy, with no history of systemic disease, anemia, or bleeding disorders. All SCA patients were treated with HU for 12 months, with a median daily dose of 20 mg/kg.

Hematological parameters

Blood samples were used for the quantification of hematological parameters (Hb, mean corpuscular volume, platelet counts, and WBC counts) before HU therapy and after 1 year of HU therapy. These hematological parameters were detected using the automated Coulter Gen 5 system 2 (Beckman-Coulter, CA). Hb fraction S was assessed using high-performance liquid chromatography using the Variant II system (Bio-Rad, CA).

Clinical parameters

Patient medical records were reviewed to determine the number of instances of VOC and ACS that occurred before treatment with HU and during the first year of HU therapy.

Flow cytometric analysis of lymphocyte subsets

Fifty microliters of the blood sample was incubated for 15 min with 5 μ L fluorescein isothiocyanate (FITC)-conjugated CD3, peridinin-chlorophyll-protein (Per-CP)-conjugated CD19, and phycoerythrin (PE)-conjugated CD16/56 (Becton Dickinson [BD] Biosciences, San Jose, CA). A FACSCalibur flow cytometer with the CellQuest software (BD Biosciences) was used for analysis. An anti-human IgG isotype-matched negative control was used with each sample. A scatter histogram was used to define the lymphocyte

Table 1. Hematological and clinical parameters of SCA patients, before and after 1 year of HU treatment, and healthy control group.

	Before HU therapy N = 30	After 1 year N = 30	Control healthy N = 30	P value ¹	P value ²	P value ³
Laboratory and hematological parameters						
Hb (g/dL)	6.06 ± 0.87	8.96 ± 1.35	12.52 ± 0.64	0.0001	0.0001	0.0001
HbF (%)	8.13 ± 0.59	11.49 ± 2.65	0.59 ± 0.54	<0.0001	<0.0001	<0.0001
HbS (%)	80.9	74.6	NA	<0.0001	NA	NA
RBCs (10 ¹² /L)	2.45 ± 0.5	3.33 ± 0.73	4.43 ± 0.62	0.002	0.0001	0.0001
Hematocrit	22.79 ± 4.66	33.13 ± 6.91	38.99 ± 5.8	0.0001	0.0001	0.011
Platelets (10 ⁹ /L)	317.2 ± 89.5	280.9 ± 52.9	282.2 ± 47.2	0.649	0.243	0.669
WBCs (10 ⁹ /L)	11.35 ± 2.72	8.92 ± 2.3	6.4 ± 2.34	0.028	0.000	0.006
Monocytes (10 ⁹ /L)	0.284 ± 0.082	0.234 ± 0.085	0.202 ± 0.099	0.313	0.049	0.514
Neutrophils (10 ⁹ /L)	5.95 ± 2.78	4.44 ± 2.27	3.38 ± 1.67	0.171	0.008	0.293
Total lymphocytes (10 ⁹ /L)	4.16 ± 0.18	3.16 ± 0.35	3.06 ± 0.29	0.0001	0.0001	0.570
Clinical variables						
VOC event/year	1.84 ± 1.58	0.91 ± 1.47	NA	0.0014	NA	NA
ACS event/year	0.35 ± 0.48	0.08 ± 0.28	NA	0.004	NA	NA

Hb hemoglobin, MCV mean cell volume, HbF fetal hemoglobin, HbS hemoglobin S, RBC red blood cell, WBC white blood cell, VOC vaso-occlusive crisis, ACS acute chest syndrome, NA not applicable.

P values were calculated by using Mann–Whitney test and considered significant when <0.05. P value¹: baseline vs. treated SDC patients, P value²: baseline vs. healthy control, P value³: treated vs. healthy control.

population. The presence of B lymphocytes, T lymphocytes, NKT cells, and NK cells was then assessed on the lymphocyte population (Fig. 1).

For the detection of T helper cells and cytotoxic T lymphocytes, 300 μ L blood sample was cultured in 300 μ L RPMI-1640 medium (1:1) in a fluorescence-activated cell sorting tube and incubated with 3 μ L phorbol myristate acetate (US Biological, MA) and 1 μ L ionomycin (US Biological), which were used as a positive non-specific stimulus, for 24 h at 37 °C in a 5% CO₂ incubator. In all, 3 μ L Brefeldin A (US Biological, MA) was added at the same time to block cytokine secretion at the Golgi, allowing for optimal detection of the molecules.¹² Next, 50 μ L of the prepared mixture was stained with Per-CP-conjugated CD8 and allophycocyanin-conjugated CD4 (BD Biosciences) for 15 min. After red blood cell (RBC) lysis and washing, a fixative solution was added, followed by incubation for 10 min. Permeabilization solution and 5 μ L of PE-conjugated interleukin 4 (IL-4) and FITC-conjugated interferon gamma (IFN γ ; BD Biosciences) were added and the cells were incubated for 20 min. For flow cytometric analysis, 50,000 events per sample were acquired. A scatter histogram was used to define the lymphocyte population. Next, the percentages of CD4+ (T helper [Th]) cells and CD8+ (T cytotoxic [Tc]) cells were assessed in the lymphocyte population. Next, the expression levels of IL-4 and IFN γ in CD4+ lymphocytes and CD8+ lymphocytes were detected (Fig. 1). Th1 cells were defined as IFN γ (+) IL-4(-) CD4(+) cells; Th2 cells were defined as IFN γ (-) IL-4(+) CD4(+) cells; Tc1 cells as IFN γ (+) IL-4(-) CD8(+) cells; and Tc2 cells as IFN γ (-) IL-4(+) CD8(+) cells. The Th1/Th2 and Tc1/Tc2 ratios were calculated.

Statistical analysis

Differences between the groups were assessed for statistical significance using Mann–Whitney test. Data are expressed as mean \pm standard deviation (SD). A P value of \leq 0.05 indicated the existence of a statistically significant difference.

RESULTS

A total of 30 children with SCA and 30 controls were enrolled. No significant differences in the mean age or sex distribution were found between the SCA patient and healthy control group. Hematological parameters indicated that, at baseline (before starting HU treatment), SCA patients had significantly lower Hb, RBCs, and hematocrit levels compared to the control group. In contrast, WBC, neutrophil, monocyte, and total and absolute lymphocyte counts were significantly higher in SCA patients compared to healthy controls, as shown in Table 1.

After 1 year of treatment with HU, Hb levels and percentages of HbF were significantly increased compared to baseline levels ($P < 0.0001$) but were still significantly lower than those of the controls ($P = 0.0001$). Likewise, the percentage of HbS was significantly reduced, and RBC and hematocrit counts were significantly increased but did not reach the normal (control) values.

WBC counts were significantly decreased in SCA patients who received HU compared to their baseline values ($P = 0.02$); however, the counts were still significantly higher than those of the control group. In contrast, platelet counts of SCA patients who received HU treatment were not significantly different compared to either baseline values or the healthy control group.

Total lymphocyte counts were significantly decreased in SCA patients after treatment, and the difference was not statistically significant compared to the value from the control group. Monocyte counts were decreased, but the difference compared to the baseline and healthy control values was not significant. In addition, we observed a significant improvement in the rate of acute complications, including VOCs and ACS, among SCA patients, as shown in Table 1.

In the analysis of lymphocyte subsets, we first compared all SCA patients studied to healthy controls. Compared to controls, our SCA patients (before starting HU treatment) demonstrated a significant increase in lymphocyte subsets, including CD3⁺, CD4⁺, and CD8⁺ T cells. In addition, the absolute count of CD19⁺ B cells was significantly increased in SCA patients compared to controls (Table 2 and Fig. 2a).

The absolute counts of both NK and NKT cells were significantly increased in our SCA patients (Table 2 and Fig. 2b). Furthermore, the percentage of Th2 cells was significantly increased, resulting in a significant decrease in the Th1/Th2 ratio. However, even before treatment, there was no significant difference in the percentage of Th1 cells compared to the healthy control group. Likewise, the percentage of Tc2 cells was significantly increased in comparison to healthy controls, but there was no significant difference in the percentage of Tc1 cells before treatment compared to healthy controls (Table 2 and Fig. 2c).

After HU treatment, there was a significant decrease in lymphocyte subset counts, including CD3⁺, CD4⁺, and CD8⁺ cells, when compared to baseline counts ($P < 0.0001$ for all), and the counts were closer to those of healthy controls, with the

Table 2. lymphocyte subsets in patients with sickle cell anemia, before HU treatment and after 1 year, and healthy control group.

	SCA patients before treatment (n = 30)	SCA patients after 1 year follow up (n = 30)	Healthy controls (n = 30)	P value ¹	P value ²	P value ³
Total lymphocytes (10 ⁹ /L)	4.16 ± 0.18	3.16 ± 0.35	3.06 ± 0.29	0.0001	0.0001	0.570
CD19 ⁺ (10 ⁹ /L)	0.62 ± 0.067	0.61 ± 0.14	0.47 ± 0.095	0.981	0.002	0.001
CD3 ⁺ (10 ⁹ /L)	2.67 ± 0.18	2.3 ± 0.36	2.22 ± 0.24	0.004	0.000	0.662
CD4 ⁺ (10 ⁹ /L)	1.63 ± 0.28	1.38 ± 0.25	1.21 ± 0.16	0.016	0.0001	0.065
CD8 ⁺ (10 ⁹ /L)	0.87 ± 0.15	0.61 ± 0.12	0.6 ± 0.096	0.0001	0.0001	0.989
CD4 ⁺ /CD8 ⁺ ratio	1.76 ± 0.35	1.82 ± 0.51	1.96 ± 0.41	0.935	0.462	0.588
NK cells (10 ⁹ /L)	0.35 ± 0.061	0.29 ± 0.043	0.29 ± 0.07	<0.0001	0.156	0.852
NKT (10 ⁹ /L)	0.11 ± 0.02	0.1 ± 0.017	0.1 ± 0.016	0.248	0.113	0.880
Th1 (%)	21.4273 ± 7.76	24.0289 ± 8.40	18.7375 ± 1.92	0.02	0.510	0.035
Th2 (%)	4.81 ± 1.41	3.19 ± 0.82	2.42 ± 0.61	0.000	0.0001	0.03
Th1/Th2	6.73 ± 2.14	9.65 ± 2.45	10.19 ± 2.08	<0.0001	0.0001	0.733
Tc1 (%)	27.38 ± 8.75	22.5 ± 9.56	24.3 ± 6.78	0.282	0.596	0.777
Tc2 (%)	3.2 ± 1.94	1.37 ± 1	1.56 ± .044	<0.0001	0.001	0.865
Tc1/Tc2	10.02 ± 10.1	17.13 ± 11.22	13.58 ± 6.38	0.007	0.569	0.463

NK natural killer cells, NKT NKT cells, Th T-helper cells, Tc T-cytotoxic cells.

P values were calculated by using Mann–Whitney test and considered significant when <0.05. P value¹: baseline vs. treated SDC patients, P value²: baseline vs. healthy control, P value³: treated vs. healthy control.

exception of CD4⁺ T cells, which did not reach the normal value observed in healthy controls. In contrast, the absolute count of CD19⁺ B cells was non-significantly increased compared to the baseline value but was significantly higher than the count in healthy controls (Table 2 and Fig. 2a).

Moreover, a significant decrease in the absolute count of NK cells was observed compared to baseline values, and the post-treatment counts were closer to those observed in healthy controls. In contrast, the NKT count was not significantly decreased when compared to baseline values and healthy control group (Table 2 and Fig. 2b).

Following treatment with HU, the percentage of Th1 cells was significantly increased compared to baseline values and was non-significantly higher than healthy control values, while the percentage of Th2 cells was significantly decreased compared to baseline values but still significantly higher than in the healthy control group; consequently, the Th1/Th2 ratio was significantly increased compared to baseline values. The percentage of Tc2 cells was significantly decreased after HU treatment and was similar to the value measured in the control group, while the percentage of Tc1 cells was not significantly different to that observed at baseline. As a result, the Tc1/Tc2 ratio was significantly increased after HU treatment (Table 2 and Fig. 2c).

DISCUSSION

HU therapy was approved >20 years ago by the United States Food and Drug Administration, and numerous studies have demonstrated its beneficial effects in improving SCA manifestations.⁹ However, a limited number of studies have focused on the impact of HU treatment on the immune profile of Egyptian SCA patients. Thus, our aim was to analyze the effect of HU on immune profiles of pediatric patients with SCA, comparing these profiles to those of healthy controls.

First, we evaluated the clinical and hematological effects of HU on children with SCA who were followed up at our university hospital. We found that children receiving regular HU therapy exhibited significant clinical and hematological improvements, as had been observed in previous studies.^{13,14} In the present study, SCA children demonstrated a considerable reduction in HbS percentage and increases in HbF, Hb, and hematocrit levels

following HU therapy. Furthermore, in agreement with previous studies, WBC and platelet counts were significantly increased.^{9,15} Similarly, some previous studies have reported a correlation between SCA and elevated WBC count, and others showed abnormal immune activation involving specific cellular subsets in patients with SCA.^{16–18} We also found that HU therapy led to significant improvement in the clinical condition in nearly all patients, as there was a marked reduction in the complication rate in SCA patients who received HU. The VOC rate in this study was significantly decreased after 1 year of HU therapy. In a recent study, this reduction was even greater than we observed (7.34 ± 6.5 to 0.05 ± 0.026).¹⁸ There was also a decrease in the rate of a second episode of ACS in SCA patients after HU therapy, which is in agreement with a previous study¹⁹ which found that the incidence of ACS was decreased in 50% of the patients, as well as another study in which the incidence of ACS dropped from 6.1 to 0.8% of the patients during therapy.¹⁵ The chief mechanism of action of HU in SCA is its ability to induce HbF production in erythrocytes, thereby inhibiting HbS polymerization and sickling. However, HU sometimes has fast benefits observed in SCD prior to the onset of HbF elevations; these benefits include decreased leukocyte counts and increased blood flow due to local vasodilation.^{20,21} A possible explanation for HU's acute effects is its nitric oxide (NO) donor property; previous studies indicate that intravascular NO generation occurs in SCD patients following HU therapy. NO, in turn, promotes vasodilation by activating intracellular cGMP signaling in smooth muscle cells and has been shown to inhibit endothelial and leukocyte activation.^{20,22} Almeida et al.²¹ demonstrated that HU has immediate beneficial effects on leukocyte adhesion recruitment and secondary red cell interactions in a murine sickle cell model of inflammatory vaso-occlusion, particularly when combined with a cGMP amplifying agent.

Lymphocytes have an important role in the regulation of proper inflammatory responses and decreases in their populations could enhance adverse inflammatory states.²³ In our study, we observed a decrease in counts of most of the immune cells measured in SCA patients after receiving HU treatment. Our finding that HU was associated with decreases in total lymphocytes and CD3⁺, CD4⁺, and CD8⁺ T lymphocytes in SCA patients agrees with the results of a recent longitudinal, placebo-controlled study of HU in young

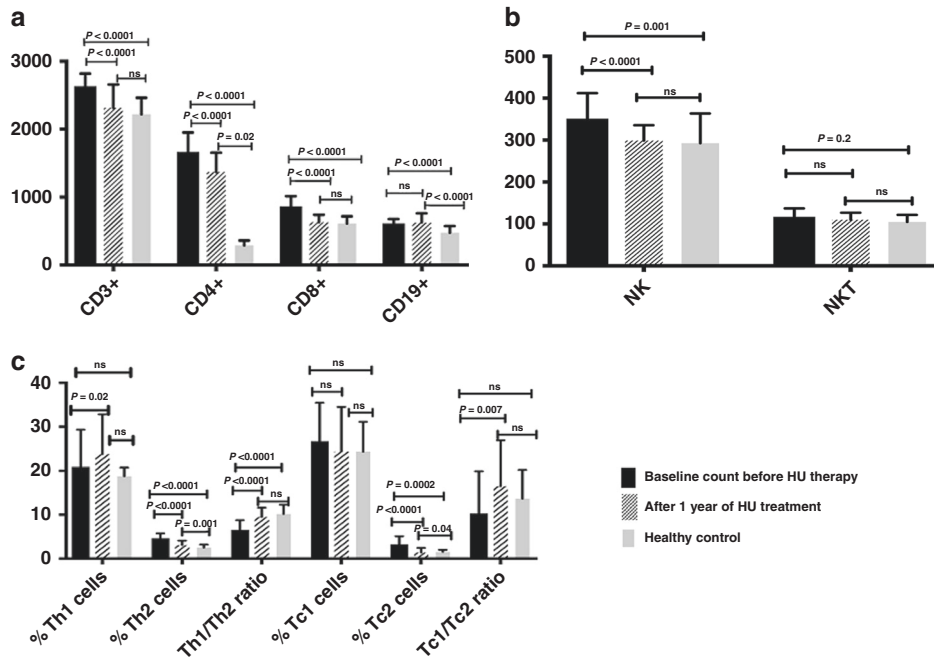


Fig. 2 Difference in immune cells frequencies and counts among patients (before and after HU) and control. The SCA patients' group at baseline before HU treatment (black bars), and after one year of receiving HU treatment (blotted bars), and the second group, which included the healthy control group (grey bars). **a** The difference of CD3+, CD4+, CD8+, and CD19+ among SCA patients and controls. **b** The difference of NK and NKT among SCA patients and controls. **c** The difference of Th1, Th2, Th1/Th2, Tc1, Tc2, and Tc1/Tc2 among SCA patients and controls.

children with SCA.²⁴ In contrast, CD19⁺ B cell counts were non-significantly increased after starting HU treatment but did not reach a normal value. HU appears to reverse the increased T-lymphocyte count, in patients with SCA, in agreement with the results of previous studies.^{7,24} Notably, HU has been shown to reduce leukocytosis in SCA patients.²⁵

Moreover, we found that, before starting HU treatment, the absolute counts of NK and NKT cells were significantly increased compared to the normal values. In addition, in a study using a murine model of SCA, activation of invariant NKT (iNKT) cells was observed. These data may indicate that iNKT cells play an essential role in maintaining inflammation in SCA mice through a pathway comprising IFN γ and the production of chemotactic CXCR3 chemokines. This novel mechanism for vaso-occlusion and subsequent inflammation may also occur in human disease.²⁶ On the other hand, a recent study reported no difference in the absolute count of NK cells between SCA patients and a healthy control group.⁷

Although the data are inadequate to analyze the effect of HU treatment on circulating NK and NKT cells, we found that HU treatment was able to decrease and normalize the absolute count of NK cells, whereas the NKT cell count was non-significantly decreased after HU treatment. Further studies are required to support our results. Such studies could elucidate the mechanisms mediating the effect of HU treatment on counts of circulating NK and NKT cells in patients with SCA.

Th1 cells are responsible for cell-mediated immune protection against intracellular pathogens. Th2 cells protect against extracellular parasites such as helminths and have a role in atopic reactions.²⁷ The Th1/Th2 balance regulates the immune system under normal conditions and is impaired in several autoimmune disorders.^{27,28} In SCA patients who had not yet started HU treatment, we observed a decrease in the percentage of Th1 cells compared to healthy controls, with a shift toward a Th2 bias. This resulted in a lower Th1/Th2 ratio in SCA patients, which may provide a way to estimate the susceptibility to bacterial infections.²⁸ These observations (significant decrease in Th1 and

consequent rise in the Th1/Th2 ratio) appear to be normalized after 1 year of HU treatment. The finding that patients on HU develop a normal Th1/Th2 ratio could be explained by the ability of hydroxycarbamide to preserve splenic function²⁹ by preserving elevated HbF levels in SCA patients,¹¹ thus reducing the predisposition to encapsulated bacterial infection. Airway hyper-responsiveness affects more than half of children with SCA even in the absence of asthma. Previous studies found that Th2 response in asthma is associated with increased expression of placenta growth factor (PIGF) and downstream PIGF genes in the epithelial cells of respiratory passages.³⁰ Eiyomo Mwa Mpollo et al.³¹ observed increased airway hyperresponsiveness and leukotriene levels in a mouse model of SCD, which were abolished by anti-PIGF Ab or the 5-lipoxygenase inhibitor zileuton. They demonstrated that the PIGF exacerbates airway hyperresponsiveness in asthma and serves as a unique link between the leukotriene and Th2 pathways. Additionally, these findings suggest that zileuton and anti-PIGF Ab may be encouraging treatments for reducing pulmonary diseases in patients with SCD.³¹

Study limitations

Although we included most of the children with SCA in our center, the sample size remains relatively small. It is necessary to conduct a larger prospective longitudinal study to more completely evaluate the effects of HU treatment on immune profiles in children with SCA.

CONCLUSION

HU appears to have the beneficial effect of normalizing the immune profile in children with SCA. Studies on lymphocyte function in patients with SCA are needed in the future.

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AUTHOR CONTRIBUTIONS

K.I.E., K.S., I.L.M., M.A.M.Y., and H.F.H. designed the study, followed the patients, analyzed the data, and drafted the manuscript. A.M.Z., A.M.A.G., H.F.H., and Z.A.M.Z. performed all laboratory investigations of the study. A.E., S.M.A.-A., and M.M.E. drafted the manuscript. All authors were involved in the critical analysis of the final version of the manuscript. All authors approved the manuscript as submitted and agree to be accountable for all aspects of the work.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All protocols and investigations of our study followed the regulations of the research ethics committee of Assiut University. All caregivers of all participants have given their informed written consent.

ADDITIONAL INFORMATION

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