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ORIGINAL ARTICLE

Effect of iron deficiency anemia and its treatment on cell mediated immunity

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Abstract Iron deficiency anemia (IDA) is one of the most prevalent micronutrient deficiencies particularly in the developing countries. While there is evidence of an altered immune profile in iron deficiency, the exact immunoregulatory role of iron is not known. Knowledge particularly in children, who are vulnerable to iron deficiency and infection, is lacking. We aimed to study the effects of IDA and its treatment with oral iron supplementation on cell-mediated immunity. The levels of T-lymphocytes, their $CD4^+$, $CD8^+$ and $CD1a^+$ subsets, transferrin receptor (CD71) and serum ferritin were evaluated in 40 iron-deficient and 40 healthy children. The impact of oral iron supplementation for three months on the same parameters was also noted in children with IDA. The level of mature T-lymphocytes ($CD4^+$ and $CD8^+$) was significantly lower ($P<0.001$) while that of the immature T-cells ($CD1a^+$) was significantly higher ($p<0.001$) in IDA children compared to the control. The mature T-cell count was significantly improved after iron therapy. In spite of significant reduction in the immature T-cells (CD1a+) level after iron supplementation, it was significantly higher than the control. The present study demonstrated that T-lymphocytes maturation was defective in IDA and improved partially after 3 months of iron supplementation. Therefore, longer time of iron therapy may be required to induce complete maturation of T-lymphocytes.

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Introduction

Iron deficiency anemia (IDA) due to nutritional deficiency is not just a disease of developing countries but it can also be seen in developed countries. It is considered to be the most prevalent micronutrient deficiency in the world. Estimates indicate that 1.2 billion people suffer mild to severe forms of anemia and up to 46% of children have IDA frequently associated with infection. Micronutrient deficiency has been suggested to impair cell-mediated immunity in particular, iron, zinc and vitamin A deficiencies, all of which have an impact on the immune system [1].

The negative consequences of IDA on behavior [2], psychomotor development [3] and growth rate are well documented, while its effect on the immune system is controversial. Experimental studies have shown that iron is a fundamental element for normal development of the immune system. Its deficiency affects the capacity to have an adequate immune response as it is necessary for immune cell proliferation and the generation of specific response to infection [4]. Despite proven reversible functional immunological defects, a clinically important relationship between states of iron deficiency and susceptibility to infections remain controversial [5].

There are several possible mechanisms that could explain the effects of iron deficiency on the immune system. In humans with intact immune systems, non-specific immunity is affected by iron deficiency in several ways. Macrophage phagocytosis is generally unaffected by iron deficiency, but bactericidal activity of these macrophages is shown to be attenuated in some studies. Neutrophils have a reduced activity of myeloperoxidase, an iron-containing enzyme,

which produces reactive oxygen intermediates responsible for intercellular killing of pathogens [6]. Moreover, in vitro studies have shown that there is a direct link between iron deficiency and impaired T-lymphocyte proliferation. There is a decrease in T-lymphocyte blastogenesis and mitogenesis in response to a number of different mitogens [4]. Cytokines are important mediators of cellular immune activity, although it has been reported that the in vitro production of interleukin-2 by lymphocytes of iron-deficient children may be impaired, little is known concerning the effects of iron deficiency on cytokines [7].

The soluble transferrin receptor CD71 (sTfR) is a clinical marker of erythropoietic activity that is used in the diagnosis of iron deficiency. Also, it represents a valuable quantitative assay of marrow erythropoietic activity as well as a marker of tissue iron deficiency. It has been introduced as a tool for diagnosing iron depletion [1]. It was suggested that the increase in sTfR in even mild iron deficiency reflects the increased density of surface TfR on iron-deprived cells [8].

CD1a has been defined as a marker of the most immature thymocyte populations. Previous studies have found that a higher proportion of circulating immature T-lymphocytes ($CD1a^+$) and a lower proportion of mature T-lymphocytes ($CD4^+$ and $CD8^+$) in malnourished children, and values have been reversed after adequate micronutrient and macronutrient repletion [9].

We undertook this study to evaluate the possible effects of ISA and its treatment with oral iron supplementation on cell-mediated immunity.

Subjects and methods

Eighty-two children with IDA were diagnosed clinically and by laboratory investigations. They were given iron supplementation (6mg/kg/day of elemental iron) for three months after treatment of parasitic infestations. Thirty cases did not complete the study. Twelve cases did not improve (clinically and by laboratory investigations) and they were exposed to further assessment. Those who did not either continue or improve were excluded from the study. So, 40 children with IDA were considered as the patients group and 40 healthy children of comparable age and sex served as the control group.

Exclusion criteria

1. Children with a history of receiving iron or multivitamins one year before the study.
2. Cases with chronic illness, chronic infection or acute infection at the time of the study.
3. Children with evident malnutrition (by anthropometric measurements and estimation of serum albumin).
4. Children with hemoglobin level below 8 gm/dL.
5. Children with a history of chronic blood loss.

Sampling: Five ml of venous blood were collected from each child under aseptic precautions and were divided as follow:

- One ml blood was placed in EDTA-containing vacutainer tube and was used to perform complete blood count.
- Two ml were placed in a plain glass tube and after clotting, the tube was centrifuged at 2000 rpm for 3 min and then the serum was separated to be used for estimation of serum iron, total iron-binding capacity (TIBC) and ferritin.
- Two ml were placed in EDTA-containing vacutainer tube and was used for flow cytometric determination of CD71, CD1a, CD4 and CD8 cells. Sampling was repeated for children of patients group only after three months of iron supplementation.

All the children included in the study were subjected to the following:

1. Thorough history taking and clinical examination.
2. Complete blood count using Advia 60 cell counter (Bayer).
3. Colorimetric estimation of serum iron and TIBC (Biomerieux-France).
4. Estimation of serum ferritin by ELISA (kit supplied by Drg. International Inc, USA.) The manufacturer`s instructions were followed. 5- Flow cytometry for determining CD71, CD1a, CD4 and CD8 cells using FACs caliber flow cytometry (BD, Bectom Dickinson). All antibodies were obtained from Bectom Dickinson.

Flow cytometry procedure

Two color analysis was performed with phycoerythrin (PE) or fluorescent isothyocyanate (FITC) conjugated monoclonal antibodies: CD2-FITC, CD3-FITC, CD1a-PE, CD71-PE, CD4-PE and CD8-PE. # 100 µL from blood samples and 10 µl of appropriate conjugated monoclonal antibodies or matched isotype controls was added each in prepared tube, then vortex and incubated in dark for 30 min on ice.

- RBCs were lysed in 450 µl FACs lyse solution and incubated in the dark for 30 min on ice.
- The samples were washed twice in 5% PBS and resuspended in PBS containing 2% formaldehyde and analysed on a fluorescence-activated cell scanner (FACs Caliber, BD). Acquisition and analysis were performed using cell Quest software.

A total of 5000 events were routinely acquired. The gate was done on the lymphoid populations and T-cells were identified being $CD2^+$.

Statistical methods

Statistical analysis was conducted using the SPSS (version 10, 2002) for windows statistical package. P-value < 0.05 was considered statistically significant.

Results

All data were collected, statistically analyzed and represented in 10 tables and 4 figures.

Table 1 shows that there was no significant difference in age, sex, weight and height between patients and control group.

At presentation, as regard to the hematological parameters, there was significant decrease in Hb concentration, RBCs count, PCV and MCV in IDA patients when compared to the control group. Also, there was significant increase in platelets count and RDW. No significant difference in TLC, lymphocytic count and reticulocyte count could be detected on comparing both groups (Table 2). Serum iron and ferritin were significantly lower in IDA patients with significant increase in TIBC (Table 5). And as regard to the immunological parameters, percentage of CD71+ and CD 1a+ lymphocytes were significantly higher in IDA group before treatment as compared to the controls (P<0.001), while the percentage of $CD4^+$ and $CD8^+$ lymphocytes were significantly lower than the control group (P<0.001) (Table 8). After three months of iron supplementation, all hematological, immunological parameters and iron studies improved significantly [Tables 3, 6 and 9]. Compared to control group, Hb concentration, RBCs count, PCV, MCV, platelet counts were normalized (Table 4). Additionally, serum iron, ferritin and TIBC were normalized after iron supplementation (Table 7). And as regard to the immunological parameters, although the percentage of $CD1a^+$ and $CD71^+$ lymphocytes were significantly decreased after iron supplementation, they did not reach the same level as the control group. Also, significant increase in the percentage of both $CD4^+$ and $CD8^+$ lymphocytes could be detected. Their percentage became comparable with those of the control group (Table 10).

Discussion

Anemia is the most common nutitional disorder in the world and iron deficiency is implicated in the majority of

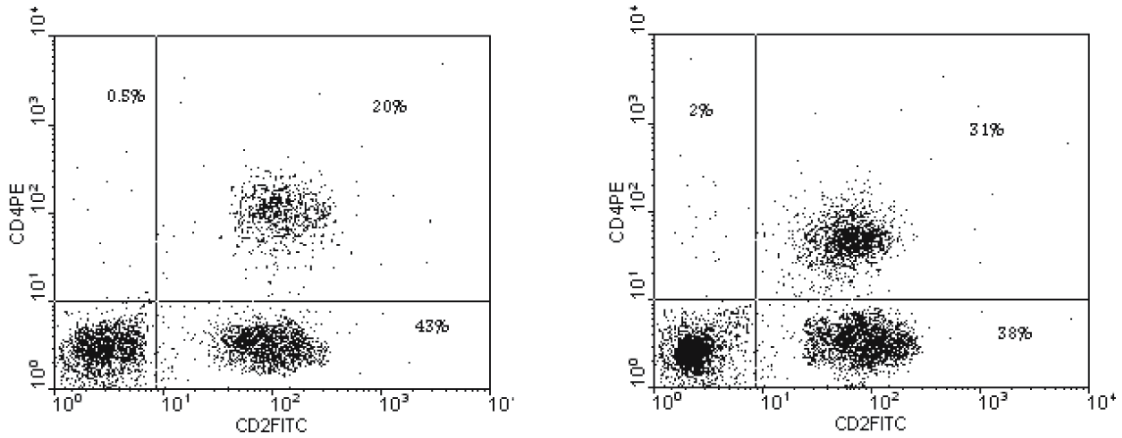

Fig. 1 Dot plot display of flow cytometry showing example of CD4+ percentage of T-lymphocytes in patient with IDA before and after iron supplementation

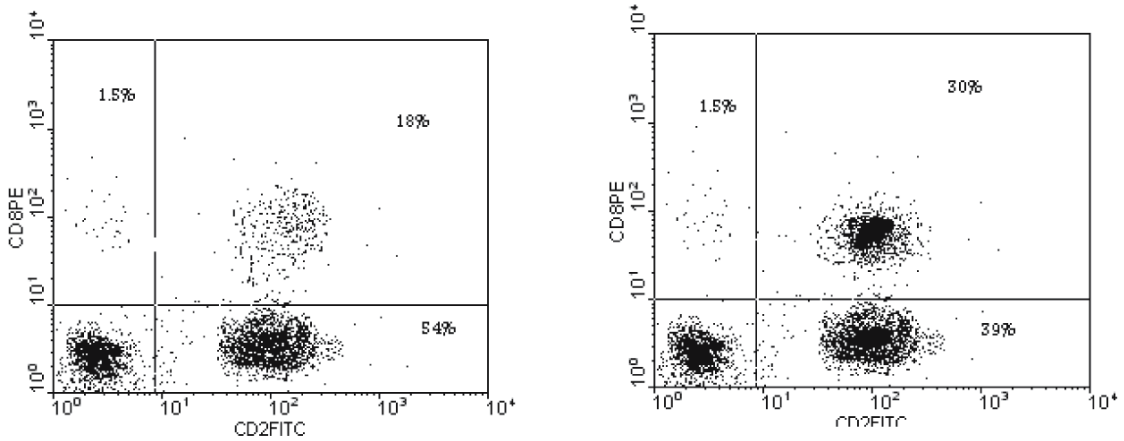

Fig. 2 Dot plot display of flow cytometry showing example of CD8+ percentage of T-lymphocytes in patient with IDA before and after iron supplementation.

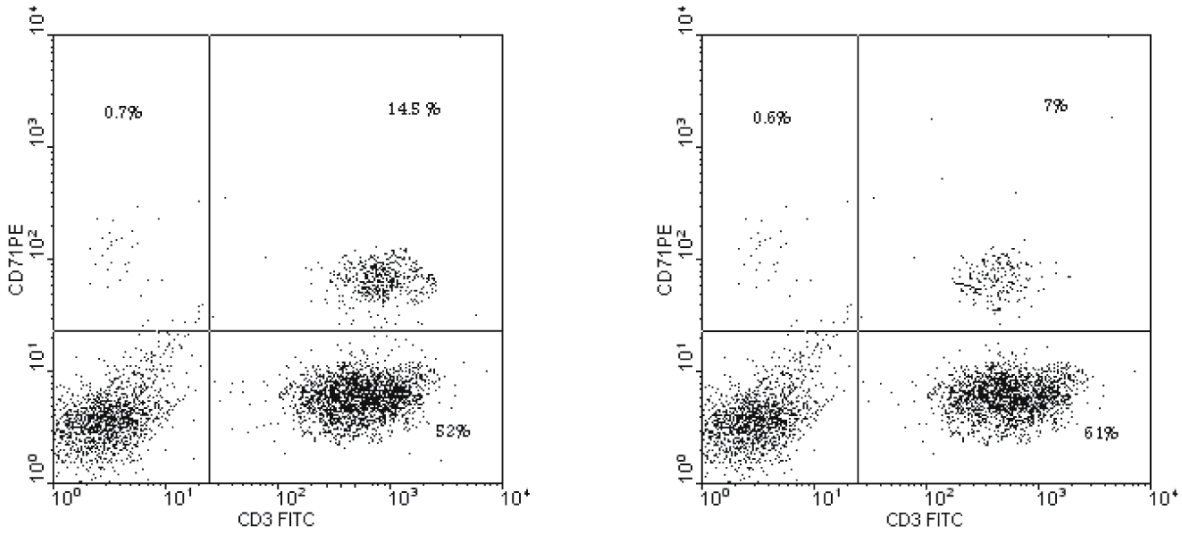

Fig. 3 Dot plot display of flow cytometry showing example of CD71+ percentage of T-lymphocytes in patient with IDA before and after iron supplementation.

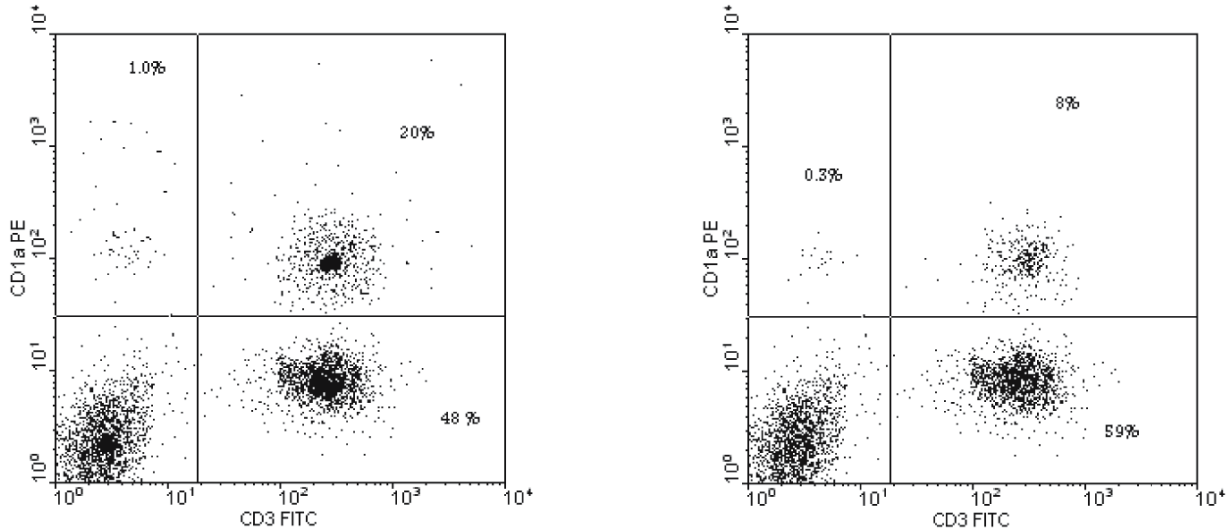

Fig. 4 Dot plot display of flow cytometry showing example of CD1a+ percentage of T-lymphocytes in patient with IDA before and after iron supplementation.

cases. While the prevalance of iron deficiency varies across the world, It is well known that children and women in the reproductive age group are especially vulnerable to iron deficiency [1]. Both experimental and some clinical studies have emphasized the importance of iron in the integrity of the immune system especially in the innate immunity and the cellular component system of active immunity [5].

In this study, the two studied groups were of comparable weight and height, thus eliminating the effect of protein energy malnutrition on cell-mediated immunity. Hematological parameters in IDA group before treatment reflect iron deficiency (significantly lower serum iron, serum ferritin and significantly higher TfR and TIBC) compared to the control group. As regard to the immunological parameters, there was higher proportion of circulating immature T-lymphocytes ($CD1a^+$) and lower proportion of mature T-lymphocytes ($CD4^+$ and $CD8^+$)in the patient group at presentation as compared to control group. The previous data reflects impaired T-lymphocyte maturation.

After iron supplementation for three months, hematological and immunological parameters improved significantly. Compared to the control group, all hematological parameters were normalized and as regard to the immunological indicators, there was significant improvement in $CD1a^+$ and $CD71^+$ lymphocyte subsets, however, they did not reach the same level as the control group.

Table 1 Clinical data of the studied groups

Variables	Control (*n*=40)		Patients (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
Age (years)	6.3–10.6	8.2 ± 1.2	6 - 9.6	7.9 ± 1.04	1.13	0.264
Weight (Kg)	21–30	24.9 ± 2.4	19.5 - 27.6	23.7 ± 2.4	2.38	0.020
Height (cm)	106–130.6	119.9 ± 8.5	105.7 - 130.2	118.9 ± 7.6	0.57	0.572
Sex	N	%	N	%	X2	P
Male	23	57.5	22	55		
Female	17	42.5	18	45	0.06	0.821

Table 2 Hematological parameters in IDA group before treatment as compared to the control group

Variables	Control (*n*=40)		IDA patients before treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
Hb (g/dL)	11–14	12.6 ± 0.8	8–10.5	9 ± 0.7	20.97	<0.001*
RBCs × 10^6/mm^3	3.9–5.4	4.5 ± 0.4	3.2–4.7	3.8 ± 0.36	8.62	<0.001*
PCV	33–42	38.1 ± 2.4	24–32	27.4 ± 2.2	21.13	<0.001*
MCV (fL)	77–94	85 ± 3.9	60–79	72.7 ± 4.6	12.89	<0.001*
RDW	7–9.8	8.4 ± 0.89	10.5–14.3	12.6 ±1.06	18.8	<0.001*
TLC/mm^3	3560–11000	6704 ± 1907	3500–10260	6288 ±1942	0.97	0.337
Total lymphocyte count/ mm^3	1092–5200	2490 ±1073	1060–5220	2428 ± 1025	0.26	0.794
Platelets count × 10^3/ mm^3	185–420	257.3 ± 64	190–510	310.1 ± 69.1	3.55	0.001*
Reticulocyte count %	1.4–2.7	2 ± 0.36	1.1–3	1.9 ± 0.5	1.1	0.277

Table 3 Changes in hematological parameters in IDA group after three months of iron supplementation

Variables	IDA before treatment (*n*=40)		IDA after treatment (*n*=40)		Paired t	P
	Range	Mean±SD	Range	Mean±SD		
Hb (g/dL)	8–10.5	9 ± 0.7	10.8–14.1	12.6 ± 0.7	32.1	<0.001*
RBCs × 10^6/mm^3	3.2–4.7	3.8 ± 0.36	3.8–4.9	4.5 ± 0.3	11.65	<0.001*
PCV	24–32	27.4 ± 2.2	33–43	38.5 ± 2.4	27.9	<0.001*
MCV (fL)	60–79	72.7 ± 4.6	79–90	84.1 ± 2.9	13.66	<0.001*
RDW	10.5–14.3	12.6 ± 1.06	8.2–12.9	10.3 ± 1.2	14.68	<0.001*
TLC / mm^3	3500–10260	6288 ± 1942	3650–10250	6271 ± 1916	0.86	0.397
Total lymphocyte count/ mm^3	1060–5220	2428 ± 1025	1083–4489	2389 ± 946	1.81	0.078
Platelets count × 10^3/mm^3	190–510	310.1 ± 69.1	183–351	241.8 ± 35.6	6.97	<0.001*
Reticulocyte count %	1.1–3	1.9 ± 0.5	2.4–7	4.8 ± 1.2	18.48	<0.001*

In agreeement with our results, Sejas et al. [1] stated that iron deficiency in school children has a significant effect on the levels of the circulating immature lymphocyte subpopulations.They found that the proportions of CD1a and CD71 markers were increased in the anemic group and decreased following iron supplementation, suggesting that iron has a positive effect on proliferation and maturation of T- lymphocytes. It was found that iron deficiency induces thymic atrophy in laboratory animals and very likely in humans by unkown mechanisms possibly by reduced cell proliferation or T-cell differentiation [10].

Also,in accordance with our findings Santos and Falcao [11] reported a decrease in total lymphocyte numbers and $CD3^+/CD4^+$ cell ratio. Luraschi et al. [12] showed decreased $CD8^+$ levels. It was also reported that in iron deficiency, T-lymphocytes showed decreased numbers, blastogenesis and mitogenesis in response to different mitogens. This alteration is largely correctable

Table 4 Hematological parameters in IDA group after treatment as compared to the control group

Variables	Control (*n*=40)		IDA after treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
Hb (g/dL)	11–14	12.6 ± 0.8	10.8–14.1	Mean±SD	0.12	0.907
RBCsx10^6/mm^3	3.9–5.4	4.5 ± 0.4	3.8–4.9	12.6 ± 0.7	0.17	0.862
PCV	33–42	38.1± 2.4	33–43	4.5 ± 0.3	0.84	0.405
MCV (fL)	77–94	85 ± 3.9	79–90	38.5 ± 2.4	1.22	0.226
RDW	7–9.8	8.4 ± 0.89	8.2–12.9	84.1 ± 2.9	7.95	<0.001*
TLC / mm^3	3560–11000	6704 ± 1907	3650–10250	10.3 ± 1.2	1.00	0.319
Total lymphocyte count / mm^3	1092–5200	2490 ± 1073	1083–4489	6271 ± 1916	0.45	0.657
Platelets count x10^3/ mm^3	185–420	257.3 ± 64	183–351	2389 ± 946	1.33	0.188
Reticulocyte count %	1.4–2.7	2 ± 0.36	2.4–7	241.8 ± 35.6	13.85	<0.001*

Table 5 Iron studies in IDA group before treatment as compared to the control group

Variables	Control (*n*=40)		IDA before treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
Serum iron (µg/dl)	54–134	91.8 ± 23.8	12. 8–45	25.5 ± 9.3	16.4	<0.001*
Ferritin (ng/ml)	16–173	83.5 ± 44.4	1.9–30	8.6 ± 6.9	10.5	<0.001*
TIBC (µg/dl)	247-540	350 ± 65.8	229–532	410.2 ±71.6	3.9	<0.001*

Table 6 Changes in iron studies in IDA group after three months of iron supplementation

Variables	IDA before treatment (*n*=40)		IDA after treatment (*n*=40)		Paired t	P
	Range	Mean±SD	Range	Mean±SD		
Serum iron (µg/dl)	12.8–45	25.5 ± 9.3	52–124	83.7 ± 22.2	24.65	<0.001*
Ferritin(ng/ml)	1.9–30	8.6 ± 6.9	30–140	87.5 ± 29.1	19.41	<0.001*
TIBC (µg/dl)	229–532	410.2 ±71.6	260–486	348.3 ± 54.3	5.3	<0.001*

Table 7 Iron studies in IDA group after treatment as compared to the control group

Variables	Control (*n*=40)		IDA after treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
Serum iron (µg/dl)	54–134	91.8 ± 23.8	52–124	83.7 ± 22.2	1.58	0.118
Ferritin(ng/ml)	16–173	83.5 ± 44.4	30–140	87.5 ± 29.1	0.48	0.634
TIBC(µg/dl)	247–540	350 ± 65.8	260–486	348.3 ±54.3	0.12	0.908

Table 8 T-lymphocyte subsets in IDA group before treatment as compared to the control group

Variables	Control (*n*=40)		IDA before treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
CD1a+%	1.6–5.2	3.18 ± 1.2	13.5–22.8	18.3 ± 2.6	33.7	<0.001*
CD71+%	1.7–4.2	3 ± 0.76	13–20.1	16.5 ± 2.4	33.9	<0.001*
CD4+%	26.7–44.5	36.2 ± 5.6	19.2–32.8	26.7 ± 4.5	8.36	<0.001*
CD8+%	20–45.6	28.7 ± 5.9	16.4–29.3	21.6 ± 2.7	6.99	<0.001*

with iron repletion [13]. In addition, Berger et al. [14] have found that the number as well as percentage of mature T cells were lower in iron deficient children as compared to controls. Also, Mullick et al. [15] observed a trend of lower $CD4^+$ cells with increasing severity of IDA.

In contrast with our findings, Ekiz et al. [5] reported that there was no change in the T-lymphocyte numbers and distribution of subgroups in cases with IDA. The reported T-cell dysfunction may be the result of functional defects of T-cells rather than quantitative defects. Several investigators showed decreased levels of several cytokines in IDA

Table 9 Changes in T-lymphocytes subsets in IDA group after three months of iron supplementation

Variables	IDA before treatment (*n*=40)		IDA after treatment (*n*=40)		Paired t	P
	Range	Mean±SD	Range	Mean±SD		
CD1a+%	13.5–22.8	18.3 ± 2.6	6.2–12.2	9.6 ± 2.0	15.9	<0.001*
CD71+%	13–20.1	16.5 ± 2.4	6.5–12	9.4 ± 1.7	13.9	<0.001*
CD4+%	19.2–32.8	26.7 ± 4.5	29.2–43.5	35.05 ± 4.0	8.58	<0.001*
CD8+%	16.4–29.3	21.6 ± 2.7	23.8–43.6	30.7 ± 5.5	9.54	<0.001*

Table 10 T-lymphocyte subsets in IDA group after treatment as compared to the control group

Variables	Control (*n*=40)		IDA after treatment (*n*=40)		t	P
	Range	Mean±SD	Range	Mean±SD		
CD1a+%	1.6–5.2	3.18 ±1.2	6.2–12.2	9.6 ± 2.0	17.33	<0.001*
CD71+%	1.7–4.2	3 ± 0.76	6.5–12	9.4 ± 1.7	21.63	<0.001*
CD4+%	26.7–44.5	36.2 ± 5.6	29.2–43.5	35.05 ± 4.0	1.06	0.291
CD8+%	20–45.6	28.7 ± 5.9	23.8–43.6	30.7 ± 5.5	1.53	0.129

and T-cell dysfunction may be the result of this low cytokine activity [16].

Also, Thibault et al. [17] reported a quantitative rather than a qualitative defect in cell-mediated immunity, They observed decreased in vitro IL-2 production by lymphocytes of IDA children, while the numbers of lymphocytes were noted to be similar in iron deplete and control groups. The discrepancy between these findings and the results of the present study can be attributed to different size, locality or presence of other factors affecting immunity as other nutritional deficiencies or infections.

After treatment of IDA group, the disturbed immunological parameters were reversed with significant decrease of $CD1a^+$ cells and significant increase of $CD4^+$ and CD8+ T-lymphocytes, confirming the proliferative and maturing effect of iron on T- lymphocytes.

On comparing IDA group after treatment with control group, most hematologic and immunologic parameters were comparable except CD71 and CD1a were still significantly higher in treated IDA group when compared to controls. CD71 is a marker of tissue iron deficiency and this may take longer time of treatment to be corrected. Skikne et al. [18] showed that sTfR increase early in iron deficiency even before development of anemia and it is a sensitive marker of tissue iron deficiency with or without anemia. Sejas et al. [1] addressed the importance of measuring CD1a as a marker of T- cell differentiation and considered measuring CD4 and CD8 as only indirectly indicating problems during T- cell maturation. The higher CD1a in treated IDA group compared to controls may indicate the need for longer time of treatment to fully achieve T-cell maturation or the need for further study of the role of other micronutrients deficiencies as vitamin A and/or zinc deficiency in those patients.

Further studies to evaluate the effect of iron deficiency on other aspects of the immune system as humoral immunity and phagocytic activity is highly recommended. Also, other factors that can influence the action of immune system in conjuction with iron deficiency can be studied as infections and other micronutrient deficiencies. From this study, it could be concluded that T-cell maturation was defective in IDA group before treatment. This defect was improved by iron supplementation for three months. Immature T-cells and sTfR were still significantly higher in IDA group after treatment compared to controls indicating the possibility of requiring more time to full recover T- cell immunity and replenish tissue iron.

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