Could the Crosstalk Between Mdscs and Tregs Have a Role in β-thalassemia?

Asmaa M Zahran (zahranam@aun.edu.eg)
Clinical Pathology Department, South Egypt Cancer Institute, Assiut University, Assiut, Egypt
Omnia El-Badawy
Medical Microbiology & Immunology Department, Faculty of Medicine, Assiut University, Assiut, Egypt
https://orcid.org/0000-0001-8445-711X
Eman R. Badawy
Clinical Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt
Marwa Ghazaly
Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt
Khaled Saad
Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt
Khalid Hashim Mahmoud
Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt
Amira Elhoufey
Department of Community Health Nursing, Faculty of Nursing, Assiut University, Assiut, Egypt. Department of Community Health Nursing, Alddrab University College, Jazan University, Jazan, Saudi Arabia.
Khalid I. Elsayh
Pediatric Department, Faculty of Medicine, Assiut University, Assiut, Egypt

Research Article

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Abstract

Background: Secondary-iron overload, alloimmunization, and increased risk of infections are common complications in β-thalassemia major (BTM) patients. Tregs and myeloid-derived suppressor cells (MDSCs) play an essential role in preventing excessive immune responses. This research aimed to investigate the interaction between Tregs and MDSCs in BTM patients and their relation with disease severity.

Methods: This case-control study included 26 patients with BTM and 23 healthy age- and sex-matched controls. All patients were investigated for complete blood picture, serum ferritin, and flow cytometric analysis of peripheral blood to detect Tregs, MDSCs, and MDSC subsets.

Results: A significant increase was observed in the frequencies of Tregs and MDSCs, particularly MO-MDSCs, in the patients compared with the controls. These cells also showed direct relations with ferritin and TLC and an inverse association with hemoglobin. Furthermore, a positive correlation was seen between Tregs and each of the total MDSCs and MO-MDSCs.

Conclusion: Our findings highlight the role Tregs and MDSCs cooperatively plays in the BTM and their importance in suppressing the high activity of the immune system found in those patients due to repeated blood transfusions and antigenic stimulation.

Introduction

Thalassemia is a genetic disorder in which there is a defect in hemoglobin production. Thalassemia major (TM) patients suffer from severe anemia and require regular blood transfusion [1]. TM is a common health problem in Mediterranean countries, especially Egypt. TM's most common complications are iron overload, heart failure, recurrent infections, and alloimmunization from repeated blood transfusion [2].

Regulatory T cells (Tregs) are CD4 + CD25 + ^{Hi} that express the transcription factor forkhead box protein 3 (Foxp3) and form up to 5–10 % of CD4 + T Cells [3]. Tregs' primary function is to induce and maintain peripheral tolerance, which plays a vital role in preventing excessive immune responses and autoimmunity [4]. The increase in antigenic stimuli that occur due to repeated blood transfusions might change the percentage of Tregs and Foxp3 in β-thalassemia [5]. Previous studies showed that Tregs have a crucial role in the magnitude and frequency of alloimmunization by suppressing the immune system [6].

Myeloid-derived suppressor cells (MDSCs) are innate immune cells known as a heterogeneous group of immature myeloid cells at a specific differentiation stage with an immunosuppressive effect. Normally they are present in low numbers and increase in certain disease conditions, psychological stress, or natural aging [7,8]. They include monocytic MDSCs (MO-MDSCs) which are HLA-DR-, CD11b+, CD33+, and CD14 + and polymorphonuclear MDSCs (PMN-MDSCs) which are HLA-DR-, CD11b+, CD33+, and CD15 +

[7]. The main function of MDSCs is to suppress immune cells, mainly T-cells, and to a lesser extent, B-cells and Natural killer (NK) cells [9].

Previous studies showed that chronic inflammatory conditions as in thalassemia, persistent tissue damage as in cancer, autoimmunity, and chronic infections increase the release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular pattern (PAMPs) and production of various cytokines that in turn increase the release of myeloid cells from marrow and induce the immunosuppressive effect of MDSCs [10]. The possibility that MDSCs play a role in immune dysregulation in hematologic malignancies, BM failure syndromes, and autoimmune disorders and their role as therapeutic targets has attracted the interest to study it in other hematological diseases [11]

Interaction between Tregs and MDSCs was studied in various conditions as tumors, allergic disorders, diabetes, and autoimmune diseases. Previous studies showed that MDSCs could control de novo development and induction of Tregs [12]. The overlap of Tregs and MDSCs target cells indicates the significance and resilience of immune suppression under pathological conditions [13]. This research aimed to study the interaction between Tregs and MDSCs in β-thalassemia major (BTM) patients and their relation with disease severity.

Patients And Methods

This case-control study was held in the Pediatric Hematology Unit on patients with BTM, Assiut University Children Hospital, Assiut, Egypt. The study was approved by Ethics Committee of Faculty of Medicine, Assiut University, Assiut, Egypt, (Approval No. 17300557). All methods were carried out in accordance with local guidance after written informed consents were obtained from the guardians of participants.

The study included 26 patients with BTM, and 23 healthy age- and sex-matched children were enrolled as controls. Patients were receiving a regular blood transfusion and chelation therapy. Patients with known diabetes, cardiac, renal, infectious, inflammatory, or pulmonary diseases, newly diagnosed and non-transfusion dependent BTM cases were excluded from the study. Any patient with a history of recent infection or any immunosuppressive medications, e.g., steroids, etc. during one month before enrollment, was also excluded.

All patients were subjected to history and physical examination, in addition to complete blood picture, serum ferritin, and flow cytometric analysis of peripheral blood to detect Tregs (CD4 + CD25 + ^{Hi}Foxp3+), MO-MDSCs (HLA-DR-, CD11b+, CD33+, CD14+) and PMN-MDSCs (HLA-DR-, CD11b+, CD33+, CD15+).

Flow cytometric detection of regulatory T cells:

Regulatory T cells were enumerated using fluorescein isothiocyanate (FITC)-conjugated Foxp3 (IQ Product the Netherland), phycoerythrin (PE) conjugated CD25 (Bioscience, USA), and Peridinium-chlorophyll-protein (Per-CP)-conjugated CD4 (Becton Dickinson (BD) Bioscience, CA, USA). Five µl of CD4

and CD25 were incubated with 50 µl of the blood sample for 15 minutes at 4 C in the dark. Following incubation, red blood cell lysis and washing with phosphate-buffered saline (PBS) were done. Then fixing solution was added, and incubation for 10 minutes was done. Afterward, cells were washed with PBS, and then the permeabilizing solution and 5 µl of Foxp3 were added and incubated for 20 minutes at 4C. After one wash, the cells were resuspended in PBS and analyzed by FACSCalibur flow cytometry with CellQuest software (Becton Dickinson Biosciences, USA). An isotype-matched negative control was used for each sample. Lymphocytes were detected according to their forward and side scatters. Then CD4 + cells were gated. Total CD4 + CD25+, CD4 + CD25 + ^{low}, CD4 + CD25 + ^{Hi} T cells and CD4 + CD25 + ^{Hi}Foxp3+ Tregs were evaluated as percentages from $CD4^+$ cells as shown in figure (1).

Flow cytometric detection of myeloid-derived suppressor cells:

MDSCs were detected by using FITC-conjugated CD11b, PE-conjugated CD33, Per-CP-conjugated CD15, Per-CP-conjugated CD14, and allophycocyanin (APC) conjugated HLA-DR (All from Becton Dickinson (BD) Biosciences, San Jose, CA, USA). Blood sample (50 µl) was incubated with 5 µl of CD33, CD11b, HLA-DR, and CD14 in one tube and CD33, CD11b, HLA-DR, and CD15 in another tube for 20 minutes at 4◦ C in the dark. Following incubation, red blood cell lysis and washing with PBS were done. Then cells were suspended in PBS and analyzed using Cell Quest software on BD FACSCalibur flow cytometer, as presented in figure (2). An isotype-matched negative control was used for each sample. HLA-DR negative cells were selected from HLA-DR and side scatter histogram. The HLA-DR negative cells were assessed for their expression of CD33 and CD11b to detect total myeloid-derived suppressor cells (MDSCs: HLA-DR-CD33 + CD11b+). Then MDSCs were assessed for their expression of CD15 and CD14 to detect MO-MDSCs (HLA-DR-CD33 + CD11b + CD14+) and PMN-MDSCs (HLA-DR-CD33 + CD11b + CD15+).

Statistical Analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS 22, IBM, USA). Data were expressed as the mean ± standard deviation of the mean (SD) or standard error (SE). The differences between the groups were examined for statistical significance using the Independent t-test. The correlation coefficient was generated by Pearson's correlation. Statistical significance was defined as $p < 0.05$.

Results

This study was conducted on 26 BTM patients with a mean age of 8.7 ± 5, and 54% were males. The healthy individuals' mean age was 8.2 ± 3, and 52% were males. Demographic data and laboratory investigations of all patients are presented in table (1). Results showed a significant increase in total leukocyte count (TLC) and ferritin than the reference ranges. On the other hand, there was a decrease in HB and platelet (PLT) count.

Table 1
Demographic data of patients

Parameters	**Patients (n = 26)**
Age	8.7 ± 5
Sex* Males	14 (54%)
Females	12 (46%)
Weight (Kg)	21.7 ± 10
Height (cm)	113.7 ± 25
Head circumference (cm)	50 ± 2
TLC ($X10^9$/L)	14.6 ± 3
PLT ($X10^9$/µl)	221.7 ± 45
HB (g/dl)	7.8 ± 1
Ferritin (ng/ml)	883.8 ± 229
n number, TLC total leukocyte count, PLT platelet count, HB hemoglobin	
*Data expressed as mean ± SD, * number (percent)*,	

Levels of Tregs and MDSCs in patients and healthy controls

A comparison of the levels of Tregs and total MDSCs between BTM patients and controls is shown in table (2). Patients with BTM had higher percentages of both CD4 + $CD25^{Hi}$ T cells and CD4 + $CD25^{Hi}$FoxP3 + Tregs compared to controls. On the line, total MDSCs, particularly the monocytic type (MO-MDSCs), significantly raised.

Table 2
Levels of Tregs and MDSCs in thalassemia patients and controls

Cells (%)	Patients (n = 26)	Control (n = 23)	*p*-value
CD4 + T cells	44.7 ± 1	43.95 ± 1	0.7
CD4 + CD25 + T cells	12.6 ± 0.3	11.6 ± 0.3	**0.02**
CD4 + $CD25^{low}$ T cells	8 ± 0.4	8.8 ± 0.3	0.2
CD4 + $CD25^{Hi}$ T cells	4.7 ± 0.6	2.9 ± 0.4	**0.03**
CD4 + $CD25^{Hi}$FoxP3 + Tregs	2.4 ± 0.2	1.3 ± 0.1	**< 0.0001**
Total MDSCs	3.6 ± 0.3	1.3 ± 0.1	**< 0.0001**
PMN-MDSCs	86.2 ± 2	88.9 ± 0.4	0.3
MO-MDSCs	13.7 ± 1	10.3 ± 0.5	**0.009**

n number, Tregs regulatory T cells. MDSCs myeloid-derived suppressor cells, PMN-MDSCs Polymorphonuclear myeloid-derived suppressor cells, MO-MDSCs monocytic myeloid-derived suppressor cells. Results expressed as mean ± SE, Independent t-test, Significant p-value < 0.05

The correlations between the frequencies of Treg cells, MDSCs, and laboratory parameters:

As illustrated in figure (3), CD4 + $CD25^{Hi}$FoxP3 + Tregs were directly related to serum ferritin level (r = 0.5, $p < 0.0001$), and TLC (r = 0.6, $p < 0.0001$), and inversely related to hemoglobin level (r= -0.6, $p < 0.0001$).

Similarly, total MDSCs have shown positive correlations with serum ferritin level (r = 0.7, $p < 0.0001$), and TLC (r = 0.5, $p < 0.0001$), and negative correlations with platelet count (r= -0.4, $p = 0.001$), and hemoglobin level (r= -0.6, $p < 0.0001$), figure (4).

MO-MDSCs were directly associated with serum ferritin level (r = 0.7, $p < 0.0001$), and TLC (r = 0.4, $p = 0.001$), and inversely associated with hemoglobin level (r= -0.4, $p = 0.005$), figure (5). Additionally, Tregs had significant positive correlations with total MDSCs (r = 0.3, $p = 0.01$) and MO-MDSCs (r = 0.3, $p = 0.02$), figure (6).

Discussion

β-thalassemia is one of the commonest hereditary blood disorders. Regular transfusions, which remain the gold standard of therapy for β-thalassemia, effectively manage thalassemia symptoms. Even though secondary-iron overload remains the main consequence of transfusion in BTM patients [14], alloimmunization and increased risk of infections are other common complications in BTM patients. Pathogenesis is not fully understood [1]. Alloimmunization was found to depend on many factors as RBC

antigen discrepancy between donor and recipient, immune status of the recipient, and the immunomodulatory effects of allogeneic blood transfusion [15]. The immune system is, therefore, a key player in the clinical features accompanying thalassemia.

Here we have studied the frequency of Tregs and MDSCs in BTM patients and assessed their relation with disease severity. Our results showed increased frequencies of Tregs and total MDSCS, particularly the MO-MDSCs compared to controls. Tregs are a component of the immune system that suppress immune responses of other cells. Earlier studies reported higher Treg levels in TM patients compared with the normal subjects [5,15]. Bozdogan and others suggested that increased Tregs in BTM patients might be due to the chronic exposure to antigenic stimulus because of frequent blood transfusions that trigger Tregs to prevent alloimmunization [5]. In line with our results, a significant positive correlation was observed between the Tregs and ferritin concentration in thalassemia patients. Consequently, as ferritin increases, it can suppress the immune system by inducing Tregs in these patients [15].

MDSCs are functionally comparable to Tregs. Several studies described a significant elevation in total MDSCS and MO-MDSCS cells in chronic inflammatory diseases and tumors [16,17] as an induced core anti-inflammatory mechanism to inhibit unwarranted immune cell activities. They negatively regulate immune function by suppressing the activity of T cells, NK cells, and B cells [18], and their rise in BTM could be implicated in increased liability to infection in this group.

Little is known about MDSCs in BTM. Siriworadetkun and colleagues reported an increased level of MO-MDSCs in BTM patients, especially the splenectomized patients, compared to the healthy controls [18]. The chronic inflammation may have triggered bone marrow to generate MDSCs causing MDSC expansion and accumulation in the circulation of BTM patients. In agreement with other studies [19,20], our BTM patients had a high level of inflammatory cells in the form of high TLC. Additionally, the direct relations observed between TLC and each of total MDSCs and MO-MDSCs may support the hypothesis mentioned above that chronic inflammation triggers bone marrow to generate these cells.

The relation between Tregs and MDSCs has been widely studied. Previous researches suggested that MDSCs release IL-10 and transforming growth factor β (TGF-β), which are critical for the induction of Tregs [21–23]. On the other hand, Tregs can enhance MDSCs function and control their differentiation through a mechanism involving TGF-β [24]. An earlier study of MDSC relation with Tregs in transient hypogammaglobulinemia suggested that its pathogenesis is based on the interplay between the MDSCs and Tregs [25]. However, till recently, the association of MDSCs and Tregs has not been examined in BTM.

Our findings demonstrated that in the BTM group, both Tregs and MDSCs, particularly the MO-MDSCs, were significantly higher than healthy control subjects. Also, these cells had shown direct relations with ferritin and TLC and an inverse association with hemoglobin. Furthermore, a positive correlation was seen between Tregs and each of the total MDSCs and MO-MDSCs.

Conclusion

Altogether, our findings highlight the role Tregs and MDSCs cooperatively plays in the BTM and their importance in suppressing the high activity of the immune system that is found in those patients due to repeated blood transfusions and antigenic stimulation.

Abbreviations

Beta-thalassemia major BTM

damage-associated molecular patterns (DAMPs)

fluorescein isothiocyanate (FITC)

forkhead box protein 3 (Foxp3)

Myeloid-derived suppressor cells (MDSCs)

pathogen-associated molecular pattern (PAMPs)

phycoerythrin (PE)

Peridinium-chlorophyll-protein (Per-CP)

polymorphonuclear MDSCs (PMN-MDSCs)

thalassemia major TM

regulatory T cells (Tregs)

Declarations

Due to technical limitations, Declaraitons section is not available for this version.

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Figures

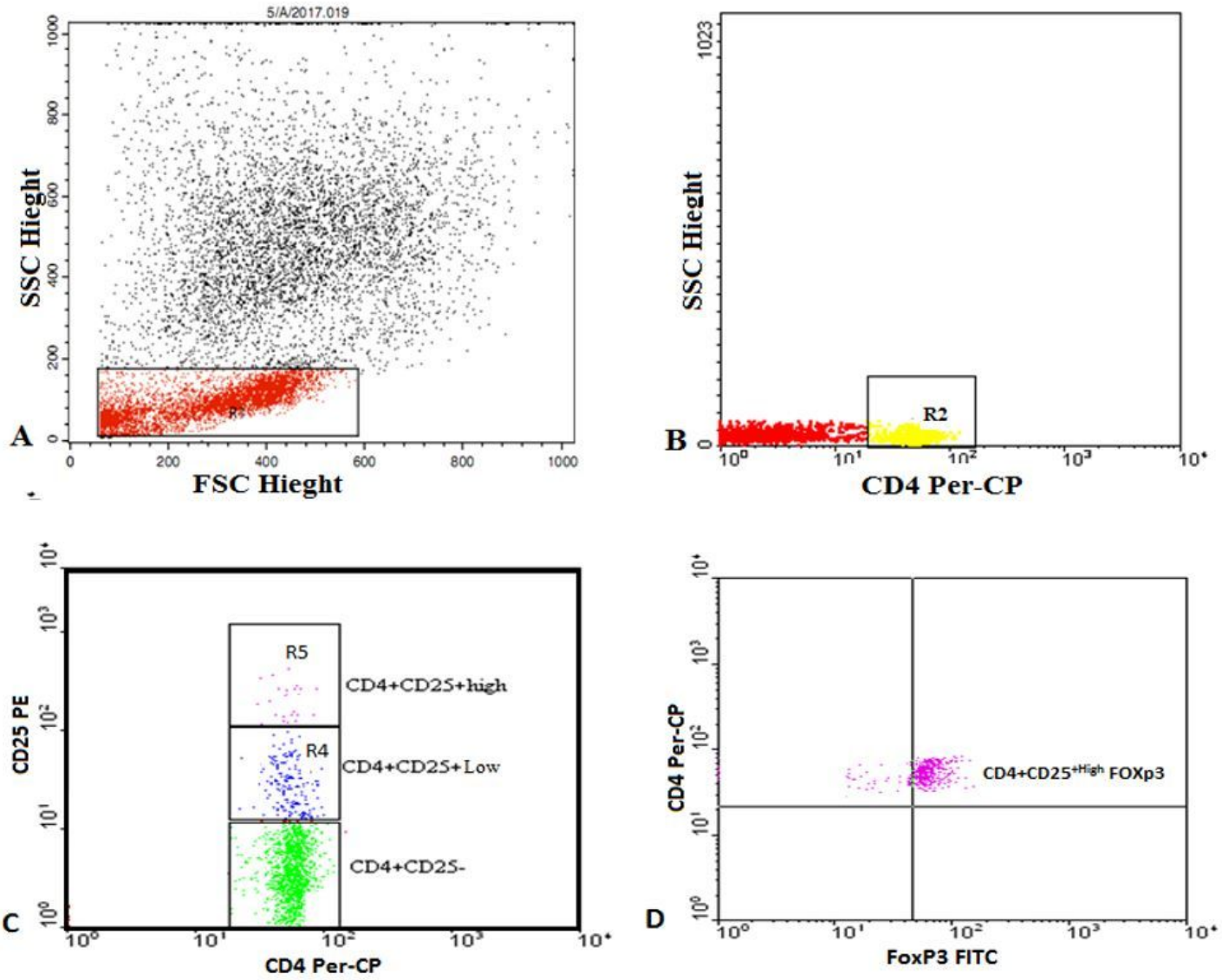

Figure 1

Flow cytometric detection of regulatory T cells. A: The lymphocyte population was defined on a forward and side scatter histogram (R1). B: The expression of CD4 on the lymphocytes population was detected, then CD4+ cells were gated (R2) for further analysis of CD25. C: Three gates were drawn to define CD4+CD25- cells (R3), CD4+CD25+low cells (R4), and CD4+CD25+hi cells (R5). D: The percentage of CD4+CD25+hiFoxp3+cells (regulatory T cells) was then assessed.

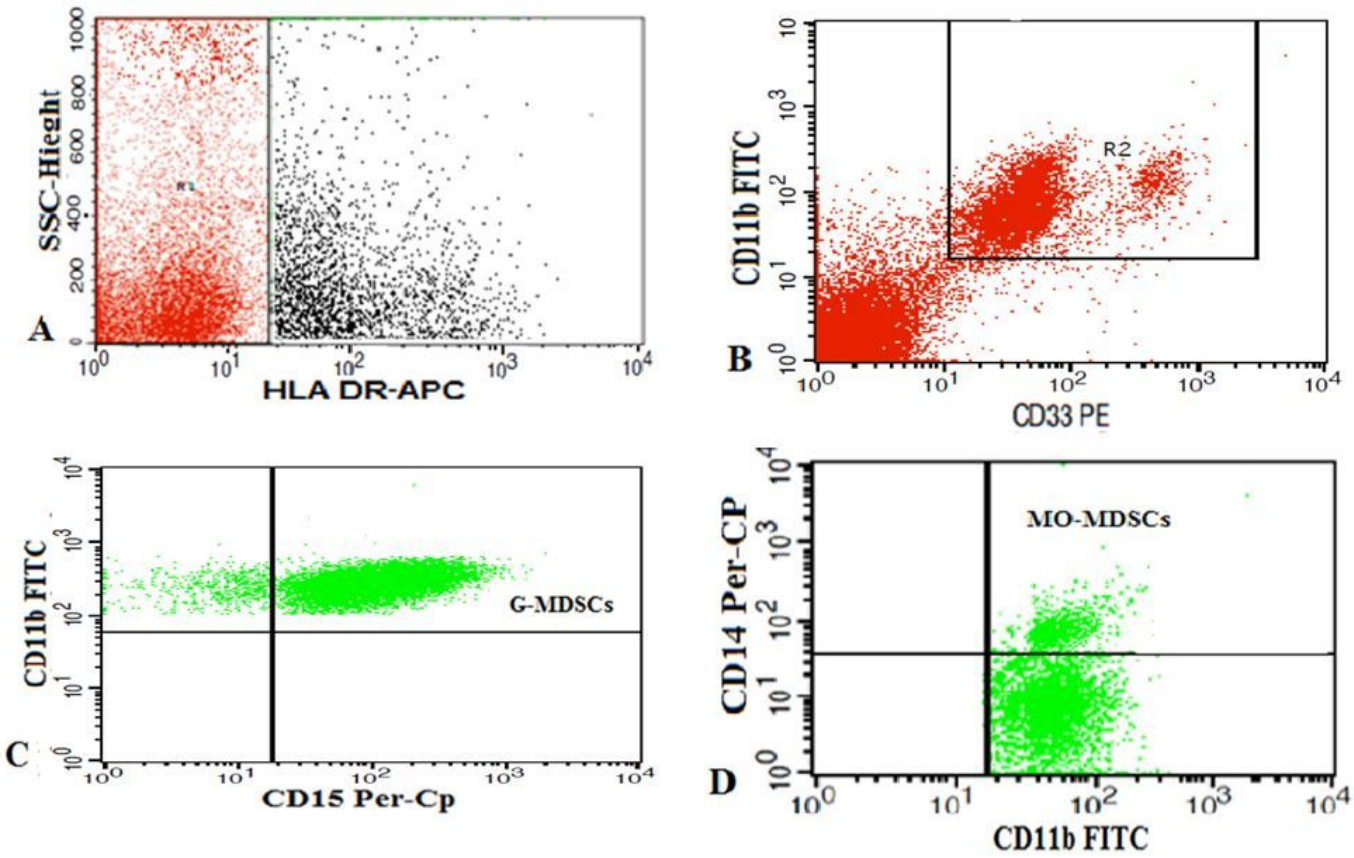

Figure 2

Flow cytometric detection of myeloid-derived suppressor cells A: HLA-DR negative cells (R1) were selected from HLA-DR and side scatter histogram. B: HLA-DR negative cells were assessed for their expression of CD33 and CD11b to detect total MDSCs (R2) (HLA-DR-CD33+CD11b+) C,D: Total MDSCs were assessed for their expression of CD15 and CD14 to detect MO-MDSCs (HLA-DR-CD33+CD11b+CD14+) and PMN-MDSCs (HLA-DR-CD33+CD11b+ CD15+)

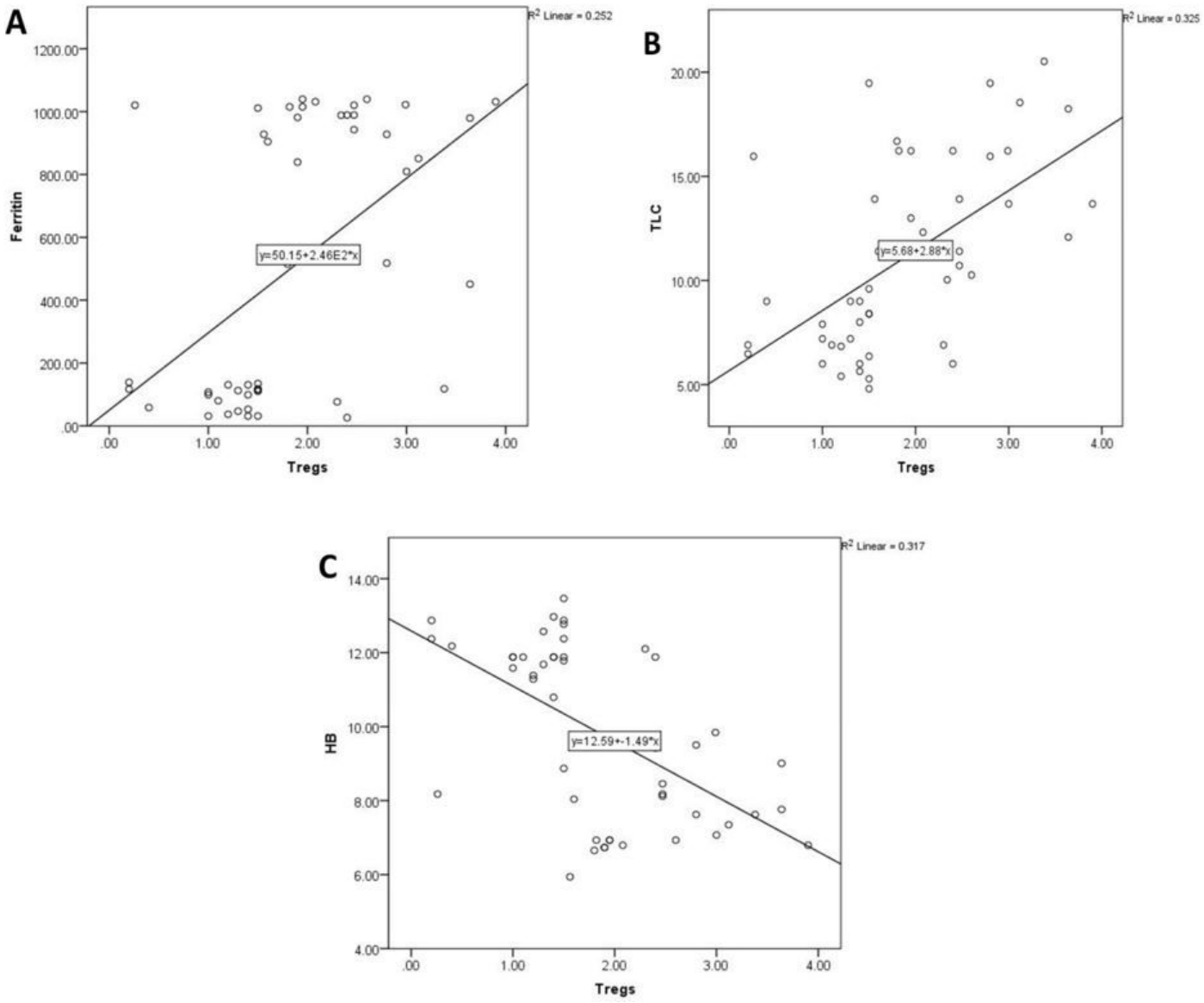

Figure 3

Correlations of CD4+CD25HiFoxP3+ Tregs with serum ferritin level (A), TLC (B), and hemoglobin level (C).

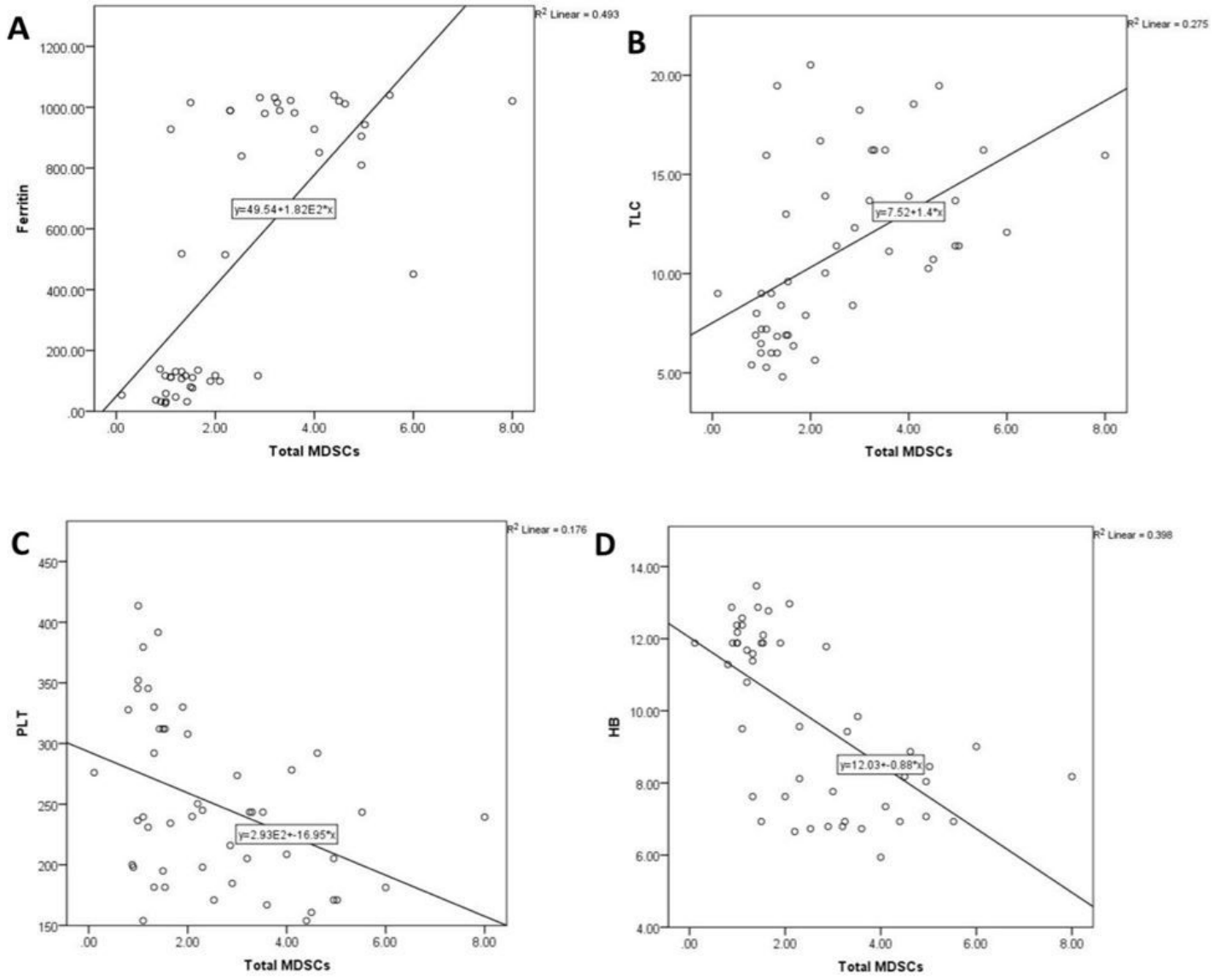

Figure 4

Correlations of total MDSCs with serum ferritin level (A), total leukocyte count (B), platelet count (C), and hemoglobin level (D).

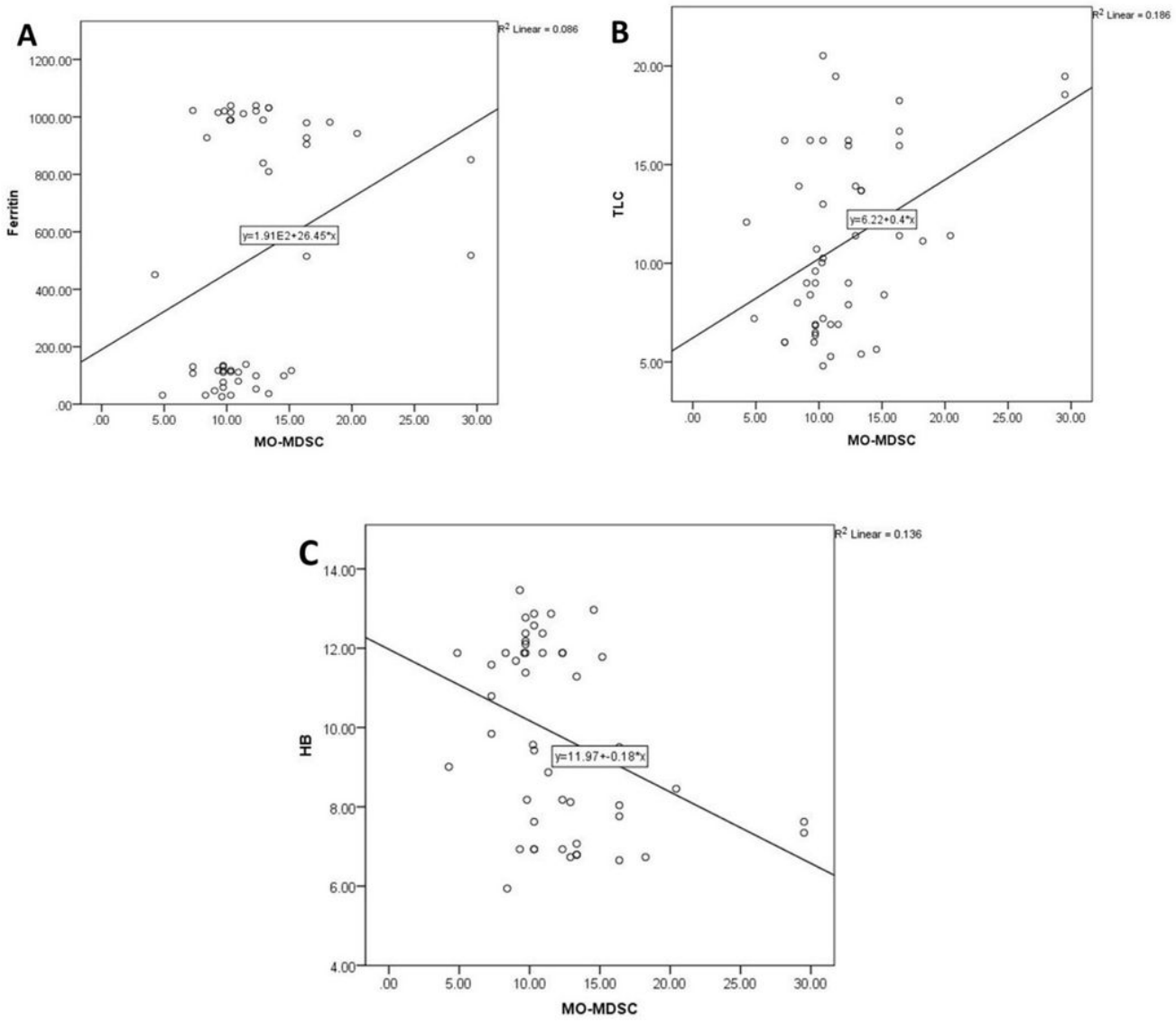

Figure 5

Correlations of MO-MDSCs with serum ferritin level (A), total leukocyte count (B), and hemoglobin level (C).

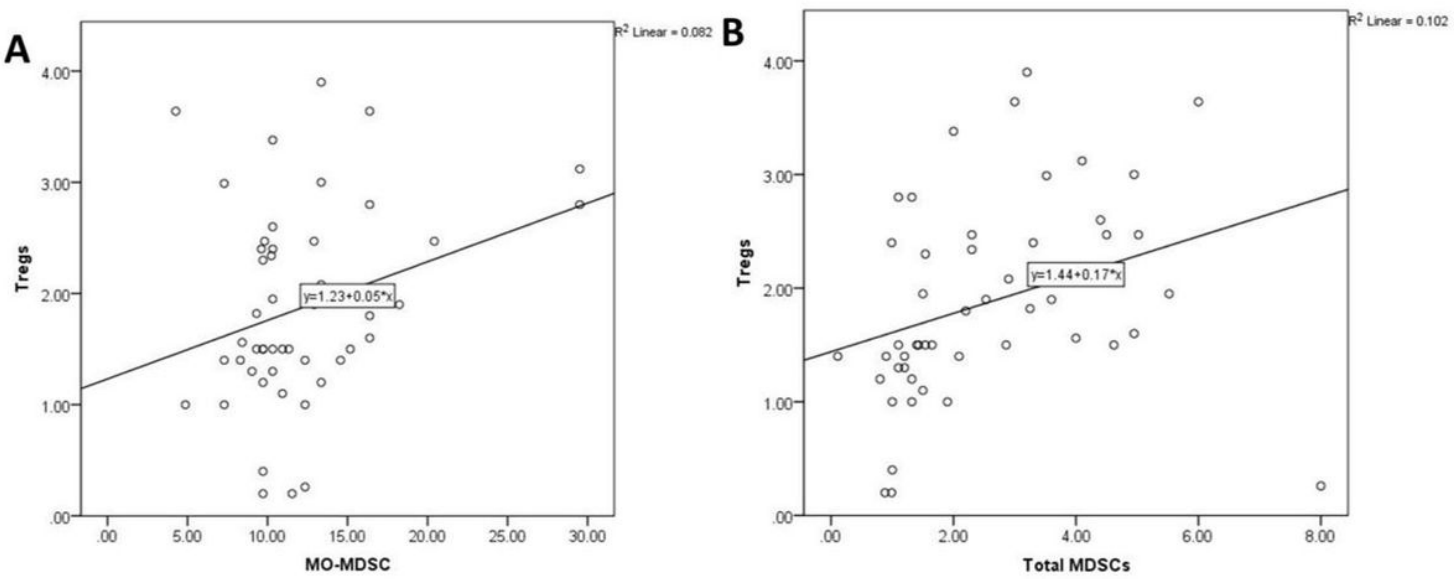

Figure 6

Correlations of CD4+CD25HiFoxP3+ Tregs with MO-MDSCs (A) and total MDSCs (B)