

Erythropoietin administration is associated with improved T-cell properties in patients with myelodysplastic syndromes



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ARTICLE INFO

Article history:

Received 3 February 2016

Received in revised form 5 October 2016

Accepted 1 November 2016

Available online 2 November 2016

Keywords:

Myelodysplastic syndrome

Erythropoietin

Immune modulation

T cells

Lymphocytes

ABSTRACT

The immune system is impaired in myelodysplastic syndromes (MDS) and plays a role in the pathogenesis of the disease. Here we show effects of recombinant human erythropoietin (rHuEPO) on T cell (CD4⁺, CD8⁺ and CD4⁺CD25⁺) number and function in MDS patients. Healthy (20 subjects), MDS patients without rHuEPO treatment ('MDS', 13), and MDS patients treated with rHuEPO ('MDS + EPO', 17) were examined. CD4⁺ and CD8⁺ T cell numbers were reduced and increased respectively in MDS compared to healthy subjects. EPO treatment normalized these levels. CD4⁺CD25⁺ cell numbers, lower in MDS, were normalized in MDS + EPO. *In vitro* activation of CD4⁺ and CD8⁺ cells with phytohemagglutinin as measured by CD69 expression, demonstrated a 7.2 fold increase in CD4⁺ activation vs 13.6 fold for MDS and MDS + EPO respectively ($p = 0.004$); and 10.2 fold (MDS) vs 18.6 fold (MDS + EPO, $p < 0.003$) for CD8⁺ T cells. Expression of the co-stimulatory marker CD28, decreased in CD4⁺ and CD8⁺ T cells in MDS, was normalized in MDS + EPO CD4⁺ T cells. Subgroup analysis of milder disease (WHO RA and RARS) and more advanced disease revealed no difference in CD4⁺ and CD8⁺ T cell numbers. However, the activation of these cells in the RA/RARS subgroup was impaired in EPO-untreated and enhanced in EPO-treated MDS patients. Our data suggest that EPO treatment improves immune abnormalities in MDS and may depend on disease severity.

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1. Introduction

Erythropoietin is produced in the kidney and circulates to the bone marrow where it stimulates erythropoiesis. Recombinant human EPO (rHuEPO) is administered routinely to patients with anemia of kidney disease [1], as well as anemia associated with cancer (most notably, hematologic cancers including multiple myeloma (MM) and myelodysplastic syndromes (MDS)) [2–6]. We and others have demonstrated additional, non-erythroid effects of EPO including an immune-mediated anti-myeloma effect [7–10].

Earlier work from our laboratory has demonstrated that patients with advanced multiple myeloma (MM) suffer from several immunological abnormalities and some of them are normalized in patients treated with rHuEPO [11]. These included: normalization of CD4:CD8 T-cell ratio, T cell phytohemagglutinin (PHA)-mediated

activation and proliferation potential, expression of the T cell co-stimulatory molecule CD28 and the inhibitory molecule CTLA-4, as well as the normalization of serum levels of IL-6.

Myelodysplastic syndromes are clonal hematopoietic stem cell disorders characterized by cytopenias and hematopoietic cellular dysfunction. Several immune abnormalities have been described in MDS including T cell lymphopenia, relative increased CD8⁺ cells, and reduced CD4⁺ cells, and increased or decreased Treg numbers depending on the site of the cells (e.g. bone marrow vs. peripheral blood) and the stage of the disease [12–15].

rHuEPO serves as a safe and major therapeutic agent for anemic MDS patients [5,16–21]. Given our previous studies demonstrating its immune-mediating properties [11,22,23], we tested several immune parameters, including numbers and ratio of CD4⁺ and CD8⁺ cells, numbers of CD4⁺CD25⁺ cells as well as T-cell markers of function – activation (CD69⁺), the co-stimulatory molecule CD28, and the inhibitory molecule CTLA-4. These were examined in MDS patients, comparing those treated and those not treated with rHuEPO, and also in comparison with healthy controls. We found that the decreased CD4⁺ and increased CD8⁺ cell populations, as well as the reduced CD4⁺CD25⁺ cell populations in MDS patients were normalized in such patients treated with rHuEPO. We also

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found that the reduced activation of CD4⁺ and CD8⁺ cells was normalized in MDS + EPO patients, and that the reduced number of cells expressing the co-stimulatory molecule CD28 was also normalized in the CD4⁺ cells of the MDS + EPO group.

2. Methods

2.1. Selection of participants

The participants were recruited in the Departments of Hematology and Internal Medicine in our institution. All patients, recruited randomly, had been diagnosed with MDS and all but one were classified as lower-risk, defined as IPSS (International Prognostic Scoring System) low or intermediate-1 (int-1). Cytogenetics reports were available for all but five of the patients, and for those patients the IPSS score was estimated based only on the blast count and the number of cytopenias. One group was treated with rHuEPO (Epoetin alpha, Eprex, Janssen Cilag, Epoetin beta, Recormon, Roch, or Darbapoetin, Aranesp, Amgen), and a second group, with a similar MDS classification was not treated with rHuEPO. In addition, a third group comprised of healthy subjects, served as control. Decisions about rHuEPO treatment were made by the treating physicians, irrespective of this study.

The study was approved by the institutional review board (Helsinki Committee) at our hospital, and patients gave their informed consent.

2.2. Immunophenotyping

Total blood: 100 μ l samples of peripheral blood were stained with varying combinations of CD3-FITC, PE-Cy5 or APC, CD4-FITC, CD8-PE-Cy5 or PERCP, CD69-PE, CD25-PE, CTLA-4(CD152)-PE, CD28-PE (Dako, Denmark Biologend, San Diego, CA). After 30 min at 4 °C, erythrocytes were lysed with Uti-lyse lysis solution (Dako, Denmark). Samples were washed with PBS, and cells were detected either with a FACSsort flow cytometer (Becton Dickinson, BD, San Jose, CA) or with a Gallios flow cytometer (Beckman Coulter, Brea, CA). Cells were analyzed using either WinMDI (Scripps Research Institute, La Jolla, CA) or Kaluza (Beckman Coulter, TreeStar, Ashland, OR) software.

Enriched mononuclear cells: Peripheral blood mononuclear cells were isolated using Ficoll-Paque PLUS reagent (General Electric, Sweden). Mononuclear cells were stained with antibodies against cell surface markers: CD19-PE, CD4-FITC, CD25-PE (Biologend, San Diego, CA) for 30 min at 4 °C to identify CD19⁺ B cells [24] and CD4⁺CD25⁺ cells which contain Tregs [25,26]. All samples were washed with PBS. Flow cytometry was performed using a FACSsort flow cytometer (Becton Dickenson). Results were analyzed using Kaluza software (Beckman Coulter).

2.3. Activation assay

Whole blood samples diluted in a 1:1 vol with RPMI medium were distributed into 24-well culture plates. The mitogen PHA was added (10 mg/ml) to induce T cell activation. The cultures were incubated for 20 h at 37 °C in humidified air with 5% CO₂. The levels of CD69 and of CTLA-4 (CD152) and CD28 were evaluated for T cells using three-color combination mAb, as previously described.

2.4. Mathematical and statistical analyses

Each time patient samples were examined (three or more) they were compared and normalized to healthy controls analyzed at the same time. This allowed for comparison of patients over time.

Data were combined by normalizing each parameter for the average of that parameter for the controls. As such, all parameters

for the controls have the value, 1.00, and those of all patients have values relative to 1.00. For the markers of activation (CD69, CD152, CD28) which were measured at baseline and after 20 h with PHA, all measures (baseline and 20 h) were normalized to the *baseline* values of the controls. Comparisons among the groups were performed using Student's *t*-test, and $p \leq 0.05$ was considered statistically significant.

3. Results

Twenty healthy individuals ("Healthy"), 13 patients with MDS not treated with rHuEPO ("MDS"), and 17 MDS patients treated with rHuEPO ("MDS + EPO") participated in the study. Table 1 demonstrates the baseline patient characteristics. Note that there is no difference in age between the MDS and MDS + EPO groups, though the healthy controls are somewhat younger. The male/female ratio is higher in the MDS group. There is no significant difference in total WBC or absolute lymphocyte counts among the groups, but as expected, the MDS patients treated with rHuEPO have a higher HB than those without such treatment. Table 2 provides detailed information about each patient. Note that all but one were classified as IPSS low or int-1, and the vast majority had a low blast count and favorable cytogenetics, but there was a variety of disease severity according to WHO (World Health Organization) classification (fourth edition) [27,28].

3.1. CD4⁺ and CD8⁺ T-cell numbers are abnormal in MDS and normalized in MDS + EPO

Fig. 1 depicts the differences among the groups in T cell numbers: CD3⁺CD4⁺ T helper cells, and CD3⁺CD8⁺ cytotoxic T cells. The individual data for each patient are shown in Table 3. As seen in the figure, CD4⁺ T cell number is reduced in MDS patients compared with healthy controls (1A, left, $p < 0.001$) and normalized in rHuEPO-treated patients ($p < 0.01$). CD8⁺ T cell number is increased in MDS patients (1A, right, $p = 0.02$) and normalized in rHuEPO-treated patients ($p = 0.05$). In healthy subjects, the ratio of CD4⁺ to CD8⁺ (Fig. 1B) is 2.2. In the MDS group the ratio of these cells is nearly equalized (1.2), but in the MDS + EPO group the ratio approaches that of the control group (1.9). Looking at the individual patient data (Table 3), one can see that in the healthy population, 76% had a ratio of at least 1.7. In the MDS group only 23% had such a ratio, while in the MDS + EPO group 73% achieved that ratio.

3.2. CD4⁺CD25⁺ T cell subset are normalized in MDS + EPO patients

Fig. 2 demonstrates the relative number of CD4⁺CD25⁺ T cells (which include regulatory T cells) in each of the three groups. The number of these cells in the blood of MDS patients is significantly lower than that of the healthy subjects. Patients treated with rHuEPO had levels similar to those of the healthy controls.

3.3. T cell activation is normalized in MDS + EPO patients

The CD69 surface marker is an indicator of cellular activation [29] (Fig. 3). T cell activation assay was performed in a subset of patients (Table 4). In healthy participants, activation of both CD4⁺ and CD8⁺ cells with PHA (white bars) is dramatic—achieving an approximately 15-fold increase in CD69⁺ cells (mean \pm SEM, CD4⁺CD69⁺: 13.3 ± 1.1 -fold; CD8⁺CD69⁺: 17.0 ± 1.6 -fold). The activation seen in the MDS group is significantly lower (CD4⁺CD69⁺: 7.2 ± 1.6 -fold; CD8⁺CD69⁺: 10.2 ± 2.2 -fold). However, the activation seen in the MDS + EPO group is similar to that of the healthy controls and significantly greater than that of the MDS group (CD4⁺CD69⁺: 13.6 ± 1.1 -fold; CD8⁺CD69⁺: 18.6 ± 1.3 -fold). For the

Table 1
Patient characteristics.

	Healthy	MDS	MDS + EPO	P (MDS vs MDS + EPO)
N	20	13	17	
Male/Female (N/N)	8/12	10/3	9/8	
Age years, mean (\pm 95%CI)	58.2 (53.9–62.5)	78.6 (74.4–82.8)	80.9 (76.6–85.1)	0.47
Blood counts				
HB (\pm SD)	13.2 (1.2)	9.4 (1.5)	11.1 (1.5)	0.01
WBC (\pm SD)	5.4 (1.5)	6.1 (2.1)	6.8 (2.2)	0.46
Lymph (\pm SD)	1.7 (0.4)	1.5 (1.2)	1.3 (0.5)	0.74

N-number, CI-confidence interval, HB-hemoglobin, WBC-white blood cell count, Lymph-lymphocytes (absolute count), all at the time of recruitment.

Table 2
Detailed Patient Characteristics.

Patients	WHO	BM blast%	Karyotype	CBC			IPSS
				HB	WBC	PLT	
MDS							
1	RA	<5%	46, XX, del5q***	8.6	8.7	563	LOW
2	RCMD	<5%	45, X,-Y [15]	8.6	5.4	245	LOW
3	RCMD	<5%	46, XX [19]	10.1	10.9	114	LOW
4	RA	<5%	N/A**	8.6	12.3	277	LOW, prob**
5	RA	<5%	46, XY [16]	8.9	7.9	729	LOW
6	RAEB	7%	46, XY [20]	10.6	5.1	24	INT-1
7	RCMD	<5%	N/A**	10.2	4.2	79	INT-1, prob**
8	RCMD	<5%	N/A**	9.2	4.4	79	INT-1, prob**
9	RA	<5%	46, XY [10]	8.5	4.2	188	LOW
10	CMML	<5%	46, XY [16]	10.1	6.6	223	LOW
11	RAEB-1	6%	46, XX [4], dup 3q [6]	10.3	6.65	219	INT-1
12	RCMD	<5%	46, XY [15]	9.6	5.3	145	LOW
13	RAEB-2	11%	43–45, XY complex [10]‡	8.1	5.3	27	HIGH
MDS + EPO							
1	RA	<5%	46, XX [20]	8.7	8.4	326	LOW
2	RCMD	<5%	N/A**	10.5	4.8	138	INT-1, prob
3	RA	<5%	45, XX [29]	10.4	5.4	203	LOW
4	RA, del5q	<5%	46, XY [13] del5q [2]	10.1	5.7	333	LOW
5	RCMD*	<5%	N/A**	9.8	9.9	237	INT-1, prob
6	RARS	<5%	46, XX [20]	9.9	8.0	495	LOW
7	RCMD	<5%	46, XX [30]	9.4	4.38	80	INT-1
8	RARS	<5%	46, XY [20]	10.3	7.75	193	LOW
9	RA	<5%	46, XX [15]	9.7	7.7	297	LOW
10	RA	<5%	46, XX [20]	10.1	6.8	212	LOW
11	RA	<5%	45, XY [2] complex [18]♦	11.9	4.8	154	INT-1
12	RARS	<5%	46, XY [13] 45, X,-Y [7]	9.6	6.9	250	LOW
13	RCMD	<5%	46XY [15]	9.9	7.5	147	LOW
14	RA	<5%	46, XY [1] 45, X,-Y [3]	10.9	9.3	177	LOW
15	CMML	<5%	46, XY [15]	9.6	9.8	140	LOW
16	RARS	<5%	46, XX [15]	11.4	5.7	229	LOW
17	RCMD	<5%	47, XX +19 [10]	12.1	3.5	92	INT-1

BM-bone marrow, WHO-World Health Organization stage, IPSS-international prognosis scoring system, RA-refractory anemia, RS-ringed sideroblasts, RCMD-refractory cytopenia with multiple dysplasia, RAEB-1 or –2–refractory anemia with excess blasts – one or two, INT-1-intermediate-one. CMML-Chronic myelomonocytic leukemia. CBC is from the time of BM biopsy/diagnosis. Number in brackets [] denotes the number of mitoses examined.

*Trilineage dysplasia, **Cytogenetics not available (N/A, requiring estimation of IPSS score; prob="probably"), ***G-Banding: no mitoses; FISH: 44/200 with del5q.

‡43–45, XY with abnormalities in chromosomes 1, 3, 5, 7, and 14.

♦45, XY, 3q+, –6, 7q+, 12q–.

comparison between the MDS and the MDS + EPO groups, $p=0.004$ for CD4⁺ and $p=0.003$ for CD8⁺ cell activation. Note that in all MDS patients whether treated with rHuEPO or not, there is a trend for increased T cell activation at baseline. Of the healthy subjects examined (Table 4), all but three of CD4⁺ and all of CD8⁺ achieved a ≥ 10 -fold activation. In the MDS group only one (CD4⁺) and three (CD8⁺) achieved that level of activation, while in the MDS + EPO group all but two of the CD4⁺ and all of the CD8⁺ cells achieved that level.

3.4. Co-stimulatory marker normalized only in CD4⁺ cells in MDS + EPO patients

The expression of the co-stimulatory marker, CD28 was decreased in MDS patients (Fig. 4) for both CD4⁺ and CD8⁺ cells

(CD4⁺CD28⁺: 0.85, $p=0.04$; CD8⁺CD28⁺: 0.57, $p<0.01$, comparing MDS patients with healthy controls). It was normalized by EPO treatment only for the CD4⁺ cells (CD4⁺CD28⁺: 1.0, $p=0.02$; CD8⁺CD28⁺: 0.58, $p=0.92$, comparing MDS + EPO with MDS).

3.5. Other cellular markers

The relative levels of the inhibitory molecule CTLA-4 (CD152) were measured in a subset of patients in both CD4⁺ and CD8⁺ cells for control, EPO treated and EPO not-treated MDS patients (CD4⁺: 3.5 ± 1.3 , 8.4 ± 1.6 , 10.8 ± 3.9 , respectively; CD8⁺: 2.4 ± 0.7 , 5.9 ± 1.9 , 4.4 ± 2.0 , respectively). There was no difference between MDS and MDS + EPO groups. B-cells (CD19⁺) were also exam-

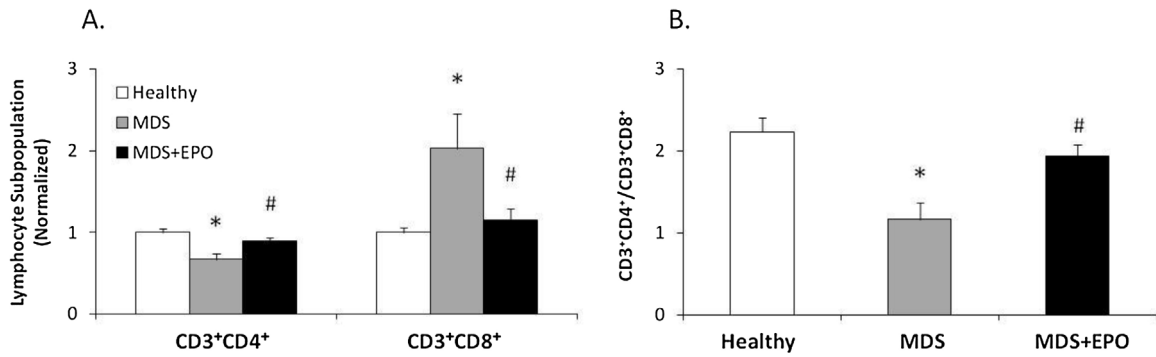


Fig. 1. T cell numbers and ratios are normalized in rHuEPO treated MDS patients.

Panel A depicts the numbers of CD3⁺ T cells that are either CD4⁺ (left portion of Fig. 1A), or CD8⁺ (right portion). In both the left and the right portions of 1A, as well as in all subsequent graphs, the cells are depicted for the Healthy control (white bars), MDS (no EPO treatment, gray bars) and MDS + EPO (black bars) groups. All values are normalized as described in the methods section. Error bars reflect the standard error of the mean (\pm SEM). Panel B depicts the ratio of CD4⁺:CD8⁺ in all three groups. * $p \leq 0.05$ for the comparison of MDS and Healthy groups. # $p \leq 0.05$ for the comparison and MDS + EPO to MDS groups.

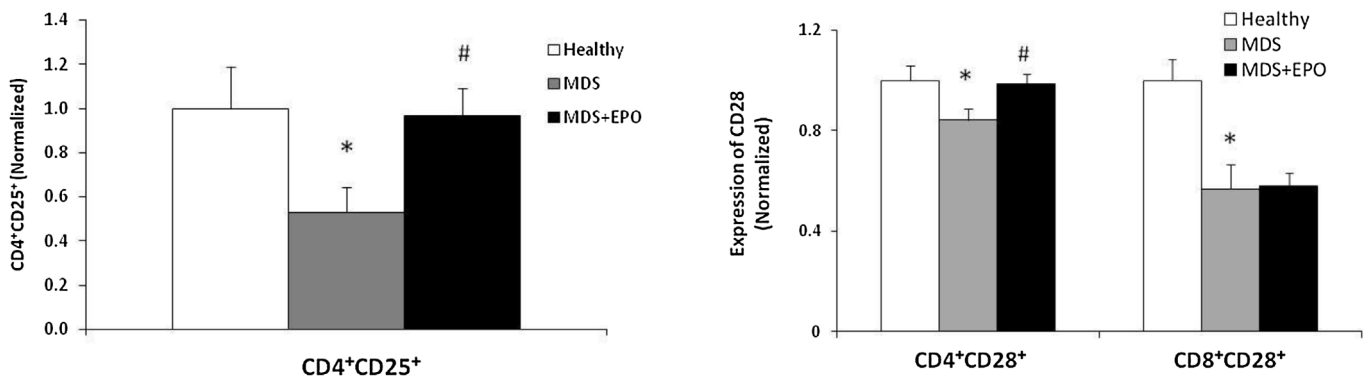


Fig. 2. CD4⁺CD25⁺ cells, reduced in MDS patients, are normalized in MDS patients treated with rHuEPO.

The cells with CD4⁺CD25⁺ which include the Tregs. All values are normalized as described in the methods section. * $p \leq 0.05$ for the comparison of MDS and Healthy groups. # $p \leq 0.05$ for the comparison and MDS + EPO to MDS groups.

Fig. 4. Expression of co-stimulatory marker, CD28, is reduced in both CD4⁺ and CD8⁺ T cells of MDS patients, but is normalized only in CD4⁺ cells of rHuEPO treated MDS patients.

The left portion demonstrates the levels of the co-stimulatory marker CD28 in CD4⁺ T cells, and the right portion demonstrates these levels in CD8⁺ T cells. * $p \leq 0.05$ for the comparison of MDS and Healthy groups. # $p \leq 0.05$ for the comparison and MDS + EPO to MDS groups.

ined and were found to be similar in numbers among the groups (1 ± 0.095 , 0.8 ± 0.14 , 0.8 ± 0.14 , respectively).

Monocytes are also affected by MDS [15] and these cells evaluated in the clinical laboratory were found to be greater in MDS patients treated with EPO ($0.98 \pm 0.26 \times 10^3/\mu\text{L}$), than in either of the other two groups (MDS: $0.46 \pm 0.09 \times 10^3/\mu\text{L}$, Healthy $0.41 \pm 0.05 \times 10^3/\mu\text{L}$). The difference between MDS + EPO and the healthy subjects was statistically significant ($p < 0.05$). The dif-

ference between the MDS + EPO and MDS (non-treated) patients was only significant ($p = 0.03$) when comparing the monocytes as a percentage of the white blood cells (Percentages for healthy, MDS and MDS + EPO groups respectively: $7.08 \pm 0.35\%$, $5.51 \pm 0.96\%$, $12.92 \pm 2.24\%$).

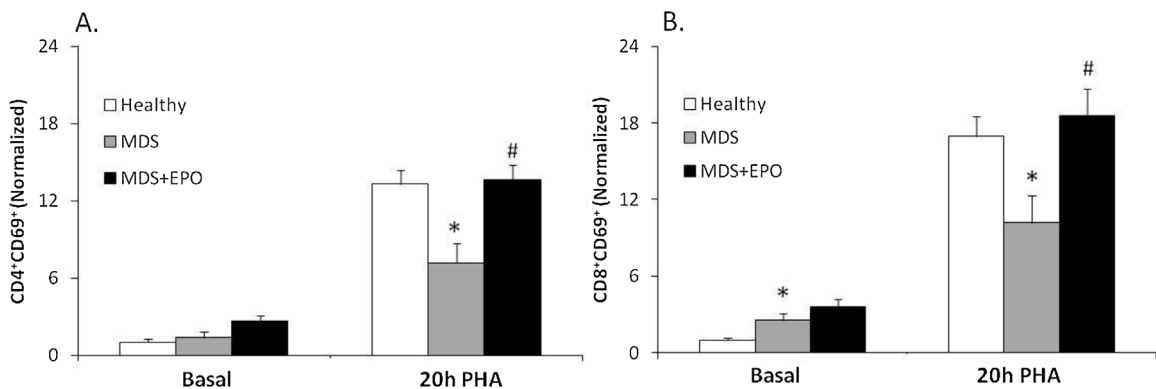


Fig. 3. T-cell activation is low in MDS but normalized in rHuEPO-treated patients: CD69 expression, as a marker of T-cell activation was studied in CD4⁺ (3A) and CD8⁺ (3B) T cells. The results are provided for basal conditions (left portion of both 3A and 3B) and following 20h of PHA exposure (right portion). All numbers are normalized to the values of the healthy controls at baseline. * $p \leq 0.05$ for the comparison of MDS and Healthy groups. # $p \leq 0.05$ for the comparison and MDS + EPO to MDS groups.

Table 3
Detailed Patient T cell data (Normalized), CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells.

Patients	*CD3 ⁺ CD4 ⁺	*CD3 ⁺ CD8 ⁺	**CD4 ⁺ :CD8 ⁺ ratio
Healthy			
1	1.03	1.05	1.93
2	0.92	1.26	1.43
3	0.87	1.01	1.71
4	0.72	0.84	1.70
5	1.28	0.92	2.73
6	1.09	0.67	3.19
7	1.04	0.76	2.72
8	1.06	1.05	2.00
9	1.09	1.26	1.71
10	0.79	1.01	1.54
11	1.09	1.12	1.91
12	1.17	0.84	2.75
13	0.85	1.22	1.38
14‡			
15‡			
16‡			
17‡			
18	1.34	1.30	3.03
19	0.52	0.46	3.33
20	1.15	1.24	2.72
Average	1.00	1.00	2.24
SEM	0.05	0.06	0.17
Patients	*CD3 ⁺ CD4 ⁺	*CD3 ⁺ CD8 ⁺	**CD4 ⁺ :CD8 ⁺ ratio
MDS			
1	0.75	0.67	2.19
2	0.64	0.84	1.50
3	0.92	1.09	1.65
4	0.53	1.18	0.89
5	0.51	0.97	1.04
6	0.81	1.22	1.31
7	0.64	0.50	2.50
8	0.96	1.05	1.80
9	0.90	4.83	0.55
10	0.79	4.65	0.50
11	0.10	2.55	0.11
12	0.67	4.08	0.49
13	0.56	2.74	0.60
Average	0.67	2.03	1.16
SEM	0.06	0.44	0.20
Patients	*CD3 ⁺ CD4 ⁺	*CD3 ⁺ CD8 ⁺	**CD4 ⁺ :CD8 ⁺ ratio
MDS ± EPO			
1	0.96	1.01	1.88
2	0.72	0.84	1.70
3	0.81	0.71	2.24
4	0.96	0.80	2.36
5	1.11	1.34	1.63
6	0.60	0.42	2.80
7	1.00	1.01	1.96
8	0.96	1.26	1.50
9	0.98	0.84	2.30
10	0.77	1.43	1.06
11	1.04	1.01	2.04
12	1.06	1.13	1.85
13	0.91	0.93	2.87
14	1.06	2.84	1.09
15‡	0.87		
16‡	0.34		
17	1.02	1.71	1.76
Average	0.89	1.15	1.94
SEM	0.05	0.15	0.14

‡Data missing for these patients owing to technical difficulties.

*Data normalized to healthy control population.

**Ratio of the non-normalized CD4⁺ and CD8⁺ counts.

3.6. Subgroup analysis

Patients were divided into subgroups based on their WHO classification, where RA (refractory anemia) and RARS (RA with ringed sideroblasts) were considered milder disease, and other categories

Table 4
Detailed Normalized Patient T cell data, Activation.

Patients	CD4 ⁺ CD69 ⁺ baseline	CD4 ⁺ CD69 ⁺ activated	CD8 ⁺ CD69 ⁺ baseline	CD8 ⁺ CD69 ⁺ activated
Healthy				
4	0.09	14.19	0.85	19.82
5	3.26	17.67	2.27	22.94
6	0.98	9.30	1.42	11.33
7	0.74	9.30	1.10	11.61
8	0.16	15.77	0.20	18.41
9‡	0.23		0.14	
10	1.40	9.30	0.85	11.33
11	1.51	13.60	1.47	19.82
12	0.47	17.44	0.85	22.66
13	1.16	13.02	0.85	15.01
Average	1.00	13.29	1.00	16.99
SEM	0.30	1.13	0.20	1.59
Patients	CD4 ⁺ CD69 ⁺ baseline	CD4 ⁺ CD69 ⁺ activated	CD8 ⁺ CD69 ⁺ baseline	CD8 ⁺ CD69 ⁺ activated
MDS				
1	2.56	2.33	3.40	4.53
2‡	2.00		3.96	
3	0.52	9.07	3.12	18.97
4‡		5.58	3.96	8.78
5	2.33	4.65	1.67	6.51
6	0.53	13.26	1.42	13.59
7	0.23	8.14	0.28	8.78
Average	1.36	7.17	2.54	10.20
SEM	0.43	1.57	0.54	2.15
Patients	CD4 ⁺ CD69 ⁺ baseline	CD4 ⁺ CD69 ⁺ activated	CD8 ⁺ CD69 ⁺ baseline	CD8 ⁺ CD69 ⁺ activated
MDS ± EPO				
1	0.84	11.63	1.13	13.31
2	3.26	22.56	4.53	26.62
3	2.33	16.28	3.40	22.09
4	1.56	11.63	2.55	16.14
5	0.93	11.63	1.39	15.86
6	4.65	13.95	4.25	16.99
7	3.49	9.30	3.68	13.31
8‡	1.37	14.65		23.51
9	4.65	13.95	6.23	18.13
10	4.65	15.12	7.08	20.67
11‡	3.26		4.25	
12	0.84	9.30	1.70	17.56
Average	2.65	13.64	3.65	18.56
SEM	0.44	1.13	0.58	1.27

‡Data missing for these patients owing to technical difficulties. Patient numbers are consistent with those of Tables 2 and 3.

were considered more advanced disease. The relative CD4⁺ and CD8⁺ T cell numbers for MDS and the MDS + EPO groups were the same as those of the whole cohort, irrespective of disease severity (Table 5). The fewer CD4⁺CD25⁺ cells seen in the MDS patients compared to the MDS + EPO patients in the whole cohort were found only in the more advanced disease subgroup (Table 5, bold numbers); There was no difference between the MDS and MDS + EPO patients in the milder disease (RA/RARS) subgroup.

The activation of the CD4⁺ and CD8⁺ cells as reflected in the marker CD69, where there was decreased activation in the MDS patients that had improved in the MDS + EPO patients, was seen only in the milder disease subgroup (Table 5, bold numbers). A closer examination of the table reveals that in the more severe subgroup, there is no difference between MDS and MDS + EPO patients, probably because the cells of the MDS patients are already activated more effectively than they are in the milder disease MDS subgroup. This phenomenon will be studied further in future experiments with larger cohorts of patients.

Table 5
Results of T cell number and function, grouped by disease severity.

	Healthy (20)	RA/RARS			More Advanced MDS		
		MDS (4)	MDS + EPO (11)	P	MDS (9)	MDS + EPO (6)	P
CD3 ⁺ CD4 ⁺ **	1	0.67	0.87	0.15	0.68	0.94	0.04
CD3 ⁺ CD8 ⁺ **	1	1.91	1.15	0.28	2.08	1.17	0.21
CD4 ⁺ :CD8 ⁺ ratio	2.24	1.17	1.91	0.06	1.16	1.98	0.06
CD4 ⁺ CD25 ⁺ **	1	0.85	0.83	0.92	0.40	1.30	0.02
CD4 ⁺ CD69 ⁺ activation**	13.29	4.19	13.31	< 0.01	10.16	14.50	0.38
CD8 ⁺ CD69 ⁺ activation**	16.99	6.61	18.55	< 0.01	13.78	18.60	0.39
CD4 ⁺ CD28 ⁺ **	1	0.88	0.98	0.31	0.82	0.99	0.09
CD8 ⁺ CD28 ⁺ **	1	0.50	0.59	0.48	0.61	0.55	0.75

RA/RARS – patients with either refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS). More advanced MDS is comprised of all other patients. Number in parentheses denotes the number of subjects in each group.

*Normalized to values of healthy patients. **Normalized to baseline values of healthy patients. Numbers are bold where there are differences depending on the disease severity.

4. Discussion

This study of patients with MDS demonstrates that some of the abnormalities in T cells are normalized in rHuEPO treated patients.

rHuEPO has long been used to treat anemia, initially and primarily of renal failure [30], but it is also used to treat anemia associated with cancer [3,4,6]. Despite the concerns that using this treatment in cancer patients might lead to increased mortality, there are still clear indications for its use as delineated in both American and European guidelines (e.g. ASCO/ASH from 2012) [2,4,5]. Among the neoplastic diseases for which rHuEPO is used are MM and low risk MDS [5], and we have shown that such patients do benefit from the treatment [31,32].

EPO receptor (EPO-R) expression on T cells is controversial [22,33]. Our quest for potential cellular mediators of EPO effects on the lymphocytes led us to the novel discovery that EPO-R are expressed in dendritic cells (DCs), the most efficient T cell primers. Hence, DCs, and other EPO-R expressing cells such as macrophages [22] may play a role in rHuEPO-mediated improvement of T cell parameters.

In our earlier study [11], we demonstrated that patients with MM who were treated with rHuEPO, had a concomitant improvement in several immune parameters, which may be markers for improved function. The abnormalities in MM – increased CD3⁺CD8⁺ cell numbers, decreased CD4:CD8 ratio, and decreased CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell activation potential as indicated by CD69 levels after activation with PHA – all improved with rHuEPO treatment.

This led to our examining similar parameters in patients with MDS and their response to EPO treatment. Other studies have found that MDS patients have deficiencies in many of the same parameters. For example, it has been found that there is increased apoptosis and therefore reduced number of T-cells [34], specifically, a decreased number of T_H1 cells [15,35]. Some studies found that CD8⁺ cells are also reduced but to a lesser degree [13]. Because of the heterogeneity of MDS there may be different responses of immune cells depending on the type of disease or even on the phase of the disease. This might explain why Meers et al., for example, found that the numbers of CD4⁺ and CD8⁺ T cells did not differ from those of healthy controls [36]. This may also be the reason that in a study of peripheral T cells in MDS, the CD4 and CD8 subpopulations were actually expanded [37].

In our study using flow cytometry, CD8⁺ cells are increased, and normalized by EPO treatment.

The variability among patients is also reflected in Treg cells which have been shown to be impaired in their function in early stage MDS, though they increase numbers and retain their function in late stage MDS [15,38,39]. Therefore, in LR-MDS, Tregs with impaired function may play a role in the selection of dysplastic

clones, while in higher-risk MDS increased Treg numbers might suppress the immune response against transformed clones and increase the likelihood of leukemic transformation [12]. In this study of MDS the number of CD4⁺CD25⁺ cells, which include Tregs, was lower in untreated MDS patients, yet normalized in the MDS patients treated with EPO. As such, since Tregs may be involved in the pathogenesis of different stages of the disease, if EPO modifies the Tregs, it may also improve the outcome of the disease itself. EPO was found to modulate the impact of Tregs in autoimmune disease, for example in experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis [40]. Further work that examines the Tregs in both mild and advanced disease will examine these differences and the effects of EPO on these cells.

What is the significance of the changes in the T cells in MDS patients? It is very likely that the immune system not only changes or is damaged in MDS, but that it plays a role in the pathophysiology of the disease [39]. For example, a study by Fozza et al. demonstrated an oligoclonal expansion of cytotoxic CD8⁺ cells, suggesting that they may be involved in the inhibition of bone marrow progenitor cells [37]. Moreover, studies have shown that treatment with cyclosporine and antithymocyte globulin (ATG) improves the outcome of approximately 33% patients with LR-MDS, further suggesting the role of the autoimmunity in the pathophysiology of the disease [41].

In the current study there were no significant differences in B-cell numbers among the three groups (though both the MDS and the MDS + EPO groups had a trend towards lower B-cell numbers than the healthy group). This is consistent with some studies but not others. Some report that B cells can undergo increased apoptosis [15,42], while others demonstrate no difference in B cell numbers [13], and one reported evidence for reduced B cell gene expression in progenitor (CD34⁺) cells [43]. Some report variable production of gammaglobulin [15], which reflects the function of the B-cell line, and perhaps the number as well.

In addition to CD69, another parameter that may be a surrogate marker of function is the expression of the T cell co-stimulatory molecule CD28 and the inhibitory molecule CTLA-4 which were therefore examined in this study as well.

EPO treatment is used for the anemia of MDS, and there have been several proposed protocols for treatment based on the likelihood of the patient to respond to the therapy. Moreover, the data show that rHuEPO is safe for MDS patients [16–20]. It may even prolong survival [18]. The data demonstrate that rHuEPO in addition to its benefit on the red cell line may improve cellular immunity.

The EPO-associated normalization of the T-cell numbers and proportions is important. If these abnormalities in MDS reflect the secondary immune deficiencies of patients with the disease, then EPO is perhaps linked with the improvement of these deficiencies. However, if the T cell abnormalities are actually part of pathophysi-

ology of MDS, then EPO treatment could theoretically be associated with improvement of the disease itself [39].

This study shares certain similarities with our MM study [11], yet the differences may reflect the nature of the MDS – that it is a stem cell disease, while MM is a disease of the plasma cells.

One wonders if the positive effect that EPO has on the immune system might be mediated by its effect on the red cell lineage as the patients treated with EPO had a higher level of HB. One study demonstrated improvement of several immune parameters in hemodialysis patients treated with EPO whose HB normalized, compared to EPO-treated patients whose HB did not fully normalize [44]. On the other hand, our MM EPO study found that there was no difference in HB levels between the EPO treated and untreated patients [11] and yet, the immune functions normalized. Similarly in our study focusing on humoral immunity in hematologic patients, we found that EPO treatment was associated with normalizing antibody levels in response to the influenza vaccine even though the group receiving EPO had a lower average HB than the untreated patients [23]. The role of HB improvement in rHuEPO induced immune normalization is yet to be determined.

In summary, we have demonstrated that in MDS, as in MM, patients have deficiencies in their cellular immunity, that these deficiencies are normalized in patients treated with rHuEPO, and that there may be differences depending on the disease severity. Further studies need to be performed in humans and in mouse models to examine more fully the immune deficiencies and the effects of EPO in milder and more advanced disease, and to determine the mechanisms involved in this phenomenon. Since it has been determined that treatment with rHuEPO (for anemia) is safe for MDS patients, and conforms to the accepted guidelines both in the United States and in Europe, EPO could be studied to determine its efficacy as an immune booster, even in those patients who do not yet satisfy criteria for rHuEPO treatment based on anemia.

Acknowledgements

We gratefully acknowledge Dr. Nathalie Ben-Califa for helpful discussions and technical support. This work was supported by a grant from The Israel Cancer Association to DN and MM. This work was carried out in partial fulfillment of the requirements for a Ph.D. degree for ND-U from the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

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