IL10 inversely correlates with the percentage of CD8+ cells in MDS patients

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1. Introduction

Myelodysplastic syndrome (MDS) is characterized by an increased programmed cell death of bone marrow (BM) cells, both clonal and nonclonal precursors, which contributes to ineffective hematopoiesis and peripheral cytopenias [1–5]. Although the complex pathogenesis of MDS remains poorly defined, several studies indicate a role for the immune system in the progression of early MDS to the advanced stage [6,7]. Low-risk MDS is characterized by excessive apoptosis in the BM and by an autoimmune disease-like profile; whereas advanced MDS is distinguished by immune evasion, lower apoptosis and secondary DNA damage, facilitating the progress into acute leukemia [7,8]. Immunosuppressive and immunomodulatory therapeutics have presented favorable results, such as abrogation of transfusion dependence for a subset of the patients [8–12].

Although regulatory (Tregs) and cytotoxic T cells are reported to be modulated during the course of MDS [13], the exact mechanism by which these cells contribute to MDS progression is not yet clear. Low numbers of Tregs in low-risk MDS are associated with T cell cytotoxicity of BM precursor cells, whereas higher frequencies of Tregs in high-risk MDS result in a suppression of immune response [8,14,15].

In an attempt to better understand the role of the immune system in MDS, we evaluated CD4+ and CD8+ lymphocyte profiles in the peripheral blood of MDS patients. These data were correlated with clinical characteristics, the expression of FOXP3 and the anti-inflammatory cytokines IL10, TGFβ1 and CTLA4. IL10 expression inversely correlated with the percentage of CD8+ cells and was higher in high-risk MDS. Our findings provide further evidence for the role of T cell-mediated IL10 production in MDS and strengthen the idea of distinct cytokine profiles in low and high-risk MDS.

2. Materials and methods

2.1. Patients and healthy donors

Peripheral blood samples, collected from 49 patients with MDS and 29 unrelated, random, and healthy individuals (median age = 39, range, 28–60), were analyzed. All patients that attended the clinic between 2010 and 2011, with a confirmed diagnosis of MDS and untreated at the time of the study were included. All healthy controls and patients provided informed written consent and the study was approved by the ethics committee of the University of Campinas. Patients’ characteristics are described in Table 1.

2.2. Peripheral blood analyses

Hematological values were determined with a CELL-DYN Sapphire automated hematology analyzer (Abbott Diagnostics, Illinois, USA). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation (Sigma, St Louis, MO). PBMC were stained with the conjugated monoclonal antibodies: allophycocyanin (APC) anti-CD3, fluorescein isothiocyanate (FITC) anti-CD8, and phycoerythrin (PE) anti-CD4. An FSC/SSC gate was created around the viable lymphocyte population for further analysis of CD3+ cells, CD3+CD4+ and CD3+CD8+ subsets. Data acquisition was performed using a FACScalibur Flow Cytometer.

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Table 1
Clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age y, median (range)</td>
<td>67 (27–89)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>24 (49)/25 (51)</td>
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<tr>
<td>WHO classification, n (%)</td>
<td>RCUD 09 (19);</td>
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<tr>
<td></td>
<td>RCMRD 23 (47);</td>
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<tr>
<td></td>
<td>RAES 8 (16);</td>
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<tr>
<td></td>
<td>RAEB1 6 (14);</td>
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<tr>
<td></td>
<td>RAEB2 2 (4)</td>
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<tr>
<td>Risk stratification by WHO, n (%)</td>
<td>Low-risk 40 (82);</td>
</tr>
<tr>
<td></td>
<td>High-risk 29 (18);</td>
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<tr>
<td>Cytogenetic risk group, n (%)</td>
<td>Good 41 (84);</td>
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<tr>
<td></td>
<td>Intermediate 3 (6);</td>
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<tr>
<td></td>
<td>Poor 2 (4);</td>
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<tr>
<td></td>
<td>No growth 3 (6);</td>
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<tr>
<td>Peripheral blood counts, median (range)</td>
<td>Hemoglobin, (g/dL) 10.5 (5.5–15.6);</td>
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<tr>
<td></td>
<td>White blood count, (×10^9/L) 3.58 (0.86–9.8);</td>
</tr>
<tr>
<td></td>
<td>Neutrophil count, (×10^9/L) 1.63 (0.16–6.51);</td>
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<tr>
<td></td>
<td>Platelet count, (×10^9/L) 158 (0.7–648);</td>
</tr>
<tr>
<td></td>
<td>Number of cytopenia, (%) 0 (6); 1 (12); 2 (41); 3 (20);</td>
</tr>
<tr>
<td></td>
<td>Bone marrow blasts, %, median (range) 1.5 (0–14);</td>
</tr>
</tbody>
</table>

RCUD indicates refractory cytopenia with multilineage dysplasia; RCMRD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RAEB-1/2, refractory anemia with excess blasts-1/2.

3. Results

3.1. Distinct profiles of peripheral blood lymphocytes exist in MDS

We observed a significant decrease in lymphocyte count in the MDS group compared to the control group after adjusting for age (P=0.002, Fig. 1A). This statistical difference remained after we classified the patients into subgroups, according to WHO, but the decreased lymphocyte count was more pronounced in the high-risk MDS (P<0.001, Fig. 1B). Lymphopenia (<1.1×10^9 cells/L) was found in 13 (26.5%) of 49 MDS patients.

Age-adjusted percentages of CD3+ cells were significantly higher in the MDS group (P=0.004, Fig. 2A). Analyses of the CD3+ cell subsets presented no statistical differences for CD3+CD4+ cells (Fig. 2C), but showed a strong trend toward an increased percentage of CD3+CD8+ cells in the MDS individuals (P=0.05, Fig. 2E). Comparisons between the subgroups of the disease showed higher CD3+ frequencies in the high-risk, compared to the low-risk MDS (P=0.02, Fig. 2B), followed by higher CD3+CD4+ frequencies in the high-risk compared to the control group (P=0.02, Fig. 2D). The frequency of CD3+CD8+ cells was significantly higher in the low-risk MDS, when compared with the control group (P=0.04, Fig. 2F).

There was no statistical difference between the MDS and the control groups with regard to the CD4:CD8 ratios (Fig. 2G), however comparison among MDS patients revealed a significantly higher CD4:CD8 ratio in the high-risk, compared to the low-risk groups (P=0.03, Fig. 2H).

We also correlated CD3+CD4+ and CD3+CD8+ cell frequencies with clinical data (age, sex, hemoglobin, leukocyte, granulocyte, platelet, number of cytopenias, percentage of blasts in BM, and karyotype risk group). Univariate analysis demonstrated that advanced age correlated with a decreased percentage of CD3+CD8+ cells (Beta =−0.28; P=0.037; R²=0.11), which was confirmed by multivariate analysis (Beta =−0.30; P=0.024; R²=0.14). There was no significant correlation between CD3+CD4+ cell frequency and the clinical parameters studied.

3.2. IL10 inversely correlates with the percentage of CD8+ cells and presents higher expression in high-risk MDS

To better understand the regulation of the anti-inflammatory cytokines IL10, TGFβ1, and CTLA4, as well as FOXP3, in MDS, the

Fig. 1. Peripheral blood absolute lymphocyte count in MDS and control groups. (A–B) Multivariate regression analysis was performed with lymphocyte count, as the dependent variable, and age and disease status as independent variables. The P value and the number of individuals are shown in the figure. Low and high-risk MDS, according to WHO classification.
Fig. 2. CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell profiles in MDS and control groups. Multivariate regression analysis was performed with %CD3⁺ (A–B), %CD3⁺CD4⁺ (C–D), %CD3⁺CD8⁺ (E–F), and log-transformed ratio of CD4:CD8 (G–H), as the dependent variables, and age and disease status as independent variables. The P value and the number of individuals are shown in the figure. Patients were subgrouped into low and high-risk MDS, according to WHO.
Patients were subgrouped into low and high-risk MDS, according to WHO. (G) Relationship between the log-transformed relative expression of *IL10/HPRT* and the frequency of CD3+CD8+ cells in the peripheral blood of MDS patients. Spearman's correlation. The *P* value and the number of individuals are shown in the Figure.
transcript expressions of these genes were analyzed in peripheral CD3+ cells. We observed a decrease in FOXP3 expression in the MDS group, when compared with the control group after adjusting for age (P = 0.009, Fig. 3A). Similar statistical differences remained when low-risk MDS was compared with the control group (P = 0.006, Fig. 3B). IL10, TGFß1, and CTLA4 correlated positively with FOXP3 expression, according to Spearman’s correlation (P = 0.008, r = 0.37; P = 0.04, r = 0.28; P = 0.01, r = 0.35, respectively). Secondly, there was a trend toward a lower IL10 expression in the MDS group, when compared with the control group (P = 0.06, Fig. 3C), probably due to the lower IL10 expression observed in the low-risk MDS, when compared to the control group (P = 0.02, Fig. 3D). Additionally, higher levels of IL10 transcripts in the high-risk, compared to the low-risk MDS, were observed (P = 0.01, Fig. 3D). No significant differences in TGFß1 and CTLA4 expressions were observed (Fig. 3E-F). IL10 transcripts inversely correlated with CD3+CD8+ frequency in MDS patients (Spearman r = −0.36; P = 0.02, Fig. 3G); however, there were no correlations between the cytokines analyzed and CD3+CD4+ frequency.

4. Discussion

There is a clear involvement of multiple myeloid cell lineages in the MDS clone, which results in the loss of the capacity of differentiation and apoptosis in the bone marrow, with consequent peripheral pancytopenia in patients [18,19]. Conversely, several studies have shown that, in most cases of MDS, the lymphocytes are not involved in the malignant clone [20–22]. In our study, there was a significant decrease in the absolute lymphocyte counts in the peripheral blood of MDS patients, which is in accordance with the literature [21,23,24] and supports the immunological abnormalities that have been extensively described in MDS patients. Lymphopenia in MDS has been suggested to occur due to a decrease in the T cell numbers, as a consequence of T cell apoptosis in the peripheral blood of MDS patients [23,25]; as well as a consequence of CD19+ cell apoptosis in both the peripheral blood and bone marrow of MDS patients [21,26]. Additionally, a decreased NK T cell number, already described in MDS patients [27], could contribute to the lower absolute lymphocyte count. Further studies addressing the absolute values of each cell type in the peripheral blood of MDS patients could clarify which specific cells are involved in the lower absolute lymphocyte counts. Although our findings show that only 26.5% of patients with lymphopenia, the most prominent decrease in lymphocyte counts was in the high-risk group, which is in agreement with the previous reports [21,28].

Data presented herein show that the increase in CD3+ cell percentage in MDS may be a reflection of CD8+ frequency in the low-risk group and CD4+ frequency in the high-risk group. The increased CD8+ frequency in the low-risk MDS is in agreement with other studies [29–35] and supports the contribution of CD8+ cells to the apoptosis of hematopoietic progenitors, since the early stages of this disease are characterized by an increased apoptotic activity [7].

In contrast to previous reports demonstrating no significant differences in the CD4+ cell frequency between patients and controls [36,37], we showed a higher CD4+ cell frequency in high-risk MDS, with a consequently increased CD4:CD8 ratio in this subgroup. In fact, studies of T cell subsets in MDS have been contradictory; a decreased CD4:CD8 ratio in MDS patients has been reported [15,38], while other studies have shown an increased CD4:CD8 ratio in intermediate and high-risk MDS [39]. It has been shown that the inversion of the CD4:CD8 ratio is associated with the response to immunosuppressive therapy (IST) and is inversely correlated with the proliferative T-cell index before IST in these patients [15]. Taken together, our results suggest the contribution of CD4+ cells to the pathophysiology of the disease.

The majority of CD4+ Tregs cells present specific FOXP3 expression, a transcription factor, which is important for the development and function of these cells [40]; however, a population of CD8+FOXP3+ T cells has been described in several autoimmune diseases, after allergen exposure and allogenic transplantation [41–46]. We found that the lower expression of FOXP3 transcripts in the peripheral CD3+ cells of MDS patients was clearly due to the lower expression of this gene in the low-risk group. The only study that reports on CD8+ Treg cells in MDS, related no difference in the number of CD8+ Treg cells between MDS groups, IPSS or disease progression [14]. Although data regarding CD4+ Treg frequency in the low-risk MDS patients are uncertain, mainly due to the different flow cytometry strategies used [47], our data for FOXP3 expression support studies that report that the number of CD4+ Tregs is lower in low-risk MDS [7,14].

Although IL10 and TGFß1 are secreted by many cell types, the production of IL10 and TGFß1, as well as the expression of CTLA4, are indications of the activation of Treg cells [40]. Our results in CD3+ cells showed a significant positive correlation between the expression of these regulatory molecules and FOXP3 expression, indicating that these transcripts are derived from Treg cells. IL10 and FOXP3 expressions were lower in the low-risk group, compared to the control group, corroborating the hypothesis of down-regulated Tregs in low-risk MDS [7].

A significant increase in IL10 expression was observed in high-risk, compared to low-risk MDS, which is in agreement with the measurement of IL10 concentrations in serum, described by Kor-dasti et al. [48]. Interestingly, we observed an inverse correlation between CD8+ cell frequency and IL10 expression, supporting data describing the recruitment of CD8+ cells in an inverse relationship with the levels of Tregs in the bone marrow of MDS patients [49]. IL10 is thought to contribute to the immune suppressive milieu, by inhibition of antigen presentation, cytokine expression and T helper cell functions [50,51], all features of the immune evasion that is characteristic of high-risk MDS [7]. We postulate that IL10, secreted by Tregs, may have a role in the prevention of MDS clone elimination, with a consequent role in MDS progression.

In conclusion, our findings provide further evidence for Treg deregulation in low-risk MDS; and most importantly, add new insight into the role of T cell-mediated IL10 production in MDS and strengthen the idea of distinct cytokine profiles in low and high-risk MDS.

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**Authors’ Contributions**

MRL: carried out all experiments and participated in the writing of the manuscript. FT, PMC and SCOG: responsible for collection of patient samples and clinical data, and participated in the edition of the manuscript. JKNP, JAMN: helped with the experiments, analysis, and edition of the manuscript. HCM: carried out all the statistical analysis and contributed to manuscript writing. STOS: contributed to the study design, data analyses, and manuscript writing. PF was the principal investigator and takes primary responsibility for the paper.
Conflict of interest

Authors have no conflicts of interest.

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