

Monocytic MDSC as a source of immunosuppressive cytokines in chronic lymphocytic leukemia (CLL) microenvironment

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Abstract

Introduction. Myeloid derived suppressor cells (MDSCs) are one of the major components of the tumor microenvironment. The accumulation of MDSCs has been demonstrated in many types of human solid tumors. However, the relevance of this heterogeneous population in hematopoietic malignancies has only recently gained stronger attention. MDSCs are a phenotypically and functionally heterogeneous group of cells. The results of recent studies indicate that the immune dysregulation in chronic lymphocytic leukemia (CLL) affects a monocytic MDSC (M-MDSC) subpopulation. This study aimed to analyze the frequency of M-MDSCs with intracellular IL-10 and TGF- β 1 expression in newly diagnosed CLL patients. We investigated the potential role of M-MDSCs in CLL by analyzing the level of IL-10 and TGF- β 1 expression in circulating M-MDSCs in correlation with clinical and laboratory parameters characterizing disease activity and patients' immune status.

Material and methods. Seventy CLL patients and 17 age-matched healthy volunteers were included in this study. Flow cytometric detection of Mo-MDSCs (CD14⁺CD11b⁺CD15-HLA-DR^{-low}) with intracellular IL-10 and TGF- β 1 expression was done.

Results. We found a significantly higher median percentage of M-MDSC with IL-10 or TGF- β 1 expression in CLL patients than in healthy volunteers. The percentage of M-MDSC with intracellular IL-10 or TGF- β 1 expression was significantly lower in CLL patients at stage 0 as compared to the stages I/II and III/IV according to Rai stages. The percentage of M-MDSC with intracellular TGF- β 1 expression was significantly higher in ZAP-70-positive and CD38-positive patients compared with ZAP-70-negative and group of CD38-negative ones. There was also a significantly higher percentage of M-MDSC positive for intracellular TGF- β expression in patients carrying the 11q22.3 and/or the 17p13.1 deletion than in patients without these genetic aberrations. The percentage of M-MDSC IL-10-positive and M-MDSC TGF- β 1-positive measured at the time of diagnosis was higher in patients requiring therapy as compared to patients without treatment during the observation period.

Conclusion. In conclusion, we have shown that an increased percentage of M-MDSC cells producing IL-10 and TGF- β 1 in CLL patients may be associated with the suppression of the immune response against CLL. It can be assumed that the increased percentage of M-MDSC with an intracellular expression of IL-10 and TGF- β 1 may be used in the future as the factor defining the group of patients with shorter time to onset of treatment. (*Folia Histochemica et Cytobiologica* 2020, Vol. 58, No. 1, 25–36)

Key words: M-MDSC; IL-10; TGF- β ; chronic lymphocytic leukemia; flow cytometry; genetic analysis

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by an abnormal expansion of mature CD5⁺ B cells in the bone marrow and their accumulation in blood and secondary lymphoid organs [1]. The neoplastic B cells in CLL patients are dependent on interactions with their microenvironment. CLL cells fluctuate together with a microenvironment, which supports leukemia

cell survival and determines disease progression in CLL [2–5]. MDSCs (myeloid-derived suppressor cells) are one of the major components of the tumor microenvironment. The accumulation of MDSCs has been demonstrated in many types of human solid tumors [6]. However, the relevance of this heterogeneous population in hematopoietic malignancies has only recently gained stronger attention [7–10]. MDSCs are a phenotypically and functionally heterogeneous group of cells. In human there are two major types of MDSCs: monocytic-MDSC (M-MDSC; expressing CD14) and polymorphonuclear MDSC (PMN-MDSC; expressing CD15) [5, 11, 12]. Both subpopulations of MDSCs express the myeloid marker CD33. Unlike monocytes, MDSCs are characterized by the lack or low expression of HLA-DR [5]. M-MDSCs produce high amounts of immunosuppressive cytokines, such as IL-10 and TGF- β [13].

In CLL, malignant B lymphocytes, serve an important role in the immune response, so their interactions with other immune cells are more complex than observed in solid tumors. The results of recent studies indicate that the immune dysregulation in CLL also affects MDSCs [14, 15]. Jitschin *et al.* [16] observed the accumulation of M-MDSCs (defined as CD14⁺HLA-DR^{lo} cells) in peripheral blood of CLL patients. Probably, some cytokines, such IL-10, contribute to the recruitment and accumulation of MDSCs in CLL microenvironment [17, 18]. In addition, it has been shown that CLL cells themselves constitutively produce immunosuppressive IL-10 [19]. The course of CLL is accompanied by a state of immunosuppression. The leukemic B cells achieve this mainly through the involvement of regulatory T (Treg) cells. In turn, MDSCs release IL-10 and TGF- β to exert their suppressive function by Tregs [8, 20]. The origin of M-MDSC is unknown. Some scientists believe that M-MDSCs are derived from emergency myelopoiesis that can occur during cancer development [12, 21, 22]. Other theories suggest that M-MDSCs are formed in the pathways of reprogramming monocytes [23]. Probably, this process is connected with some cytokine milieu (e.g. IL-10) [22, 24].

In a recent study, we found the accumulation of intermediate and non-classical monocytes in CLL patients. The non-classical monocytes constituted the majority of cells lacking HLA-DR expression. Moreover, non-classical monocytes presented low CD163 expression [25]. HLA-DR^{low/-}CD163^{low/-} phenotype is also characteristic for M-MDSC [23].

This study aimed to analyze the frequency of M-MDSCs with intracellular IL-10 and TGF- β 1 expression in newly diagnosed CLL patients. We investigated the potential role of M-MDSCs in CLL by

analyzing the level of IL-10 and TGF- β 1 expression in circulating M-MDSCs in correlation with clinical and laboratory parameters characterizing disease activity and patients' immune status.

Material and methods

Patients and samples. The study group comprised 70 patients with CLL diagnosis, which was based on criteria from the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) [26]. All subjects were newly diagnosed. Peripheral blood (PB) samples were collected at the time of diagnosis and prior to any anticancer therapy. CLL patients were recruited between January 2016 and June 2019 in the Department of Hematooncology and Bone Marrow Transplantation of the Medical University of Lublin (Lublin, Poland). Clinical stage was determined according to the Rai classification system [27]. Thirty-seven patients were Stage 0, 11 patients were Stage I, 12 patients were Stage II, 7 patients were Stage III and 3 patients were Stage IV. The CLL group was further divided into three risk groups: Low risk (stage 0), intermediate-risk (stage I or II), and high-risk (stage III or IV). Characteristics of patients at the time of diagnosis are summarized in Table 1. Control PB samples were obtained from 17 healthy volunteers (HVs; 8 females and 9 males, aged from 35–74 years, median 56 years).

Peripheral blood (PB) samples were collected into EDTA-coated tubes and immediately processed. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation on Gradisol L (Aqua-Med, Lodz, Poland) for 25 min. at 400 g at room temperature (RT). Interphase cells were removed, washed twice and resuspended in phosphate-buffered saline (PBS).

Ethics statement. This study was approved by the Ethics Committee of the Medical University of Lublin (No. KE-0254/107/2013 and KE-0254/49/2016). Written informed consent was obtained from all patients with respect to the use of their blood for scientific purposes.

Detection of M-MDSC and analysis of intracellular IL-10 or TGF- β 1 expression. Flow cytometry analysis of M-MDSC (defined as CD14⁺CD11b⁺CD15⁺HLA-DR⁻/low cells) was performed on PBMCs. The samples were stained with combination of fluorescent-labelled monoclonal antibodies (MoAbs): mouse anti-human 14 FITC (Clone M ϕ P9), mouse anti-human CD11b V450 (Clone ICRF44), mouse anti-human HLA-DR PE-Cy7 (Clone L243) and CD15 APC (Clone HI98) (BD Biosciences, Franklin Lakes, NJ, USA). Cells were incubated for 20 min at RT. Following membrane staining, cells were fixed with Cytofix/Cytoperm and permeabilized with Perm/Wash buffer (BD Biosciences) according to the manufacturer's protocol. Cells were then intracellularly stained (20 min at RT) with PE anti-human

Table 1. Characteristics of the patients at CLL diagnosis

Features	No. patients (%)
Sex	
Female (%)	33 (47.1)
Male (%)	37 (52.9)
Rai Stage	
0 (%)	37 (52.9)
I (%)	11 (15.7)
II (%)	12 (17.1)
III (%)	7 (10.0)
IV (%)	3 (4.3)
ZAP-70 (cut-off 20%)^a	
Positive (%)	24 (34.3)
Negative (%)	46 (65.7)
CD38 (cut-off 20%)^b	
Positive (%)	25 (35.7)
Negative (%)	45 (64.3)
Cytogenetic abnormalities	
del(17p13.1) (%)	1 (1.4)
del(11q22.3) (%)	9 (12.9)
Without del(17p13.1) and del(11q22.3) (%)	60 (85.7)
Patients requiring therapy	17 (24.3)
Untreated patients	53 (74.7)
	median (range)
Age at diagnosis (years)	65 (46–85)
WBC count [G/L]	21.92 (10.11–290.46)
Lymphocyte count [G/L]	16.57 (5.21–284.9)
β_2 M [mg/dl]	2.42 (1.35–5.39)
LDH [IU/l]	362.0 (178.0–492.0)
Hemoglobin [g/dl]	13.95 (8.7–16.8)
Platelets [G/L]	187.0 (23.0–414.0)
% CD19 ⁺ /CD5 ⁺ /ZAP-70 ⁺ cells ^a	18.0 (0.2–50.0)
% CD19 ⁺ /CD5 ⁺ /CD38 ⁺ cells ^b	16.1 (0.02–88.7)

CLL — chronic lymphocytic leukemia; WBC — white blood cell; LDH — lactate dehydrogenase; β_2 M — β_2 microglobulin. ^aPatients with ZAP-70 expression lower or higher than 20% were classified as ZAP-70 negative or positive, respectively. ^bPatients with CD38 expression lower or higher than 30% were classified as CD38 negative or positive, respectively.

LAP (TGF- β 1) antibody (Clone TW4-2F8) or PE anti-human IL-10 antibody (Clone JES3-19F1) (BioLegend, San Diego, CA, USA).

Flow cytometry analysis. Samples were analyzed by flow cytometry directly following preparation. Data acquisition was performed on a FACSCanto II instrument with FACS-Diva Software (BD Biosciences). For each analysis, 100,000

events were acquired and analyzed. Kaluza 2.1.1 (Beckman Coulter, Miami, FL, USA) was used for the data analysis. An acquisition gate was put on lymphocytes according to the forward scatter (FSC) and side scatter (SSC) properties. The gating strategy to identify M-MDSC with IL-10 or TGF- β 1 expression is shown in Figure 1A–E. The results are expressed as the percentage of M-MDSC with intracellular IL-10 or TGF- β 1 expression. Furthermore, within M-MDSC population, IL-10 and TGF- β 1 cytokines were quantified regarding their mean fluorescence intensity (MFI). To establish the gating strategy, fluorescence minus one (FMO) control was used. The FMO control tube included all antibodies that were used for M-MDSC cell staining, except for the one (IL-10 PE or TGF- β 1 PE) that was measured.

Analysis of T regulatory cells (CD4+CD25+FoxP3+). Treg cells were evaluated *via* analysis of the surface expression of CD4 and CD25 antigens, as well as intracellular expression of FoxP3 by flow cytometry. Tregs were stained with Human Treg Flow Kit (FOXP3 Alexa Fluor 488/CD4 PE/Cy5/CD25 PE) (BioLegend) according to the manufacturer's instructions.

Analysis of CD38 and ZAP-70 expression in CLL cells. CLL cells were stained for CD38 antigen and ZAP-70 protein expression (as described previously [28]). Fresh PB samples were stained with FITC mouse anti-human CD19 (Clone SJ25C1), PE-Cy5 mouse anti-human CD5 (Clone UCHT2) and CD38 FITC (Clone HIT2) or anti-ZAP-70 PE (Clone 1E7.2) (BD Biosciences). A cut-off point for ZAP-70 positivity in leukemic cells was $\geq 20\%$. Patients with CD38 expression lower or higher than 30% were classified as CD38 negative or positive, respectively.

I-FISH analysis. Detection of del(17p13.1) and del(11q22.3) was a part of the routine diagnostic practice. We used a previously described method [29].

Statistical analysis. The Kruskal-Wallis test with Dunn correction or U Mann-Whitney test were used for comparative analysis of the variables. The Spearman rank correlation coefficient was used in correlation tests. Differences were considered statistically significant with p-value ≤ 0.05 . Statistical analysis was calculated with Statistica 13 PL (Statistica, Krakow, PL). Graphs were processed using GraphPad Prism version 5.

Results

The percentage of M-MDSC was significantly increased in patients with CLL in comparison to the HVs (median [IQR], 2.84% [1.25–4.50%] vs. 8.605 [6.82–11.12%], $p < 0.001$). Moreover, we found significantly higher median percentage of M-MDSC

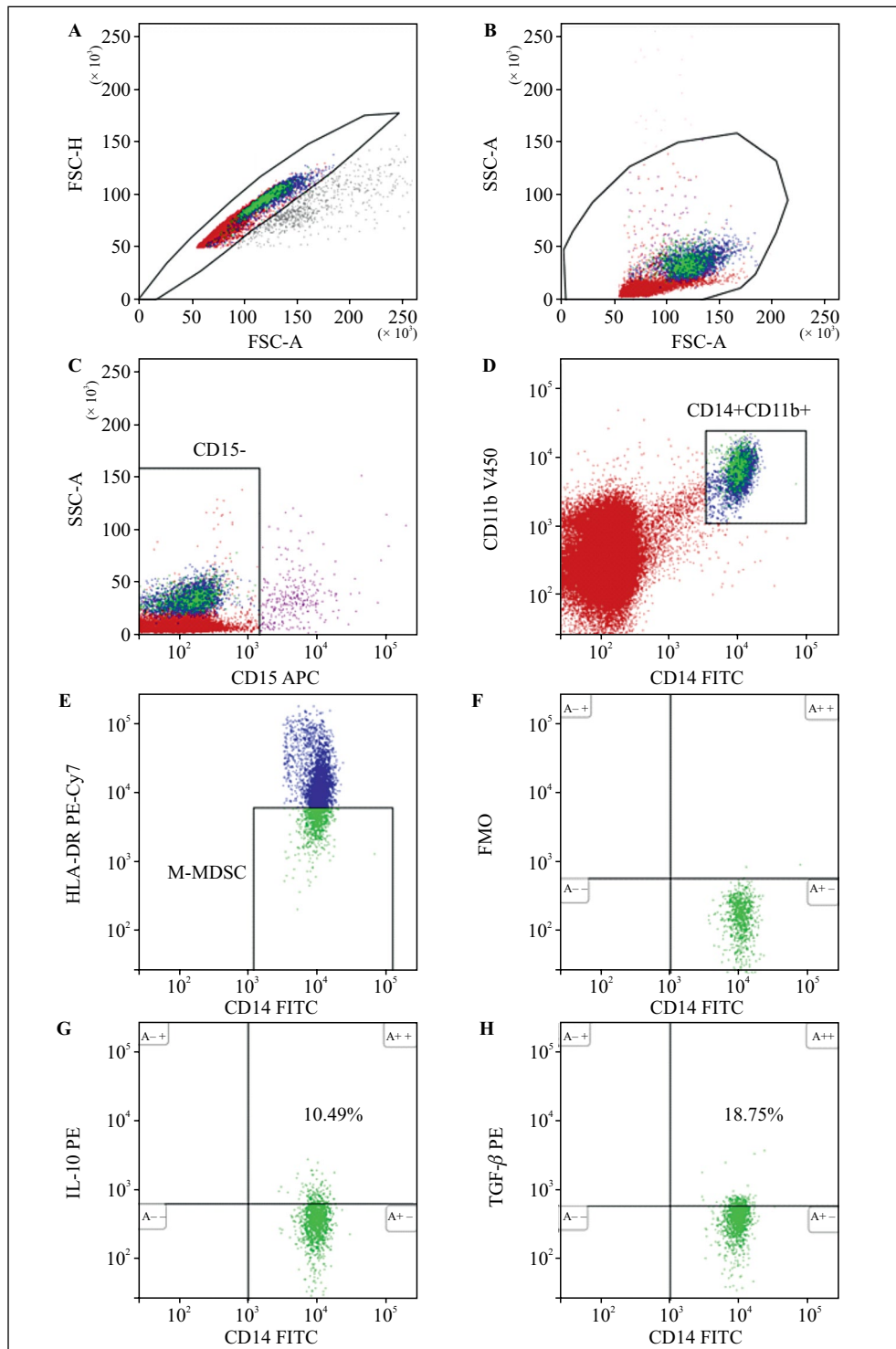


Figure 1. Representative dot plots illustrating the analytic method for the identification of M-MDSCs with the intracellular expression of IL-10 and TGF- β 1. **A.** FSC-A vs. FSC-H dot plot: doublets' discrimination. **B.** After gating a singlet (P1 region) mononuclear cells (PBMCs) were selected based on their SSC/FSC properties. **C.** CD15 APC vs. SSC-A dot plot. Discrimination of CD15⁻ from CD15⁺ cells. **D.** Selected CD15⁻ cells were analyzed for CD14 FITC and CD11b V450 staining. **E.** Selected CD14⁺CD11b⁺ cells were analyzed for HLA-DR PE-Cy7 expression. The dot plots (CD14 FITC vs. HLA-DR PE-Cy7) indicate CD14⁺CD11b⁺CD15⁻HLA-DR^{low} cells (M-MDSC). **F.** Dot plots indicating a FMO (*Fluorescence minus one*) control, which contains all fluorochromes in a panel except for IL-10 PE or TGF- β PE. The FMO control identifies any spread of fluorochrome in an unlabeled channel and places the gates in the correct place. **G, H.** Selected CD14⁺CD11b⁺CD15⁻HLA-DR^{low} cells were analyzed for IL-10 PE (**G**) or TGF- β PE staining (**H**). A++ quadrant in dot plot (**G**) shows the percentage of M-MDSCs IL-10-positive. A++ quadrant in dot plot (**H**) shows M-MDSCs with the intracellular expression of TGF- β . Data were analyzed using Kaluza 2.1.1 software.

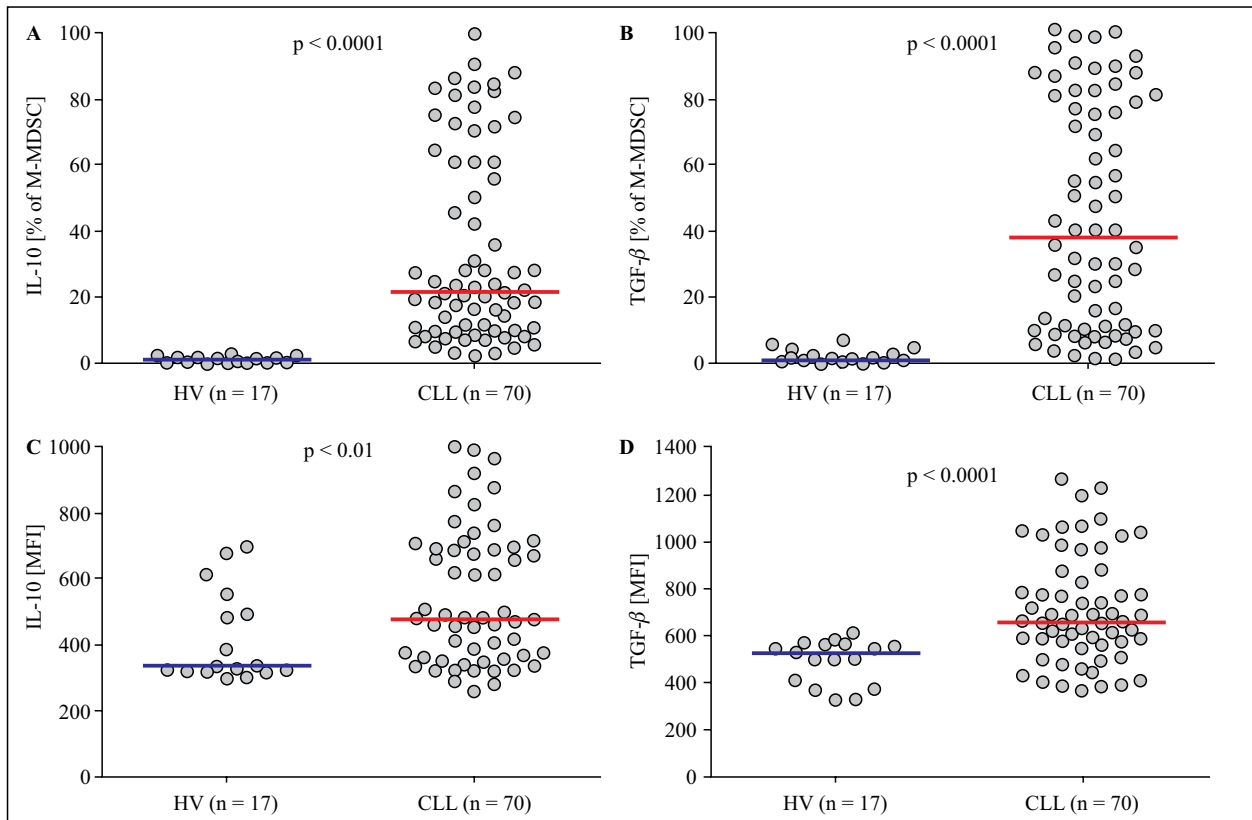


Figure 2. IL-10 and TGF- β expression in M-MDSCs from CLL patients and healthy volunteers (HV). (A) percentage of IL-10 positive M-MDSCs; (B) mean fluorescence intensity (MFI) of IL-10 in M-MDSCs. (C) percentage of TGF- β positive M-MDSCs; (D) MFI of TGF- β in M-MDSCs. The U Mann-Whitney test was used for comparative analysis.

with intracellular IL-10 expression in CLL patients than in healthy volunteers (median [IQR], 21.72% [9.81–60.93%] vs. 0.57% [0.06–1.31%], $p < 0.0001$; Fig. 2A). Likewise, percentage of M-MDSC with TGF- β 1 (median [IQR], 37.36% [9.93–78.37%]) expression was significantly higher in comparison to the HVs (median [IQR], 1.17% [0.33–3.68%]) (Fig. 2B). Furthermore, within M-MDSC population, IL-10 and TGF- β 1 cytokines were quantified regarding their mean fluorescence intensity (MFI). CLL patients increased IL-10 expression level (median [IQR], 477.0 [355.3–689.3] MFI), when compared to healthy controls (median [IQR], 333.0 [318.2–522.2] MFI) ($p < 0.01$) (Fig. 2C). Likewise, TGF- β 1 expression level indicated by MFI was significantly higher in M-MDSC from CLL patients when compared to HV (median [IQR], 658.4 [563.6–865.2] MFI vs. 526.60 [389.6–565.8] MFI, $p < 0.001$) (Fig. 2D).

The percentage of M-MDSC with intracellular IL-10 expression showed low inter-individual variability in healthy controls while in CLL patients it was much more diverse and significantly lower in patients at stage 0 as compared to the stages I/II and III/IV ac-

cording to Rai stages (Fig. 3A, Table 2). Likewise, MFI was lower in patients at stage 0 (median, 456.0 MFI) as compared to the stages I/II (median, 481.90 MFI) and III/IV (median, 757.30 MFI) (Fig. 3B, Table 2). Similarly the percentage of M-MDSC with intracellular TGF- β 1 expression was significantly lower in patients at stage 0 as compared to the stages I/II and III/IV according to Rai stages (Fig. 3C, Table 2). Likewise, MFI was lower in patients at stage 0 as compared to the stages I/II and III/IV (Fig. 3D, Table 2).

The percentage of M-MDSC with intracellular TGF- β 1 expression was significantly higher in ZAP-70-positive patients compared with ZAP-70-negative ones (Fig. 4A, Table 3) ($p < 0.05$). Likewise, higher intracellular TGF- β 1 expression determined by MFI was observed in ZAP-70+ than in ZAP-70- patients ($p < 0.05$) (Fig. 4B, Table 3). We also observed a higher percentage of M-MDSC with TGF- β 1 expression in CD38-negative patients than in CD38-positive ones ($p < 0.05$) (Fig. 4C, Table 3). Likewise, MFI was higher in CD38+ than in CD38- patients; however, the difference was not statistically significant ($p > 0.05$) (Fig. 4D, Table 3). Opposing

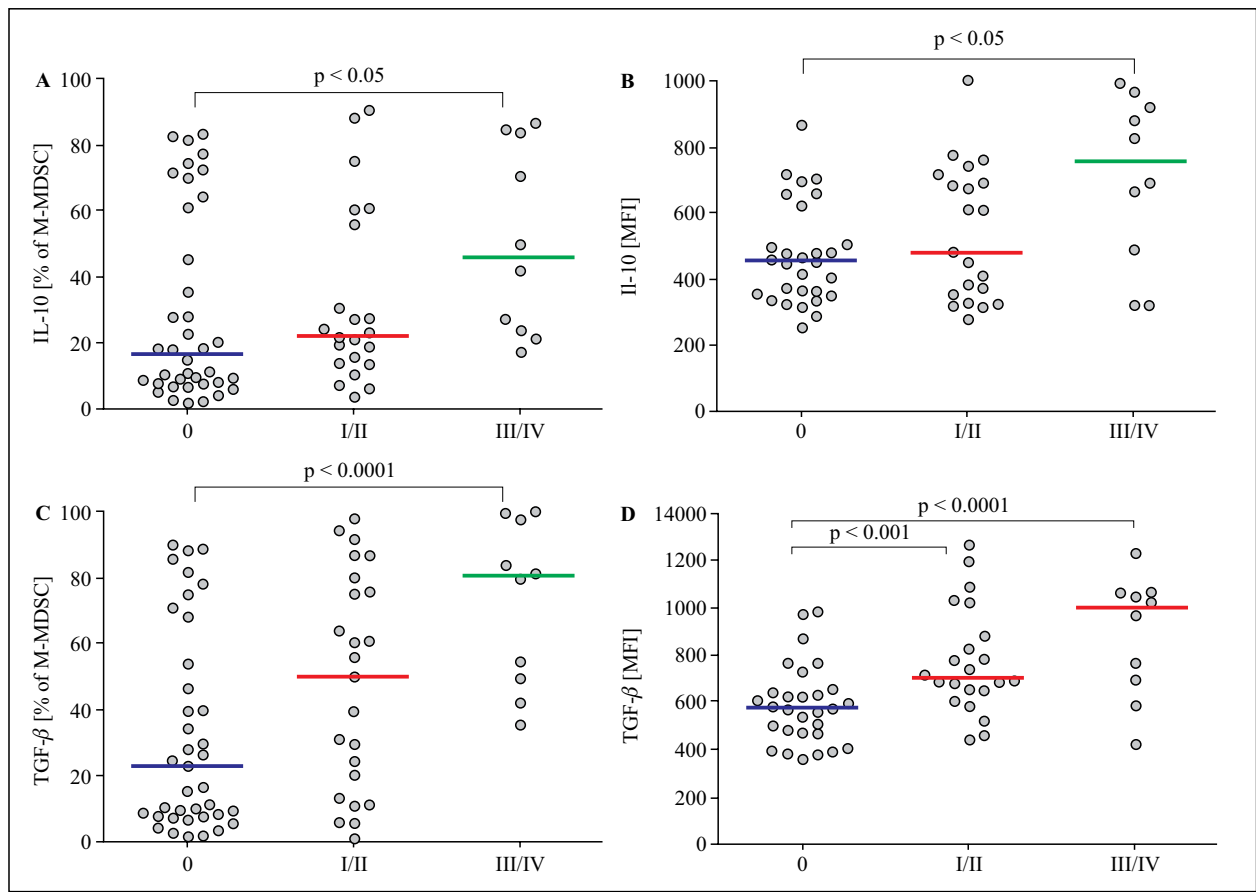


Figure 3. IL-10 and TGF- β expression in M-MDSCs from CLL patients in different disease stages. (A) percentage of IL-10 positive M-MDSCs; (B) mean fluorescence intensity (MFI) of IL-10 in M-MDSCs. (C) percentage of TGF- β positive M-MDSCs; (D) MFI of TGF- β in M-MDSCs. The Kruskal-Wallis test with Dunn correction was used for comparison or comparative analysis.

Table 2. M-MDSC with intracellular IL-10 or TGF- β 1 expression in CLL patients at various disease stages (three risk groups)

CD14 ⁺ CD11b ⁺ CD15 ⁺ HLA-DR ^{low} IL-10 ⁺						
	Stage 0 (n = 37)		Stage I/II (n = 23)		Stage III/IV (n = 10)	
	%	MFI	%	MFI	%	MFI
Median	17.18	456.0	22.80	481.90	46.14	757.30
Minimum	2.32	258.2	4.28	279.20	17.51	318.70
Maximum	83.77	864.6	90.63	1000.00	86.40	991.50
IQR	53.65	212.6	42.85	357.6	60.39	484.00
CD14 ⁺ CD11b ⁺ CD15 ⁺ HLA-DR ^{low} TGF- β 1 ⁺						
	%	MFI	%	MFI	%	MFI
Median	22.89	582.70	50.10	704.90	80.43	1000.00
Minimum	1.29	365.20	1.27	445.70	35.36	428.8
Maximum	89.41	987.90	97.63	1269.0	99.48	1230.0
IQR	53.01	175.60	66.76	261.70	49.69	393.40

MFI — Mean fluorescence intensity

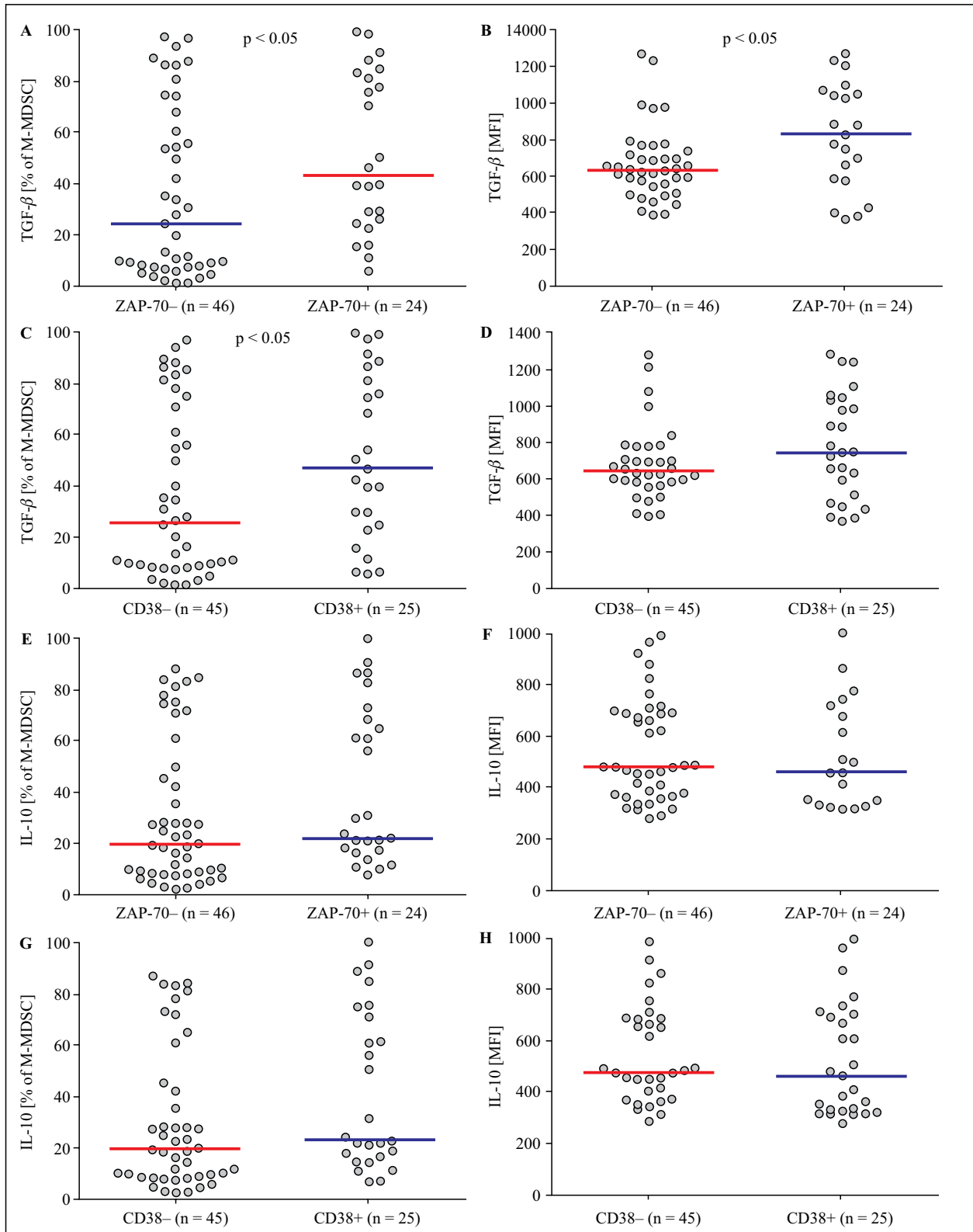
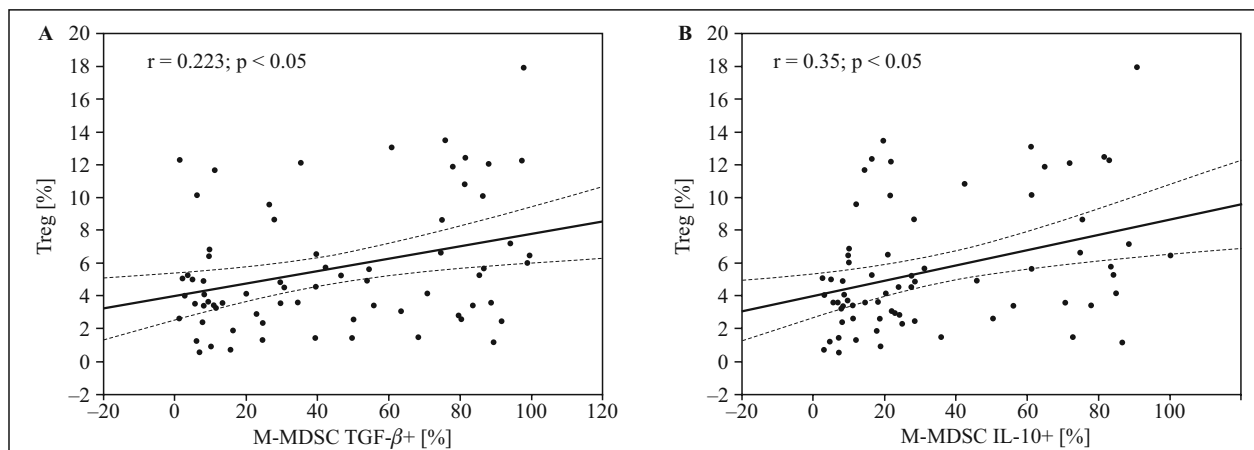


Figure 4. IL-10 and TGF- β expression in M-MDSCs from CLL patients analyzed by adverse prognostic factors. **A.** The percentage of CD14⁺CD11b⁺CD15⁺HLA-DR^{-low}TGF- β ⁺ cells in ZAP-70-negative patients compared with ZAP-70-positive patients. **B.** TGF- β expression levels indicated by the MFI (mean fluorescence intensity) in M-MDSCs from ZAP-70-negative and ZAP-70-positive patients. **C.** The percentage of CD14⁺CD11b⁺CD15⁺HLA-DR^{-low}TGF- β ⁺ cells in CD38-negative patients compared with CD38-positive patients. **D.** TGF- β expression levels indicated by the MFI in M-MDSCs from CD38- and CD38+ patients. **E.** The percentage of CD14⁺CD11b⁺CD15⁺HLA-DR^{-low}IL-10⁺ cells in ZAP-70-negative patients compared with ZAP-70-positive patients. **F.** IL-10 expression levels indicated by the MFI in M-MDSCs from ZAP-70-negative and ZAP-70-positive patients. **G.** The percentage of CD14⁺CD11b⁺CD15⁺HLA-DR^{-low}IL-10⁺ cells in CD38-negative patients compared with CD38-positive patients. **H.** IL-10 expression levels indicated by the MFI in M-MDSCs from CD38- and CD38+ patients. The U Mann-Whitney test was used for comparative analysis.

Table 3. Percentage of M-MDSC with the expression of IL-10 and IL-10 MFI in CLL patients classified into groups: ZAP-70+, ZAP-70-, CD38+, CD38-

M-MDSC IL10-positive								
	ZAP-70+ (n = 24)		ZAP-70- (n = 46)		CD38+ (n = 25)		CD38- (n = 45)	
	%	MFI	%	MFI	%	MFI	%	MFI
Median	21.95	458.20	20.02	478.00	22.91	465.60	19.67	478.00
Minimum	7.70	316.60	2.32	279.20	6.640	279.20	2.320	290.70
Maximum	99.84	1000.00	88.24	991.50	99.84	1000.00	86.40	991.50
IQR	48.33	382.90	41.80	318.70	55.59	380.50	36.32	296.50
M-MDSC TGF- β 1-positive								
	%	MFI	%	MFI	%	MFI	%	MFI
Median	43.28	830.20	24.56	631.80	25.51	738.70	46.71	639.40
Minimum	6.18	365.20	1.27	387.70	1.27	365.20	5.57	392.90
Maximum	99.48	1269.00	97.63	1265.0	97.14	1269.00	99.48	1265.0
IQR	57.85	476.80	60.33	189.90	60.20	532.20	63.41	197.20

**Figure 5.** The relationship between the percentages of Treg lymphocytes and M-MDSCs with intracellular TGF- β (A) or IL-10 (B) expression. The Spearman rank correlation coefficient was used in correlation tests.

to what was observed for TGF- β 1 differences in percentage of M-MDSC with IL-10 expression and IL-10 MFI levels were not statistically significant between ZAP-70+ and ZAP-70- patients (Fig. 4E, 4F). Likewise, the difference between CD38-negative patients and CD38-positive ones was not statistically significant ($p > 0.05$) (Fig. 4G, 4H).

The percentage of M-MDSC with TGF- β 1 expression correlated positively with the peripheral blood WBC count ($r = 0.272$; $p < 0.05$) and lymphocyte count ($r = 0.260$; $p < 0.05$). In the group of patients with CLL, no significant correlations were identified between the frequency of M-MDSC TGF- β 1-positive and platelet counts, hemoglobin concentration, serum LDH and β 2-microglobulin levels or the age of the patients.

There was a significant correlation between the percentage of M-MDSC with intracellular TGF- β 1 expression and Treg cells ($r = 0.223$; $p < 0.05$) (Fig. 5A). Likewise, percentage of M-MDSC cell with IL-10 expression correlated with frequency of Treg ($r = 0.35$; $p < 0.05$) (Fig. 5B).

Moreover, there was a significant difference in the median percentage of M-MDSC with intracellular IL-10 expression between patients carrying the 11q22.3 and/or the 17p13.1 deletion (median [IQR], 31.41% [21.29–76.96%]) and patients without these genetic aberrations (median [IQR], 20.67% [10.80–58.36%]) ($p < 0.05$) (Fig. 6A). We also observed a significantly higher percentage of M-MDSC positive for intracellular TGF- β expression in patients carrying the 11q22.3 and/or the 17p13.1 deletion (median [IQR], 55.10%

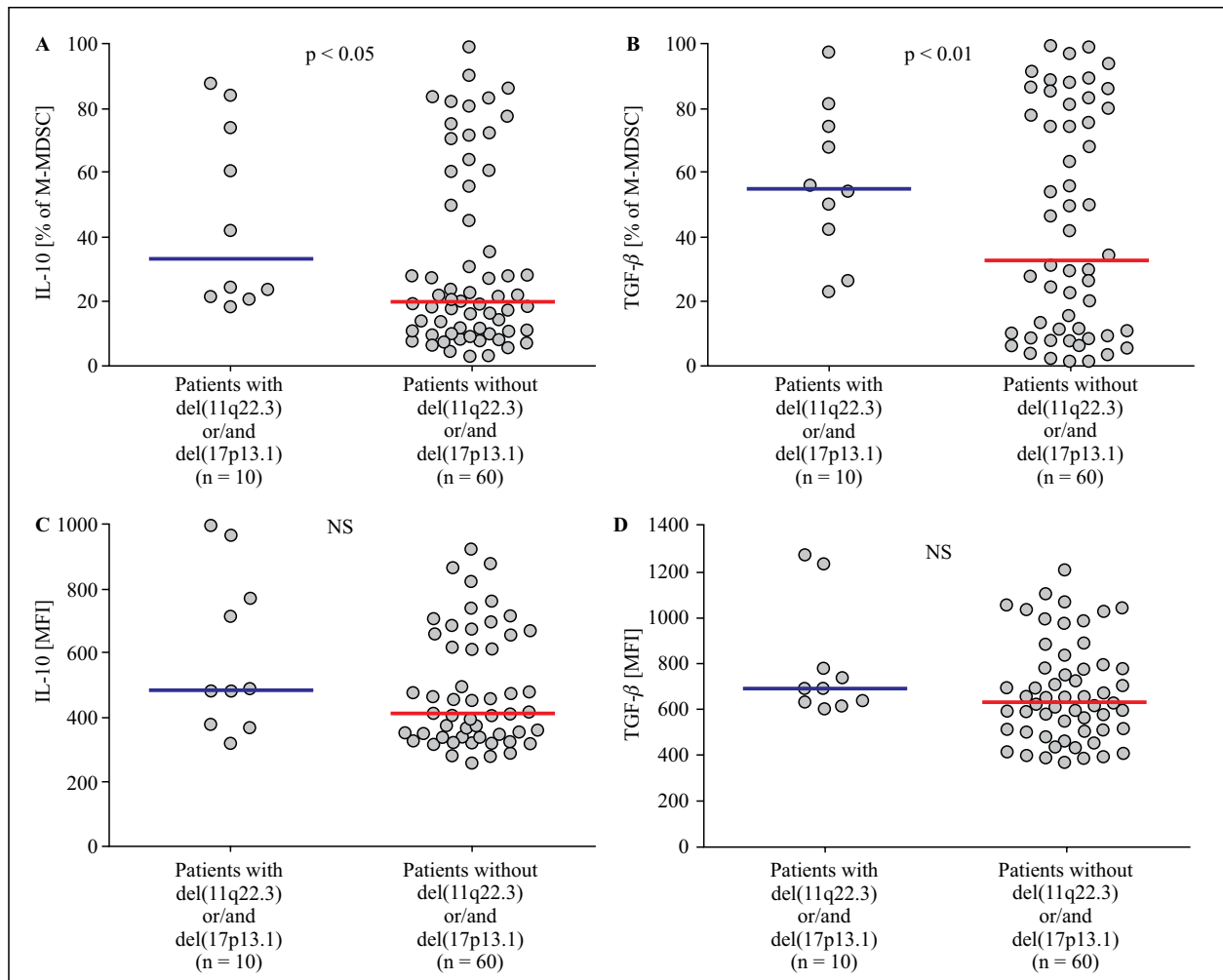


Figure 6. IL-10 and TGF- β expression in M-MDSCs from CLL patients carrying the 11q22.3 and/or the 17p13.1 deletion and patients without these genetic aberrations. (A) percentage of IL-10 positive M-MDSCs; (B) percentage of TGF- β positive M-MDSCs; (C) mean fluorescence intensity (MFI) of IL-10 in M-MDSCs; (D) MFI of TGF- β in M-MDSCs. The U Mann-Whitney test was used for comparative analysis.

[38.30–76.18%]) than in patients without these genetic aberrations (median [IQR], 32.60% [9.82–80.53%]) ($p < 0.01$) (Fig. 6B).

Likewise, TGF- β 1 and IL-10 expression levels indicated by the MFI were compared between patients carrying the 11q22.3 and/or the 17p13.1 deletion (TGF- β 1: median [IQR], 674.4 [571.5–903.0] MFI; IL-10: median [IQR], 470.8 [366.2–822.8] MFI) and patients without these genetic aberrations (TGF- β 1: median [IQR], 629.5 [495.5–808.0] MFI; IL-10: median [IQR], 411.8 [334.2–656.7] MFI). However, the difference was not statistically significant ($p > 0.05$) (Fig. 6C, 6D).

During the follow-up period (range 1–55 months), 17 patients required therapy (Table 1). The percentage of M-MDSC IL-10-positive and M-MDSC TGF- β 1-positive measured at the time of diagnosis

was statistically higher in patients requiring therapy as compared to patients without treatment during the observation period (IL-10: median [IQR], 27.95% [11.73–99.84%] vs. 21.48% [18.08–49.01%] TGF- β 1: median [IQR], 49.76% [24.56–83.46%] vs. 39.35% [13.77–77.72%]) ($p < 0.05$). However, MFI level was not statistically significant between both groups (IL-10: median [IQR], 477.0 [372.2–696.0] MFI vs. 375.3 [319.3–774.9] MFI; TGF- β 1: median [IQR], 735.7 [477.1–927.3] MFI vs. 651.5 [575.5–876.9] MFI; $p > 0.05$).

Discussion

The presence of M-MDSC in the microenvironment has recently aroused great interest. M-MDSC, like regulatory T cells, is thought to contribute to the

effective reduction of anti-tumor response [30]. Unfortunately, there are very few studies available on M-MDSCs in CLL patients [16, 31–33]. Jitschin *et al.* [16] identified increased numbers of MDSCs (CD14⁺HLA-DR^{lo}) in untreated patients with CLL. This finding is supported by our results showing the M-MDSC (defined as CD14⁺CD11b⁺CD15⁺HLA-DR^{-low} cells) accumulation in CLL patients.

M-MDSCs have been described to express IDO (indoleamine-2,3-dioxygenase). IDO activity is an important mechanism by which effector T cells can be induced and converted to Tregs. The IDO-mediated depletion of tryptophan and subsequent production of immunosuppressive products may also lead to T cell suppression through the down-regulation of the TCR-CD3- ζ chain [34]. IDO^{high} MDSCs suppress both T-cell activation and proliferation in untreated CLL patients [16]. M-MDSCs have also been described to secrete TGF- β and IL-10, which exert direct immunosuppressive effects on T effector cells or induce the generation of Treg cells [35].

Alhakeem *et al.* [19] observed that CLL patients have a significantly higher level of IL-10 in plasma than healthy donors. They reported that IL-10 affects CLL growth indirectly by suppressing anti-CLL T-cells. In their study IL-10 reduced the generation of effector CD4 and CD8 T-cells [19]. Still little is known about the mechanisms that lead to the increasing of M-MDSC, especially in the microenvironment of leukemia [20] and lymphoma [24]. Lee *et al.* [36] found that TGF- β induced the expansion of the monocytic MDSC population, expression of immunosuppressive molecules by MDSCs, and the ability of MDSCs to suppress CD4⁺ T cell proliferation. In our study, we found a significantly higher percentage of M-MDSC with intracellular IL-10 or TGF- β expression in CLL patients than in healthy volunteers. Moreover, we reported a significant correlation between the percentage of M-MDSC with TGF- β or IL-10 expression and the frequency of Treg cells. In general, the accumulation of Treg lymphocytes in CLL is well documented. Their increased incidence in patients with CLL affects the severity of the disease and worse prognosis [37].

Recently, Zahran AM *et al.* [33] reported that the abundance of CD14⁺HLA-DR^{lo} cells is associated with frequency of ZAP-70 and CD38 cells. Expression of CD38 and ZAP-70 in cancer cells of CLL patients are typical immunophenotypic unfavorable prognostic markers of patients' life expectancy [38]. It is interesting that M-MDSC from ZAP-70-positive and CD38-positive cases expressed higher TGF- β or IL-10 levels. What is more, we were able to demonstrate a significant association of TGF or IL-10 expression

in M-MDSC with del(11q22.3) and/or del(17p13.1). Deletions in the long arm of chromosomes 11 [del(11q)] or in the short arm of chromosome 17 [del(17p)] are recognized as high-risk genetic lesions [26]. Del(17p) is associated with an aggressive clinical course. Patients with leukemia cells that have del(17p) are resistant to standard chemotherapy regimens containing alkylating agents and purine analogs [26, 39, 40]. Likewise, the presence of del(11q) is associated with a clinically progressive disease in almost all CLL cases [41]. Patients with leukemia cells that have del(11q) have been associated with shorter TTFT (time to first treatment), shorter remission durations, and shorter OS (overall survival) following standard chemotherapy compared to cases without this genetic aberration [41].

Gustafson *et al.* [31] reported that the abundance of CD14⁺HLA-DR^{lo} cells is associated with a shorter time to CLL progression. Likewise, Liu *et al.* [32] reported that upregulation of CD14⁺HLA-DR^{low/-} MDSCs correlated with CLL progression and a poor prognosis for CLL patients. Recently, Zahran AM *et al.* [33] reported that the survival time was significantly shorter in patients expressed high levels of M-MDSC when compared to those with low levels. In our study patients who required therapy had a higher percentage of M-MDSC with TGF- β or IL-10 expression. Moreover, the percentage of CD14⁺HLA-DR^{-low} cells with TGF- β or IL-10 expression increased with more advanced clinical stage of CLL. Undoubtedly, the existence of a relationship between the number of CD14⁺HLA-DR^{-low} cells and the stage of clinical advancement of the disease indicates the possible involvement of CD14⁺HLA-DR^{-low} cells in CLL progression. According to the data presented by Jitschin *et al.* [16], patients with a higher M-MDSC percentage have a more rapid accumulation of leukemia lymphocytes. In our study, the percentage of M-MDSC with TGF- β expression correlated positively with the WBC count and PB lymphocyte count.

This is the first report investigated the potential role of M-MDSCs in CLL by analyzing the level of IL-10 and TGF- β 1 expression in circulating M-MDSCs in correlation with clinical and laboratory parameters characterizing disease activity and patients' immune status. We have shown that an increased percentage of M-MDSC cells producing IL-10 and TGF- β 1 patients may be associated with suppression of the immune response against leukemic cells. It can be assumed that the increased percentage of M-MDSC with intracellular expression of IL-10 and TGF- β 1 may in the future be the factor defining the group of patients with shorter time to onset of treatment.

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Authors' contributions

ABJ – designed research, performed research, analyzed and interpreted data, wrote the paper, critically revised the manuscript. WK — performed research, collected medical data, wrote the paper and critically revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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