

Expression of CD163 and HLA-DR molecules on the monocytes in chronic lymphocytic leukemia patients

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Abstract

Introduction. Human peripheral blood monocytes are the part of the leukemia microenvironment. We examined three monocyte subgroups: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺) monocytes. As these subpopulations can be also characterized by different levels of HLA-DR and CD163, we evaluated their expression on monocyte subpopulations of patients with chronic lymphocytic leukemia (CLL) and healthy individuals.

Material and methods. The monocyte subsets in peripheral blood of CLL patients (n = 40) and healthy controls (n = 10) were evaluated by flow cytometry. The monoclonal antibodies: anti-CD14 FITC, anti-CD16 PE-Cy5, anti-CD163 PE, anti-HLA-DR PE were used.

Results. The percentage of CD16⁺-positive monocytes was significantly higher in CLL patients than in healthy donors. The highest percentage of CD163⁺ monocytes is in the ‘classical’ (CD14⁺⁺CD16⁻) population. In turn, the non-classical monocytes constituted the majority of cells lacking HLA-DR expression. In CLL patients, there was no statistically significant relationship between the percentage of each monocyte subpopulation and the stage according to Rai Staging of CLL.

Conclusions. The presence of CD163 on classical monocytes suggests that these cells have anti-inflammatory properties. Besides, the low expression of HLA-DR on non-classical monocytes may result in impaired ability to stimulate the immune system. (*Folia Histochemica et Cytobiologica* 2020, Vol. 58, No. 1, 17–24)

Key words: monocyte; subpopulations; CD163; HLA-DR; chronic lymphocytic leukemia

Introduction

Chronic lymphocytic leukemia (CLL) patients suffer from the immunological dysfunctions that refer not only to B cells, but also to other elements of the immune system, including T cells, NK cells, neutrophils and monocytes/macrophages [1]. The observed inhibition of antitumor response during CLL may be ascribed to cells that form the tumor microenvironment, which favor the clonal expansion of B lymphocytes and promote their survival [2]. Monocytes are mononuclear cells involved in the innate immune responses. In CLL they could have a significant influence on the regulation of the growth

or elimination of cancer cells [3]. Based on differences in the expression of surface markers CD14 (receptor for lipopolysaccharide, *i.e.* LPS, LPS-R) and CD16 (FcγRIII receptor), three subpopulations of monocytes can be identified: the classical (CD14⁺⁺CD16⁻), the intermediate (CD14⁺⁺CD16⁺) and the non-classical (CD14⁺CD16⁺⁺) monocytes [4–6]. Under physiological conditions, the percentage of classical monocytes constitutes about 95% of all monocytes circulating in the peripheral blood. The role of the remaining 5% of CD16⁺ monocytes has not been clearly defined [7].

The disproportion between the percentage of CD16⁻ and CD16⁺ monocytes was observed in cancer patients [8]. It is believed that these abnormalities may have a significant impact on the proangiogenic and anti-tumor capacities of CD16⁺ monocytes [9]. A higher expression of HLA-DR and CD86 molecules was observed on classical monocytes (CD16-negative) [10]. On the other hand, among CD16-positive mono-

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cytes, the intermediate monocytes (CD14⁺⁺CD16⁺) have a high expression of the CD163 [11]. It is currently believed that the CD163 protein is involved in the uptake of the hemoglobin-haptoglobin complex and the regulation of inflammatory processes [12, 13]. CD163 may also be in a soluble form (soluble CD163, sCD163) [14]. The role of the sCD163 molecule has not been exactly explained. However, it seems to be involved not only in the removal of hemoglobin-haptoglobin complexes but also in the anti-inflammatory response [13]. Its higher level was found in hematological cancers, including chronic lymphocytic leukemia [15]. Moreover, CD163 is certainly a marker of macrophage activity and is generally thought to be associated with downregulation of inflammation, but its biological role still has not been fully elucidated [14, 15]. Monocytes are one of the least known immune cells with a potentially important role in the pathogenesis of CLL.

The aim of our study was to evaluate the expression of CD163 and HLA-DR molecules on the monocyte subpopulations in CLL patients and healthy individuals.

Materials and methods

Characteristics of the study group. The study group consisted of 40 patients with newly diagnosed CLL at the Department of Hematooncology and Bone Marrow Transplantation of the Medical University of Lublin, Poland. The diagnosis of CLL was based on the clear recommendations developed by the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) [16].

The age of patients ranged from 39 to 82 years (median: 63 ys). The study group included 24 men and 16 women. According to the Rai classification [17], patients were divided into 3 groups: the low risk group (stage 0) — 22 patients, intermediate-risk group (stage I/II) — 11 patients, high-risk group (stage III/IV) — 7 patients. Detailed characteristics of the examined group of CLL patients are presented in Table 1. The control group consisted of 10 healthy volunteers (7 men, 3 women). Age of donors ranged from 24 to 54 years (median: 47 ys).

This study was approved by the Bioethics Committee of the Medical University of Lublin (No. KE-0254/49/2016). All patients gave their written consent to participate in the research.

Blood collection and preparation of samples for flow cytometry. Approximately 5 ml of venous blood was collected into an EDTA-coated tube from each patient and healthy person. The material for the study was immediately processed. Subpopulations of monocytes circulating in the peripheral blood of CLL patients and healthy controls were

Table 1. Characteristics of chronic lymphocytic leukemia (CLL) patients

| The criteria for differentiating patients | Number of patients |
|--|---------------------------------------|
| Sex | |
| Female (%) | 16 (40%) |
| Male (%) | 24 (60%) |
| Rai stage | |
| 0 | 22 (55%) |
| I/II | 11 (27.5%) |
| III/IV | 7 (17.5%) |
| ZAP-70 (cut-off point 20%) | |
| ZAP-70-positive | 17 (42.5%) |
| ZAP-70-negative | 23 (57.5%) |
| CD38 (cut-off point 30%) | |
| CD38-positive | 11 (27.5%) |
| CD38-negative | 29 (72.5%) |
| | Median value (minimum-maximum) |
| Age at diagnosis (years) | 63 (39–82) |
| WBC [G/l] | 25.2 (10.1–112.5) |
| LYM [G/l] | 18.8 (5.5–106.3) |
| β_2 M [mg/dl] | 2.4 (1.36–5.39) |
| LDH [IU/l] | 373 (266–619) |
| HGB [g/dl] | 14.00 (8.1–17.2) |
| CD19 ⁺ CD5 ⁺ ZAP-70 ⁺ (%) | 13.41 (0.2–44.3) |
| CD19 ⁺ CD5 ⁺ CD38 ⁺ (%) | 8.43 (0.3–80.9) |
| CD19 ⁺ CD5 ⁺ (%) | 3.23 (0.9–16.6) |

Abbreviations: WBC — white blood cell count; LYM — absolute lymphocyte count; LDH — lactate dehydrogenase; β_2 M — β_2 -microglobulin; HGB — hemoglobin

determined by flow cytometry. Monocyte cell surface antigen assessments were performed with the following monoclonal antibodies conjugated with fluorochromes: anti-CD14 FITC (BD Pharmingen); anti-CD16 PE-Cy5 (BD Pharmingen); anti-HLA-DR PE (BioLegend); anti-CD163 PE (BioLegend). 100 μ l of blood was taken into vials and labeled with monoclonal antibodies.

Samples were incubated for 20 minutes at room temperature. The next step was RBC lysis with FACS Lysis Solution (Becton Dickinson) for 10 minutes at room temperature. Right after centrifugation at 700 \times g for 5 minutes, supernatant was poured out and the marked cells were rinsed twice with phosphate buffered saline PBS (700 \times g).

A BD FACSCaliburTM flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with CellQuestPro software (BD Biosciences) were used for cytometric analysis and data evaluation.

Statistical analysis. The statistical analysis was carried out with GraphPad Prism 5.0. software (GraphPad Software,

Inc. La Jolla, California, USA). U Mann-Whitney test or Wilcoxon test were used. Median, minimal and maximal values and IQR were used for data description. The Spearman rank correlation coefficient was used in correlation tests. The level of significance was set at $p < 0.05$.

Results

The percentage of classical monocytes in patients with CLL was significantly lower in relation to the control group ($p < 0.0001$), while the percentage of CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes was significantly higher compared to the control group ($p < 0.01$) (Fig. 1, Table 2). The percentage of CD14⁺⁺CD16⁻ classical monocytes (median: 75.56%) was significantly higher than CD16-positive monocytes in CLL patients. The latter group includes the CD14⁺⁺CD16⁺ intermediate subset (median: 6.34%) and non-classical CD14⁺CD16⁺⁺ (median: 10.65%) subpopulation ($p < 0.01$) (Fig. 2, Table 2).

Of all subpopulations, classical monocytes accounted for the highest percentage of CD163⁺ monocytes (median: 96.73%), while non-classical monocytes presented the lowest percentage of CD163⁺ cells (median: 5.12%) (Fig. 3, Table 3).

The non-classical CD14⁺CD16⁺⁺HLA-DR⁺ monocytes had the lowest percentage of HLA-DR⁺ cells (median: 73.36%) compared to classical HLA-DR⁺ (median: 97.73%) and intermediate HLA-DR⁺ cells (median: 96.77%) ($p < 0.0001$) (Fig. 4, Table 4).

In addition, the study showed that the percentage of subpopulations of classical, intermediate and non-classical monocytes expressing CD163 does not correlate with leukocytosis, lymphocytosis and serum $\beta 2$ microglobulin concentration ($p > 0.05$). The percentage of CD14⁺⁺CD16⁻CD163⁺ monocytes showed a negative correlation with the concentration of CD5⁺CD19⁺ leukemia cells ($r = -0.51$; $p < 0.05$). According to the results, the percentage of CD14⁺HLA-DR^{low/neg} monocytes showed a negative correlation only with the percentage of CD14⁺⁺CD16⁻CD163⁺ monocytes

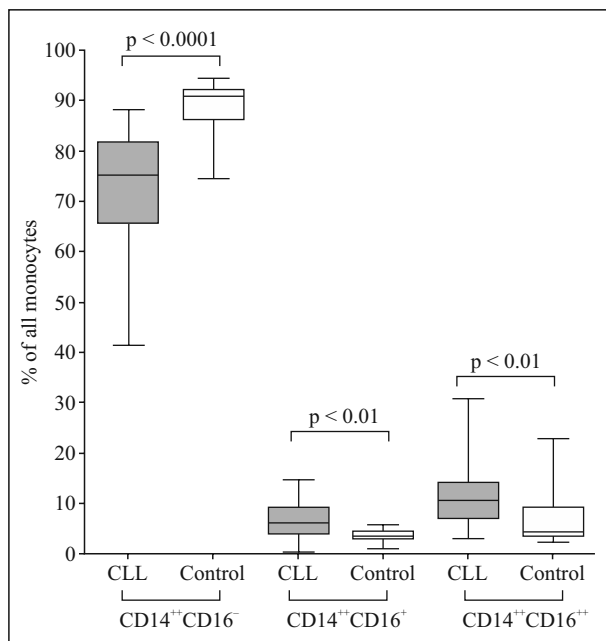


Figure 1. Comparison of the percentage of individual monocyte subpopulations: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺) among all monocytes in CLL patients and healthy volunteers studied by flow cytometry as described in Methods. *P* values were calculated using the non-parametric U Mann-Whitney test. Bars, line and whiskers represent median, maximum, minimum and IQR, respectively. Abbreviations: CLL — chronic lymphocytic leukemia; IQR — interquartile range.

among all subpopulations of monocytes ($r = -0.55$; $p < 0.05$).

Discussion

Among the populations of immune cells, monocytes are one of the least known cells with potentially important role in the pathogenesis of CLL [18]. Since monocytes appear to play an important role in the pathogenesis of CLL, the present study presents the

Table 2. Statistical analysis of the percentage of monocytes with the phenotypes CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ in CLL patients and in the control group

| Variables | CD14 ⁺⁺ CD16 ⁻ (%) | | CD14 ⁺⁺ CD16 ⁺ (%) | | CD14 ⁺ CD16 ⁺⁺ (%) | |
|-----------|--|---------|--|---------|--|---------|
| | CLL | Control | CLL | Control | CLL | Control |
| Median | 75.56 | 91.01 | 6.34 | 3.17 | 10.65 | 4.55 |
| Min | 41.42 | 74.66 | 0.24 | 1.14 | 3.03 | 2.25 |
| Max | 88.35 | 94.54 | 14.58 | 5.68 | 30.70 | 22.75 |
| IQR | 16.29 | 6.18 | 5.54 | 1.78 | 7.59 | 5.89 |

Abbreviations: CLL — chronic lymphocytic leukemia; MIN — minimum; MAX — maximum; IQR — interquartile range

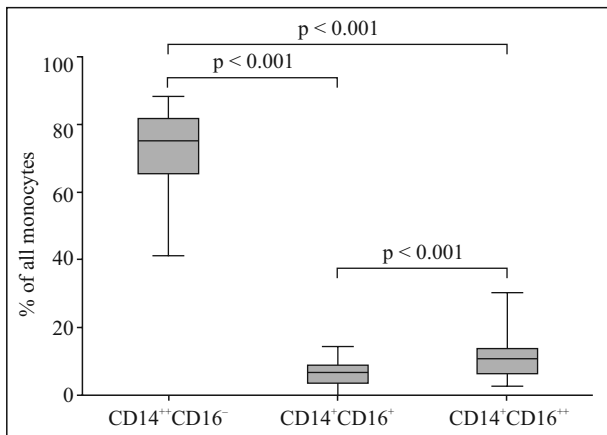


Figure 2. Comparison of the percentage of individual monocyte subpopulations among all monocytes in CLL patients was performed as described in the Legend to Figure 1. *P* values were calculated using the non-parametric Wilcoxon test. Bars, other graphical symbols and abbreviations as in the description of Figure 1.

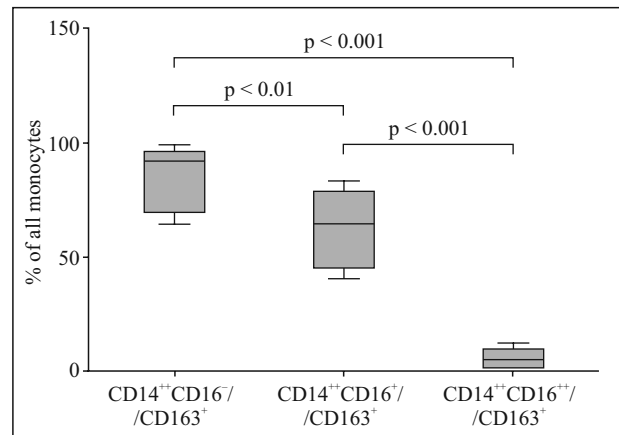


Figure 3. Comparison of the percentage of monocyte subpopulations expressing CD163 in CLL patients was performed as described in the Legend to Figure 1. *P* values were calculated using the non-parametric Wilcoxon test. Bars, other graphical symbols and abbreviations as in the description of Figure 1.

Table 3. Statistical analysis of the percentage of monocytes with the phenotypes CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ expressing the CD163 molecule in the studied group of CLL patients

| Variables | CD14 ⁺⁺ CD16 ⁻ /CD163 ⁺ (%) | CD14 ⁺⁺ CD16 ⁺ /CD163 ⁺ (%) | CD14 ⁺ CD16 ⁺⁺ /CD163 ⁺ (%) |
|-----------|--|--|--|
| Median | 96.73 | 63.70 | 5.12 |
| Min | 62.10 | 44.87 | 0.30 |
| Max | 100.0 | 78.64 | 12.99 |
| IQR | 26.88 | 29.98 | 9.13 |

Abbreviations as in the description of Table 2.

evaluation of the proportion of the monocytes subpopulation in CLL. Although the discovery of three different subpopulations of monocytes suggests the existence of the functional differences between these cells their properties are still not fully understood. Monocytes analyzed in CLL patients were divided into classical monocytes (CD14⁺⁺CD16⁻) and monocytes expressing CD16 molecules, which are differentiated into two subpopulations: the intermediate monocytes CD14⁺⁺CD16⁺ and the non-classical monocytes CD14⁺CD16⁺⁺ [6].

It is assumed that the increased expression of CD16 on the surface of monocytes may correlate with their activation process [8]. An increase in the percentage of CD16-positive monocytes observed in the present study may indicate that CLL lymphocytes stimulate CD16⁺ monocytes and lead to their activation. This is in line with Maffei *et al.* [19], who concluded that the increase in the percentage of non-classical monocytes may result from their stimulation by leukemic lymphocytes. According to the latter study, the CD14⁺CD16⁺⁺

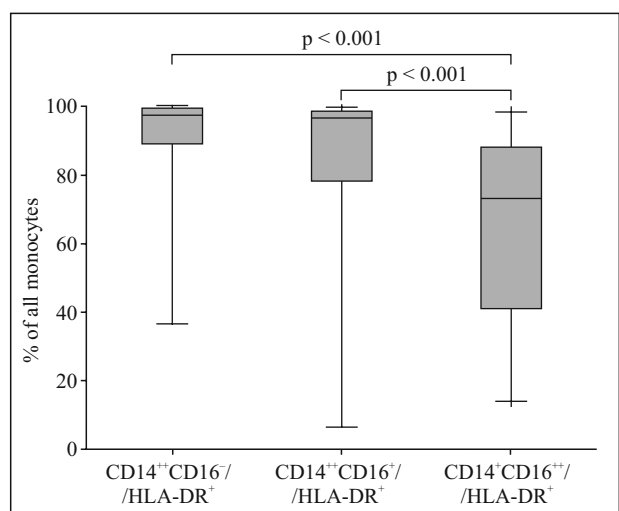


Figure 4. Evaluation of the percentage of individual HLA-DR positive monocyte subsets in patients with CLL was performed as described in the Legend to Figure 1. *P* values were calculated using the non-parametric Wilcoxon test. Bars, other graphical symbols, and abbreviations as in the description of Figure 1.

Table 4. Comparison of the percentage of classical, intermediate and non-classical monocytes with HLA-DR expression in CLL patients

| Variables | CD14 ⁺⁺ CD16 ⁻ /HLA-DR ⁺ (%) | CD14 ⁺⁺ CD16 ⁺ /HLA-DR ⁺ (%) | CD14 ⁺ CD16 ⁺⁺ /HLA-DR ⁺ (%) |
|-----------|---|---|---|
| MEDIAN | 97.73 | 96.77 | 73.36 |
| MIN | 36.47 | 6.270 | 14.38 |
| MAX | 100.0 | 100.0 | 100.0 |
| IQR | 10.22 | 20.45 | 47.37 |

Abbreviations as in the description of Table 2.

monocytes do not produce cytokines in response to LPS and are unable to phagocytose [19].

In this study we observed that patients with CLL possess a higher percentage of non-classical monocytes CD14⁺CD16⁺⁺ and a significantly higher percentage of intermediate monocytes CD14⁺⁺CD16⁺ as compared to the healthy group. Italiani *et al.* [5] also showed a similar percentage of monocytes circulating in the peripheral blood of CLL patients, classical monocytes (85%), intermediates (5%) and non-classical (10%) [5].

It is worth mentioning that the increase in the percentage of monocytes expressing CD16 was also observed in other pathological conditions, *e.g.* during bacterial, viral or parasitic infections [20] and in acute or chronic conditions such as sepsis or atherosclerosis [8], chronic liver disease [21], rheumatoid arthritis [22], and renal diseases [23, 24].

The role of monocytes expressing CD16 during the mobilization of the immune system is not fully understood. One theory explains the differentiation of monocytes by the process of maturation and differentiation of these cells into the peripheral blood macrophages [8]. The differences in the percentage of each subpopulation of monocytes observed in CLL patients, compared to the proportion observed in healthy subjects may be due to the fact that classical monocytes may differentiate into tissue macrophages TAMs (Tumor Associated Macrophages) [25, 26].

The differences relate to, among others, the degree of expression of HLA-DR, CD86 and CD1d, which determine their ability to present antigens [8]. Furthermore, the CD14⁺⁺CD16⁺ intermediate monocytes have a high expression of CD163 molecule [14] that inhibits activation and proliferation of T cells [27]. It is also a specific marker of monocytes/macrophages exhibiting strong anti-inflammatory properties [28]. Recent studies have shown that anti-inflammatory monocytes CD14⁺⁺CD16⁺ are also associated with significant expression of TGF- β . This cytokine enables them to inhibit T-dependent response, and to stimulate proliferation of T regulatory cells [1].

Of all subpopulations, classical monocytes accounted for the highest percentage of CD163⁺ monocytes, while non-classical monocytes had the lowest percentage of CD163⁺ cells. Moniuszko *et al.* [9] also observed high expression of the CD163 molecule on classical monocytes. The percentage of CD14⁺⁺CD16⁺CD163⁺ monocytes was higher than the percentage of non-classical CD163⁺ monocytes. It is believed that the presence of CD163 on the surface of monocytes affects the ability of these cells to inhibit the inflammatory response [8] as the CD163⁺ monocytes may have anti-inflammatory properties [29]. Therefore, it is assumed that due to the disturbed antigen presentation process and abnormal maturation of dendritic cells, the activity of the immune system in patients with CLL decreases [6, 19]. Moreover, monocytes have reduced ability to stimulate the immune system, due to impaired antigen presentation to T cells [30]. Furthermore, the researchers suggest that a subpopulation of monocytes characterized by increased percentage of CD163-positive cells present in inflamed or tumor tissues can polarize to macrophages M2, in particular subtype M2d called TAM [31–33]. It is worth recalling that the presence of macrophages with the expression of CD163 in the tumor microenvironment in patients with breast cancer is unfavorable prognostic factor. Results of a recent Polish study suggest the positive correlation between the presence of CD163 macrophage and tumor size [34]. Perhaps the classical or intermediate CD163-positive monocytes differentiate into TAMs. Our studies also assessed the expression of HLA-DR on monocytes in CLL patients. The analysis showed that the percentage of cells expressing HLA-DR among classical monocytes (CD14⁺⁺CD16⁻) is significantly higher compared with the percentage of non-classical CD14⁺CD16⁺⁺HLA-DR⁺. Additionally, the percentage of CD14⁺⁺CD16⁺HLA-DR⁺ monocytes among intermediate monocytes was significantly higher than the percentage of CD14⁺CD16⁺⁺HLA-DR⁺. The majority of non-classical monocytes exhibit only low expression of HLA-DR (HLA-DR^{-low}).

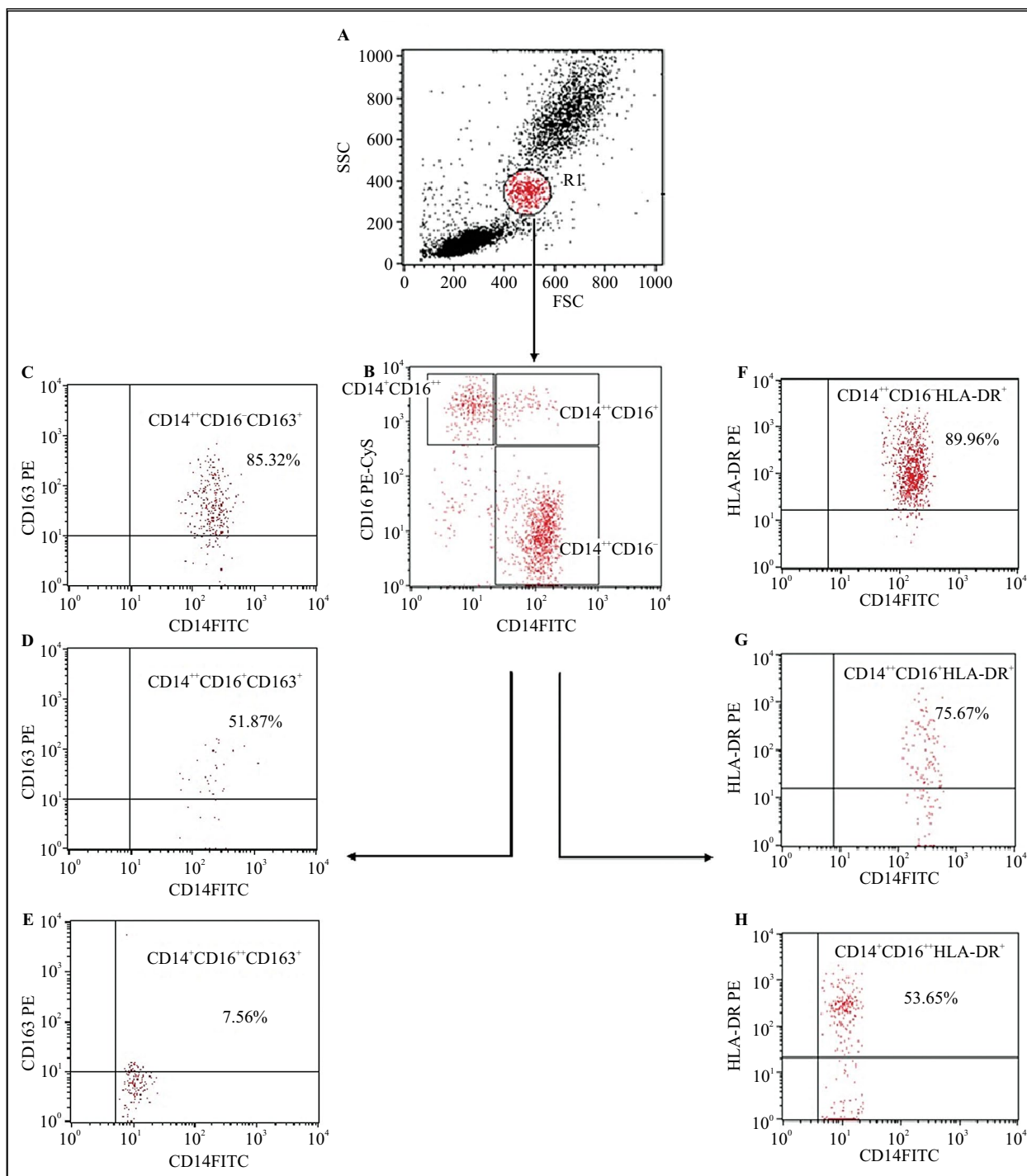


Figure 5. The dot plots of representative data from one chronic lymphocytic leukemia patient illustrate the analysis method for the identification of monocyte subpopulations in peripheral blood following three-color staining. (A) Monocyte population was gated (Region R1) using FSC and SSC plot. (B) Next, the monocytes accumulated in the R1 were analyzed for staining of monocyte subpopulations. We used dot plots of CD14 FITC versus CD16 PE-Cy5. The dot plot shows classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺) monocytes. Expression of CD163 (C, D, E) and HLA-DR (F, G, H) was assessed in each of these subpopulations. Abbreviations: FSC — forward scatter; SSC — side scatter.

It suggests that these cells have a reduced ability to stimulate the immune system, due to disturbed presentation of antigens to T cells [30]. The increased

number of CD14⁺HLA-DR^{low/neg} cells in the peripheral blood is present in many cancers and is related to the stage, grade, gender and tumor size [35]. Gustafson

et al. [10] observed an increased percentage of HLA-DR^{low} monocytes in patients with CLL compared to the control group (18.6% ± 11.5% vs. 9.9% ± 6.4%). The CD14+ HLA-DR^{low/neg} monocytes secrete large amounts of IL-10 and TGF- β that stimulate regulatory T cells (Treg) proliferation and cause an alteration in the maturation of dendritic cells [10]. Moreover, an increased percentage of monocytes with low HLA-DR expression was noted in patients with acute hepatic failure, which may result in higher susceptibility to infection due to the significantly reduced immune function [21]. Monocytes exhibiting high expression of CD163 and low expression of HLA-DR may be described as a subpopulation with anti-inflammatory properties and the ability to inhibit the anti-tumor response [30, 36].

Conclusions

A statistically significant increase in the percentage of intermediate and non-classical monocytes was found in CLL patients in comparison to the control group. The highest percentage of CD163+ cells was observed in “classical” monocytes. The presence of this receptor suggests that these cells have anti-inflammatory properties. In addition, in CLL patients the highest percentage of HLA-DR^{neg/low} cells expression was observed among non-classical monocytes, which means that these cells have probably impaired ability to stimulate the immune system.

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