FEATURES OF CD163+ AND HLA-DR+ EXPRESSION ON BLOOD MONOCYTES ASSOCIATED WITH BREAST CANCER

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Circulating monocytes are important actors of the tumor growth pathogenesis. It has been shown that some features of the monocyte populations expressing endocytosis receptors or the major histocompatibility complex components are observed in blood of patients with breast cancer. The study was aimed to assess the relationship of tumor parameters and blood cytokine profile with the composition of circulating monocyte populations in patients with localized and locally advanced breast cancer. The study has shown that the circulating monocytes' phenotypic characteristics are correlated to the clinical and morphological features of the neoplastic process. The content of populations with the CD14+CD16++CD163+ and CD14++CD16+CD163+ phenotypes positively correlates with the disease stage, while larger primary tumor size is associated with lower CD14+CD16++ monocyte levels. Elevated serum levels of IL8 and MCP-1 are observed in breast cancer patients. High IL6 levels in patients with breast cancer are associated with the reduced percentage of CD14++CD16-HLA-DR+, CD14+CD16++HLA-DR+ and CD14++CD16-CD163+ monocytes. Thus, CD163+ and HLA-DR+ monocytes are associated with the clinical and morphological parameters and blood levels of cytokines, which suggests that these populations are involved in breast cancer progression and indicates that further research is advisable for translation of the findings into clinical practice.

Keywords: monocytes, CD163, HLA-DR, breast cancer, IL6, IL8, MCP-1

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ОСОБЕННОСТИ ЭКСПРЕССИИ CD163+ И HLA-DR+ НА МОНОЦИТАХ КРОВИ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Циркулирующие моноциты — значимые участники патогенеза опухолевого роста. Показано, что в крови больных раком молочной железы наблюдаются особенности популяций моноцитов, экспрессирующих рецепторы эндоцитоза, либо компонентов главного комплекса гистосовместимости. Целью данной работы было проведение анализа взаимосвязи параметров опухоли и цитокинового профиля крови с популяционным составом циркулирующих моноцитов больных локализованными и местно-распространенными формами рака молочной железы. В исследовании было показано, что фенотипические характеристики циркулирующих моноцитов взаимосвязаны с клинико-морфологическими особенностями опухолевого процесса. Содержание популяций с фенотипом CD14+CD16++CD163+ и CD14++CD16+CD163+ имело положительную корреляцию со стадией заболевания, в то время как больший размер первичного опухолевого узла ассоциирован с более низким содержанием CD14+CD16++-моноцитов. У больных РМЖ увеличено содержание IL8 и MCP-1 в сыворотке крови. Высокий уровень содержания IL6 у больных РМЖ ассоциирован со снижением доли CD14++CD16-HLA-DR+-моноцитов, CD14+CD16++HLA-DR+-моноцитов и CD14++CD16-CD163+-моноцитов. Таким образом, CD163+ и HLA-DR+-моноциты связаны с клинико-морфологическими параметрами и уровнем цитокинов крови, что свидетельствует о вовлечении данных популяций в прогрессию рака молочной железы и говорит о целесообразности дальнейших исследований для трансляции полученных результатов в клиническую практику.

Ключевые слова: моноциты, CD163, HLA-DR, рак молочной железы, интерлейкин 8, интерлейкин 6, фактор миграции моноцитов

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Monocytes are the most important effectors of nonspecific immunity involved in numerous disease processes associated with chronic inflammation. The malignant neoplasm emergence and development is one of such processes characterized by monocyte involvement in all phases of tumor pathogenesis. To date, it is known that the monocyte pool consists of three major populations: classical monocytes with the $CD14^{++}CD16^{-}$ phenotype, non-classical monocytes with the $CD14^{+}CD16^{++}$ phenotype, and intermediate $CD14^{++}CD16^{+}$ monocytes [1]. Furthermore, $CD14^{++}CD16^{-}$ monocytes are the source to replenish the pool of tumor-associated macrophages, dendrite cells and myeloid-derived suppressor cells in the tumor tissue [2]. The capability of maintaining endothelial integrity and absorbing foreign particles is typical for $CD14^{+}CD16^{++}$ cells [2, 3]. The role of intermediate population is poorly understood, however, it is assumed that this population occupies an intermediate position between the other two populations [4].

In addition to division into three major populations, monocytes are distinguished by expression of protein receptors related to certain cell functions. Thus, the monocyte capability of endocytosis involving the haptoglobin-hemoglobin complex uptake via CD163 receptor reflects the capability of absorbing particles and is enhanced under monocyte exposure to mediators reducing pro-inflammatory activity of such cells [5]. It is also known that cells of the macrophage-monocyte lineage possess functional plasticity and express the receptors related to the antigen-presenting function of monocytes and macrophages [6]. The increase in the counts of monocytes showing low HLA-DR expression is considered to be associated with immunosuppression observed in individuals with cancer and infectious diseases [7]. Thus, it is worthwhile to investigate the population structure of monocytes not only based on markers of the classical, intermediate, and non-classical population, but also considering additional functional markers.

The dynamics of malignant neoplasm development are considered to be closely related to two major factors: individual characteristics of the organism and biological properties of the tumor. In particular, it has been shown that the age of BC manifestation, genetic predisposition, and the presence of germline mutations can determine the clinical course of the disease in the patient [8]. Since biological processes in breast tissue largely depend on the woman's hormonal health, BC is often associated with impaired sex hormone regulation and, therefore, is correlated to menstrual status [9]. Furthermore, it has been confirmed that endocrine disorders and body's metabolic status are associated with production of biological factors, cytokines and chemokines, regulating the immune cell functional activity, by the tumor [10]. Body mass index (BMI), one of the metabolic status criteria, can be also associated with the course of BC [11]. The relationship between body weight increase and adult-onset BC has been demonstrated, where the increase by 5 kg/m^2 corresponds to the increase in the risk of breast cancer by 2% in women [11, 12].

In addition to the patient's individual characteristics, clinical and morphological parameters, such as primary tumor size, regional neoplastic process extension and tumor molecular subtype, are important factors associated with the BC clinical course [13, 14].

Cytokines and the major immune response regulators [15]. The circulating blood cytokine profile reflects body's systemic homeostasis [15]. In individuals with malignant neoplasms, the tumor itself can contribute to the body's abnormal homeostasis by affecting the immune system components [15]. It has been found that monocyte programming under exposure to interleukin 6 (IL6) and interleukin 8 (IL8) is an essential step of inducing their inflammatory phenotype [16]. Migration of monocytes from the bone marrow or spleen being the site of monocyte deposition is controlled by the major monocyte migration factor (MCP-1 or CCL2). Identification of the association of monocyte population structure with the disease stage and the patients' individual characteristics makes it possible to describe monocyte involvement in BC pathogenesis.

Given the fact that cells of the macrophage-monocyte lineage are important for BC development, the study was aimed to assess the association of $CD163^{+}$ and $HLA\text{-}DR^{+}$ monocyte counts with clinical and morphological characteristics of the disease and blood cytokine profile in individuals with BC.

METHODS

A total of 50 patients with stage I–III primary breast cancer, T1-3N0-3M0, aged 52.0 [46.0–63.0] years were enrolled. The diagnosis was confirmed by morphological assessment. The histologic tumor type corresponded to invasive carcinoma of no special type in all cases. A conventional panel of ERα, PR and HER2 immunohistochemical markers was used for breast tumor classification in accordance with molecular subtypes. The clinical and anamnestic data were acquired by analysis of the patients' medical history and outpatient charts. The characteristics of patients enrolled are provided in Table 1. BMI was calculated for each patient according to the following formula: square of the ratio of body weight (kg) to body length (m). Exclusion criteria were as follows: patients having multiple primary malignant tumors, history of cancer of other localization, previous breast surgery.

The control group included healthy women (average age 61.0 [50.0–69.0] years). Exclusion criteria were as follows: history of cancer, exacerbation of chronic disorder.

Determining the tumor molecular subtype

Expression of estrogen receptors (ER), progesterone receptors (PR), HER2/neu status (HER2), proliferative activity (Ki-67 expression) were estimated to determine the BC molecular subtype by immunohistochemistry performed using the standard method. Antibodies (Dako; Denmark) against estrogen receptors (clone 1D5, RTU, mouse), progesterone receptors (clone PgR636, RTU, mouse), c-erbB-2 protein (HER2/neu, working dilution 1:500, rabbit), Ki-67 (clone MIB-1, RTU, mouse) were used. Expression of the sex hormone receptors was assessed by quantitative Histo-Score method. Ki-67 expression was estimated as a percentage of positively stained cells of invasive breast carcinoma of no special type (in 10 fields of view per 1000 cells at 400x magnification). Molecular DC subtypes were determined based on the combination of estrogen and progesterone receptor expression, HER2/neu status and Ki-67: luminal B HER2– (ER+ and/or PR+, HER2– and Ki-67 ≥ 20%), luminal B HER2+ (ER+ and/or PR+, HER2+), triple negative (ER–, PR– and HER2–), and HER2/neu-overexpressing subtype (ER–, PR–, $HER2^{+}$).

Peripheral blood monocyte phenotyping

The $CD163^{+}$ (a scavenger receptor) and $HLA\text{-}DR^{+}$ expression on monocytes of classical $CD14^{++}CD16^{-}$, non-classical $CD14^{+}CD16^{++}$ and intermediate $CD14^{++}CD16^{+}$ populations in blood of patients of the BC group was assessed. For that venous blood was collected from patients and donors into the K3-EDTA vacuum blood sampling systems. A total of 100 μL

Table 1. Clinical and morphological parameters of patients with breast cancer

Clinical and pathological parameters	N (%) (n = 50)
Age	59.3 ± 10.4
BMI	27.2 ± 5.54
Menstrual status:	
preserved	24 (48.2)
menopause	26 (51.8)
Stage	
I	7 (14.0)
II	23 (46)
III	20 (40)
Molecular subtype	
Luminal B	25 (50.0)
Triple-negative subtype	19 (38.0)
HER2-positive	6 (12)
Lymph node metastasis	
present	22 (46.5)
absent	28 (53.5)

of white blood cell fraction were collected to determine the population structure of blood monocytes. Then the cells were suspended in 150 µL of the staining buffer (Cell Staining Buffer, Sony; Japan). Cells were counted with the Luna II cell counter (Logos Biosystems, Inc.; Korea). After that the cell concentrate was added 5 µL of Human TruStain FcX™ (Biolegend; USA) to block nonspecific binding and incubated for 10 min. Then it was stained using the set of labeled antibodies against markers CD45, CD14, CD16, CD163 and HLA-DR. The following antibodies were used in the study: CD45–APC-Cy7 (BD Bioscience, catalogue number 557833; USA), CD56–PE-Cy7 (eBioscience, Thermo Fisher Scientific; USA), CD14–FITC (BD Bioscience, catalogue number 345784; USA), CD16 –APC (BD Bioscience, USA, catalogue number 561248), CD163–PE (BD Bioscience, USA, catalogue number 556018), HLA-DR–PE-Cy5 (BD Bioscience, catalogue number 555813; USA), Isotype PE-Cy™5 (BD Bioscience, catalogue number 555750; USA), Isotype PE (BD Bioscience, catalogue number 556018; USA). Cell viability was assessed using the 7-AAD nucleic acid dye (BD Bioscience; USA). Staining was performed by adding antibodies to a ratio of 5 µL per 10^6 cells. The samples supplemented with the same concentration of appropriate isotopic control were used as negative controls. These samples were incubated at room temperature in the dark for 20 min. Then each stained or unstained sample was added 900 µL of VersaLyse Lysing Solution (Beckman Coulter; USA). Samples were analyzed in the NovoCyte cytometer (ACEA Bioscience; USA). The gating tactics is provided in Fig. 1. The data obtained were processed using the NovoExpress SoftWare (Acea; USA).

Assessment of serum cytokine levels

Serum cytokine levels were assessed using the Bio-Plex multiplex magnetic bead immunoassays (Bio-Rad, Hercules, CA; USA). The Human Milliplex MAG panel was used for analysis of three analytes: IL6, IL8 and MCP-1; 50 µL of serum from each case and control were analyzed using the Luminex 200 analyzer with the MasterPlex CT software for control and MasterPlex QT software for analysis (MiraiBio; USA). The calibration curves were plotted using the manufacturer's standards. Serum samples of patients with BC and healthy individuals were inactivated by heating prior to analysis. During the study we assessed the effects of heat inactivation on the cytokine stability; impossibility of normalization was the criterion for exclusion from the analysis.

Statistical data processing methods

Statistical processing was performed with the Statistica 8.0 software for Windows (StatSoft Inc.; USA). The pattern of the studied variables distribution was tested for normality using the Kolmogorov–Smirnov test. The numeric data obtained were presented in the following format based on the testing results: Me [LQu–UQu]. The Mann–Whitney U test was used to determine significant differences in independent groups. The correlation analysis involved calculation of the Spearman's rank correlation coefficient. The results for serum cytokine concentrations were presented in the GraphPad Prism 8 SoftWare (GraphPad Soft Inc; USA). In all tests, significant differences were reported at the following significance levels: $p < 0.001$, $p < 0.01$ and $p < 0.05$.

RESULTS

In the studied group, the patients' age was not associated with the blood monocyte population structure. At the same time, a moderate correlation between BMI and the percentage of $CD14^{++}CD16^{+}HLA\text{-}DR^{+}$ cells with the correlation coefficient of 0.409, $p \leq 0.05$, was reported (Table 2).

It is well-known that preservation or loss of menstrual function in individuals with BC is directly related to the patients' hormonal status and body's homeostasis. The levels of $CD14^{++}CD16^{+}CD163^{+}$ monocytes were slightly decreased in women with preserved menstrual function compared to the menopausal/postenopausal group: 96.01 [83.29–98.46]% vs. 99.49 [89.79–100.00]% at the trend level, $p = 0.056$.

The population structure of monocytes in affected individuals was correlated to the tumor clinical and morphological characteristics. The increase in the neoplastic process clinical stage positively correlated with the levels of classical $CD14^{++}CD16^{-}CD163^{+}$ cells (moderate correlation), while weak correlation was reported for the intermediate $CD14^{++}CD16^{+}CD163^{+}$ population (r = 0.52 and 0.356, respectively, $p < 0.05$). In contrast, T (tumor size) showed a weak negative correlation with the content of cells of the non-classical $CD14^{+}CD16^{++}$ population (r = –0.389 and $p < 0.05$). As for N parameter reflecting the regional lymph node metastasis, no significant correlations with the characteristics of the phenotype pattern of blood monocytes (Table 2).

When comparing two groups of patients with the tumor size of T1–2 and T3–4, it was found that the $CD14^{-}CD16^{+}$ monocyte counts significantly decreased with increasing tumor size (Table 3).

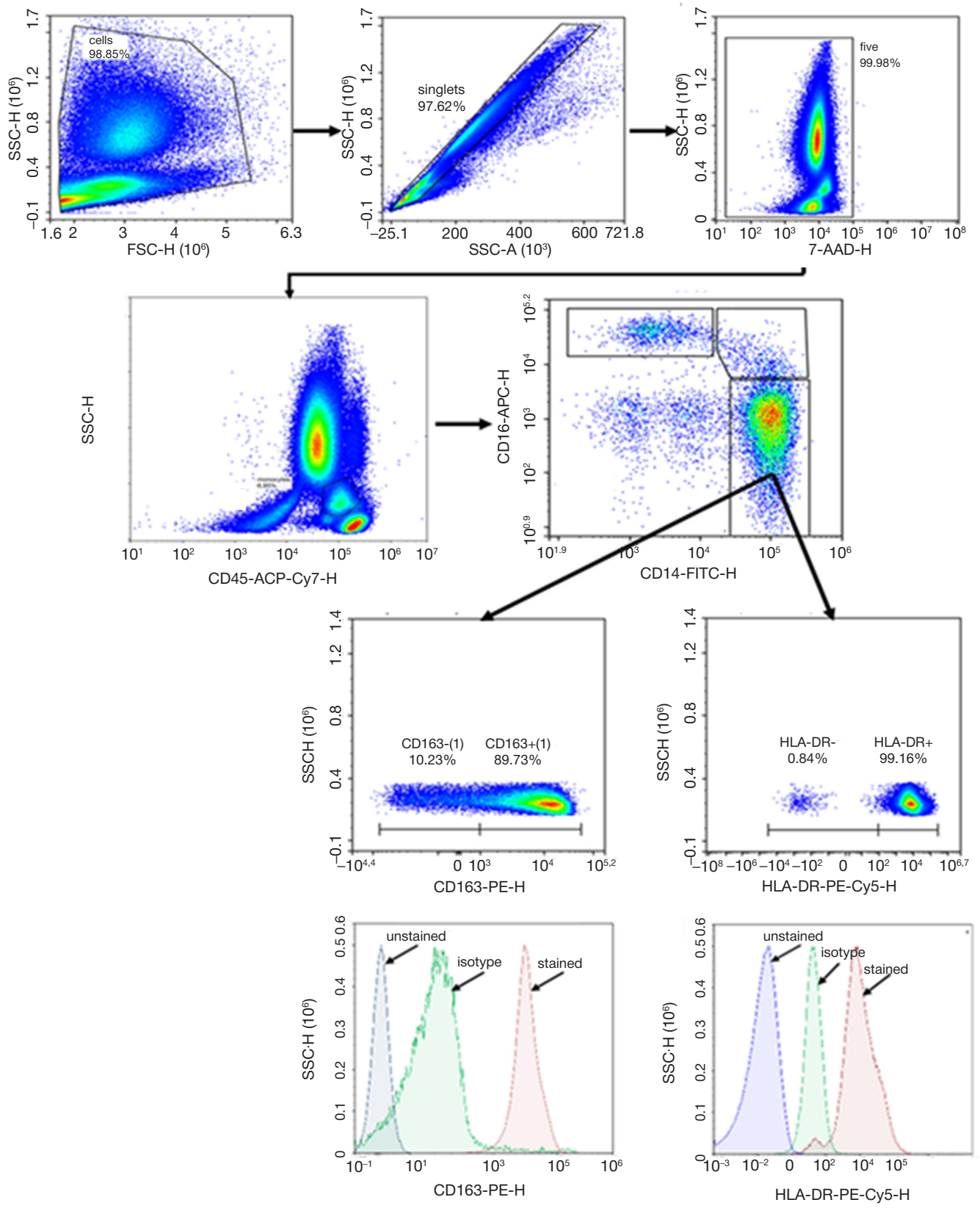

Fig. 1. Cytometry gating strategy for identification of monocyte subpopulations

At the same time, the $CD14^{+}CD16^{++}CD163^{+}$ monocyte counts decreased at the trend level from 98.77 [77.94–99.97]% at T1–2 to 69.81 [41.36–99.1]% at T3–4 (p = 0.09). The monocyte population structure was also associated with the degree of regional lymph node involvement. The content of $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ reported for patients with N0-1 was higher than that reported for the group with N2–3 by more than 4%. The disease stage as an integral indicator of the neoplastic process extent was associated with the monocyte distribution across the major populations. We compared the group of patients with the neoplastic process showing no regional lymph node involvement (cancer stage I–IIA) with the group showing tumor extension to the regional lymph nodes (stage IIB–IIIC). Thus, in the group of patients with stage I–IIA cancer, the share

Table 2. Correlation of individual features and tumor clinical and morphological parameters with the monocyte subpopulations in patients with BC

	Age	BMI	Stage	T	N
$CD14^+16^-$, %	$r = 0.135$ $p \geq 0.05$	$r = -0.073$ $p \geq 0.05$	$r = 0.228$ $p \geq 0.05$	$r = 0.206$ $p \geq 0.05$	$r = 0.149$ $p \geq 0.05$
$CD14^-16^+$, %	$r = -0.123$ $p \geq 0.05$	$r = 0.120$ $p \geq 0.05$	$r = -0.087$ $p \geq 0.05$	**$r = -0.389$ $p \leq 0.05$**	$r = -0.129$ $p \geq 0.05$
$CD14^+16^+$, %	$r = -0.122$ $p \geq 0.05$	$r = -0.043$ $p \geq 0.05$	$r = -0.028$ $p \geq 0.05$	$r = -0.026$ $p \geq 0.05$	$r = -0.036$ $p \geq 0.05$
$CD14^+16^-163^+$, %	$r = 0.038$ $p \geq 0.05$	$r = -0.017$ $p \geq 0.05$	**$r = 0.521$ $p \leq 0.05$**	$r = -0.082$ $p \geq 0.05$	$r = 0.198$ $p \geq 0.05$
$CD14^+16^-HLA\text{-}DR^+$, %	$r = -0.127$ $p \geq 0.05$	$r = 0.211$ $p \geq 0.05$	$r = 0.301$ $p \geq 0.05$	$r = -0.114$ $p \geq 0.05$	$r = -0.196$ $p \geq 0.05$
$CD14^-16^+163^+$, %	$r = 0.027$ $p \geq 0.05$	$r = -0.061$ $p \geq 0.05$	$r = 0.153$ $p \geq 0.05$	$r = -0.272$ $p \geq 0.05$	$r = -0.021$ $p \geq 0.05$
$CD14^-16^+HLA\text{-}DR^+$, %	$r = 0.069$ $p \geq 0.05$	$r = 0.082$ $p \geq 0.05$	$r = 0.093$ $p \geq 0.05$	$r = 0.159$ $p \geq 0.05$	$r = -0.085$ $p \geq 0.05$
$CD14^+16^+163^+$, %	$r = -0.250$ $p \geq 0.05$	$r = 0.195$ $p \geq 0.05$	**$r = 0.356$ $p \leq 0.05$**	$r = -0.171$ $p \geq 0.05$	$r = 0.217$ $p \geq 0.05$
$CD14^+16^+HLA\text{-}DR^+$, %	$r = 0.082$ $p \geq 0.05$	**$r = -0.409$ $p \leq 0.05$**	$r = 0.091$ $p \geq 0.05$	$r = 0.106$ $p \geq 0.05$	$r = 0.079$ $p \geq 0.05$

Note: BMI — body mass index.

of $CD14^{++}CD16^-$ cells was 89.4 [83.48–94.18]%, while in the group with stage IIB-IIIC cancer it was 94.2 [92.4–96.69]%, $p = 0.05$. These indicators for monocytes with the CD14-$CD16^+$ phenotype were higher in individuals with stage I-IIA compared to individuals with stage IIB–IIIC. The levels of $CD14^{++}CD16^-$ $CD163^+$ cells in patients with stage I–IIA were also higher than that of patients with stage IIB-IIIC, $p = 0.03$.

Parameters of $CD163^+$ and $HLA\text{-}DR^+$ expression on various monocyte populations were associated with the tumor molecular subtypes (Table 4). Thus, patients with triple-negative subtype (TNBC) were distinguished from the group of patients with luminal B and $Her2^+$ subtypes by reduced $CD14^+CD16^{++}HLA\text{-}DR^+$ cell counts. $Her2^+$ subtype, in turn, was characterized by elevated levels of $CD14^{++}CD16\text{-}CD163^+$ and $CD14^{++}CD16^+CD163^+$ cells compared to the same indicators of the joint group of patients with luminal/TNBC subtype ($p < 0.05$). The $CD14^+CD16^{++}HLA\text{-}DR^+$ content was also significantly higher in patients with $Her2^+$ tumors than in patients with luminal B and TNBC subtypes ($p < 0.05$). The group of patients with luminal B tumor subtype was distinguished from patients with luminal B and $Her2^+$ subtypes only by the reduced $CD14^{++}CD16^+CD163^+$ monocyte levels associated with luminal B cancer ($p < 0.05$).

The circulating blood cytokine profile is among important characteristics of the body determining its system-level functioning. Initially, serum levels of IL6, IL8 and MCP-1 cytokines were assessed in patients and healthy women. The analysis of MCP-1, IL6 and IL8 levels in blood serum showed that the group with BC was distinguished by elevated MCP-1 levels, specifically 0.32 [0.07–0.41] μg/mL, relative to the values of healthy women, 0.14 [0.12–0.27] μg/mL, $p = 0.002$ (Fig. 2).

The serum IL8 level of patients with BC was 70.1 [25.6–300.0] pg/μL, this value exceeded the value reported for the group of healthy women, 46.0 [16.3–113.0] pg/μL, $p =$

Table 3. Association of the disease stage with phenotypic characteristics of monocytes in patients with BC

	T1–2 ($n = 29$)	T3–4 ($n = 21$)	N0–1	N2–3	Stage I–IIA	Stage IIB–IIIC
$CD14^+16^-$, %	92.2 [83.7–94.58]	94.2 [92.1–96.88]	92.85 [83.7–96.69]	93.21 [89.28–96.13]	89.4 [83.48–94.18]	94.2 [92.4–96.69]
	$p = 0.15$		$p = 0.55$		$p = 0.05$	
$CD14^-16^+$, %	4.63 [1.3–10.23]	2.01 [1.24–2.41]	2.56 [1.3–9.13]	4.1 [1.51–5.8]	4.7 [2.56–11.41]	2.01 [1.44–2.44]
	$p = 0.04$		$p = 0.6$		$p = 0.01$	
$CD14^+16^+$, %	2.42 [1.3–10.23]	1.5 [1.24–5.49]	2.01 [1.24–5.16]	2.05 [0.48–5.6]	2.42 [1.13–5.6]	2.01 [1.28–3.42]
	$p = 0.63$		$p = 0.79$		$p = 0.54$	
$CD14^+16^-163^+$, %	94.63 [89.23–99.67]	96.74 [76.9–99.73]	94.63 [89.23–99.85]	96.74 [55.35–99.67]	92.94 [89.12–98.34]	97.98 [94.9–99.99]
	$p = 0.92$		$p = 0.65$		$p = 0.03$	
$CD14^+16^-$ $HLA\text{-}DR^+$, %	99.26 [97.34–99.9]	99.01 [96.65–99.81]	99.6 [98.08–99.9]	95.09 [87.7–99.01]	99.26 [97.34–99.99]	99.26 [98.09–99.93]
	$p = 0.48$		$p = 0.02$		$p = 0.93$	
$CD14^-16^+163^+$, %	98.77 [77.94–99.97]	69.81 [41.36–99.1]	99.6 [98.08–99.9]	50.0 [25.6–99.21]	89.89 [65.41–99.29]	98.08 [50.0–99.86]
	$p = 0.09$		$p = 0.15$		$p = 0.64$	
$CD14^-16^+$ $HLA\text{-}DR^+$, %	68.92 [54.06–87.73]	79.32 [55.08–93.31]	96.87 [69.81–99.97]	60.03 [37.0–79.32]	69.58 [54.06–85.91]	76.15 [60.03–93.13]
	$p = 0.37$		$p = 0.55$		$p = 0.36$	
$CD14^+16^+163^+$, %	98.0 [89.79–99.9]	98.7 [77.86–99.81]	76.15 [55.12–93.31]	98.7 [31.27–99.81]	96.19 [83.79–99.9]	99.18 [94.58–99.9]
	$p = 0.44$		$p = 0.16$		$p = 0.23$	
$CD14^+16^+$ $HLA\text{-}DR^+$, %	93.04 [80.43–98.44]	96.88 [89.85–98.47]	95.24 [88–98.47]	84.51 [55.0–97.5]	93.04 [86.99–98.28]	96.88 [84.51–98.44]
	$p = 0.48$		$p = 0.74$		$p = 0.86$	

Table 4. Phenotypic characteristics of blood monocytes depending on the tumor molecular subtype

	TNBC	Lum B/Her2+	*p*
CD14+16-, %	89.28 [79.79–96.13]	93.21 [92.1–96.69]	0.09
CD14-16+, %	4.1 [1.04–11.41]	2.41 [1.44–5.45]	0.1
CD14+16+, %	2.05 [1.39–6.43]	2.01 [1.13–3.89]	0.31
CD14+16-163+, %	94.63 [89.15–99.67]	95.34 [90.87–99.85]	0.76
CD14+16-HLA-DR+, %	99.26 [90.5–100.00]	99.26 [98.08–99.9]	0.23
CD14-16+163+, %	84.28 [50.00–99.29]	98.77 [53.47–100.0]	0.15
CD14-16+HLA-DR+, %	62.6 [53.4–84.06]	84.51 [60.03–96.33]	0.02
CD14+16+163+, %	98.06 [87.6–100.0]	98.00 [83.79–99.97]	0.19
CD14+16+HLA-DR+, %	95.67 [80.43–99.03]	93.14 [84.51–98.28]	0.54
	Her2+	Lum B/TNBC	
CD14+16-, %	94.2 [92.1–96.88]	92.4 [83.7–96.13]	0.63
CD14-16+, %	2.01 [0.15–4.7]	3.8 [1.51–9.13]	0.09
CD14+16+, %	1.28 [1.00–5.49]	2.05 [1.33–5.16]	0.08
CD14+16-163+, %	**99.67 [99.48–100.00]**	**94.3 [89.12–98.34]**	**0.04**
CD14+16-HLA-DR+, %	99.6 [99.26–99.00]	99.01 [96.65–99.99]	0.33
CD14-16+163+, %	99.86 [69.81–100.00]	91.28 [50.00–99.29]	0.62
CD14-16+HLA-DR+, %	**93.31 [60.03–98.81]**	**68.92 [54.06–85.91]**	**0.03**
CD14+16+163+, %	**100.00 [98.86–100.00]**	**96.19 [82.79–99.81]**	**0.02**
CD14+16+HLA-DR+, %	96.88 [80.43–99.71]	93.14 [84.51–98.44]	0.08
	Lum B	TNBC/Her2+	
CD14+16-, %	93.09 [88.64–94.58]	92.1 [86.7–96.13]	0.24
CD14-16+, %	2.56 [1.69–5.8]	3.65 [1.04–8.3]	0.55
CD14+16+, %	2.01 [1.24–3.3]	2.05 [1.28–5.6]	0.86
CD14+16-163+, %	94.3 [89.08–96.74]	98.34 [90.74–99.73]	0.09
CD14+16-HLA-DR+, %	99.01 [97.34– 99.93]	99.6 [98.09–99.99]	0.71
CD14-16+163+, %	98.08 [53.47–99.78]	89.89 [65.4–99.86]	0.65
CD14-16+HLA-DR+, %	76.15 [55.12–93.01]	69.58 [55.08–90.5]	0.09
CD14+16+163+, %	**95.83 [82.79–98.1]**	**99.81 [92.79–100.00]**	**0.04**
CD14+16+HLA-DR+, %	93.04 [84.51–98.06]	96.79 [86.99–99.03]	0.35

Note: TNBC — triple-negative breast cancer, Lum B — luminal B breast cancer, Her2+ –Her2neu-positive breast cancer

0.01. In contrast, blood levels of IL6 in patients were reduced from that of healthy individuals (23.3 [17.2–30.4] vs. 28.3 [24.3–33.4] pg/μL), but just at the trend level with $p = 0.06$.

The group of patients was divided into two subgroups with low (< Me) and high (≥ Me) biological factor levels in order to assess the nature of the relationship between serum MCP-1, IL8, IL6 levels and phenotypic features of monocytes associated with BC. No significant differences were revealed for MCP-1 and IL8, however, serum levels of IL6 were correlated to the monocyte phenotype. High IL6 levels were associated with the reduced share of CD14++CD16-CD163+, CD14++CD16-HLA-DR+ and CD14+CD16++HLA-DR+ monocytes, these were 91.29 [75.03–97.89]% vs. 99,12 [94.3–100.0]%, 98.09 [95,65–99,43]% vs. 99.93 [99.34–100.0]% and 48.06 [32.1–78.3]% vs. 93,01 [75.41–98.81]%, respectively. Thus, reduced HLA-DR expression on monocytes was typical for individuals with the decreased serum IL6 levels (Fig. 2B).

DISCUSSION

The neoplastic process extension (tumor size increase, regional lymph node or other organ metastasis) is a criterion of cancer progression. In our study the counts of CD14+CD16++ monocytes belonging to the non-classical population negatively correlated with the tumor size (Table 2). When assessing the association between the population structure and the stage we noted that a more advanced disease stage was characterized by lower CD14+CD16++ cell counts. An opposite trend was reported for the CD14++CD16- population predominating in blood: the CD14++CD16- counts increased with increasing disease stage (Table 3). Thus, the levels of CD163+ and HLA-DR+ monocytes were associated with a number of BC progression key factors, specifically with the disease stage and the presence of regional lymph node metastasis. Noteworthy is the fact that the levels of CD14+CD16++ cells potentially possessing antitumor activity are reduced. Thus, non-classical monocytes demonstrate the ability to suppress metastasis in murine models of lung cancer [17]. At the same time, the counts of CD14+CD16- cells being the source for replenishment of the tumor-associated macrophage pool increase [2, 4].

The CD14++CD16-HLA-DR+ monocyte population was associated with the regional lymph node involvement in the neoplastic process (Table 3). It is well-known that the CD14++CD16- population is recruited to perform the function of antigen-presenting cells in inflammatory response [18]. In individuals with BC, the regional lymph node involvement

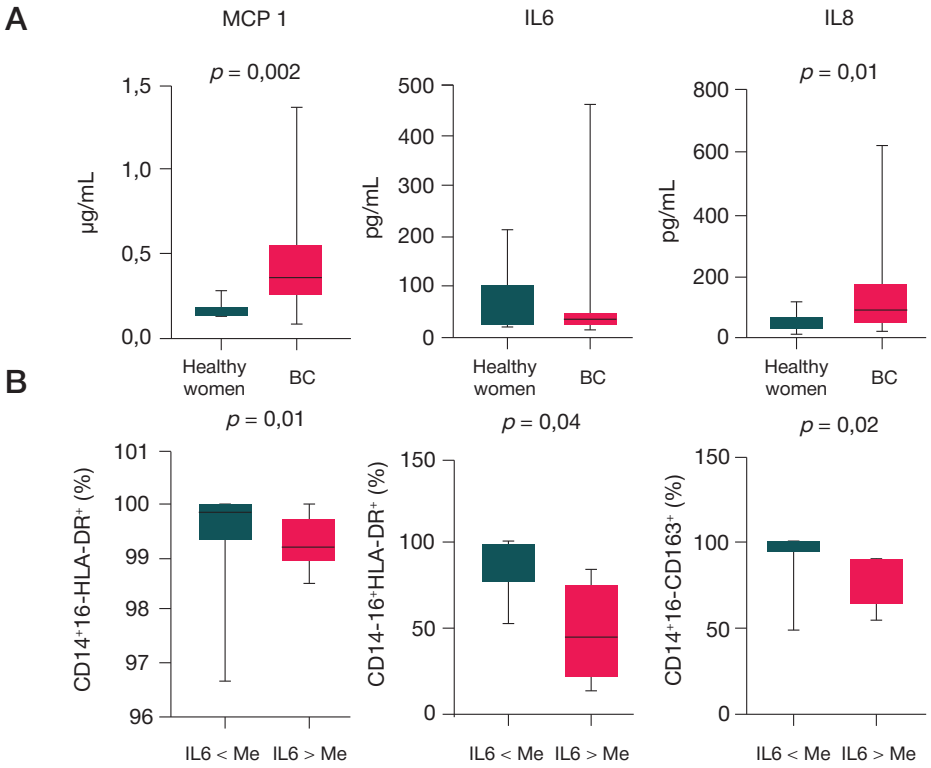

Fig. 2. Association of blood cytokine levels with circulating monocyte phenotype in breast cancer. **A**. Serum cytokine levels in patients with breast cancer and healthy women. **B**. Correlation of serum IL6 levels with the CD163+ and HLA-DR+ monocyte counts in breast cancer

is likely to stimulate generation of the $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ monocytes capable of maintaining the antitumor immune response. At the same time the disease progression expressed in the increase in malignancy stage is associated with the increase in the percentage of $CD14^{++}CD16^{-}CD163^{+}$ and $CD14^{++}CD16^{+}CD163^{+}$ cells, while the $CD14^{+}CD16^{++}CD163^{+}$ monocyte counts decrease with increasing tumor size (Tables 2, 3). It is noteworthy that the levels of $CD14^{++}CD16^{-}$ monocytes also positively correlate with the disease stage. We have earlier shown that the CD163 molecule expression is increased in monocytes of patients with BC, while in macrophages of the breast tumor this indicator increases in response to chemotherapy [19]. Furthermore, redistribution of monocyte counts between the $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ populations can be critical, which determines multidirectional changes in the counts of cells of these populations with increasing disease stage and tumor size. Among monocytes of classical population, the CD163 receptor-expressing cells have shown the association with clinical and morphological parameters. The latter suggest a pivotal role in BC pathogenesis played by the CD163 receptor-mediated mechanisms.

It has been shown that BC molecular subtypes show different immunogenicity [20]. Thus, the vast majority of TNBC subtypes are characterized by the increased mutational load and the content of tumor infiltrating lymphocytes being the criteria for prescription of immunotherapy drugs [21]. We have noted that monocyte population structure is correlated to the tumor molecular subtype. Thus, TNBC was characterized by the 1.4-fold decrease in the $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ cell counts (Table 4). At the same time, the $Her2^{+}$ subtype was characterized by the increased $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ monocyte counts. Thus, the differences between BC subtypes can be detected at the blood monocyte population level.

The soluble factors circulating in blood create a certain information environment capable of ensuring functional polarization of effectors and regulators of the immune system [22]. Assessment of the relationship between the serum levels of certain cytokines (IL6, IL8 and MCP-1) and the characteristics of monocyte population structure was performed for this purpose. Noteworthy is the increase in the concentration of the major MCP-1 monocyte migration factor in affected individuals, which is possibly associated with activation of monocyte recruitment to the tumor tissue (Fig. 2A). Despite the fact that we have noted no changes in blood levels of IL6 associated with BC, the relationship between the IL6 levels and the monocyte population structure has been revealed (Fig. 2A). It is interesting that the CD163 receptor expression on the $CD14^{++}CD16^{-}$ cells decreased along with the HLA-DR molecule expression, which had not been earlier reported in patients with BC and required further research. Patients with high IL6 levels had reduced $CD14^{++}CD16^{-}HLA\text{-}DR^{+}$ and $CD14^{+}CD16^{++}HLA\text{-}DR^{+}$ counts (Fig. 2B). IL6 exerts multidirectional effects on malignant neoplasms, it can both promote and inhibit tumor growth [23]. It has been shown that elevated serum levels of IL6 are a negative prognostic factor in patients with BC [23, 24]. Perhaps, the association between the levels of this cytokine and the decrease in the levels of monocytes performing antitumor functions can represent a possible explanation of the IL6 negative impact on the BC course (Fig. 2B). There is some evidence that monocytes showing low HLA-DR expression can differentiate into myeloid-derived suppressor cells suppressing the immune response [25]. The association of IL6 levels with the $HLA\text{-}DR^{+}$ monocyte counts is likely to be one of the mechanisms underlying the development of breast tumors and requires further research.

CONCLUSIONS

Thus, the study has shown that the disease progression is followed by redistribution of the major monocyte populations towards an increase in the classical population. Furthermore, elevated CD163 receptor expression on monocytes of the

classical population is typical for the advanced disease stage, while elevated HLA-DR expression, by contrast, is observed in individuals with no or minimal regional lymph node metastasis. The breast tumor molecular subtype is also related to the $CD163^{+}$ and $HLA\text{-}DR^{+}$ monocyte distribution across classical, intermediate, and non-classical populations. The decrease in HLA-DR expression on the $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocytes and CD163 expression on the $CD14^{++}CD16$ monocytes is associated with elevated serum IL6 levels in affected individuals. We believe that determination of monocyte population structure in patients with BC can contribute to the development of additional criteria for treatment tactics shaping. Further translational research focused on assessing the possibility of using the indicators of monocyte population characteristics in clinical practice for patients with BC is appropriate.

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