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Assessment of peripheral blood and bone marrow T, NK, NKT and dendritic cells in patients with multiple myeloma*

Ocena limfocytów T, NK, NKT oraz komórek dendrytycznych we krwi obwodowej i szpiku kostnym chorych na szpiczaka plazmocytowego

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Symptoms of multiple myeloma (MM) include bone destruction with pathological fractures, kidney failure and frequent infections, which are the major causes of patient mortality. In our recent research, we demonstrated that the degree of dendritic cell (DC) subpopulation deficit could be related to MM progression, which in consequence may contribute to the MM--related impairment of the immune responses. In the present study, we determined by flow cytometry the frequencies of CD4+ and CD8+ T cells, NK, and NKT-like cells as well as their correlation with myeloid and lymphoid populations of DCs in patients with MM. The study involved 50 patients diagnosed with MM at the Department of Hematology in the Holycross Cancer Center in Kielce. The research samples were collected after the MM diagnosis and before the initiation of anticancer therapy. The obtained results revealed the relations between the percentages of DC subpopulations and lymphocyte subsets, especially the activated ones, in the peripheral blood (PB) and bone marrow (BM). The described role of DCs in the process of the immunological response, either adaptive or innate, leads us to conclude that the decrease of the number or percentage of these cells may have a negative impact on the process of activation of effector cells and, consequently, on the effectiveness of a response to foreign as well as neoplastic antigens in patients with MM.

Keywords: multiple myeloma • myeloid dendritic cells • lymphoid dendritic cells • natural killer cells • T cells

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Abbreviations: **BDCA** – blood dendritic cell antigen, **CD** – cluster of differentiation, **CMP** – common myeloid progenitors, **CTLA** – cytotoxic T-lymphocyte antigen, **DC** – dendritic cells, **DC-SIGN** – dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, **Flt3L** – FMS-like tyrosine kinase 3 ligand, **GM-CSF** – granulocyte-macrophage colony-stimulating factor, **HLA** – human leukocyte antigen, **IL** – interleukin, **IFN** – interferon, **iNKT** – invariant natural killer T-cells, **MHC** – major histocompatibility complex, **MM** – multiple myeloma, **MUC-1** – mucin 1, cell surface associated, **NK** – natural killer, **NKT** – natural killer T-cells, **NO** – nitrous oxide, **PGE2** – prostaglandin E2, **Tc** – T cytotoxic, **TCR** – T-cell receptor, **TGF** – transforming growth factor, **Th** – T helper, **TNF** – tumor necrosis factor, **TRANCE** – TNF-related activation-induced cytokine, **VEGF** – vascular endothelial growth factor.

Introduction

Both humoral and cellular responses participate in anti-cancer immunity. Immunological mechanisms engaged in the anti-cancer reaction include: the function of NK and NKT cells, cytotoxic functions of T lymphocytes, macrophages, neutrophils, cytokines produced by the immune cells, and antibody-dependent cellular cytotoxicity [22,35]. T helper lymphocytes and antigen-presenting cells, e.g. dendritic cells, constitute a crucial element of the anti-cancer response [21,27]. Dendritic cells (DCs) are defined as professional cells presenting an antigen and are able to present either a foreign antigen or an autoantigen [1,5,21].

DCs exhibit surface expression of the following antigens: CD1a, CD40, CD54, CD58, CD80, CD83, CD86, DC-SIGN. They are also characterized by high expression of MHC molecules class I and II [5,21]. There are two major subpopulations of DCs: lymphoid DCs (plasmacytoid) and myeloid DCs. The ontogenesis of DCs is not fully discovered. Either myeloid or lymphoid DCs can stem from common myeloid progenitors (CMP) of the bone marrow. However, these cells do not constitute a homogeneous group. Lymphoid DCs produce mainly IFN-α, IFN-β, and IL-4, whereas myeloid DCs produce IL-12. Dendritic cells determine the type of immune response. Lymphoid DCs stimulate the differentiation of Th0 lymphocytes to Th2 lymphocytes and induce a humoral response. Myeloid DCs stimulate the differentiation of Th0 lymphocytes to Th1 lymphocytes and Th17 lymphocytes and they induce a cellular response. However, it should be emphasized that lymphoid DCs produce IFN-α and IFN-β, which influence the activation of Tc (CD8+) lymphocytes and NK cells. As a consequence, it is difficult to distinguish the function of lymphoid and myeloid DCs [13,19,21,41]. The degree of maturity of DCs is also significant as far as their function is concerned. Mature DCs induce an immunological response; however, immature cells can lead to the development of tolerance to certain antigens. Immature DCs are characterized by low expression of costimulatory molecules, which, instead of stimulating lymphocytes, can lead to their anergy. Among the factors which inhibit the maturation process of DCs are cytokines produced by tumor cells (e.g. IL-10) [10,11,27].

Factors which stimulate the growth and differentiation of immature DCs are IL-4, Flt3L, GM-CSF, TNF, and TRANCE [19,21]. In contrast, IL-6, IL-10, VEGF and also a variety of other substances produced by neoplasms, e.g. PGE2, NO, a soluble receptor for IL-12 and many other factors, have an inhibiting influence on the differentiation of DCs [12,16,21,27,42]. IL-6 and VEGF, high concentrations of which can be observed in multiple myeloma, inhibit the differentiation and maturation of DCs [12,16,18,27,46].

Our recent study demonstrated a significant reduction in the percentages of both myeloid and lymphoid DCs in MM patients, more pronounced in those with the worse prognosis as determined by the high levels of β2 microglobulin [33]. Accordingly, a marked decrease in the proportions of both myeloid and lymphoid DCs in the bone marrow (BM) of patients with an advanced clinical stage (III) compared to earlier stages (I+II) of disease was also found. Our results suggest that the degree of DC subpopulation deficit could be related to the MM progression, which in consequence may contribute to the MM-related impairment of the immune responses [33].

In the present research, an attempt to determine alterations in examined subpopulations of the immune cells (Tc, Th, NK, NKT) and their correlation with myeloid and lymphoid populations of DCs in patients with MM was made.

Material and methods

Study and control group

The study involved 50 patients diagnosed with MM at the Department of Hematology in the Holycross Cancer Center in Kielce. The research samples were collected after the MM diagnosis before the initiation of anticancer therapy.

The average age of patients was 64 (ranging from 34 to 81). In the study group, there were 27 women and 23 men.

The diagnosis of multiple myeloma was based on the criteria of the International Myeloma Working Group. At the time of diagnosis patients were staged according to the Durie-Salmon classification, as follows: stage I – 14 patients, stage II – 8 patients and stage III – 28 patients. The research protocol was approved by the Ethics Committee of the Medical University of Lublin and all patients gave written informed consent.

Patients with the coexistence of other chronic diseases, such as allergic diseases, autoimmune diseases, other cancer diseases, serious infections, as well as those treated with immunosuppressants and immunomodulators or receiving transfusion of blood products in the past 6 months, were excluded from the study. The control group consisted of 25 persons (15 women, 10 men) undergoing hip replacement surgery due to osteoarthritis from whom the bone marrow and peripheral blood samples were collected at the time of surgery. The median age of subjects in the control group was 63.7 (ranging from 33 to 79).

Immunophenotyping

Peripheral blood (PB) and bone marrow (BM) samples were collected into heparinized tubes and immediately processed. Peripheral blood mononuclear cells were separated by density gradient centrifugation and then stained with monoclonal antibodies (mAbs) conjugated with relevant fluorochromes: Fluorescence IsoThioCyanate (FITC), PhycoErythrin (PE) and CyChrome (Cy).

Percentages of NK and NKT-like cells were evaluated with flow cytometry using mAbs – anti-CD3/FITC, CD16CD56/PE, CD45/PerCP (BD Biosciences) – which allowed for simultaneous assessment of T ($CD3^+$) lymphocytes and NK ($CD16^+CD56^+$) cells. During analysis, the $CD3^+CD16^+CD56^+$ population was also determined. A standard, whole-blood assay with erythrocyte cell lysis was used to prepare the PB samples.

Immunofluorescence studies on T cell subsets were performed using a combination of the following mAbs: CD3/FITC, CD19/PE, CD8/FITC, CD4/PE, CD25/Cy, CD69/Cy, and CD3/FITC, HLA-DR/PE, purchased from R&D Systems, and CD45RA/PE, CD45RO/PE from Pharmingen.

To assess the percentages of DC subpopulations the following mAbs were used: IgG1/FITC, IgG2a/PE, IgG2a/PE-Cy5 – as a negative control for the fluorescent green, orange and red, anti-CD45/FITC, anti-CD14/PE – in order to determine precisely the goal for a population of mononuclear cells, anti-BDCA-1/FITC, anti-CD19/PE-Cy5 – in order to determine the percentage of immature, myeloid-lineage DCs, anti-BDCA-2/FITC, anti-CD123/PE – to determine the percentage of immature DC lymphoid lines. "Cleanness" of the lymphocyte and monocyte gateway was evaluated by examining the distribution of cells in the coordinates of CD45/CD14. The expression of surface antigens on cells was determined by flow cytometry. BDCA-1^+CD19^- cells were considered to be myeloid DCs, whereas BDCA-2^+CD123^+ cells were considered to be lymphoid DCs.

The percentage of positive cells was measured from a cut-off set using an isotype-matched nonspecific control antibody. Three-color immunofluorescence analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with 488 nm argon laser. A minimum of 10 000 events were acquired and analyzed using CellQuest software. The percentages of cells expressing surface markers were analyzed.

Statistical analysis

Statistical analysis of the results was conducted using Statistica 9.0. Deviation from normality was evaluated by the Kolmogorov–Smirnov test. Data were expressed as the mean value ± SD, median, minimum and maximum. Differences between groups were assessed using the Mann–Whitney U test. Pearson or Spearman correlation analyses were used to analyze the correlation. A p value less than 0.05 was considered statistically significant.

Results

Percentages of subpopulations of T lymphocytes and NK cells in the PB of patients with MM and healthy individuals

The mean percentage of $CD3^+$ T lymphocytes in the PB of the patients with MM was 67.91% ± 16.56% (median 71.47%), and it was significantly higher than the value in the control group (p=0.02), where the mean percentage of these cells was 51.7% ± 24.12% (median 59.53%) (Figure 1).

The mean proportion of $CD3^+$HLA-DR^+ T lymphocytes in the PB of the patients with MM was 3.85% ± 3.64% (median 2.73%) and was significantly lower (p=0.01) than in the control group, where the mean percentage of these cells was 6.01% ± 2.84% (median 7.01%) (Figure 2).

The mean percentage of $CD8^+$ T lymphocytes in the PB of the patients with MM was 28.89% ± 11.68% (median 26.4%) and was significantly higher (p=0.001) than in

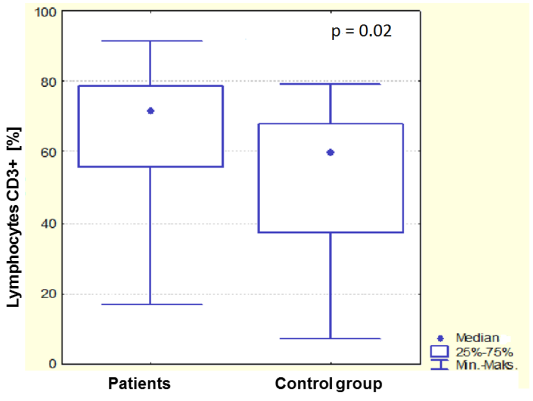

Fig. 1. Median percentage of $CD3^+$ T lymphocytes in peripheral blood of patients with MM and individuals from the control group

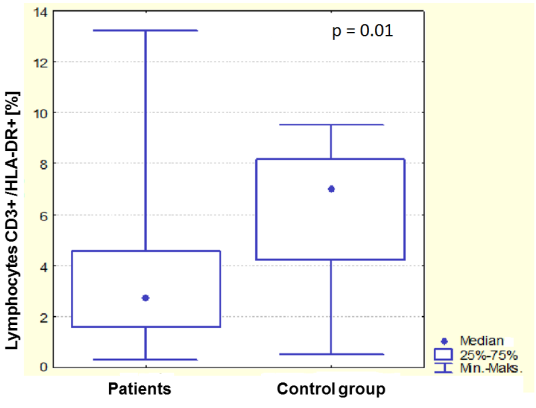

Fig. 2. Median percentage of $CD3^+HLA\text{-}DR^+$ T lymphocytes in peripheral blood of patients with MM and individuals from the control group

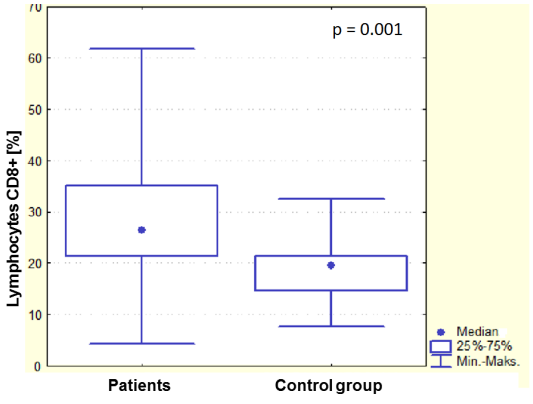

Fig. 3. Median percentage of $CD8^+$ T lymphocytes in the peripheral blood of patients with MM and individuals from the control group

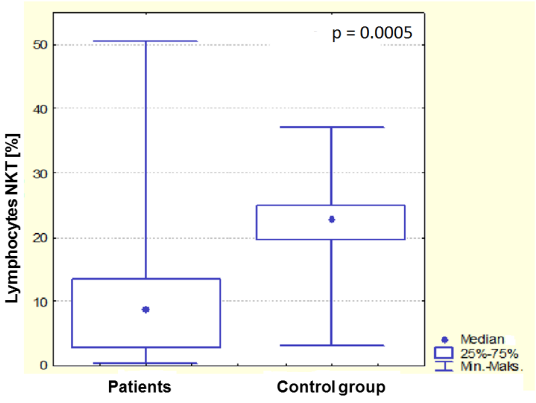

Fig. 4. Comparison of the median percentage of NKT cells in the peripheral blood of patients with MM and individuals from the control group

the control group, in which the mean percentage was 18.72% ± 6.86% (median 19.6%) (Figure 3).

The mean frequency of $CD3^+CD56^+16^+$ NKT cells in the PB of the patients with MM was 10.93% ± 11.02% (median 8.59%), and this value was significantly lower (p=0.0005) than in the control group, where the mean percentage of these cells was 21.15% ± 9.08% (median 22.8%) (Figure 4).

The mean proportion of PB $CD56^+16^+$ NK cells in the group of patients was 14.67% ± 6.89% (median 14.73%) and was significantly higher (p=0.000002) than in the control group, where the mean percentage was 4.71% ± 2.99% (median 3.8%) (Figure 5).

No statistically significant differences concerning the mean percentage of $CD4^+$, $CD4^+CD69^+$, $CD4^+CD25^+$, $CD8^+CD69^+$, $CD8^+CD25^+$ T lymphocytes were demonstrated between the group of patients and the control group (data not shown).

Percentages of subpopulations of T lymphocytes and NK cells in the bone marrow of patients with MM and of healthy individuals

The mean percentage of $CD3^+$ T lymphocytes in the BM of the patients was 38.64% ± 23.4% (median 37.92%) and was significantly higher (p=0.01) than in the control group, in which the mean value was 21.89% ± 13.17% (median 19.17%) (Figure 6).

The mean frequency of $CD8^+$ T lymphocytes in the BM of the patients with MM was 19.36% ± 13.44% (median 17.95%) and was significantly higher (p=0.007) than in the control group, where the mean percentage of these cells was 9.51% ± 6.65% (median 8.42%) (Figure 7).

The mean proportion of $CD56^+16^+$ NK cells in the BM of the patients with MM was 15.1% ± 12.61% (median 11.88%) and was significantly higher than in the control group (p = 0.006), in which it was 6.32% ± 3.37% (median 5.66%) (Figure 8).

No significant differences concerning the percentage of $CD3^+HLA\text{-}DR^+$, $CD4^+$, $CD4^+CD69^+$, $CD4^+CD25^+$, $CD8^+CD69^+$, $CD8^+CD25^+$ T lymphocytes, and $CD3^+CD56^+16^+$ NKT cells between the group of patients and the control group were observed (data not shown).

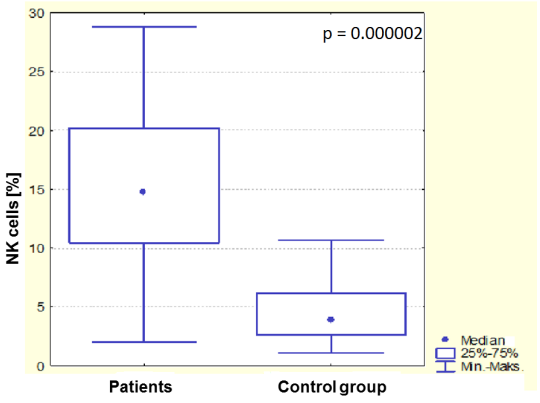

Fig. 5. Comparison of the median percentage of NK cells in the peripheral blood of patients with MM and individuals from the control group

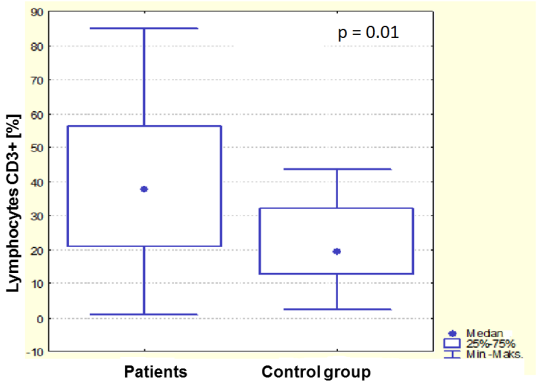

Fig. 6. Comparison of the percentages of $CD3^+$ T lymphocytes in the bone marrow of patients with MM and individuals from the control group

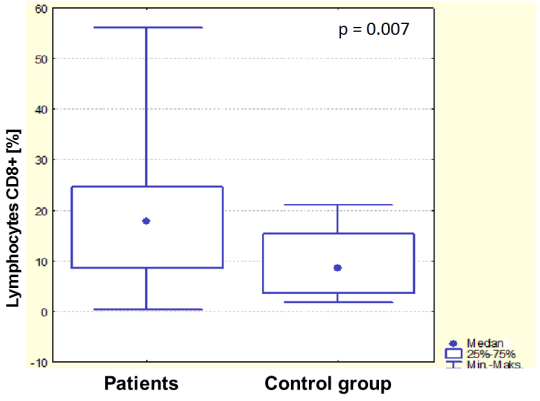

Fig. 7. Comparison of the median percentage of $CD8^+$ T lymphocytes in the bone marrow of patients with MM and individuals from the control group

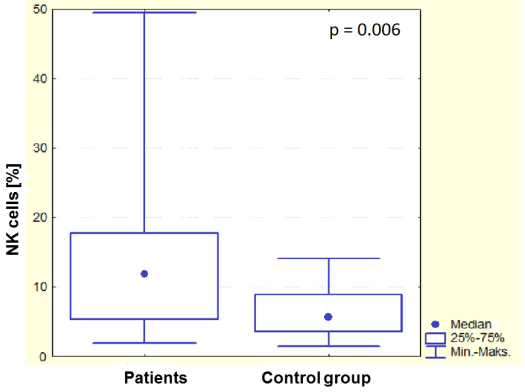

Fig. 8. Comparison of the median percentage of NK cells in the bone marrow of patients with MM and individuals from the control group

Correlations between studied populations of lymphocytes and myeloid and lymphoid DCs in the bone marrow of patients with MM

There were no statistically significant correlations between $CD3^+$, $CD3^+HLA\text{-}DR^+$, $CD4^+$, $CD4^+CD69^+$, $CD4^+CD25^+$, $CD8^+$, $CD8^+CD69^+$, $CD8^+CD25^+$ T lymphocytes, $CD3^+CD56^+16^+$ NKT cells, $CD3^-CD56^+16^+$ NK cells and myeloid DCs in the BM.

A positive correlation between the percentages of $CD3^+HLA\text{-}DR^+$ T lymphocytes and the proportions of $BDCA2^+CD123^+$ lymphoid DCs in the BM was demonstrated; the relation was statistically significant (p=0.001) (Figure 9).

Furthermore, a significant positive correlation was found between the percentages of $CD3^+CD56^+CD16^+$ NKT cells and the frequencies of $BDCA2^+CD123^+$ lymphoid DCs in the BM (p=0.04) (Figure 10).

No statistically significant correlations were found between the percentages of $CD3^+$, $CD4^+$, $CD4^+CD69^+$, $CD4^+CD25^+$, $CD8^+$, $CD8^+CD69^+$, $CD8^+CD25^+$ T lymphocytes, $CD56^+CD16^+$ NK and the percentages of lymphoid DCs in bone marrow of the patients.

Discussion

Immunological alterations are responsible for the increased incidence of infections as well as the progression of the disease, thus playing an important role in pathogenesis and the clinical course of MM. The activation of the immune cells and the production of cytokines observed during the course of an infection are capable of creating an environment convenient for the growth and proliferation of MM cells. So far, these mechanisms have been only partially explored [6,9,20,35]. In this context, it seems to be extremely significant to investigate the interactions between the tumor and the cells of the immune system.

While we were performing the tests, significant alterations were found in the cellular populations examined. In the PB as well as BM of the patients, a higher percentage of T lymphocytes was found in comparison to the control group; however, the frequency of activated PB T cells ($CD3^+HLA\text{-}DR^+$) was significantly lower than

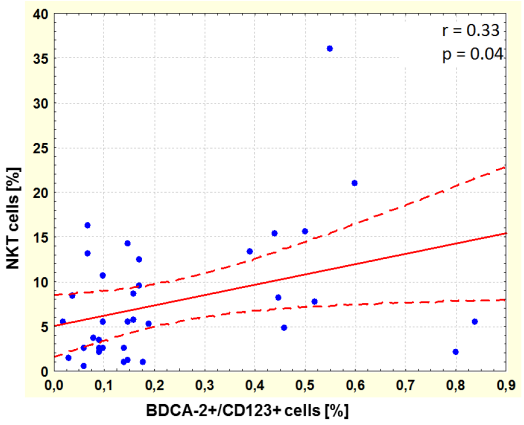

Fig. 9. Correlation between the percentages of $CD3^+HLA\text{-}DR^+$ T lymphocytes and the percentages of lymphoid DCs in bone marrow of patients with MM

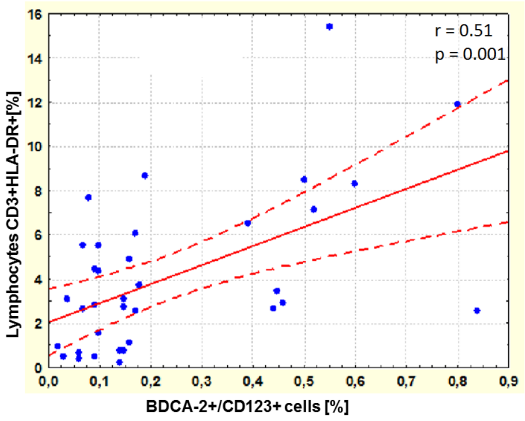

Fig. 10. Correlation between the percentage of $CD3^+CD56^+16^+$ NKT cells and the percentage of lymphoid DCs in bone marrow of patients suffering from multiple myeloma

that observed in the control group. This result suggests that the ability of circulating T cells to respond to tumor antigens is diminished, thus supporting the previous notion about T cell impairment in MM. In line with our observation, the decrease of the percentage of $CD3^+HLADR^+$ cells in MM was also found by Schütt et al. [38]. Although Kuriyama et al. observed a lowered percentage of T cells in patients with MM, they did not test activation markers on these cells [29]. It is highly probable that despite the increased percentage, T cells from MM patients can exert an impaired effector function. Accordingly, Binachi et al. demonstrated a disrupted signaling pathway from the TCR/CD3 complex to the inside of the cell in patients with MM, likely due to prolonged contact of T cells with malignant plasmocytes, thus allowing them to exhibit a phenotype of exhausted cells [2,3].

Also, worthy of note was a significantly higher percentage of cytotoxic cells, including CD8+ T cells and NK cells, in both the PB and the BM of MM patients in comparison to the control group. Notably, among markedly expanded CD8+ T cells, the percentage of activated cells did not differ significantly compared to controls, which leads to the conclusion that activation mechanisms in cytotoxic T cells are dampened during MM development. The activation of T lymphocytes requires, apart from the recognition of the antigen by the TCR receptor, co-stimulatory signals induced by the CD28 molecule [4,21]. Mozaffari et al. demonstrated that the expression of CD28 is decreased on T lymphocytes of patients with MM, with simultaneously increased expression of the CTLA-4 molecule, which transmits the inhibiting signal for T cells [30]. This fact, in combination with a decreased percentage of DC populations recently found by us in the same MM patients [33], may result in the insufficient activation of $CD8^+$ T cells. Among $CD8^+$ T lymphocytes, many populations of clonal cells with $CD8^+CD57^+$ phenotype and with the presence of perforin in the cytoplasm have been demonstrated. According to Sze et al., the appearance of such a population in patients with MM can improve the prognosis [40]. A similar notion concerning the positive impact of clonal cytotoxic T lymphocytes ($CD8^+$) for overall survival was presented independently by Raitakari et al. (2003) [36] and Perez-Andres et al. (2006) [34]. In contrast, Kay et al. demonstrated a relation between the lowered percentage of $CD4^+$ and $CD8^+$ T cells and a worse prognosis in patients with MM [24,25,26]. Therefore, it cannot be excluded that the higher $CD8^+$ T cell population found in the MM patients enrolled in the current study may indirectly predict a stable clinical course of the disease.

Our tests also demonstrated significantly higher percentages of NK cells in the PB and BM of the patients with MM. As described previously, NK cells play a crucial role in the antitumor immune response. They can destroy malignant cells, even when they do not demonstrate the expression of MHC molecules. The relations between DCs and NK cells in various types of neoplasm, including MM, are complex and reciprocal [18,23,28,31]. It has been demonstrated that the interaction between DCs and NK cells is crucial for DC maturation with subsequent induction of MM-specific cytotoxic T lymphocytes. It is possible that abnormalities in DC populations reported by us in the same group of MM patients [33] can also be caused by the impaired activity of NK cells. In fact, Fauriat et al. demonstrated drastically lower expression of the co-receptor 2B4 (CD244) on NK cells in patients with MM, which plays an important role in the activation of these cytotoxic cells [15]. Altogether, the above results indicate that NK cells, although expanded in MM, might sustain functional impairment concerning their role in both antitumor responses and DC function.

In addition, our current research demonstrated a significantly lower percentage of NKT cells in the PB of the patients in comparison to the control group. NKT cells, beside the expression of T and NK cell markers, are able to recognize antigens in the context of the CD1d molecule [43], but not MHC, and play a role in the anti-tumor and anti-infection cytotoxic responses. Spano-

udakis et al. demonstrated that MM cells at the early stages of disease development have positive expression of the CD1d molecule on their surface, which decreases with progression of the disease [39]. In addition, Dhodapkar et al. proved that as MM develops, the function of NKT cells becomes impaired, and this defect correlates with disease progression [14]. In our own research, a direct relation between the frequency of NKT cells and DCs in the PB of the patients was not demonstrated; however, both cell populations were significantly smaller than in the control group. Therefore, the possibility that alterations in DC subpopulations may negatively influence the percentage of NKT cells in the PB of the patients with MM should be taken into consideration.

From the presented results and available data, we can conclude that the development of MM is related to immune alterations, including a decrease of the percentages of the DC subpopulations and disturbed T lymphocyte activation. Hence, the treatment of patients with MM should also focus on the reversion of the normal DC populations and normalization of the proportions among lymphocyte subpopulations and other effector cells of the immune system. Hajek et al. proved that in patients who underwent autotransplantation of hematopoietic stem cells during a period of six months, subpopulations of PB DCs become comparable to those found in healthy individuals [17]. It is likely that immunotherapy with the use of DCs will become a novel approach to MM treatment; in fact, the cells *ex vivo* stimulated with protein isolated from malignant plasmocytes can induce *in vivo* efficient MM-specific T cell responses [18,32,37,47].

Our finding of abnormalities in immune cell distribution in PB and BM from MM patients strongly suggests that MM development and progression has a considerable impact on the dysregulation of the immune system. However, the presented data only partially explain the role of DCs in the observed alterations and pathogenesis of MM, and there are still various unclear and disputable issues which require further research. Regardless of the final role of DCs in the development of this disease, it seems that the correlation, demonstrated in our current research, of the decreased percentage of DCs with affected frequencies/activation of the effector T cells may influence the process of effector function, regarding the response to foreign and neoplastic antigens in the course of MM.

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