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Research article

Decreased circulating myeloid-derived suppressor cell count at the engraftment is one of the risk factors for multiple myeloma relapse after autologous hematopoietic stem cell transplantation

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ABSTRACT

Recent studies demonstrated that myeloid-derived suppressor cells (MDSCs) are involved in the pathogenesis and progression of multiple myeloma (MM). Nevertheless, data on the quantitative and functional changes in MDSCs during standard MM treatment remain poorly understood. Here, we determined that monocytic MDSCs (M-MDSC; $CD14^{+}HLA\text{-}DR^{low/-}$) and granulocytic MDSCs (PMN-MDSC; $Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{+}$) in MM patients in remission following induction therapy (IT) were significantly increased, while early MDSCs (E-MDSCs; $Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{-}$) were decreased compared to the donor group. In progression, MM patients had the most pronounced decrease in E-MDSCs and enhanced levels of PMN-MDSCs. IT was accompanied with a decrease in the expression of arginase-1 (Arg-1). In MM patients with relapse or resistance to IT, $Arg\text{-}1^{+}$ cell frequency in M-MDSCs and E-MDSCs, as well as $PD\text{-}L1^{+}$ M-MDSCs, was increased, which may facilitate tumor immunosuppression. G-CSF administration led to a significant increment in the MDSC subsets. At the engraftment, circulating M-MDSC and PMN-MDSCs were temporarily increased, with a gradual decline to the pre-transplant levels in 12 months. The percentage of E-MDSCs was decreased at the leukocyte recovery. Patients with a higher (>Me) M-MDSC count at the engraftment had a shorter post-transplant leukopenia duration (Me 11 vs. 13 days; $p_U = 0.0086$). The advanced MM stage, depth of response, and lower relative count of circulating E-MDSCs at the engraftment were independent risk factors associated with a lower progression-free survival.

The obtained data allow us to hypothesize that MDSCs may play a positive role at the stage of leukocyte recovery by ameliorating the long-term anti-tumor response in MM.

1. Introduction

Recent studies have shown that myeloid-derived suppressor cells (MDSCs) play a pivotal role in the regulation of the immune response in many pathologies, including tumors, chronic infections, trauma, sepsis, autoimmune diseases, etc. [1]. The accumulation of MDSCs in these pathologies has been described in the bone marrow (BM), peripheral blood (PB), spleen, lymph nodes, as well as in the tumor microenvironment [2–4] and is associated with a poorly controlled chronic inflammatory process [5].

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According to previous studies, the induction of MDSCs is regulated by the sequential action of two types of signals (a two-signal model) [6,7]. The first group of signals (M-CSF, G-CSF, and GM-CSF) controls myelopoiesis. The second group includes pro-inflammatory cytokines of tumor origin or another inflammatory process (IFNγ, IL-4, IL-6, IL-13, IL-17, etc.), which trigger prolonged inflammation and divert differentiation of myeloid precursors towards immature myeloid cells – MDSCs, – by activating the expression of specific genes in them [7,8]].

Two main subsets of MDSCs have been described in humans: 1) monocytic MDSCs (M-MDSCs) with a morphology and phenotype similar to monocytes but characterized by down-regulation of the MHC class II molecule HLA-DR ($CD14^{+}HLA\text{-}DR^{low/-}$) [9]; and 2) granulocytic, or polymorphonuclear MDSCs (PMN-MDSCs), phenotypically resembling neutrophils ($CD11b^{+}CD33^{+}CD14^{-}CD15^{+}(CD66b^{+})HLA\text{-}DR^{low/-}$), but detected by a lower density gradient and a pronounced suppressor activity. In addition, the third type has been recently described – early-stage MDSCs (E-MDSCs), with the Lin^{-} (CD3, CD14, CD15, CD19, CD56) $HLA\text{-}DR^{-}CD33^{+}$ phenotype, whose functional features are still poorly investigated [10].

Due to gene and protein expression profiles different from those of mature myeloid cells, MDSCs inhibit cells of innate and adaptive immunity *in vitro* and *in vivo,* with the most pronounced suppressive activity against T cells [11]. The suppressive effect of MDSCs is mediated by various mechanisms, including depletion of L-arginine and L-tryptophan in the microenvironment due to the expression of arginase-1 (Arg-1) and indolamine-2,3-dioxygenase (IDO), respectively, generation of oxygen free radicals, secretion of TGF-β and IL-10, and induction of regulatory T cells (Treg) [12,13]. Each of the MDSC subsets can undermine the T cell response through different mechanisms.

An increased number of circulating MDSCs is considered as a predictive biomarker of progression and poor outcome in various types of solid tumors (cervical, colorectal, gastric, pancreatic, squamous cell, non-small cell lung, breast, and head and neck cancers) as well as a predictor of response to immunotherapy [14–17]. At the same time, despite ongoing research, MDSC roles in hematological malignancies remain unclear and even controversial. On the one hand, an increased number of circulating M-MDSCs is a negative predictive marker in chronic myeloid leukemia [18], multiple myeloma [19], diffuse large B-cell lymphoma [20], and several non--Hodgkin's lymphomas [21]. Several authors point to the prognostic value of increased PMN-MDSC counts in Hodgkin's lymphoma and B-cell non-Hodgkin's lymphomas [22].

On the other hand, MDSCs may have a positive immunoregulatory effect in allogeneic hematopoietic stem cell transplantation (allo-HSCT). The standard protocol for the mobilization of hematopoietic stem cells (HSCs) with recombinant human granulocyte colony-stimulating factor (G-CSF) drugs indirectly induces the release of $CD34^{+}$ HSCs and, apart from other effects, exerts the expansion of MDSCs in PB and, consequently, in leukopheresis products [23]. M-MDSCs of allografts from healthy donors have suppressive activity against allo-T cells and increase Treg activity, and therefore may alleviate acute graft-versus-host disease [24].

Multiple myeloma (MM) is a B-cell tumor with the expansion of monoclonal plasma cells in BM and the accumulation of dysfunctional monoclonal immunoglobulins in the PB. Multiple myeloma accounts for approximately 1% of all cancers and, despite advances in the field of therapy, remains one of the most incurable malignant tumors [25]. The bone marrow microenvironment plays an important role in the MM pathogenesis, including the formation of plasma cell niches that provide escape from immune surveillance, tumor chemoresistance, and angiogenesis [26]. At the same time, there is growing evidence that MDSCs are also involved in the pathogenesis and progression of MM [27–29]. Thus, PMN-MDSCs maintain the stem properties of MM tumor cells and promote tumor growth in experimental models *in vitro* and *in vivo* [30]. Simultaneously, data on the frequencies of various MDSC subsets in MM are few and contradictory, as well as the pathogenetic and predictive significance of distinct MDSC subsets at various stages of MM treatment are not completely clear.

Despite the implementation of novel targeted anticancer agents, high-dose chemotherapy (HDCT) with autologous hematopoietic stem cell transplantation (auto-HSCT) remains an effective approach in the treatment of MM. There are few studies showing a higher number of M-MDSCs before auto-HSCT is associated with a worse post-transplant outcome and a lower time to progression [19]. However, the role of MDSCs at post-transplant remains virtually unexplored. Meanwhile, re-infused with graft or *de novo* expanded MDSCs may contribute to the regulation of T cell recovery, implemented by homeostatic proliferation of memory T cells and thymic generation of naive T cells. Since homeostatic proliferation results in a narrowing of the T cell receptor (TCR) repertoire, limiting its overexpression with MDSCs may have a beneficial effect by promoting a broad TCR repertoire diversity. On the other hand, hindering the homeostatic proliferation or thymic emigrant expansion during the restoration of the T cell pool under the action of MDSCs at a later time may have negative consequences, in particular by increasing the risks of tumor recurrence and opportunistic infections. Besides, the disease itself and treating agents can significantly affect the functional parameters of MDSCs, altering their regulatory properties. However, quantitative and functional changes in MDSCs (e.g., their expression of suppressive molecules) before HDCT and after auto-HSCT and the possible predictive significance of these changes remain poorly understood.

The present study characterized the features of MDSC subsets (M-MDSCs, PMN-MDSCs, and E-MDSCs), including their counts and expression of suppressive molecules Arg-1, IDO, and PD-L1, depending on clinical parameters and stages of therapy. The expansion and functional alterations of MDSCs in MM patients with G-CSF-mediated HSC mobilization were demonstrated. In addition, we showed that MDSC frequency at the engraftment was associated with early leukocyte recovery (for M-MSDCs) and was another risk factor (E-MSDCs) for MM recurrence.

2. Results

2.1. Counts of circulating M-MDSCs and PMN-MDSCs were increased in MM patients

Multiple myeloma patients pre-treated with induction therapy (IT) and who had achieved remission were characterized by a

statistically significant almost two-fold increase in relative and absolute counts of circulating M-MDSCs ($p_U = 0.001$ and $p_U = 0.0003$, respectively), as well as a relative count of PMN-MDSCs ($p_U = 0.049$) compared with healthy donors (Fig. 1B). While the frequency of E-MDSCs in MM patients and donors did not differ significantly, the absolute E-MDSC count in PB of pre-treated patients was significantly reduced (pU = 0.03; Fig. 1B) accompanied with comparable absolute counts of PBMCs (Supplementary Fig. 1).

Increased M-MDSC frequencies were detected in both patients with stage II and stage III MM (Fig. 1B). However, only patients with stage III MM had higher absolute counts of M-MDSCs ($p_U = 0.046$), relative and absolute counts of PMN-MDSCs ($p_U = 0.05$ and $p_U = 0.036$, respectively), and lower absolute counts of E-MDSCs ($p_U = 0.04$) compared to healthy donors (Fig. 1B). In addition, patients with stage III MM had significantly lower frequencies of E-MDSCs than patients with stage II MM ($p_U = 0.034$).

Thus, quantitative changes in all three populations of MDSCs (an increase in the counts of M-MDSCs and PMN-MDSCs and a decrease in E-MDSCs) were the strongest and statistically significant in patients with stage III MM. The exception was M-MDSCs; their significant increase was already detected in patients with stage II.

2.2. Induction therapy regimens affect circulating MDSCs in MM patients

Multiple myeloma patients included in the study had been treated with different regimens of IT, which could have an impact on MDSCs. In this regard, two groups were distinguished: 1) patients who achieved remission during therapy with BTZ-based regimens (n = 29); 2) patients resistant to therapy with BTZ-containing regimen and treated with the immunomodulatory drug lenalidomide (LEN-treated; n = 18). Patients of these groups did not differ in the ratio of stages II and III, which in group 1 (BTZ regimens) was 10–19 patients, respectively, and in group 2 (LEN-treated) - 6 to 12 patients, respectively (Yates corrected $\chi 2 = 0.06$; $p = 0.81$). To evaluate the effect of therapy on MDSCs, a group of untreated patients with newly diagnosed MM was also studied (n = 6).

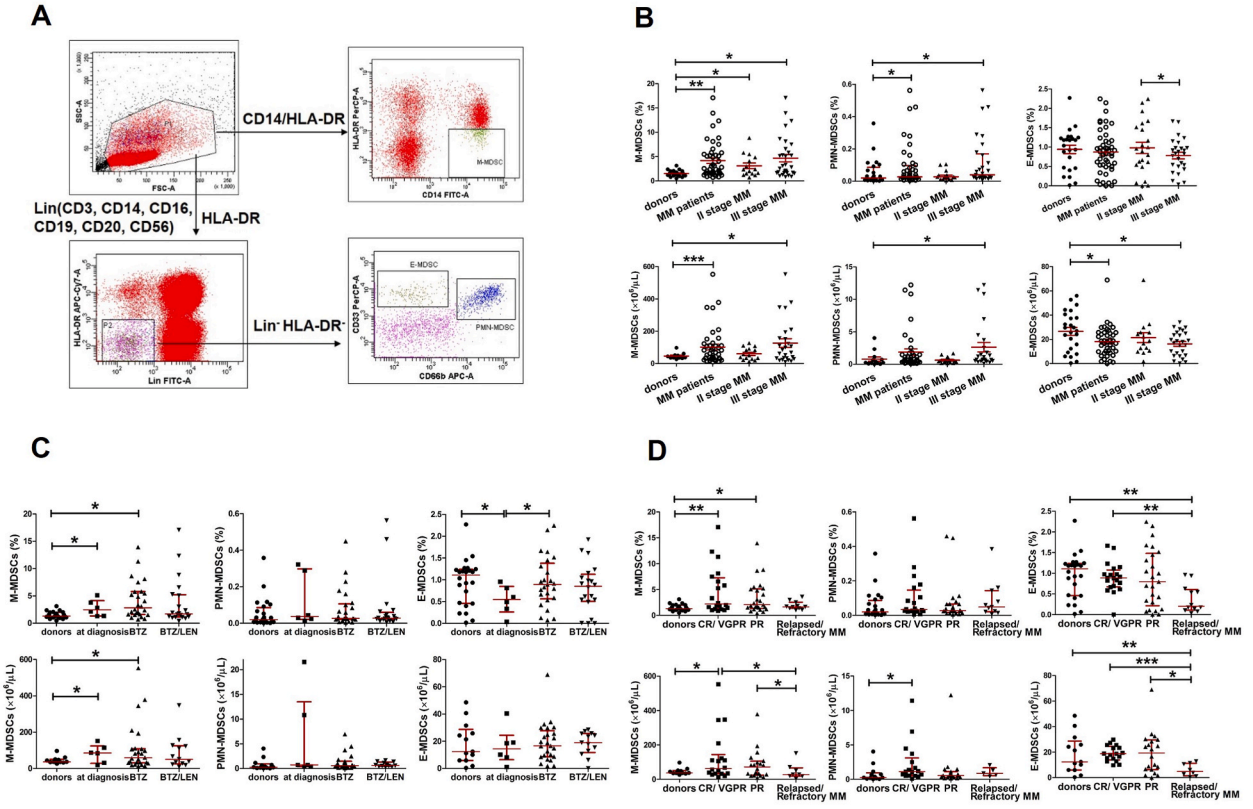

Fig. 1. Myeloid-derived suppressor cells in peripheral blood of patients with multiple myeloma **A.** Gating strategy for the identification of M-MDSC, PMN-MDSC, and E-MDSC subsets among PBMCs. **B.** Relative (upper rows) and absolute (bottom rows) counts of M-MDSC, PMN-MDSC, and E-MDSC are shown as individual values, Me and IQR depending on MM stage in patients with remission following IT (n = 47) and healthy donors (n = 23). **C.** Relative (upper rows) and absolute (bottom rows) counts of MDSC subsets are shown as individual values, Me and IQR depending on previous lines of induction therapy. BTZ – patients who achieved remission during therapy with BTZ-based regimens (n = 29); BTZ/LEN – patients resistant to therapy with BTZ-regimens and treated with lenalidomide (n = 18); at diagnosis – untreated patients with newly diagnosed MM (n = 6). **D.** Relative (upper rows) and absolute (bottom rows) counts of MDSC subsets are shown as individual values, Me and IQR depending on the response to IT. CR/ VGPR – Complete response/Very good partial response (n = 22); PR – Partial response (n = 25); relapsed/refractory MM – MM patients with progression or resistant to ongoing IT (n = 11).
* – p_U value < 0.05; ** – p_U value < 0.01; *** – p_U value < 0.001.

As shown in Fig. 1C, MM patients at diagnosis were characterized by significantly increased relative (*vs.* donors $p_U = 0.05$) and absolute numbers of M-MDSCs (*vs.* donors $p_U = 0.006$). Monocytic MDSC counts during therapy with BTZ-based regimens did not change compared with their counterparts at diagnosis and remained significantly higher (the relative and absolute counts *vs.* donors $p_U = 0.001$ and $p_U = 0.05$, respectively). In LEN-treated patients, these rates did not differ significantly from donor values.

The percentage of E-MDSCs in patients at diagnosis was more than 2 times lower than in donors ($p_U = 0.048$). In patients with BTZ-based regimens, the relative number of E-MDSCs was higher than in individuals at diagnosis ($p_U = 0.05$) and comparable with donors. In the group of LEN-treated patients, E-MDSC counts were detected at the same level as in BTZ-based regimens.

PMN-MDSC counts remained intact in MM patients at diagnosis and in those receiving different IT regimens and were comparable with donor values.

Thus, it can be concluded that MM patients at diagnosis are characterized by increased M-MDSC counts and reduced count of E-MDSCs, while BTZ-based regimens are associated with still high level of M-MDSCs but comparable E-MDSC counts to donor values. In patients receiving LEN-based regimens in combination with BTZ, both M-MDSC and E-MDSC counts were approximately equal to donor values.

2.3. Counts of MDSC subsets are associated with response to therapy and change in relapsed/refractory MM

To evaluate whether the number of MDSCs in MM patients is associated with response to therapy and the achievement of remission, we compared MDSC counts in patients who responded to IT with a complete response (CR) or a very good partial response (VGPR) ($n = 22$), or with a partial response (PR; $n = 25$). In addition, we analyzed circulating MDSCs in relapsed or resistant to ongoing IT patients (relapsed/refractory MM, $n = 11$, including three deaths developed in the coming months).

Patients with CR/VGPR and PR did not differ in MDSC subsets (Fig. 1D). Given the increased levels of M-MDSCs at diagnosis and in the treated patients, we anticipated seeing a further increment in MDSCs in relapsed/refractory MM. However, we saw a trend towards a decrease in the frequency of M-MDSCs compared to patients with CR/VGPR ($p_U = 0.10$) and a significant decrease in absolute M-MDSC counts by more than 2 times compared to patients with CR/VGPR and PR (for both $p_U < 0.05$; Fig. 1D).

In the group of patients with relapsed/refractory MM (Fig. 1D), we found a diminished relative and absolute E-MDSC counts compared to patients with CR/VGPR ($p_U = 0.002$ and $p_U = 0.0004$, respectively) and PR ($p_U = 0.038$ and $p_U = 0.011$, respectively), as well as compared with healthy donors ($p_U = 0.008$ and $p_U = 0.006$, respectively). At the same time, relapsed/refractory MM patients had the highest levels of relative and absolute G-MDSC counts, which exceeded donor levels.

Thus, the progression of MM was not associated with an increase in circulating MDSC counts but, on the contrary, was related to a decrease in both E-MDSC frequency and absolute M-MDSC count with persistently elevated counts of G-MDSCs.

Given the heterogeneity of the analyzed patients depending on the stage and IT courses, which in turn could also affect the content of MDSCs in patients in remission and with relapsed/refractory MM, we performed a multivariate analysis of variance. As can be seen from Table 1, the MM stage was the most significant factor in the expansion of E-MDSCs in peripheral blood.

2.4. Frequencies of M-MDSCs expressing intracellular Arg-1 are reduced in MM patients with remission

In the healthy donor group, more than 90% of PMN-MDSCs intracellularly expressed Arg-1 (Fig. 2). Nearly a third of PMN-MDSCs also expressed intracellular IDO and surface PD-L1, which could potentially mediate their suppressive activity. In M-MDSCs and E-MDSCs, the highest frequencies were found for PD-L1$^+$ cells (Me 20.5 and 20.2%, respectively), while relative counts of Arg-1$^+$ and IDO$^+$ cells were lower.

Arginase-1$^+$ cells among M-MDSCs were significantly diminished in MM patients after IT compared with donors ($p_U = 0.045$; Fig. 2A). The proportion of these cells in patients with stage II was decreased as a trend ($p_U = 0.12$), while in patients with stage III, the decline was more pronounced and significant ($p_U = 0.045$). In other MDSC subsets, there was no significant differences in the proportion of Arg-1$^+$ cells. The content of Arg-1$^+$ cells in E-MDSCs did not differ from their counterparts in healthy donors. The frequencies of IDO$^+$ and PD-L1$^+$ cells in MDSC populations did not differ between MM patients and donors (Fig. 2A).

Thus, of all the suppressor molecules analyzed, the only marker associated with MM severity was a decrease in the proportion of

Table 1
Multivariate analysis of clinical parameters for frequency of circulating MDSCs in MM patients.

Factorial ANOVA	M-MDSCs		PMN-MDSCs		E-MDSCs	
	F-value	p-value	F-value	p-value	F-value	p-value
MM Stage	1.09	0.30	1.19	0.28	5.62	0.023
Response	0.68	0.41	0.09	0.76	0.58	0.45
IT regimens	0.18	0.67	0.61	0.44	0.04	0.84
MM Stage* Response	1.66	0.20	0.68	0.41	5.28	0.027
MM Stage* IT regimens	0.61	0.44	0.11	0.74	4.27	0.046
Response* IT regimens	0.03	0.85	0.14	0.71	0.66	0.42
MM Stage* Response* IT regimens	0.07	0.79	0.35	0.55	0.37	0.55

ANOVA – Analysis of variance; M-MDSCs – monocytic myeloid-derived suppressor cells; PMN-MDSCs – polymorphonuclear myeloid-derived suppressor cells; E-MDSCs – early-stage myeloid-derived suppressor cells; IT – Induction therapy.

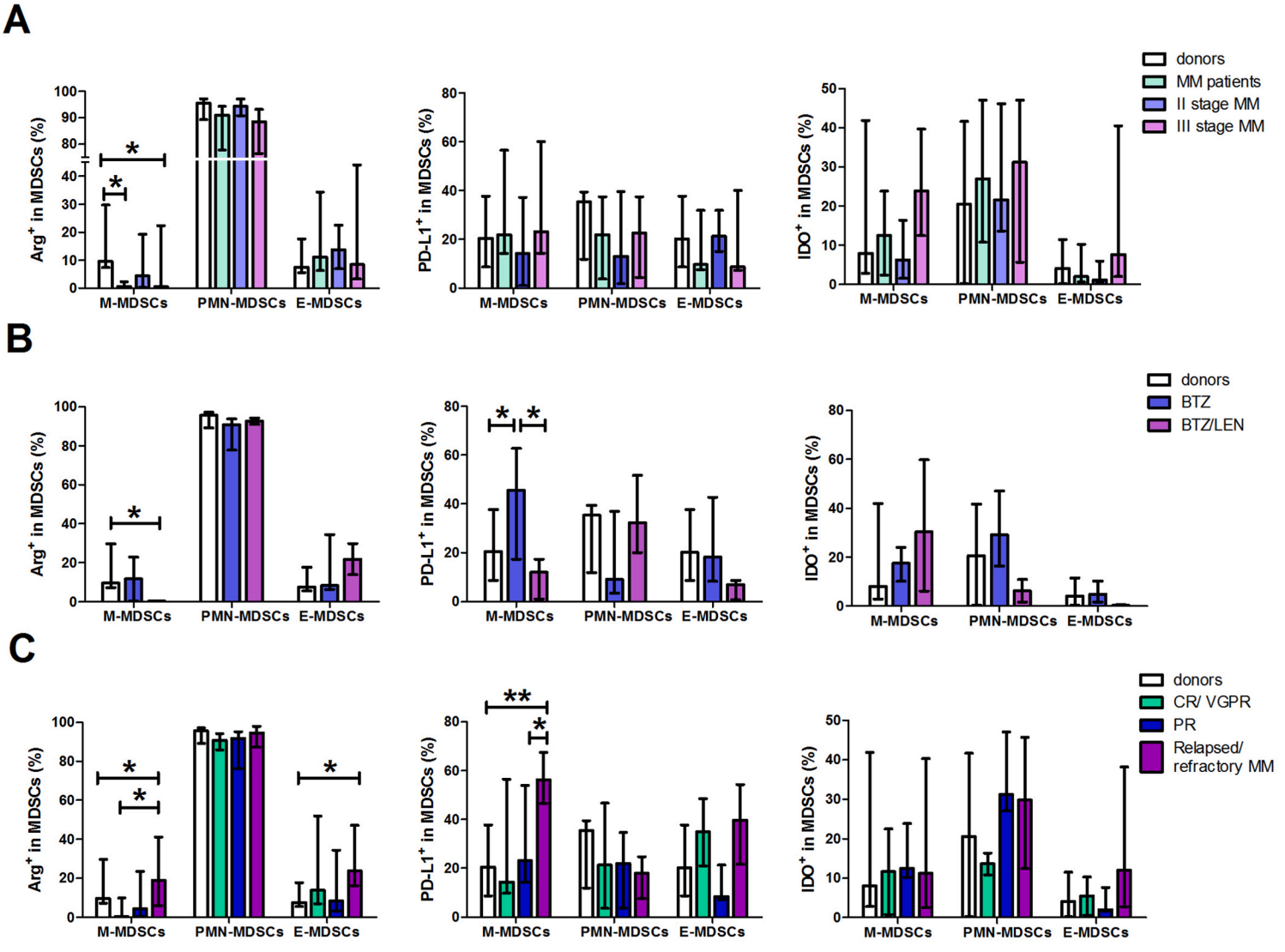

Fig. 2. Suppressor molecule expression in myeloid-derived suppressor cells of patients with multiple myeloma **A.** Expression of Arg1, PD-L1 and IDO molecules in MDSC subsets of patients with remission following IT depending on MM stage (II stage MM, n = 7; III stage MM, n = 13) and healthy donors (n = 13). **B.** Expression of Arg1, PD-L1 and IDO molecules in MDSC subsets of MM patients depending previous lines of induction therapy; BTZ regimens, n = 7; BTZ/LEN regimens, n = 13. **C.** Expression of Arg1 and PD-L1 molecules in MDSC subsets of MM patients depending on the response to IT; CR/VGPR, n = 8; PR, n = 12; relapsed/refractory MM, n = 9. Data on suppressor molecule expression are presented as Me and IQR of the percentage of positive cells among MDSC subsets. * – p_U value < 0.05; ** – p_U value < 0.01.

Arg-1$^+$ cells in M-MDSCs.

2.5. Frequencies of M-MDSCs expressing intracellular Arg-1 are decreased in MM patients in remission who received lenalidomide-based induction therapy, while an increase in Arg-1 and PD-L1 expression in M-MDSCs and E-MDSCs is associated with relapsed/refractory MM

Further, we analyzed the expression of suppressive molecules depending on the type of therapy. The frequency of M-MDSCs expressing Arg-1 was significantly lower in the group of LEN-treated patients compared to healthy donors (Me 0.27 *vs.* 11.7%; $p_U = 0.016$). No decrease in Arg-1$^+$ M-MDSCs was observed in patients treated with BTZ. At the same time, a feature of this group was a more than 2-fold increase in the number of PD-L1$^+$ cells in M-MDSCs (*vs.* donors $p_U = 0.03$; Fig. 2B). Patients treated with LEN-based regimens showed a significantly declined count of PD-L1$^+$ cells in M-MDSCs ($p_U = 0.013$) compared with the group of patients who received BTZ-based regimens.

We did not reveal any significant differences in the expression of suppressive molecules between patients with CR/VGPR and PR (Fig. 2C).

In contrast to remission, Arg-1$^+$ cells in M-MDSCs and E-MDSCs were increased in patients with MM progression compared with healthy donors ($p_U = 0.05$ and $p_U = 0.026$, respectively; Fig. 2C). The number of Arg-1-expressing M-MDSCs in patients with relapsed/refractory MM was also significantly higher compared with patients with CR/VGPR (Me 18.9 vs. 0.35%; $p_U = 0.02$).

Another feature was the increased expression of PD-L1 in M-MDSCs in patients with relapsed/refractory MM. More than half of M-MDSCs in patients of that group expressed PD-L1 (Me vs. donors, 56.2 vs. 20.5%, $p_U = 0.009$). The proportion of PD-L1$^+$ M-MDSCs in these patients was also significantly higher than in patients with PR ($p_U = 0.042$).

Thus, decreased expression of Arg-1 in M-MDSCs in patients with remission is associated primarily with LEN-based IT regimens. With progression (relapsed/refractory MM), the suppressor potential of MDSCs increases, which is manifested by an increase in the number of Arg-1$^+$ cells in both M-MDSCs and E-MDSCs, as well as an increase in the proportion of PD-L1$^+$ M-MDSCs.

Using multivariate analysis of variance, we attempted to identify the most significant factors associated with the suppressive potential of MDSCs in MM. As can be seen from Table 2, the treatment regimen was a significant factor for PD-L1$^+$ M-MDSC frequency as well as a trend for Arg-1$^+$ M-MDSCs. The combination of MM stage factors and response to ongoing therapy had an interaction effect on Arg-1 expression in E-MDSCs as a trend.

2.6. G-CSF-induced mobilization of HSCs is associated with the expansion of all types of MDSCs and an increase in the percentage of PMN-MDSCs and M-MDSCs expressing Arg-1 and PD-L1

Administration of G-CSF in combination with cyclophosphamide during HSC mobilization led to a significant increase in all three analyzed MDSC subsets in PB of MM patients. As can be seen from Fig. 3A, the most pronounced increase in relative (more than 100-fold) and absolute MDSC counts was observed for PMN-MDSCs. The relative number of circulating M-MDSCs directly correlated with their counterparts in the apheresis product (Fig. 3D; $R = 0.571$, $p = 0.021$; other MDSCs in the apheresis product were not determined due to their sensitivity to cryopreservation [31]).

As shown in Fig. 3B, an increase in the frequency of M-MDSCs in peripheral blood after G-CSF-induced stimulation was accompanied by a more than 20-fold increment in Arg-1$^+$ cells ($p_W = 0.015$). The expression of other suppressive molecules in M-MDSCs did not change. Although there was an increase in the amount of inhibitory molecules in E-MDSCs, it did not reach statistical significance. In PMN-MDSCs, on the contrary, a decrease in the expression of all analyzed suppressive molecules (statistically insignificant) was detected, which may be associated with the accumulation in the circulation of immature granulocytes with the same phenotype as PMN-MDSC. However, the total proportion of circulating cells with PMN-MDSC phenotype and M-MDSCs expressing Arg-1 and PD-L1 were increased after G-CSF-induced mobilization ($p_W = 0.0013$ and $p_W = 0.0002$, respectively, for Arg$^+$ cells and $p_W = 0.008$ and $p_W = 0.01$, respectively, for PD-L1$^+$ cells) (Fig. 3C). No changes were found for IDO expression (data not shown in the figure).

Thus, it can be concluded that G-CSF-induced HSC mobilization is accompanied by the expansion of all types of MDSCs, including cells with the phenotypes Lin$^-$DR$^-$CD33$^+$CD66$^+$Arg$^+$ and Lin$^-$DR$^-$CD33$^+$CD66$^+$PD-L1$^+$, CD14$^+$HLA-DR$^{low/-}$Arg$^+$ and CD14$^+$HLA-DR$^{low/-}$ PD-L1$^+$, as well as up-regulation of Arg-1 expression in M-MDSCs. Since these cells, primarily M-MDSCs, are further collected into the apheresis product, it can be assumed that MDSCs may affect auto-HSCT outcomes in MM patients.

Table 2
Multivariate analysis of clinical parameters for frequency of circulating Arg- and PD-L1-expressing MDSCs in MM patients.

Factorial ANOVA	Arg-1$^+$ cells in M-MDSCs (%)		Arg-1$^+$ cells in E-MDSCs (%)		PD-L1$^+$ cells in M-MDSCs (%)	
	F-value	p-value	F-value	p-value	F-value	p-value
MM Stage	0.09	0.77	1.66	0.24	0.29	0.60
Response	0.12	0.73	0.74	0.42	0.12	0.73
IT regimes	2.76	0.12	0.0005	0.98	5.20	0.046
MM Stage* Response	0.80	0.38	3.05	0.13	0.41	0.54
MM Stage* IT regimens	0.0004	0.98	0.0011	0.97	0.09	0.77
Response* IT regimens	0.30	0.59	–	–	0.38	0.55

ANOVA – Analysis of variance; M-MDSCs – monocytic myeloid-derived suppressor cells; E-MDSCs – early-stage myeloid-derived suppressor cells; IT – Induction therapy.

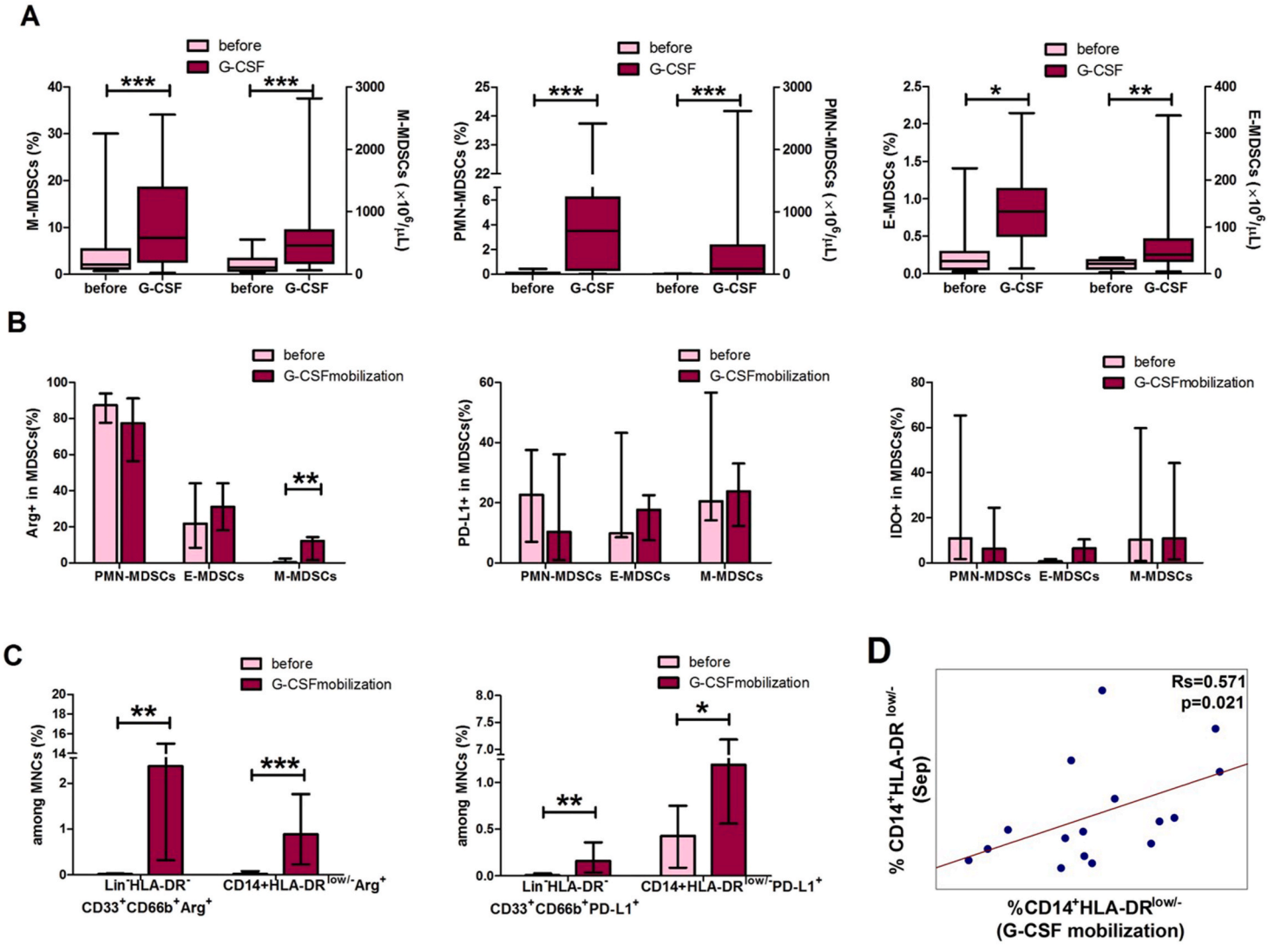

Fig. 3. Effect of G-CSF-induced HSC mobilization on myeloid-derived suppressor cells in patients with multiple myeloma **A.** Relative (left Y-axis) and absolute (right X-axis) counts of MDSC subsets are shown as Me and IQR values in peripheral blood of MM patients before HSC mobilization (before; n = 33) and after systemic administration of G-CSF with cyclophosphamide (G-CSF; n = 33). **B.** Expression of Arg1, PD-L1 and IDO molecules in MDSC subsets are shown as Me and IQR values in peripheral blood of MM patients before and after HSC mobilization (n = 14). **C.** Total proportion of circulating cells with PMN-MDSC and M-MDSC phenotypes expressing Arg-1 (left graphic) and PD-L1 (right grapfic) among MNCs are shown as Me and IQR values in peripheral blood of MM patients before and after HSC mobilization (n = 14). **D.** Correlation between percentage of M-MDSCs in peripheral blood after G-CSF-induced mobilization and in apheresis product (Sep).
* – p_W value < 0.05; ** – p_W value < 0.01; *** – p_w value < 0.001.

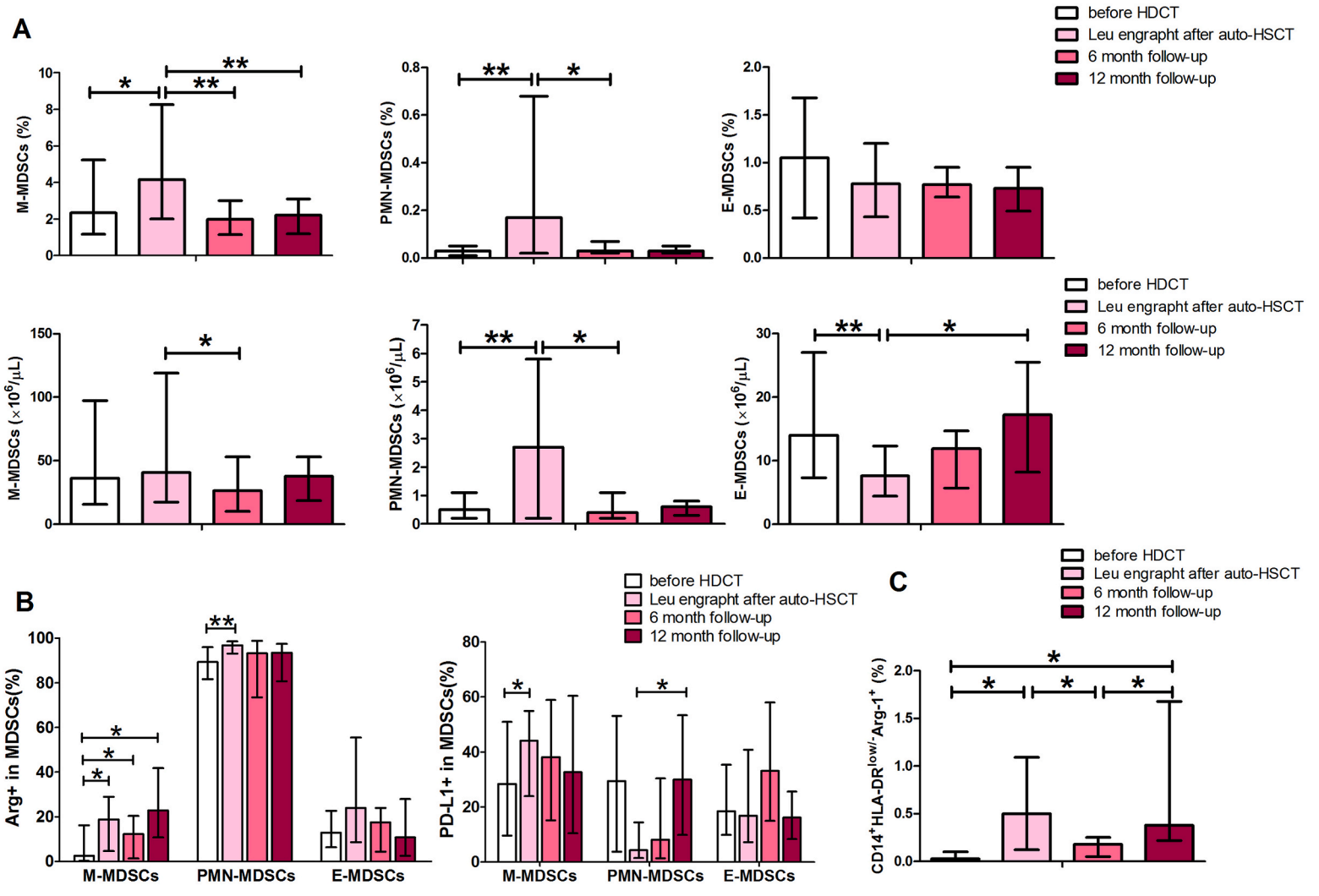

Fig. 4. Myeloid-derived suppressor cells in peripheral blood of multiple myeloma patients underwent auto-HSCT **A.** Relative (upper rows) and absolute (bottom rows) counts of M-MDSC, PMN-MDSC, and E-MDSC are shown as Me and IQR values in peripheral blood of MM patients before HDCT (n = 39), at the leukocyte recovery after auto-HSCT (peripheral leukocyte counts $<1 \times 10^9$/L; Leu engrapht; n = 56), after 6 (n = 21) and 12 months (n = 17) after auto-HSCT. B. Expression of Arg1 (left graphic) and PD-L1 (right graphic) molecules in MDSC subsets are shown as Me and IQR values in peripheral blood of MM patients before HDCT (n = 14), at the leukocyte recovery after auto-HSCT (Leu engrapht; n = 35), after 6 (n = 13) and 12 months (n = 10) after auto-HSCT. C. Total proportion of circulating cells with M-MDSC phenotype expressing Arg-1 ($CD14^+HLA\text{-}DR^{low/-}Arg\text{-}1^+$) among MNCs are shown as Me and IQR values in peripheral blood of MM patients before HDCT and after auto-HSCT.

* – p_U value < 0.05; ** – p_U value < 0.01.

2.7. Auto-HSCT is associated with transient changes in the proportions of circulating MDSCs with the subsequent recovery following 12 months to pre-transplant values

At the next stage of the study, we evaluated the dynamics of MDSC recovery in patients who received high-dose chemotherapy and auto-HSCT. Of note, from the time of the HSC mobilization until HSC transplantation (pre-HSCT), the percentage and absolute number of all MDSC populations among PBMCs significantly decreased to baseline values of MM patients prior to G-CSF-induced HSC mobilization.

After patients underwent auto-HSCT, relative counts of circulating M-MDSCs and PMN-MDSCs increased almost 2-fold by the time of leukocyte recovery ($p_U = 0.038$ and $p_U = 0.003$, respectively; Fig. 4A). The absolute count of PMN-MDSCs also significantly increased ($p_U = 0.005$), despite the reduced number of PBMCs compared to the pre-transplant period (pre-HSCT) ($p_U < 0.0001$).

The correlation analysis did not reveal any relationship between the counts of M-MDSCs in the apheresis product and in PB at post-transplant, which could evidence the contribution of re-infused progenitor cells to the formation of the total pool of M-MDSCs after auto-HSCT. Thus, it can be assumed that the recovery of MDSCs at early post-transplant is not associated with the expansion of re-infused autograft MDSCs.

At 6-month follow-up post-transplant, the proportions of circulating M-MDSCs and PMN-MDSCs significantly decreased ($p_U = 0.007$ and $p_U = 0.024$, respectively) to pre-transplant values and remained at the same level at 12-month follow-up.

The recovery dynamics of the absolute M-MDSC and PMN-MDSC counts at post-transplant was similar; however, significant changes were found only for PMN-MDSCs. At 6 months post-transplant, during the recovery of the absolute PBMC count, the absolute number of PMN-MDSCs declined ($p_U = 0.02$) and in the next 6 months it remained without significant changes.

On the contrary, the relative and absolute counts of E-MDSCs at the engraftment were decreased in comparison with their counterparts before HDCT (statistically significant for absolute values, $p_U = 0.004$). The proportion of circulating E-MDSCs among PBMCs remained at the same level during the 12-month post-transplant period, while the absolute count of E-MDSCs also increased in parallel with a gradual increment in the absolute PBMC count (at the engraftment vs. 12-month follow-up, $p = 0.032$), but did not exceed pre-transplant values.

In general, after HDCT and auto-HSCT, while PBMCs are decreased at the engraftment, the frequency of M-MDSCs and the relative and absolute counts of PMN-MDSCs are increased. At 6 months after transplantation, in patients with a sustained clinical response, the counts of M-MDSCs and PMN-MDSCs are decreased to pre-transplant values and retain this level for the next 6 months. The content of E-MDSCs is reduced at the engraftment, the relative count remains at the same level up to 12 months post-transplant, and the absolute number is increased at 12 months after transplantation to the initial pre-transplant values.

2.8. The proportions of arginase-1-expressing PMN-MDSCs and M-MDSCs are increased in the post-transplant period

As noted above, M-MDSCs and E-MDSCs of MM patients at the time of mobilization were characterized by reduced Arg-1-expressing cells; their level then significantly increased after G-CSF administration. However, by the time of HDCT and auto-HSCT, monocytic and early MDSCs downregulated Arg-1 production (vs. G-CSF post-administration, $p_U = 0.066$ and $p_U = 0.17$, respectively). After auto-HSCT, the proportion of Arg-1-positive M-MDSCs at the engraftment was increased ($p_U = 0.026$; Fig. 4B). By the time of 6-month and 12-month follow-up, the number of Arg-1-positive M-MDSCs remained significantly higher compared to baseline values before HSC mobilization (Me 12.3 and 22.1% vs. 2.6%, $p_U = 0.046$ and $p_U = 0.025$, respectively). In addition, while the total proportion of circulating M-MDSCs declined, the frequency of 14^+HLA-$DR^{low/-}$Arg-1^+ cells was increased. After 12 months post-transplant, relative counts of M-MDSCs became significantly higher compared to the pre-transplant level (Me(IQR) 0.38 (0.22–1.68) vs. 0.03 (0.01–0.1) %; $p_U = 0.0015$; Fig. 4C), and reached donor values ($p_U = 0.24$).

The proportion of Arg-1-positive cells among PMN-MDSCs was increased significantly at the engraftment (vs. before HDCT, $p = 0.007$; Fig. 4B) and, within 12 months, it remained consistently high (>90%). The proportion of Arg-1-positive E-MDSCs was transiently increased at the engraftment, with a subsequent gradual decline after 6 and 12 months to the initial values; however, these changes did not reach statistical significance.

Table 3
Duration of leukopenia depending on the content of circulating MDSCs at the engraftment in MM patients.

M-MDSCs (%)				M-MDSC (× 10^6/μl)		
	Higher Me	Lower Me	p_U	Higher Me	Lower Me	p_U
Duration of leukopenia, days	11 (11–12)	13 (11–15)	0.0086	11 (10–12)	13 (12–15)	0.0004
PMN-MDSCs (%)				***PMN-MDSC (× 10^6/μl)***		
	Higher Me	Lower Me	p_U	Higher Me	Lower Me	p_U
Duration of leukopenia, days	12 (10–14)	12 (11–14)	0.46	12 (10–13)	12 (11–14)	0.48
E-MDSCs (%)				***E-MDSC (× 10^6/μl)***		
	Higher Me	Lower Me	p_U	Higher Me	Lower Me	p_U
Duration of leukopenia, days	12 (12–14)	12 (11–14)	0.39	12 (10–12)	13 (11–14)	0.08

M-MDSCs – monocytic myeloid-derived suppressor cells; PMN-MDSCs – polymorphonuclear myeloid-derived suppressor cells; E-MDSCs – early-stage myeloid-derived suppressor cells; Me – Median values.

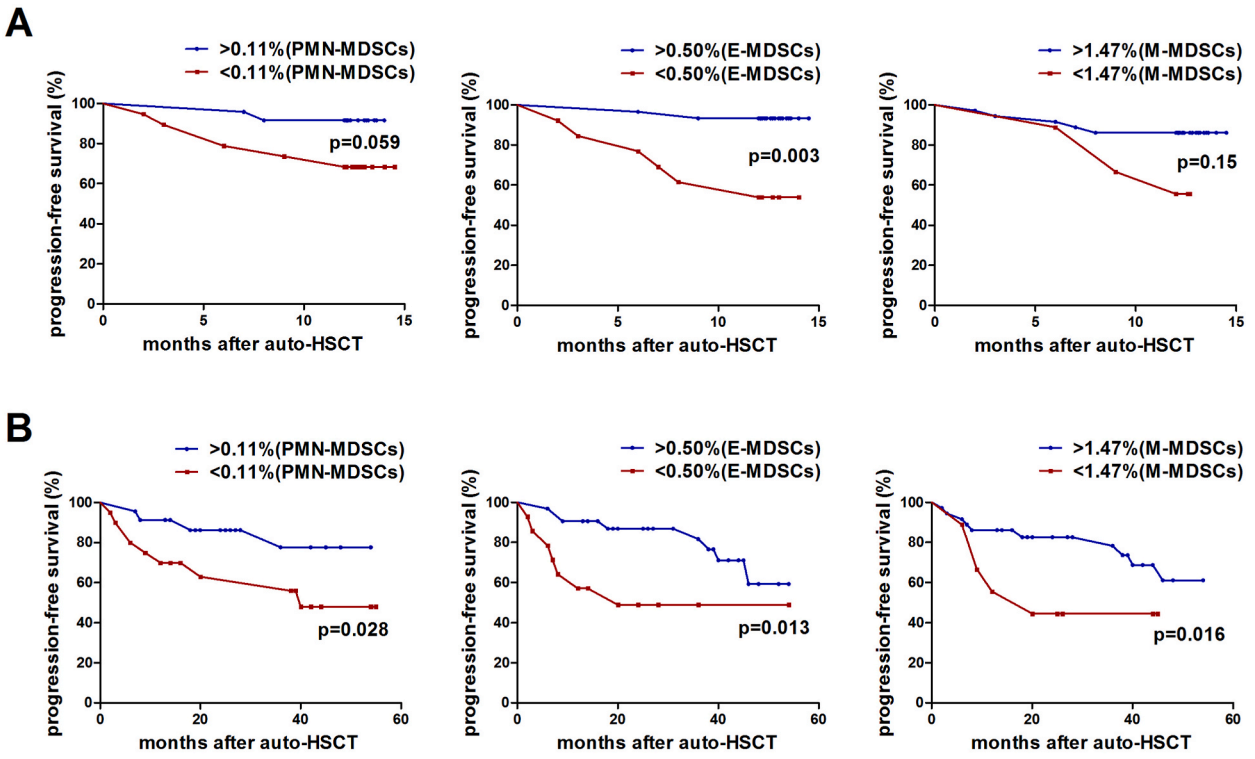

Fig. 5. Progression-free survival in multiple myeloma patients with auto-HSCT **A.** Survival to MM relapse/progression after HDCT followed with auto-HSCT at 12-month follow-up regarding with relative number of MDSC subsets at the leukocyte engraftment. B. Survival to MM relapse/progression after HDCT followed with auto-HSCT at total follow-up regarding with relative number of MDSC subsets at the leukocyte engraftment. >Me – patients with the values of the indicated MDSC subsets above the median at the engraftment (blue curve); <Me – patients with the values of the indicated MDSC subsets below the median at the engraftment (red curve). p – significance between the curves.

Table 4
The Cox proportional hazards regression analysis of post-transplant MM relapse dependence on clinical and laboratory factors.

Variable	Univariate Cox analysis			Multivariate Cox analysis		
	B (SE)	HR	p	B(SE)	HR	p
MM Stage (II vs III)	0.84 (0.67)	0.900	0.21	1.96 (0.86)	1.610	0.02
Depth of response (CR vs PR)	1.09 (0.57)	4.56	0.057	1.79 (0.84)	9.01	0.03
Lymphopenia duration (<12d vs > 12d)	−0.20 (0.60)	0.903	0.74	−0.28 (0.76)	1.77	0.72
MRD before HDCT (+ vs –)	−1.37 (0.94)	0.003	0.15	0.01 (1.18)	0.02	0.99
Maintenance therapy (BTZ vs LEN)	0.59 (0.59)	1.10	0.32	−0.41 (0.75)	0.39	0.58
M-MDSCs (>1.47 vs ≤ 1.47%)	1.16 (0.62)	4.26	0.059	1.39 (0.89)	4.79	0.12
PMN-MDSCs (>0.11 vs ≤ 0.11%)	1.13 (0.58)	4.58	0.049	1.33 (0.71)	3.28	0.06
E-MDSCs (>0.50 vs ≤ 0.50%)	1.29 (0.54)	5.11	0.017	1.38 (0.64)	3.06	0.03

B – regression coefficient; SE – Standard Error; HR – hazard ratio; BTZ – patients who achieved remission during therapy with BTZ-based regimens; BTZ/LEN – patients resistant to therapy with BTZ-regimens and treated with lenalidomide; CR – Complete response; PR – partly response; MRD – Minimal residual disease status; HDCT – high-dose chemotherapy.

The relative count of PD-L1-positive M-MDSCs was significantly increased at the engraftment ($p_U = 0.029$), and M-MDSCs were the main population among circulating MDSCs that expressed this molecule (Fig. 4B). Subsequently, PD-L1 expression in M-MDSCs was moderately decreased ($p_U = 0.076$). On the contrary, in PMN-MDSCs the proportion of PD-L1-positive cells at the engraftment was declined (statistically insignificant); however, in the next 12 months the level of these cells was restored to pre-transplant values. E-MDSCs were characterized by transient changes in PD-L1 expression; however, we did not find statistically significant differences between time points. At 12-month follow-up, PD-L1-expressing subsets of all three MDSC populations were comparable with the corresponding pre-transplant values.

The expression of IDO in MDSCs after HDCT and auto-HSCT changed transiently. A significant increase in IDO-positive cells at the engraftment was observed only in E-MDSCs (vs. before HDCT, $p_U = 0.025$), and their counts were significantly decreased at the following time points. In general, frequencies of IDO-expressing in MDSCs after 6 and 12 months did not differ significantly from their counterparts before HDCT and auto-HSCT (Suppl_Figure 2).

So, the counts of Arg-1-positive M-MDSCs and PMN-MDSCs, PD-L1-positive M-MDSCs, and IDO-positive E-MDSCs are increased at the engraftment in MM patients. During the next 12 months, the expression of Arg-1 in M-MDSCs and PMN-MDSCs retains consistently high levels, while the expression of other molecules in MDSCs generally reaches pre-transplant values, not exceeding those in the donor group.

2.9. A higher content of circulating M-MDSCs at the engraftment is associated with a shorter period of critical leukopenia after HDCT and auto-HSCT

To evaluate a possible relationship between MDSC content and leukocyte recovery after HDCT and auto-HSCT, we compared the duration of leukopenia in patients with high (>Me) and low (<Me) counts of circulating MDSCs. As can be seen from Table 3, patients with higher levels of M-MDSCs (relative and absolute) were characterized by a shorter period of leukopenia.

Multiple regression analysis showed the significant influence (coefficient of determination $R^2 = 0.83$, F-criterion = 2.66, $p = 0.049$) of the duration of lymphopenia after HDCT and auto-HSCT in patients with MM on the relative count of circulating M-MDSCs at the engraftment ($p = 0.014$). The proportion of circulating PMN-MDSCs and E-MDSCs, according to regression analysis, did not affect the duration of leukopenia ($p = 0.14$ and $p = 0.85$, respectively). For the absolute number of M-MDSCs, a similar pattern was revealed, but at the trend level ($p = 0.073$).

Generally, it can be assumed that a higher content of MDSCs, primarily M-MDSCs, at the stage of early reconstitution is associated with a more effective leukocyte recovery after HDCT and auto-HSCT.

2.10. A lower content of E-MDSCs at the engraftment is one of the risk factors for MM recurrence after HDCT and auto-HSCT

Considering the evaluated relationship between the content of MDSCs and the efficacy of leukocyte recovery, we finally analyzed the progression-free survival depending on MDSC counts at the engraftment.

Using ROC analysis, optimal threshold values were determined for the relative and absolute counts of each MDSC subset at the engraftment (Supplementary Table 2). It is noteworthy that for each MDSC subset, the threshold values were below the medians. Next, using the selected thresholds, a Kaplan-Meier disease-free survival analysis with the log-rank test was performed.

Fifty-five patients were included in the progression-free survival analysis; ten individuals had not yet reached the 12-month follow-up, and nine individuals had MM recurrence in the first 12 post-transplant months; two and four patients relapsed in the second year (after 18 and 20 months) and more than 36 months after HDCT and auto-HSCT, respectively. The median observation period after auto-HSCT was 26 months (IQR 14–44 months).

In the 12-month follow-up group (Fig. 5A), patients with higher MDSC counts at the engraftment were characterized by lower rates of early relapses. Thus, the maintenance of remission in the group with PMN-MDSCs >0.11% was recorded more often than in patients with PMN-MDSCs ≤0.11% (91.3% vs. 68.4%; $p_{log\text{-}rank} = 0.059$). Similarly, the proportion of patients with a sustained response in the group with E-MDSCs >0.50% was higher than in patients with E-MDSCs ≤0.50% (93.1% vs. 53.8%; $p_{log\text{-}rank} = 0.003$). For populations of M-MDSCs, a similar association of higher cell count (>1.47%) with a low recurrence rate in 12 post-transplant months was established (86.5% vs. 42.9%, $p_{log\text{-}rank} = 0.016$). In the group of patients with the content of M-MDSCs below the cut-off threshold (≤1.47%), a median survival time (10.5 months) was achieved.

The revealed patterns generally retained when analyzing progression-free survival in the general group with the observation period from 2 to 60 months (Fig. 5B).

Differences in relapse-free rates between patients with PMN-MDSCs >0.11% and PMN-MDSCs ≤0.11% reached statistical significance (73.7 vs. 45.7%, $p_{log\text{-}rank} = 0.028$). In the patients with PMN-MDSC count below the cut-off threshold, a median survival time (38.9 months) was achieved. Differences in the frequency of MM patients with remission depending on the content of E-MDSCs above and below the cut-off, retained statistical significance (93.1 vs. 53.8%, $p_{log\text{-}rank} = 0.028$). The median survival time (12.3 months) was also achieved in the group of patients with E-MDSCs ≤0.50%. Progression-free survival in patients with M-MDSCs ≤1.47% was lower at the trend level compared to the patients with M-MDSCs >1.47% (42.9 vs. 58.4%, $p_{log\text{-}rank} = 0.15$).

The Cox regression analysis (Table 4) showed that more advanced MM stage, depth of response (PR) before HDCT and auto-HSCT, and the relative count of circulating E-MDSCs at the engraftment (≤0.50%) were independent significant risk factors associated with lower progression-free survival.

3. Discussion

In our study, we tried to assess the clinical and prognostic significance of MDSCs in MM by evaluating the content of various subsets of these cells and their functional potential, depending on the clinical course of the disease and treatment regimens. Firstly, it should be emphasized that there were multidirectional changes in distinct MDSC subsets. The increase in counts of M-MDSCs and PMN-MDSCs was accompanied by the decrease in E-MDSCs. Changes in all three subsets were more pronounced in patients with stage III, while in stage II they reached statistical significance only for M-MDSCs. These data indicate an association of MDSCs with disease severity, which was confirmed by multivariate analysis for E-MDSCs. Comparison of MDSC counts in patients at diagnosis and in remission after IT made it possible to evaluate the effect of BTZ and LEN therapy on MDSCs. Based on the data obtained, BTZ therapy led to the increment in E-MDSCs (to normal values) with no changes in the number of M-MDSCs or PMN-MDSCs, while the use of LEN (in the case of resistance to BTZ) was associated with the increase in E-MDSCs and the decline in M-MDSCs (to normal values). When comparing MDSC counts in patients with remission and relapsed/refractory MM, we did find a direct association of progression with a significant

decrease in E-MDSC count, which apparently reflects the fact of more aggressive therapy in these patients. Simultaneously, relapsed or refractory MM patients displayed the highest levels of PMN-MDSCs, with a content of M-MDSCs comparable to donors. Previous studies of MDSCs in MM predominantly detected increased counts of PMN-MDSCs at diagnosis and in patients with relapsed/refractory MM [32–36]. Besides, Favaloro et al. demonstrated an increased PMN-MDSC content in patients with complete response [37], which is consistent with our data. Fewer publications reported increased numbers of M-MDSCs in MM [32,38,39]. Moreover, in distinct studies, the authors found no changes in the content of M-MDSCs, not only in patients with remission but also in primary MM [34,40]. The absence of differences may be due to the features of M-MDSCs gating, in particular, the evaluation of these cells during cytometric analysis among myeloid cells or monocytes [39]. Thus, Wang Z. et al. noted an increased monocyte count even in stable disease [39]. It is possible that the differences in M-MDSC frequencies between donors and MM patients would be more sufficient when analyzing them in a total PBMC population.

Several publications indicate that the number of PMN-MDSCs and M-MDSCs is associated with the severity of MM (ISS stage, renal function) [35,39], which is consistent with our data. At the same time, we for the first time characterized in details a population of E-MDSCs, which had been described much poorer in MM patients. Increased counts of M-MDSCs and PMN-MDSCs and a reduced number of immature E-MDSCs at the more advanced MM stage (stage III) may be a consequence of a higher tumor burden, accompanied by an increment in concentration of various cytokines, including IL-6, IL-10, and TGFβ, which are important for the generation of MDSCs [41,42]. Given that MDSC populations originate from a common myeloid progenitor, these findings may be explained by the more pronounced production of tumor-derived factors that stimulate pathological activation and differentiation of MDSCs into more mature forms at the advanced stages of MM. We showed that E-MDSCs were drastically decreased in patients with newly diagnosed MM and then were leveled in patients who had achieved remission. A similar effect of induction therapy on the recovery of absolute E-MDSCs was described by Lee S.-E. et al. [19]. *In vitro* studies showed that LEN in PBMC culture hindered MDSC generation by simultaneously activating the transcription factor regulating interferon 8 (IFR8), which directs the differentiation of myeloid precursors towards monocytes, and blocking the CCL5/CCR5 axis involved in MDSC expansion during MM [43].

The second important finding of our study was that the increased content of M-MDSCs after induction therapy was accompanied with an attenuating of their suppressive potential due to a decrease in Arg-1 expression. A decline in Arg-1^{+} cells in M-MDSCs was established for patients who received the LEN-containing IT regimen and was not detected with BTZ therapy. Similar data were

Table 5
Patient characteristics.

Characteristics	Patients n (%) (total n = 112)
Age, years, median (range)[a]	55 (50–60)
Patient gender (female/male)	66/46
Serum M-protein	
IgG, kappa	50 (45%)
IgG, lambda	17 (15%)
IgG, kappa + lambda	3 (3%)
IgA, kappa	11 (10%)
IgA, lambda	4 (4%)
n.a.	10 (9%)
Bence-Jones protein	16 (14%)
Durie-Salmon stage	
II	32 (28%)
III	77 (69%)
n.a.	3 (3%)
Renal function	
Normal renal function (A group)	91 (81%)
Renal inadequacy (B group)	13 (12%)
n.a.	8 (7%)
Pre-transplant induction therapy	
Bortezomib-based (PAD, VCD, VD)	67 (60%)
Lenalidomide-based (VRD, RD, RCD, monotherapy)	26 (23%)
At diagnosis	6 (5%)
n.a.	13 (12%)
Response to induction therapy (%)	
CR/VGPR	37 (35%)
PR	58 (55%)
Relapsed/Refractory MM	11 (10%)
Duration from diagnosis to auto-HSCT, months, median (range)	10.0 (8.0–14.5)

Data are presented as number (n) of patients with frequencies (percentage) in parentheses. [a] – patient age at the time of performing experiments.
n.a., not applicable/not assessed.
CR – complete remission; PR – partial response; VGPR – very good partial response.
PAD – Bortezomib, Dexamethasone, Doxorubicin; VCD – Bortezomib, Cyclophosphamide, Dexamethasone; VD – Bortezomib and Dexamethasone; VRD – Bortezomib, Lenalidomide, Dexamethasone, RD – Lenalidomide and Dexamethasone; RCD – Lenalidomide, Cyclophosphamide, Dexamethasone.

obtained by Romano A. et al., who demonstrated a decrease in Arg-1 expression in PMN-MDSCs in MM patients receiving LEN but not BTZ, which may be associated with the immunomodulatory effect of LEN [32]. In turn, the progression of MM, according to our data, was characterized by increased proportions of Arg-1$^+$ cells in E-MDSCs and Arg-1$^+$ and PD-L1$^+$ cells among M-MDSCs, while counts of E-MDSCs and M-MDSCs themselves were declined. This may be sufficient for tumor immunosuppression and progression. Our data partially agree with previously published works. Increased Arg-1 production was found in PMN-MDSCs of PB and BM in relapsed/refractory MM patients [32,36]. Incremented PD-L1 expression in relapsed/refractory MM was shown for BM M-MDSCs [44]. According to our multivariate analysis, the most significant factors controlling the number of CD14$^+$HLA-DR$^{low/-}$PD-L1$^+$ cells were the regimen of therapy as well as a combination of regimen and response to therapy. The PD-1/PD-L1 signaling pathway plays a substantial role in MM by undermining the antitumor T cell response and is considered as a target for MM immunotherapy [44]. Expression of PD-L1 was detected both on immune cells and on MM cells [45]. The role of the PD-L1/PD-1 axis in T cell suppression has been described for MDSCs in various pathologies, and this mechanism seems to be intrinsic for M-MDSCs [20,46]. As patients with relapsed/refractory MM receive more aggressive regimens of therapy, the increase in PD-L1 expression in relapsed/refractory MM, on the one hand, indicates the resistance of the PD-L1/PD-1 axis to the therapy and, on the other hand, designates new potential targets for check-point inhibitors in MM.

Next, we showed the expansion of all MDSC populations, including PMN-MDSCs and M-MDSCs expressing Arg-1 and PD-L1, following G-CSF-induced mobilization of HSCs in MM patients. Previous studies have also demonstrated an increase in MDSCs after G-CSF administration [23,47]. Since the surface markers for identifying PMN-MDSCs and neutrophils overlap, it is possible that the pool of PMN-MDSCs also included immature neutrophils, despite the fact that we used a low density gradient to isolate PBMCs. This is also indicated by our data on slightly lower proportions of Arg-1-, PD-L1-, and IDO-expressing cells only in the pool of PMN-MDSCs. In any case, given the sensitivity of granulocytes to cryopreservation [31], M-MDSCs seem to be the only MDSC subset in the autograft that retains its activity after thawing and reinfusion.

In our opinion, the most important findings of the study were the associations of MDSCs with both the efficiency of leukocyte restoration and transplantation outcomes in MM patients. The counts of circulating M-MDSCs and PMN-MDSCs were transiently increased at the engraftment, followed by a gradual decrease to pre-transplant levels by the 12th post-transplant month. However, it is only the higher numbers of M-MDSCs at engraftment that are associated with a reduction in critical leukopenia. According to previous studies, CD34$^+$ cell count in the auto-graft [48–50], early disease stage at diagnosis [49], conditioning regimen, and patient age [51] were among the clinical factors affecting the efficiency and duration of hematopoietic recovery (assessed by neutrophil and platelet counts) after auto-HSCT in MM patients. Apparently, the content of M-MDSCs and leukopenia resolving is another biomarker associated with the efficiency of hematopoiesis recovery. The positive role of M-MDSCs in this case may be implemented by the formation/restoration of the HSC niche in BM of patients after HDCT.

In addition, we for the first time demostrated data on the association of a higher MDSC content at the engraftment with a lower frequency of relapses and, accordingly, a longer progression-free survival. Our results disagree with the findings of Lee S.-E. et al., who had shown an association between increased numbers of pre-transplant M-MDSCs and poor auto-HSCT prognosis. At the same time, the number of MDSCs after transplantation did not have prognostic significance [19]. Such discrepancies may be explained by differences in the induction therapy regimens and the clinical and laboratory characteristics of patients.

According to the Cox multivariate analysis performed, independent significant risk factors associated with a lower progression-free survival in our study were the depth of response (PR) before auto-HSCT and a reduced frequency of circulating E-MDSCs at the engraftment ($\leq$0.50%). Clinical studies have shown that the response to anticancer therapy is an important prognostic factor for MM, and the achievement of CR after the induction therapy is considered as a favorable prognostic factor [52,53]. Simultaneously, the prognostic significance of the increased content of circulating E-MDSCs at the stage of early reconstitution as a favorable factor in the outcome of auto-HSCT was demonstrated here for the first time.

Our results also indicate an increase in the proportion of Arg-1-expressing M-MDSCs after auto-HSCT. Apparently, cells with immunosuppressive potential seems to play a positive role during leukopenia recovery, in accordance with the trend we found towards lower proportions of M-MDSCs expressing Arg-1 in patients with early disease relapse (data not shown). In the study of Lee S.-E et al., M-MDSCs and E-MDSCs after transplantation did not have a suppressive effect on autologous T cells and NKT cells [19], which is inconsistent with our data on the expression of suppressor molecules in MDSCs. According to generally accepted the role of MDSCs, the presence of cells with immunosuppressive potential can be important for the prognosis and prediction of relapse. For example, the PD-L1/PD-1 signaling pathway may be involved in the induction of Treg generation [54]. However, the expression of PD-L1 in MDSCs by the 12-month period point did not exceed that in the donor group. Considering the data of Hoechst B. et al. [13] that M-MDSCs induce Treg via Arg-1-independent mechanisms, Arg-1 expression in M-MDSCs after auto-HSCT requires further study.

In our study, we did not evaluate a direct inhibitory activity of MDSCs against autologous T cells to elucidate the relationship between the time of post-transplant relapse and the MDSC suppressive capacity, which is a limitation of the study. In addition, if MDSCs are suspected to be involved in shaping the HSC niche, then BM MDSCs are also of interest. However, our data allowed us to formulate a new hypothesis, according to which MDSCs at the engraftment ameliorate the recovery of subsequent antitumor response in MM patients. At early post-transpalant, leukopenia launches homeostatic proliferation of re-infused mature T cells, which process is accompanied by a narrowing of the TCR repertoire. In this regard, we speculate that the attenuation of homeostatic proliferation will facilitate the subsequent reconstitution of the T cell compartment, preventing the uncontrolled expansion of rapidly proliferating clones of mature T cells and therefore sustaining the formation of a wide TCR repertoire of *de novo* generated naive T cells. The latter hinders tumor recurrence and opportunistic infections. To confirm this assumption, further studies of MDSC effects on homeostatic proliferation and expansion of T cells are required. These investigations will elucidate the role of MDSCs in the pathogenesis of MM relapses and their prognostic significance for assessing the outcome of auto-HSCT.

4. Material and methods

4.1. Patient characteristics

The study included 112 Caucasian patients with MM aged 38–72 years (median 55 years), who were examined and treated at the Department of Hematology with Bone Marrow Transplantation Unit of Immunopathology Clinic, Research Institute of Fundamental and Clinical Immunology from August 2017 to March 2023. The clinical characteristics of patients are presented in Table 5. Six patients were with newly diagnosed MM; the rest had already been receiving induction anticancer therapy (pre-treated patients) by the time the study began. Responses were defined according to International Myeloma Working Group criteria [55].

The first line treatment was performed with bortezomib (BTZ)-containing regimen (PAD, VCD, VD). In cases of relapse or resistance to BTZ programs, lenalidomide programs (VRD, RD, RCD, lenalidomide monotherapy) were used as the 2nd line therapy. At the time of the study, complete remission (CR) or very good partial response (VGPR) was achieved in 35 patients, partial response (PR) was in 57 patients; 11 patients were in the progression of MM or resistant to ongoing therapy, three patients subsequently dropped out of the study. The median time from the beginning of therapy to transplantation was 10 months.

The patients were mobilized with cyclophosphamide (2–4 g/m^2) plus G-CSF (5 μg/kg/day for 4–6 days). Apheresis sessions (n = 1–3) were performed with ASTEC 204 (Fresenius) or Spectra LRS 07 (COBE) cell separators on the 4–6th days of G-CSF administration until ≥2.0 × 10^6 CD34$^+$CD45$^+$ cells/kg were reached. The median dose of CD34$^+$CD45$^+$ HSCs was 4.5 × 10^6/kg (3.3–5.7 × 10^6/kg).

Autologous HSCs were transfused after conditioning with melphalan 200 mg/m^2 or 140 mg/m^2 (in patients with renal insufficiency). The duration of critical post-transplant cytopenia (peripheral leukocyte and platelet counts <1 × 10^9/L and 50 × 10^9/L, respectively) varied from 8 to 18 days (Me = 12 days, IQR 11–14 days).

Twenty-three age-matched, sex-matched, and race-matched healthy donors were enrolled in the study. All healthy individuals and patients gave informed consent in accordance with the Declaration of Helsinki of 1975; the local ethics committee of Research Institute of Fundamental and Clinical Immunology approved the study protocol (no. 28, dated August 15, 2017).

4.2. Blood sample collection and isolation of peripheral blood mononuclear cells

A total of 230 heparinized peripheral blood samples were obtained from 112 MM patients at diagnosis (n of samples = 6), following induction therapies (before cyclophosphamide administration for HSC mobilization; n = 47), following HSC mobilization (at the day of the 1st apheresis session; n = 33), prior to the conditioning with melphalan (n = 39), at the engraftment (the 2nd day of peripheral leukocyte count >1 × 10^9/L; n = 56), following six (n = 21) and 12 months (n = 17). In addition, 11 samples of individuals with relapsed/refractory MM were assessed. Due to technical limitations, several patients skipped some follow-ups of the study. Patients at different time points did not differ in age, gender, pretreatment, stage of MM, or response to therapy (Suppl. Table 1).

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood (10 mL) by Ficoll density gradient (ρ = 1.077) centrifugation and underwent immediate flow cytometric analysis. If necessary, erythrocytes were lysed with VersaLyse Lysing Solution (Beckman Coulter, Marseille, France) according to manufacturer instructions.

4.3. Flow cytometric analysis of MDSCs from PBMCs

Relative counts of PMN-MDSCs (Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^+$), M-MDSCs (CD14$^+$HLA-DR$^{low/-}$), and E-MDSCs (Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^-$) were assessed in freshly isolated PBMCs with flow cytometry. The following anti-human monoclonal antibodies were used: anti-Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56; FITC, «BD Biosciences», USA), anti-CD14 (FITC, «BD Biosciences»), anti-CD33 (PerCP-Cy5.5, «BD Biosciences», USA), anti-CD66b (APC, «BioLegend», USA), anti-HLA-DR (APC-Cy7, «BD Biosciences», USA; PerCP, «BD Biosciences»). Isotype antibodies conjugated to the same fluorochromes were used as negative controls.

The study was performed in accordance with the recommendations of Bronte et al. [10] using the parameters of forward vs. side scatter, the gating area included the region of singlet PBMCs (lymphocytes and monocytes). The gating strategy is presented in Fig. 1A. Briefly, to assess PMN-MDSCs and E-MDSCs, singlet cells were isolated from all PBMCs followed by gating with forward scatter and side scatter (FSC-A vs. SSC-A; P1), and the cell population without surface expression of linear markers and HLA-DR was examined. Next, the expression of CD33 and CD66b molecules in Lin$^-$HLA-DR$^-$ cells was assessed. CD33$^+$CD66b$^+$ and CD33$^+$CD66b$^-$ cells were identified as PMN-MDSCs and E-MDSCs, respectively. Relative counts of Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^+$ PMN-MDSCs and Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^-$ E-MDSCs are presented as the percentages of PBMCs. To estimate the frequencies of M-MDSCs, the expression of CD14 and HLA-DR molecules in singlet PBMCs (P1) was analyzed, and cells with the CD14$^+$HLA-DR$^{low/-}$ phenotype were assigned to M-MDSCs. The analysis was performed after the accumulation of at least 10,000 events in the region of Lin$^-$HLA-DR$^-$ cells, or, if assessing M-MDSCs, at least 10,000 events in the region of CD14$^+$ HLA-DR$^{low/-}$ cells.

Absolute counts of studying MDSC subsets were calculated by the following formula:

$$[\text{percentage of MDSC subset} \times \text{PBMC absolute count}] / 100\ \%.$$

4.4. Analysis of suppressive molecule expression in MDSCs

Surface expression of the PD-L1 molecule on MDSCs was assessed using PE-labeled anti-PD-L1 monoclonal antibodies (BD PharMingen, USA) in PMN-MDSCs (Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^+$), E-MDSCs (Lin$^-$HLA-DR$^-$CD33$^+$CD66b$^-$) and M-MDSCs(14+HLA-DR$^{low/}$

$^{-}$) according to standard flow cytometric approaches for surface antigens.

To assess the intracellular expression of Arg-1 and IDO, PBMCs were incubated with fluorochrome-conjugated monoclonal antibodies to PMN-MDSCs, E-MDSCs, and M-MDSCs as described above. Next, the cells were permeabilized using Transcription Factor Buffer Set (Becton Dickinson, USA) and stained with PE-conjugated anti-Arginase-1 (BD PharMingen, USA) or PE-conjugated anti-IDO (BD PharMingen, USA) monoclonal antibodies. Relative counts of MDSCs expressing Arg-1, IDO, and PD-L1 were assessed among $Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{+}$, $Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{-}$, and $CD14^{+}HLA\text{-}DR^{low/-}$ cells. Isotype antibodies conjugated with the same fluorochromes were used as a negative control.

4.5. Statistical analysis

Statistical analysis was performed using Statistica 6 (StatSoft, Inc., Tulsa, OK, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) software. Data in the text were presented as median (Me) and interquartile ranges (IQR). The Mann-Whitney *U* test was used to calculate differences between groups of patients. The sign test was applied to determine differences within dependent (paired) groups of patients. Correlations between variables were evaluated using the Spearman rank correlation test. Linear regression and multivariate analysis of variance were performed to identify a relationship between MDSCs and various clinical parameters. Survival analysis was analyzed according to the Kaplan-Meier method, log-rank test was used to assess the statistical significance. Various predictors of auto-HSCT outcome were evaluated with the Cox regression analysis. $P < 0.05$ was considered statistically significant.

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Data availability statement

The data and supportive information are available within the article. Data associated with the study has not been deposited into a publicly available repository; it will be made available on request from the corresponding author.

CRediT authorship contribution statement

Tamara Tyrinova: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Egor Batorov:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Tatyana Aristova:** Resources, Investigation. **Galina Ushakova:** Resources. **Svetlana Sizikova:** Resources. **Vera Denisova:** Supervision, Resources. **Elena Chernykh:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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