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Immunosuppressive capacity of circulating MDSC predicts response to immune checkpoint inhibitors in melanoma patients.

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LIST OF ABBREVIATIONS

APC	antigen-presenting cells
ATP	adenosine triphosphate
BRAF	B-rapidly accelerated fibrosarcoma
CR	complete response
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
EVs	extracellular vesicles
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIF-1α	hypoxia-inducible factor-1α
ICI	immune checkpoint inhibitor
IFN-γ	interferon-γ
IL	interleukin
irAE	immune related adverse events
iRECIST	Immunotherapy Response Evaluation Criteria in Solid Tumours
LAG-3	lymphocyte activating gene 3
LOX-1	lectin-type oxidized LDL receptor-1
M-CSF	macrophage colony-stimulating factor
MDSC	myeloid-derived suppressor cells
MEK	mitogen-activated protein kinase
MHC I	major histocompatibility complex class I
MM	malignant melanoma
NK cells	natural killer cells
NO	nitric oxide
NRAS	neuroblastoma RAS viral oncogene homolog
ORR	overall response rate
OS	overall survival
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PFS	progression-free survival
PGE-2	prostaglandin E2
ROS	reactive oxygen species
SARS-CoV2	severe acute respiratory syndrome coronavirus type 2

SNL	sentinel lymph-node
TAM	tumor-associated macrophages
TAN	tumor-associated neutrophils
TCR	T cell receptor
TGF-β	tumor growth factor-β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	tumor-infiltrating leukocytes
TIM-3	T-cell immunoglobulin and mucin-containing domain-3
TME	tumor microenvironment
Treg	regulatory T cells
T-VEC	talimogene laherparepvec
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 Cancer and immune system

The concept shaping our understanding of cancer immunobiology today is called cancer immunoediting. It describes how immune cells interact with cancer cells and includes three phases: elimination, equilibrium and escape (1,2). The environment around the tumor (tumor microenvironment (TME)) consists of blood vessels, immune cells (such as tumor-infiltrating lymphocytes (TIL), antigen-presenting cells (APC), tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), regulatory T cells (Tregs), fibroblasts, signalling molecules and extracellular matrix, which all play a crucial role in tumor survival and progression (3,4). Furthermore, TME influences the efficacy of anti-cancer treatments, for example high immune cell infiltration in the primary melanoma skin cancer positively correlated with an overall survival and improved response to immune checkpoint inhibitor (ICI) therapy (5,6). Nevertheless, under chronical inflammatory conditions in the TME, immune cells are modified in the way that they lose their ability to supress and eliminate tumor cells (this concept is called immunosurveillance and it is correspondent to the equilibrium phase) but rather support tumor growth and its escape from the immune system (7,8). Furthermore, tumor cells downregulate major histocompatibility complex (MHC) I expression, which makes them less recognisable by T effector cells, secrete inhibitory factors like tumor growth factor-β (TGF-β) and upregulate the expression of co-inhibitory molecules such as the programmed cell death-ligand 1 (PD-L1), causing T cells anergy and apoptosis (9). These principals of cancer immunoediting and immunosurveillance are addressed by modern immunotherapies such as ICI. ICI agents block co-inhibitory signal transmission between tumor and T cells, thereby counteracting the tumor escape (10).

1.2 Malignant melanoma

Malignant melanoma (MM) is the most aggressive skin cancer that arises from pigment-producing melanocytes. Worldwide, melanoma cased 0,6% of all cancer deaths in year 2020 and is the most common cause of skin-cancer related deaths (11). While the incidence of MM is increasing, the mortality rates tend to fall (12,13). Besides the congenital risk factors as fair skin color, presence of melanocytic or dysplastic naevi or positive family history, the strongest acquired risk factors for melanoma development are severe sunburns induced by ultraviolet radiation (14,15). Notably, melanoma can occur not only on the skin, but also in the mucosa of inner organs or uvea (16). These types of melanoma together with acral melanoma (MM on palms, soles and nails) are considered to undergo non sun-induced development, since they arise in body parts protected from direct UV light (15). For the majority of MM, which are sun-induced cutaneous melanomas, the dual pathway hypothesis of melanoma formation has been described (17). Melanomas occurring on the trunk in young individuals without chronic sun damage pattern of the skin undergo a nevus prone pathway initiated by early sun exposure and promoted by intermittent sun exposure. These melanomas are characterized by early activation of B-rapidly accelerated fibrosarcoma (BRAF) proto-oncogene serine/threonine-kinase. While in so-called chronic sun exposure pathway, melanoma lesions are characterized by accumulated sun exposure and NRAS proto-oncogene GTPase (NRAS) activation (17).
Clinically, melanoma is classified according to the AJCC 2017 guidelines (18–21). The most crucial factors associated with poor prognosis are melanoma thickness, mitoses rate, presence of ulceration, and anatomic location on the head and neck (22,23).

1.2.1 Overview of clinically used therapies

The main treatment of primary melanoma is surgical resection with safety margins depending on melanoma thickness. After the resection, it is recommended to perform the sentinel lymph-node (SNL) biopsy for melanomas with thickness at least 1,0 mm or for thinner melanomas with ulceration. In accordance with new multicentre randomized clinical trials, regional lymph node dissection should only be performed in case of clinically detectable lymph node affection and is not recommended immediately after the SNL-positive biopsy, since complete regional lymph node dissection in these cases improved only regional nodal relapse-free survival but had no effect on relapse-free and overall survival (24–26). Therefore, SNL basin should be periodically

controlled with ultrasound. According to guidelines (20), the patients with high-risk melanomas of at least 4,0 mm depth or with suspected lymph node metastasis should be examined with CT and MRI to exclude metastatic spread.

1.2.1.1 Targeted therapy

In cases of proven tumor spread and inoperable metastatic disease, systemic therapies are used in palliative setting. Since melanoma is a highly immunogenic tumor characterized by high mutational burden (27–29), the mutational analysis of the resected melanoma is crucial for the therapy decision (30). The most common mutation (appr. 50% of the melanomas) is BRAF V600E mutation, causing an activation of mitogen-activated protein kinase (MEK) pathway, which can be targeted by BRAF- (dabrafenib, encorafenib, or vemurafenib) and MEK-inhibitors (trametinib, binimetinib, or cobimetinib) (31). Due to the rapid clinical response (days to weeks regardless of tumor burden) and the objective response rate of 70%, BRAF and MEK inhibitors are approved as a first-line therapy for BRAF-driven advanced stage melanomas (32). However, most patients develop resistance after about 1 year of therapy due to activation of alternative signaling pathways (30,33). Patients with brain metastases and high tumor burden after failure of BRAF/MEK-inhibitor treatment still have very poor prognosis (3-year survival lies by under 10%) (34). Patients with metastatic melanoma without detectable BRAF V600E mutation should be treated with immunotherapy, for example with anti-PD-1 and anti-CTLA-4 antibodies (20). Notably, even though increased PD-L1 expression on melanoma cells is associated with prolonged survival and better therapy response, PD-L1 expression has no impact on the therapy decision (35–37).

1.2.1.2 Triple therapy

The so-called triple therapy with the combination of BRAF-, MEK- and anti-PD-1 antibodies is currently under investigation (STARBOARD, phase III) (38). Melanoma patients receiving triple therapy showed prolonged median progression-free survival (PFS) (17,0 months vs. 9,9 months in combination with placebo) and median overall survival (OS) (46,3 months vs. 26,3 months) (39). At the same time, the triple therapy was described to cause more overall toxicity (grade 3-5 adverse events 70% triple therapy arm vs. 45% in placebo-controlled arm) (40).

1.2.1.3 Radiation therapy

Another clinically widely used melanoma treatment is radiation therapy, which can be used both locally and systemically. Radiotherapy has found its place in the treatment of primary tumor (e.g. unresectable lentigo maligna or if safety margins after resection are not reached), as adjuvant treatment after lymphadenectomy and as therapy for locoregional and distant metastases (spinal cord, skin, lymph nodes, liver, lung and brain) (20). Since radiation can modulate immune cell functions in the TME, the combinational treatment with ICI was investigated, and improved clinical outcomes were observed (41,42).

1.2.1.4 T-VEC

By presence of cutaneous/subcutaneous non-resectable locoregional metastases modified oncolytic herpes virus called talimogene laherparepvec (T-VEC) can be used for intertumoral injections (43). T-VEC contains GM-CSF gene, and its replication in the tumor tissue reinforce antitumor immune response, causing melanoma cell lysis (44). Randomized phase III OPTiM trial had shown that T-VEC treatment resulted in prolonged overall survival, durable response rate and objective response rate compared to intertumoral GM-CSF injections (45,46). Moreover, the combination of T-VEC injections with ICI was associated with very high response rates (ORR 61,9% and CR 33%) in melanoma patients (47).

1.2.1.5 Chemotherapy

Chemotherapy has taken a back seat in clinical practice today (20). Chemotherapeutic agents induce cytotoxicity, and their clinical use is limited not only due to less favourable response rates compared to immunotherapy but also due to high numbers of adverse events (48).

1.2.1.6 Adjuvant therapies

In cases where SNL-positive lymph node has been removed and no distant metastases were detectable, or in cases where a limited number of operable metastases have been removed and no other measurable lesions are detectable, systemic therapies can be applied as an adjuvant treatment. Since an adjuvant therapy has been shown to prolong distant metastasis-free survival and recurrence-free survival, such therapy with BRAF/MEK-inhibitors is recommended for patients with BRAF mutation in AJCC2017 stages IIIA-D and therapy with PD-1 inhibitors is

recommended for melanoma patients in AJCC2017 stages III-IV with no evidence of disease (20,49–52).

1.2.2 Immunotherapy as a first-line treatment? Response rates, advantages, and limitations

Melanoma lesions are not only characterised by high mutational burden but also by extensive immune cell infiltration, indicating a great potential for positive response to immunotherapy (53). The most used immunotherapeutic agents in melanoma are immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1 antibodies.

1.2.2.1 Underlying mechanism of immune checkpoint inhibitor treatment

It is known that T cell activation requires several distinct signals (54). Firstly, the T cell receptor (TCR) interacts with an antigen, presented on MHC I or II molecules on APC or tumor cells. Secondly, T cell activation requires a co-stimulatory signal, such as interaction of CD28 on T cell with CD80/CD86 on APC (55). Thirdly, certain cytokines (IL-2, IL-4, IL-6, IL-12, IFN- γ and others) determine into which subtype of T cells they will develop (56,57). It is known that not only co-stimulatory, but also co-inhibitory signals play a role in the activation of T cells. In physiological conditions, co-inhibitory molecules expressed on APC such as PD-L1/PD-L2 and CD80/CD86 interact with PD-1 and CTLA-4 expressed on T cells, respectively. This interaction play a vital role in the maintenance of self-tolerance by inhibition of autoreactive T cells (58,59). Under the chronic inflammatory conditions in the TME, characterized by elevated levels of pro-inflammatory cytokines and especially IFN-γ, tumor cells and immunosuppressive cells gain the ability to upregulate negative checkpoint molecules as PD-L1 and CD80/CD86 (60). These inhibitory signals block T cells activation, induce T cell anergy and apoptosis, lead to immune exhaustion in the TME and cancer escape (61,62). Inhibitors of the negative immune checkpoint molecules such as anti-PD-1 (nivolumab, pembrolizumab) or anti-CTLA-4 antibodies (ipilimumab) block co-inhibitory molecules on T cells and prevent the inactivation of T cells, thereby reactivating anti-tumor immune response (63,64).

1.2.2.2 Logn-term outcomes of ICI in clinical trials

Indisputable advantages of ICI are improved survival and stable and long-lasting response rate. In the phase III CheckMate 067 trial overall survival was 72,1 months

for combination of CTLA-4 and PD-1 inhibitor therapy, 36,9 months for PD-1 inhibition and 19,9 months for CTLA-4 inhibition, while 6,5-year OS rates were 57%, 43%, and 25% respectively (65). However, despite the success of ICI, 60% of melanoma patients show no response to PD-1 monotherapy and approximately 40% are resistant to CTLA-4/PD-1 combinational treatment (66,67). Factors such as an impaired formation of memory T cells and insufficient formation or dysfunction of anti-tumor effector T cells have been described as factors, increasing the resistance to ICI (68).

1.2.2.3 Immune related adverse events

Since ICI activate the immune system, they can lead to immune related adverse events (irAE), which limits their usage (69). Most common irAE under anti-CTLA-4 treatment are colitis and other gastrointestinal symptoms as well as hypophysitis, whereas anti-PD-1 therapy may induce pneumonitis and thyroiditis (70,71). In case of combinational treatment severe irAE (grade 3-4) occur in 59% and in case of PD-1 monotherapy in 24% of melanoma patients (72). Interestingly, the occurrence of vitiligo in melanoma patients under ICI was associated with better clinical outcomes (73).

1.2.2.4 New ICI molecules

New ICI that target other negative checkpoint molecules are currently coming to light. The combination of PD-1 and lymphocyte activating gene 3 (LAG-3) inhibition showed 20% improved response and prolonged PFS compared to PD-1 monotherapy (10,1 months vs. 4,6 months) (74). IrAE rate for this new combinational treatment corresponded to 19% (74).

1.2.3 Assessment of ICI efficacy and biomarkers of response

The conventional response assessment used in the clinic is restaging, which is performed 8 to 12 weeks after treatment initiation. Tumor leasions are assesed according to Immunotherapy Response Evaluation Criteria in Solid Tumours (iRECIST) criteria (75). Phenomenon of initiall ≥ 25% increase in tumor burden on the first restaging that was not confirmed as progressive disease at next assessment has been called pseudoprogression and was firsly observed in patients treated with ipilimumab (76). This phenomenon can be explained by immune infiltration into the tumor and needs to be confirmed by the following radiological assessment in another 8 to 12 weeks to prevent the premature cessation of treatment (77).

As the response rates to ICI remain limited, there is an urge need to understand the mechanisms of resistance and to identify new biomarkers of response that can be assesed in a non-invasive way and can be easily integrated into clinical practice. Currently investigated potential prognostic markers assess melanoma genetics by analyzing neo-epitopes or overall mutational load (35,78), immunosuppressive TME by evaluating TIL and PD-1 expression on CD8 T cells (79–82), exosomal PD-L1 expression (83,84), T cell–inflamed gene expression profile, including interferon-γ (IFN-γ) signaling, the expression of immune checkpoint molecules and inflammatory mediators (like IL-6 and IL-8) (85–87), the composition of the gut microbiome (88) and the treatment with antibiotics prior to ICI (89). Interestingly, such factors as the gut microbiome was shown not only to have an impact on the efficiency of the ICI therapy in melanoma (88) but also have an impact on occurrence of irAE (90).

It has been shown that increased frequency of MDSC in the peripheral blood of advanced melanoma patients correlates with reduced PFS, OS and poor response to immunotherapy (91–93). Moreover, an increased number of MDSC in peripheral blood can be used as a biomarker for the resistance to PD-1 therapy (94).

1.3 MDSC - one of the reasons why ICI does not always work

MDSC is a heterogeneous population of myeloid cells, which derive from myelopoietic progenitors in the bone marrow under the influence of chronic inflammatory mediators and then migrate into lymphatic organs (lymph nodes, spleen) or TME (95–97). This alteration of myelopoiesis in cancer can also occur extramedullary in the spleen (98). Moreover, it has been shown that MDSC could uprise from mature myeloid cells such as monocytes driven by tumor-derived extracellular vesicles (EVs) (99).

In humans, MDSC can be categorized in two strongly immunosuppressive populations: monocytic MDSC (M-MDSC) (described as $CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}CD66b^{-}$) and polymorphonuclear MDSC (PMN-MDSC) ($CD33^{dim}HLA\text{-}DR^{low/-}CD14^{-}CD66b^{+}Lin^{-}$) as well as small population without immunosuppressive capacity, called early-stage MDSC (e-MDSC) ($CD33^{dim}HLA\text{-}DR^{low/-}CD66b^{-}Lin^{-}$) (97,100). Cells with typical MDSC phenotype but lacking immunosuppressive activity are also present in healthy individuals in much smaller numbers and called MDSC counterparts (100). The main challenge in MDSC investigation is to distinguish between PMN-MDSC and tumor-associated neutrophils (TAN) as well as M-MDSC and tumor-associated macrophages (TAM), since the latter share a similar phenotype with PMN- and M-MDSC and can

exert protumorigenic activities (101–103). In contrast to M-MDSC, TAM represent mature cells characterised by macrophage-specific markers like CD68 and CD163, absence of S1000A9 and prostaglandin E2 (PGE-2) expression (95). However, more current research revealed that M-MDSC can further differentiate in the TME into macrophages characterised by high expression of S100A9 and immunosuppressive activity (104). These fundings emphasise the complexity of the intertumoral landscape of myeloid cells. The differentiation between TAN and PMN-MDSC remains challenging since they share the same phenotype (105). An new marker lectin-type oxidized LDL receptor-1 (LOX-1) was proposed to identify PMN-MDSC (106). However, elevated expression of LOX-1 could not be confirmed on melanoma samples due to relatively low accumulation of PMN-MDSC (106).

1.3.1 MDSCs recruitment, activation, and functions

The tumor microenvironment is characterized by a constant presence of growth factors and inflammatory mediators known as cancer-related inflammation or the seventh hallmark of cancer (107,108). As a first step, factors such as interleukin (IL)-1β, IL-6, IL-8, vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by melanoma and host cells in the TME induce generation and expansion of MDSC in bone marrow and spleen and cause MDSC recruitment the tumor site (109,110). Next, MDSC are activated by the direct influence of IL-1β, IL-6, IL-8, IL-4, IL-13, IFN-γ and PGE2 in the tumor site (110,111). These two phases are partially overlapping.

The main characteristic of MDSC is their strong ability to suppress anti-tumor T and NK cells (95,96,109), which could be measured in the inhibition of T cell proliferation assay (100,112). MDSC cause T cell anergy and apoptosis due to high expression of PD-L1 and Fas ligand (FasL) (113). TGF-β and hypoxic conditions in the TME induce hypoxia-inducible factor-1α (HIF-1α), which lead to the expression of ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ectonucleotidase (CD73) on MDSC (114). These enzymes convert extracellular adenosine triphosphate (ATP) into adenosine that additionally inhibits effector T cell functions (115). Moreover, MDSC express arginase-1, produce reactive oxygen species (ROS) and nitric oxide (NO) causing T cell anergy and downregulation of TCR ζ-chain (116,117). Notably, M-MDSC was also reported to rapidly convert into TAM in the tumor tissues, which are known for inhibition of immune response and promotion of tumor growth (118,119).

All above mentioned MDSC-mediated mechanisms of immunosuppression support the tumor escape and reduce response to various melanoma therapies, including ICI treatment.

1.3.2 MDSC in chronical inflammatory conditions other than cancer

MDSC were first described in cancer, but they are present in a variety of other diseases characterized by chronic inflammation such as autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis, type I diabetes, multiple sclerosis, inflammatory bowel disease), unresolved infections (biofilm formation by *Staphylococcus aureus*, infection with *Mycobacterium tuberculosis*, several *Candida* species, hepatitis, human immunodeficiency and severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection), allergic asthma, sepsis and obesity (120,121). The restricting effect of MDSC on overactivated immune system in these diseases demonstrates the complexity of the role that MDSC play in the organism (96). Moreover, MDSC were shown to suppress T cell responses during pregnancy, maintaining maternal–fetal tolerance (122).

All these findings highlight the crucial role that MDSC play in human immune system and emphasize the importance of understanding MDSC function and developing new MDSC-targeted and combinational treatments to improve clinical outcomes and prolong patient survival.

2 AIM OF THE STUDY

Malignant melanoma is characterized by high mortality rate, especially when diagnosed in an advanced stage. Due to high immunogenicity of the tumor, treatment with ICI revolutionized therapeutical options and represents first-line treatment for unresectable melanoma. However, approximately 40% of patients fail to respond to immunotherapy, suggesting the need to develop further therapeutical options, to identify new biomarkers of response and to better understand the mechanisms leading to treatment failure.

First, I aimed to review the existing evidence on the impact of major TME players such as MDSC, TAM and Tregs and the role of gut microbiome on tumor growth and ICI treatment outcome. I discussed the current evidence supporting the use of these TME actors as biomarkers of response and other approaches to response prediction such as PET-CT, tumor biopsy and liquid biopsy (especially analysis of soluble or expressed in the extracellular vesicles PD-L1 or soluble inflammatory factors in the patient's plasma). To obtain a comprehensive overview over the therapeutic options currently used or investigated in advanced melanoma patients, I reviewed the available data on combination treatment of ICI with radiotherapy, T-VEC or targeted therapy; with agents targeting tumor neoantigens, causing depletion of immunosuppressive MDSC and targeting other ICI-molecules such as LAG-3, TIM-3 and TIGIT. I focused on the impact of these combination treatments on ICI effectiveness. The results of these investigations are published in the review manuscript "Modern Aspects of Immunotherapy with Checkpoint Inhibitors in Melanoma".

Furthermore, the aim of this investigation was to expand the existing knowledge about MDSC, their function and the dynamic changes that MDSC undergo in patients with malignant melanoma. Even though the role of MDSC in cancer is well investigated, ICI-related changes in freshly isolated circulating MDSC from melanoma patients are not sufficiently studied, especially not in patients with no evidence of disease (non-metastatic patients), receiving ICI as an adjuvant treatment to the surgical tumor resection. Furthermore, many studies describe MDSC frequency, immunosuppressive pattern, and functional activity before and after the initiation of ICI therapy, but the dynamic changes in these parameters during ongoing ICI treatment are poorly understood.

Therefore, I isolated PBMC from the peripheral blood of 29 advanced melanoma patients and 10 age-matched healthy donors. MDSC frequencies (and their non-immunosuppressive MDSC-counterparts in healthy donors) and the expression of immunosuppressive molecules on their surface were analyzed by multicolor flow cytometry. To investigate the immunosuppressive function of MDSC, I isolated two MDSC populations by FACS sorting and co-incubated them with T cells isolated from the same patient. Furthermore, to better understand the mechanisms of MDSC activation and action, the concentration of MDSC-related inflammatory mediators in the patient's plasma was analysed by bio-plex assay. To deepen our knowledge of the dynamic changes that MDSC undergo during ICI therapy, all of the above mentioned measurements were performed for each patient at four different time points: before therapy initiation and after the first three applications of ICI treatment up to the time point of the first staging (approximately 3 months after therapy initiation). It is important to emphasize that all above mentioned analyses of MDSC frequency, phenotype and suppressive activity and the concentration of MDSC-related chemokines were performed in metastatic patients receiving ICI as a palliative treatment and in non-metastatic patients receiving ICI in adjuvant setting. Moreover, to investigate these MDSC characteristics as possible biomarkers of therapy response, I compared these parameters in metastatic patients showing complete, partial response or stable disease (classified as responders) to metastatic patients with progressive disease (non-responders) defined by radiological staging commonly used in the clinic. Most results of this study were published in the manuscript "Immunosuppressive capacity of circulating MDSC predicts response to immune checkpoint inhibitors in melanoma patients".

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4 PUBLICATIONS

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2. Petrova V, Groth C, Bitsch R, Arkhypov I, Simon SCS, Hetjens S, Müller V, Utikal J, Umansky V. Immunosuppressive capacity of circulating MDSC predicts response to immune checkpoint inhibitors in melanoma patients. *Front Immunol* (2023) 14:1–11. doi: 10.3389/fimmu.2023.1065767

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Review

Modern Aspects of Immunotherapy with Checkpoint Inhibitors in Melanoma

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Abstract: Although melanoma is one of the most immunogenic tumors, it has an ability to evade anti-tumor immune responses by exploiting tolerance mechanisms, including negative immune checkpoint molecules. The most extensively studied checkpoints represent cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and programmed cell death protein 1 (PD-1). Immune checkpoint inhibitors (ICI), which were broadly applied for melanoma treatment in the past decade, can unleash anti-tumor immune responses and result in melanoma regression. Patients responding to the ICI treatment showed long-lasting remission or disease control status. However, a large group of patients failed to respond to this therapy, indicating the development of resistance mechanisms. Among them are intrinsic tumor properties, the dysfunction of effector cells, and the generation of immunosuppressive tumor microenvironment (TME). This review discusses achievements of ICI treatment in melanoma, reasons for its failure, and promising approaches for overcoming the resistance. These methods include combinations of different ICI with each other, strategies for neutralizing the immunosuppressive TME and combining ICI with other anti-cancer therapies such as radiation, oncolytic viral, or targeted therapy. New therapeutic approaches targeting other immune checkpoint molecules are also discussed.

Keywords: melanoma; immunotherapy; immune checkpoint inhibitors; immunosuppression; tumor microenvironment

1. Introduction

The concept of cancer immunosurveillance is based on the fact that tumor cells can be recognized and eliminated by immune system [1,2]. Immunogenicity of malignant melanoma is based on a high ultraviolet-driven mutational burden [3]. This leads to the overexpression of tumor specific antigens enabling the formation of the antigen specific immune response [4,5]. However, development of aggressive metastatic melanoma shows that tumors are edited by the immune system, and selected resistant variants could escape the immune control [6,7]. Therefore, several immune-based therapeutic approaches such as vaccination [8], adoptive transfers [9] and immune checkpoint-blockade [10] were applied, aiming at reinvigorating anti-tumor immune response and improving survival of advanced-stage melanoma patients [11].

The most studied negative immune checkpoint molecules and broadly accepted targets for immunotherapy are cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 is upregulated on the T cell surface early during activation in lymph nodes,

binds to CD80/CD86 reducing co-stimulation through CD28 and functions as a negative downstream loop for T cell receptor (TCR) signaling [12]. PD-1 interaction with its ligands PD-L1 and PD-L2 inhibits effector T cell functions in peripheral tissues [13]. Playing a pivotal role in the maintenance of self-tolerance under physiological conditions, these checkpoint molecules could be exploited by tumors to evade the immune responses. Hence, inhibiting such interactions could reactivate anti-tumor immune reactions [14]. Moreover, the combination of anti-CTLA-4 and anti-PD-1 antibodies was shown to work synergistically by expanding activated effector CD8 T cells [15,16]. Another approach was shown to implicate the combination of PD-L1-CD80 heterodimerization and the suppression of the CTLA-4/CD80 axis [17]. Currently used antibodies to target CTLA-4 are ipilimumab and tremelimumab, to target PD-1 are nivolumab, pembrolizumab, cemiplimab and to target PD-L1 are atezolizumab and avelumab [14,18,19].

This review will focus on current achievements in the therapy with immune checkpoint inhibitors (ICI) in melanoma and will discuss the strategies to improve of treatment efficacy by combining ICI with other therapies.

2. Therapeutic Effects of Immune Checkpoint Inhibitors

Latest clinical guidelines on melanoma management consider immune checkpoint blockade (anti-PD-1 alone or in combination with anti-CTLA4) as a first-line treatment option for unresectable stage III and IV melanoma patients [20,21]. In cases of resectable melanoma, anti-PD1 agents are prescribed as well in an adjuvant setting [22]. This treatment is currently investigating in a neoadjuvant setting [23].

Since the responses of tumors to immunotherapy and chemotherapy are different, immune-related response criteria and immune-response evaluation criteria in solid tumors were developed [24,25]. Such criteria improve the evaluation of additional response patterns during immunotherapy such as pseudoprogression. Currently achieved response to ICI treatment of melanoma patients reached 52% for pembrolizumab and 58% for combination of nivolumab and ipilimumab [26–28]. The 5-year survival rate was reported to be 41% and 52% in these two trials, respectively. These therapeutic achievements were associated with a high toxicity up to 59% of grade 3 and 4 adverse events in patients treated with the combination of nivolumab and ipilimumab [27]. Another trial studied a ipilimumab combination with pembrolizumab, which does not yet belong to the approved settings. The objective response was achieved by 61% of patients, 1-year overall survival (OS) was 89%, and 1-year progression-free survival was 69%. Grade 3 and 4 adverse events occurred in 27% of patients [29]. These data represent a favorable effect of such combinations with increased response values and less high-grade adverse effects.

However, many patients remained resistant to ICI therapy since tumor cells could develop resistance to anti-tumor immune reactions or induce a profound immunosuppression in the tumor microenvironment [30].

3. Tumor Cells Evade Immune Responses

A characteristic gene profile was described for melanoma cells resistant to ICI. It includes the repression of genes, which control antigen presentation and interferon (IFN)-γ signaling as well as the induction of genes regulating epithelial-mesenchymal transition, remodeling of extracellular matrix, cell adhesion and angiogenesis [31–34]. Interestingly, down-regulation of major histocompatibility complex (MHC) class I protein expression was found to be associated with the resistance to anti-CTLA-4, but not to anti-PD-1 therapy [35]. In the same work, MHC class II expression in >1% melanoma cells was shown to predict response to anti-PD-1, but not to anti-CTLA-4 therapy. This suggests that tumor cells disrupt antigen presentation limiting the efficient anti-tumor response. In fact, anti-PD-1 blockade before antigen priming of T cells leads to accumulation of the dysfunctional $PD\text{-}1^{+}CD38^{hi}CD8^{+}$ cells abolishing the effects of the therapy [36]. Moreover, tumor cells can prevent the formation of anti-tumor

T cell memory in the draining lymph node by secreting PD-L1-bearing extracellular vesicles (EV), contributing to the resistance to anti-PD-1 antibodies [37].

4. Immunosuppressive Tumor Microenvironment as an Important Factor of ICI Treatment Failure

A deeper investigation of the immunosuppressive networks within the TME could help to understand the limitations of ICI treatment and to develop strategies for increasing treatment efficiency. Immunosuppression in the TME is mediated by various cells and soluble factors described below.

4.1. Myeloid-Derived Suppressor Cells (MDSC)

MDSC represent a heterogeneous population of immunosuppressive myeloid cells, generating under chronic inflammation conditions and cancer and accumulating in the TME [38]. In humans, three MDSC subsets have been described: $CD11b^+CD14^+HLA\text{-}DR^{low/-}CD15^-Lin^-$ monocytic (M-MDSC), $CD14^-CD11b^+CD15^+HLA\text{-}DR^{low/-}Lin^-$ polymorphonuclear (PMN) MDSC, and $HLA\text{-}DR^{low/-}CD33^{dim}CD66b^-Lin^-$ early-stage MDSC (e-MDSC) [39]. MDSC could inhibit anti-tumor functions of T and natural killer (NK) cells via different mechanisms. They can express PD-L1 and FasL and cause T cell anergy and apoptosis [40]. The induction of hypoxia-inducible factor-1α (HIF-1α) through transforming growth factor-β (TGF-β) and hypoxic conditions leads to the upregulation of the ectoenzymes CD39 and CD73, producing immunosuppressive adenosine in the extracellular space [40,41]. Reactive oxygen species (ROS) and nitric oxide (NO) produced by MDSC induce T cell apoptosis and the down-regulation of TCR ζ-chain expression [41,42]. Furthermore, MDSC can stimulate regulatory T cell (Treg) activity [43].

Previous studies demonstrated that high frequency of MDSC in the peripheral blood of advanced melanoma patients correlated with disease progression, decreased overall and progression free survival as well as decreased efficacy of immunotherapy, making them a promising therapeutic target [44–47]. There are different ways to suppress the immunosuppressive activity of MDSC [48]. Normalization of myelopoiesis and depletion of immunosuppressive MDSC could be achieved by using all-trans retinoic acid (ATRA) [49,50], tyrosine-kinase inhibitors [51,52] or some chemotherapeutic agents such as gemcitabine or paclitaxel [53,54].

Another approach of targeting MDSC represents an inhibition of their immunosuppressive activity. Based on the preclinical data showing that phosphodiesterase (PDE)-5 inhibitor sildenafil could suppress MDSC activity, enhance T cell functionality and prolong survival of melanoma-bearing mice [55,56], another PDE-5 inhibitor tadalafil was applied in advanced, therapy-resistant melanoma patients. Therapy was well-tolerated, and 25% of treated patients showed stable disease (SD) with the progression free survival (PFS) of 4.6 months [57]. Moreover, patients with SD showed increased infiltration of activated $CD8^+$ T cells in the metastasis as compared to non-responding patients.

Since the main immunosuppressive effect of MDSC is observed in the TME, the inhibitors of their recruitment to the tumor were tested. Small molecule inhibitor of C-X-C motif chemokine receptor (CXCR) 1 and CXCR2 SX-682 was demonstrated to suppress PMN-MDSC migration and activity, and enhance the efficiency of ICI therapy in mouse oral carcinoma and Lewis lung carcinoma model [58]. In human, SX-682 has been recently applied to advanced melanoma patients alone or in combination with pembrolizumab (Table 1). This table contains ongoing clinical trials, including the combination of ICI with targeting of various immunosuppressive cells (MDSC, CAF, TAM, Treg) and tumor cells as well as with targeting of processes and molecules such as hypoxia, microbiome, neoantigens, and epigenetic mutations. In addition, we included trials combining classical ICI with targeted therapies and new immune checkpoint molecules as well.

Table 1. Ongoing combinatorial clinical trials.

Targets	Trial Number	Intervention	Disease	Trial Phase
MDSC	NCT03200847	ATRA (Vesanoid) + pembrolizumab	Advanced melanoma	I, II
	NCT02403778	ATRA + ipilimumab	Advanced melanoma	II
	NCT03161431	SX-682 alone or in combination with pembrolizumab	Melanoma (III, IV)	I
	NCT02259231	RTA 408 (Omaveloxolone) + nivolumab or ipilimumab	Unrespectable or metastatic melanoma	Ib, II
CAF	NCT03875079	RO6874281 + pembrolizumab	Metastatic melanoma	Ib
TAM	NCT01363206	GM-CSF (Leukine, Sargramostim) + ipilimumab	Unresectable metastatic melanoma	II
Treg	NCT02203604	Aldesleukin (IL-2) + ipilimumab	Metastatic melanoma (IIIA–IV)	II
	NCT02983045	NKTR-214 (PEGylated IL-2) + nivolumab with or without ipilimumab	Advanced malignancies, including melanoma	I, II
	NCT03548467	NKTR-214 after prior anti-PD-1 therapy	Advanced malignancies, including melanoma	I, II
	NCT03635983	NKTR-214 + nivolumab or nivolumab alone	Untreated, inoperable or metastatic melanoma	III
	NCT03138889	NKTR-214 + pembrolizumab	Advanced malignancies, including melanoma	I, II
	NCT03435640	Intratumoral NKTR-262 + systemic NKTR-214 with or without nivolumab	Melanoma and other cancer types	I, II
	NCT03635983	NKTR-214 + nivolumab or nivolumab alone	Untreated, inoperable or metastatic melanoma	III
Microbiome	NCT03341143	Fecal microbiota transplant (FMT) + pembrolizumab	Advanced melanoma patients, non-responders	II
	NCT03817125	Vancomycin or placebo pretreatment + nivolumab + SER-401 or placebo	Unresectable or metastatic melanoma	Ib
	NCT03772899	FMT for a healthy donor a week before approved melanoma treatment (pembrolizumab/nivolumab)	Advanced melanoma	I
	NCT03643289	Comparison of gut microbiome before and during anti-PD-1 therapy (till week 9)	Advanced melanoma stage IV	Observational
Hypoxia	NCT03311308	Metformin + pembrolizumab or pembrolizumab alone	Advanced, unresectable melanoma stage III or IV	I
	NCT03171064	Exercise + nivolumab or pembrolizumab	Metastatic melanoma	II
Tumor cells	NCT02799901	Hypofractionated radiation therapy (RT) (27 Gy over 3 fractions) + nivolumab	Advanced melanoma	II
	NCT03693014	Hypofractionated RT + Ipilimumab, Nivolumab or Pembrolizumab, continued according to the standard schedule	Metastatic cancer, including melanoma	II
	NCT02406183	Ipilimumab + RT	Metastatic melanoma	I
	NCT04042506	Nivolumab + RT	Metastatic melanoma	II
	NCT04017897	Anti-PD1 (pembrolizumab or nivolumab) + RT	Unresectable, naive metastatic melanoma (IIIB to IVM1c)	II
	NCT01449279	Ipilimumab + RT	Metastatic melanoma	II

Table 1. *Cont.*

Targets	Trial Number	Intervention	Disease	Trial Phase
Tumor cells	NCT01689974	Ipilimumab + RT or ipilimumab alone	Metastatic melanoma	II
	NCT01769222	Ipilimumab + RT or ipilimumab alone	Recurrent malignancies, including melanoma	I, II
	NCT02659540	Nivolumab + ipilimumab in combination with conventional or hypofractionated RT	Unresectable melanoma stage IV	I
	NCT02263508	Pembrolizumab + T-VEC or placebo	Stage IIIB-IVM1c melanoma	III
	NCT04068181	Pembrolizumab + T-VEC after progression on anti-PD-1 therapy	Stage IIIB-IVM1d melanoma	II
	NCT01740297	Ipilimumab + T-VEC or ipilimumab alone	Stage IIIB–IV metastatic melanoma	I, II
	NCT02965716	Pembrolizumab + T-VEC	Stage IIIB–IV metastatic melanoma	II
	NCT03842943	Neoadjuvant pembrolizumab + T-VEC	Resectable stage 3 melanoma	II
Tumor mutations	NCT02902042	Encorafenib + binimetinib + pembrolizumab	Metastatic BRAF V600 mutant melanoma	I, II
	NCT02910700	Nivolumab + trametinib with or without dabrafenib	BRAF-mutated or wild type metastatic stage III-IV melanoma	II
	NCT02908672	Cobimetinib + vemurafenib with atezolizumab or placebo	Metastatic BRAF V600 mutant melanoma	III
	NCT02303951	Vemurafenib + cobimetinib + atezolizumab	BRAF V600 mutant stage IIIC-IV melanoma	II
	NCT01767454	Dabrafenib + ipilimumab or dabrafenib + trametinib + ipilimumab	Metastatic or unresectable BRAF V600 mutant melanoma	I
Epigenetic modifications	NCT03765229	Entinostat + pembrolizumab	Stage III–IV metastatic melanoma	II
	NCT02437136	Entinostat + pembrolizumab	Advanced malignancies, including melanoma	Ib, II
Neoantigens	NCT03929029	NeoVax + Montanide® with nivolumab + ipilimumab	Advanced melanoma	Ib
	NCT02385669	Peptide Vaccine + Ipilimumab	Stage IIA–IV melanoma (advanced, adjuvant, neoadjuvant)	I, II
	NCT03047928	PD-L1/IDO peptide vaccine + nivolumab	Metastatic melanoma	I, II
	NCT03633110	GEN-009 Adjuvant Vaccine + pembrolizumab or nivolumab	Solid tumors, including melanoma	I, II
	NCT04072900	Personalized neoantgen peptide vaccine + anti-PD-1 + rhGM-CSF + Imiquimod 5% Topical Cream	Metastatic melanoma	I
	NCT04091750	Nivolumab + ipilimumab + cabozantinib followed by nivolumab + cabozantinib	Advanced melanoma	II
Other immune checkpoint molecules	NCT02676869	IMP321 + pembrolizumab	Stage III–IV advanced melanoma	I
	NCT02519322	Nivolumab + relatimab or + ipilimumab or alone before surgery	Stage IIIb–IV advanced melanoma	II

Table 1. *Cont.*

Targets	Trial Number	Intervention	Disease	Trial Phase
Other immune checkpoint molecules	NCT03743766	Relatimab + nivolumab or each drug alone followed by relatimab + nivolumab in all subjects	Unresectable or metastatic melanoma	II
	NCT03470922	Relatimab + nivolumab or nivolumab alone	Unresectable or metastatic melanoma	II, III
	NCT03652077	INCAGN02390 antibody against TIM-3 alone	Advanced malignancies, including melanoma	I
	NCT04139902	Neoadjuvant therapy with PD-1 inhibitor dostarlimab (TSR-042) or dostarlimab (TSR-042) + TSR-022 (TIM-3 inhibitor)	Stage IIIB–IV advanced melanoma	II
	NCT03708328	RO7121661, bispecific anti-PD-1 and anti-TIM-3 antibody	Advanced malignancies, including melanoma	I
	NCT02817633	TSR-022 (anti-TIM-3) alone or + TSR-042 (anti-PD-1) or triple combination of TSR-022 (anti-TIM-3), TSR-042 (anti-PD-1) and TSR-033 (anti-LAG3)	Advanced malignancies, including melanoma	I
	NCT03628677	AB154 (anti-TIGIT) alone or + AB122 (anti-PD-1)	Advanced malignancies, including melanoma	I
	NCT03119428	OMP-313M32 (anti-TIGIT) alone or + nivolumab	Advanced malignancies, including melanoma	I

4.2. Neutrophils

Exposed by high amounts of TGF-β, granulocyte-colony stimulating factor (G-CSF) and IFN-β, tumor associated neutrophils (TAN) lose their anti-tumor functions and start to support tumor progression [59]. TAN have been described to enhance tumor angiogenesis and promote metastasis [60]. High neutrophil to lymphocyte ratio (≥4) at the baseline is considered as a powerful prognostic factor associated with reduced PFS and OS in melanoma patients treated with immune checkpoint inhibitors [61,62].

4.3. Cancer-Associated Fibroblasts (CAF)

CAF are a major component of the tumor stroma [63]. They produce different cytokines such as TGF-β, fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF), which lead to the tumor progression [64]. Moreover, an accumulation of CAF was described to correlate with low efficiency of anti-PD-1 therapy [65]. CAF secret fibroblast activation protein (FAP), which suppresses T cells function and recruitment [66,67]. In addition, FAP was reported to be a negative prognostic marker in the absence of immunotherapy but a positive indicative biomarker in ICI treated melanoma patients with a positive impact on PFS and OS [65]. In the murine melanoma model it was shown that stromal fibroblast matrix metalloproteinase-9 mediated surface PD-L1 cleavage, thus leading to the anti-PD-1 therapy resistance [68]. There is an ongoing trial (NCT03875079) to investigate the activity of the FAP-targeting agent RO6874281 in combination with pembrolizumab.

4.4. Tumor-Associated Macrophages (TAM)

TAM are known to produce interleukin (IL)-1β, cyclooxygenase-2, angiotensin, IFN-γ promoting tumorigenesis [69]. These cells can recruit regulatory T cells (Treg) and inhibit effector T cells by secreting IL-10 and expressing PD-L1 [70]. $CD68^+$ TAM in tumor cell nests were described to be associated with a negative prognosis and recurrence in cutaneous melanoma [70]. Furthermore, the ratio of $CD8^+$ T cells to $CD68^+$ macrophages was shown to predict a disease specific survival in

melanoma [71]. CD163$^+$ macrophages were reported to accumulate in the TME of melanoma patients resistant to ICI therapy and to play a role in the maintenance of the immunosuppression. The depletion of CD163$^+$ macrophages led to the invasion of activated T cells and inflammatory monocytes into the tumor, resulting in tumor regression [72,73].

4.5. Regulatory T Cells

Treg represent another important part of TME. It has been shown that the amount of forkhead box protein P3 positive (FOXP3$^+$) Treg is upregulated in the peripheral blood of melanoma patients [74]. Furthermore, the frequency of circulating FOXP3$^+$ Treg is associated with a poor prognosis in melanoma [75]. Tumor infiltrating Treg have been described to be a predominant cluster of the cells with high CTLA-4 expression [76]. It was found that the therapy with common anti-CTLA-4 antibodies (ipilimumab) did not deplete Treg in the tumor [77], however, Fc-engineered anti-CTLA-4 antibodies can specifically deplete FOXP3$^+$ Treg and promote CD8$^+$ T cell expansion, suggesting their higher clinical efficiency than the widely used non-Fc-engineered ipilimumab [76]. In another study, it was reported that the presence of Fcγ receptor-expressing macrophages within the TME is critical for the depletion of tumor-infiltrating Treg [78].

The application of NKTR-214, an engineered cytokine with biased IL-2 receptor binding, was demonstrated to selectively stimulate CD8$^+$ T cells and to deplete Treg in patients with advanced or metastatic solid tumors [79].

5. Role of Microbiome in the ICI Therapy of Melanoma

It has recently been clearly demonstrated that the microbiome could influence the ICI therapy in melanoma patients [80]. Although oral microbiome showed no effect on the response to cancer immunotherapy, an enrichment of *Clostridiales*, *Ruminococcaceae*, and *Faecalibacterium* in the gut was associated with response, while an enrichment of *Bacteroidales* was observed in non-responders and associated with increased risk of relapse [80]. The same study demonstrated that a favorable gut microbiome composition at the baseline was associated with increased CD8$^+$ T cell infiltration and anti-tumor immune responses. Furthermore, the fecal transplantation from melanoma patients responding to ICI to germ-free mice led to a better response to anti-PD-1 therapy as compared to mice, receiving gut transplants from non-responding patients [80]. Another study demonstrated that the presence of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* was associated with a better prognosis in melanoma patients [81]. Moreover, the anti-cancer immunity was described to be affected by the alteration in the metabolism of specific bacterial species but not by their presence [82]. There are several ongoing clinical trials dealing with the gut microbiota transplantation in melanoma patients (Table 1).

6. Predicting the Response to the ICI Therapy

Since the response rates to ICI treatment are still restricted [26–29,83], the identification of response-biomarkers before or shortly after the therapy initiation is one of the biggest challenges in the immuno-oncology. Current approaches to predict response to ICI in melanoma are based on the radiology, tumor biopsy and liquid biopsy [84,85].

Radiological imaging (body computer tomography (CT) scan, head magnetic resonance imaging (MRI)) is used to assess the response to ICI treatment in melanoma patients and is routinely performed three months after the start of treatment. Prediction of response in the earlier time points is possible by using ^{18}F-FDG PET/CT, where response criteria were developed using the scans made at 21 to 28 days after the start of treatment [86]. This approach was also shown to be beneficial in long-term response prediction and guidance of ICI withdrawal [87–89].

As a part of PD-1/PD-L1 axis, amount of PD-L1 expression on tumor cells was thought to be a distinct predictive marker for therapy response. Although PD-L1 overexpressing tumors showed an association with the higher response to ICI, durable responses could be also observed in PD-L1 negative

tumors [90,91]. Therefore, complementary approaches are needed to improve the prognostic value of tumor PD-L1, including a dynamic monitoring of PD-L1 expression or PD-L1 RNA sequencing [92,93].

Further interest attracts the measurement of PD-L1 (soluble and expressed in extracellular vesicles, EV) in liquid biopsies. Soluble PD-L1 is a splice variant without a transmembrane domain capable to directly inhibit T cell proliferation and IFN-γ production [94]. Elevated basal levels of soluble PD-L1 in the plasma of melanoma patients was associated with progressive disease [95]. Furthermore, the measurement of PD-L1 in EV could help to predict the response to ICI, demonstrating an advantage of the detection in EV over tumor biopsies [96]. Melanoma patients responding to pembrolizumab could be distinguished from non-responders by increased levels of EV PD-L1 at 3 to 6 weeks after the start of therapy [97]. In another study, it was shown that exosomal PD-L1 mRNA levels decreased during nivolumab or pembrolizumab treatment of melanoma patients with complete or partial response, while in patients with progressive disease EV PD-L1 expression was increased [98].

Besides PD-L1, soluble CD163 and macrophage-related chemokines (e.g., C-X-C motif chemokine ligand (CXCL) 5, 10) were reported to predict efficacy of ICI [85]. Decreased serum levels of IL-8 at 2 to 4 weeks after the start of ICI treatment were associated with the response in patients even with the initial pseudoprogression [99]. Induction of CXCR3 ligands in murine melanoma model was described to increase the response to the therapy with anti-PD1 antibodies, and elevated CXCR3 levels were observed in plasma of responding melanoma patients [100].

Another predictive marker could be the amount of tumor-infiltrating T cells. It has been shown that T cells dominated among other immune cells, accumulated in human melanoma metastatic tissue [101]. Strong pre-existing T cell infiltration, IFN-γ–related gene expression signatures in the tumor and high serum level of IFN-γ were reported to be associated with a good clinical prognosis and to predict the response to anti-PD-1 therapy in melanoma patients [101–105]. It was reported that 98% of PD-L1$^+$ tumors were associated with high TIL numbers and the PD-L1$^+$ melanoma cells were localized adjacent to TILs [106].

7. Increasing Effectiveness of ICI Therapy

In order to enhance the beneficial therapeutic effect of ICI, this treatment was combined with other anti-tumor therapies. Since radiation therapy (RT) is used in melanoma patients and can induce antigen release from tumors, its combination with immunotherapy was applied, leading to the T cell activation and improvement of OS without increasing the number of adverse events [107,108]. In a retrospective study with 208 melanoma patients with brain metastasis treated with anti-PD-1 antibodies and RT, the survival rates at 6 and 12 months after the start of treatment were 77% and 70%, respectively [109]. There are numerous ongoing trials investigating the combination of immuno- and radiation therapy in metastatic melanoma patients (Table 1).

Another promising approach to increase the efficiency of ICI is to combine it with metformin, a drug for type II diabetes. Metformin was shown to induce not only cell cycle arrest in melanoma cells, leading to their autophagy and apoptosis, but also to affect the TME [110]. It is known that metformin activates AMP-activated protein kinase a (AMPKa) in mitochondria, which lead to the downregulation of HIF-1α expression, resulting in reduced intratumoral hypoxia. Metformin was also reported to promote T cell activity in the combination with ICI, leading to B16 melanoma rejection in mice [111]. In a clinical trial, it was shown that the combination of ICI and metformin increased objective response rate (ORR), disease control rate (DCR), PFS and OS in comparison with the group treated with ICI alone [112]. However, due to a small patient cohort, these changes were not statistically significant.

Interestingly, the reduction of tumor hypoxia could be achieved by a physical exercise as well. In B16F10 mouse melanoma model, voluntary wheel running resulted in the epinephrine-dependent, IL-6-sensitive NK cell activation and increased migration of NK and T cells into the tumor [113]. In addition, a physical activity prior to tumor cells inoculation led to a strong reduction of primary tumor growth and numbers of lung metastasis in those mice. Other study demonstrated that the growth of B16F10 melanoma in mice on high-fat diet was accelerated as compared to mice receiving a

balanced diet [114]. Importantly, this growth increase was significantly reduced by continuous physical exercise that was associated with the lymphocyte proliferation [114]. In melanoma patients, exercises undertaken before diagnosis were not significantly correlated with a reduction in cancer-related or overall mortality [115]. However, in patients with unresectable stage III or IV melanoma undergoing immunotherapy, the reduction of fatigue was shown to be the main positive impact of physical activity [116]. The ongoing combinational trial is represented in the Table 1.

Targeted therapies (BRAF and MEK-inhibitors) are known to be effective in patients with BRAF-V600 mutation and achieve rapid response with a high response rate [117]. The median maintenance of response to this therapy is approximately one year because of the development of acquired resistance [118], while ICI have been described to induce durable response. It was reported that 33% of melanoma patients achieved complete response when treated with the combination of dabrafenib and trametinib with spartalizumab (anti-PD-L1-antibody); the 1-year OS was 86%; however, the number of grade ≥3 adverse events was 75% [119]. In another study, dabrafenib and trametinib were combined with pembrolizumab (triple therapy) or placebo (double therapy) [120]. The median duration of response in tripled therapy group was 18.7 months and 12.5 months in double therapy group. PFS was 16.0 months in triple and 10.3 months in double therapy. In a smaller patient's cohort, an objective response was achieved in 73% of patients, and 40% maintained the response at a median follow-up of 27.0 months [121]. 73% of patients from the same cohort developed grade 3 and 4 adverse events. Another trial, investigating the combination of atezolizumab (anti-PD-L1-anibody), cobimetinib and vemurafenib showed similar results with an objective response rate of 71.8% and median duration of response of 17.4 months; 39.4% of patients maintained response for 29.9 months of follow-up [122]. These data suggest that this combination therapy can increase the maintenance of the response, but the high grades of adverse events need to be taken into account. Ongoing trials to the triple combination are shown in Table 1.

ICI could also be combined with the oncolytic virus talimogen laherparepvec (T-VEC) that was approved for melanoma immunotherapy. T-VEC is a genetically modified virus, which replicates in tumor cells causing cancer cell lysis [123]. It has been reported that the intratumoral T-VEC injection in combination with pembrolizumab led to increased $CD8^+$ T cells infiltration associated with the ORR rate of 62% and the CR in 33% of patients [124].

Combination of all-trans retinoic acid (ATRA) with ipilimumab was reported to decrease frequency of circulating MDSC as well as the expression of PD-L1, IL-10, and indoleamine 2,3-dioxygenase by MDSC, whereas in the ipilimumab monotherapy group the MDSC frequency increased during the treatment [125]. Furthermore, patients receiving combinational treatment tend to have an increased activated $CD107a^+$ IFN-γ^+CD8^+ T cell numbers compared to the patients treated with ipilimumab alone.

Combination of NKTR-214 and Nivolumab was shown to achieve response rates of 53%, which correlated with high IFN-γ levels [126]. Furthermore, the accumulation of IFN-γ and $CD8^+$ TIL in tumor tissue had been seen in favorable as well as in unfavorable tumor microenvironment. The ongoing trials investigating the combination of NKTR-214 with ICI in metastatic melanoma patients are listed in Table 1.

It was demonstrated that epigenetic modulation induced by the histone deacetylase inhibitor entinostat (MS-275) could enhance the antigen presentation in tumor cells and inhibit immunosuppressive activity of MDSC and Treg [127,128]. After combining entinostat with the anti-PD-1 antibodies, 19 % of non-responding to anti-PD-1 therapy melanoma patients, achieved objective response [129]. These data represent a new approach to overcome resistance using epigenetics. Other ongoing trials using this combination are listed in Table 1.

A new approach of targeting different TME components using nanoparticles has been recently proposed [130]. In melanoma mouse models, nanoparticles were shown to potentiate the efficiency of PD-1 blockade [131–133], to reduce the tumor volume and to prolong mouse survival [134].

8. Other ICI in Malignant Melanoma

In addition to PD-L1 and CTLA-4, several other immune checkpoint molecules have been investigated during the last decade. Among them are lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin- and mucin domain- containing molecule 3 (TIM-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT). All these molecules were reported to be highly expressed on immune cells in the TME, especially on TILs and Treg, which makes them a promising target for cancer immunotherapy [135].

LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ T cells, Treg, B and NK cells as well as DC [136]. It interacts with MHCII molecules on APC or with Galectin-3 and liver sinusoidal endothelial cell lectin (LSECtin) on cancer cells, leading to the inhibition of $CD4^+$ and $CD8^+$T cell proliferation and decreased cytokine secretion [137]. Such inhibition of T cell function was found to be associated with the promotion of tumor growth and tumor escape [138,139]. LAG-3 blocking could be achieved by LAG-3-Ig fusion protein or LAG-3 targeting antibody (relatlimab). The treatment of melanoma patients with relatlimab resulted in the ORR of 16% and DCR of 45% [140]. Interestingly, only 9% of patients had grade 3 or 4 adverse events that was comparable to the therapy with nivolumab.

TIM-3 is expressed on $CD4^+$ and $CD8^+$ T cells, Treg, B cells, NK cells, DC, mast cells and macrophages. Under physiological conditions, it serves as a negative regulator of Th1 response and Th1 related production of TNF and IFN-γ; therefore, its blockade could lead to autoimmune disease [141]. Interaction of TIM-3 with Galectin-9 expressed on tumor cells was reported to result in CD8 TIL apoptosis in colon cancer [142]. In melanoma high expression of TIM-3 was associated with CD8 T cell exhaustion [143].

TIGIT was reported to be involved in the inhibition of $CD8^+$T cells and modulation of DC activity, resulting in the upregulation of IL-10 and downregulation of IL-12 production [144,145]. Moreover, TIGIT was demonstrated to play a crucial role in the maturation of naïve T cells to $Foxp3^+$ Treg [146]. $TIGIT^+$ Tregs showed higher immunosuppressive potential than their $TIGIT^-$ counterparts [147]. In malignant melanoma, the co-expression of PD-L1, LAG-3, TIM-3 and TIGIT was demonstrated to induce $CD8^+$ TILs with most exhausted phenotype [125,126]. Double blockage of PD-1 and TIGIT in melanoma led to an increased proliferation and cytokine production of $CD8^+$ TIL and was considered to be a promising approach in immunotherapy [148]. The ongoing clinical trials evaluating the efficiency of LAG-3, TIM-3 and TIGIT blockade are shown in Table 1.

9. Conclusions

Despite of melanoma immunogenicity, this tumor develops immune escape mechanisms that stimulate a fast melanoma progression. Such mechanisms include impaired antigen presentation by tumor cells, accumulation of dysfunctional effector T cells and generation of the immunosuppressive TME represented by MDSC, TAN, CAF, TAM, and Treg. Therefore, numerous approaches were developed to reinvigorate the anti-tumor immune response. Recently approved immunotherapies with ICI (anti-PD-1, anti-PD-L1 and anti-CTLA-4 antibodies) have revolutionized the treatment of melanoma. This treatment significantly increased the survival of melanoma patients and provided a durable control of the disease [26–28]. However, the response rates to ICI are still restricted. Thus, further efforts should be undertaken to maximize the efficacy of ICI treatment. This aim could be achieved by improving the selection of patients who might benefit from the ICI therapy, by applying early radiological findings and by measuring predictive markers from tumor and liquid biopsies. Furthermore, the combination of different ICI (such as ipilimumab and nivolumab), their combination with targeting of the immunosuppressive TME or with other anti-cancer therapies could significantly improve the efficacy of tumor immunotherapy. Furthermore, targeting of other immune checkpoints (such as LAG-3, TIM-3, TIGIT) and its combination with approved ICI are currently under investigation (Table 1). Approved ICI, their targets, and targets for combined treatments are summarized in the Figure 1.

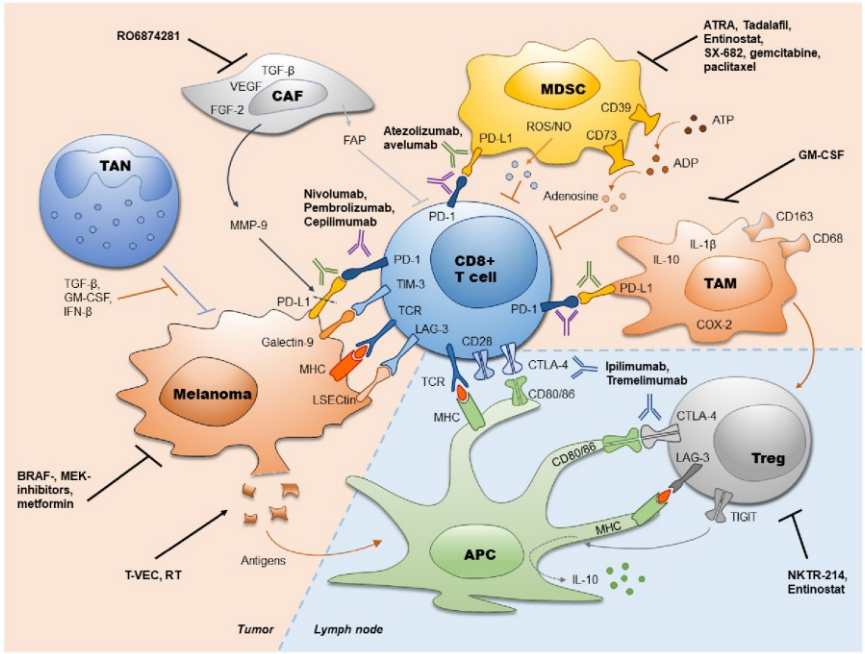

Figure 1. Immune checkpoint inhibitors in melanoma and their combination with other therapies. Currently used antibodies against PD-1 (atezolizumab, avelumab), PD-L1 (nivolumab, pembrolizumab, cepilimumab) and CTLA-4 (ipilimumab, tremelimumab) as well as strategies to increase the efficiency of immune checkpoint inhibitors (ICI) are presented. ADP: adenosine diphosphate; APC: antigen presenting cell; ATP: adenosine triphosphate; ATRA: all-trans retinoic acid; CAF: cancer-associated fibroblasts; COX-2: cyclooxygenase-2; CTLA-4: cytotoxic T lymphocyte-associated protein-4; FAP: fibroblast activation protein; FGF-2: fibroblast growth factor 2; GM-CSF: granulocyte-macrophage colony stimulating factor; IFN-β: interferon-β; IL: interleukin; LAG-3: lymphocyte activation gene-3; LSECtin: liver sinusoidal endothelial cell lectin; MDSC: myeloid-derived suppressor cells; MHC: major histocompatibility complex; MMP-9: matrix metallopeptidase 9; NO: nitric oxide; PD-1: programmed cell death protein 1; PD-L1: programmed cell death ligand 1; ROS: reactive oxygen species; RT: radiation therapy; TAM: tumor-associated macrophages; TAN: tumor associated neutrophils; TCR: T-cell receptor; TGF-β: transforming growth factor-β; TIGIT: T cell immunoreceptor with Ig and ITIM domains; TIM-3: T-cell immunoglobulin- and mucin domain- containing molecule 3; Treg; regulatory T cells; T-VEC: talimogen laherparepvec; VEGF: vascular endothelial growth factor.

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Abbreviations

ADP	adenosine diphosphate
AMPKa	AMP-activated protein kinase a
APC	antigen presenting cell
ATP	adenosine triphosphate
ATRA	all-trans retinoic acid
CAF	cancer-associated fibroblasts
COX-2	cyclooxygenase-2
CT	computer tomography
CTLA-4	cytotoxic T lymphocyte-associated protein-4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DCR	disease control rate
EV	extracellular vesicles
FAP	fibroblast activation protein
FGF-2	fibroblast growth factor 2
$FOXP3^+$	forkhead box protein P3
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HIF-1α	hypoxia-inducible factor-1α
ICI	immune checkpoint inhibitors
IFN	interferon
IL	interleukin
LAG-3	lymphocyte activation gene-3
LSECtin	liver sinusoidal endothelial cell lectin
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MMP-9	matrix metallopeptidase 9
MRI	magnetic resonance imaging
NK	natural killer
NO	nitric oxide
ORR	objective response rate
OS	overall survival
PD-1	programmed cell death protein 1
PDE	phosphodiesterase
PD-L1	programmed cell death ligand 1
PD-L2	programmed cell death ligand 2
PFS	progression free survival
PMN	polymorphonuclear
ROS	reactive oxygen species
RT	radiation therapy
SD	stable disease
TAM	tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T-cell receptor
TGF-β	transforming growth factor-β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T-cell immunoglobulin- and mucin domain- containing molecule 3
TME	tumor microenvironment
Treg	regulatory T cells
T-VEC	talimogen laherparepvec alimogen laherparepvec
VEGF	vascular endothelial growth factor

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Immunosuppressive capacity of circulating MDSC predicts response to immune checkpoint inhibitors in melanoma patients

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Purpose: Although the treatment of advanced melanoma patients with immune checkpoint inhibitors (ICI) significantly increased the therapeutic efficiency, many patients remain resistant to ICI that could be due to immunosuppression mediated by myeloid-derived suppressor cells (MDSC). These cells are enriched and activated in melanoma patients and could be considered as therapeutic targets. Here we studied dynamic changes in immunosuppressive pattern and activity of circulating MDSC from melanoma patients treated with ICI.

Experimental design: MDSC frequency, immunosuppressive markers and function were evaluated in freshly isolated peripheral blood mononuclear cells (PBMC) from 29 melanoma patients receiving ICI. Blood samples were taken prior and during the treatment and analyzed by flow cytometry and bio-plex assay.

Results: MDSC frequency was significantly increased before the therapy and through three months of treatment in non-responders as compared to responders. Prior to the ICI therapy, MDSC from non-responders displayed high levels of immunosuppression measured by the inhibition of T cell proliferation assay, whereas MDSC from responding patients failed to inhibit T cells. Patients without visible metastasis were characterized by the absence of MDSC immunosuppressive activity during the ICI treatment. Moreover, non-responders showed significantly higher IL-6 and IL-8 concentrations before therapy and after the first ICI application as compared to responders.

Conclusions: Our findings highlight the role of MDSC during melanoma progression and suggest that frequency and immunosuppressive activity of circulating MDSC before and during the ICI treatment of melanoma patients could be used as biomarkers of response to ICI therapy.

KEYWORDS

MDSC, melanoma, immunosuppression, immune checkpoint inhibitors, cytokines

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Introduction

Malignant melanoma is characterized by high mutational burden and increased immunogenicity (1, 2) but also by a profound immunosuppression (3). The latter represents one of the major reasons for poor therapy responses (4, 5). The application of immune checkpoint inhibitors (ICI), including antibodies against programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte protein 4 (CTLA-4) significantly increased survival and response rates for advanced melanoma patients (6). However, many patients fail to respond to ICI. In particular, only 20% melanoma patients respond to anti-CTLA-4 treatment, 30-40% respond to anti–PD-1 antibodies and 58% show clinical response to the combination therapy with these antibodies (7). Since the rate of the non-responsiveness to ICI is high, there is an urgent need to understand the underlying mechanisms of immunosuppression and to find biomarkers predicting clinical responses to ICI.

One of the major players in the immunosuppressive tumor microenvironment (TME) are myeloid-derived suppressor cells (MDSC). This heterogeneous population of myeloid cells emerges under chronic inflammatory conditions typical for cancer and is characterized by a strong ability to suppress anti-tumor T and NK cells *via* different mechanisms (8–10). In humans, three MDSC subsets have been described: $CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}CD66b^{-}$ monocytic (M-MDSC) and $CD33^{dim}HLA\text{-}DR^{low/-}CD14^{-}CD66b^{+}Lin^{-}$ polymorphonuclear (PMN-MDSC) that are strongly immunosuppressive as well as $CD33^{dim}HLA\text{-}DR^{low/-}CD66b^{-}Lin^{-}$ early-stage MDSC (e-MDSC), which fail to show immunosuppressive function (11, 12). Cells with typical MDSC phenotype are also present in healthy individuals in much smaller numbers and called non-immunosuppressive MDSC counterparts (12).

Generation, expansion, and recruitment of MDSC to the TME is influenced by cytokines, chemokines and growth factors produced by melanoma and host cells such as interleukin (IL)-1β, IL-6, IL-8, vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (9, 13). MDSC immunosuppressive function is stimulated by IL-1β, IL-6, IL-8, IL-4, IL-13, interferon γ (IFN-γ) and prostaglandin E2 (PGE2) (13).

It has been demonstrated that MDSC strongly express PD-L1, leading to T cell suppression of in the circulation and TME (14). MDSC display ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ectonucleotidase (CD73) expression, catalyzing the conversion of extracellular ATP into adenosine that inhibits effector T cell functions (15). The combination of CD39/CD73 targeting and ICI was reported to stimulate anti-tumor immunity in preclinical models (16). Moreover, MDSC produce reactive oxygen species (ROS) and nitric oxide (NO), which cause T cell anergy by the down-regulation of TCR ζ-chain expression (17, 18). All these mechanisms of immunosuppression mediated by MDSC support the tumor escape and reduce response to different melanoma therapies, including the ICI treatment. Even though the role of MDSC is well investigated, ICI-related changes in freshly isolated circulating MDSC from melanoma patients are not sufficiently studied, especially in patients with no evidence of disease (non-metastatic patients), receiving ICI in adjuvant setting. Here we analyzed the characteristics and function of MDSC as well as MDSC-related inflammatory mediators in the peripheral blood of 29 melanoma patients before and during ICI treatment. We found that elevated baseline frequency of MDSC, their high immunosuppressive activity as well as increased baseline levels of IL-6 and IL-8 are associated with unfavorable response to ICI treatment in metastatic patients. In contrast, MDSC from responding metastatic patients tend to lose their ability to suppress T cell functions under the ICI treatment. We suggest that the combination of ICI and MDSC targeting could improve the efficiency of melanoma immunotherapy.

Materials and methods

Patients and healthy donors

For this study, peripheral blood samples were collected from 19 metastatic and 10 non-metastatic melanoma patients receiving ICI at the Skin Cancer Center (University Medical Center Mannheim, Germany). Metastatic patients received ICI as a palliative treatment and non-metastatic patients as an adjuvant therapy (Figure 1). To simplify the narration, patients receiving ICI in palliative or adjuvant regimen will be called metastatic and non-metastatic patients respectively. This study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee (2010-318N-MA). Peripheral blood from 10 age- and gender-matched healthy donors (HD) without indications of immune-related diseases was obtained according to the Ethics Committee approval (2010-318N-MA) and used as controls. The collection of samples and clinical data was performed after written informed consent.

Clinical data

Metastatic patients with unresectable stage IIIC-IV melanoma (according to the AJCC 2017 classification) were treated with nivolumab, 480 mg (fixed dose) every 4 weeks or pembrolizumab, 200 mg, every 3 weeks. Metastatic patients with presence of brain metastasis were treated every 3 weeks with a combination of nivolumab, 1 mg/kg body weight, and ipilimumab, 3 mg/kg body weight. One patient from the metastatic group deceased before the therapy start. Non-metastatic melanoma patients with stage IIIB-IIID without current evidence of disease were treated with nivolumab, 3mg/kg body weight, every 2 weeks or pembrolizumab, 200 mg, every 3 weeks. In this group, 3 patients received nivolumab, 480 mg, every 4 weeks before the new dosage for adjuvant treatment was approved. All recruited patients received no immunotherapy before the study onset. Treatment efficacy was assessed by contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography CT (PET-CT) based on the Immunotherapy Response Evaluation Criteria in Solid Tumors (iRECIST) 3 months after the first administration of ICI. Based on the response at this time point, metastatic patients were divided into

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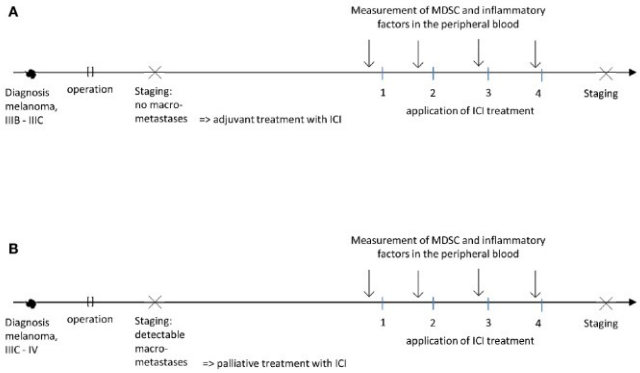

FIGURE 1
Schematic representation of adjuvant **(A)** or palliative **(B)** treatment settings (patients with no evidence of disease and those with distant metastases respectively).

responders showing complete response (CR), partial response (PR) or stable disease (SD) and non-responders (progressive disease, PD). Patient characteristics are summarized in Table 1.

Analysis of peripheral blood samples

Peripheral blood was collected from melanoma patients before (baseline) and after the 1st, 2nd, and 3rd application of ICI with trisodium citrate as an anticoagulating agent. Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation with Biocoll (Biochrom) and applied for flow cytometry and cell sorting. After the PBMC removal, plasma was collected and stored at -80°C.

Flow cytometry

Freshly prepared PBMC were treated with FcR Blocking Reagent (130-111-568, Miltenyi Biotec) according to the manufacturer's protocol and stained with fixable viability dye 700 (BD Biosciences) followed by the incubation with monoclonal antibodies (mAbs) for 30 min at 4°C. The following fluorescently labeled mAbs were used for the surface staining: CD66b-PerCPCy5.5 (clone G10F5), CD14-APCCy7 (clone MΦP9), HLA-DR-V500 (clone G46-6), lineage cocktail (LIN) (CD3/19/20/56)-APC, CD33-PE-Cy7 (clone P67.6), CD39-FITC (clone TU66), PD-L1-BV421 (clone MIH1, all from BD Biosciences) and CD73-BV605 (clone AD2, Biolegend). Intracellular ROS and NO were detected using hROS Detection Kit (Cell Technology) and diaminofluorescein-FM diacetate (Cayman Chemical) according to the manufacturer's instructions. Acquisition was performed by 10-color flow cytometry using BD FACSLyric with FACSuite software (BD Biosciences). FlowJo V 10 software (BD Biosciences) was used to analyze at least 10^6 events. Positive surface markers were gated according to the fluorescence minus one (FMO) control.

Inhibition of T cell proliferation assay

Immunosuppressive activity of MDSC during the ICI treatment was evaluated according to the standardized Mye-EUNITER protocol (12). Briefly, $CD3^+$ T cells were isolated from PBMC by magnetic-activated cell sorting (MACS, Miltenyi Biotec) according to the manufacturer's protocol. CD3 depleted PBMC were sorted for $HLA\text{-}DR^-/CD33^{high}$ M-MDSC and $HLA\text{-}DR^-/CD33^{dim}/CD66b^+/LIN^-$ PMN-MDSC. CD3 T cells were labeled with 20 µM cell proliferation dye eFluor 450 (CP-Dye405, eBioscience) and were cultured alone or with sorted PMN- or M-MDSC (T cells:MDSC ratio = 1:1) in a 96 well round bottom plate (Sarstedt) in L-lysine and L-arginine low RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS at 37°C. The plate was precoated for 3 hours with CD3 (clone OKT-3, eBioscience) and CD28 antibodies (clone CD28.2, Beckman Coulter). The proliferation of $CD8^+$ T cells was assessed after 96 h of co-culture by measuring CPDye405 dilution at the BD FACSLyric™ flow cytometer.

Bio-Plex assay

Concentrations of cytokines and chemokines in the serum of melanoma patients and HD were measured by the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad) using the manufacturer's protocol. Acquisition and data analysis were performed by bio-plex Manager™.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (Version 8.1.2). Data showing a Gaussian distribution were compared with the unpaired two-tailed Student's t test and not normally distributed data with Mann-Whitney test. Mixed-effects

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TABLE 1 Clinical characteristics of melanoma patients treated with ICI in palliative and adjuvant settings.

	Palliative treatment (n=19)	Adjuvant treatment (n=10)	Healthy donors (n=10)
Median age, years (range)	64 (41-84)	67 (34-84)	60 (38-73)
Sex, n			
Male	13	8	5
Female	6	2	5
AJCC stage, n			
IIIB	0	6	
IIIC	3	3	
IIID	0	1	
IV	16	0	
Primary melanoma site, n			
Cutaneous	13	10	
Uveal	1	0	
Unknown	5	0	
Therapy, n			
Nivolumab	12	9	
Pembrolizumab	1	1	
Nivolumab + ipilimumab	5	0	
Deceased before therapy start	1	0	
Therapy outcome, n			
Responder	12		
Complete response	1		
Partial response	8		
Stable disease	3		
Non-responder (progressive disease)	6		

analysis with multiple comparisons was used to compare treatment groups and to investigate dynamic changes during the treatment. Survival curves were generated using the Kaplan-Meier method, and the statistical comparison was done by the log rank (Mantel-Cox) test.

Results

Patient characteristics

29 melanoma patients receiving ICI and 10 HD gave informed consent to participate in a prospective clinical study (Table 1). In the adjuvant treatment group, eight patients remained relapse-free (80%) and two patients (20%) developed new metastases after the first staging. In the palliative treatment group one patient showed CR (6%), eight patients have PR (44%) and three patients showed SD (17%). Six individuals who showed PD (33%) were classified as non-responders. One patient died shortly before the therapy start.

Immunosuppressive pattern and function of MDSC at the baseline

First, we analyzed $CD33^{dim}HLA\text{-}DR^{low/-}CD66b^{+}Lin^{-}$ PMN- and $CD33^{+}HLA\text{-}DR^{low/-}CD14^{+}$ M-MDSC in PBMC from melanoma patients and their non-suppressive MDSC counterparts from HD by flow cytometry. The gating strategy is shown in Supplementary Figure 1. We found a significant increase in the frequency of both PMN- and M-MDSC in metastatic melanoma patients (palliative therapy setting) and patients without metastases (adjuvant setting) as compared to their counterparts in HD (Figure 2A). The maximum of PMN-MDSC frequency was observed in metastatic patients with advanced disease (Figure 2A). To investigate a possible association between the frequency of circulating PMN- and M-MDSC within PBMC and the overall survival (OS) and progression-free survival (PFS) of melanoma patients, we distributed metastatic patients in the groups with low and high frequencies of PMN- and M-MDSC, using their median values (0.54% and 0.73% of live PBMC respectively) as a cutoff (19). We demonstrated that a high frequency of PMN-MDSC before therapy begin (>0.54%) was associated with the tendency for reduced OS and PFS (Figure 2B, Supplementary Figure 2A). High M-MDSC frequency (>0.73%) also showed a tendency to correlate with decreased OS (Figure 2C, Supplementary Figure 2B).

Next, we investigated an immunosuppressive pattern of different MDSC subsets in both metastatic and non-metastatic patients at baseline. Metastatic melanoma patients showed elevated frequencies of $PD\text{-}L1^{+}$ M-MDSC as compared to non-metastatic patients (Figure 2D). Furthermore, the frequency M-MDSC expressing PD-L1 was higher that of PMN-MDSC (Figure 2D). However, we failed to observe any differences in the production of NO and ROS by MDSC from non-metastatic and metastatic patients (data not shown). CD39 and CD73 were stronger expressed on circulating M- than PMN-MDSC from melanoma patients (Figure 2E). While testing MDSC function using the inhibition of T cell proliferation assay, we observed that MDSC isolated from non-metastatic patients showed no immunosuppressive activity (Figure 2F). In contrast, metastatic patients formed two groups according to the immunosuppressive function of PMN- and M-MDSC: one group with high and another with low suppressive activity of MDSC. Interestingly, there was no difference between the immunosuppressive potential of PMN- and M-MDSC (Figure 2F).

Next, we studied MDSC frequency and function at baseline in metastatic patients who responded or failed to respond to ICI. We observed a slight tendency for accumulation of PMN- and M-MDSC before the therapy start in non-responders as compared to responders (Figure 3A). Although we found no significant differences in the expression of immunosuppressive molecules PD-L1, CD39 and CD73 on MDSC between these two groups (Supplementary Figure 3), we determined a clear difference in immunosuppressive activity of these cells. PMN- and M-MDSC isolated from non-responders showed a strong inhibition of T cell proliferation (Figures 3B, D), whereas MDSC from responders were not immunosuppressive (Figures 3C, D) except

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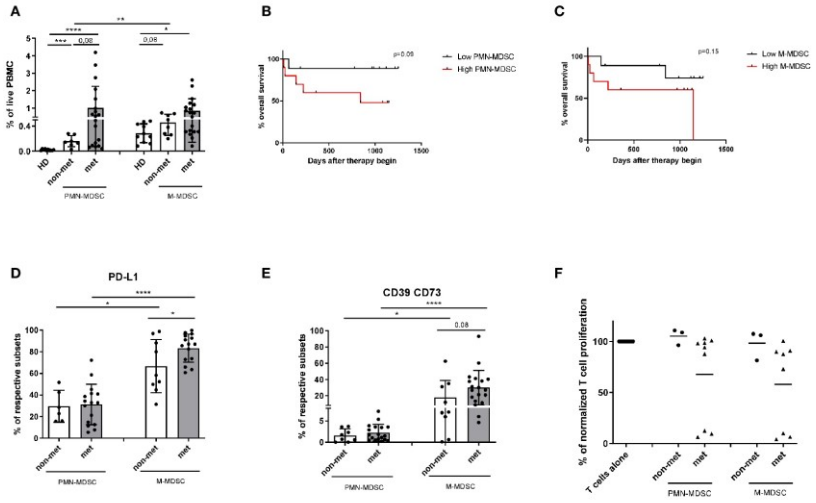

FIGURE 2
Characteristics of PMN- and M-MDSC from melanoma patients before ICI therapy. PBMCs were isolated from the peripheral blood of melanoma patients and HD. MDSC and their counterparts in HD were assessed by flow cytometry. **(A)** The results in metastatic (n=19) and non-metastatic (n=8) patients as well as their counterparts in HD (n=10) are presented as the percentage of HLA-DR$^{low/-}$CD33^{dim}CD66b^{+}Lin^{-} PMN- and HLA-DR$^{low/-}$CD33^{high}CD14^{+} M-MDSC among live PBMC. **(B)** OS of metastatic melanoma patients with high (>0.54% of live PBMC; n=10) and low (<0.54%; n=9) PMN-MDSC frequencies at the baseline is shown as a Kaplan-Meier curve. **(C)** OS of metastatic melanoma patients with high (>0.73%; n=10) and low (<0.73%; n=9) M-MDSC frequencies at the baseline is shown as a Kaplan-Meier curve. **(D, E)** Expression of PD-L1 and ectoenzymes CD39 and CD73 on PMN- and M-MDSC from metastatic and non-metastatic patients was shown as the percentage of PD-L1^{+} cells **(D)** or CD39^{+}CD73^{+} cells **(E)** among the respective MDSC subset. **(F)** Immunosuppressive capacity of PMN- and M-MDSC was determined upon the co-culture with activated CD3 T cells labeled with CP-Dye405. After 96 h of incubation, T cell proliferation was assessed by CP-Dye405 dilution measured by flow cytometry. Cumulative data for T cell proliferation are presented as the percentage of divided T cells normalized (norm.) to the respective control of stimulated T cells alone (n=3-8). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

one patient whose M-MDSC displayed immunosuppressive activity at the baseline but not after the 1st ICI application (Figure 3D).

Inflammatory factors before the start of ICI treatment

To evaluate the MDSC-related cytokine and chemokine profile in the peripheral blood of melanoma patients, we performed a bio-plex assay. We found that IL-6, IL-8, TNF-α and CCL5 were significantly increased in the plasma of metastatic and non-metastatic melanoma patients as compared to HD (Figures 4A, B). Moreover, metastatic patients showed significantly higher IL-8 concentrations in plasma than non-metastatic patients (Figure 4A). Our analysis revealed no differences in the concentration of other MDSC-related inflammatory factors (such as CCL2, CLL3, CCL4) between metastatic and non-metastatic groups (Supplementary Figure 4A). Interestingly, we found a correlation between increased levels of IL-6, IL-8, TNF-α and the accumulation of circulating PMN-MDSC in metastatic (Figures 4C–E) but not in non-metastatic patients (Supplementary Figures 4B–D), underlining their role in the melanoma progression. Similarly, augmented IL-6 concentration was associated with an increased frequency of M-MDSC only in patients with metastases (Figure 4F) but not in those without metastases (Supplementary Figure 4E).

To evaluate the potential of investigated soluble factors as predictive markers for the response to ICI, we compared their plasma concentrations in responders and non-responders. IL-6 and IL-8 were significantly increased at the baseline in non-responders as compared to responders, whereas TNF-α and CCL5 did not display such predictive capacity (Figure 4G).

Patients with more advanced disease exhibit stronger MDSC activity

Analyzing immunosuppressive characteristics of metastatic melanoma patients before the treatment, we observed that some patients were characterized by increased MDSC frequency and immunosuppression as well as by high concentrations of MDSC-related cytokines (IL-6, IL-8). All these patients (except one who deceased before the therapy start) displayed high metastatic load and received combinational therapy with anti-CTLA-4 and anti-PD-1 antibodies. We observed that abovementioned patients with a very severe disease were also characterized by MDSC activation associated with the accumulation of IL-6 and IL-8 as well as by the non-responsiveness to combinational ICI treatment (Table 2).

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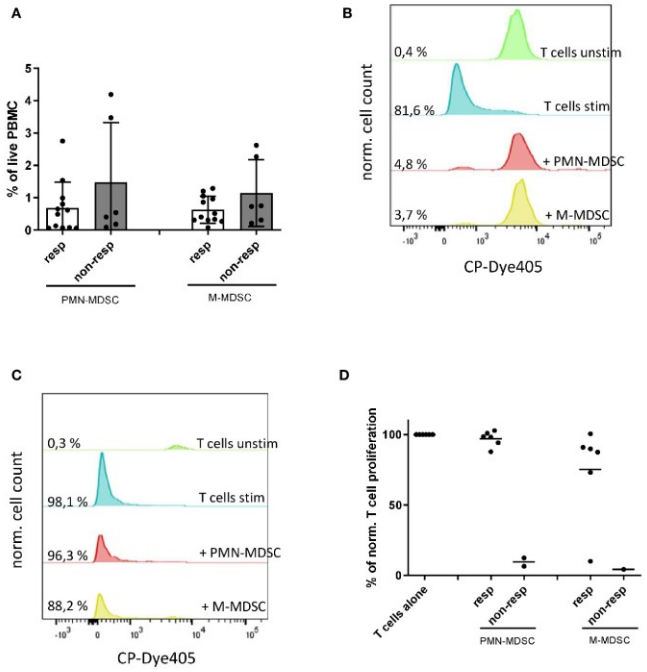

FIGURE 3
Baseline characteristics of PMN- and M-MDSC from responders and non-responders. **(A)** Results are presented as the frequency of circulating PNM- and M-MDSC among live PBMC from responders (n=12) and non-responders (n=6). Representative histograms for the proliferation of unstimulated (unstim) and stimulated (stim) T cells incubated alone or in the presence of isolated PMN- or M-MDSC from a non-responding **(B)** and responding patient **(C)**. **(D)** Immunosuppressive capacity of PMN- and M-MDSC was determined upon the co-culture with activated CD3 T cells labeled with CP-Dye405. Cumulative data for T cell proliferation are shown as the percentage of divided T cells normalized (norm.) to the respective control of stimulated T cells alone (n=2-8).

Dynamic changes in MDSC characteristics and soluble factors under ICI treatment

We performed the dynamic assessment of MDSC frequency and immunosuppressive function as well as MDSC-related soluble factors in metastatic and non-metastatic melanoma patients from the time point before therapy up to the first staging (baseline, after the 1st, 2nd, and 3rd ICI application). We found that the frequency of PMN-MDSC in metastatic melanoma patients showed a tendency to be higher than in non-metastatic patients through the first three ICI injections (Figure 5A). However, there were no differences between the kinetics of M-MDSC frequency upon the therapy in metastatic and non-metastatic patients (Supplementary Figure 5). We also found no changes in the expression of PD-L1, CD39 and CD73 on PMN- and M-MDSC from these two groups due to high interpersonal variance (Supplementary Figures 5B–E). Interestingly, MDSC isolated from non-metastatic patients showed no suppressive activity towards T cells during the first three ICI applications, while metastatic patients displayed high immunosuppressive potential at baseline and after the 1st ICI injection, which they tend to lose after 2nd and 3rd ICI application (Figure 5B).

Next, we analyzed the association between dynamic changes in MDSC frequency and immunosuppressive phenotype with the patients' response to ICI. In addition to the analysis of the single time points, we compared responders and non-responders using mixed-effects model. This model allows to compare repeated measurements (before, after the 1st, 2nd, and 3rd ICI application) where every patient acts as its own control and the model can handle missing values. It was found that PMN-MDSC frequency in PBMC of non-responders remained significantly higher than that in responders during first three ICI injections (Figure 5C). The highest frequency of PMN-MDSC was observed in non-responders after the 2nd ICI injection (Figure 5C). Similarly, non-responders showed significantly increased M-MDSC frequency under ICI treatment as compared to responders (Figure 5D). Moreover, we found a significant difference in PD-L1 expression on PMN- and M-MDSC between responders and non-responders with the main difference between two groups after the 2nd ICI injection (Figures 5E, F). Regarding ectonucleotidase expression, we found no significant differences in $CD39^+CD73^+$ MDSC between responders and non-responders (Supplementary Figures 6A, B).

Furthermore, we demonstrated low and nearly unchanged immunosuppressive activity of both PMN- and M-MDSC

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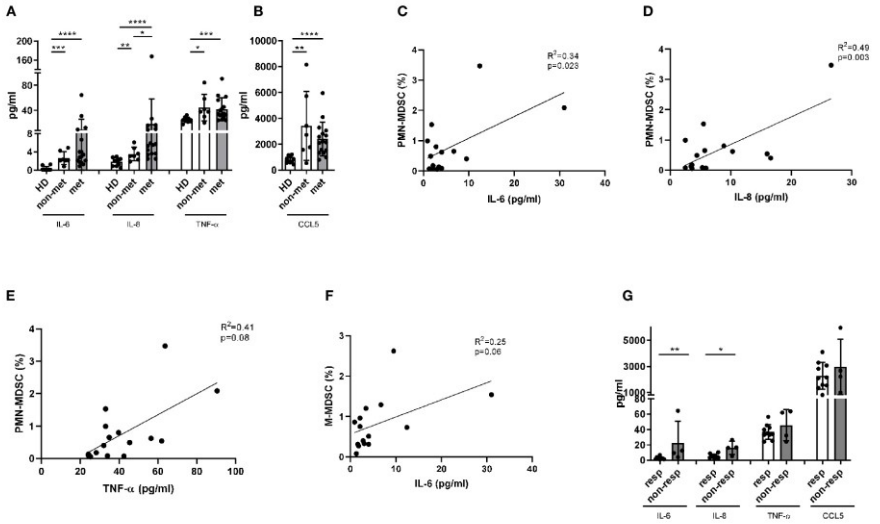

FIGURE 4
Production of inflammatory factors in melanoma patients at the baseline. Concentrations of IL-6, IL-8, TNF-α **(A)** and CCL5 **(B)** were detected in plasma of metastatic (n=16) and non-metastatic (n=7) patients as well as HD (n=10) by bio-plex assay and expressed as pg/ml. The frequency PMN-MDSC among PBMC were plotted against the level of IL-6 **(C)**, IL-8 **(D)** and TNF-α **(E)** in metastatic melanoma patients (n=15). The correlation was evaluated by a linear regression analysis. **(F)** The frequency M-MDSC within PBMC were plotted against the level of IL-6 in metastatic melanoma patients (n=15). The correlation was evaluated by a linear regression analysis. **(G)** Concentrations of IL-6, IL-8, TNF-α and CCL5 in plasma from metastatic patients, responding (n=10) and non-responding (n=4) to the ICI treatment are expressed as pg/ml. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

isolated from responders through three months of treatment (Figure 5G). In contrast, MDSC from non-responders were highly immunosuppressive at baseline and after the first injection (Figure 5G).

Interestingly, whereas the concentration of IL-6 in responders were constantly at a very low level, its levels in non-responders tended to decrease under the ICI treatment (Figure 6A). Significantly elevated plasma levels of IL-6 and IL-8 at baseline and after the first ICI injection were characteristic for non-responders (Figures 6A, B). Importantly, the mixed-effects model revealed a significant difference in concentrations of IL-6 and IL-8 between responders and non-responders (p=0.0068 for IL-6 and p=0.0024 for IL-8); however, no association with the time point could be found (Figures 6A, B). In contrast, plasma levels of TNF-α and CCL5 did not significantly differ between responders and non-responders over the therapy course (Figures 6C, D).

Discussion

We analyzed the frequency, immunosuppressive pattern, and function of MDSC subsets and MDSC-related soluble inflammatory mediators in the peripheral blood of melanoma patients receiving ICI. Our study provides a comprehensive analysis of dynamical changes in MDSCs phenotype and function at baseline and through three months of treatment (until the first staging) not only in advanced melanoma patients but also in those with no evidence of disease who were treated with ICI.

We observed that patients with progressive disease (in contrast to responders) tend to accumulate PMN- and M-MDSC in the peripheral blood from the baseline and through the first three months of treatment. Elevated PMN-MDSC frequency was associated with poorer OS and PFS in metastatic melanoma patients. These data are in agreement with other publications showing that elevated frequency of circulating MDSC in advanced melanoma patients correlates with decreased OS, PFS and less favorable therapy outcome (20, 21). Moreover, significantly lower PMN-MDSC amount in responding patients at the baseline as compared to non-responders was reported (22). In addition, low frequency of M-MDSC prior to the ICI treatment was previously reported to correlate with better response and increased OS (23).

Investigating the immunosuppressive capacity of MDSC, we demonstrated that circulating MDSC from non-responders exhibited the ability to inhibit T cell proliferation at baseline, whereas MDSC from responders failed to suppress T cell activity. This could be due to an enhanced T cell activation in responders (24), indicating that MDSC were not able to suppress these cells. Importantly, both PMN- and M-MDSC isolated from patients without metastases failed to inhibit T cell proliferation at any studied time point that could be explained by the deficiency of cytokines as IL-6, IL-8 and TNF-α supporting MDSC immunosuppressive function (13). Interestingly, in our study MDSC from both responders and non-responders tended to have reduced ability to suppress T cell proliferation after three doses of ICI. This could be due to the fact that anti-PD1 therapy reduces MDSC-related anergy of T cells mediated by PD1/PD-L1 interaction (25).

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TABLE 2 Baseline MDSC and cytokine levels in patients with metastatic melanoma who received combinational anti-PD-1 and anti-CTLA-4 treatment.

Pat.	PMN-MDSC frequency (cutoff -0.54% of live PBMC)	M-MDSC frequency (cutoff -0.73% of live PBMC)	PMN-MDSC immune suppression	M-MDSC immune suppression	IL-6 concentration (cutoff - 3.21 pg/ml)	IL-8 concentration (cutoff - 5.6 pg/ml)	Response to anti-PD-1 + anti-CTLA-4 combinational treatment	Metastases
1	high	high	+	+	high	high	Deceased before therapy begin	Liver, suprarenal gland
2	high	high	+	not determined	high	high	Non-responder	Lung, liver, brain, spleen
3	low	high	+	+	high	high	Non-responder	Lung, liver, brain, orbital cavity
4	high	low	not determined	not determined	high	high	Non-responder	Lung, liver, spinal bone, suprarenal gland
5	low	high	–	+	high	low	Responder	LN with esophagus invasion, liver

The patients were distributed in the groups with low and high frequencies of PMN- and M-MDSC as well as low and high cytokine concentrations, using their respective median values as a cutoff (19). "+" indicates the presence and "-" shows the absence of MDSC-mediated immunosuppression.

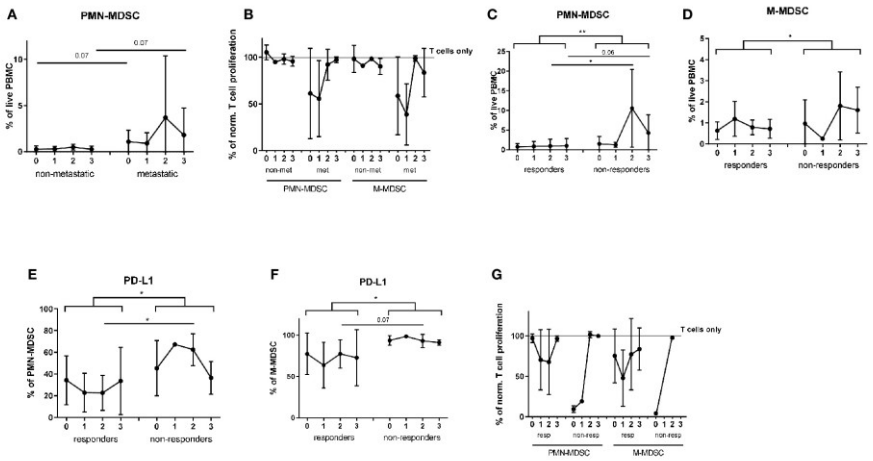

FIGURE 5
Analysis of MDSC in melanoma patients during the ICI therapy. PBMC were isolated from metastatic (n=16) and non-metastatic (n=5) patients before each ICI application (point 0 - prior the treatment; point 1 - after the first infusion; point 2 - after the second infusion; point 3 - after the third infusion) and assessed by flow cytometry. **(A)** Levels of circulating PMN-MDSC in metastatic and non-metastatic patients are expressed as the percentage within live PBMC. **(B)** Immunosuppressive capacity of PMN- and M-MDSC was determined upon the co-culture with activated CD3 T cells labeled with CP-Dye405. Cumulative data for T cell proliferation are shown as the percentage of divided T cells normalized to the respective control of stimulated T cells alone (n=5-16). Levels of circulating PMN- **(C)** and M-MDSC **(D)** in metastatic patients, responding (n=12) and non-responding (n=4) to the ICI therapy are expressed as the percentage of corresponding subsets among live PBMC. PD-L1 expression on PMN- **(E)** and M-MDSC **(F)** in responders (n=12) and non-responders (n=4) is presented as the percentage of PD-L1$^+$ cells among the respective MDSC subset. **(G)** Immunosuppressive activity of PMN- and M-MDSC was measured at different time points during the ICI therapy upon the co-culture with activated CD3 T cells labeled with CP-Dye405. Cumulative data for T cell proliferation are shown as the percentage of divided T cells normalized to the respective control of stimulated T cells alone (n=1-10). $^*P < 0.05$, $^{**}P < 0.01$.

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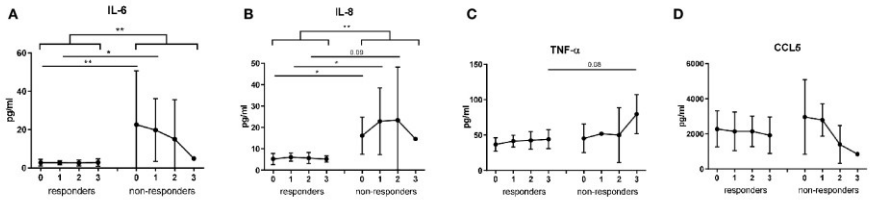

FIGURE 6
Evaluation of cytokine and chemokine concentrations in melanoma patients during the ICI treatment. Levels of IL-6 **(A)**, IL-8 **(B)**, TNF-α **(C)** and CCL5 **(D)** were measured in plasma of metastatic patients responding (n=12) and non-responding (n=4) to the ICI therapy (point 0 - before the treatment; 1 - after the first injection; 2 - after the second injection; 3 - after the third injection) by bio-plex assay and expressed as pg/ml. $*P < 0.05$, $**P < 0.01$.

To decipher the mechanisms of MDSC accumulation in the peripheral blood of metastatic patients, we investigated soluble inflammatory factors involved in MDSC activation and migration like IL-6, IL-8, TNF-α and CCL5 (26). In particular, IL-6 is known to upregulate PD-L1 expression on MDSC, to lead to their activation and accumulation (27) and to cause a poor response to immunotherapy (28, 29). Moreover, IL-6 upregulates the expression of C-motif chemokine receptor (CCR) 5 on MDSC leading to their recruitment to the tumor site and enhanced inhibitory activity towards $CD8^+$ T cells (30). IL-8 was shown to attract human PMN- and M-MDSC in a dose-dependent manner (31). In addition, its neutralization decreased MDSC migration (32, 33). Similarly to IL-6, high IL-8 concentration was shown to be associated with tumor progression, worse responsiveness to the ICI therapy (34) and identified as an independent biomarker of poor ICI therapy outcome (35). Furthermore, IL-8 is not only an important clinical marker of progression, but also a biomarker to monitor the clinical benefit of ICI, since early decrease in IL-8 indicated response to ICI therapy in melanoma patients and unmasked true response in cancer patients showing pseudoprogression (36). While investigating advanced melanoma patients with high tumor burden, we observed a significant increase in MDSC frequency and suppressive functions as well as in concentration of inflammatory factors in these patients, in particular IL-6 and IL-8. These findings are in line with a recent study, showing that patients with high IL-6, IL-8 concentrations and elevated MDSC frequency had worse OS (37).

Although PD-L1 expression on MDSC was reported to be significantly increased in melanoma patients with shorter PFS and worse OS (23), we did not observe such correlation in our study, which could be due to relatively low patient numbers. Interestingly, we found a tendency for the accumulation of $CD39^+CD73^+$ M-MDSC at the baseline in metastatic compared to non-metastatic patients, indicating a stronger immunosuppressive phenotype of these cells. A high expression of both CD39 and CD73 on MDSC was described to be associated with cancer progression in NSCLC (15). In addition, an increased soluble CD73 concentrations in serum of melanoma patients undergoing ICI was associated with shorter PFS and OS and was identified as an independent prognostic factor for PFS and OS in melanoma patients (38).

Our study has several limitations, including a small patient cohort and missing values at some time points that affected the power of statistical analysis. Due to a short lifespan of PMN-MDSC, we were not able to isolate these cells from each patient to perform the functional assay.

Taken together, our study highlights the role of PMN-MDSC, M-MDSC and MDSC-related inflammatory factors in melanoma progression and the outcome of ICI immunotherapy and confirms the importance of MDSC targeting together with ICI treatment on order to increase the efficiency of ICI in advanced melanoma patients. We suggest that the combination of elevated frequency and high immunosuppressive activity of circulating MDSC and increased IL-6 and IL-8 concentrations in plasma could be considered as promising prognostic biomarkers of resistance to ICI in advanced melanoma patients. These findings should be validated based on a larger patient cohort in the future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Das Klinische Ethikkomitee (KEK) der Universitätsmedizin Mannheim 2010-318N-MA. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VP, CG, RB, JU, and VU designed the study. VP, CG, and IA performed experiments and analyzed data. VP, RB, CG, SH, JU, and VU interpreted data and contributed to the discussion. SS, VM, and JU provided clinical expertise. VP and VU wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1065767/full#supplementary-material

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5 APPENDIX

Supplementary material of:

Petrova V, Groth C, Bitsch R, Arkhypov I, Simon SCS, Hetjens S, Müller V, Utikal J, Umansky V. Immunosuppressive capacity of circulating MDSC predicts response to immune checkpoint inhibitors in melanoma patients. Front Immunol (2023) 14:1–11. doi: 10.3389/fimmu.2023.1065767

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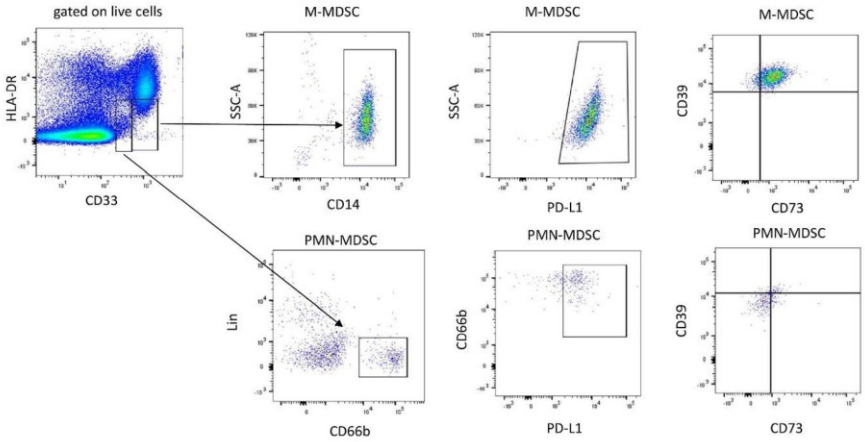
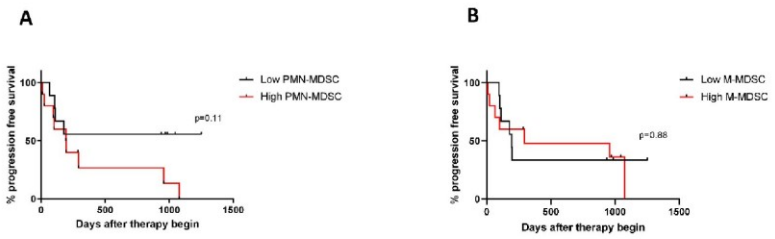
Supplementary Material

Supplementary Figure 1



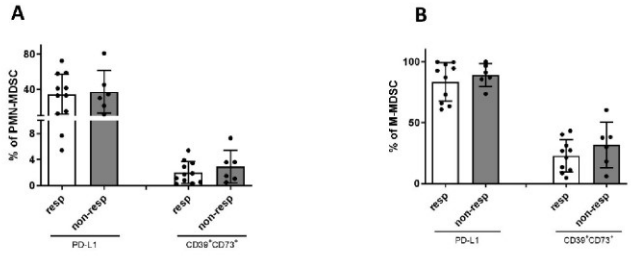
Supplementary Figure 1. Gating strategy for PMN- and M-MDSC from melanoma patients. Representative dot plots for HLA-DR$^{low/-}$CD33^{high}CD14^{+} M-MDSC and HLA-DR$^{low/-}$CD33^{dim}CD66b^{+}Lin^{-} PMN-MDSC, expressing PD-L1, CD39 and CD73.

Supplementary Figure 2



Supplementary Figure 2. Circulating MDSC and progression free survival (PFS) of metastatic melanoma patients. (A) PFS of patients with high (>0.54%; n=10) and low (<0.54%; n=9) PMN-MDSC frequencies at the baseline is shown as a Kaplan-Meier curve. (B) PFS of patients with high (>0,73 %: n=10) and low (<0,73 %, n=9) M-MDSC frequencies at the baseline is presented as a Kaplan-Meier curve.

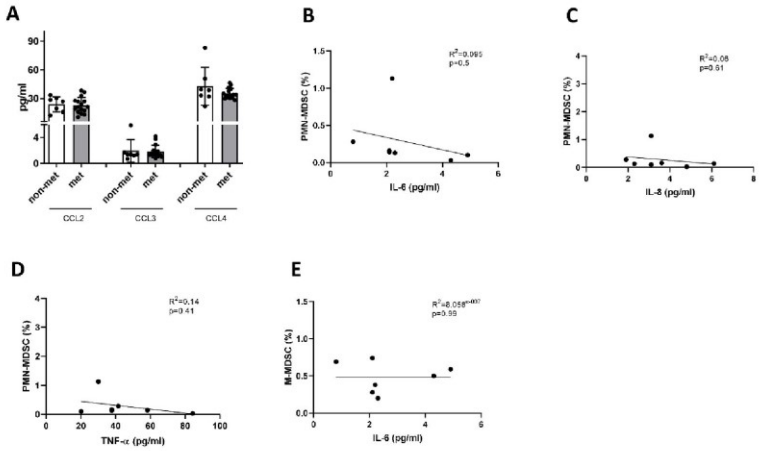
Supplementary Figure 3



Supplementary Figure 3. Immunosuppressive pattern of circulating MDSC from melanoma patients during the ICI treatment. Expression of PD-L1, CD39 and CD73 was measured on PMN- (A) and M-MDSC (B) from responders (n=11) and non-responders (n=6) by flow cytometry and expressed as the frequency of PD-L1^{+} and CD39^{+}CD73^{+} cells within respective MDSC subsets.

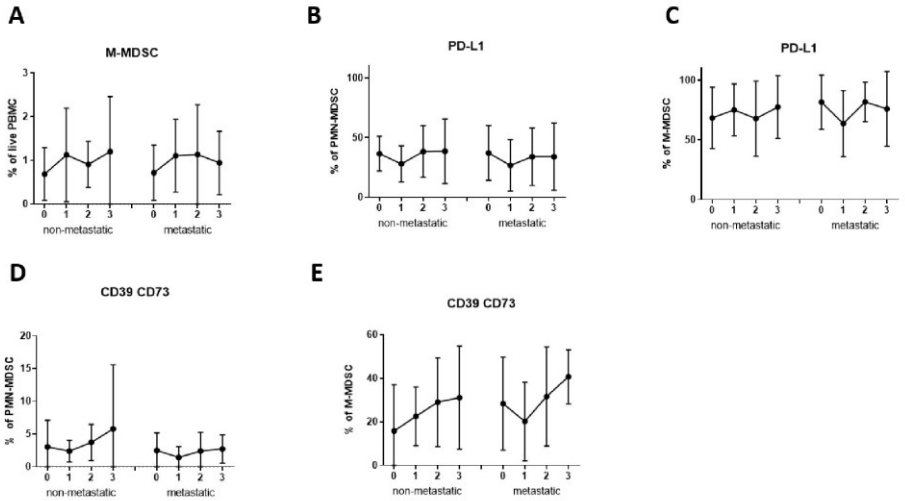
3

Supplementary Figure 4



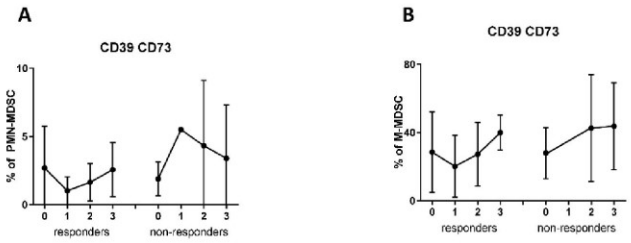
Supplementary Figure 4. Evaluation of inflammatory mediators and circulating MDSC in melanoma patients at the baseline. Concentrations of CCL2, CCL3 and CCL4 (A) were detected in plasma of metastatic (n=16) and non-metastatic (n=7) melanoma patients by bio-plex assay and expressed as pg/ml**.** The frequency of PMN-MDSC within PBMC were plotted against the level of IL-6 (B), IL-8 (C) and TNF- α (D) in non-metastatic melanoma patients (n=7). The frequency M-MDSC within PBMC were plotted against the level of IL-6 (E) in non-metastatic melanoma patients (n=7). The correlation was evaluated by a linear regression analysis.

Supplementary Figure 5



Supplementary Figure 5. Analysis of MDSC in melanoma patients during the ICI treatment. PBMC were isolated from metastatic (n=16) and non-metastatic (n=10) patients before each ICI application (point 0 - prior the treatment; point 1 - after the first; point 2 - after the second; point 3 - after the third injection) and evaluated by flow cytometry. (A) Levels of circulating M-MDSC in are expressed as the percentage within live PBMC. PD-L1^{+} PMN- (B) or PD-L1^{+} M-MDSC (C) are presented as the percentage among total respective subsets. CD39^{+}CD73^{+} PMN- (D) or CD39^{+}CD73^{+} M-MDSC (E) are presented as the percentage within respective total subsets.

Supplementary Figure 6



Supplementary Figure 6. Ectonucleotidase expression on MDSC during the ICI therapy. PBMC were isolated from patients, responding (n=12) or non-responding (n=4) to ICI (point 0 - prior the treatment; point 1 - after the first injection; point 2 - after the second injection; point 3 - after the third injection) and measured by flow cytometry. Data are expressed as the percentage of $CD39^{+}CD73^{+}$ PMN- (A) or $CD39^{+}CD73^{+}$ M-MDSC (B) within respective total MDSC subpopulations.

6 CURRICULUM VITAE

Personal Information

Name und surname:	Vera Petrova
Date of birth:	14.10.1995
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School Education

09/02 – 06/11	Secondary school education at lyceum №5, Ufa, Russian Federation
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University Education

09/13 – 06/15	Medical studies at Bashkir State Medical University Ufa, Russian Federation (average score: 1.0)
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02/18, 06/18	Participation in the Junior Scientific Masterclass, Medical Faculty Mannheim, Ruperto Carola University Heidelberg
11/18 – expect. 06/24	Doctoral dissertation in immunoncology at the Graduate school „Hallmarks of skin cancer”, Mannheim, Germany
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