Aus der Klinischen Kooperationseinheit Dermatoonkologie des Deutschen Krebsforschungszentrums (DKFZ) an der Klinik für Dermatologie, Venerologie und Allergologie der Medizinischen Fakultät Mannheim
(Direktor: Prof. Dr. med. Jochen Utikal)

The study on myeloid-derived suppressor cells (MDSCs) and T cells in melanoma patients with adverse events after immunotherapy immune checkpoint inhibitors

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Alisa Helen Lepper

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Dekan: Prof. Dr. med. Sergij Goerdt
Referent: Prof. Dr. med. Jochen Utikal

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LIST OF ABBREVIATIONS

ALM	acral lentiginous melanoma
AMP	adenosine monophosphate
APC	antigen-presenting cell
ATP	adenosine triphosphate
BRAF	V-Raf murine sarcoma viral oncogene homolog B
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDR3	complementary determining region 3
CMP	common myeloid progenitor cell
CR	complete response
CRP	C-reactive protein
CT	computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTLA-4	cytotoxic T-lymphocyte antigen
DC	dendritic cell
e.g.	for example
e-MDSC	early stage MDSC
FMO	fluorescence minus one
FOXP3	forkhead box P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte/macrophage progenitor cell
ICI	immune checkpoint inhibitor
IDO	indolamine-2,3-dioxygenase
IFN	interferon
IL	interleukin
irAE	immune-related adverse event
LAG-3	lymphocyte-activation gene 3
LDH	lactate dehydrogenase
LMM	lentigo maligna melanoma
M-CSF	macrophage colony-stimulating factor
M-MDSC	monocytic-MDSC
MAPK	mitogen-activated protein kinase

MDSC	myeloid-derived suppressor cell
MEK	MAPK kinase
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NLR	neutrophil-to-lymphocyte ratio
NM	nodular melanoma
NO	nitric oxide
OS	overall survival
PBMC	peripheral blood mononuclear cell
PD	progressive disease
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PET-CT	positron emission tomography-CT
PFS	progression-free survival
PMN-MDSC	polymorphonuclear-MDSC
PR	partial response
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	stable disease
SSM	superficial spreading melanoma
T-VEC	Talimogene laherparepvec
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th1	T helper 1 cell
Th17	T helper 17 cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	tumor-infiltrating leukocytes
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TME	tumor microenvironment
TNF-α	tumor necrosis factor-α
TP	time point

Treg	regulatory T cell
UV	ultraviolet
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 Malignant melanoma

Malignant melanoma is a type of skin cancer that originates from melanin-producing cells called melanocytes, which are localized in the basal layer of the human skin. Melanoma can develop either de novo, without any detectable precursor lesion, or in 20-30% of cases, evolve from a previously benign melanocytic lesion[1]. When atypical melanocytes are confined to the basal layer, the type of melanoma is termed melanoma in-situ. However, once melanoma cells infiltrate deeper layers of the skin, the tumor becomes invasive and has the potential to metastasize through different pathways.

The main pathways of metastatic development include satellite or in-transit metastases, lymphatic metastases, and distant metastases[2]. Distant metastases usually occur in the lung, liver, bone, and brain[3]. As soon as metastases occur, melanoma is characterized by its rapid progression[4]. Therefore, early detection of melanoma lesions is indispensable, and a systematic skin cancer-screening program can reduce tumor burden as well as mortality rates[5].

1.1.1 Epidemiology

Melanoma is considered as one of the deadliest forms of skin cancer, accounting for 90% of skin cancer-related deaths and the tumor is attributed to 2% of all cancer cases diagnosed in Germany[6]. Over the past decades, incidence rates augmented, especially among fair-skinned populations[7]. The highest risk for melanoma development is reported in Australia and New Zealand, where incidence rates are two to three times higher than in other countries[7].

Annually, there are over 320.000 new cases of melanoma diagnosed worldwide, resulting in over 57.000 deaths per year globally[8]. Research by Whiteman et al. predicts an overall increasing number of patients being diagnosed with melanoma up to 2031[9].

Melanoma predominantly occurs in young and middle-aged people, with the median age of diagnosis being 57 years[10].

1.1.2 Risk factors

Melanoma is recognized as a multifactorial disease, including both host-associated and exposure-associated risk factors. Among these, exposure to ultraviolet (UV) light is the most important and potentially avoidable risk factor attributed to melanoma development, as UV light can induce DNA mutations[10-12].

Another identified risk factor is the number of moles and dysplastic nevi. A previous analysis showed that patients with more than 100 nevi experience a sevenfold increased risk of developing melanoma[10]. Similarly, studies have found that patients with five or more dysplastic lesions are at a tenfold higher risk[13].

Additional host-associated risk factors include light skin and eye color[14], immunosuppression[15], and a family history of melanoma[10].

While most cases of melanoma occur sporadically without a known family history, about 10% are familial diseases[16]. Familial melanoma is often linked to mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, or less commonly, mutations in the cyclin-dependent kinase 4 (CDK4) gene[13, 16]. Other conditions associated with an elevated risk of melanoma include xeroderma pigmentosum, familial retinoblastoma, Lynch syndrome type II, and Li-Fraumeni cancer syndrome[16].

1.1.3 Classification of melanoma lesions

Malignant melanoma can be classified into four main subtypes: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM)[17].

SSM: SSM is the most prevalent subtype of melanoma, accounting for about 70% of melanoma cases[12]. Usually, it occurs as a flat, slowly growing lesion with varying colors, including brown, grey, black, pink, or bluish[10, 17]. It frequently develops on sun-exposed areas of the body, often appearing on the backs of men and women's legs[10].

NM: NM comprises approximately 5% of all diagnosed melanoma cases[12]. The tumor presents as a smoothly shaped nodule or papule colored brown or black-brown. It predominantly affects patients over the age of 50 and is often located on the trunk or limbs[10]. It is characterized by limited radial growth and a rapid progressive vertical growth phase, contributing to its aggressive nature[18]. Therefore, the rapid growth pattern often leads to a poor prognosis, and the tumor strongly contributes to melanoma-related deaths[19].

LMM: LMM accounts for 4-15% of all melanoma lesions and is characterized by its slow progression, commonly developing in sun-exposed areas (e.g., face, head, etc.)[12]. The tumor typically appears as a large flat macule and is most often found in older patients[17]. It frequently arises from an in-situ lesion confined to the epidermis, known as lentigo maligna[20].

ALM: ALM, representing approximately 5% of melanomas, typically manifests on the palms, soles, and subungual spaces[12]. It is a prevalent melanoma subtype in the Asian and African American population[17, 20].
A retrospective analysis performed by Greaves et al. showed that BRAF mutations were less prevalent in acral melanomas (16.2%) compared to non-acral cutaneous lesions (51.4%)[21].

1.2 Melanoma-mediated immune escape

In the 1890s, William B. Coley proposed that the immune system might play a role in treating cancer by investigating that unresectable cancer diseases could be treated by injecting streptococcal bacteria into the tumor site[22]. Later, Lewis Thomas and Frank Macfarlane Burnet postulated the concept of cancer immunosurveillance, suggesting that the immune system can recognize and eliminate cancer cells[23]. Dunn et al. expanded the immunosurveillance hypothesis and introduced the concept of immunoediting[24]. The term immunoediting includes both the tumor-preventing and tumor-promoting roles of the immune system[25].

Cancer immunoediting involves three stages: elimination, equilibrium, and escape[24, 26]. First, during elimination phase, the immune system eliminates cancer cells, and this stage corresponds to the concept of immunosurveillance. However, some cancer cells evade the immune system and survive the elimination phase, entering the next stage: the equilibrium phase[25]. Equilibrium is the longest process of cancer immunoediting, characterized by a continuous process of eliminating malignant cells and the formation of resistant tumor cells [27]. The final stage in cancer immunoediting is the escape phase, during which the tumor can grow and spread in an uncontrolled manner[24].

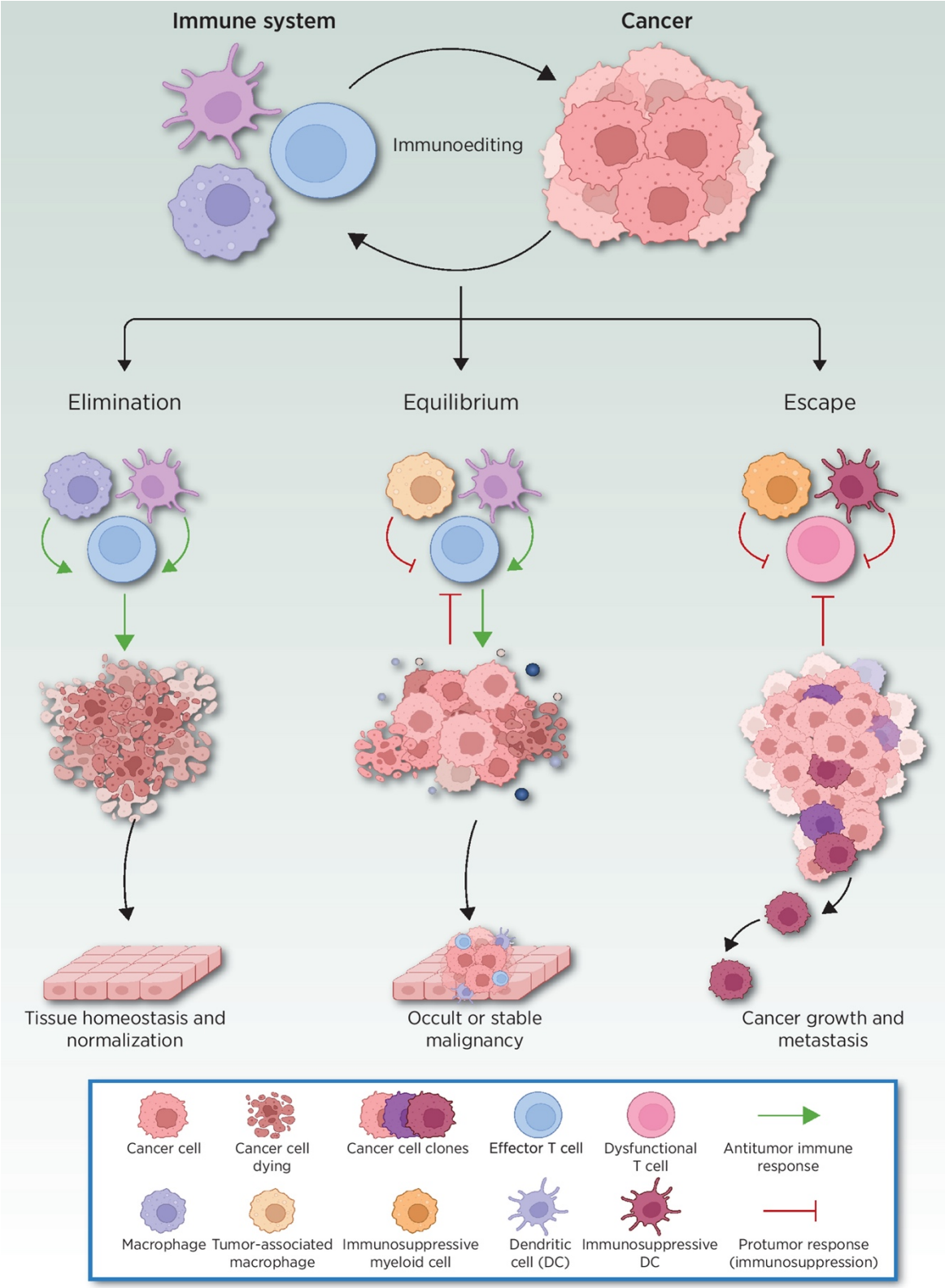

Figure 1. The cancer immunoediting hypothesis. The framework of cancer immunoediting involves the elimination, equilibrium, and escape stage. During the elimination stage, the immune system targets the cancer leading to the elimination of tumor cells. However, if cancer cells survive the first phase, they proceed to enter the equilibrium stage. During the equilibrium phase, remaining cancer cells can acquire the ability to escape the immune system by establishing an immunosuppressive tumor microenvironment (TME). Figure taken from Gubin et al.[25]

Moreover, studies have confirmed the association between the immune system and cancer, showing that the presence of tumor-infiltrating lymphocytes (TILs) predicts a favorable outcome for melanoma patients[28, 29].

However, tumor cells, including melanoma cells, can acquire mechanisms to escape the immune system. Proposed mechanisms include defective immune recognition, overexpression of immune checkpoint molecules, and the generation of an immunosuppressive tumor microenvironment (TME) characterized by infiltrating immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs)[30].

1.2.1 Defective immune recognition

$CD8^+$ T cells are typically activated through binding to major histocompatibility complex class-I (MHC-I) molecules exhibited by antigen-presenting cells (APCs). The MHC-I complex is loaded with antigens derived from pathogens after proteasomal degradation[31]. Upon activation, $CD8^+$ T cells secrete cytokines and chemokines and eliminate cancer cells by releasing granzymes and perforin[31].

As a result, impaired antigen presentation or suppressed cytotoxic activity of $CD8^+$ T cells may lead to defective immune recognition[32]. Melanoma cells can secrete vascular endothelial growth factor (VEGF), interleukin (IL)-8, and IL-10, which inhibit the maturation and priming of APCs, particularly dendritic cells (DCs), crucial for an appropriate cytotoxic activity[30].

Another suggested mechanism for immune evasion involves the loss of MHC-I antigen presentation machinery[33]. Downregulation of MHC has been linked to poor overall survival (OS) and progression-free survival (PFS)[34]. Several mechanisms associated with MHC-I downregulation have been described, including loss of transcriptional factors, epigenetic silencing of gene regulatory molecules, and loss of MHC-I polymorphism[34, 35].

1.2.2 Overexpression of checkpoint molecules

The overexpression of immune checkpoint molecules and their respective ligands has been identified as a key mechanism driving immune evasion in melanoma. This overexpression leads to T cell exhaustion or dysfunction, resulting in defective cytokine production and impaired cytotoxicity[36].

Key regulatory elements include the expression of cytotoxic T-lymphocyte antigen (CTLA-4) and programmed cell death protein 1 (PD-1) or its ligand (PD-L1)[37]. CTLA-4 is expressed on activated effector T cells and is constantly expressed on Tregs[38].

Naïve or resting T cells do not exhibit CTLA-4 on their cell surface. Instead, CTLA-4 is sequestered in intracellular compartments. Upon T cell receptor (TCR) stimulation, CTLA-4 is expressed on the cell surface[37]. CTLA-4 competes with the costimulatory homolog, CD28, to interact with CD80 (B7-1) and CD86 (B7-2) on APCs[39]. CTLA-4 usually binds with a stronger affinity than CD28[39]. Consequently, increased CTLA-4:B7 binding can lead to limited IL-2 production and reduced proliferation and survival of T cells[40].

The critical role of CTLA-4 in preventing autoimmunity has been supported by several in vivo and in vitro experiments[38]. A study on CTLA-4 knockout mice revealed that these mice developed lymphoproliferative diseases with extensive lymphatic infiltration and died three to four weeks later[41]. Accordingly, inhibition of CTLA4:B7 interaction can promote cardiac graft rejection in CD28-deficient mice[42].

PD-1 can be expressed on T cells, natural killer (NK) cells, and B cells[43]. PD-1 interacts with PD-L1 or PD-L2 on cancer cells and other immune cells. This interaction leads to an attenuated T cell function mainly in the peripheral tissue by inhibiting interferon-γ- (IFN-γ), tumor necrosis factor-α- (TNF-α), and IL-2 production, which hampers the host antitumor immune response[37, 40]. In contrast to CTLA-4 signaling, PD-1 exerts its immunosuppressive effect during a later stage of T cell activation[44]. Activated T cells exhibit PD-1, and PD-1 expression can be induced by inflammatory signals in peripheral tissue[37].

In many types of cancer, including melanoma, PD-1 is upregulated on TILs. Furthermore, Chapon et al. found that the expression of PD-1 on TILs correlated with disease progression[45].

Accordingly, PD-1 expression was also identified as a crucial factor in maintaining peripheral tolerance, as demonstrated by studies on knockout mice, which subsequently developed autoimmune diseases[46, 47].

1.2.3 Tumor microenvironment (TME)

The TME is a complex network of interactions containing elements of the tumor and components of the host's immune system, playing a critical role in determining tumor growth and progression. It comprises cellular and non-cellular compartments. The non-cellular compartment consists of components of the extracellular matrix such as collagen, hyaluronan, or laminin[48]. The cellular compartment includes tumor cells, stromal cells like fibroblasts, and immune cells like macrophages or lymphocytes, collectively shaping the TME[48]. Additionally, TME formation depends on interactions between the tumor type and the host's immune system.

Tumors display distinct immune phenotypes based on the activation and infiltration of immune cells into the tumor site: "hot" tumors exhibit high T lymphocyte infiltration and numerous inflammatory markers, whereas "cold" tumors are characterized by minimal immune cell infiltration and limited release of inflammatory signals[43]. Melanoma, for instance, represents a "hot" tumor, which is characterized by its high mutational load[49, 50]. Mutations presented by MHC molecules and recognized by T cells are termed neoantigens[51]. Moreover, the number of immunogenic mutations may predict tumor response to immunotherapies[51].

In the TME, melanoma cells release various inflammatory mediators such as transforming growth factor-β (TGF-β) and indolamine-2,3-dioxygenase (IDO), thereby establishing chronic inflammation and promoting the accumulation of immunosuppressive cells like Tregs, MDSCs, and tumor-associated macrophages (TAMs)[30].

1.2.3.1 Myeloid-derived suppressor cells (MDSCs)

The term MDSC was first introduced by Gabrilovich et al. in 2007 and describes a heterogeneous population of immunosuppressive cells characterized by their myeloid origin[52]. In healthy individuals, hematopoietic progenitor cells develop via common myeloid progenitor cells (CMP) and granulocyte/macrophage progenitor cells (GMP) to terminally differentiated cells, namely DCs, macrophages, or neutrophils[53].
However, in pathological conditions like cancer[54], chronic inflammation[55, 56], or obesity[57], the differentiation of hematopoietic stem cells into myeloid cells can be altered, resulting in an accumulation and expansion of MDSCs. Many cancer types are associated with MDSC accumulation, including melanoma[58], breast cancer[59], pancreatic cancer[60], hepatocellular cancer[61], and bladder cancer[62]. Moreover, MDSCs are involved in tumor progression across various cancer entities, including melanoma[59, 61, 63].

The activation of MDSCs can be induced by the prolonged release of inflammatory signals such as myeloid growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), IL-6, and adenosine signaling[64].

There are two main subsets of MDSCs described in humans and mice: monocytic (M-MDSCs) and granulocytic MDSCs (PMN-MDSCs), distinguished by their monocytic or granulocytic myeloid origin, respectively[64]. The phenotype of M-MDSCs and PMN-MDSCs varies between humans and mice. In mice, MDSCs are phenotypically characterized as $Gr1^+CD11b^+$ and further defined as $CD11b^+Ly6C^+Ly6G^-$ cells for M-MDSCs and $CD11b^+Ly6G^+Ly6C^{low/-}$ cells for PMN-MDSCs[65]. In humans, MDSCs are identified as $CD33^+CD11b^+HLA\text{-}DR^{low/-}$ cells, and M-MDSCs further characterized as $CD14^+CD15^-$ and PMN-MDSCs as $CD15^+CD14^-CD66b^+$ cells[65]. Another subpopulation of early stage MDSCs (e-MDSCs) has been identified in humans[64].

Various mechanisms of MDSC-mediated immunosuppressive activities within the TME have been elucidated[43].
First, MDSCs play a crucial role in the accumulation of immunosuppressive cells within the TME[66]. Precisely, they induce the development of Tregs[53, 66]. Moreover, MDSCs can foster other immune cells towards a more immunosuppressive phenotype. For

instance, MDSCs can promote the differentiation of macrophages into a more immunosuppressive M2 phenotype[67].

Second, MDSCs lead to the generation of immunosuppressive adenosine within the TME. Initially, CD39 converts extracellular adenosine triphosphate (ATP) into adenosine monophosphate (AMP), followed by another dephosphorylation process mediated by CD73, a membrane-bound nucleotidase expressed on MDSCs, resulting in adenosine production[68]. Adenosine prevents priming of naïve T cells and suppresses T cell-mediated antitumor immune responses[68, 69]. Additionally, extracellular adenosine might modulate the generation and immunosuppressive activity of MDSCs[70].

Another potent mechanism mediated by MDSCs involves the expression of PD-L1, which, upon binding with PD-1 on T cells, promotes T cell anergy and apoptosis[53].

Furthermore, MDSCs can secrete reactive oxygen species (ROS) and reactive nitrogen species (RNS), particularly nitric oxide (NO), which contribute to reduced TCR expression, T cell apoptosis, and inhibition of T cell proliferation[53].

Finally, MDSCs may contribute to the formation of a premetastatic environment through the generation of VEGF and facilitate extracellular matrix degradation through the production of matrix metalloproteinases (MMPs)[71].

1.2.3.2 Regulatory T cells (Tregs)

Tregs are an immunosuppressive subset of T cells, identified as $CD4^+CD25^+FOXP3^+$ cells[72]. The transcription factor forkhead box P3 (FOXP3) is specifically exhibited in Tregs and is crucial for their maturation and development[72].
Tregs are widely recognized as the primary cell population responsible for maintaining peripheral immune tolerance. Particularly, they play a pivotal role in preventing autoimmune diseases and limiting chronic inflammatory conditions such as type 1 diabetes or asthma[73]. Mutations in the FOXP3 gene have been linked to the development of severe autoimmune disorders[74, 75].

Nevertheless, Tregs also suppress antitumor immune responses[73]. In many cancer patients such as melanoma, lung, and gastric cancer, Tregs are significantly elevated among circulating $CD4^+$ T cells compared to healthy individuals[76]. Apart from the primary tumor, Tregs are also enriched within metastatic melanoma lymph nodes[77]. Moreover, a high number of tumor-infiltrating Tregs and an increased ratio of Tregs/$CD8^+$ T cells within the TME have been associated with a markedly shorter OS in several tumor types, including melanoma[78, 79]. Indeed, a critical step in TME formation is the recruitment of Tregs to the tumor tissue following chemokine release by melanoma cells and surrounding immune cells[80].

Tregs promote the establishment of an immunosuppressive TME and mediate immunosuppressive activities on T effector cells, NK cells, monocytes/macrophages, and APCs[76]. Hence, Tregs utilize various mechanisms to establish an immunosuppressive environment and inhibit anti-tumor immune responses (Figure 2)[73].
These cells release inhibitory molecules like IL-10 or TGF-β, which suppress T cell activity[81]. Notably, a study on mice lacking the TGF-β receptor revealed that these mice subsequently developed lethal autoimmune phenomena[82].
Another described mechanism of Treg-mediated immunosuppression involves the direct induction of effector T cell cytolysis through the secretion of granzyme and perforin[83].
Furthermore, Tregs can induce immunosuppression through metabolic disruption, impairing the metabolism of effector T cells via several mechanisms. First, Tregs express the ectoenzymes CD39 and CD73, leading to the production of

immunosuppressive adenosine[73]. Second, Tregs have been found to exert immunosuppressive activity on T cells by inducing IL-2 depletion among Tregs[84].

As mentioned above, Tregs drive immunosuppression by expressing immune checkpoint molecules like CTLA-4, PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), or lymphocyte-activation gene 3 (LAG-3)[76]. Thus, these regulatory molecules can interact with their respective ligands, thereby inhibiting DC function and diminishing the activation of effector T cells[76].

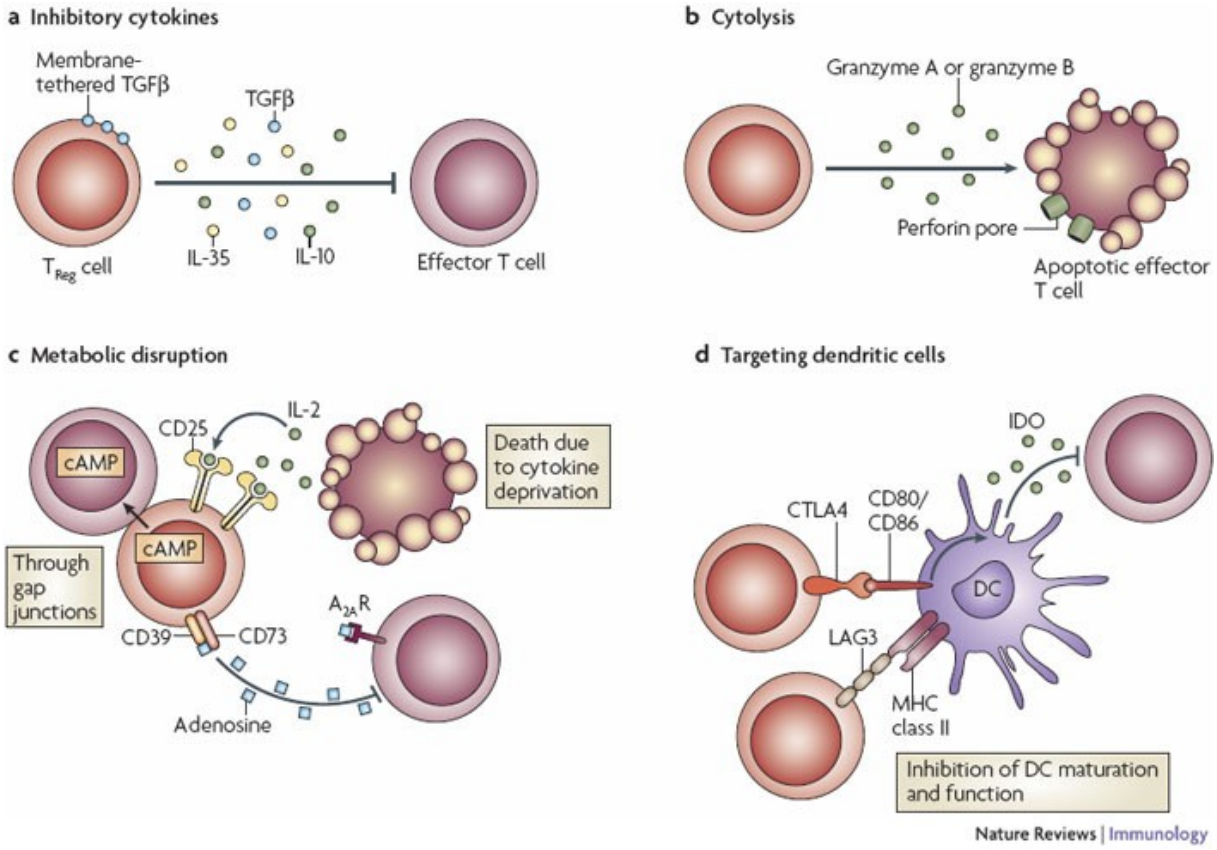

Figure 2. Overview depicting Treg-mediated mechanisms of immunosuppression. Tregs employ various strategies to promote the formation of an immunosuppressive TME. These mechanisms primarily include: (A) the secretion of inhibitory cytokines such as TGF-β, IL-35, and IL-10, (B) cytotoxicity induced by granzyme and perforin, (C) disruption of metabolic pathways, and (D) modulation of DC function through the upregulation of checkpoint molecules. Figure is taken from Vignali et al.[73].

1.3 Melanoma therapies

Melanoma treatment strategies depend on the tumor stage. In early stages, melanoma can often be treated by surgical resection. However, the occurrence of metastases presents a greater challenge, as surgery alone may not be sufficient. Additionally, conventional chemotherapeutic agents typically show limited efficacy against melanoma[85]. Dacarbazine, which was the standard therapeutic drug in the past, has shown a median survival of five to eleven months and a one-year survival rate of 27% for metastatic melanoma patients[86].
In recent years, significant advancements in melanoma treatment have been made. Particularly noteworthy is the introduction of immunotherapies and targeted therapies, which have revolutionized the landscape of melanoma treatment options. These treatment approaches will be further elaborated below.

1.3.1 Immunotherapies

The understanding of the host immune system involved in cancer progression has led to the development of immunotherapies[50]. Unlike conventional chemotherapeutic agents, which target cancer cells directly, immunotherapies aim to modulate the immune system to treat neoplastic diseases.

1.3.1.1 Immune checkpoint inhibitors (ICI)

To date, one of the most successful approaches in treating melanoma has been achieved by immune checkpoint inhibitors (ICI). As previously mentioned, CTLA-4 and PD-1 are important immune checkpoint molecules, and the principle of ICI is to target these regulatory molecules. Therefore, ICI aim to activate the host immune system against the tumor and enhance immune activation to combat tumor cells[87].

Ipilimumab, the first ICI, was approved for melanoma treatment in 2011. The treatment is a monoclonal antibody that targets CTLA-4 on T cells. By blocking CTLA-4, CD28 can recognize APCs via binding to their ligands CD80 and CD86, thereby enhancing effector T cell response[44, 88]. Moreover, inhibition of CTLA-4 can improve anti-tumor immunity by depleting Tregs, as these cells express high levels of CTLA-4[89]. Thus, CTLA-4-mediated anti-tumor effects are diverse but largely T cell-dependent.

Clinical trials have shown that patients treated with ipilimumab + dacarbazine exhibited significantly improved OS compared to those treated with dacarbazine + placebo (median OS 11.2 versus 9.1 months)[90].

Nivolumab and pembrolizumab are monoclonal antibodies that target PD-1. By blocking the PD-1 axis with anti-PD-1 or anti-PD-L1/2 antibodies, respectively, they inhibit the interaction of PD-1 on activated T cells with the corresponding ligand and thereby restore anti-tumor immunity[91]. Various studies have validated that blocking PD-1 can promote T cell expansion[92, 93], which improves memory T cell function and mediates tumor cell killing mechanisms[44] (Figure 3B).

Several clinical studies have shown that targeting the PD-1/PD-L1 axis or applying a combination of anti-PD-1/CTLA-4 antibodies is even more beneficial than CTLA-4 blockade alone[94, 95]. A follow-up study revealed a two-year overall survival of 63.8% in the combination therapy group compared to 53.6% in the anti-CTLA-4 monotherapy group[95].

ICI treatment is characterized by a long-lasting response. A pooled analysis of the CheckMate 069 phase II trial and CheckMate 067 phase III trial indicates that patients may continue to benefit from ICI even after treatment discontinuation due to adverse events[96]. However, around 60-65% of melanoma patients do not respond to ICI[97, 98]. Mechanisms of ICI resistance in melanoma patients include insufficient generation of neoantigens[99], dysfunction of APCs by an altered MHC I complex[100], TME formation with immunosuppressive cells like MDSCs[101], or the presence of alternative checkpoint molecules (e.g., LAG-3 or TIM-3)[102, 103].

Recently, the new checkpoint inhibitor relatlimab, an anti-LAG-3 antibody, has emerged and has shown a prolonged PFS when administered as a combination therapy with anti-PD-1 antibodies compared to anti-PD-1 monotherapy in advanced melanoma patients[104]. LAG-3 is a checkpoint molecule expressed on several lymphocyte subsets such as activated T cells, B cells, and NK cells[105]. It usually inhibits T cell activation by competing with the CD4 receptor for MHC II binding[105].

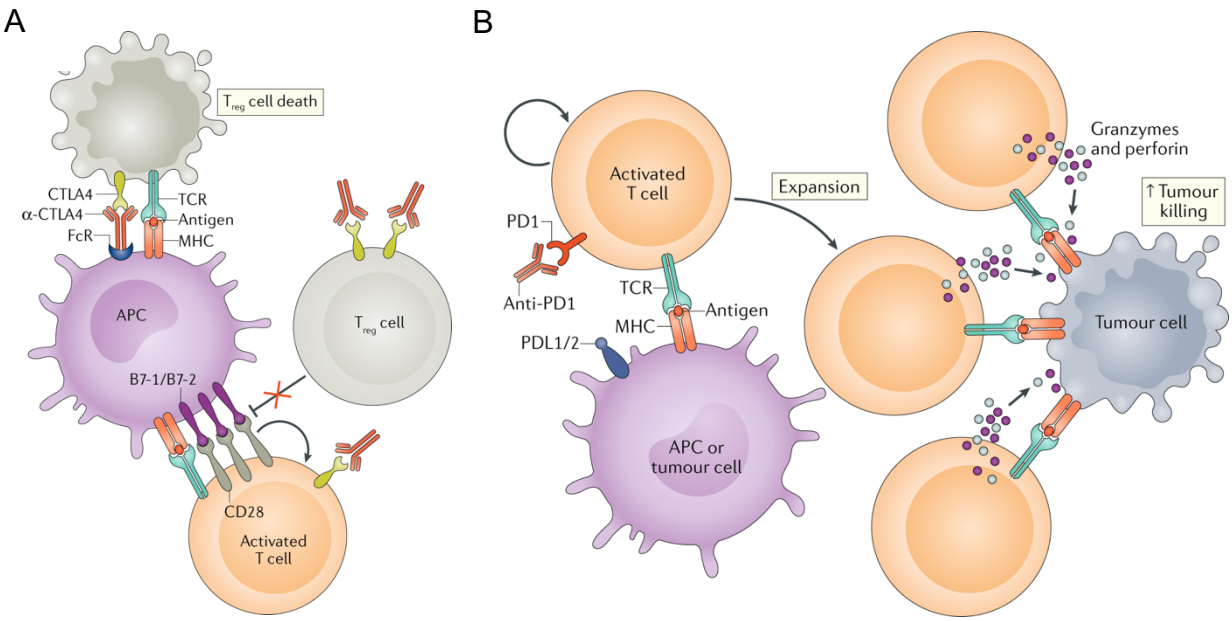

Figure 3. Mechanisms of ICI. (A) Inhibition of CTLA-4 enhances T cell activation and effector function by improving CD28:B7 interaction. Furthermore, blocking CTLA-4 on Tregs can induce antibody-dependent cellular cytotoxicity, further enhancing anti-tumor immune responses. (B) Blocking PD-1 reinvigorates T cell function, modulating T cell expansion, and enhancing memory T cell function. Figure adapted from Waldman et al.[44]

1.3.1.2 Toxicity of ICI

Despite the great success of ICI in melanoma treatment, its usage is currently limited by toxicity. By upregulating the immune system, ICI can trigger an unspecific overactivation followed by immunological side effects termed as immune-related adverse events (irAE). These irAE can involve various organs, including the skin, liver, endocrine system, gastrointestinal tract, and lungs[106, 107]. Furthermore, rare forms of irAE with a broad spectrum of clinical presentations have also been described[108].

The incidence rates of irAE vary among different ICI agents. Adverse events occur in approximately 60% of patients treated with ipilimumab[109] and 30-40% of patients receiving anti-PD-1 antibodies[110, 111]. Moreover, combination therapy with anti-CTLA-4/PD-1 has been associated with more severe adverse events compared to monotherapy with either anti-PD-1 or anti-CTLA-4 antibodies[94, 95].

A pooled analysis revealed that ICI-induced adverse events usually manifest within two to 15 weeks after ICI treatment initiation, with high grade irAE tend to develop earlier after anti-CTLA-4 monotherapy or anti-CTLA-4/PD-1 combination therapy compared to patients treated with anti-PD-1 monotherapy[112]. While most irAE appear to develop within weeks following treatment initiation, there are also cases of late-onset irAE that occur during or even after treatment discontinuation[113]. The development of late-onset irAE could be attributed to residual immunological changes following ICI treatment supported by the fact that the half-life of ipilimumab is approximately 14 days[112]. Currently, identifying late-onset irAE remains challenging for clinicians, and consequently, their prevalence may still be underreported[114].

IrAE can be fatal, and in some cases, treatment discontinuation or temporary interruption, along with the application of immunomodulating drugs, might be indicated. Most cases of irAE respond to steroid treatment and are considered steroid-sensitive, typically resolving within six to twelve weeks[115]. However, there are also steroid-refractory adverse events where the administration of immunomodulatory drugs such as TNF-α antagonists, azathioprine, or mycophenolate mofetil could be successful[115]. The decision to reintroduce ICI after irAE resolution remains challenging, as around 26-43% of patients experience recurrences of prior irAE, and 13-26% of patients develop new irAE upon re-administration[116].

1.3.1.3 Other immunotherapies

Talimogene laherparepvec (T-VEC) is a genetically modified oncolytic herpes simplex virus that is approved for the intralesional treatment of primary melanoma lesions or cutaneous metastases[117]. The virus preferentially replicates in melanoma cells, inducing tumor cell lysis and subsequent release of tumor-derived antigens, thereby promoting anti-tumor immune responses[118]. Additionally, anti-tumor immunity is enhanced by including GM-CSF-encoding genes in the treatment[117].

In recent years, promising results have been published from studies investigating mRNA-based vaccines in murine models for cancer treatment[119, 120]. These vaccines can enhance the host's immune response by encoding tumor antigens, thereby facilitating antigen presentation by DC[120]. A recently published clinical study investigated mRNA vaccines in combination with ICI for melanoma patients[121]. Therein, the combination therapy, including mRNA vaccination and ICI, showed superior recurrence-free survival compared to ICI monotherapy[121].

1.3.2 Targeted therapies

Mutations in the V-Raf murine sarcoma viral oncogene homolog B (BRAF) gene have been identified as a major driver of the oncogenic mitogen-activated protein kinase (MAPK) cell signaling pathway[122]. BRAF encodes the B-raf protein, a serine/threonine protein kinase, and mutations in this gene lead to constitutive activation of the kinase[123]. BRAF mutations are observed in 50% of patients with cutaneous melanoma, making this mutation more prevalent among melanoma patients compared to other cancer entities[123, 124]. In other melanoma subtypes, such as acral or mucosal melanoma, the prevalence of BRAF mutations is lower, ranging around 6-20%[125]. The most frequently reported BRAF mutation in melanoma, known as BRAFV600E, develops at the 600th position, where valine is substituted by glutamic acid[122]. The identification of the BRAF mutation has led to the development of new targeted therapies.

For example, vemurafenib is a targeted therapy that can effectively inhibit altered B-raf kinase activity. A randomized controlled phase 3 trial comparing the treatment outcomes of vemurafenib with the chemotherapeutic agent dacarbazine demonstrated a significantly improved PFS among patients receiving vemurafenib[126].
Therefore, the identification of BRAF mutations has become a crucial factor in melanoma diagnostics and the planning of therapeutic strategies.

In addition to vemurafenib, other BRAF inhibitors such as dabrafenib or encorafenib have emerged and have been approved for melanoma treatment in recent years. All BRAF inhibitors share the same adverse event profile. Common side effects include dermatologic manifestations like rash, cutaneous squamous-cell carcinomas, photosensitivity, hyperkeratotic lesions, keratoacanthomas, alopecia, and hand-foot

syndrome[127, 128]. Other agents that target the MAPK kinase (MEK) molecule, a downstream target of the MAPK pathway, have also been developed, namely cobimetinib, trametinib, or binimetinib.

However, monotherapy with a BRAF inhibitor carries the risk of acquired resistance, which is commonly mediated by the MAPK pathway[129]. To delay the development of resistance, a MEK inhibitor can be added to treatment[130]. Combined BRAF/MEK inhibitor treatment has demonstrated considerably improved PFS in BRAF-mutated melanoma patients compared to BRAF monotherapy[130, 131].

1.4 Aim of this thesis

To date, ICI have revolutionized melanoma treatment. However, the onset of irAE remains a significant limitation of ICI treatment since irAE can be life-threatening or even lethal in some cases. Several mechanisms have been elucidated to be involved in the pathogenesis of irAE, including the presence of pre-existing autoantibodies[132], cytokine release[133], microbiome composition[134], and genetic predisposition[135]. Furthermore, some studies propose that irAE onset might be the result of an imbalanced immune system following ICI treatment[136].

This immune system imbalance may possibly be mediated by activated T cells and impaired function of immunosuppressive subsets such as Tregs and M-MDSCs[136, 137]. However, longitudinal studies investigating immunological changes at different time points during ICI treatment are lacking, and there are currently no approved blood-based biomarkers that reliably predict irAE occurrence.

In the present study, the aim was to identify an immune signature in the peripheral blood of melanoma patients following ICI treatment and establish a blood-based immune panel associated with an increased risk of irAE development. For this purpose, I analyzed peripheral blood mononuclear cells (PBMCs) from patients with and without irAE regarding T cell activation (expression of CD25, CD69, and TCR ζ-chain), immunosuppressive subsets such as Tregs and M-MDSCs, and the immunosuppressive capacity mediated by M-MDSCs (expression of PD-L1, CD73, ROS, and NO). Several time points were included for flow cytometry analysis: before ICI initiation, during ICI treatment, and the time point of irAE onset.

Previously published data have suggested that immunosuppressive drugs like corticosteroids might influence circulating immune cells towards a more immunosuppressive state[138]. Therefore, I also investigated how immunosuppressive drugs applied following irAE occurrence could modulate Tregs and M-MDSCs.
Finally, I evaluated data from routine blood tests, including complete blood count, serum lactate dehydrogenase (LDH), and C-reactive protein (CRP) levels, to determine if changes in these parameters could serve as biomarkers predicting irAE onset.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Chemicals and reagents

Reagent	Company	Catalog no.
Albumin	Carl Roth	3737.3
Benzonase® Nuklease	Merck	E1014-25KU
Biocoll Separating Solution	Biochrom AG	L6715
CellROX Deep Red reagent	Thermo Fisher	A1049201
DAF-FM DA	Cayman Chemical	18767
Dimethyl sulfoxide (DMSO)	Carl Roth	A994.1
Dulbecco's phosphate-buffered saline (DPBS)	Sigma-Aldrich	D2650
FcR blocking reagent, human	Miltenyi Biotec	130-59-901
Fetal bovine serum (FBS)	Pan Biotech	P30-3702
Fixable viability stain 700	BD Biosciences	564997
RPMI 1640 medium	Thermo Fisher	11875101
Türk's solution	Sigma-Aldrich	1092770100
UltraPure 0.5 M EDTA	Thermo Fisher	15575020
X-VIVO 20	Lonza	BE04-448Q

2.1.2 Conjugated antibodies

Specificity	Conjugate	Clone	Company	Catalog no.	Dilution
CD3	V500	SP34-2	BD Biosciences	560770	1:50
CD4	APC-Cy7	RPA-T4	BD Biosciences	557871	1:50
CD8	APC	RPA-T8	BD Biosciences	555369	1:20
CD14	PerCP-Cy5.5	MΦP9	BD Biosciences	562692	1:50
CD25	BV421	M-A251	BD Biosciences	562442	1:50
CD69	PE-Cy7	FN50	BD Biosciences	557745	1:25
CD73	BV605	AD2	Biolegend	344024	3:50
FOXP3	Alexa 488	259D/C7	BD Biosciences	560047	1:50

HLA-DR	V500	G46-6	BD Biosciences	561224	1:50
PD-1	PerCP-Cy5.5	EH12.1	BD Biosciences	561273	1:50
PD-L1	PE-Cy7	MIH1	BD Biosciences	558017	1:50
TCR ζ-chain	PE	3ZBR4S	Biolegend	12-2478-42	1:50

2.1.3 Solution, medium

Solution/medium	**Composition**
FACS buffer	DPBS
	2% FBS
	0.2% NaN_3
Freezing medium 1	60% FBS
	40% X-VIVO 20
Freezing medium 2	80% FBS
	20% DMSO
MACS buffer	DPBS
	0.5% BSA
	0.5 mM EDTA

2.1.4 Kits

Product	**Company**	**Catalog no.**
FoxP3/Transcription Factor Fixation/Permeabilization Kit	eBioscience	00-5523-00

2.1.5 Routine laboratory material

Product	**Company**	**Catalog no.**
15 mL tube	Sarstedt	62.554.502
50 mL tube	Sarstedt	62.547.254
96-well plate round bottom	Sarstedt	83.3925
Cryovial, 2 mL	Sigma Aldrich	72.379
Leucosep 50 mL tube	Greiner Bio-one	227290, E14051MC
Pasteur pipettes, graduated, 3.4 mL, sterile	Carl Roth	562692

Pasteur pipettes without cotton plug, 2.5 mL	Carl Roth	4518.1
Pipette tips 10 µL	Sarstedt	70.1130.600
Pipette tips 200 µL	Sarstedt	70.762.100
Pipette tips 1000 µL	Sarstedt	70.760.452
Reaction tube 1.5 mL	Eppendorf	0030120086
Reaction tube 2 mL	Eppendorf	0030120094
Serological pipette 5 mL	Sarstedt	86.1253.001
Serological pipette 10 mL	Sarstedt	86.1254.001
Serological pipette 25 mL	Sarstedt	86.1685.001

2.1.6 Laboratory equipment

Device	**Company**
Centrifuge BiofugeprimoR	Heraeus
Centrifuge MEGAFUGE 40R	Heraeus
Centrifuge Labofuge 400R	Heraeus
Counting chamber Neubauer improved	Brand
Flow cytometer FACS Lyric	BD Biosciences
Freezing container „Mr. Frosty“	Thermo Fisher
Fridge	Liebherr
Ice machine	Manitowoc
Laminar flow hood Hera safe	Heraeus
Light microscope DM IL	Leica
N_2 tank BIOSAFE	Cryotherm
Pipettes Transferpette	Brand
Refrigerator (-20 °C)	Liebherr
Refrigerator (-80 °C)	Heraeus
Vortexer REAX Top	Heidolph
Water bath DC3	HAAKE, GFL

2.1.7 Computer software

Software	Provider
FACSuite™	BD Biosciences
FlowJo V10	BD Biosciences
GraphPad Prism	GraphPad Software

2.2 Methods

2.2.1 Patient cohort and sample collection

The study related to this thesis was approved by the local ethics committee (2010-318N-MA). Before analysis, all patients provided their informed written consent.

Peripheral blood samples were obtained from 31 stage III and IV melanoma patients who were treated with ICI between January 2018 and September 2021. All patients underwent ICI treatment at the Skin Cancer Center, University Medical Center Mannheim, Germany. The adjuvant treatment group involved 16 patients, while 15 patients were treated with ICI in a palliative treatment regimen. Adjuvant treatment followed a protocol of either 3 mg/kg body weight nivolumab every three weeks or 200 mg pembrolizumab every three weeks. Palliative treated patients received either monotherapy with 480 mg nivolumab every four weeks or 200 mg pembrolizumab every three weeks. Additionally, nine palliative treated melanoma patients underwent prior combination therapy, including 1 mg/kg body weight nivolumab and 3 mg/kg body weight ipilimumab, every three weeks for up to four cycles followed by nivolumab monotherapy.

Treatment response was determined using contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography-CT (PET-CT) every twelve weeks after ICI start. Palliative treated patients were categorized as responders or non-responders based on the iRECIST criteria, considering the best overall response during the observation period. Therapy responses were classified as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD), with PD indicating non-response.

Patients undergoing ICI treatment were regularly monitored for irAE at the Skin Cancer Center through clinical examinations and routine blood analysis. Based on clinical observations, patients were assigned to either the irAE or the no irAE group. IrAE severity was evaluated according to the Common Terminology Criteria of Adverse Events (CTCAE) Version 5.0 published by the National Cancer Institute[139]. The grading system ranges from grade 1 to grade 5, while grade 1 represents the mildest reaction and grade 5 is the worst reaction and death due to irAE, respectively. IrAE of grade 3 or higher were considered severe.

Relevant clinical data, including gender, age, histopathological characteristics, AJCC staging, irAE characteristics, ICI treatment response with radiological evaluations, and type and duration of ICI treatment, were retrospectively collected from patients' medical records at the Department of Dermatology, University Medical Center Mannheim. Furthermore, information on routine blood analysis, containing complete blood count, serum LDH, and CRP levels, was obtained from patients' medical records and included in my analysis. Data from routine blood tests were collected concurrently with PBMC analysis at indicated time points, as shown below.

2.2.2 Time points (TPs) of sample analysis

The time points for flow cytometry analysis were retrospectively selected based on clinical evaluations. Figure 4 shows the time points included for the analysis.

For the irAE group: TP 0 – before the start of ICI therapy; TP 1 - before irAE occurrence; TP 2 - during irAE onset; TP 3 - during immunosuppressive therapy to manage irAE.

Regarding the no irAE group: TP 0 – before the start of ICI therapy; TP 1 – during ICI treatment; TP 2 – during ICI treatment. TPs 1 and 2 in the no irAE group were adjusted according to the calculated median time of TPs 1 and 2 in the irAE group: TP 1 (48.5 and 52.5 days for irAE and no irAE groups, respectively) and TP 2 (80.5 and 108 days for irAE and no irAE groups, respectively).

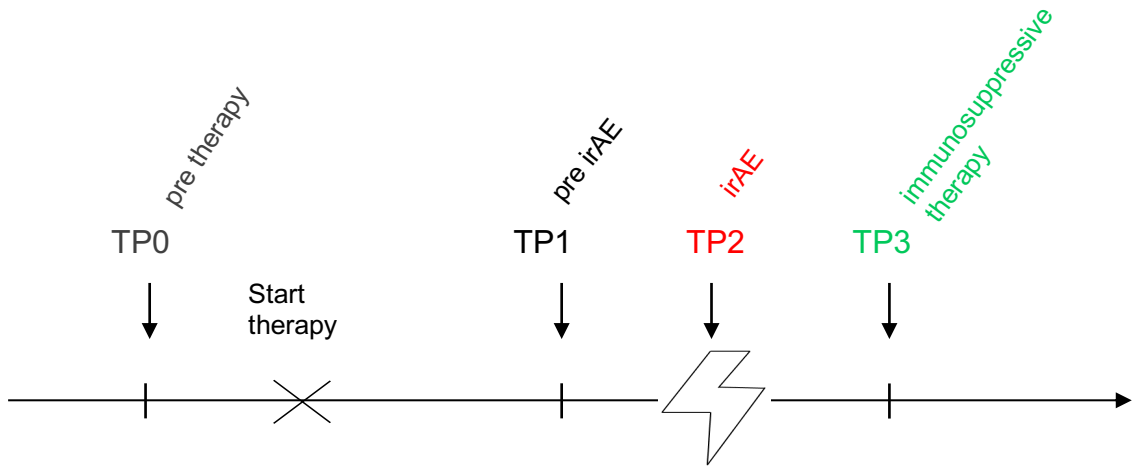

Figure 4. Overview illustrating selected time points (TPs) for analyses of peripheral blood mononuclear cells (PBMCs) analysis and routine blood tests.

2.2.3 Cell counting

To evaluate the cell number, 5 µL of a single cell suspension were diluted 1:20 with Türk's solution. For cell counting, 10 µL of the solution were dispensed into a Neubauer camber. The total number of live cells was calculated using the following formula:

$$Total\ cell\ number\ per\ ml = \frac{counted\ number\ of\ alive\ cells}{number\ of\ squares\ used\ for\ counting} * \ dilution\ factor * \ 10^4$$

2.2.4 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from lithium heparin blood samples.

Before PBMC isolation, 50 mL Leucosep tubes were prepared by adding 15.5 mL Biocoll (Biochrom) and centrifuging at 1400 rpm for 30 sec. After centrifugation, the upper layer containing any remaining Biocoll was gently discarded, and 10 mL PBS was added to each Leucosep tube.

For PBMC isolation, the patients' blood was transferred into the prepared Leucosep tubes and centrifuged at 2000 rpm for 20 min. at room temperature without break. Following centrifugation, the serum was carefully removed up to 1 cm above the cell pellet. The mononuclear cell layer was then transferred to a 50 mL Falcon tube and thoroughly mixed.

Subsequently, the cell pellet was resuspended in 50 mL of RPMI and centrifuged at 1400 rpm for 10 min. at room temperature. The supernatant was discarded, and 5 mL X-VIVO 20 was added, followed by centrifugation at 1400 rpm for 5 min. at room temperature.

2.2.5 Cryopreservation of PMBCs

After centrifugation, the supernatant was removed, and the cells were resuspended in freezing medium I (500 µL per 10 x 10^6 cells). Next, 500 µL of the cell suspension were added to each cryotube. Subsequently, the cryotubes were gently enriched with 500 µL of the freezing medium II, added drop by drop, and mixed thoroughly. The PBMCs were then stored overnight at – 80°C before being transferred to the liquid nitrogen

tank within 48 hours for long-term preservation. Each cryotube contained approximately 10 x 10^6 cells (at least 5 x 10^6 cells).

2.2.6 Thawing of PBMCs

A thawing medium, consisting of 10 mL X-VIVO 20 + 50 U/mL Benzonase per cryovial, was prepared in 50 mL Falcon tubes. Frozen cells were thawed in a preheated 37°C water bath. Immediately afterwards, 0.5 mL of the preheated thawing medium were carefully added drop by drop into each cryotube. Subsequently, the cells were completely transferred into the 50 mL Falcon tubes enriched with the preheated thawing medium and centrifuged at 400 x g for 10 min. at room temperature. After removing the supernatant, the cell pellet was resuspended in 10 mL of RPMI. This was followed by centrifugation at 400 x g for 10 min. at room temperature, and the supernatant was discarded.

2.2.7 Flow cytometry analysis

2.2.7.1 Extracellular staining

Around 1x10^6 cells were dispensed per well into a 96-well plate round bottom. The cells were then washed with 150 µL human MACS buffer (300 x g, 5 min., 4°C). For extracellular staining, master mixes containing the respective conjugated antibodies were prepared and diluted in human MACS buffer. To identify live cells, Fixable viability stain Ax700 was added to the master mixes at a concentration of 1:500, and to reduce unspecific antibody binding, FcR Blocking Reagent was added at a dilution of 1:25, respectively. After washing, the cells were resuspended with 100 µL of the respective master mixes and incubated for 30 min. at 4°C in the dark. Following incubation, the cells were washed with 150 µL of human MACS buffer (300 x g, 5 min., 4°C). Finally, for FACS analysis, the cell pellets were resuspended in 100 µL of human MACS buffer. Acquisition was conducted using FACS Lyric (BD Biosiences) and data was analyzed using the FlowJo software (BD Biosiences).

2.2.7.2 Intracellular staining

Analysis of intracellular markers was performed using the FOXP3/Transcription Factor Fixation/Permeabilization kit. Following extracellular staining, each cell pellet was resuspended with 150 µL of Fix-Perm solution (Fixation/Permeabilization concentrate + Fixation Permeabilization diluent at a ratio of 1:4). For fixation and permeabilization, the cells were then incubated for 30 min. at room temperature in the dark, followed by two washing steps (300 x g, 5 min., at room temperature) with 150 µL Perm-Wash (Permeabilization Buffer + ddH_2O at a ratio of 1:10). The respective antibodies for intracellular staining were diluted in Perm-Wash, and each cell pellet was resuspended with 100 µL of the antibody mix. For staining, the cells were incubated for 30 min. at room temperature in the dark. After two additional washing steps with 150 µl of Perm-Wash, the cells were resuspended in 100 µL Perm-Wash for FACS analysis.

2.2.7.3 ROS and NO analysis

ROS production was determined using the CellROX™ Deep Red Reagent kit, and the reagent was diluted in a 1:500 ratio with human MACS buffer. For NO detection, 100 µL of a 1:100 dilution of DAF-FM Diacetate in human MACS buffer was added to the cells. ROS/NO staining was performed concurrently with the extracellular staining process and incubated at 4°C for 30 min. in the dark. After washing with 150 µL of human MACS buffer, the cells were resuspended with 100 µL human MACS buffer for subsequent FACS analysis, which was performed within one hour after the staining process.

2.2.8 Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software, Version 8.1.2. The Shapiro-Wilk test was employed to evaluate the data for a Gaussian distribution. For data following a normal distribution, I used a paired or unpaired two-tailed student's t-test. For data not following a normal distribution, the Wilcoxon signed-rank test was utilized to compare paired samples, and the Mann-Whitney U-test for unpaired variables, respectively. Correlation analysis of two variables was performed using the Pearson correlation coefficient, followed by a two-tailed p-value evaluation.

PFS was displayed as a Kaplan-Meier curve, and statistical analysis was conducted using the log-rank test. PFS was calculated as the time between treatment start until tumor relapse. For patients without any sign of tumor progression, data were censored based on the date of last contact.

The Fisher's exact test was performed to evaluate the following clinical characteristics: gender, age, AJCC stage, primary site of the tumor, treatment regimen, treatment outcome, and administered ICI agent.

3 RESULTS

3.1 Patient cohort

3.1.1 Clinical characteristics

In this study, I included 31 patients with malignant melanoma receiving ICI therapy at the Department of Dermatooncology, Skin Cancer Center, University Medical Center Mannheim. IrAE were observed in 17 patients (55%), while 14 patients (45%) remained free from irAE during the observation period. Overall, the cohort consisted of 19 males (61%) and twelve females (39%) with a median age of 63 years, ranging from 31 to 85 years. Table 1 provides a summary of clinical characteristics. Patients' data were collected during irAE occurrence or the reference TP 2, respectively. Upon analyzing the clinical characteristics, I found no significant differences in age or gender when comparing irAE versus no irAE.

Melanoma staging was classified according to the AJCC stage (2018). My study included: one patient with AJCC stage IIIA, six patients with AJCC stage IIIB, eleven patients with AJCC stage IIIC and 13 patients with AJCC stage IV. Most patients were diagnosed with cutaneous melanoma (28 patients, 90%), while three patients (10%) had a melanoma of unknown primary. Adjuvant ICI treatment was administered to 16 patients (52%), and 15 patients (48%) received ICI in a palliative treatment setting. Monotherapy with anti-PD-1 inhibitors was applied to 22 patients (71%), while combination therapy, containing up to four application cycles of anti-CTLA-4/anti-PD-1 inhibitors, was administered to nine patients (29%). Additionally, I found no significant differences comparing the clinical characteristics (primary site, treatment group, or treatment agent) of patients with and without irAE.

Table 1. Clinical characteristics of patients with irAE and without irAE.

Characteristic	IrAE (n=17)	No irAE (n=14)	P value
Median age, years (range)	61 (32-85)	66 (31-85)	0,9335
Gender, n			>0,9999
Male	10	9	
Female	7	5	
AJCC stage, n			
IIIA	1	0	>0,9999
IIIB	3	3	>0,9999
IIIC	5	6	0,4775
IV	8	5	0,7168
Primary site, n			0,5764
Cutaneous	16	12	
Unknown	1	2	
Treatment group, n			0,7224
Adjuvant	8	8	0,6084
Relapse	4	6	
Relapse-free	4	2	
Palliative	9	6	
CR	1	0	>0,9999
PR	4	1	0,3445
SD	2	1	>0,9999
PD	2	4	0,3697
Therapy, n			>0,9999
Anti-PD-1/CTLA-4	5	4	
Anti-PD-1	12	10	

3.1.2 Characteristics of irAE

My study identified 21 cases of irAE among 14 patients. Table 2 presents a comprehensive overview of all observed irAE and their corresponding grading according to the CTCAE criteria. The median time for irAE development was 80.5 days after ICI initiation. The earliest irAE occurred 24 days after treatment start, while the latest reported irAE emerged 669 days after ICI initiation. Within my study cohort, one patient experienced two distinct irAE entities at different time points: hypophysitis and, three months later, colitis. Both adverse events, hypophysitis and colitis, were considered as separate incidents for FACS analysis. Additionally, another patient experienced multiple irAE simultaneously, including hepatitis, acute kidney injury, hypophysitis, and pancreatitis.

Frequently occurring irAE included colitis (four cases, 19%), thyroiditis (four cases, 19%), hypophysitis (three cases, 14%), and hepatitis (three cases, 14%). Most of the reported irAE (16 cases, 76%) were classified as mild to moderate (grade 1-2), while severe irAE (grade ≥3) occurred in five cases (24%). No patients with a CTCAE grading of 4 or 5 were included in my study. Furthermore, I observed that three out of five reported severe irAE occurred following prior combination treatment with anti-PD-1/CTLA-4 antibodies. However, the administration of combination therapy did not show a significant association with the occurrence of grade ≥3 irAE (p=0.26).

Table 2. Reported immune-related adverse events.

Toxicity	**Reported events**		
	Grade 1	**Grade 2**	**Grade 3**
Hepatitis	0	1	2
Colitis	2	1	1
Pancreatitis	0	1	0
Acute kidney injury	0	0	1
Thyroiditis	0	4	0
Hypophysitis	0	3	0
Arthritis	0	2	0
Peripheral sensory polyneuropathy	0	0	1
Rash	1	0	0
Eye disorder – other, specify	0	1	0

3.2 Progression-free survival (PFS) and treatment outcome

First, I aimed to investigate the association between irAE onset and treatment outcome. To achieve this, I analyzed PFS and response rates among patients with and without irAE. My study revealed a notably elevated PFS, with a p-value of 0.02, among patients experiencing adverse events (Figure 5A). To further dissect potential differences in PFS among patients receiving ICI in adjuvant or palliative treatment regimen, I performed separate analyses of treatment outcomes and PFS for both groups.

Within the adjuvant treatment group, eight patients experienced irAE, with four (50%) of them showing tumor relapse, while the remaining four patients (50%) stayed relapse-free during the observation period. In the no irAE group, six patients (75%) experienced tumor relapse, while only two patients (25%) displayed no sign of progression (Figure 5B). Notably, in the adjuvant cohort, the percentage of relapse-free patients did not show a significant difference between the irAE and no irAE group (p=0.61). However, patients with irAE tended to demonstrate an improved PFS, though this finding did not reach statistical significance (Figure 5C).

Among patients undergoing palliative ICI therapy and experiencing irAE, seven patients (78%) were classified as responders (comprising one patient with complete response (CR), four patients with partial response (PR), and two patients with stable disease (SD)), while two patients (22%) were categorized as non-responders. In contrast, in the no irAE group, the majority of patients presented as non-responders (four patients, 66%), while two patients (33%) were identified as responders (Figure 5D). Consequently, in the palliative treatment cohort, patients with irAE tended to exhibit improved response rates compared to those without irAE (p=0.14) (Figure 5E). Additionally, patients experiencing irAE after palliative ICI treatment demonstrated a significantly prolonged PFS.

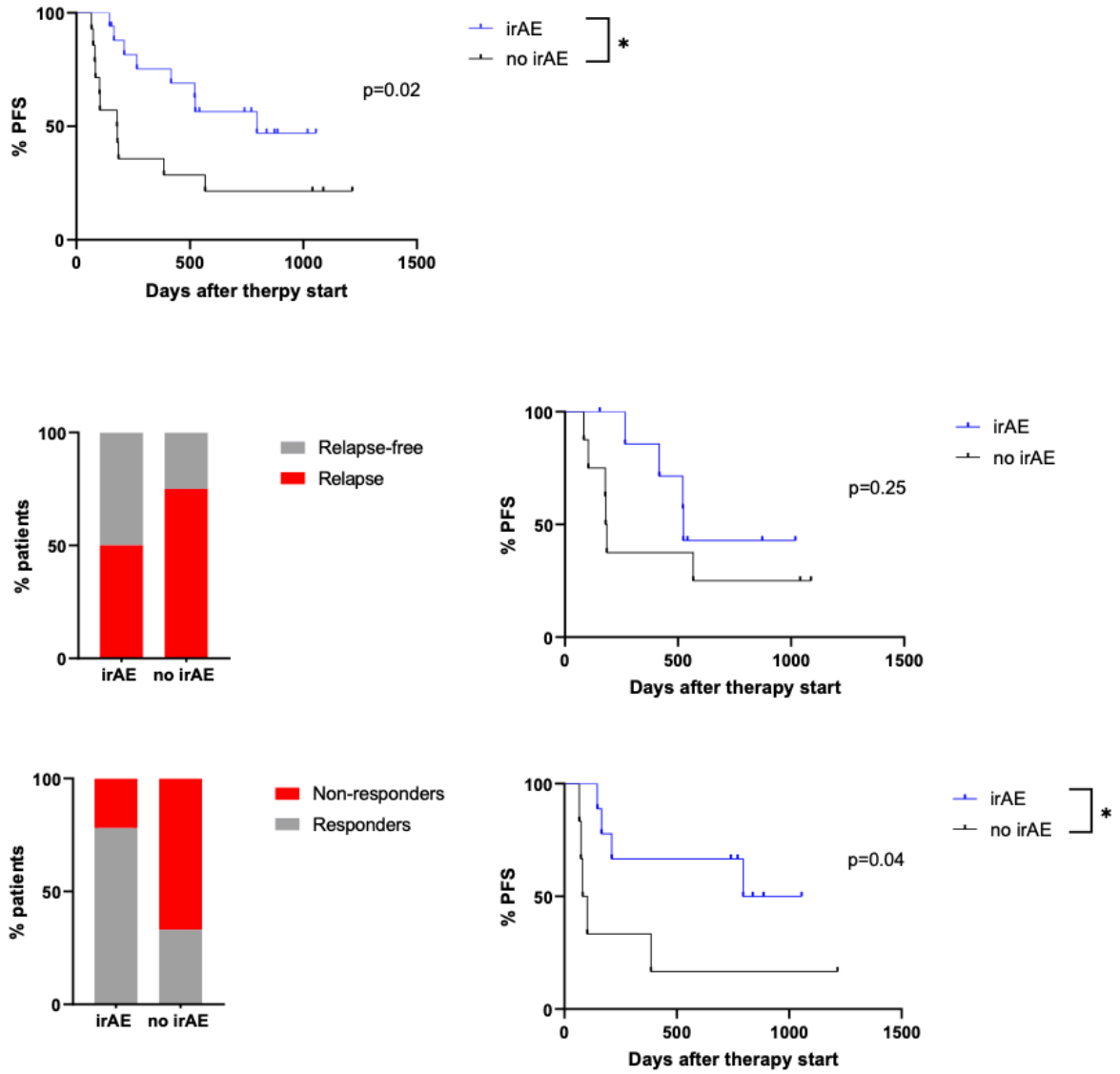

Figure 5. Progression-free survival (PFS) and clinical outcome after ICI treatment in the irAE versus no irAE group. (A) PFS of the total patient cohort displayed as a Kaplan-Meier curve (n=31). (B) Clinical outcomes in the adjuvant treatment group (n=16), including the percentage of relapse and relapse-free patients among those with and without irAE. (C) PFS analysis of adjuvant treated patients (n=16), comparing patients with and without irAE. (D) The percentage of responders and non-responders following palliative ICI treatment (n=15) among patients with and without irAE. (E) PFS analysis of patients treated in a palliative treatment setting (n=15), comparing irAE and no irAE.

3.3 Analysis of T cells

To evaluate T cell characteristics, I measured the expression of T cell activation markers (CD25, CD69, TCR ζ-chain), PD-1 expression, and the number of Tregs, using flow cytometry. The gating strategy for identifying $CD4^+$ and $CD8^+$ T cell is shown in Figure 6A.

3.3.1 IrAE development is associated with an elevated frequency of certain activated T cell subsets.

All measurements of T cells were conducted using flow cytometry, and the analysis was performed with reference to the respective fluorescence minus one (FMO) control, as displayed in Supplementary Figure 1.

I observed an upregulation of the frequency of $CD69^+$ cells among $CD8^+$ T cells during irAE onset compared to the previous time point. When comparing TP 2 of the irAE and the no irAE groups, the frequency of $CD8^+CD69^+$ T cells appeared higher in the irAE group (Figure 6B). Although my data suggested a potential association between activated $CD8^+CD69^+$ T cells and irAE onset, correlation analysis between irAE severity and circulating $CD8^+CD69^+$ T cells did not reveal any significant correlation (Figure 6C).

The activation marker CD25 was found to be upregulated on $CD8^+$ T cells during irAE compared to the previous time point (Figure 6D). In addition, the frequency of $CD8^+CD25^+$ T cells seemed to increase during irAE onset compared to TP 0. However, the frequency of $CD8^+CD25^+$ T cells at TP 2 was also elevated in the no irAE group.

Finally, I evaluated TCR ζ-chain expression, a crucial subunit of the T cell receptor involved in receptor assembly, expression, and T cell signaling[140]. Here, I failed to detect any association between TCR ζ-chain expression levels in $CD8^+$ T cells and irAE occurrence (Figure 6E). Moreover, I observed no differences in expression levels between both groups throughout the treatment period.

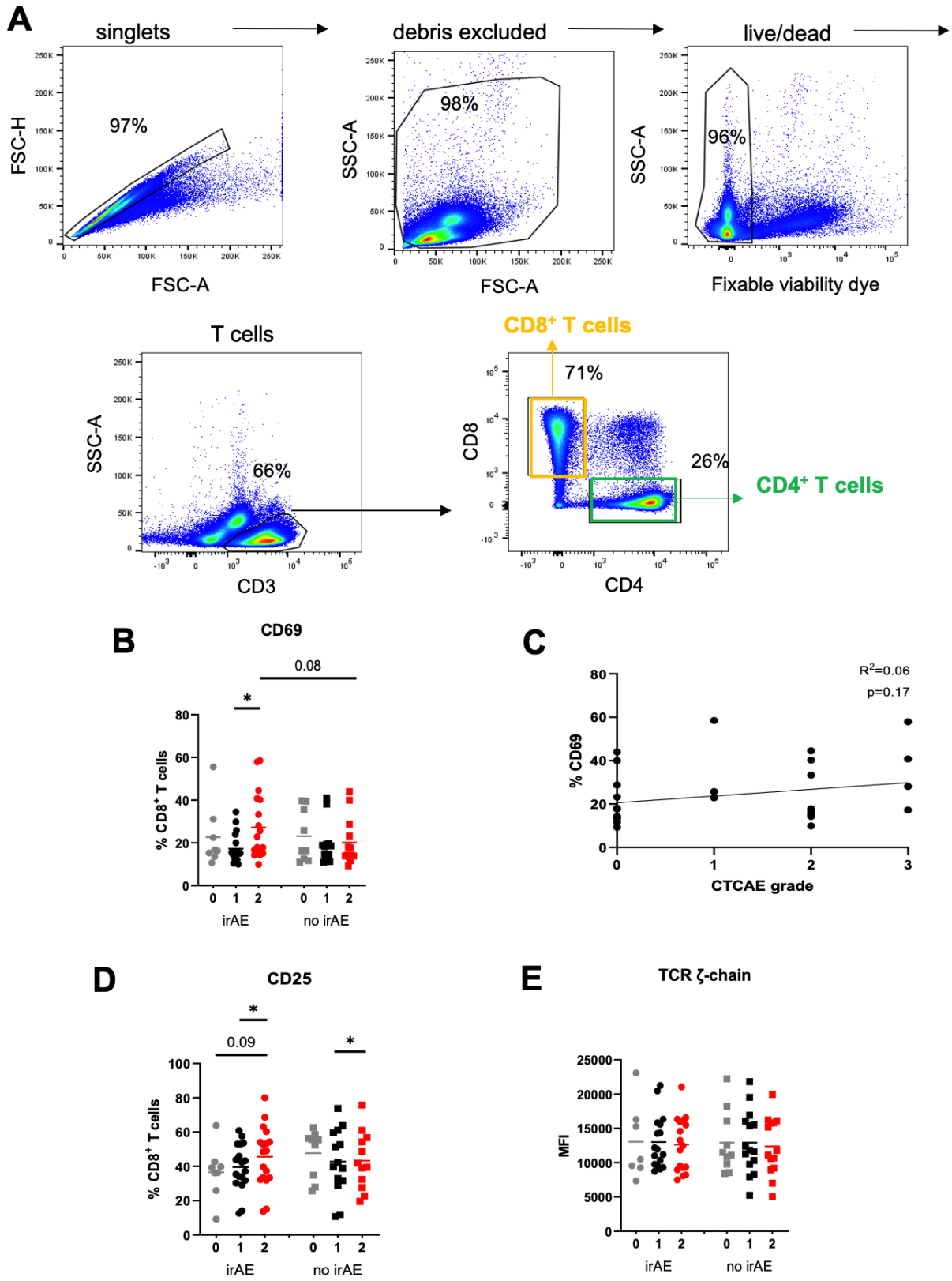

Figure 6. Analysis of the activation markers CD69, CD25, and TCR ζ-chain on CD8⁺ T cells. (A) The gating strategy for flow cytometry analysis of live CD8⁺ and CD4⁺ T cells is presented. First, single cells were discriminated by gating forward scatter height (FSC-H) against area (FSC-A). Subsequently, exclusion of debris from the cell population was performed by plotting cell size (FSC-A) versus granularity (SSC-A). Dead cells were then excluded from the analysis using the Fixable viability dye. Following gating on CD3, the two T cell subsets, CD8⁺ and CD4⁺ T cells, were identified.

(B) Frequency of $CD8^+CD69^+$ T cells in the irAE group (n=8-18) and no irAE group (n=9-14). (C) Correlation analysis between the percentage of circulating $CD8^+CD69^+$ T cells and irAE severity (n=31). IrAE severity was evaluated according to the CTCAE criteria. The correlation analysis was assessed by linear regression. No irAE was considered as CTCAE grade 0. For patients with more than one observed irAE, the highest examined irAE was chosen. (D+E) Analysis of CD25 and TCR ζ-chain expression levels on $CD8^+$ T cells among the respective T cell subsets in patients with (n=8-18) and without irAE (n=9-14). TCR ζ-chain expression was determined as the median fluorescence intensity (MFI). (Individual values and means are shown, *$p<0.05$)

Next, I analyzed the activation status of circulating $CD4^+$ T cells. CD69 expression was determined according to the respective FMO control, as shown in Supplementary Figure 2. Unlike my findings on $CD8^+$ T cells, I did not observe an association between irAE onset and CD69 expression levels on $CD4^+$ T cells (Figure 7A).

Furthermore, I investigated the frequency of activated $CD4^+CD25^+FOXP3^-$ T cells. The gating strategy is demonstrated in Figure 7B. In the irAE group, I found a transient decrease in the frequency of $CD4^+CD25^+FOXP3^-$ T cells following treatment initiation. However, during irAE, I observed a significant increase in activated T cells (Figure 7C). In contrast, the proportion of this cell subset remained stable in the no irAE group. Here, I did not demonstrate any significant changes in CD25 expression levels when comparing irAE versus no irAE.

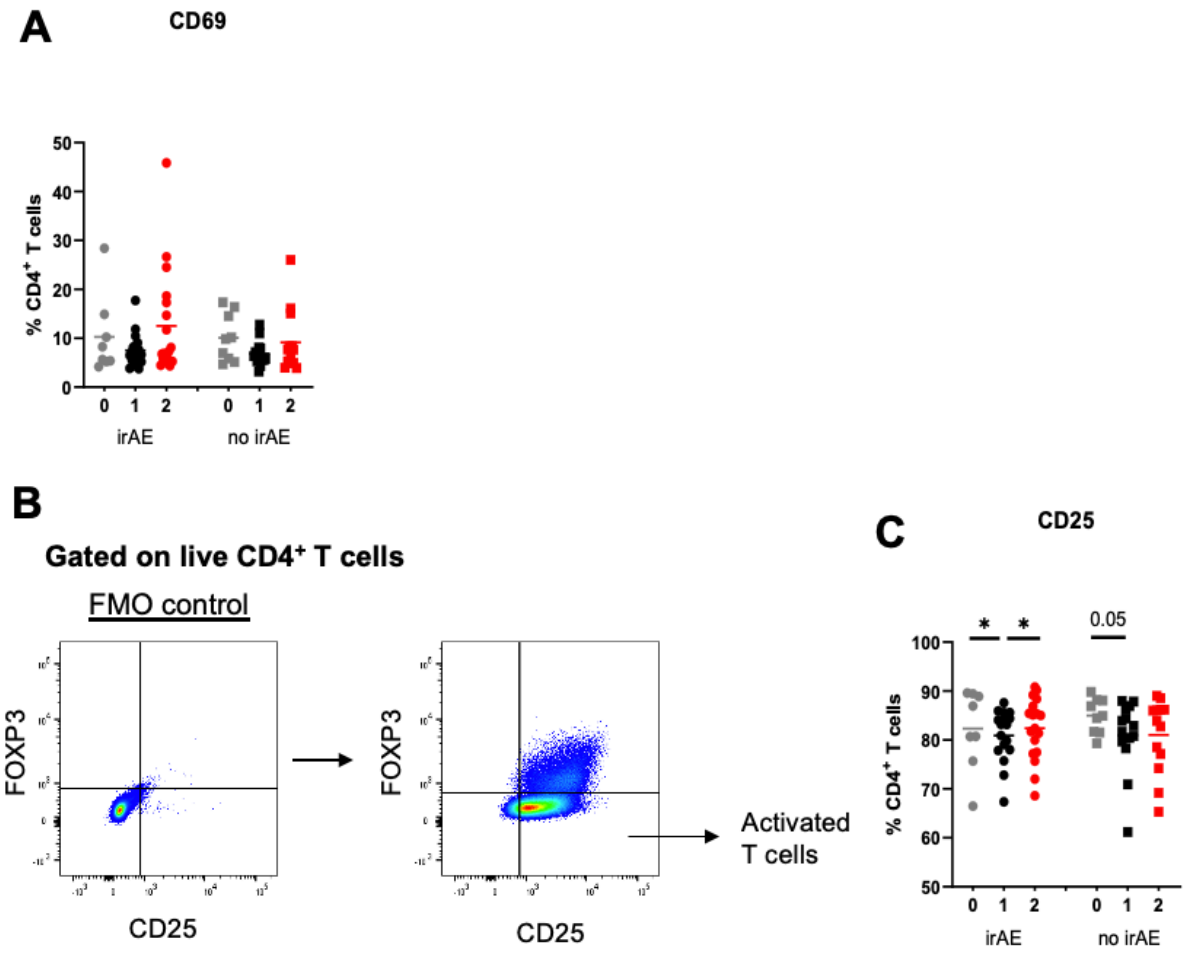

Figure 7. Analysis of the activation markers CD69 and CD25 on CD4⁺ T cells. (A) Comparison of the percentage of $CD4^+CD69^+$ T cells between patients with irAE (n=8-18) and those without irAE (n=9-14). (B) Gating strategy for identifying activated $CD4^+CD25^+FOXP3^-$ T cells.
(C) Corresponding figure showing the changes in $CD4^+CD25^+FOXP3^-$ T cells among patients with (n=8-18) and without irAE (n=9-14). (Individual values and means are shown, *p<0.05)

3.3.2 Decline in PD-1 expression levels following ICI treatment initiation.

Finally, I measured PD-1 expression levels on $CD8^+$ and $CD4^+$ T cells at TP 0, 1, and 2. My investigations revealed a notable decrease in PD-1 expression following ICI initiation, observed in patients with reported irAE and those without. Moreover, regardless of irAE onset, the percentage of $CD8^+PD\text{-}1^+$ and $CD4^+PD\text{-}1^+$ T cells within total $CD8^+$ and $CD4^+$ T cells respectively remained consistently low throughout the treatment period, as displayed in Figure 8.

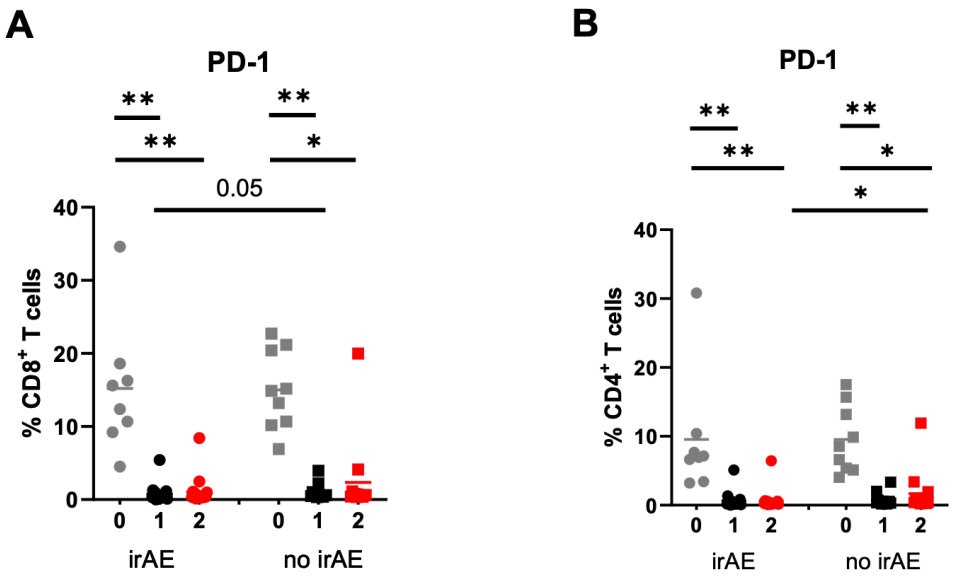

Figure 8. PD-1 expression on CD8⁺ and CD4⁺ T cells. (A, B) Results from patients with irAE (n=8-18) and without irAE (n=9-14) are presented as the proportion of circulating CD8⁺PD-1⁺ and CD4⁺PD-1⁺ T cells among total live CD8⁺ and CD4⁺ T cells, respectively. (Individual values and means are shown, *p<0.05, **p<0.01, ***p<0.001)

3.3.3 The frequency of circulating Tregs correlates with irAE onset

First, I investigated the percentage of circulating Tregs at TP 0, 1, and 2 for patients with and without irAE. Tregs represent a subset of T cells known for their immunosuppressive properties and their ability to restrain autoimmune diseases[73]. In my study, I used a panel of CD3, CD4, CD25 and FOXP3 antibodies to identify Tregs as CD4⁺CD25⁺FOXP3⁺ T cells[141]. The gating strategy is depicted in Figure 9A.

In the irAE group, I observed a transient but significant increase in Tregs during TP 1. However, upon further analysis, I demonstrated a decreased frequency of Tregs at the onset of adverse event compared to the preceding time point (Figure 9B). In contrast, for patients without irAE, I found a significant increase in Tregs during ICI treatment compared to the baseline. When comparing TP 2 between the irAE and no irAE groups, the data revealed a significantly lower percentage of Tregs in patients experiencing adverse events. Interestingly, I was able to demonstrate that a diminished frequency of CD4⁺CD25⁺FOXP3⁺ T cells correlated with the occurrence and severity of irAE, respectively (Figure 9C).

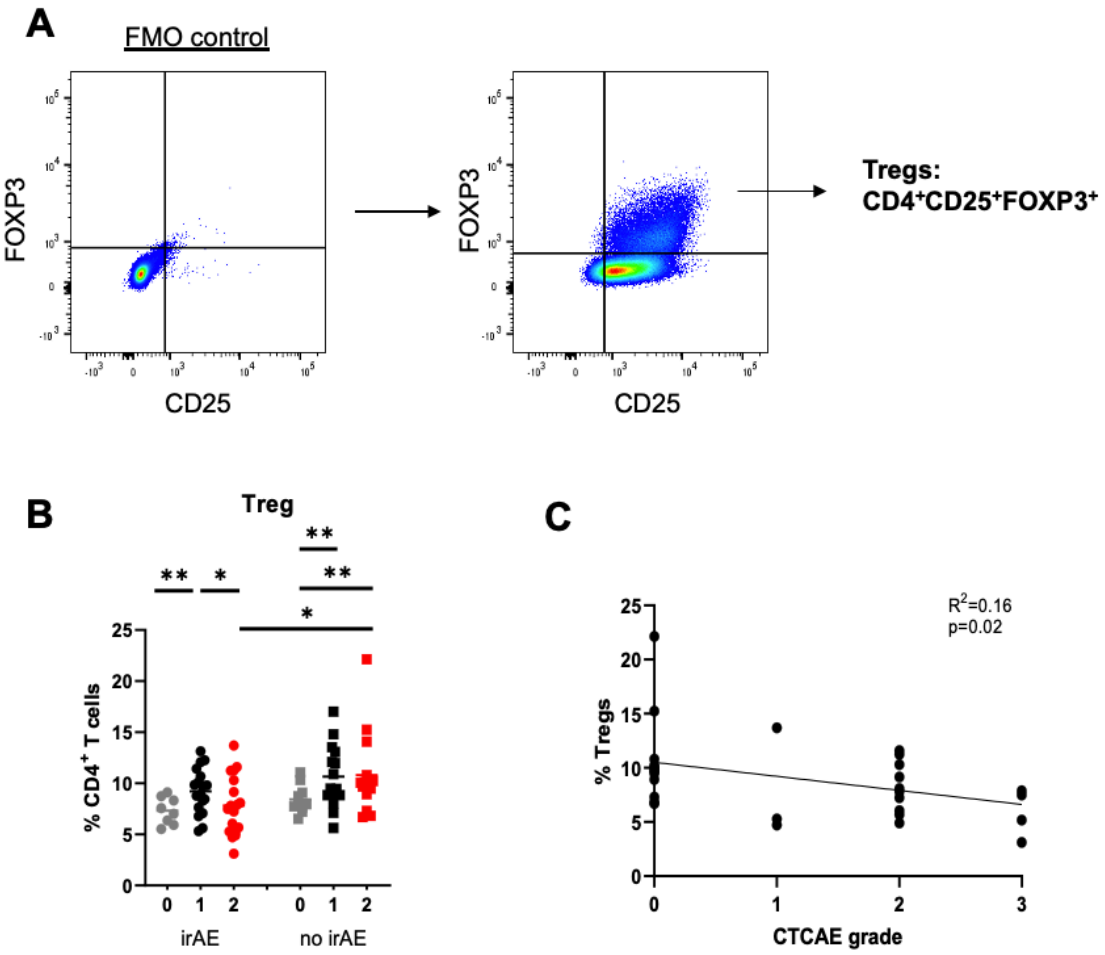

Figure 9. Analysis of Tregs. (A) Representative dot plots showing the identification of circulating Tregs as $CD4^+CD25^+FOXP3^+$ T cells. (B) The frequency of circulating Tregs at indicated time points is presented for patients with irAE (n=8-18) and without irAE (n=9-14). (C) The percentage of Tregs within total $CD4^+$ T cells is plotted against the CTCAE grading (n=31). Correlation analysis was performed using linear regression. No irAE was classified as CTCAE grade 0. In cases where patients experienced multiple irAE, the highest examined irAE was selected. (Individual values and means are shown, *p<0.05, **p<0.01)

3.4 Analysis of M-MDSCs

To identify M-MDSCs after flow cytometry analysis, I employed a gating strategy as presented below in Figure 10A. M-MDSCs were characterized as $CD14^{+}/HLA\text{-}DR^{low/-}$ cells. Only frozen PBMCs were used for FACS analysis. Consequently, I did not need to include other antibodies to define PMN-MDSCs, as these cells would not survive the freezing process.

3.4.1 Study of M-MDSCs and their immunosuppressive capacity for irAE onset

In the study cohort, I did not observe any difference in M-MDSC frequencies between irAE and no irAE (Figure 10B). However, within the no irAE group, I found a significant increase in M-MDSCs comparing TP 0 and TP 1, while patients with irAE did not show any significant changes during the treatment period or irAE, respectively.

Furthermore, I aimed to characterize the immunosuppressive pattern of M-MDSCs. Therefore, I evaluated the following immunosuppressive markers: PD-L1, CD73, ROS, and NO. The expression levels were assessed according to the corresponding FMO control, as depicted in Supplementary Figure 3.

I did not detect any significant changes in PD-L1 and CD73 expression levels on M-MDSCs comparing irAE versus no irAE, nor over the treatment period within both groups (Figure 10C+D). However, in the irAE group, the expression level of CD73 tended to decrease from TP 0 to TP 2.

For ROS and NO detection, I measured the released ROS and NO production by M-MDSCs as median fluorescence intensity (MFI) at indicated time points for patients with and without irAE (Figure 10E+F).
Likewise, my data did not indicate any differences in ROS and NO production comparing irAE and no irAE. Interestingly, I revealed an increased ROS production during ICI treatment among patients without irAE onset. In contrast, the measured NO production decreased throughout the treatment period for patients without reported irAE. For patients with irAE, the detected ROS and NO production remained stable at indicated time points.

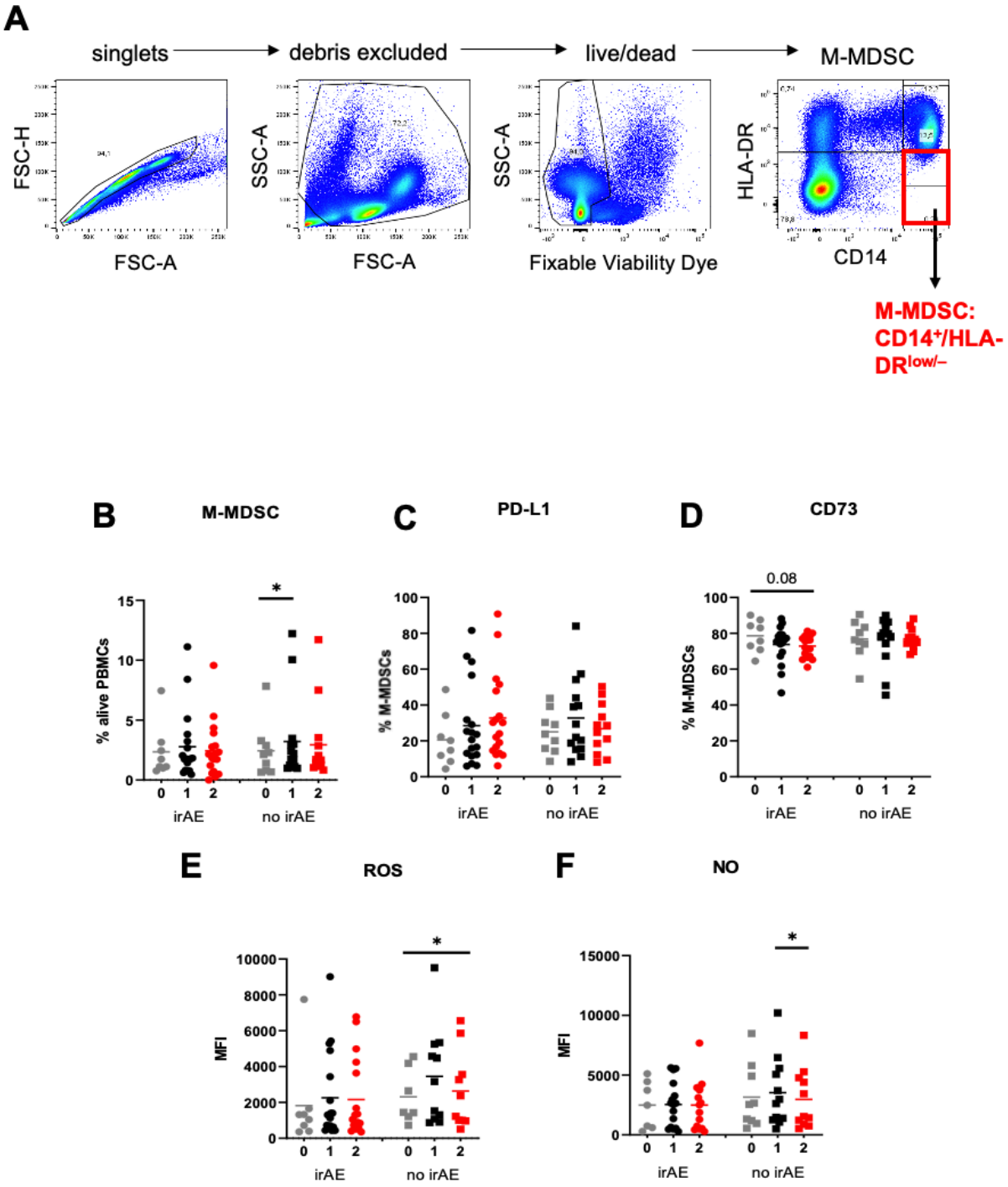

Figure 10. Characterization of circulating M-MDSCs in ICI-treated patients during TP 0, 1, and 2. **(**A) A representative gating strategy to discriminate M-MDSCs is shown. First, doublets, debris, and dead cells were excluded. Second, the markers CD14 and HLA-DR were used to identify M-MDSCs as $CD14^{+}$/$HLA\text{-}DR^{low/-}$ cells. (B) Results of circulating M-MDSCs in patients with (n=8-18) and without irAE (n=9-14) are displayed as the percentage within live PBMCs. (C+E) The expression levels of PD-L1 and CD73 on M-MDSCs are presented as the frequency of $PD\text{-}L1^{+}$ and $CD73^{+}$ M-MDSCs among total live M-MDSCs comparing irAE (n=8-18) versus no irAE (n=9-14). (E+F) The production of ROS and NO by M-MDSCs is displayed as MFI for patient with irAE (n=7-18) and no irAE (n=7-12). (Individual values and means are shown, *p<0.05)

3.5 Potential impact of immunosuppressive treatment on circulating Tregs and M-MDSCs

In my study, I analyzed PBMCs from five patients who underwent immunosuppressive treatment to manage irAE. My objective was to investigate the effect of immunosuppressive drugs on circulating immune cells, particularly M-MDSCs and Tregs. The study cohort included four patients who received methylprednisolone at TP 3: Three patients received doses ranging from 10 to 30 mg, while one patient received a high dose of 120 mg methylprednisolone. The fifth patient received a dose of 1000 mg mycophenolate mofetil and 15 mg hydrocortisone as adrenal replacement therapy during TP 3.

In four out of five patients, I found an increase in circulating Tregs as well as M-MDSCs when comparing TP 2 and TP 3 (Figure 11A+B). However, this observation did not reach statistical significance. Additionally, I evaluated the immunosuppressive profile of M-MDSCs characterized by the percentage of PD-L1$^+$ and CD73$^+$ M-MDSCs among total live M-MDSCs. Surprisingly, I found a tendency for the expression levels to decrease after immunosuppressive treatment, as displayed in Figure 11C+D.

Interestingly, one patient treated with a high dose of 120 mg methylprednisolone at TP 3 following immune-related hepatitis, showed a massive expansion of M-MDSCs. The gating strategy of M-MDSCs and the corresponding figure showing the changes in M-MDSCs from TP 0 to TP 3 of this patient are displayed in Figure 11E+F.

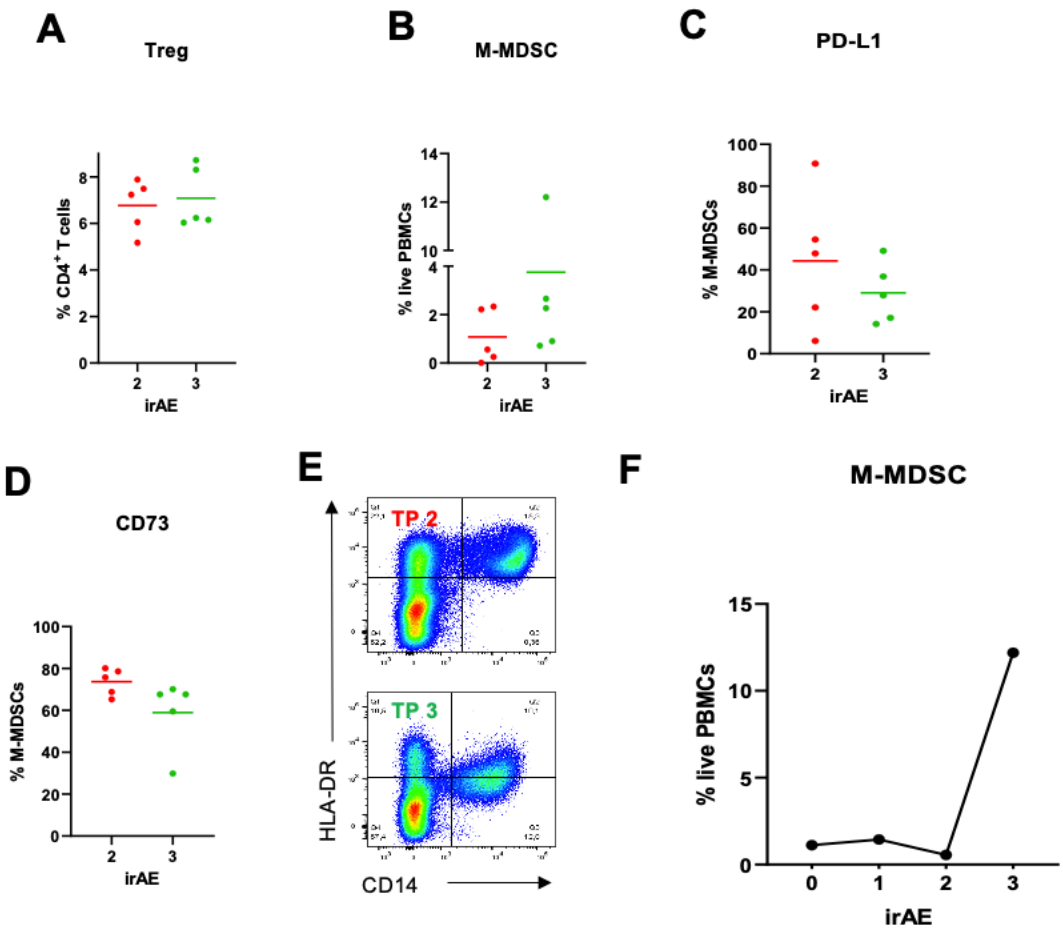

Figure 11. Investigating the role of immunosuppressive treatment on Tregs and M-MDSCs. Immunosuppressive therapy is indicated as TP 3. (A, B) The percentage of Tregs and M-MDSCs at the time point of irAE (TP 2) versus during immunosuppressive treatment (TP 3) in patients with irAE (n=5) is shown. (C+D) PD-L1 and CD73 expression levels on M-MDSCs within the total live M-MDSC population (n=5). (Individual values and means are shown).
(E+F) Example of a patient treated with high dose methylprednisolone following immune-related hepatitis, including representative dot plots showing the gating strategy for M-MDSCs at TP 2 and TP 3, and the changes in M-MDSCs observed during TP 0, 1, 2, and 3.

3.6 Analysis of routine blood tests

The absolute leukocyte count and subsequent leukocyte subsets were evaluated at TP 0, 1, and 2 for patients with and without irAE, as shown in Figure 12A-E. Statistical analysis was conducted within both groups and between the irAE and the no irAE groups, respectively.

In the irAE group, the absolute number of leukocytes remained stable throughout the treatment period and during irAE onset. In contrast, patients without irAE exhibited a decrease in absolute leukocytes after treatment initiation (Figure 12A).

Patients experiencing irAE showed a decrease in lymphocytes during ICI treatment. Notably, my data revealed a significantly higher baseline absolute lymphocyte count in the irAE group compared to the no irAE group (Figure 12B).

In terms of eosinophils, an increase was observed during ICI therapy for patients without irAE, while the eosinophil count remained unchanged in the irAE group. Moreover, no significant differences in eosinophils were found when comparing irAE versus no irAE (Figure 12C).

Neutrophil analysis showed a notable decrease at TP 1 compared to TP 0 in patients without irAE. However, no significant changes were observed in the irAE group or when comparing irAE versus no irAE (Figure 12D).

Finally, I analyzed the absolute number of monocytes. Here, my data did not indicate an association between changes in monocyte counts and irAE onset. In addition, there were no significant differences in monocyte levels throughout ICI treatment in the no irAE group (Figure 12E).

Further analysis included changes in serum LDH and CRP levels (Figure 12F+G). Serum LDH levels were examined during routine blood tests at indicated time points. A significant increase in LDH levels was found at TP 2 among patients with irAE compared to the preceding time points, and these TP 2 levels were significantly higher than those observed in the no irAE group.

Next, CRP levels, a commonly used biomarker indicating inflammation, were evaluated. The irAE group showed elevated CRP levels during adverse event compared to TP 1. Conversely, CRP levels remained unchanged during ICI treatment for patients without irAE. When comparing irAE versus no irAE, no significant differences in CRP levels were observed.

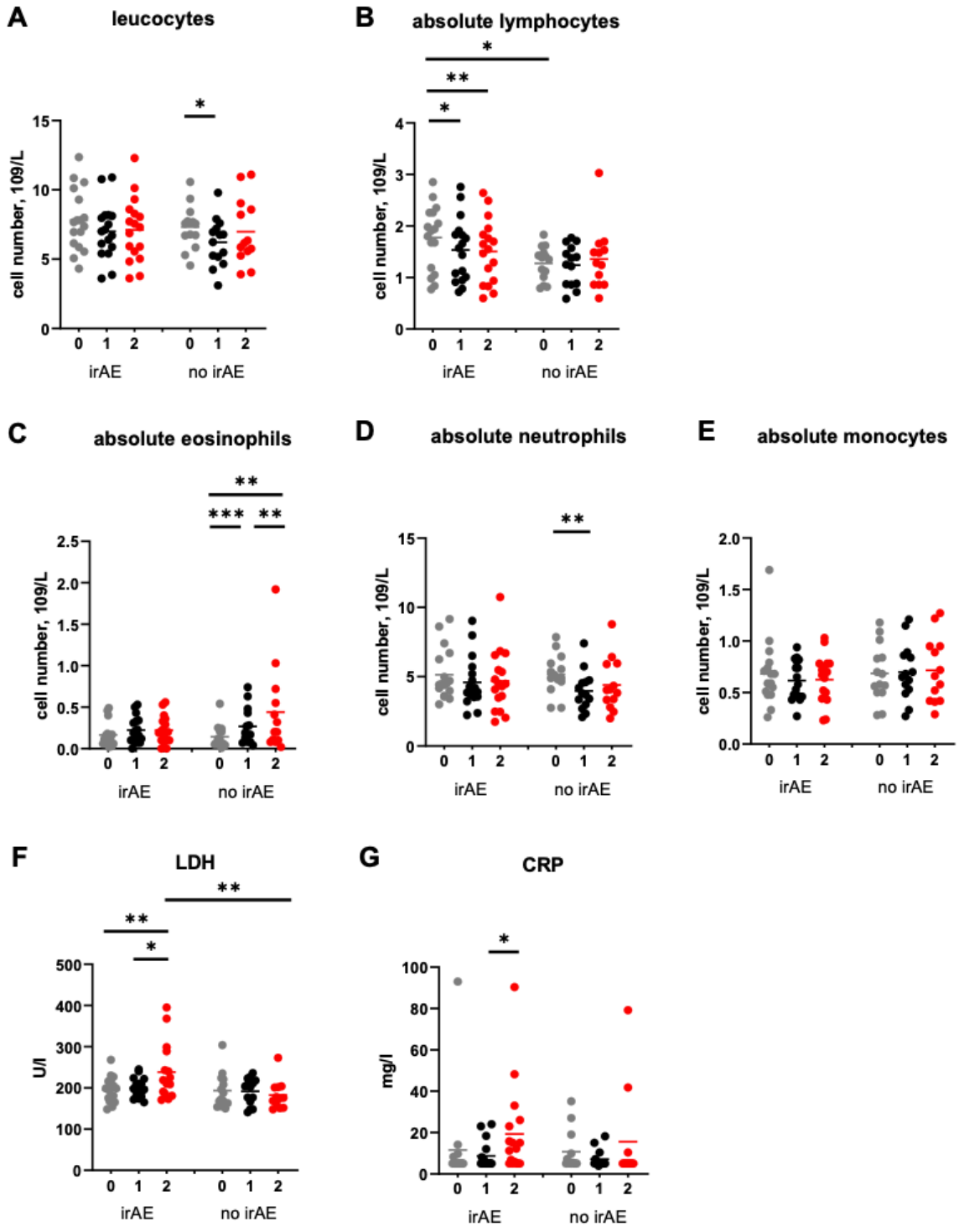

Figure 12. Analysis of routine blood counts, LDH, and CRP serum levels. The results present the absolute count of leukocytes (A), lymphocytes (B), eosinophils (C), neutrophils (D), and monocytes (E) in patients with (n=17) and without irAE (n=14). Changes in LDH (F) and CRP levels (G) for patients with (n=16-17) and without irAE (n=11-14) at TP 0, 1, and 2 are demonstrated. (Individual values and means are shown, *p<0.05, **p<0.01, ***p<0.001).

4 DISCUSSION

The study presents a comprehensive analysis of immunological alterations during irAE and potential predictive factors favoring irAE onset. This analysis included T cell markers (CD69, CD25, TCR ζ-chain, PD-1), the frequency of Tregs and M-MDSCs, as well as the immunosuppressive phenotype of M-MDSCs.

4.1 Summary of patients' characteristics, irAE, and treatment outcome

Commonly observed irAE in my study included thyroiditis, colitis, hypophysitis, and hepatitis. Accordingly, these irAE entities are also described as common adverse events in the literature[106].

Furthermore, I found a significantly elevated PFS among patients with irAE. Subsequently, I investigated PFS for patients treated in adjuvant and palliative treatment regimen separately. The analysis within the palliative treated patients confirmed an increased PFS among those with irAE. This finding is in line with several observational studies demonstrating an association between irAE and improved treatment outcomes, including PFS and OS[142]. Thus, irAE onset itself may serve as a predictive marker for ICI treatment response[143, 144].

The CheckMate study 067 revealed that irAE development was preferentially seen after anti-CTLA-4/PD-1 combination therapy compared to anti-PD-1 monotherapy[145]. However, I did not observe different incidence rates of irAE when comparing patients treated with anti-CTLA-4/PD-1 combination therapy versus anti-PD-1 monotherapy. Regarding irAE severity, I showed severe adverse events after anti-CTLA-4/PD-1 treatment (three reported cases) and anti-PD-1 treatment (two reported cases). The missing effect of different treatment regimens (monotherapy versus combination therapy) favoring irAE development might be explained by the small study cohort.

4.2 IrAE onset is characterized by a distinct T cell profile

In the literature, T cells are recognized to be involved in the pathophysiology of irAE [146]. Previous studies have found an association between irAE occurrence and the clonal expansion of $CD8^+$ T cells[147, 148]. Robert et al.[149] suggested an increased diversity of the TCR V-beta Complementary Determining Region 3 (CDR3) in patients with irAE after anti-CTLA-4 antibody treatment, indicating TCR richness among these patients.

Moreover, Nuñez et al.[150] found an expansion of proliferating T cell, including $CD8^+CD38^+Ki\text{-}67^+$ T cells and $CD4^+CD38^+Ki\text{-}67^+$ conventional T cells, soon after ICI initiation in melanoma patients with irAE. Their study also revealed that patients with a massive expansion of $CD8^+CD38^+Ki\text{-}67^+$ T cells tended to develop adverse events earlier, suggesting a potential correlation between T cell activity and the timing of irAE onset[150].

A recently published immune monitoring study on melanoma patients demonstrated an induction of activated $CD8^+$ and $CD4^+$ T cells, precisely $CD4^+CD38^+HLA\text{-}DR^+$ T cells and $CD8^+CD38^+$ T cells, during irAE occurrence[151]. Similarly, Kovacsovics-Bankowski et al.[152] identified activated T cells as an inducible marker for the onset of severe irAE, demonstrating an expansion of $CD4^+CD38^+$, $CD8^+CD39^+$, and $CD8^+HLA\text{-}DR^+$ T cells at the peak of irAE.

Consistent with these findings, the study demonstrated an activation of $CD8^+$ T cells during irAE indicated by an upregulation of CD69. Moreover, CD69 expression levels on $CD8^+$ T cells tended to be higher in patients at the time point of irAE compared to the reference time point in the no irAE group. CD69 serves as an activation marker on T cells that can be detected 2-3 h after stimulation, and its expression is faster than CD25 appearance[153]. CD69 upregulation has also been observed in various chronic inflammatory conditions like autoimmune thyroiditis, arthritis, or multiple sclerosis[154].

Investigating $CD4^+$ T cells, an elevation of activated $CD4^+CD25^+FOXP3^-$ T cell during irAE was found.

However, conflicting results were published by Benesova et al., who found a stronger expansion of $CD8^+CD25^+$and $CD8^+CD69^+$ T cells in patients without irAE[155]. Nevertheless, this study focused on the immunological profile of patients with musculoskeletal irAE[155].

Regarding PD-1 expression levels on $CD8^+$ and $CD4^+$ T cells, I noted a considerable decline in PD-1 for both T cell subsets after ICI initiation. This decline was observed in patients with and without irAE, with PD-1 levels remaining consistently low throughout the observation period. When analyzing baseline PD-1 expression levels on $CD8^+$ and $CD4^+$ T cells, I could not detect any differences in patients with versus without irAE.

All patients in the study were treated with anti-PD-1 antibodies, either as monotherapy or combination therapy. Thus, I hypothesized that PD-1 is not downregulated but rather blocked following the administered anti-PD-1 treatment. Similar results were reported by Reschke et al.[151] who detected a strong downregulation of PD-1 on $CD3^+$, $CD8^+$, and $CD4^+$ T cells after treatment start with anti-PD-1 antibodies. Moreover, they found that baseline PD-1 expression levels on $CD8^+$ T cells were significantly higher in responders compared to non-responders[151]. Consequently, these findings suggest that baseline PD-1 status may serve as a predictive marker for ICI-associated treatment response rather than for irAE occurrence.

My investigation on $CD4^+CD25^+FOXP3^+$ Tregs demonstrated a transient elevation of Tregs during TP 1 for patients with irAE, followed by a decrease during irAE occurrence. In contrast, patients without irAE showed an increase in Tregs over the treatment period. A recent longitudinal analysis revealed a temporary increase in Tregs one to two weeks after treatment start in patients with irAE, followed by a significant decrease just before irAE onset in patients with non-small cell lung cancer[150]. A potential hypothesis for this temporary Treg expansion could be that an increased Treg population might be necessary to control ICI-triggered inflammation.

Further analysis on Tregs revealed a significantly lower frequency of Tregs in patients with adverse events compared to the reference time point in the no irAE group. Additionally, I demonstrated that the occurrence of severe adverse events correlated with a decreased frequency of Tregs. Corresponding with my data, Chaput et al.[156]

reported a lower number of Tregs at baseline in patients with immune-related colitis following ICI compared to the no irAE group. Another study observed a total increase in effector Tregs characterized as $CD4^+CD127^{lo}CD45RA^-FOXP3^{hi}$, in patients with thymic epithelial tumor and non-small cell lung cancer following anti-PD-1 treatment, although patients with severe irAE showed a smaller expansion of effector Tregs[157].

Tregs generate an immunosuppressive TME through the expression of PD-1 and the constitutive expression of CTLA-4[158]. Thus, targeting the immune checkpoints CTLA-4 and PD-1 on Tregs might reduce Treg activity and favor autoimmunity[137, 159]. Hatzioannou et al. reviewed that ICI therapy might promote a destabilized phenotype of Tregs, characterized by a loss of FOXP3, or a fragile subtype of Tregs still expressing FOXP3[160].

In addition to the checkpoint inhibitors CTLA-4 and PD-1, Tregs can express the checkpoint molecule T cell immunoreceptor with Ig and ITIM domains (TIGIT). A recent study has described lower baseline levels of $TIGIT^+$ Tregs as predictive for irAE onset[152].

Further insights into the Treg gene profile in patients with adverse events were provided by Grigoriou et al.[161]. They identified a distinct gene signature in Tregs among patients with irAE, associated with proinflammatory pathways like IFN-γ-, IFN-α response, and TNF-α signaling[161]. However, they failed to demonstrate a difference in Treg frequencies when comparing irAE versus no irAE, but it should be considered that Grigoriou et al. performed PBMC analysis at a specific time point (third cycle of anti-PD-1 infusion), not during irAE[161].

Tregs play an important role in the immunosuppressive TME[76]. A lower number of these cells has been associated with an improved clinical outcome in melanoma patients and various other cancer types[76, 78]. Hence, the lower percentage of Tregs among patients experiencing irAE could also be attributed to the beneficial treatment outcomes observed in these patients, as confirmed by PFS analysis, which showed an improved ICI response among patients with irAE.

4.3 Changes in M-MDSCs are not associated with irAE onset

The role of monocytes in irAE development has been previously discussed in the literature. Previous studies indicated an association between elevated circulating monocytes and irAE onset[162, 163]. However, my analysis of routine blood tests investigating monocyte counts did not confirm this association.

In addition to Tregs, MDSCs contribute to the generation of an immunosuppressive TME. As mentioned earlier, MDSCs can apply their immunosuppressive activity through the expression of PD-L1, CD73, or the release of ROS and NO[53].

The role of M-MDSCs in tumor progression, particularly in melanoma, has been extensively studied. Recent publications have highlighted the association between increased MDSC frequencies and decreased OS or PFS[101, 164]. Furthermore, MDSCs have been observed to accumulate at baseline before ICI start in patients with tumor progression, suggesting that MDSCs accumulation might indicate resistance towards ICI therapy[101, 165].

The role of MDSCs in autoimmune disorders remains controversial and not fully understood. Autoimmune diseases are characterized by the activation of autoreactive $CD8^+$ T cells, expansion of $CD4^+$, Th1, and Th17 cells, along with the inhibition of Tregs[65]. Currently existing literature presents conflicting studies proposing disease-promoting and disease-preventing roles of MDSCs for various autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease[65, 166]. Some studies suggest that MDSCs mediate disease-limiting effects in autoimmunity by inducing Tregs or inhibiting Th17 cells[167, 168]. Paradoxically, other authors propose that MDSCs might promote autoimmunity through Th17 cell differentiation, which in turn fosters the release of cytokines like Interferon-γ and IL-17, known to be associated with the development of severe irAE[157, 167, 169, 170].

To date, only a limited number of studies have investigated the role of circulating MDSCs in irAE development. In the present study, I failed to detect any association between MDSC frequencies and irAE onset. Moreover, I found no differences in the expression levels of immunosuppressive molecules, namely PD-L1 and CD73, as well as ROS and NO, when comparing irAE versus no irAE. In line with my findings, Damuzzo et al.[171] also did not demonstrate any association between irAE and MDSC frequencies. However, a review published by McCrae et al.[172] revealed that an elevated number of total $CD33^{+}HLA\text{-}DR^{-}$ MDSCs might promote the occurrence of immune checkpoint inhibitor-associated thrombosis.

In the no irAE group, I observed an increase in M-MDSCs and ROS production during the treatment course, while CD73 expression levels on M-MDSCs tended to decrease among patients with irAE. These observations could be attributed to the improved treatment outcomes for patients with irAE, as MDSC accumulation and increased immunosuppressive activity were predominantly observed in non-responders[101, 164].

4.4 Role of immunosuppressive treatment on circulating immune cells

Immunosuppressive drugs, including corticosteroids and other agents, are commonly used in the management of irAE[115]. Existing literature suggests that corticosteroids have an effect on circulating immune cells, potentially counteracting the effects of ICI on the immune system[138]. While ICI enhance the activation of immune cells and proinflammatory cytokines, steroids suppress these mechanisms[138]. Moreover, concerns haven been raised whether immunosuppressive drugs might compromise the anti-tumor immune response, leading to the question about how corticosteroids or other immunomodulating drugs may influence the TME. Yet, it remains unclear whether the administration of these drugs could erase the anti-tumor effect of ICI, as existing data are controversial[173-175]. In the case of irAE, previous publications have shown that systemic steroids administered due to irAE management were not associated with worsened survival or tumor response[176, 177]. However, high doses of steroids correlated with shorter OS[178]. These findings support the hypothesis that the effect of steroids regarding ICI treatment efficacy may vary depending on the purpose of their administration[179].

Furthermore, Goodman et al.[138] stated that also the timing of steroid application is important, and steroid use at baseline or shortly after ICI initiation might be unfavorable.

In the present study, I investigated the changes of circulating M-MDSCs and Tregs during the administration of immunosuppressive drugs following irAE. My data suggested a tendency for increased M-MDSC frequencies following immunosuppressive treatment. However, this effect was not statistically significant, most likely due to the limited number of patients included in this study following immunosuppressive treatment. It is noteworthy to mention that I observed a strong expansion of M-MDSCs in one patient who was treated with a high dose of methylprednisolone at TP3. Similar to my findings, other publications have demonstrated corresponding results[180]. Wang et al.[180] investigated PMBCs of patients with multiple sclerosis before and after the administration of methylprednisolone and observed an association between the application of steroids and the expansion of MDSCs, precisely PMN-MDSCs rather than M-MDSCs. Moreover, the exogenous steroid dose positively correlated with the number of MDSCs[181] . In vitro experiments confirmed that supplementing dexamethasone for MDSC generation could increase the number and immunosuppressive function of in vitro-generated MDSCs[182]. This effect was not limited to glucocorticoids alone, as Cyclosporin A was also found to increase the number of MDSCs in coculture experiments[183].

Regarding Tregs, it was proposed that glucocorticoid treatment mediates anti-inflammatory effects via the induction of Tregs[184, 185].

Overall, I can conclude that the administration of corticosteroids could potentially modulate circulating immunosuppressive cell subsets like MDSCs. However, the impact of immunosuppressive drugs following irAE on the TME and towards ICI therapy efficacy remains incompletely understood.

4.5 Results of routine blood tests predicting irAE onset

The investigation of changes in circulating blood counts to predict irAE development is of significant interest. Chennamadhavuni et al.[163] reviewed higher baseline absolute lymphocyte, absolute monocyte, and absolute eosinophil counts as biomarkers indicating irAE onset. Moreover, other publications have revealed an increased absolute lymphocyte count two weeks following ICI therapy initiation as a blood-based biomarker predicting irAE occurrence[186].

Additionally, a low neutrophil-to-lymphocyte ratio (NLR) was more frequently observed among patients with irAE[163]. An observational study also demonstrated an increase in the NLR during irAE[187].

In line with these data, I found an increased number of circulating lymphocytes at baseline in patients with irAE compared to patients without irAE. However, regarding other leukocyte subsets such as monocyte and eosinophil counts, I could not verify an association with irAE development, possibly due to the limited number of patients enrolled in the study.

Importantly, many reviews and publications focus only on baseline blood analysis, whereas blood count analysis at different time points and during irAE onset, as demonstrated in the present study, are currently lacking.

In addition to peripheral blood counts, CRP levels and LDH serum levels were determined during routine blood tests. Elevated LDH levels are usually found in pathological conditions such as hemolysis, liver disease, skeletal muscle disease, or various cancer types[188]. A high serum LDH in cancer patients could be explained due to a shift in glucose metabolism in cancer cells towards elevated glycolytic activity and tumor necrosis[189]. In particular, LDH is used as an established biomarker for routine follow-up in melanoma patients. CRP is typically considered as an unspecific inflammatory marker, and higher CRP levels have been associated with an unfavorable treatment response as well as shorter survival in melanoma patients[190].

My results showed an association of irAE development with increased serum LDH. Furthermore, the data revealed elevated CRP levels during irAE. These findings are in contrast with the improved PFS observed among patients with irAE and the role of LDH and CRP as biomarkers predicting tumor burden and survival rates[191].

The longitudinal study by Husain et al.[192] supports my results on increased CRP and LDH levels among patients with irAE. Particularly, an elevated LDH level was found just before irAE onset, and increased CRP and IL-6 levels were detected during the peak of adverse events[192]. Notably, severe irAE were reported to correlate with high LDH levels[193]. However, other studies which only considered baseline LDH measurements failed to detect any association between irAE and elevated LDH levels[162, 194].

Altogether, I can conclude that LDH and CRP may not serve as reliable biomarkers indicating tumor burden at the time point of irAE.

4.6 Strengths and limitations

The present study has several strengths.

First, the study provides a longitudinal analysis demonstrating dynamic immunological changes of circulating immune cells in patients with and without irAE. Here, the study includes different time points for analysis: before ICI initiation, during ICI treatment, at the onset of adverse events, and during immunosuppressive treatment. In contrast, most published papers only consider baseline measurements or single time point analyses.

Second, all patients received ICI at the Skin Cancer Center, University Medical Center Mannheim, and underwent regular examinations for upcoming irAE. Thus, the patients' records regarding the treatment course and irAE should be comprehensive.

Third, staging examinations were discussed in a multidisciplinary tumor board and confirmed by a multidisciplinary team of clinicians.

Fourth, I performed whole PBMC analysis of one patient within one day to generate comparable data for each patient.

However, my study also presents several limitations.

First, I need to consider the heterogenous study population. In particular, the study included different irAE entities as well as various treatment regimens. This could be an important factor because several studies suggest that some irAE arise through exclusive mechanisms[108, 195, 196]. For instance, vitiligo, a cutaneous autoimmune disorder, is described as an organ-related and cancer-specific irAE, which is preferentially found in melanoma patients[195]. The underlying mechanism, known as epitope spreading, hypothesizes that vitiligo arises through cross-reactivity by the expression of shared epitopes in tumor cells and healthy melanocytes[195]. Another example is immune-related myocarditis, which can also develop through the presence of shared antigens[196]. An agent-specific mechanism of irAE development is reported for anti-CTLA-4-antibody-related hypophysitis, probably induced by direct binding of anti-CTLA-4 antibodies[108].

Second, my study is limited by the small sample size. Hence, I could not conduct further subgroup analyses among different treatment regimens, treatment agents, or irAE entities due to the limited sample size and heterogenous study population. Future studies should address these above-listed limitations to identify immunological characteristics and mechanisms among different subgroups.

Third, this study may be biased by the retrospective study design, as the time points for FACS analysis were selected retrospectively. Unfortunately, PBMCs from several time points, especially TP 0, were missing, and I cannot provide a complete longitudinal analysis from TP 0 to TP 2 for all patients. Additionally, due to the retrospective study design, I missed identifying patients with pre-existing circulating antibodies. This is important because multiple analyses have shown that the presence of autoimmune disease may be associated with an increased risk for irAE onset[197, 198]. Associated irAE include the onset of new autoimmune disorders or the development of flare-ups of already existing autoimmune diseases.

Moreover, the presence of pre-existing autoantibodies may predispose to develop irAE. Patients without any sign of disease activity for autoimmune disorders who were positive for rheumatoid factor before ICI initiation showed an increased risk of experiencing irAE[199]. Corresponding results were published for the development of thyroiditis, where the presence of pre-existing anti-thyroglobulin antibodies and anti-thyroid peroxidase antibodies was associated with the development of immune-related thyroiditis[132].

4.7 Conclusion

Taken together, the present study revealed an enhanced treatment outcome among patients experiencing irAE compared to those without irAE as demonstrated by PFS analysis.

Furthermore, this findings on Tregs and activated T cells suggest that PBMC analysis using flow cytometry holds promise as a potential approach for irAE monitoring. Particularly, I observed dynamic alterations in immune cell subsets, especially during irAE onset. I identified a distinct T cell profile during irAE, characterized by a diminished proportion of Tregs and elevated frequencies of certain activated T cell subsets.

However, despite these findings, my attempts to identify baseline markers predicting adverse event development before ICI administration were unsuccessful.

Moreover, this immune monitoring study did not show conclusive results regarding M-MDSCs and their immunosuppressive capacity (PD-L1, CD73, ROS and NO) as reliable markers predicting irAE occurrence. Instead, these findings may represent improved tumor control among patients with irAE.

Nevertheless, my data revealed a tendency towards M-MDSC expansion following immunosuppressive treatment after irAE, notably after the application of high dose corticosteroids.

5 SUMMARY

Immune checkpoint inhibitors (ICI) represent a remarkable breakthrough in melanoma treatment. The therapy could considerably improve treatment outcomes, particularly for advanced melanoma patients. However, despite the great success of ICI therapy, approximately 30-60% of patients experience immunological side effects, known as immune-related adverse events (irAE). IrAE can be triggered by an overactivation of the immune system following ICI treatment. These adverse events can sometimes be fatal, leading to lasting organ damage, the requirement for systemic immunomodulatory treatment, and even treatment discontinuation. Therefore, biomarkers capable of predicting irAE onset or identifying patients at risk of experiencing irAE are crucial for effective diagnosis and management.

IrAE development might be attributed to an imbalanced immune system, which can be mediated by increased T cell activity or a loss of function of immunosuppressive cell subsets like regulator T cell (Tregs).

In the present study, I aimed to establish an immune profile in the peripheral blood of melanoma patients associated with irAE onset. For this purpose, I conducted routine laboratory tests and flow cytometry analysis of peripheral blood samples from 31 melanoma patients treated with anti-PD-1 monotherapy or anti-PD-1-/anti-CTLA-4 combination therapy. I investigated the activation status of T cells and the role of immunosuppressive subsets like Tregs and monocytic myeloid-derived suppressor cells (M-MDSCs) in patients with and without irAE. My analysis included different time points: before ICI start, during ICI treatment, at the onset of irAE, and during immunosuppressive treatment to manage irAE.

Overall, I observed a significantly improved progression-free survival (PFS) among patients with irAE. Additionally, I demonstrated an activation of $CD8^+$ T cells indicated by an upregulation of the early activation marker CD69, and an increased frequency of activated $CD4^+$ T cells ($CD4^+CD25^+FOXP3^-$) during irAE. Furthermore, I revealed a decrease in Tregs during irAE occurrence. Moreover, lower frequencies of Tregs correlated with more severe adverse events.

Another aim of this study was to evaluate the impact of immunomodulatory drugs following irAE on circulating immune cell subsets. Here, I observed that the number of M-MDSCs and Tregs tended to be elevated during immunosuppressive treatment.

Analysis of routine blood laboratory tests found increased LDH and CRP serum levels during adverse events.

Taken together, the present study identified that certain activated T cell subsets and the decrease of Tregs may lead to an imbalanced immune homeostasis, which could potentially promote the occurrence of irAE.

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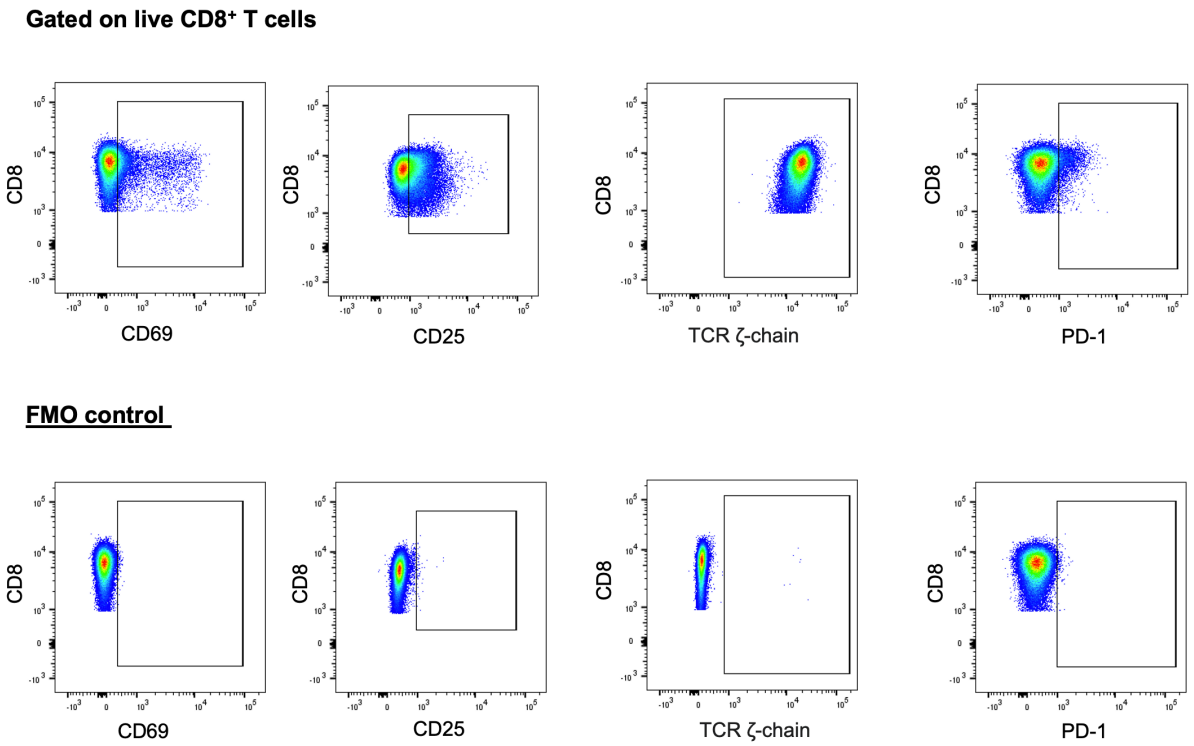
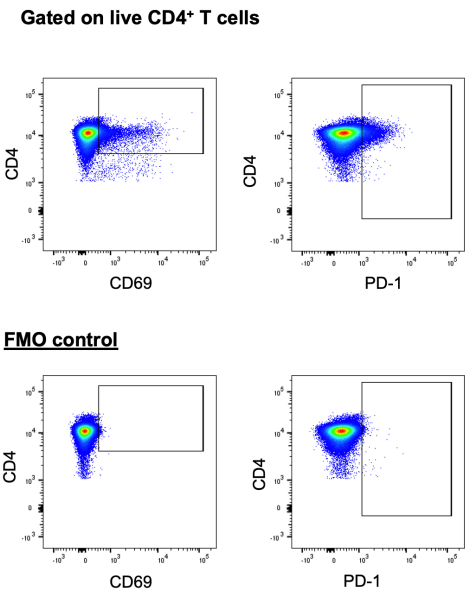
7 APPENDIX

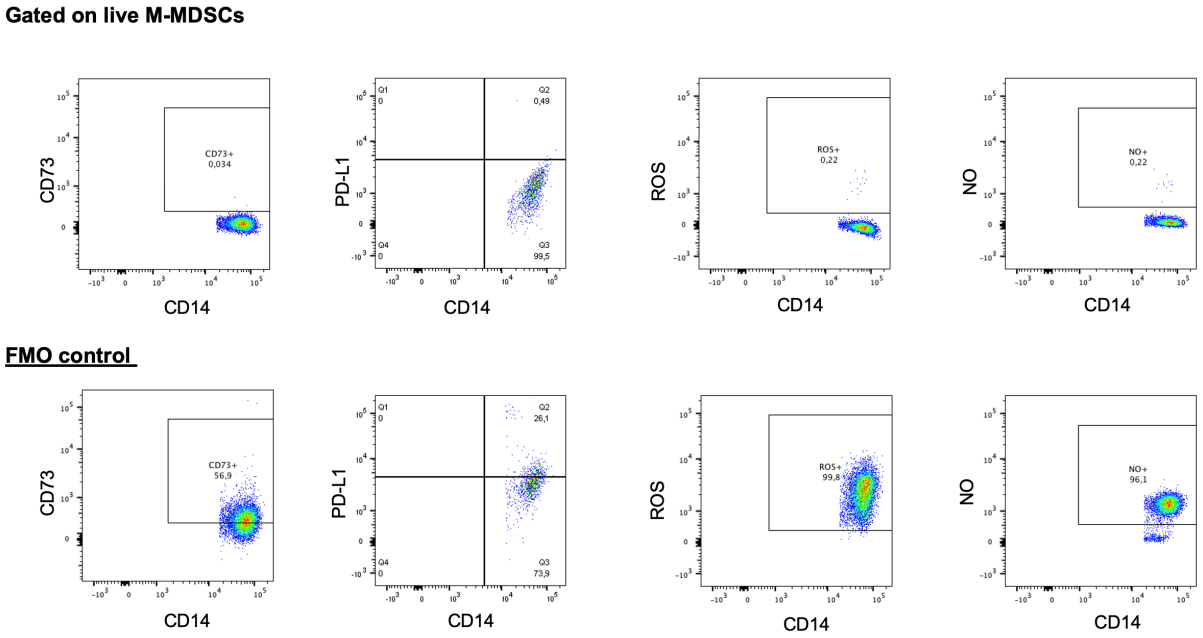


Supplementary Figure 1. Representative dot plots are shown to identify the expression levels of CD69, CD25, TCR ζ-chain and PD-1 on $CD8^+$ T cells according to the respective FMO control. Live $CD8^+$ T cells were identified after exclusion of doublets, debris, and dead cells, followed by CD3 gating to discriminate all T cells.



Supplementary Figure 2. Gating strategy for $CD4^+CD69^+$ and $CD4^+PD\text{-}1^+$ T cells is displayed. After exclusion of doublets, debris and dead cells, the cells were gated for the T cell marker CD3.

The frequency of $CD4^+CD69^+$ and $CD4^+PD\text{-}1^+$ T cells was determined according to the corresponding FMO control.



Supplementary Figure 3. Representative dot plots showing the gating strategy of $PD\text{-}L1^+$an $CD73^+$ M-MDSCs as well as ROS and NO production by M-MDSCs according to the respective FMO control.

8 CURRICULUM VITAE

PERSONAL DETAILS

Name:	Lepper, Alisa Helen
Date of birth:	02.02.1998
Place of birth:	Gießen

FORMAL EDUCATION

2008 – 2016	High school: Landgrafs-Ludwigs-Gymnasium Gießen
Jun. 2016	High school diploma (Abitur)

HIGHER EDUCATION

WS2016/17	Start medical studies at the Medical Faculty Mannheim, University Heidelberg
Sept. 2018	First state examination (Erster Abschnitt der Ärztlichen Prüfung, M1)
Oct. 2022	Second state examination (Zweiter Abschnitt der Ärztlichen Prüfung, M2)
Nov. 2023	Third state examination (Dritter Abschnitt der Ärztlichen Prüfung, M3)

9 LIST OF OWN PUBLICATIONS

Lepper A, Bitsch R, Ozbay Kurt FG, Arkhypov I, Lasser S, Utikal J, et al. Melanoma patients with immune-related adverse events after immune checkpoint inhibitors are characterized by a distinct immunological phenotype of circulating T cells and M-MDSCs. Oncoimmunology. 2023;12(1):2247303.

Ozbay Kurt FG, **Lepper A**, Gerhards C, Roemer M, Lasser S, Arkhypov I, et al. Booster dose of mRNA vaccine augments waning T cell and antibody responses against SARS-CoV-2. Front Immunol. 2022;13:1012526.

Arkhypov I, Ozbay Kurt FG, Bitsch R, Novak D, Petrova V, Lasser S, et al. HSP90alpha induces immunosuppressive myeloid cells in melanoma via TLR4 signaling. J Immunother Cancer. 2022;10(9).

Bitsch R, Kurzay A, Ozbay Kurt F, De La Torre C, Lasser S, **Lepper A**, et al. STAT3 inhibitor Napabucasin abrogates MDSC immunosuppressive capacity and prolongs survival of melanoma-bearing mice. J Immunother Cancer. 2022;10(3).

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