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ORIGINAL RESEARCH

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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

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ABSTRACT

Treatment with immune checkpoint inhibitors (ICIs) has improved the prognosis of melanoma patients. However, ICIs can cause an overactivation of the immune system followed by diverse immunological side effects known as immune-related adverse events (irAE). Currently, the toxicity of irAE is limiting the usage of ICIs. Here, we studied circulating monocytic myeloid-derived suppressor cells (M-MDSCs) and T cells in course of irAE after the ICI therapy. Our longitudinal study involved 31 melanoma patients with and without adverse events during anti-PD-1 monotherapy or anti-CTLA-4/PD-1 combination therapy. Peripheral blood samples were analyzed before ICI start, during ICI treatment, at the time point of irAE and during immunosuppressive treatment to cure irAE. We observed an enhanced progression-free survival among patients with irAE. In patients with irAE, we found an upregulation of CD69 on $CD8^+$ T cells and a decreased frequency of regulatory T cells (Tregs). Moreover, lower frequencies of Tregs correlated with more severe side effects. Patients treated with immunomodulatory drugs after irAE manifestation tend to show an elevated number of M-MDSCs during an immunosuppressive therapy. We suggest that an activation of $CD8^+$ T cells and the reduction of Treg frequencies could be responsible for the development of irAE.

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Introduction

Since immune checkpoint inhibitors (ICIs) such as anti-PD-1 and anti-CTLA-4 have been approved for metastatic melanoma therapy, the prognosis of stage III and IV melanoma patients could be remarkably improved.[1,2] This treatment targets the key regulatory molecules cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death (PD-1) which are involved in the attenuation of T cell function.[3,4]

ICI can trigger autoimmune reactions termed immune-related adverse events (irAE).[5] Preferentially, adverse events develop in the skin, the gastrointestinal tract, the endocrine system, and the liver.[6,7] In some cases, adverse events are life-threatening or require a premature treatment discontinuation and the application of immunomodulatory drugs.[8] Response toward the ICI therapy is achieved by T cell activation and by modulating the tumor microenvironment (TME) with immunosuppressive cells such as regulator T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Tregs are indispensable for immune homeostasis. Their depletion might facilitate autoimmune diseases, whereas the expansion may promote tumor progression.[9] The role of MDSCs in tumor progression is well studied in several cancer entities, particularly in melanoma.[10–13] These cells exert an immunosuppressive effect on T cells and NK cells via several mechanisms, including the programmed cell death-ligand 1 (PD-L1) and the generation of adenosine by ectonucleotidase CD73.[10]

To evaluate the response to the ICI treatment, several markers such as the S100 protein, lactate dehydrogenase (LDH), C-reactive protein (CRP) and various leukocyte subsets were used as predictive biomarkers.[13–15]

A variety of different mechanisms is known to be involved in the development of irAE such as genetic predisposition,[16] autoantibodies,[17] changes in cytokine production[18] and microbiome.[19] However, the role of MDSCs in irAE development is poorly investigated.

In our study, we performed an analysis of T cells and MDSCs as well as some blood biomarkers (including S100, CRP and different leucocyte subsets) in melanoma patients treated with ICI, addressing the question if they could be associated with irAE onset. We observed a reduction in the frequency of Tregs and an enrichment of activated $CD69^+CD8^+$ T cells during irAE. Analyzing monocytic MDSCs (M-MDSCs), we found no

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significant changes in their frequency and immunosuppressive pattern in patients with irAE.

Materials and methods

Patients and sample collection

Peripheral blood samples from melanoma stage III and IV patients treated with ICIs were collected between January 2018 and September 2021. All patients received ICI therapy at the Skin Cancer Center (University Medical Center Mannheim, Germany). This study was approved by the local ethics committee (2010-318N-MA) and all participants gave their informed written consent prior to analysis. 16 patients were treated in an adjuvant and 15 patients in a palliative setting. The palliative treatment involved either a monotherapy with 480 mg nivolumab every 4 weeks or 200 mg pembrolizumab every 3 weeks. For some patients with metastatic melanoma, a combination therapy with 1 mg/kg body weight nivolumab and 3 mg/kg body weight ipilimumab every 3 weeks (≤4 cycles) followed by nivolumab monotherapy was applied. The adjuvant treatment setting included 3 mg/kg body weight nivolumab every 3 weeks or 200 mg pembrolizumab every 3 weeks. Peripheral blood mononuclear cells (PBMCs) were isolated from lithium heparin blood samples by density gradient centrifugation with Biocoll (Biochrom) followed by a cryopreservation with X-VIVO medium (Lonza) enriched with 30% fetal bovine serum (FBS, Pan Biotech) as well as with 10% DMSO (Thermo Fisher) and stored in liquid nitrogen.

Clinical assessment

Routine blood analysis and clinical examinations were regularly conducted to check for upcoming irAE. According to the clinical observations, the patients were assigned either to irAE or no irAE groups, respectively. Time points for sample analysis were chosen retrospectively based on the reported irAE. The severity of irAE was assessed by the Common Terminology Criteria for Adverse Events 5.0 (CTCAE). Different time points (TP) were included for our analysis: TP 0 - before the ICI therapy start; TP 1 - before irAE occurred; TP 2 - during irAE onset; TP 3 - during immunosuppressive therapy to treat adverse events. For the no irAE group, at least 2 time points during ICI therapy were analyzed. The TP 1 and the TP 2 in no irAE group were adjusted according to the calculated median time of TP 1 and TP 2 for 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).

The treatment responsiveness and tumor relapse were evaluated by contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography-CT (PET-CT) every 12 weeks after ICI start. Patients under palliative ICI were characterized regarding their overall response according to the iRECIST criteria as responders or non-responders based the best overall response during the considered period. Complete response (CR), partial response (PR) and stable disease (SD) were considered as a therapy response, whereas progressive disease (PD) was classified as a non-response.

Flow cytometry

PMBCs were thawed in Benzonase Nuclease (Sigma-Aldrich) /X-VIVO medium (Lonza), washed with RPMI medium and stained with the following fluorochrome-conjugated antibodies: anti-CD14 – PerCP-Cy5.5 (BD, clone MΦP9), anti-HLA-DR – V500 (BD, clone G46–6), anti-PD-L1 – PE-Cy7 (BD, clone MIH1), anti-CD73 – BV605 (Biolegend, clone AD2), anti-CD3 – V500 (BD, clone SP34–2), anti-CD4 – APC-Cy7 (BD, clone RPA-T4), anti-CD8 – APC (BD, clone RPA-T8), anti-CD69 – PE-Cy7 (BD, clone FN50), anti-PD-1 – PerCP-Cy5.5 (BD, clone EH12.1) and anti-CD25 – BV421 (BD, clone M-A251). Dead cells were discriminated with the Fixable Viability Stain A×700 (BD). To reduce unspecific antibody binding, FcR Blocking Reagent (Miltany) was added. Analysis of the intracellular marker FOXP3 – Alexa 488 (BD, clone 259D/C7) were conducted using the FOXP3/Transcription Factor Fixation/Permeabilization kit (ThermoFisher). Acquisition was performed by 10-color flow cytometry using BD FACSLyric with FACSuite software (BD Biosciences). FlowJo V 10 software (BD Biosciences) was used to analyze at least 10^6 events. Positively stained cells were gated according to the fluorescence minus one (FMO) control.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (Version 8.1.2). The data were evaluated for a Gaussian distribution by the Shapiro-Wilk test. Normally distributed variables were analyzed by a paired or an unpaired two-sample t-test. The Wilcoxon signed-rank test was used to compare paired samples and the Mann-Whitney U-test for unpaired data respectively. Correlation analysis was performed by the Pearson correlation coefficient followed by two tailed p value. Progression-free survival (PFS) was displayed as a Kaplan-Meier curve and analyzed by the log-rank test. For PFS, the time between therapy start until tumor relapse was examined. If a patient showed no sign of tumor progression, the data was censored regarding the date of last contact. Fisher's exact test was used to compare the percentage of responders vs. non-responders (palliative treatment group) and relapse vs. relapse-free patients (adjuvant treatment group).

Results

Patient characteristics and irAE

The characteristics of the 31 stage III and IV melanoma patients, including 17 patients with irAE (55%) and 14 patients without irAE (45%), are displayed in Table 1. An anti-PD-1 monotherapy was administered in 22 patients (71%); 9 patients (29%) received a prior combination therapy consisting of ≤ 4 cycles anti-CTLA-4/PD-1 inhibitors. Table 2 summarizes all detected irAE entities during the investigated period. Two patients experienced more than one irAE entity. The majority of irAE was mild to moderate, whereas severe irAE (grade >2)

Table 1. Clinical characteristics of patients following ICI with 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	

Table 2. Observed immune-related adverse events after ICI treatment.

	Reported events		
Toxicity	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

Adverse events are classified according to the CTCAE 5.0 grading criteria. Some patients experienced more than one immune-related adverse event.

were reported in 5 cases (24%). All severe side effects (grade >2) appeared after a prior combination therapy with anti-CTLA-4/PD-1 inhibitors. Frequently reported irAE in our study involved colitis (4 cases, 19%), thyroiditis (4 cases, 19%), hypophysitis (3 cases, 14%) and hepatitis (3 cases, 14%).

Improved PFS and therapy outcome among patients with irAE

Patients with irAE displayed a significantly improved PFS compared to the no irAE group (Figure 1a). Patients treated in a palliative setting displayed an elevated PFS (Supplementary Figure S1A), whereas the adjuvant treated patients showed no significant difference in PFS when comparing irAE vs. no irAE group (Supplementary Figure S1B). Moreover, 78% palliative treated patients with irAE responded to the therapy, whereas in the no irAE group, only 33% of patients were responders ($p = 0.14$) (Figure 1b). Regarding the adjuvant treatment setting, there was no significant difference in the number of patients with tumor relapse between both groups (Figure 1c).

IrAE onset was accompanied by diminished Treg frequency and an elevated frequency of CD69$^+$CD8$^+$ T cells

We performed the measurement of T cell subsets by flow cytometry at different time points during ICI treatment as displayed in Figure 2a. First, we studied the frequency of circulating Tregs defined as CD4$^+$CD25$^+$FOXP3$^+$ cells (Figure 2b). We found a significant reduction in the frequency of Tregs during irAE, whereas the patients without adverse events displayed no changes in Tregs during the ICI treatment (Figure 2c). Furthermore, the frequency of Tregs in irAE group was significantly lower than that in no irAE group measured at the corresponding time point (TP 2) (Figure 2c). In addition, decreased Treg frequencies demonstrated a weak but statistically significant correlation ($p = 0.02$) with more severe side effects or no irAE development (Figure 2d).

Next, we analyzed the status of CD4$^+$ and CD8$^+$ T cells by flow cytometry (Suplementary Figure S2). We found an increase in the frequency of CD69$^+$CD8$^+$ T cells among total CD8$^+$ cells during irAE (TP 2) as compared to the previous time point (TP 1) (Figure 3a). Moreover, the frequency of CD69$^+$CD8$^+$ T cells during irAE showed a tendency for the elevation as compared to the corresponding time point (TP 2) in patients without irAE (Figure 3a). Measuring CD4$^+$ T cells, we observed no upregulation of CD69 in patients with or without adverse events (Figure 3b). Furthermore, the severity of irAE did not significantly correlate with an increased frequency of CD69$^+$CD8$^+$ T cells (Figure 3c).

To further characterize the activation status of CD8$^+$ and CD4$^+$ T cells, we measured the expression of CD25. Our findings showed a significant increase in the frequency of CD8$^+$CD25$^+$ activated T cells during ICI treatment for patients with irAE and without irAE (Figure 3d). Investigating CD4$^+$CD25$^+$FOXP3$^-$ activated T cells, we found an elevation of these cells in the irAE group after the onset of the adverse events (TP 2) as compared to the previous time point (TP 1) (Figure 3e).

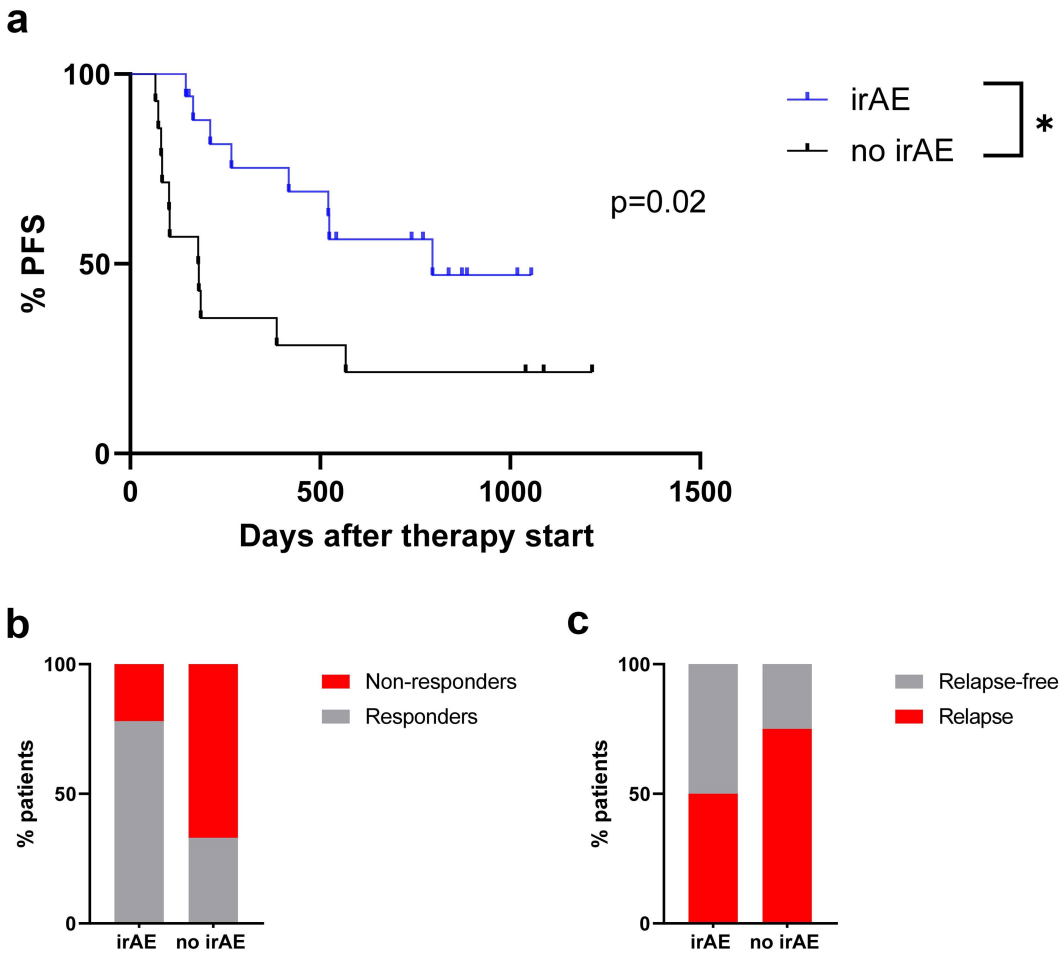

Figure 1. Clinical outcome of the ICI therapy of melanoma patients with and without irAE. (a) PFS of patients is displayed as a Kaplan–Meier curve. (b) the percentage of responders and non-responders among palliative treated patients ($n = 15$) with and without irAE. Responders were defined as CR, PR, and SD. (c) the percentage of adjuvant treated patients ($n = 16$) showing tumor relapse and relapse-free in the irAE and no irAE group. *$P < .05$.

In addition, we measured a similar low frequency of $CD8^+PD\text{-}1^+$ and $CD4^+PD\text{-}1^+$ T cells among total $CD8^+$ and $CD4^+$ T cells, respectively, in ICI-treated patients both with and without irAE (Figure 3f,g). Supplementary Figure S3 displays a detailed analysis of Tregs and T cell characteristics for adjuvant and palliative treated patients.

Analysis of M-MDSC in patients with and without irAE

Analyzing circulating M-MDSCs that we defined as $CD14^+HLA\text{-}DR^{low/-}$ cells (Figure 4a), we found no changes in their frequencies as well as in the expression of PD-L1 and CD73 upon the irAE onset (TP 2) as compared to the TP 1 (Figure 4b-d, Supplementary Figure S4). In addition, no significant difference in M-MDSC frequencies and immunosuppressive pattern were detected when comparing irAE vs. no irAE groups (Figure 4b-d). An analysis of M-MDSC frequency in distinct patients from the adjuvant and palliative treatment groups is demonstrated in Supplementary Figure S5.

Next, we investigated the impact of immunosuppressive therapies on circulating immune cells. In our study cohort, we analyzed PMBCs of five patients during immunosuppressive treatment (TP 3) and compared the results with those before such treatment (TP 2). Four patients were treated with methylprednisolone at TP 3 (three patients with 10–30 mg and one patient with a high dose of 120 mg). The fifth patient received 1000 mg mycophenolate mofetil and 15 mg hydrocortisone during TP 3. After an immunosuppressive treatment, the number of circulating M-MDSCs and Tregs tend to increase in almost all patients (Figure 4e, Supplementary Figure S6A). Investigating the immunosuppressive pattern of M-MDSC at TP 2 and TP 3 characterized by PD-L1 and CD73, we found that the level of their expression showed a tendency to decrease at TP3 (Supplementary Figure S6B, C). Interestingly, one patient, who received a high dose of methylprednisolone to manage an immune-related hepatitis, displayed a massive expansion of M-MDSCs at TP 3 (Supplementary Figure S7).

Analysis of leucocyte subsets as well as the levels of LDH and CRP

Absolute counts of circulating leucocyte subsets were measured during ICI therapy and irAE. In the no irAE group, the absolute number of leucocytes decreased after ICI start, whereas it remained stable among patients with irAE during therapy and at the time of irAE (Supplementary Figure S8A).

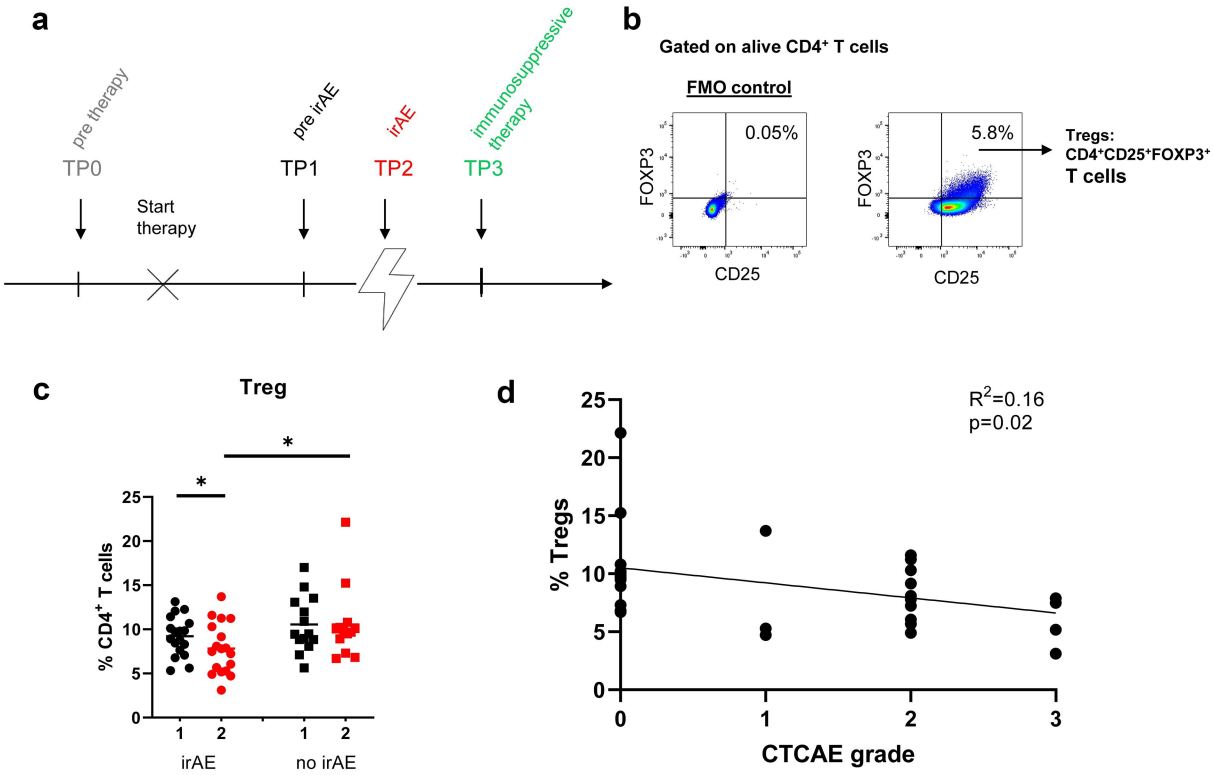

Figure 2. Characterization of Tregs in melanoma patients during irAE onset. (a) Peripheral blood samples were collected and PBMCs were isolated at indicated time points: Time point TP 0 - before ICI therapy start, TP 1 - before irAE onset (irAE group)/during ICI therapy (no irAE group); TP 2 - at the time of irAe/during ICI treatment (no irAE group); TP 3 - during immunosuppressive therapy to treat irAE. (b) Representative dot plots for $CD4^+CD25^+FOXP3^+$ Tregs. (c) results are presented as the frequency of circulating Tregs among live total $CD4^+$ T cells from patients with irAE ($n = 18$) and without irAE ($n = 13–14$) (d) the frequency of Tregs within total $CD4^+$ T cells is plotted against the CTCAE grading. No irAE is defined as CTCAE grade 0. For patients with more than one irAE at the same time, the highest examined CTCAE grade is chosen. *$P < .05$.

The absolute lymphocyte count at baseline was significantly higher in the irAE group than in no irAE group. We observed a decrease of lymphocyte numbers. During treatment and during irAE (Supplementary Figure S8B). For eosinophils, we detected an elevation of absolute eosinophils during treatment in no irAE group. However, we could not find a difference of eosinophils comparing irAE vs. no irAE (Supplementary Figure S8C). Regarding the neutrophil subpopulation, we observed a decrease after therapy start among patients without irAE (Supplementary Figure S8D). The monocyte subset did not show any major changes during treatment and at the time of irAE (Supplementary Figure S8E).

Furthermore, we analyzed serum LDH and CRP levels at the indicated time points. We found that LDH was significantly elevated during irAE compared to the previous time points. Correspondingly, comparing the time point of irAE onset and time point 2 in no irAE group, we observed a significantly higher level of LDH among patients with irAE (Supplementary Figure S8F). The time point of the IRAE was also associated with a significant elevated CRP level (Supplementary Figure S8G).

Discussion

Our clinical data suggest that the irAE onset is an important factor to predict treatment response toward ICI in patients treated in a palliative setting. These observations are in agreement with recent publications, showing an elevated PFS and overall survival in stage III/IV melanoma patients with irAE.[20,21] We did not observe any association between the therapeutic agent and the irAE grading. This might be explained by a low number of patients involved in our study. However, all severe irAE appeared after the application of combination therapy.

Immune cells, including T lymphocytes[22,23] and monocytes[24,25] have been reported as a major hallmark for the irAE pathology. However, previous studies did not investigate immunological changes during irAE occurrence. Here, we performed a comprehensive analysis of the phenotype of circulating T cell subsets and M-MDSCs in melanoma patients, including the time point of adverse event manifestation.

Investigating $CD4^+CD25^+FOXP3^+$ Tregs, we could demonstrate that irAE occurrence was associated with a lower frequency of these cells. Moreover, decreased Treg frequencies showed a weak but statistically significant correlation with the irAE severity or the absence of irAE. Similar findings were described earlier in patients with immune-related colitis following ICI who had a lower frequency of Tregs at the baseline compared to patients without irAE.[26] Another paper reported that patients with thymic epithelial tumor and non-small cell lung cancer, showed a strong increase of $CD4^+CD127^{lo}CD45RA^-FOXP3^{hi}$ effector Tregs during anti-PD-1 therapy.[27] However, patients developing severe irAE displayed a lower increase of these cells

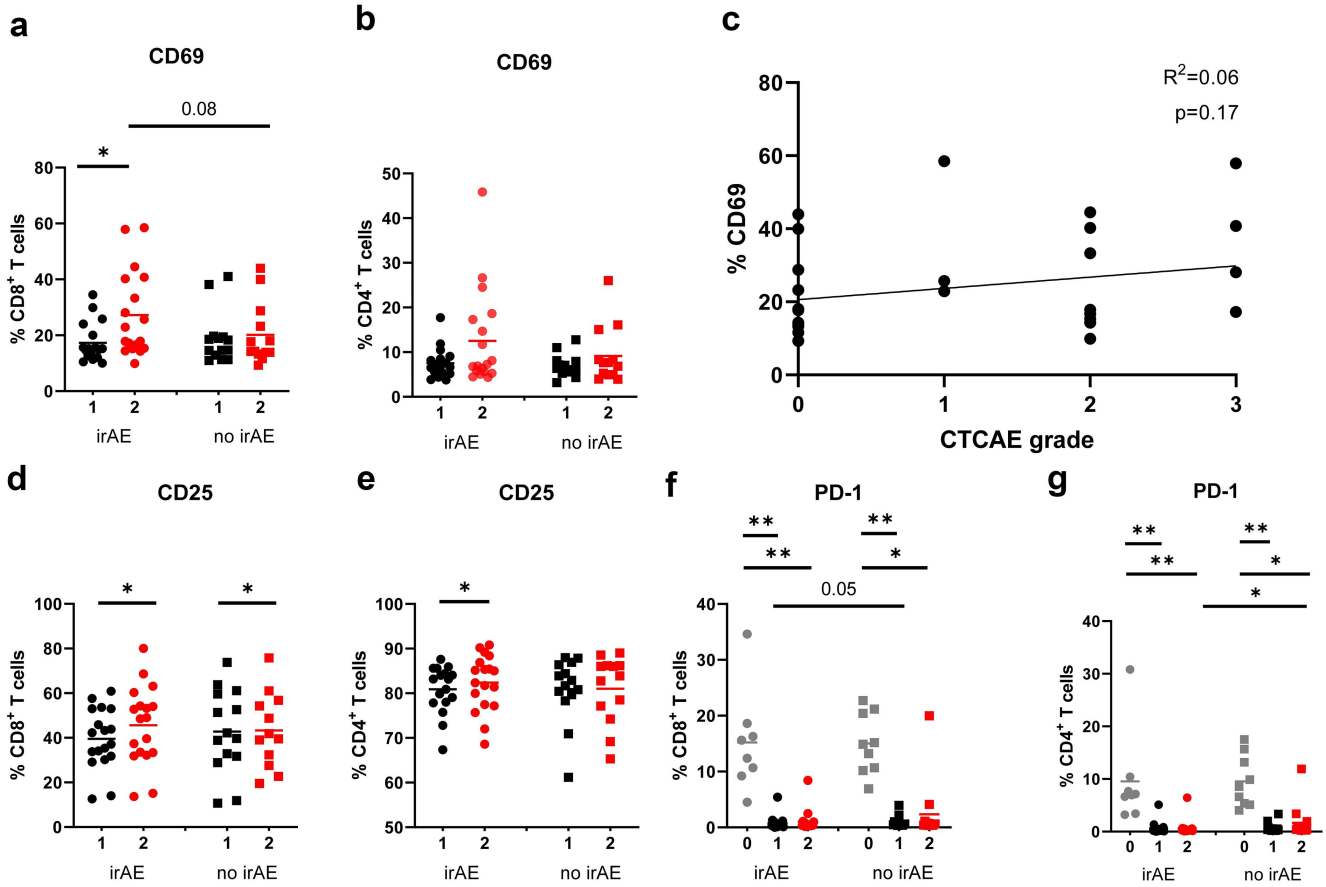

Figure 3. Analysis of $CD8^+$ and $CD4^+$ T cells in melanoma patients with and without irAE after ICI treatment. Peripheral blood samples were taken at indicated time points and measured by flow cytometry. Expression of CD69 on $CD8^+$ (a) and $CD4^+$ T cells (b) from patients with ($n = 18$) and without irAE ($n = 13–14$) is shown as the percentage of $CD69^+$ cells among the respective T cell subset. (c) the frequency of $CD69^+CD8^+$ T cells within total $CD8^+$ T cells is plotted against the CTCAE grading ($n = 31$). The correlation was evaluated by a linear regression analysis. Patients without observed irAE are defined as CTCAE grade 0. For patients with more than one reported irAE, the highest examined CTCAE grade is chosen. CD25 expression levels from patients with ($n = 17–18$) and without irAE ($n = 12–14$) and the PD-1 status in patients with adverse event ($n = 8–18$) vs. no irAE ($n = 9–14$) are shown as the percentage of $CD25^+$ cells (d, e) or $PD\text{-}1^+$ cells (f, g) among the respective T cell subsets. $^*P < .05$, $^{**}P < .01$.

throughout the treatment.[27] A reduction of Tregs in the irAE group induced by targeting co-inhibitory receptors CTLA-4 and PD-1 on these cells was shown to alter an immune homeostasis, leading to autoimmunity.[28] Tregs might be converted into dysfunctional immunosuppressive cells, which favor a destabilized phenotype accompanied by a loss of FOXP3 or lead to a fragile Treg phenotype still expressing FOXP3.[29] Moreover, immunotherapies are reported to promote this destabilized phenotype in Tregs.[29] However, the lower frequency of Tregs in our study among patients with adverse events could also be explained by the improved therapy outcome in the irAE group. In particular, a lower number of Tregs correlated with a beneficial clinical outcome in most tumor types, including melanoma.[30] Furthermore, Tregs as well as MDSCs might be involved in the prediction of ICI efficacy since these cells played a key role in the generation of immunosuppressive TME through the release of soluble molecules, hampering anti-tumor T cell functions.[31] It was demonstrated that the accumulation of functionally strong circulating MDSCs before the ICI therapy was typical for non-responders and in contrast to responders[11]. Moreover, an increased tumor infiltration of $CD8^+$ T cell was reported to be crucial for the successful ICI response.[32]

Patients with irAE were reported to show a clonal expansion of $CD8^+$ T cells.[33,34] Moreover, it was described an association of circulating activated T cells with the occurrence of irAE.[35] In accordance to these data, we found an enrichment of activated $CD8^+$ and $CD4^+$ T cells during irAE indicated by an upregulation of activation markers CD69 and CD25. The increase of CD69 expression has been identified in patients with various inflammatory diseases such as arthritis, autoimmune thyroiditis, or multiple sclerosis.[36] A stronger expansion of CD25 and CD69 on $CD8^+$ T cells in patients without irAE compared to patients experiencing musculoskeletal irAE was shown by Benesova et al.[37] In contrast to their analysis, we measured the changes of $CD25^+CD8^+$ as well as $CD69^+CD8^+$ T cells for particular time points: before irAE vs. during irAE.

Regarding PD-1 expression, we assume that PD-1 is not downregulated, but rather blocked by anti-PD-1 antibodies applied for the treatment of patients evaluated in our study.

We failed to detect any association between the frequency of circulating M-MDSC and the irAE onset. Similar results were reported by Damuzzo et al.[38] who compared MDSC subsets in patients experiencing irAE with those patients without irAE after the treatment with ipilimumab. Furthermore, we observed a tendency for an increased

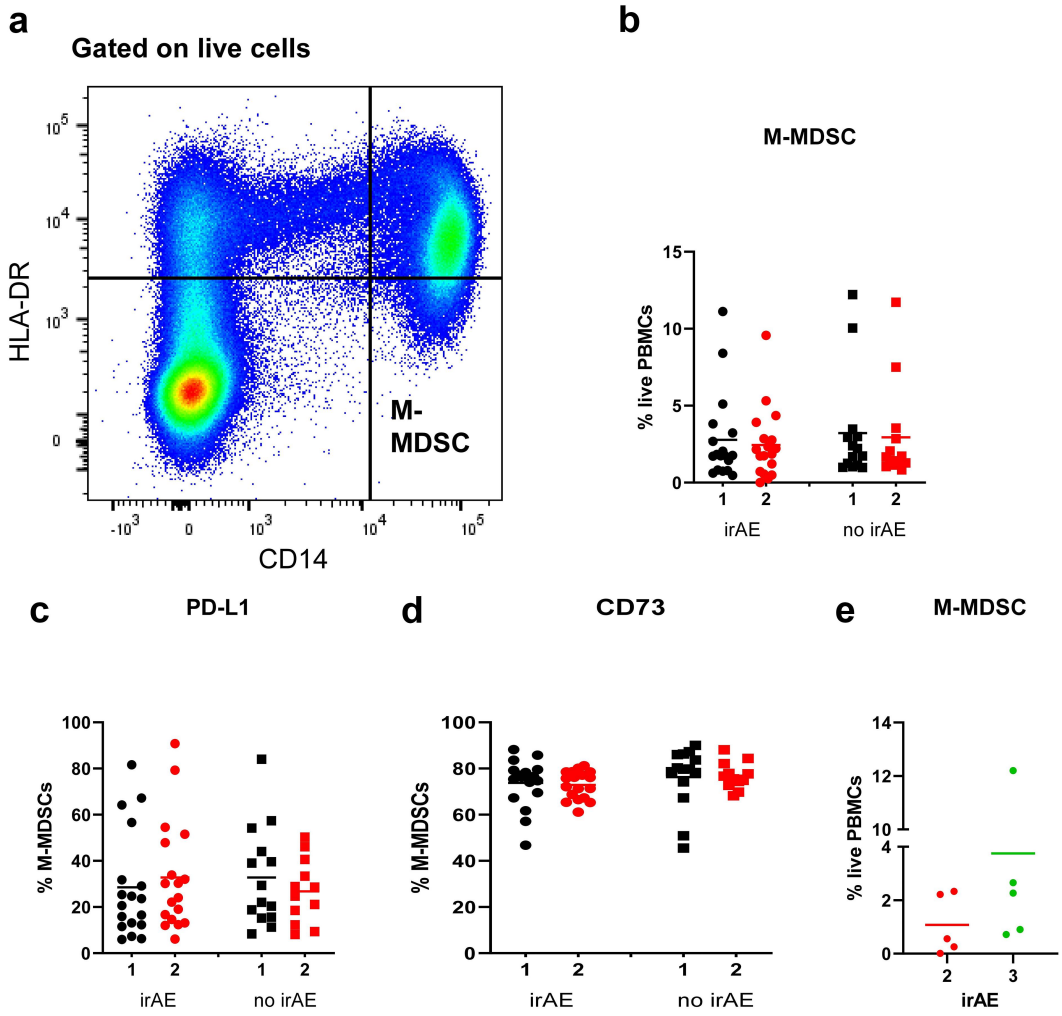

Figure 4. Circulating M-MDSCs in ICI-treated patients from irAE and no irAE groups. Peripheral blood samples were taken at indicated time points and analyzed by flow cytometry. (a) Representative dot plots for circulating $CD14^{+}HLA\text{-}DR^{low/-}$ M-MDSCs after exclusion of debris, doublets, and dead cells (b) frequencies of circulating M-MDSCs in patients with ($n = 18$) and without irAE ($n = 13$–14) after ICI therapy are presented as their percentage within live PBMCs. PD-L1 and CD73 expression on M-MDSCs in patients with ($n = 18$) and without irAE ($n = 12$–14) are shown as the percentage of $PD\text{-}L1^{+}$ (c) or $CD73^{+}$ cells (d) among the total MDSCs. (e) M-MDSC frequencies in patients with irAE (TP 2) and during the treatment with immunosuppressive drugs (TP 3).

number of M-MDSCs in most patients after the therapy with immunosuppressive drugs. This finding is in agreement with a recent publication reported an expansion of MDSCs in patients with multiple sclerosis after a therapy with methylprednisolone.[39]

Chennamadhavuni et al.[40] analyzed some biomarkers of irAE. Similar to our observations, they found that increased absolute lymphocyte counts at the baseline were associated with a higher risk of irAE development.[40] However, in contrast to this paper, we could not demonstrate that elevated absolute eosinophil and monocyte counts could be predictive for irAE.

The levels of LDH and CRP levels are usually applied as prognostic factors for melanoma therapy. In particular, augmented LDH and CRP levels were described to be associated with worse ICI response and shorter OS.[15] However, we observed increased LDH and CRP concentrations within the irAE group, especially during irAE onset. Other studies also reported elevated concentrations of LDH[41,42] as well as CRP and IL-6 in patients with irAE.[41] These findings of us and others may indicate that during irAE onset, both LDH and CRP levels could not be used as a predictive factor for ICI response.

Our study has several limitations. First, this includes a small patient cohort. Second, our study is characterized by a group of patients with multiple irAE types and different time points of adverse event onset. Many irAE entities are known to arise through different mechanisms. In particular, dermatologic adverse events could occur due to the presence of shared antigens in the melanoma lesion and the site of inflammation, whereas thyroid dysfunction might be associated with preexisting autoantibodies.[43] Future studies should consider above-mentioned limitations to specify the immunological profile of patients with certain irAE entities.

Altogether, our data support an important role of CD8 T cell activation and Treg reduction in the peripheral blood of melanoma patients treated with ICI for the development of irAE.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

A.L., R.B., J.U., V.U. designed the study. A.L., R.B. performed experiments and analyzed data. A.L., R.B., F.G.Ö.K., I.A., S.L., J.U., V.U. interpreted data and contributed to the discussion. J.U. provided clinical expertise. A. L., V.U. wrote the manuscript with input from all authors. All authors read and approved the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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