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Peripheral CD8 effector memory type 1 T-cells correlate with outcome in ipilimumab-treated stage IV melanoma patients

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Abstract

The role of the assessment of peripheral T-cell phenotypes in predicting overall survival (OS) after ipilimumab treatment is unclear. Here, we analyzed mononuclear cells in the blood before and at different time points during treatment with ipilimumab in 137 late stage melanoma patients. The proportions of baseline naïve and memory T cells were measured by flow cytometry and correlated with OS, with an emphasis on PD-1 expression. High frequencies (>13%) of CD8 effector-memory type 1 (EM1) T-cells at baseline correlated with longer OS ($p=0.029$) and higher clinical response rates ($p=0.01$). The frequency of these EM1 cells and the M category had independent impacts on OS (hazard ratio [HR]=1.5, $p=0.033$; and HR=1.9, $p=0.007$). In contrast, high baseline frequencies of late stage-differentiated effector memory CD8 cells (>23.8%) were negatively associated with OS ($p=0.034$) but did not correlate with clinical response. Following treatment, a decrease of CD8 PD1+EM1 cells from baseline to the time of the second drug dose and at later time points was strongly and consistently correlated with a high clinical response rate. Our observations thus suggest an important predictive role of baseline CD8 EM1 cells and changes in CD8 PD1+EM1 cells for clinical response of ipilimumab. Further validation of these biomarker candidates is warranted.

Keywords

melanoma; prognosis; ipilimumab; biomarker; effector memory cells

Introduction

Despite breakthroughs in efficacy of systemic treatments in the field of targeted therapy (1, 2) and immune checkpoint blockade (3–5) metastatic melanoma still accounts for the vast majority of deaths due to skin cancer. Ipilimumab, a monoclonal human IgG1 antibody inhibiting engagement of Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) was the first agent leading to improved overall survival (OS) in metastatic melanoma (3) and to a rate of approximately 20% long-term survivors (6). Only a subset of patients benefits from treatment but for responders, the duration may be long-lasting (3). Nonetheless, severe side effects can occur (6, 7).

CTLA-4 is expressed on CD4 and CD8 T-cells after initial activation (8) as well as constitutively on regulatory T-cells (Treg) (9). It is a key element in tolerance regulation and competes with a higher affinity than CD28 for binding with its ligands B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells (10–12). CTLA-4 ligation induces T-cell tolerance and anergy without the induction of cell death (13, 14). Physiologically, these interactions contribute to the maintenance of the equilibrium of T-cell reactivity against foreign pathogens but tolerance to self-antigens and thus protect normal tissues (13).

In melanoma, both CD4 T-helper (15) and CD8 cytotoxic T-cells (16) as well as those targeting melanoma associated antigens (17) or neo-antigens (18, 19) are able to attack cancer cells. Peripheral blood CD27+CD28+ CD8 effector cells have been associated with melanoma rejection mechanisms (20). Moreover, we recently found that frequencies of CD27+CD28+CD45RA– CD8 T-cells correlated positively with prognosis of stage IV melanoma patients (21). However, the detailed mode of action of ipilimumab and the impact of the differentiation or maturation state of immune cell compartments has not been fully elucidated. An increase in absolute lymphocyte counts (ALC) in the periphery is associated with a beneficial course of disease when administering ipilimumab (22, 23), although there are also some conflicting data on this issue (24). Disparate data have been reported for the role of Tregs on the outcome of ipilimumab treatment as well (25, 26). Ipilimumab engages antibody-dependent cellular cytotoxicity (ADCC)-mediated lysis of Tregs through non-classical monocytes, according to *ex vivo* experiments (27). Some studies suggest that the activation of human peripheral blood effector T-cells rather than inhibition of regulatory T-cells may contribute to therapeutic effects of ipilimumab (28, 29).

A likely direct mode of action of ipilimumab is enhanced anti-tumor function of T-cells after CTLA-4 blockade (30). A delayed increase of both CD4 and CD8 T-cell frequencies correlated with OS and response after the first dose of ipilimumab (31). In mice, it has been shown that CTLA-4 blockade has effects on the differentiation signature and function of CD8 T-cells (32–34). Recently, Felix et al. described an early increase in circulating central and effector memory T-cells in ipilimumab-treated melanoma patients showing disease control (35). A prominent surface marker that is mostly expressed by CD8 effector memory T-cells is Programmed cell death protein-1 (PD-1) (36). PD-1 on CD8 tumor infiltrating lymphocytes (TIL) has been shown to be used as a marker for tumor-reactive cells (37). Indeed, peripheral blood PD1+CD8 T-cells can also express neo-antigen recognizing T-cell receptors (TCR) (38). In view of these findings, we analyzed CD4 and CD8 differentiation signatures prior to and during the administration of ipilimumab to investigate whether the abundance of various effector memory T-cell subsets could identify patients that would experience positive outcome. Moreover, the role of PD-1 expression on OS associated differentiation phenotypes was investigated.

Materials and Methods

Patients

Seven sites provided clinical data and cryopreserved peripheral blood mononuclear cells (PBMC) from a total of 137 patients. Blood (baseline) was taken within 4 weeks prior to initiation of ipilimumab therapy. Additional PBMC samples after initiation of treatment were available at the time point of the 2nd dose (days 19–23 after 1st dose), the 3rd dose (days 40–44) and thereafter (>days 45). An individual increase or decrease of the respective parameter was defined by any change (independent of its magnitude) comparing the baseline value to that obtained either at the 2nd, the 3rd or after the 3rd cycle. Uveal or mucosal melanomas were excluded. Clinical responses were evaluated at the respective clinical center and categorized as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) according to immune-related response criteria (irRC) (39). The

best overall response rate (BORR) was defined as the proportion of patients experiencing CR or PR considering all tumor assessments from the start of ipilimumab treatment to the date of PD or start of a new systemic treatment (whichever occurred first). All patients gave their written informed consent for biobanking, use of biomaterials and clinical data for scientific purposes. This study was approved by the Ethics Committee, University Medical Center Tübingen (approval 524/2012B01).

Flow Cytometry

Cryopreserved PBMC samples were thawed and immediately stained with monoclonal antibodies against the markers of interest. A detailed staining protocol is described in our OMIP-20 panel (40). Antibodies used in this study are listed in Table A1. Briefly, after exclusion of dead cells, T-cells were identified by expression of CD3 in the lymphocyte population. Antibodies directed against CD4 and CD8 detected the major T-cell subsets, which were further subdivided into differentiation states by staining for CD45RA, CCR7, CD27, and CD28. Additionally, the abundance of PD-1 was assessed on the populations of interest. Data were acquired with an LSR-II flow cytometer (Becton Dickinson) running FACSDiva software v6.1.3 (Becton Dickinson). Compensation of the flow data was automatically calculated by using single color controls. A biological control derived from the same batch of cryopreserved PBMCs from a healthy donor was included in each analytical run to ensure comparability between results from different runs. Analysis of flow data was performed with FlowJo v10.1.5 (Tree Star). A threshold of a minimum of 100 events was established for analysis of cell subsets. An example of the gating strategy is displayed in Figure A1. All samples were centrally processed and analyzed in the Center for Medical Research, University Medical Center, Tübingen.

Statistics

Disease-specific survival probabilities were estimated using the Kaplan-Meier method and compared by log-rank testing. Death due to melanoma was considered as an “event”, whereas deaths due to other reasons were censored. Dichotomization of the entire cohort was performed for survival analysis using the median frequency of the cell populations of interest as the cut-off point for categorization. Correlations between BORR and proportions of cellular compartments were analyzed by χ^2 and Fisher’s exact tests (two-sided). Cox regression analysis was applied to test for the relative impact of factors. Hazard ratios (HR) and the Wald test were used for statistical evaluation. Nonparametric Spearman correlations were used to test for correlations between the identified phenotypes. P-values <0.05 were considered significant throughout the study. Statistical calculations were performed with SPSS 22 (IBM) and Prism 6.h (GraphPad).

Results

Patients

The baseline differentiation states of CD4 and CD8 T-cells were investigated in a total of 137 stage IV melanoma patients receiving ipilimumab in 7 different clinical centers. The M category was assigned according to the American Joint Committee on Cancer (AJCC) recommendations (41). Ninety-eight patients were assigned to category M1c (72%), 25 in

M1b (18%) and 13 in M1a (9%), whereas no definite classification was possible for one patient. One hundred four patients received all four doses of ipilimumab, whereas in the remaining patients the treatment was stopped after 1 to 3 doses. Median OS was 8.8 months after initiation of therapy. Median follow-up was 15.9 months for patients who were alive at the last follow-up, and 7.0 months for those who died. Clinical response data were available for 130 patients. Ninety-six (74%) patients had SD or PD whereas 34 (26%) experienced PR or CR. The median age was 58 years, 45% were female. Details are displayed in Table 1.

Proportions of T-cell differentiation states in melanoma patients at baseline

The differentiation states of CD4 and CD8 T-cells were assessed using a common model based on differential expression of CD45RA, CCR7, CD27 and CD28 (Figure A1), as described by Romero et al (42). Naïve (CD45RA+CCR7+CD27+CD28+) CD4 T-cells were present at a median frequency of 38.2% in this subset, while only 23.3% of CD8 T-cells had this phenotype. The central memory (CM) phenotype (CD45RA–CCR7+CD27+CD28+) constituted 26.5% of CD4 but only 2.5% of CD8 T-cells whereas the abundance of the effector memory (EM) compartment (CD45RA–CCR7–) was 23.6% for CD4 and 20.0% for CD8 cells. EM subtypes were identified by differential expression of CD27 and CD28 with EM1 defined as CD27+CD28+, EM3 as CD27–CD28–, and EM4 as CD27–CD28+. Cells with the putative terminally-differentiated phenotype (EMRA cells, CD45RA+CCR7–CD27–CD28–) were present at 23.8% among CD8 T-cells but were completely absent in CD4 cells (Table 2).

Associations of T-cell memory compartments with overall survival

OS was found to be positively associated with frequencies of peripheral blood CD8 EM1 cells at baseline when applying dichotomization using the median frequency as the cut-off (Figure 1A: $p=0.029$). Patients with >13.0% of CD8 EM1 cells had a median survival of 307 days, and a 1-year OS rate of 46.4%. Patients in the reciprocal group (<13.0%) had a shorter median survival of 227 days with a 1-year OS rate of 35.4% (Table 2). Additionally, the frequency of CD8 EMRA cells was negatively associated with OS (Figure 1B: $p=0.034$). Patients with >23.8% of EMRA cells had a median survival of only 224 days, and a 1-year OS rate of 35.0%. Patients in the reciprocal group (<23.8%) had a median survival of 309 days and a 1-year OS rate of 46.7% (Table 2). No further associations with OS were found for the remaining four CD8 and five CD4 differentiation phenotypes.

Correlations of the baseline CD8 EM1 and EMRA phenotypes with clinical response

In the CD8 compartment a significant correlation was observed between the abundance of the EM1 subset and BORR (Figure 1C: $p=0.01$): 36.5% of patients (23 of 63) with >13% of circulating EM1 cells achieved a CR or PR, whereas this was only the case for 16.4% (11 of 67) of those with <13% of circulating EM1 cells. In contrast, there was only a trend for the correlation between BORR and the proportion of the EMRA subset (Figure 1D: $p=0.073$). 33.3% of patients (22 of 66) in the prognostically favorable group (<23.8% of circulating EMRA cells) were classified as CR or PR, and 18.8% (12 of 64) in the reciprocal group (>23.8% of circulating EM1 cells).

CD8 EM1 and EMRA phenotypes in relation to the M category

As expected, the M category alone was an important factor associated with OS. Patients staged as M1a/b had a longer median OS (Figure A2 A: $p=0.004$) and a trend towards a higher BORR compared to M1c patients (Figure A2 B: $p=0.078$). A significant independent impact on OS was observed for the EM1 frequency and the M category, if considered in combination (HR 1.5, $p=0.033$ and HR 1.9, $p=0.007$, respectively). Patients with M1a/b and high EM1 frequencies had a 1-year-OS probability of 67.6% and a BORR of 45.5% in marked contrast to M1c patients and low EM1 frequencies whose 1-year-OS and BORR was 46.9% and 13.7%, respectively (Figure A2: C and D). No independent impact on OS was observed for the EMRA frequencies (HR 1.4; $p=0.092$) in addition to the M category (HR 1.4; $p=0.092$).

Changes in the proportion of CD8 EM1 and EMRA compartments during ipilimumab treatment

Next, relative alterations in the proportions of the CD8 EM1 and EMRA compartments were investigated at the time of the second or third drug dose, or after the third dose. Increases/decreases in frequencies compared to baseline were correlated with BORR. No significant associations were observed at any of the three time points during treatment, neither for alterations in the EM1 nor in the EMRA compartment (all $p>0.05$; Figure 2).

PD-1 expression on CD8 EM1 and EMRA cells

Finally, the expression of PD-1 was investigated on the two OS-associated CD8 memory subsets before and during/after the administration of ipilimumab. While no expression of PD-1 was detectable on the EMRA subset, the median frequency of PD1+EM1 cells was 0.8% of all CD8 T-cells (Figure A3 A). The abundance of PD-1+EM1 cells among CD8 cells correlated with the frequency of the entire EM1 compartment (Figure A3 B: $p<0.001$; $r=0.575$). No correlation of the frequency of PD-1+EM1 neither with BORR (Figure 3 A: $p=0.550$) nor with OS (Figure A4 A: $p=0.139$) was observed at baseline. Interestingly, a decrease at the time of the second, third and after the third administration of ipilimumab correlated significantly with BORR (Figure 3 B–D: $p=0.045$; $p=0.007$ and $p=0.045$, respectively). The analysis of associations with OS was equivocal. A separation of survival curves was observed comparing patients with increase versus those with decreases of PD-1+EM1 cells if analyzed at ($p=0.047$) or after ($p=0.053$) the 3rd cycle but not at the time of the 2nd infusion ($p=0.631$) of ipilimumab (Figure A4 B–D).

Discussion

Two observations prompted us to investigate clinical correlations of the differentiation phenotypes of CD8 and CD4 T-cells in melanoma patients treated with ipilimumab: First, we found increases in the CD4 and CD8 T-cells to be associated with OS and response under ipilimumab (31). Second, we previously reported a prognostic impact of frequencies of peripheral effector CD27+CD28+CD45RA[−] CD8 T-cells in a discovery cohort of late stage melanoma patients (21). The prognostic impact of frequencies of other circulating immune cell subsets such as myeloid-derived suppressor cells, Tregs and $\gamma\delta$ T-cells was reported by us before in partially overlapping cohorts (31, 43, 44).

In the current study, we found that the abundance of peripheral blood CD8 but not CD4 memory cells prior to ipilimumab was associated with OS in late-stage melanoma patients; specifically, the abundance of CD8 EM1 cells was positively, and the CD8 EMRA phenotype negatively, associated with OS. In line with these findings, Felix et al. reported lower frequencies of CD8 EM1 cells and a tendency for higher frequencies of EMRA cells in melanoma patients prior to ipilimumab treatment, relative to healthy controls, but no differences were observed for the CD4 compartment at baseline (35).

In addition to the association of the CD8 EM1 phenotype with OS we observed that a high frequency of these cells in the peripheral blood was also correlated with patients' responses to ipilimumab treatment. Finally, the peripheral frequency of these cells at baseline when combined with the M category was an independent predictor of OS. In contrast, although the frequency of CD8 EMRA cells was negatively associated with OS, it was not significantly correlated with BORR nor was it an independent factor related to OS in multivariate analysis. Our findings emphasize that the frequency of CD8 EM1 T-cells may be a candidate biomarker for ipilimumab outcomes. Here, we have only analyzed associations with peripheral blood markers, but comparative TIL analysis and functional testing of the EM1 cells is now required to characterize the mechanistic interactions necessary for a positive in-vivo effect of ipilimumab. Analyzing changes of CD8 EM1 and EMRA compartments during treatment, we did not observe correlations with response. Felix et al. (35) observed small but significant increases in CD4 and CD8 EM and EMRA compartments in patients with disease control at later time points. The diverging results between the two studies regarding changes in these cell populations might be due to the comparably low sample sizes, the differently defined end points, or the used analysis approaches. Felix et al. used fresh, unfrozen samples for their analysis, while the present report is based on frozen samples. This may also impact findings and limit the possibility to compare both studies. Thus, the clinical relevance of these changes needs to be further addressed in future studies.

Several recent studies highlight the role of T-cell responses targeting neo-epitopes for a favorable clinical response to ipilimumab (38, 45, 46). Kvistborg et al. postulated that anti-CTLA-4 treatment broadens the spectrum of melanoma-specific CD8 T-cell responses, while pre-existing melanoma and virus responses remain unaltered (45). Recently, Gros et al. reported that circulating neoantigen-specific CD8 T-cells show surface expression of PD-1 in melanoma (38). Moreover, PD-1 expression is associated with high affinity melanoma-specific T-cell clones (47). Based on these observations and following the hypothesis that PD-1+EM1 CD8 cells might serve as a surrogate for melanoma-antigen specific T-cells, we analyzed if their abundance at baseline or their changes under treatment correlated with clinical responses after ipilimumab. Before starting therapy, no correlation between of PD1+EM1 CD8 T-cells and BORR was observed. However, after treatment initiation, a decrease in the proportion of these cells in the peripheral blood relative to baseline was strongly and consistently correlated with BORR at all three analyzed time-points during ipilimumab treatment. Notably, this decrease was already taking place at the time of the second ipilimumab dose: 3 of 5 patients who had decreasing CD8 PD1+EM1 cells at the time of the second dose experienced a response. In marked contrast, no responses were observed among seven patients with increasing frequencies at this time point. To the best of our knowledge, this finding has not been reported before, and emphasizes an important

potential role of PD1+EM1 CD8 T-cells for a positive response to ipilimumab treatment. Successful trafficking of activated melanoma-specific T-cells and relocation from the peripheral blood to the tumor tissue in patients with good outcome after ipilimumab may explain the identified association. Ipilimumab treatment results in increased expression of the chemokine receptors CXCR3, CCR4 and CCR6 on T-cells in melanoma that are associated with immune cell migration towards the tumor (35). Chemokine receptor expression on T-cells has been associated with OS (48) and disease control (49) in melanoma. Future investigation of the chemokine receptor profile on the PD-1+ EM1 cells and analysis of the frequency of these cells among TILs will offer a possibility to confirm or reject this hypothesis. Moreover, it is not clear, based on the results of our study, which proportions of EM1 or PD-1+EM1 cells are melanoma-specific and/or if they target neo-epitopes. Further, PD-1 is expressed on antigen-specific T-cells in response to antigen exposure and is thus not restricted solely to cancer-reactive T-cells. Finally, PD-1 expression is regulated through a highly complex multifactorial network including, for example, factors that are not or only indirectly TCR-dependent, such as the cytokine microenvironment (50). Nevertheless, investigation of targets of these cell populations is warranted as the analysis of their frequencies might represent an easily accessible surrogate for the abundance of melanoma- or neo-antigen-specific T-cells, which were shown to be key effectors for immunological rejection in several studies (15, 17, 51).

Clinically, the frequency of CD8 EM1 T-cells at baseline and the decrease of PD1+EM1 cells during therapy represent biomarker candidates for outcome of ipilimumab treatment. However, these findings still need to be validated in independent patient cohorts. Further investigations in patients treated with anti-PD-1 antibodies either alone or in combination with ipilimumab is warranted.

In conclusion, the proportion of circulating CD8 EM1 T-cells at baseline represents a biomarker candidate for clinical outcome of ipilimumab treatment. Moreover, a decrease of CD8 PD1+EM1 cells in the peripheral blood during therapy compared to baseline is strongly associated with BORR and thus may represent an early surrogate marker. These results require confirmation and validation in independent patient cohorts. Our study emphasizes a potentially important role for CD8 PD1+EM1 cells for immunological rejection of melanoma. The decrease of the latter in the peripheral blood of patients experiencing a clinical response might represent successful cell trafficking to the tumor tissue. Further correlative studies of TILs and the analysis of target epitopes of PD1+EM1+ cells is now required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med*. 2014; 371(20): 1877–88. [PubMed: 25265492]
2. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011; 364(26):2507–16. [PubMed: 21639808]
3. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010; 363(8):711–23. [PubMed: 20525992]
4. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med*. 2015; 372(26):2521–32. [PubMed: 25891173]
5. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med*. 2015; 373(1):23–34. [PubMed: 26027431]
6. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol*. 2015; 33(17):1889–94. [PubMed: 25667295]
7. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011; 364(26):2517–26. [PubMed: 21639810]
8. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol*. 2002; 3(7):611–8. [PubMed: 12087419]
9. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009; 30(6):899–911. [PubMed: 19464196]
10. Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, et al. The interaction properties of costimulatory molecules revisited. *Immunity*. 2002; 17(2):201–10. [PubMed: 12196291]
11. Wolchok JD, Saenger Y. The mechanism of anti-CTLA-4 activity and the negative regulation of T-cell activation. *Oncologist*. 2008; 13(Suppl 4):2–9. [PubMed: 19001145]
12. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med*. 1991; 174(3):561–9. [PubMed: 1714933]
13. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol*. 2001; 19:565–94. [PubMed: 11244047]
14. Greenwald RJ, Boussiotis VA, Lorschach RB, Abbas AK, Sharpe AH. CTLA-4 regulates induction of anergy in vivo. *Immunity*. 2001; 14(2):145–55. [PubMed: 11239447]
15. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, et al. Treatment of metastatic melanoma with autologous CD4⁺ T cells against NYESO-1. *N Engl J Med*. 2008; 358(25):2698–703. [PubMed: 18565862]
16. Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, et al. Integrated NY-ESO-1 antibody and CD8⁺ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A*. 2011; 108(40):16723–8. [PubMed: 21933959]
17. Weide B, Zelba H, Derhovanessian E, Pflugfelder A, Eigentler TK, Di Giacomo AM, et al. Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis. *J Clin Oncol*. 2012; 30(15):1835–41. [PubMed: 22529253]
18. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med*. 2014; 371(23):2189–99. [PubMed: 25409260]
19. van Rooij N, van Buuren MM, Philips D, Velds A, Toebes M, Heemskerk B, et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol*. 2013; 31(32):e439–42. [PubMed: 24043743]

20. Powell DJ Jr, Dudley ME, Robbins PF, Rosenberg SA. Transition of latestage effector T cells to CD27+ CD28+ tumor-reactive effector memory T cells in humans after adoptive cell transfer therapy. *Blood*. 2005; 105(1):241–50. [PubMed: 15345595]
21. Wistuba-Hamprecht K, Di Benedetto S, Schilling B, Sucker A, Schadendorf D, Garbe C, et al. Phenotypic characterization and prognostic impact of circulating gammadelta and alphabeta T-cells in metastatic malignant melanoma. *Int J Cancer*. 2016; 138(3):698–704. [PubMed: 26383054]
22. Delyon J, Mateus C, Lefeuvre D, Lanoy E, Zitvogel L, Chaput N, et al. Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. *Ann Oncol*. 2013; 24(6):1697–703. [PubMed: 23439861]
23. Ku GY, Yuan J, Page DB, Schroeder SE, Panageas KS, Carvajal RD, et al. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. *Cancer*. 2010; 116(7):1767–75. [PubMed: 20143434]
24. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: immune correlates associated with clinical benefit in patients with resected high-risk stage IIIc/IV melanoma. *Clin Cancer Res*. 2011; 17(4):896–906. [PubMed: 21106722]
25. Tarhini AA, Edington H, Butterfield LH, Lin Y, Shuai Y, Tawbi H, et al. Immune monitoring of the circulation and the tumor microenvironment in patients with regionally advanced melanoma receiving neoadjuvant ipilimumab. *PLoS One*. 2014; 9(2):e87705. [PubMed: 24498358]
26. Simeone E, Gentilcore G, Giannarelli D, Grimaldi AM, Caraco C, Curvietto M, et al. Immunological and biological changes during ipilimumab treatment and their potential correlation with clinical response and survival in patients with advanced melanoma. *Cancer Immunol Immunother*. 2014; 63(7):675–83. [PubMed: 24695951]
27. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. *Proc Natl Acad Sci U S A*. 2015; 112(19):6140–5. [PubMed: 25918390]
28. Huang RR, Jalil J, Economou JS, Chmielowski B, Koya RC, Mok S, et al. CTLA4 blockade induces frequent tumor infiltration by activated lymphocytes regardless of clinical responses in humans. *Clin Cancer Res*. 2011; 17(12):4101–9. [PubMed: 21558401]
29. Maker AV, Attia P, Rosenberg SA. Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. *J Immunol*. 2005; 175(11):7746–54. [PubMed: 16301685]
30. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat Rev Cancer*. 2011; 11(11):805–12. [PubMed: 22020206]
31. Martens A, Wistuba-Hamprecht K, Yuan J, Postow MA, Wong P, Capone M, et al. Increases in absolute lymphocytes and circulating CD4+ and CD8+ T cells are associated with positive clinical outcome of melanoma patients treated with ipilimumab. *Clin Cancer Res*. 2016
32. Pedicord VA, Montalvo W, Leiner IM, Allison JP. Single dose of anti-CTLA- 4 enhances CD8+ T-cell memory formation, function, and maintenance. *Proc Natl Acad Sci U S A*. 2011; 108(1):266–71. [PubMed: 21173239]
33. Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med*. 1996; 183(6):2533–40. [PubMed: 8676074]
34. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med*. 2009; 206(8):1717–25. [PubMed: 19581407]
35. Felix J, Lambert J, Roelens M, Maubec E, Guermouche H, Pages C, et al. Ipilimumab reshapes T cell memory subsets in melanoma patients with clinical response. *Oncoimmunology*. 2016
36. Baitsch L, Legat A, Barba L, Fuertes Marraco SA, Rivals JP, Baumgaertner P, et al. Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. *PLoS One*. 2012; 7(2):e30852. [PubMed: 22347406]

37. Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, et al. PD-1 identifies the patient-specific CD8(+) tumor-reactive repertoire infiltrating human tumors. *J Clin Invest*. 2014; 124(5):2246–59. [PubMed: 24667641]
38. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. *Nat Med*. 2016; 22(4):433–8. [PubMed: 26901407]
39. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res*. 2009; 15(23):7412–20. [PubMed: 19934295]
40. Wistuba-Hamprecht K, Pawelec G, Derhovanessian E. OMIP-020: phenotypic characterization of human gammadelta T-cells by multicolor flow cytometry. *Cytometry A*. 2014; 85(6):522–4. [PubMed: 24756989]
41. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009; 27(36):6199–206. [PubMed: 19917835]
42. Romero P, Zippelius A, Kurth I, Pittet MJ, Tuvrey C, Iancu EM, et al. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J Immunol*. 2007; 178(7):4112–9. [PubMed: 17371966]
43. Martens A, Wistuba-Hamprecht K, Foppen MG, Yuan J, Postow MA, Wong P, et al. Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab. *Clin Cancer Res*. 2016
44. Wistuba-Hamprecht K, Martens A, Haehnel K, Geukes Foppen M, Yuan J, Postow MA, et al. Proportions of blood-borne Vdelta1+ and Vdelta2+ T-cells are associated with overall survival of melanoma patients treated with ipilimumab. *Eur J Cancer*. 2016; 64:116–126. [PubMed: 27400322]
45. Kvistborg P, Philips D, Kelderman S, Hageman L, Ottensmeier C, Joseph-Pietras D, et al. Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Sci Transl Med*. 2014; 6(254):254ra128.
46. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science*. 2015; 350(6257):207–11. [PubMed: 26359337]
47. Simon S, Vignard V, Florenceau L, Dreno B, Khammari A, Lang F, et al. PD- 1 expression conditions T cell avidity within an antigen-specific repertoire. *Oncoimmunology*. 2016; 5(1):e1104448. [PubMed: 26942093]
48. Mullins IM, Slingluff CL, Lee JK, Garbee CF, Shu J, Anderson SG, et al. CXC chemokine receptor 3 expression by activated CD8+ T cells is associated with survival in melanoma patients with stage III disease. *Cancer Res*. 2004; 64(21):7697–701. [PubMed: 15520172]
49. Jacquelot N, Enot DP, Flament C, Vimond N, Blattner C, Pitt JM, et al. Chemokine receptor patterns in lymphocytes mirror metastatic spreading in melanoma. *J Clin Invest*. 2016; 126(3):921–37. [PubMed: 26854930]
50. Bally AP, Austin JW, Boss JM. Genetic and Epigenetic Regulation of PD-1 Expression. *J Immunol*. 2016; 196(6):2431–7. [PubMed: 26945088]
51. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science*. 2016; 351(6280):1463–9. [PubMed: 26940869]

Highlights

- Analysis of circulating T-cell memory compartment in melanoma at baseline of ipilimumab
- Higher levels of CD8 EM1 cells in blood are predictive for good outcome
- Low abundance of peripheral CD8 TEMRA cells associate with good outcome
- Decreasing PD1+EM1 cells correlated with response following ipilimumab treatment

Figure 1.

Kaplan-Meier analysis of associations of the CD8 EM1 A) and EMRA B) T-cell compartments with OS at baseline (n=137 for both analyses). Censoring is indicated by vertical lines; p-values were calculated by log-rank statistics. Correlations of BORR with the OS-associated CD8 EM1 C) and the EMRA D) compartments were calculated using the χ^2 test (n=130 for both analyses).

Figure 2.

Correlations between changes in frequency of differentiation phenotypes during ipilimumab treatment and BORR (CR/PR). Alterations in the CD8 EM1 compartment at the 2nd (n=14), 3rd (n=31) and after the 3rd (n=34) cycle compared to baseline are displayed in A). Changes in the CD8 EMRA compartment at the respective time points compared to baseline (n=14; n=31; n=28) are shown in B). Statistical evaluation was performed by using the χ^2 test.

Figure 3.

Analysis of associations between the CD8 PD-1+ EM1 subset and BORR (PR/CR). Correlations were calculated for baseline frequencies (n=130) and for changes during ipilimumab treatment compared to baseline at the time of the 2nd (n=12), 3rd (n=31) and after the 3rd (n=34) cycle of ipilimumab. The χ^2 test was used to determine statistically relevant differences.

Table 1

Patients characteristics

Variable	Category	Patients (n=137)	
		n	%
Center	Amsterdam	44	32
	Essen	11	8
	Lausanne	10	7
	Nantes	6	4
	Naples	18	13
	New York	40	29
	Siena	8	58
Gender	Male	76	55
	Female	61	45
Age	40 years	19	14
	> 40 years	57	42
	> 60 years	31	23
	> 70 years	30	22
	Median age	58 years	
Number of doses	1	7	5
	2	8	6
	3	18	13
	4	104	76
M category (AJJC)	M1a	13	10
	M1b	25	18
	M1c	98	72
	Unknown	1	n.c.
Treatment background	CA-184–169 (3 or 10 mg/kg)	3	2
	Early access program (3 mg/kg)	68	50
	Regular prescription (3 mg/kg)	66	48

Variable	Category	Patients (n=137)	
		n	%
Best clinical response (irRC)	Complete /partial response	34	26
	Stable /progressive disease	96	74
	Unknown	7	n.c.

Table 2

Phenotype	Total n	Categories	n	%	% Dead	Median survival (days)	Survival analysis 1-year survival rate (95% CI)		P value [dicho]
CD4	naïve	38.2 %	72	52.6	75.0	309	44.1%	(29.3%; 52.9%)	0.601
		> 38.2 %	65	47.4	73.8	224	40.5%	(28.7%; 52.3%)	
	T CM	26.5 %	69	50.4	75.4	230	39.8%	(28.0%; 51.6%)	0.411
		> 26.5 %	68	49.6	73.5	280	41.8%	(29.8%; 53.8%)	
	T EM1	17.5 %	69	50.4	73.9	224	39.8%	(28.0%; 51.6%)	0.642
		> 17.5 %	68	49.6	75.0	273	41.8%	(29.8%; 53.8%)	
	T EM3	3.9 %	72	52.6	68.1	299	43.7%	(32.1%; 55.3%)	0.056
		> 3.9 %	65	47.4	81.5	227	37.7%	(25.7%; 49.7%)	
	T EM4	0.2 %	69	50.4	69.6	255	40.7%	(28.7%; 52.7%)	0.930
		> 0.2 %	68	49.6	79.4	269	40.9%	(29.1%; 52.7%)	
CD8	naïve	23.3 %	69	50.4	75.4	269	43.3%	(31.6%; 55.2%)	0.560
		> 23.3 %	68	49.6	73.5	255	37.7%	(25.7%; 49.7%)	
	T CM	2.5 %	67	50.0	74.6	269	44.1%	(32.1%; 56.1%)	0.611
		> 2.5 %	67	50.0	73.1	280	39.6%	(27.8%; 51.4%)	
	T EM1	13.0%	69	50.4	81.2	227	35.4%	(23.8%; 47.0%)	0.029
		> 13.0 %	68	49.6	67.6	307	46.4%	(34.4%; 58.4%)	
	T EM3	1.5 %	69	50.4	71.0	280	39.7%	(27.9%; 51.5%)	0.410
		> 1.5 %	68	49.6	77.9	227	41.9%	(29.9%; 53.9%)	
	T EM4	1.9 %	70	51.1	75.7	227	37.9%	(26.3%; 49.5%)	0.339
		> 1.9 %	67	48.9	73.1	299	43.8%	(31.6%; 56.0%)	
	TEMRA	23.8 %	69	50.4	68.1	309	46.7%	(34.7%; 58.7%)	0.034
		> 23.8 %	68	49.6	80.9	224	35.0%	(23.6%; 46.4%)	