Immunological correlates of treatment and response in stage IV malignant melanoma patients treated with Ipilimumab

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ABSTRACT

Introduction: Ipilimumab is effective in the treatment of metastatic malignant melanoma, but few biomarkers reliably predict treatment response. Methods: Patients were treated with Ipilimumab for metastatic malignant melanoma. Blood and serum samples were collected before and during treatment. Mononuclear cells in peripheral blood were subjected to immune phenotypic analyses and cytokine levels were measured in serum samples. Results were correlated with clinical data. Results: A total of 40 patients were included in the analyses. Clinical response were associated with an increase after one series of treatment in absolute lymphocyte count (ALC) (p = 0.008), absolute T cell count (p = 0.02) and the absolute number of activated T cells in peripheral blood (p = 0.003). A high frequency of myeloid derived suppressor cells (MDSC) and a higher level of IL6 were associated with treatment failure, though not significantly. Levels of IL6 in serum above the median showed a tendency to associate with reduced survival by the 4th treatment series. Finally, treatment with Ipilimumab led to a decreased frequency of FOXP3+ regulatory T cells (p = 0.009). Conclusion: Ipilimumab leads to increased ALC, T cell count and T cell activation in malignant melanoma patients responding to treatment. A high baseline frequency of myeloid-derived suppressor cells and high levels of IL6 is associated with a reduced chance of responding to therapy.

Introduction

Over the past decade, treatment of metastatic melanoma has been revolutionized by new blocking antibodies targeting check point molecules widely expressed throughout the immune system. Notable examples of these include the anti-Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) antibody Ipilimumab and the anti-Programmed Cell Death 1 (PD-1) receptor antibody Pembrolizumab, both of which are US Food and Drug-approved in the treatment of metastatic malignant melanoma.1 T cell activation during priming of immune responses is tightly linked to the balance between stimulatory and inhibitory inputs to the cell. CTLA-4 is a co-inhibitory receptor that regulates activation of T cells during priming and maintenance of adaptive immune responses.2 It has structural homology with the co-activating receptor CD28 and shares ligand restriction, though with 10–20 times higher affinity. Upon binding by molecules B7.1 and B7.2 (CD80 and CD86 respectively), expressed on antigen presenting cells, CTLA-4 inhibits T cell function and proliferation.3 Early work by Allison, J. P. et al., who pioneered in the use of anti-CTLA-4 antibodies in cancer treatment, have demonstrated, that T cell activation can be dramatically augmented ex vivo upon treatment with anti-CTLA-4 antibody.4

Ipilimumab is a fully human IgG1 antibody specific for CTLA-4, with clinical efficacy against metastatic melanoma proven in a phase III clinical trial.5 The drug may have a modest antibody dependent cellular cytotoxicity effect in CTLA-4-expressing melanoma cell lines in vitro6 but Ipilimumab is generally not believed to have any significant tumoricidal effect per se. Instead the action is believed to be linked to the dis-inhibiting effect on T cells, promoting T cell mediated killing of tumor cells.7 In support of this theory, prospective studies have reported a dramatic infiltration of T cells in the tumor during treatment, and intratumoral changes in the ratio between CD8+ T cells and regulatory T cells may be associated with extend of tumor necrosis.8,9 Furthermore, an interesting recent report demonstrated a close correlation between the number of tumor-mutations giving rise to neo-antigen-epitopes and clinical response, thereby underscoring the role of cytotoxic T cells in the mechanism of action.10 The exact mechanism by which anti-CTLA-4 antibodies promotes this immune mediated tumor-killing is still not completely understood. Some studies have pointed toward an induction of tumor specific CD8+ T cells,11,12 while other authors have speculated that effect might be conveyed though inhibition of regulatory T cells.13 In addition to that, levels of circulating MDSC have recently been reported to be reduced by Ipilimumab treatment, a phenomenon which reportedly is augmented in clinical responders.14

Treatment of metastatic melanoma with Ipilimumab is associated with an overall response rate approximately 10% and a clinical benefit rate of nearly 30% in a large phase III study5 and subsequent analyses have indicated that responses may be durable and long lasting. As a rare thing in solid cancers Ipilimumab may induce durable complete responses indicating that
a cure is possible, even in the metastatic setting. However, there is still a majority of patients not responding to therapy with Ipi-
limumab and as treatment is associated with potentially dele-
terous side effects and the drug is overtly costly, biomarkers
predicting response to subsequent treatment and methods of
improving the effect of treatment are in demand. Several recent
reports have focused on identification of biomarkers prior to
and during therapy that is correlating with treatment response.
Changes in CD4+ T cell expression of inducible T cell co-sti-
umulator (ICOS) in bladder cancer and level of MDSC in me-
lanoma has been identified as on-treatment markers associated
with clinical response.14,15

In this study, we scrutinized possible baseline and treat-
ment-related biomarkers in patients treated for metastatic
malignant melanoma with Ipi-limumab. Prospectively collected
peripheral blood mononuclear cell (PBMC) and serum samples
were subjected to multicolor flow cytometry and cytokine
measurements.

Results

Patient characteristics

A total of 40 patients receiving at least two doses of Ipi-limumab
were included in the study. Patients were treated at the Herlev
Hospital, Copenhagen University (n = 28) and Aarhus Univer-
sity Hospital (n = 12), two of three designated melanoma treat-
ment centers in Denmark. Patients were included between
October 2012 and July 2014. Baseline patient characteristics are
presented in Table 1.

Absolute lymphocyte count, CD4+ and CD8+ T cells

Clinical response to Ipi-limumab has previously been linked to
an early increase in ALC during therapy.16 In our cohort of
patients we observed a small but significant increase in the ALC
during treatment (Fig. 1a, repeated measure ANOVA p = 0.05).
When dichotomizing into patients with unequivocal progression
and patients with stable disease (SD) at 1st evaluation or better,
we saw significantly higher increases in patients responding to
therapy compared to non-responders, with regard to changes in
ALC from baseline to the time of 2nd dose of treatment
(Fig. 1b, p = 0.008), thus confirming previous reports.

As Ipi-limumab affects numerous aspects of T cell biology,
17,18 we assessed whether this increase could be attributed to
increased frequencies of T cells or a more general increase in
lymphocytes. As shown in Fig. 1c, at least part of the early
increase in ALC in responding patients could be accounted for
by an increased absolute T cell count (p = 0.02). In line with
this, change in absolute T cell count from baseline to 2nd treat-
ment dose was significantly higher in patients responding to
therapy (Fig. 1d, p = 0.03). We observed no changes in the dis-
tribution of CD4+/CD8+ T cells (Fig. 1e and f).

Ipi-limumab induces a shift from naive to effector T cells

Based on expression of a number of different extracellular
markers, several maturation states of T cells are recognized. We
used CD45RA, chemokine receptor 7 (CCR7) and CD27 to
distinguish between these effector-stages, which has previously
been identified as markers of maturation, telomere-length and
replicative potential.19

| Table 1. Patient demographics. |
|--------------------------------|
| Characteristic              | Age—median (range) | Gender—no. (%) |
| Stage of disease (AJCC*)—no. (%) | 67.25 (35–83)     | male female |
| M1a                           | 16 (40)           | 24 (60) |
| M1b                           | 6 (15)            |       |
| Prior systemic therapy—no. (%) | 20 (50)           |       |
| IL2                           | 15 (37.5)         |       |
| Temozolomide                  | 16 (40)           |       |
| BRAF inhibitor                 | 8 (20)            |       |
| Series of Ipi-limumab—no. (%) | 1 (2.5)           |       |
| 1                             | 3 (7.5)           |       |
| 2                             | 2 (5)             |       |
| 3                             | 35 (87.5)         |       |
| RECIST response—no. (%)       | 1 (2.5)           |       |
| 1st                            | 15 (37.5)         |       |
| 2nd                            | 24 (60)           |       |
| 3rd                            | 1 (2.5)           |       |
| Lactate dehydrogenase—no. (%) | 31 (77.5)         |       |
| > sULN                        | 9 (22.5)          |       |
| > 2x sULN                     | 1 (2.5)           |       |
| Gender—no. (%)                | male female       |       |
| Male                          | 16 (40)           |       |
| Female                        | 24 (60)           |       |
| Line of therapy—no. (%)       | 1 (2.5)           |       |
| 1st                            | 15 (37.5)         |       |
| 2nd                            | 24 (60)           |       |
| 3rd                            | 1 (2.5)           |       |
| BRAF inhibitor                 | 1 (2.5)           |       |
| IL2                            | 8 (20)            |       |
| Temozolomide                  | 16 (40)           |       |
| M1a                            | 14 (35)           |       |
| M1b                            | 6 (15)            |       |
| Prior systemic therapy—no. (%) | 20 (50)           |       |
| IL2                            | 15 (37.5)         |       |
| Temozolomide                  | 16 (40)           |       |
| BRAF inhibitor                 | 8 (20)            |       |
| Series of Ipi-limumab—no. (%) | 1 (2.5)           |       |
| 1                             | 3 (7.5)           |       |
| 2                             | 2 (5)             |       |
| 3                             | 35 (87.5)         |       |
| RECIST response—no. (%)       | 1 (2.5)           |       |
| 1st                            | 15 (37.5)         |       |
| 2nd                            | 24 (60)           |       |
| 3rd                            | 1 (2.5)           |       |
| Lactate dehydrogenase—no. (%) | 31 (77.5)         |       |
| > sULN                        | 9 (22.5)          |       |
| > 2x sULN                     | 1 (2.5)           |       |
| Gender—no. (%)                | male female       |       |
| Male                          | 16 (40)           |       |
| Female                        | 24 (60)           |       |

T cell PD-1 expression

T cell activation is tightly linked to the balance between signal-
ing through activating and inhibitory receptors. We sought to
assess whether treatment with Ipi-limumab had an impact on
four markers; B- and T-lymphocyte attenuator (BTLA), Lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin mucin 3 (TIM-3) and programmed death receptor 1 (PD-1), all considered to be inhibitory receptors. The majority of cells did not express any of these markers; mean 86.3% of CD4+ T cells and 82.9% of CD8+ T cells. No significant change in the fraction of cells negative for inhibitory markers was observed during therapy (data not shown). As seen on Fig. 3a, we did observe small dynamic changes within subpopulations defined by combinations of the scrutinized markers. However, due to large inter-patient variability, none of these changes were significant for subpopulations possibly reflecting fluctuations in T

![Image](image1.png)

**Figure 1.** Absolute lymphocyte and T cell count. Absolute Lymphocyte Count (ALC). a) ALC increased from baseline to 4th series of treatment. b) Change in ALC from baseline to 2nd series in patients progressing despite treatment and in responding patients (SD, PR and CR). c) Absolute T cell count increased during treatment. d) Change in absolute T cell count from baseline to 2nd series in patients progressing despite treatment and in responding patients (SD, PR and CR). e+f) Proportion of CD4+ and CD8+ cells in T cells throughout treatment, which remained unchanged despite therapy.

![Image](image2.png)

**Figure 2.** T cell phenotype. a) Phenotype of CD4+ and CD8+ T cells according to expression of CCR7, CD27 and CD45RA. b) The proportion of naive CCR7+CD27+CD45RA+ cells decreased in both CD4+ and CD8+ T cells during treatment (p < 0.0001 and p = 0.009 respectively. The proportion of effector memory CCR7-CD27-CD45RA- cells increased significantly in CD4+ T cells (p = 0.0001) and borderline significant in CD8+ T cells (p = 0.2). c) The proportion of HLA-DR+ T cells out of lymphocytes increased from baseline to 2nd and 4th series (both p = 0.002). The increase from baseline to 2nd series in the absolute number of HLA-DR+ T cells was significantly higher in responding patients (SD, PR and CR) as compared to non-responders (p = 0.003).
cell dynamics. In both CD4+ and CD8+ T cells, the predominant inhibitory receptor positive population was PD-1+LAG-3−TIM-3−BTLA−, but though neither of the subpopulations of PD-1 positive cells changed significantly as a result of treatment, we did observe a modest increase in the level of CD4+ T cells expressing this antigen from a mean of 6.6% at baseline to 8.4% (p = 0.03, Fig. 3b).

**Regulatory T cells decrease during therapy**

Regulatory T cells have been suggested as a possible target for Ipilimumab-treatment, as Tregs reportedly express high levels of CTLA-4. We set out to investigate whether the frequency, in peripheral blood, of this particular cell-type was affected by treatment. As illustrated in Fig. 4a, the proportion of CD4+ T cells displaying a Treg phenotype, did decrease significantly during treatment (repeated measures ANOVA, p < 0.0005). This effect was evident already by the 2nd treatment, though not yet significant (p = 0.26), and even more pronounced after three doses of Ipilimumab (p = 0.001). We observed no significant differences in terms of neither frequency of Tregs or change in frequency during therapy between responders and non-responders (data not shown).
Additionally, we scrutinized dynamics of subpopulations of regulatory T cells based on expression of activation-marker CD39 and transcription-factor Helios, as illustrated in Fig. 4b. Though we did observe small reciprocal changes in both double-positive and double-negative subsets (not significant), only the double-positive compartment increased significantly (Fig. 4c, repeated measures ANOVA, $p = 0.04$) (mean values from baseline to the 2nd and to the 4th 38.7% vs. 41.5% vs. 42.6% respectively, $p < 0.0005$ and $p = 0.1$ respectively).

**Frequencies of MDSC are associated with response but remain unchanged during therapy**

MDSC are a diverse group of inhibitory cells of myeloid origin, frequently found to be increased in frequency in peripheral blood of melanoma patients negatively correlating with prognosis. Moreover, treatment with Ipilimumab has been reported to reduce the frequency of MDSC. We quantified the frequency of monocytic MDSC only, as the granulocytic counterpart is sensitive to cryopreservation. MDSC were defined as Lin$^+$ HLA$^+$ DR$^{low/−}$ CD14$^+$ CD11b$^+$ CD3$^+$ (Lin defined as CD3, CD19 and CD56), and frequency was assessed out of PBMC (a representative example of gating strategy is presented in Fig. 4d). Results are presented in Fig. 4e. As seen, we did not observe any consistent treatment-related changes in the frequency of MDSCs. Interestingly, we observed higher baseline frequency of MDSC in patients with continued PD after Ipilimumab, though this difference was only borderline significant (Fig. 4f, $p = 0.1$) but no difference between responding and non-responding patients with regard to change in frequency throughout therapy (data not shown). We measured the concentration of interleukin 6 (IL6) in patient sera (see later), and sought to correlate this concentration to the frequency of MDSC before and during treatment. These analyses did not indicate any significant correlations between these parameters (data not shown).

**NK cell were not affected by treatment**

Though it is generally agreed that the mechanism-of-action of Ipilimumab is a disinhibiting effect on tumor-reactive T cells, the drug has been shown to exert some degree of NK cell mediated antibody-dependent cellular cytotoxicity (ADCC) in vitro. Additionally, NK cells may play a role in tumor immune surveillance. We therefore assessed the frequency of NK cells, defined as CD56$^{bright}$ lymphocytes not positive for CD3 and CD19, in peripheral blood. No consistent changes in the frequency of NK cells were observed during treatment (data not shown).

**On-treatment IL6 levels correlate with survival**

In cancers, including melanoma, tumor cell and stromal production of IL6 may serve as an autocrine/paracrine stimulator of tumor-growth. Numerous reports have focused on IL6 as a prognostic marker in metastatic melanoma and two relatively large studies have demonstrated a significant negative prognostic value of cytokine levels. Other studies, however, have yielded conflicting results.

We measured serum-levels of IL6 at baseline, after the 1st and 3rd dose of Ipilimumab using a cytokine bead array. We observed a high degree of inter-patient variation in cytokine-levels ranging at baseline from 2.3–49.4 pg/mL, a significant increase in the level of IL6 during treatment ($p = 0.046$) (Fig. 5a) and a widening of the range of IL6 at baseline as compared to non-responding patients ($p = 0.09$). To test a possible prognostic role of IL6 levels, we dichotomized the serum-levels by the median and compared survival according to IL6 above or below median. Results are plotted in Fig. 5b. Baseline-levels did not significantly predict survival (log rank, $p = 0.5$, data not shown), whereas higher levels of IL6 by the time of the 4th dose of Ipilimumab was associated with shorter survival though not significantly (log rank, $p = 0.07$). In order to determine if this might reflect a general unfavorable profile, a possible correlation between the level of lactate dehydrogenase (LDH) and IL6 was scrutinized. No correlation between IL6 and LDH was found either at baseline or by the 4th treatment ($R^2 = 0.003$ and $R^2 < 0.001$ respectively, data not shown), suggesting an independent value of this cytokine.

**Changes in levels of cytokines throughout treatment**

We measured serum-concentration of six different cytokines: IL2, IL4, IL10, IL17A, interferon (IFN)$γ$ and tumor necrosis factor (TNF)$α$ using cytokine bead array. As shown in Fig. 5c, most of these cytokines were detectable in variable concentrations in the majority of patients. Neither IL2, IL4, IL10, IL17A, IFN$γ$, nor TNF-$α$ showed any significant changes during therapy. However, IL17A and TNF-$α$ showed great inter-patient variation. The level of these two cytokines did not correlate to the frequency of CD4$^+$ and CD8$^+$ T cells staining positive for IL17A and TNF-$α$ in PMA/ionomycin stimulated PBMC samples (data not shown).

**Discussion**

Ipilimumab is only effective in a minority of patients, and in addition to that, rather costly. In a pursuit of appropriate biomarkers for a better selection of patients likely to respond to therapy, several have been identified correlating with treatment efficacy, but until recently non useful as predictive biomarkers.

It has previously been shown that early increases in ALC after treatment initiation correlate with treatment response. In order to confirm these data, we dichotomized our patient-cohort into patients with unequivocal progression at first evaluation and patients with SD or better and assessed ALC after one cycle of Ipilimumab. We were able to demonstrate similar ALC increases in patients with clinical response, thereby confirming previous findings. Furthermore, we saw that at least part of this increase could be accounted for by increases in the absolute T cell count. As Ipilimumab is directed at a broadly expressed target, CTLA-4, treatment is likely to have
on-target effects on non-tumor-specific T cells, and these data could represent proliferation or mobilization of both T cells specific for viral or other pathogens in addition to tumor associated antigens (TAA). Previous studies have demonstrated that Ipilimumab induces proliferation of peripheral T cells, and data suggest a propensity in responding patients to react to treatment with proliferation of T cells, possibly reflecting a responsive state of the immune system less pronounced in non-responders. Furthermore, we found a general increase in the proportion of activated HLA-DR C T cells as previously shown. Interestingly, this translated into generally higher increases in the absolute number of HLA-DR C T cells after the first series of Ipilimumab in responding patients, further pointing to higher responsiveness of T cells in patients obtaining treatment response.

When looking further into the differentiation stage of the T cells a highly significant reduction in the frequency of naive triple-positive T cells in both CD4 C and CD8 C T cells during treatment was demonstrated. This was paralleled by increased frequency of triple-negative cells, i.e., cells with an intermediate or late effector state, partly confirming previous findings. This could either be due to a differentiation of naive cells into effector-cells or proliferation of cells in the latter compartment. It has previously been shown that treatment with anti-CTLA-4 antibodies induces a broadening of the T cell repertoire, and that the majority of the clones displaying altered abundance are derived from the non-naive compartment of cells. Though the mentioned study only assessed the relative frequency of clones in the upper quartile with regard to abundance, it indicate that the increased frequency of experienced cells evident in our patient cohort is likely due to proliferation in this compartment rather than cell-death in the naive compartment.

Some controversy has gathered on the mechanism-of-action of Ipilimumab. Two theories dominate: one including a direct disinhbiting effect on tumor-reactive T cells while the other point at inhibition or killing of Tregs in the tumor-microenvironment. The two mechanisms are difficult to distinguish experimentally, and it is not unlikely that the action is dependent on both mechanisms. Adding to this, a recent study by Romano et al demonstrated the presence in peripheral blood of a CD16 C monocyte population exerting ADCC on CTLA-4 positive Tregs in Ipilimumab treated patients. We demonstrated a highly significant decline in the frequency of Tregs identified as CD4 C CD25 C Foxp3 C, and further showed a slight enrichment of CD39 C Helios C Tregs, i.e., activated naturally occurring Tregs. The enrichment of CD39 C Helios C Tregs may suggest that the overall decrease in the frequency of Tregs is primarily derived from one or more of the other non-CD39 C Helios C Tregs subsets, though none of these showed any significant decrease. Different studies have reported conflicting results regarding how Ipilimumab affects Tregs in peripheral blood. Tarhini et al. report increased frequencies correlating with improved progression-free survival. Notably, these authors identified Tregs with a similar panel of markers as used in the current study, but patients were treated with a higher dose (10 mg/kg) of Ipilimumab, which could explain the discrepancy. A different study by Sarnaik et al. where patients were treated with either 3 or 10 mg/kg, found no impact on Tregs, whereas a third study, treating patients with 3 mg/kg as used in our cohort, found...
reduced frequencies.⁴⁹ Though the mechanism is unclear, it seems that Ipilimumab might have opposite effects on the frequency of Tregs when administered in low and high doses. Furthermore, timing of blood sampling in relation to the course of treatment might have an impact on the findings; in a small-scale study by de Coaña et al.,⁴⁰ where blood samples were acquired at identical time points as in the current study, and found a similar decrease in Treg frequencies. In the current study, a small decrease in the frequency of Tregs was found after one dose of treatment and significant reduction by the 4th Ipilimumab dose. In consequence, this effect of Ipilimumab might have been missed if samples obtained late in the treatment course had not been included. A different subset of inhibitory cells, that have received a lot of attention in relation to Ipilimumab, is MDSC, which is a diverse group of cells implicated in the prognosis of several different cancers including melanoma.¹⁴,⁴¹ A number of more or less well-defined subtypes have been identified, including a monocytic CD14⁺ and a granulocytic CD15⁺ type, both present in peripheral blood. We chose to focus only on the monocytic counterpart, as the granulocytic is highly cryo-sensitive.⁷⁶ In contrast to previous findings,⁸ we found no indication that the frequency of MDSC were affected by treatment in neither responders or non-responders. We did, however, find a trend toward higher frequencies of MDSC at baseline in patients not responding to treatment, which is in accordance with previous findings.¹⁴ In a patient cohort diagnosed with various gastrointestinal cancers, a correlation between CD15⁺ MDSC and IL6 concentration in patient serum has been reported, but a similar correlation did not seem to apply to the CD14⁺ monocytic MDSC.⁴² In esophageal cancer, however, significant correlation between IL6 and CD14⁺ MDSC has been reported.⁴³ In the present study, we did not observe any significant correlation between the frequency of MDSC and IL6 at any of the assessed time points.

To this end, different gating-strategies may yield very different results. We decided to assess the MDSC frequency out of live singlet PBMC, which, in our hands, give the most reproducible results, as MDSC do not fall in a well-circumscribed gate in a forward/side scatter plot. However, this approach is inevitably sensitive to fluctuations in other PBMC subsets. Furthermore, MDSC is not an easily gated population, partly because the difference between HLA-DR-negative, low and positive is somewhat fluent, as at least in some patients, the level of expression seems to be a continuum. This leaves room for technical error biased by e.g., monocyte count.

In line with the presumed mechanism of action, most studies have focused on cellular markers in relation to treatment with Ipilimumab. We performed analyses of seven different cytokines in serum obtained at baseline and during therapy. IL6 levels showed interesting correlations to clinical data. During treatment, the level of this cytokine increased significantly in a subset of patients introducing a widening of the range of concentration. IL6 has previously been linked to an adverse prognosis in malignant melanoma.⁴⁰,⁴¹ and in accordance with this, we found a non-significant reduced survival in patients with the highest IL6 levels by the 4th treatment. In order to determine whether the concentrations of this cytokine might just reflect a general unfavorable prognostic profile in patients with above median IL6 level, we scrutinized a possible correlation between the concentration of IL6 and LDH. The latter is a known negative prognostic factor correlating with aggressiveness of disease and negatively correlating with chance of responding to Ipilimumab therapy.⁴⁴,⁴⁵ We found no indication of a correlation, possibly indicating that the underlying adverse mechanism inducing an increased level of IL6 is different from the mechanism leading to increased LDH, thereby suggesting IL6 as an independent prognostic marker.

In conclusion, we show that response to Ipilimumab is associated to inductions in the absolute levels of circulating T cells, the level of activation of T cells and that Ipilimumab treatment may reduce the frequency of regulatory T cells. Additionally, we saw higher frequencies of MDSC and IL6 in non-responding patients.

**Patients and methods**

**Patients**

Patients were treated for American Joint Committee on Cancer (AJCC) stage IIIc or stage IV malignant melanoma with Ipilimumab as part of standard-of-care at either of two centers: Herlev Hospital, Copenhagen University, Denmark or Aarhus University Hospital, Aarhus, Denmark. Patients were eligible for the study if > 18 y of age and fulfilling standard criteria for treatment with Ipilimumab including histologically verified locally advanced and metastatic skin, ocular or mucosal malignant melanoma, Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1 or 2, and acceptable blood counts, liver and renal function tests. Patients were not eligible for treatment if prior medical history was notable of significant autoimmune diseases, known infection with human immunodeficiency virus, hepatitis B or C virus and current medical need for high-dose immunosuppressant(s). Treatment with Ipilimumab was given as a total of four treatments 3 weeks apart, at a fixed dose of 3 mg/kg body weight. Participation in the study was strictly voluntary and patients signed an informed consent form prior to inclusion. A total of three blood samples were acquired from each patient. Samples were obtained at baseline (before initiation of treatment), before the 2nd and 4th dose of treatment, simultaneously with routine blood samples and a total of 108 mL was obtained per sampling event. The protocol was approved by the Ethics Committee for The Capital Region of Denmark (H-2–2012–058). The study was conducted in accordance with the Helsinki Declaration of 1975.

**Blood processing**

PBMCs were purified from heparinized blood using lymphoprep™ (StemCell Technologies) density gradient centrifugation in LeucoSep™ tubes (Greiner Bio-One). After processing, cells were frozen in Nunc® 1.8 mL CryoTube (thermo Scientific) and stored at −150°C in 90% human AB serum (Sigma Aldrich) and 10% dimethyl sulphoxide (Herlev Hospital Pharmacy). Patient-to-processing time was sought kept as low as possible and processing was in general initiated within 4 h.
Blood for collection of serum was collected in a 8 mL Vacuette® gel-tube containing clot activator (Greiner Bio-One). Serum was aliquoted in Nunc® 1.8 mL CryoTube (thermo Scientific) and stored at −80°C until analyses.

**Assessment of clinical response**

Clinical response was assessed using positron emission tomography and computed axial tomography (PET-CT) or CT alone evaluated according to Response Evaluation Criteria In Solid Tumors (RECIST v. One.). Patients were evaluated within 4 weeks before the first treatment and every 3 mo thereafter until progression. SD had to be confirmed on two independent evaluations. At the treating physician’s discretion, patients with apparent increased tumor-volume at first evaluation could be evaluated for pseudo-progression usually with a 8-week treatment-free interval.

**Flow cytometry**

PBM C samples were thawed in 37°C RPMI 1640 medium (Lonza) supplemented with 2.5 mL DNAse-containing Pulmozyme (Roche) and 0.26 mmol MgCl (Herlev Hospital Pharmacy) per 100 mL buffer. All stainings were done in phosphate buffered saline (PBS) (Lonza) containing 0.5% bovine serum albumin (Sigma-Aldrich). The following antibodies were used: CD80-PerCP (Abcam), FoxP3-PE, IL9-PE, HLA-DR-HV500, CD3-PE-Cy7, CD19-PE-Cy7, CTLA-4-PE, PD-1-PE-Cy7, CD4-HV500, CD11b-APC, CD3-APC (purchased from BD Biosciences), CD33-FITC, CD56-PE, CD124-PE (purchased from BD Pharmigen), CCRI-PE-Cy7, BTLA-PE, CD27-PerCP, HELIOS-PerCP-Cy5.5, TNF-α-PE-Cy7, CD14-BV421, CD86-BV421, CD8-BV421, CD25-BV421 (all purchased from Biolegend), CD56-PE-Cy7, CD16-FITC, CD45RA-FITC, CD127-FITC, IFNγ-FITC, IL17A-PerCP-Cy5.5, CD39-PE-Cy7 (purchased from eBioscience), LAG-3-FITC (LifeSpan Biosciences) and TIM-3-PerCP (R&D Systems). Additionally, all samples were stained with the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Life technologies). Intracellular cytokine staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm Kit according to the manufacturer’s instructions. Intracellular staining of transcription factors was carried out using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Cells were acquired on a FACScanto II using FACSDiva Software version 6.1.3 (BD Biosciences). Analysis of flow data was done using FlowJo software version 10 OSX (TreeStar, Inc., Ashland, OR). Data-analysis was done after filtering of dead cells and doublets.

**In vitro activation**

For intracellular cytokine staining, cells were thawed day zero and rested overnight in RPMI 1640 medium (Lonza) supplemented with 5% human AB serum (Sigma Aldrich) in 24-well plates at 37°C, 5% CO₂ in a humidified atmosphere. Day 1, 2 μL/mL cell suspension Leukocyte Activation Cocktail (Phorbol 12-Myristate 13-Acetate and Ionomycin) with BD GolgiPlug™ (Brefeldin A) was added or as a control, 1 μL/mL cell suspension of GolgiPlug™ (both purchased from BD Biosciences). Cells were stimulated for a total of 5 h before staining and acquisition.

**Cytokine bead array**

Concentrations of IL2, IL4, IL6, IL10, IL17A, TNF and IFNγ in serum from patients before and during treatment was measured using the BD™ Human Th1/Th2/Th17 CBA Kit (BD Biosciences) following the manufacturer’s instructions. Cytokine concentrations were measured in thawed undiluted serum-samples. Concentration of cytokines was calculated using the FCAP Array™ software version 3.0 (BD SoftFlow).

**Statistical analysis**

All statistical calculations were done in GraphPad Prism (GraphPad Software, La Jolla, CA) and SPSS software (Version 22, IBM Corporation). Scatter plots are presented with mean (horizontal line) and standard error of the mean (error bars). All tests were performed two-sided and a p value ≤ 0.05 was considered significant. Between group comparisons were performed without correction for multiple comparisons in this exploratory study. p values for within group assessments were calculated using Bonferroni correction. When applicable, differences between groups and differences over time were assessed using students t-test. Overall differences within groups assessed on multiple time points were assessed using repeated measures ANOVA with the Greenhouse–Geisser Correction. If data did not meet model assumptions for parametric testing, Wilcoxon matched-pairs signed rank test was used to assess differences over time. A difference in survival was assessed using the log-rank test and survival estimates were calculated using Kaplan–Meier’s method. Linear correlation was carried out using Spearman’s method. Regression-plots are presented with the best fitted line and 95% confidence intervals of the fitted line.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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