Immune checkpoint inhibitors in mesothelioma

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Chapter 8

Immune cells in mesothelioma microenvironment simplistic marker of response to nivolumab plus ipilimumab?

Short communication

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Abstract

Introduction

Malignant pleural mesothelioma (MPM) is a malignant disease of the pleura which recently can be treated with immune checkpoint inhibitors (ICI). To optimize this treatment, a better understanding of the tumor micro environment is needed. We investigated subgroups of immune cells in subsequent tumor biopsies of patients treated with ICI.

Methods

Biopsies from MPM patients included in two clinical ICI trials (nivolumab alone and an ipilimumab/nivolumab combination) were examined. At baseline and after 6 weeks of treatment, pleural biopsies were taken to examine the tumor microenvironment (CD20+, CD4+, CD8+, FoxP3+ and PD-1+). Cell density was defined as the number of marker positive cells per mm^2. Radiological responses were evaluated as partial response, stable disease or progressive disease according to modified RECIST criteria.

Results

Thirty-four and 36 patients were included in the nivolumab and ipiliumumab/nivolumab trial respectively. In the nivolumab trial, no significant differences in cell densities were seen in baseline biopsies of patients with partial response versus progressive disease. In contrast, in the ipilimumab/nivolumab trial, a higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells at baseline was significantly correlated with partial responses. On-treatment biopsies of both trials did not show significant changes when compared to baseline biopsies.

Conclusion

Biopsies from patients responding to nivolumab plus ipilimumab treatment show a significant higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells, without a change after 6 weeks of treatment. This observation is a first step in exploring the tumor microenvironment as predictor of response in ICI treatment in MPM.

Introduction

Malignant pleural mesothelioma (MPM) is a rare malignant tumor arising from the mesothelial cells of the pleura. It is mainly caused by exposure to asbestos, with a latency time between exposure and diagnosis of 30 to 50 years.(1)

For decades, standard systemic treatment for MPM was combination chemotherapy consisting of platinum plus pemetrexed. But recently immune checkpoint inhibitor (ICI) treatment with nivolumab (anti-PD-1 antibody) and ipilimumab (anti-CTLA-4-antibody) was approved as first line therapy, following the results of the phase III Checkmate 743 trial. This study showed a survival benefit of combination ICI treatment over standard chemotherapy (18.1 versus 14.1 months, HR 0.74 (96.6% CI 0.60-0.91, p=0.002)).(2)

Unfortunately, ICI treatment is not effective in all patients and may lead to side effects. A better understanding of MPM and its microenvironment is needed to select the proper patients for ICI treatment. The tumor micro-environment (TME) plays an important role in the response to ICI therapy. The TME in MPM is composed of stromal, endothelial and immune cells and has a heterogenous distribution in the pleural cavity.

We investigated the possible impact of subgroups of immune cells in subsequent tumor biopsies of patients treated with ICI.

Materials and methods

Patients from the Nivomes (NCT02497508) (3) and Initiate (NCT03048474) (4) clinical trials were included in this analysis. In these two single center phase II trials, patients with recurrent MPM were treated with nivolumab monotherapy (Nivomes) or nivolumab plus ipilimumab (Initiate). In both trials pleural biopsies were taken at baseline and after 6 weeks of treatment and stored formalin-fixed paraffin-embedded (FFPE).

For nivolumab treated patients, two multiplex immunofluorescence panels were used. Panel 1 included antibodies against CD4, FOXP3, CD68, CD163, pancytokeratin (panCK) and DAPI to identify all nucleated cells. Panel 2 included antibodies against CD8, PD-1, CD20, panCK and DAPI. Macrophage markers (CD68 and CD163) of panel 1 could not be validated and evaluated. For the nivolumab plus ipilimumab trial, immunohistochemistry staining was performed for CD4, CD8, FoxP3 and PD-1.

The stained slides were annotated and analyzed using HALO software for counting and calculating the percentage of all nucleated cells. Cell density was defined as the number

of marker positive cells per mm2. Details about stainings and HALO software are provided in the supplementary methods.

In both trials, PD-L1 staining was performed. In the nivolumab trial, the PD-L1 expression on tumor cells (TCs) and tumor infiltrating immune cells (ICs) was assessed using the 28-8 antibody (EnVisio, Agilent Dako, Santa Clara, Ca). In the nivolumab plus ipilimumab trial, PD-L1 expression was assessed using the 22C3 antibody (pharmDx Agilent Technologies, Santa Clara, CA). In both trials, expression on TCs and ICs was scored as negative (<1% PD-L1 positive cells) or positive (≥1% PD-L1 positive cells) and as a percentage. Readers were blinded to patient outcomes.

Responses were monitored via computed tomography (CT) scans and evaluated according to modified Response Evaluation Criteria in Solid Tumors (mRECIST) for mesothelioma (5) and reported as partial response (PR), stable disease (SD) and progressive disease (PD). Responses were evaluated at 24 weeks. Patients were monitored every six weeks thereafter (every eight weeks after 24 weeks of treatment) to calculate the median progression free survival (mPFS) and median overall survival (mOS).

A Wilcoxon rank-sum test was used to test for response group similarity based on cell densities, where a rejection region of $p < 0.05$ was regarded significant. Multiple testing correction was performed on all P-values where applicable, using the Bonferroni correction.

Results

Thirty-four patients treated with nivolumab and thirty-six treated with nivolumab plus ipilimumab, were included. At the time of analysis, median follow-up time for the Nivomes trial was 58.6 months. The updated results show a mPFS of 2.6 months (95% CI: 2.2 – 5.5) and a mOS of 11.8 months (95% CI: 9.7 – 15.7). Median follow-up time for the Initiate patients was 46 months (95% CI 44.2 – 46.4 months). The updated results show a mPFS of 6.2 months (95% CI 4.2 – 11.0) and a median OS of 22.9 months (95% CI 12.6 – 32.6).

At baseline, pleural biopsies were obtained from all patients. After 6 weeks of treatment, 31 and 32 on-treatment biopsies were taken from respectively nivolumab and nivolumab plus ipilimumab treated patients. Not all on-treatment biopsies were evaluable: some only contained muscle tissue, others only fibrotic connective tissue or necrosis.

Baseline biopsies

At baseline, in the nivolumab alone group, no significant differences in cell densities of CD20+, CD4+, CD8+ and FoxP3+ were seen in biopsies of patients with partial response versus progressive disease. (suppl fig 1)

In contrast, in the nivolumab plus ipilimumab trial, a significant higher cell density of CD4+ (p=0.002), CD8+ (p=0.001), FoxP3+ (p=0.001) and PD-1+ (p=0.012) cells was observed in patients achieving a partial response compared to those with progressive disease. (fig 1)

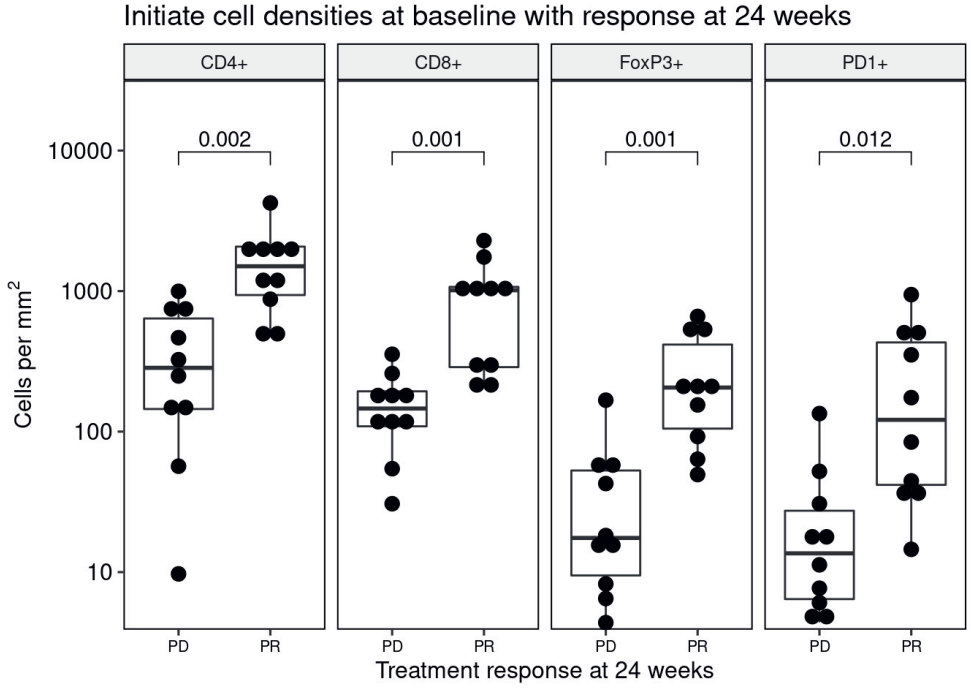

Figure 1. number of CD4+, CD8+, FoxP3+ and PD-1+ cells per mm2 at baseline in the nivolumab plus ipilimumab trial, comparing patients with progressive disease (PD) with partial response (PR) at 24 weeks.

On-treatment biopsies

Cell densities of CD20+, CD4+, CD8+ and FoxP3+ in the nivolumab trial showed no significant change nor difference after six weeks of treatment, not for all responses taken together, nor for partial response and progressive disease separately. On-treatment biopsies in nivolumab trial showed no difference between patients having PR or PD (data not shown).

In the nivolumab plus ipilimumab trial no significant change was seen in cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells in patients having progressive disease or partial response (fig 2).

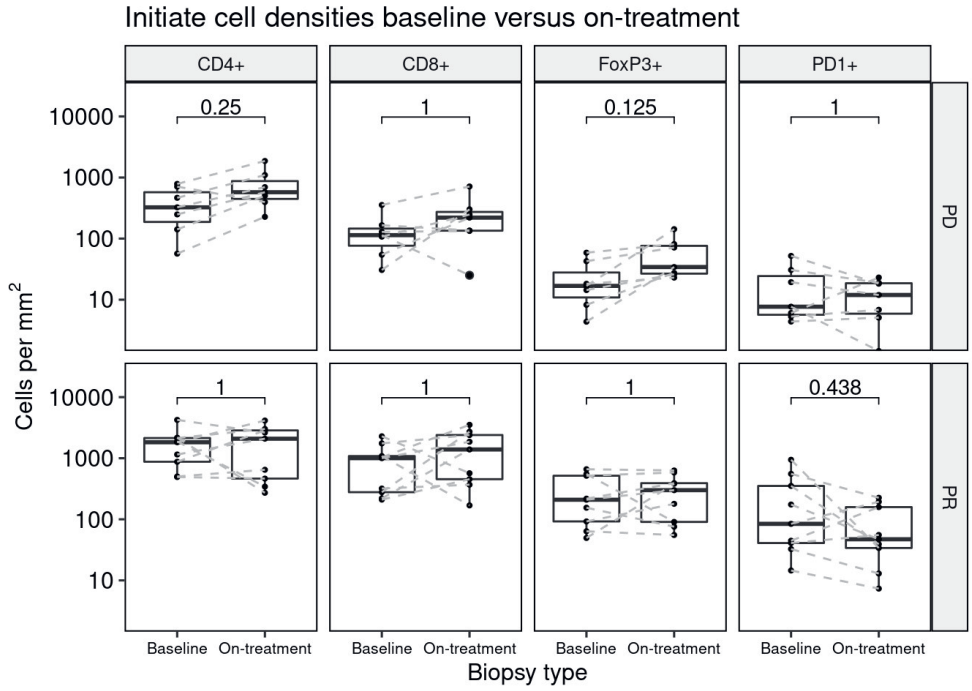

Figure 2. The number of CD4+, CD8+, FoxP3+ and PD-1+ positive cells per mm2 in the nivolumab plus ipilimumab trial, comparing baseline with 6 weeks on-treatment biopsies in patients with progressive disease (PD)(upper plots) and partial response (PR) (lower plots) at 24 weeks.

PD-L1 expression

PD-L1 expression on tumor cells or immune cells was not significantly correlated with PFS or OS (data not shown). Positive PD-L1 expression (≥1 %) on immune cells was correlated with a higher cell density of CD4+, CD8+ and FoxP3+ positive cells in both nivolumab as nivolumab plus ipilimumab group. (suppl Fig 2) This correlation was not observed when looking at PD-L1 expression on tumor cells.

Discussion

In the nivolumab plus ipilimumab study, biopsies of patients with a partial response have a higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells, as compared to biopsies from patients having progressive disease. This is not seen in patients treated with nivolumab alone.

Immune cells in the TME can influence tumor growth and mediate response to therapy. In different tumor types it is shown that the density of tumor-infiltrating lymphocytes (TILs) is associated with response to anti-PD-1 treatment.(6) Not only cell density itself, but also the type of immune cells is important; for example, infiltration of CD8+ cytotoxic T cells is associated with higher ORR, longer disease free and overall survival in NSCLC.(7)

The TME in MPM is known to be highly immune suppressive, with the presence of a large amount of tumor associated macrophages, myeloid derived suppressor cells and regulatory T cells. Conflicting data on T cell subsets exists. Some studies in MPM suggest that higher levels of CD8+ T cells have a favourable prognostic impact while others found that higher levels are associated with a lower survival.(8,9) Higher levels of CD4+ and CD20+ cells and lower levels of FoxP3+ cells are linked to a better outcome, irrespective of therapy.(8,10) Until now, no prospective study has been performed with analysis of biopsies in MPM patients treated with ICI.

In our trial we hoped to identify changes in the TME but no significant change in immune cell subsets was observed after 6 weeks of treatment with nivolumab (plus or minus ipilimumab). Therefore, on- treatment biopsies of mesothelioma do not seem to add information on prediction of effect of ICI treatment, in contrast to melanoma, where adaptive immune signatures in early treatment biopsies are predictive of response to ICIs. (11)

In this study we focused on the extreme responses, progressive disease and partial response, to find a signal in studies with a relatively low number of patients. We deliberately excluded patients with stable disease since response analysis in patients with MPM is notoriously difficult. Mesothelioma spreads around the pleura in a circular way making treatment response difficult to determine with unidimensional measurements via modified RECIST criteria.

In our Initiate trial, PD-L1 expression on tumor cells and immune cells was predictive of response to nivolumab plus ipilimumab (4), but did not correlate with PFS or OS. In larger phase III trials, PD-L1 expression on tumor cells was not predictive of response to ICI treatment in MPM.(2,12) Expression on immune cells was, however, not reported. We demonstrated that positive PD-L1 expression (≥1 %) on immune cells but not on tumor cells is correlated with a higher cell density of CD4+, CD8+ and FoxP3+ positive cells in both the nivolumab and in the nivolumab plus ipilimumab group, pointing to a more inflamed environment. Which of the immune-cells co-expressed PD-L1 is not known from our studies.

The prognostic or predictive value of TIL infiltration or specific T cell subsets alone may be a too simple reflection of reality; integrating expression of proliferation markers, inhibitory receptors, cytokines, sequencing or gene expression data is needed to provide more detailed information on the TME and effect of ICI treatment.

Limitations of this study may be the sample size and limited number of representative on-treatment biopsies. In some patients having a partial response, it was not possible to take a biopsy anymore, or only necrosis was found. Also the timing of the biopsy after 6 weeks of treatment could have influenced the effect. In peripheral blood of lung cancer patients, changes in CD8 subsets are already seen within 4 weeks of PD-1 treatment.(13)

Although comparable patient groups were included in both ICI trials, they were not designed to be compared with each other. Besides that, different staining techniques were used for the biopsies. The immunofluorescence technique in the nivolumab trial was performed many years ago and was hard to validate, and not all markers (that is CD68 and CD163) could be used. Therefore, for the successive nivolumab plus ipilimumab trial, immunohistochemistry was used. This makes it difficult to compare both trials.

Based on recent publications, it would be interesting to focus on the non-epithelioid subgroup, since that has a different micro-environment (9) and a larger benefit of ICI treatment compared to the epithelioid subgroup.(2) Brockwell found high proportions of T lymphocytes and CD45RO+ cells in sarcomatoid MPM having prolonged progression free and overall survival to ICI treatment.(14) In our study there were not enough biopsies available to draw any conclusions on the subgroup of non-epithelioid MPM.

In conclusion, biopsies from patients responding to nivolumab plus ipilimumab treatment show a significant higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells at baseline, but no specific changes after 6 weeks of treatment. This observation is a first step in exploring the TME as a predictor of response to guide ICI treatment in MPM. Larger studies are needed, with more detailed analyses of the TME.

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Supplementary data

Supplementary methods

Prior to multiplex staining 3µm slides were cut on DAKO Flex IHC slides. Slides were then dried overnight and stored in +4°C. Before a run was started slides were baked for 30 minutes at 70°C in an oven.

Staining was performed on a Ventana Discovery Ultra automated stainer, using the Opal 7-Color Manual IHC Kit (50 slides kit, Perkin Elmer, cat NEL81101KT). Protocol starts with baking for 28 minutes at 75°C, followed by dewaxing with Discovery Wash using the standard setting of 3 cycles of 8 minutes at 69°C. Pretreatment was performed with Discovery CC1 buffer for 32 minutes at 95°C, after which Discovery Inhibitor was applied for 8 minutes to block endogenous peroxidase activity. Specific markers were detected consecutively on the same slide with the following antibodies, Anti-CD68 (Clone KP1, Cat M0814, Dako, 1/500 dilution 1 hour at RT), Anti-CD8 (Clone C8/144B, Cat M7103, Dako, 1/250 dilution, 1 hour at RT), anti-FoxP3 (Clone 236A/47, Cat AB20034, AbCam, 1/50 dilution, 2 hours at RT), Anti-CD163 (Clone 10D6, Cat NCL-CD163, Leica, 1/500 dilution, 1 hour at RT), Anti-CD4 (Clone SP35, Cat 104R-15, Cell Marque, 1/50 dilution, 2 hours at RT), Anti-PanCytoKeratin (Clone AE1AE3, Cat MS-343-P, Thermo Scientific, 1/1000 dilution, 1 hour at RT), Anti-CD3 (Clone SP7, Cat M3074, Spring Bioscience, 1/400 dilution 1 hour at RT), Anti-PD1 (Clone NAT105, Abcam, 1/100 dilution 1 hour at RT), Anti-CD20 (L26, DAKO, 1/1600 dilution 32 minutes at RT), Anti-PDL1 (Clone E1L3N, Cell signaling Technologies, 1 hour at RT). Each staining cycle was composed of four steps: Primary Antibody incubation, Opal polymer HRP Ms+Rb secondary antibody incubated for 32 minutes at RT, OPAL dye incubation (OPAL520, OPAL540, OPAL570, OPAL620, OPAL650, OPAL690, 1/50 or 1/75 dilution as appropriate for 32 minutes at RT) and an antibody denaturation step using CC2 buffer for 20minutes at 95°C. Cycles were repeated for each new antibody to be stained. At the end of the protocol slides were incubated with DAPI (1/25 dilution in Reaction Buffer) for 12 minutes.

After the run was finished slides were washed with demi water and mounted with Fluoromount-G (SouthernBiotech, cat 0100-01) mounting medium.

For the Initiate trial, immunohistochemistry of the FFPE tumor samples was performed on a BenchMark Ultra autostainer or Discovery Ultra autostainer (CD3-CD56 double staining). Briefly, paraffin sections were cut at 3 um, heated at 75°C for 28 minutes and deparaffinised in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 32 minutes at 950C (CD4, CD8,PD1) or 64 minutes at 950C (FOXP3). CD4 was detected using clone SP35 (1/25 dilution, 32 minutes at 370C, Cell Marque), CD8 clone C8/144B (DAKO /

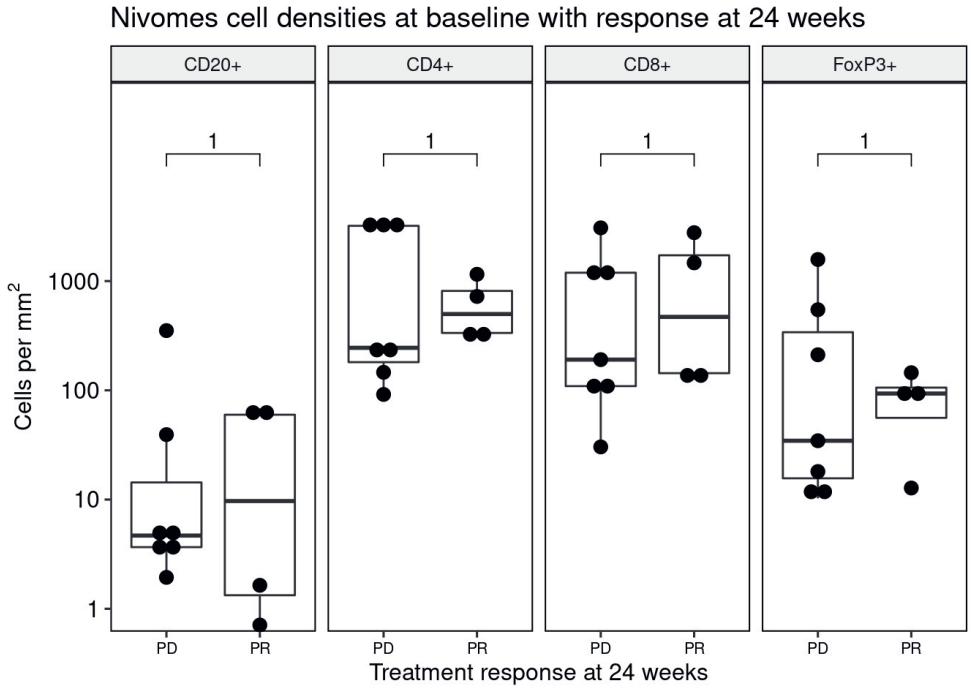
Agilent) using 1/200 dilution 32 minutes at 370C, FOXP3 using clone 236A/E7 (Abcam) at 1/200 dilution for 2 hours at RT, PD1 clone NAT105 (1/1600 dilution, 32 minutes at 370C, Abcam). Bound antibody was detected using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems).

The stained slides were annotated and analyzed using HALO software (V3.0.311.346, Indica Labs). A pathologist marked the regions of interest (ROI), consisting of (residual) tumor area and immune cells. To prevent variations in size and annotated area, consecutive slides were superimposed using the image registration tool with synchronized navigation. ROI were annotated using the brush and flood annotation tools. The Indica Labs Multiplex IHC v2.0.3 analysis algorithm was used as a template, with adjusted settings mentioned in suppl. table 1. All annotation layers were analyzed and both the summary data and object data were exported in comma separated value files using the export manager in HALO.

S1 Algorithm settings for analysis on DAB-stained slides. Indica Labs Multiplex IHC v2.0.3 was used as template, with the following adjustments.

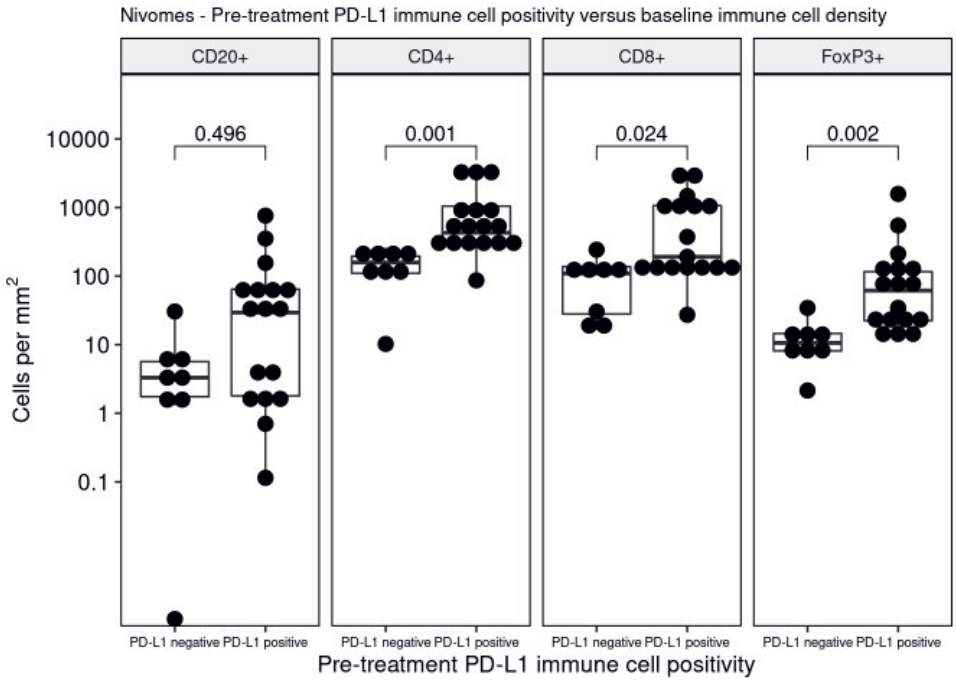
Analysis Magnification	**1**
Hematoxylin Nuclear detection weight	1.1
DAB Nuclear detection weight	1.5
Nuclear contract threshold	0.52
Minimum nuclear optical density	0.347
Nuclear size	10,118.93
Nuclear segmentation aggressiveness	0.6
Fill nuclear holes	False
Hematoxylin Markup color	124,137,180
Hematoxylin nucleus positive threshold	0.15
DAB markup color	62,39,35
DAB nucleus positive threshold	0.185
Minimum tissue OD	0.037

Supplementary figures

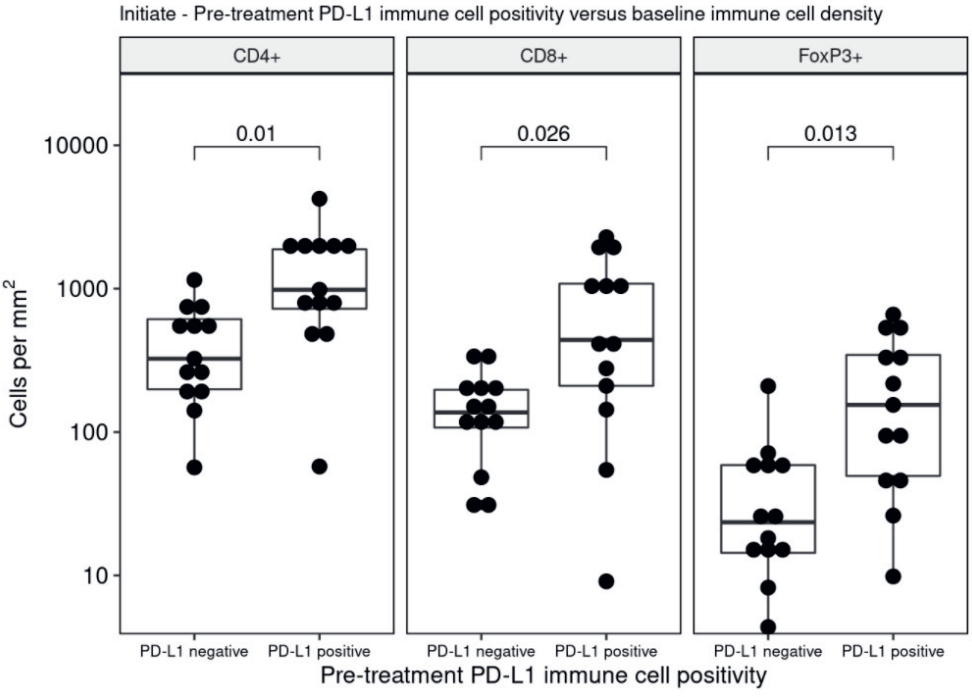


Supplementary figure 1. number of CD20+, CD4+, CD8+ and FoxP3+ cells per mm2 at baseline in nivolumab trial, comparing patients with progressive disease (PD) with partial response (PR) at 24 weeks.

A


Nivomes - Pre-treatment PD-L1 immune cell positivity versus baseline immune cell density
CD20+
CD4+
CD8+
FoxP3+
0.496
0.001
0.024
0.002
10000
1000
100
10
1
0.1
Cells per mm2
PD-L1 negative PD-L1 positive
Pre-treatment PD-L1 immune cell positivity

B


Initiate - Pre-treatment PD-L1 immune cell positivity versus baseline immune cell density
CD4+
CD8+
FoxP3+
0.01
0.026
0.013
10000
1000
100
10
Cells per mm2
PD-L1 negative
PD-L1 positive
Pre-treatment PD-L1 immune cell positivity

Supplementary figure 2: number of CD4+, CD8+ and FoxP3+ cells per mm2 in PD-L1 positive versus PD-L1 negative immune cells at baseline in nivolumab (A) and nivolumab plus ipilimumab (B) trial.