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Research Article

# Evidence That Circulating T Cells at Treatment Onset Predict Response to PD-1 Inhibitors

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## Abstract

The present study aims to identify the potential role of circulating lymphocytic subpopulations as biomarkers for response to anti-PD-1 immunotherapy. Twenty-one cancer patients who were about to start treatment with either nivolumab or pembrolizumab and eight healthy donors were enrolled. Peripheral blood mononuclear cells were obtained for flow cytometric analysis at five consecutive time points up to six months. Total CD4+lymphocytes were significantly decreased, whereas T helper 17 and regulatory T lymphocytes were significantly increased within non-responders compared to healthy donors at treatment onset, indicating their potential significance for predicting non-response to CPI immunotherapy. However, further validation is required.

**Keywords:** Checkpoint inhibitors; PD-1 inhibitors; prognostic biomarkers; circulating lymphocytes.

## Introduction

Checkpoint inhibitors (CPIs) are antibodies that target crucial molecules of the immune system that normally downregulate immune responses. These molecules include the programmed cell death 1 (PD-1) protein, expressed by T, B, natural killer T lymphocytes, monocytes, and dendritic cells, and its ligand (PDL-1), expressed in various tissues, including malignant cells [1,2]. The PD-1/PDL-1 axis is a major mechanism responsible for tumour cell evasion of immune surveillance; thus, its blockade leads to remarkable responses in certain types of tumours [3]. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is another negative regulator of effector T-cell responses. CTLA-4 is expressed by activated T cells and binds to co-stimulatory molecules on the surface of antigen-presenting cells, such as CD80 and CD86, preventing thus further T cell stimulation [4].To date, CPIs approved on a clinical basis include nivolumab, pembrolizumab, cemiplimab, and dostarlimab, which are anti-PD-1 agents; atezolizumab, avelumab, and durvalumab, which target PDL-1; and ipilimumab and tremelimumab, two anti-CTLA-4 antibodies, whereas new therapeutic targets are emerging, such as the lymphocyte activation gene 3 protein (LAG3 protein) [5]. Showing promising results in terms of patients' overall survival (OS) and progression-free survival (PFS) rates, CPIs have revolutionized our standard therapeutic approach in oncology and are currently approved as a standard of care in different advanced malignancies, including melanoma [6,7], non-driver-mutated non-small cell lung carcinoma (NCSLC)[8,9], urothelial carcinoma (UC) [10], renal cell carcinoma (RCC)[11], and head and neck squamous carcinoma (HNSCC) [12,13]. Despite excellent responses, treatment toxicity, occasionally severe and potentially lethal, may represent a cause for CPI discontinuation [14,15]. It is proposed that the dysregulation of immune homeostasis and the shifting of immune balance towards effector T cell responses versus regulatory ones represents a mechanism for the adverse events observed. These immune-related adverse events (irAEs) can affect almost every organ and may include gastrointestinal manifestations [16], endocrinopathies [17], cutaneous toxicities [18], rheumatic [19,20], neurologic[21] and pulmonary [22] manifestations. Autoimmune pneumonitis, myocarditis, and colitis are among the most common lethal irAEs [23]. Although irAEs have been correlated with favourable CPI responses in various studies, pre-treatment stratification of patients prone to irAEs and appropriate screening for irAEs during treatment could eventually reduce immune-related toxicities and mortality rates within CPI-treated patients. Among prognostic biomarkers, PDL-1 expression by tumour cells is of utmost significance and is positively correlated with CPI responses [8]. It is noteworthy, however, that neither all patients highly expressing PDL-1 respond to CPIs, nor a high PDL-1 expression is always reported within responders. Tumour mutational burden, DNA mismatch repair genes, and tumour-infiltrating immune cells (TIICs) are also important potential prognostic biomarkers for CPI immunotherapy [24–26]. However, they are all related to either tumour genomics or the tumour microenvironment, and their use remains limited. Peripheral blood biomarkers may provide cheap and easily accessible prognostic tools; lactate dehydrogenase levels, platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, and total eosinophil counts are considered to have prognostic significance for CPI immunotherapy [27–32]. However, findings regarding the potential role of circulating T-cell subpopulations as prognostic biomarkers remain controversial. As divergent responses in CPI treatment are noted, a personalized approach is needed so that patients who are more likely to respond are better stratified. In this study, we aimed to explore whether alterations in T and NK cell populations in the peripheral blood of patients receiving anti-PD-1 immunotherapy during treatment can be used as simple markers for clinical outcomes or for the development of irAEs.

## Patients and Methods

### Patients and healthy donors

Twenty-one adult patients with a confirmed diagnosis of NSCLC, UC, RCC, or HNSCC were enrolled in this single-Center prospective study. All included patients were at the initiation of treatment with a PD-1 inhibitor, either nivolumab or pembrolizumab, as a first- or next-line treatment. The study was approved by the University Hospital of Patras Ethics Committee and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice standards of care. Written informed consent was obtained from all participants before enrolment in the study. Eight age- and sex-matched healthy donors without a history of malignancy or autoimmune disease were also enrolled in the study, representing the control group.

### Methods

Patients were evaluated every 2-3 weeks at their scheduled visits for CPI administration, starting from the first infusion. The evaluation was completed six months or earlier, in cases of immunotherapy discontinuation due to disease progression or serious adverse events. All new symptoms, particularly possible immune-related AEs, were recorded. Clinically stable patients underwent CT scans at 12 and 24 weeks so that the best outcome on immunotherapy could be assessed according to the immune response evaluation criteria in solid tumours (iRECIST) [33]. Peripheral blood samples were analysed at sequential time points immediately before drug infusion. Briefly, heparinized peripheral blood was collected before the first (tp0; time point 0), second (tp1; time point 1), and third (tp2; time point 2) cycles of immunotherapy, as well as at three (tp3; time point 3) and six (tp4; time point 4) months from treatment initiation. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using a density gradient centrifugation medium according to the manufacturer's instructions (LYMPHOSEP, Biowest). PBMCs were stored in liquid nitrogen until flow cytometric analysis was performed. Flow cytometry was performed to evaluate the number of Natural Killer cells (CD3-CD16/56+; NK), total CD3+CD4+, IFN-γ-producing T helper 1 (CD3+CD4+IFN-γ+; Th1), T helper 17 (CD3+CD4+IL17A+; Th17), and regulatory T cells (CD4+CD127loCD25hiFOXP3+; Tregs) in patients and healthy donors at the time points mentioned above. The fluorochrome-conjugated antibodies and isotype controls are shown in the Supplemental Table. Antibodies against intracellular targets (INF-γ, IL-17A, FOXP3) were added after fixation and permeabilization of the cells (True-NuclearTM Transcription Factor Buffer Set, Bio Legend). The correlation between alterations in NK and T cell subpopulations and response to CPIs or irAEs was examined. Patients with disease progression according to iRECIST were considered non-responders, whereas patients with either partial or complete responses and those with stable disease were considered responders [Table 1].

### Statistical Analysis

We employed an unpaired t-test to compare data between different groups (responders, non-responders, or healthy donors) and a one-way repeated-measures analysis of variance (ANOVA) to analyze sequential data within the same group of patients (responders or non-responders) (GraphPad Prism 5, GraphPad Software Inc.). Statistical significance was set at P <0.05.

## Results

### Patients: Response to treatment and adverse events

Among the patients (N=21), all were Caucasians, 6 were females and 15 males, 15 were diagnosed with NCSLC, 4 with UC, one with RCC, and one with HNSCC. The mean age of the patients at the time of the first CPI administration (years ± SD) was 64.43 ± 9.26. Seven patients received pembrolizumab at the standard dose of 200 mg IV q3Weeks, whereas 14 patients received nivolumab at the standard dose of 240 mg IV q2Weeks. Eighteen patients received CPI as second-line treatment because of prior treatment failure, two patients with inoperable NSCLC and PD-L1 expression > 50%, received pembrolizumab as first-line treatment (patients 13 and 15, Table 1), and one patient with squamous NSCLC received pembrolizumab along with chemotherapy as first line treatment (patient 14, Table 1). Eleven of 21 patients (n=11) continued immunotherapy for more than 6 months as they were responders at this time point, and no serious adverse events were reported. Nine out of 21 patients (n=9) were non-responders; six of them were deceased due to disease progression after having received 2-3 doses of CPI, and three patients switched to another regimen due to disease progression diagnosed on scheduled CT scans (two switched at 3 months and one at 6 months). One patient died of autoimmune pneumonitis 3 months after the initiation of CPI treatment without being classified as a responder or non-responder at this time point. Regarding autoimmune complications of immunotherapy during the first six months, one patient receiving pembrolizumab developed lethal pneumonitis non-responsive to steroid pulses, two patients receiving nivolumab developed hypothyroidism three months after treatment initiation, for which L-thyroxine substitution was administered, and one patient on pembrolizumab reported asymptomatic persistent diarrhea classified as grade 1, which was improved with anti-motility agents [Table 1]. Twenty out of 21 patients had an ANA screening available at time point 0, and four out of 20 patients were found to be ANA-positive without a history of autoimmune disease. Interestingly, half of the patients who eventually developed irAEs (2 out of 4) had a positive ANA test result before treatment initiation, and the one who developed autoimmune pneumonitis had the highest ANA titer at baseline (1/640). Flow cytometric analysis was performed in 18 out of the 21 patients enrolled; three patients (Table 1; patients 8, 14, and 15) were excluded due to non-evaluable blood samples at baseline.

### Decreased total CD4+ lymphocytes in non-responders compared to HDs at baseline and post-treatment

Non-responders had significantly decreased total CD3+CD4+ lymphocytes compared to HDs at time point 0 (mean ± SEM of HDs VS Non-resp(tp0) 43.49 ± 3.358 N=8 VS 20.74 ± 4.843 N=8, p=0.002), whereas differences between responders and HDs at baseline were not of statistical significance (mean ± SEM of HDs VS Resp(tp0) 43.49 ± 3.358 N=8 VS 31.44 ± 5.378 N=9, p=0.09). CD4+ T cells were also significantly decreased at all-time points in the non-responder group compared to HDs (mean ± SEM of HDs VS Non-resp(tp1) 43.49 ± 3.358 N=8 VS 19.51 ± 5.322 N=8, p=0.002) (mean ± SEM of HDs VS Non-resp(tp2) 43.49 ± 3.358 N=8 VS 24.80 ± 7.790 N=7, p=0.04) (mean ± SEM of HDs VS Non-resp(tp3) 43.49 ± 3.358 N=8 VS 19.03 ± 12.32 N=3, p=0.02). Performing a repeated-measures one-way ANOVA to compare CD3+CD4+ lymphocytes at all time points in the non-responder group, no significant impact of immunotherapy on the T helper compartment was noted (F= 0.12, p= 0.89) [Image 1]. In contrast, in the responder group, the repeated-measures one-way ANOVA revealed a statistically significant impact of immunotherapy on the T helper compartment (F=7.023, p=0.0005); after an initial increase at time point 1, CD3+CD4+ percentages gradually decreased up to 3 months and then a significant increase was noted at 6 months. Differences were significant between time points 1 and 3 (95% confidence interval (CI) [4.146, 35.49]), between time points 2 and 4 (95% CI [-33.33, -1.988]), and between time points 3 and 4 (95% CI [-41.63, -10.29]) [Image 1].

### Increased Th17 lymphocytes in non-responders at treatment initiation

Non-responders had higher Th17 lymphocytes (expressed as % of total CD3+CD4+ lymphocytes) at baseline than both HDs (mean ± SEM of HDs VS Non-resp (tp0) 1.220 ± 0.2860 N=8 VS 3.444 ± 0.8417 N=8, p=0.03) and responders (mean ± SEM of Resp(tp0) VS Non-resp(tp0) 1.579 ± 0.3245 N=9 VS 3.444 ± 0.8417 N=8, p=0.047). Th17 lymphocytes remained significantly increased in the non-responder group compared to HDs after one cycle of CPI treatment (time point 1) (mean ± SEM of HDs VS non-responders (tp1) 1.220 ± 0.2860 N=8 VS 2.793 ± 0.6414 N=8, p=0.04). Regarding time points 2 and 3, Th17 percentages in non-responders were numerically higher than those in HDs and responders. In contrast, HDs and responders had comparable percentages of Th17 cells at all-time points [Image 2A-D]. Although the number of patients analysed was too small to reach a definite conclusion, it was shown that in those who developed irAEs (n=4), Th17 lymphocytes gradually increased during treatment, peaking at time point 2 [Images 2E, F].

### Increased regulatory T cells in non-responders at treatment initiation

Next, we examined the % of CD4+CD25hiCD127lo FOXP3+ cells, which represent the regulatory T cell compartment (Treg). Our experiments showed that in the non-responder group, Tregs (expressed as % of total CD4+ lymphocytes) were significantly increased at baseline compared to HDs and responders (mean ± SEM of Non-resp(tp0) VS HDs 2.250 ± 0.6388 N=8 VS 0.5238 ± 0.1258 N=8, p=0.02) (mean ± SEM of Non-resp(tp0) VS Resp(tp0) 2.250 ± 0.6388 N=8 VS 0.7567 ± 0.09298 N=9, p=0.03). In non-responders, Tregs did not change significantly during treatment (F=1.027, p=0.39); Tregs continued to be significantly increased compared to HDs at time points 1 (mean ± SEM of Non-resp(tp1) VS HDs 1.685 ± 0.4373 N=8 VS 0.5238 ± 0.1258 N=8, p=0.02) and 2 (mean ± SEM of Non-resp(tp2) VS HDs 2.766 ± 0.9437 N=7 VS 0.5238 ± 0.1258 N=8, p=0.03) [Image 3]. In responders, Tregs gradually increased numerically after the first cycle of CPI treatment. Hence, although the pre-treatment percentages of Tregs in the responder group were comparable to those of HDs (mean ± SEM of HDs VS Resp(tp0) 0.5238 ± 0.1258 N=8 VS 0.7567 ± 0.09298 N=9, p=0.15), at time point 2 responders had significantly increased Tregs compared to HDs (mean ± SEM of Resp(tp2) VS HDs 1.488 ± 0.3137 N=9 VS 0.5238 ± 0.1258 N=8, p=0.02) [Image 3].

**Decreased IFN-γ producing CD4+ T cells in responders at treatment initiation**

Our results showed that responders had significantly decreased IFN-γ-producing CD4+ T cells (expressed as % of total CD3+CD4+ lymphocytes) at time point 0 compared with HDs (mean ± SEM of HDs VS Resp(tp0) 1.186 ± 0.1231 N=8 VS 0.8144 ± 0.1089 N=9, p=0.04) and non-responders (mean ± SEM of Resp(tp0) VS Non-resp(tp0) 0.8144 ± 0.1089 N=9 VS 1.825 ± 0.4050 N=8, p=0.02). A post-treatment numerical increase in CD3+CD4+IFNγ+ lymphocytes within responders was noted, followed by a significant decrease at the timepoint of six months (mean ± SEM of HDs VS Resp(tp4) 1.186 ± 0.1231 N=8 VS 0.7038 ± 0.08974 N=8, p=0.007) [Image 4].

**Increased NK cell numbers in all patients compared to healthy controls at treatment initiation**

Patients had more NK cells (expressed as % of gated lymphocytes) compared to HDs at all-time points; statistically significant differences between HDs and CPI-treated patients (pts) were noted at time points 0 (mean ± SEM of HDs VS pts(tp0) 5.918 ± 0.9519 N=8 VS 14.91 ± 2.638 N=18, p=0.04), 1 (mean ± SEM of HDs VS pts(tp1) 5.918 ± 0.9519 N=8 VS 15.24 ± 1.965 N=18, p=0.005), 2 (mean ± SEM of HDs VS pts(tp2) 5.918 ± 0.9519 N=8 VS 12.26 ± 1.925 N=17, p=0.04) and 4 (mean ± SEM of HDs VS pts(tp4) 5.918 ± 0.9519 N=8 VS 18.39 ± 3.448 N=9, p=0.005). A similar trend in NK alterations was also observed in each one of the groups of responders and non-responders. Responders had significantly higher NK cells than HDs at time points 0 (mean ± SEM of HDs VS Resp(tp0) 5.918 ± 0.9519 N=8 VS 12.93 ± 2.722 N=9, p=0.04), 1 (mean ± SEM of HDs VS Resp(tp1) 5.918 ± 0.9519 N=8 VS 12.51 ± 2.257 N=9, p=0.02), 2 (mean ± SEM of HDs VS Resp(tp2) 5.918 ± 0.9519 N=8 VS 12.71 ± 2.680 N=9, p=0.04), and 4 (mean ± SEM of HDs VS Resp(tp4) 5.918 ± 0.9519 N=8 VS 17.52 ± 3.783 N=8, p=0.01). Regarding non-responders, NK cells were significantly increased compared to HDs at time points 0 (mean ± SEM of HDs VS Non-resp(tp0) 5.918 ± 0.9519 N=8 VS 18.55 ± 4.871 N=8, p=0.02) and 1 (mean ± SEM of HDs VS Non-resp(tp1) 5.918 ± 0.9519 N=8 VS 17.93 ± 3.487 N=8, p=0.005). NK cell percentages were comparable between responders and non-responders at all time points. Repeated measures one-way ANOVA analysis revealed no significant impact of immunotherapy on NK cells in either group of responders (F=2.25, p=0.09) or non-responders (F=3.65, p=0.47) [Image 5]. Patients who developed irAEs (irAEs-pts) (n=4) had comparable percentages of NK cells to those of HDs at time points 0 and 1 (p=0.17 and p=0.12, respectively). In contrast, CPI-treated patients who did not develop irAEs (non-irAEs pts) had significantly increased NK cells compared to HDs at time point 0 (mean ± SEM of HDs VS non-irAEs pts(tp0) 5.918 ± 0.9519 N=8 VS 16.40 ± 3.200 N=14, p=0.03) and at time point 1 (mean ± SEM of HDs VS non-irAEs pts(tp1) 5.918 ± 0.9519 N=8 VS 16.74 ± 2.268 N=14, p=0.002). At time point 3, in the irAEs-patient group, NK cells were numerically decreased compared to HDs (mean ± SEM of irAEs-pts(tp3) VS HDs 2.905 ± 0.7387 N=4 VS 5.918 ± 0.9519 N=8, p=0.07) and significantly decreased compared to the non-irAEs patients (mean ± SEM of irAEs-pts(tp3) VS non-irAEs(tp3) 2.905 ± 0.7387 N=4 VS 9.840 ± 1.499 N=8, p=0.01). In contrast, in the non-irAEs patient group, NK cells were significantly increased compared to HDs (mean ± SEM of HDs VS non-irAEs(tp3) 5.918 ± 0.9519 N=8 VS 9.840 ± 1.499 N=8, p=0.04) [Image 6].

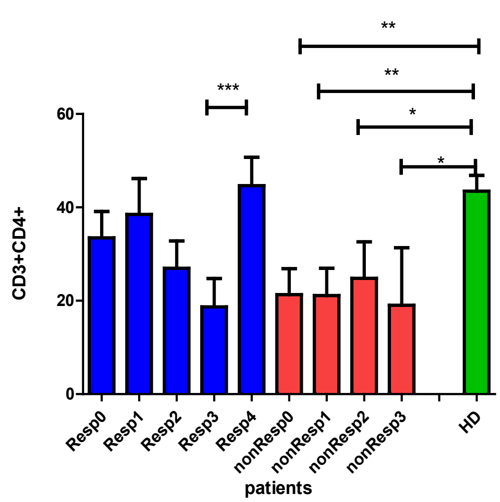



**Figure 1A:** Non-responders had significantly decreased total CD4+ lymphocytes compared to HDs at baseline (p=0.002) and at timepoints 1 (p=0.002), 2 (p=0.04) and 3 (p=0.02). Significant alterations in CD4+ lymphocytes during treatment were found only within responders (F=7.023, p=0.0005); after an initial post-treatment decrease up to 3 months, T helper lymphocytes increased significantly between the time points 3 and 4 (95% C.I.= [-41.63, -10.29]).

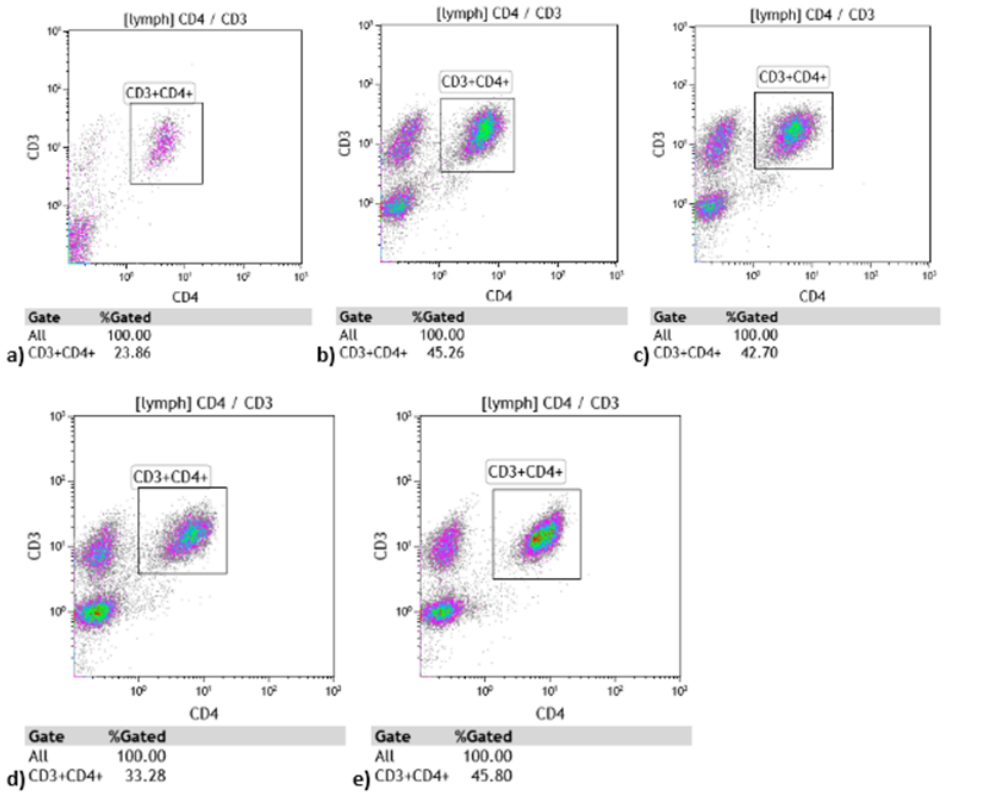



**Figure 1B:** sequential alterations in CD4+ T cells in one responder patient starting from timepoint 0 (graph a) to timepoint 4 (graph e).

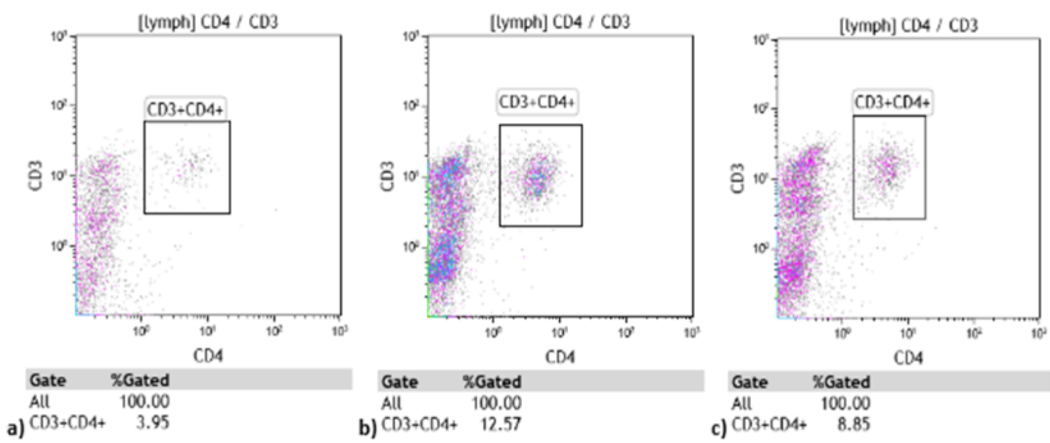



**Figure 1C:** sequential alterations in CD4+ T cells in one non-responder patient starting from timepoint 0 (graph a) to timepoint 3 (graph d).

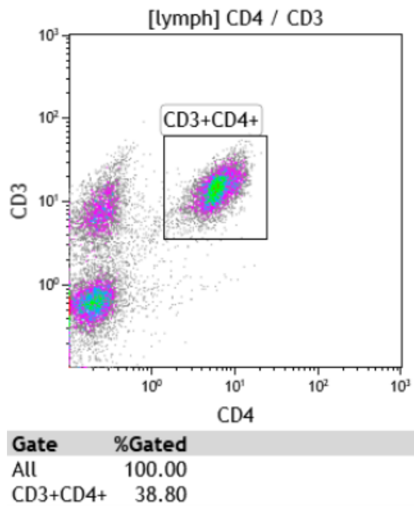
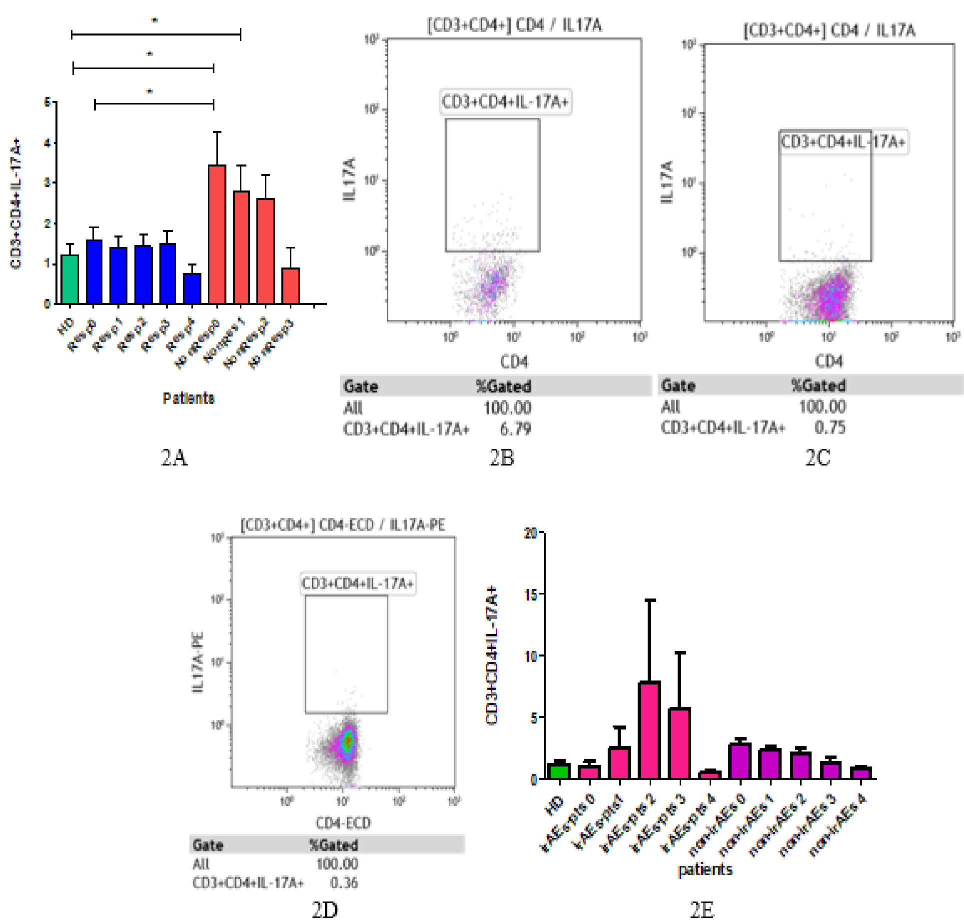
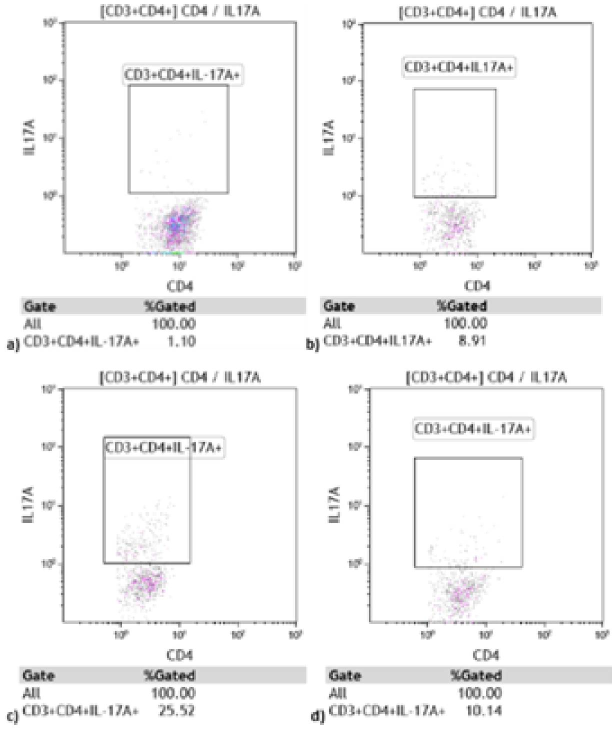


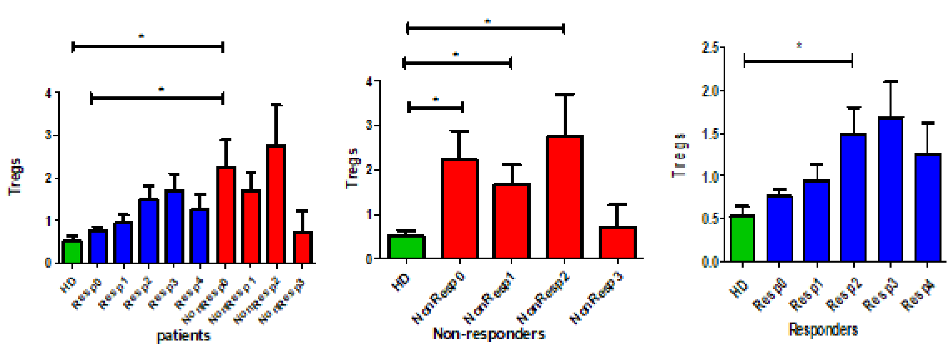

**Figure 1D:** CD4+ T cells in one HD.





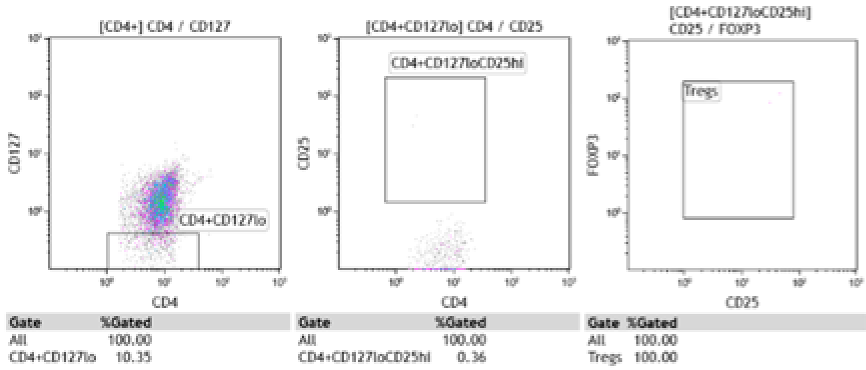

2E

**Figure 2A:** Non-responders had significantly increased Th17 lymphocytes compared to HD (p=0.03) and responders (p=0.047) at treatment initiation, and at timepoint 1 compared to HD (p=0.04). 2B,C,D: Th17 lymphocytes at timepoint 0 in one non-responder, one responder patient and one HD respectively. 2E: in patients who eventually developed irAEs (irAEs-pts) Th17 lymphocytes gradually increased up to the timepoint of 3 months, whereas no increases were noted in patients who did not develop irAEs (non-irAEs). 2F: Sequential alterations in Th17 lymphocytes from tp0 to tp3 (graphs a-d) in the autoimmune pneumonitis patient. The maximum increase in Th17 took place at tp2 (Graph c).

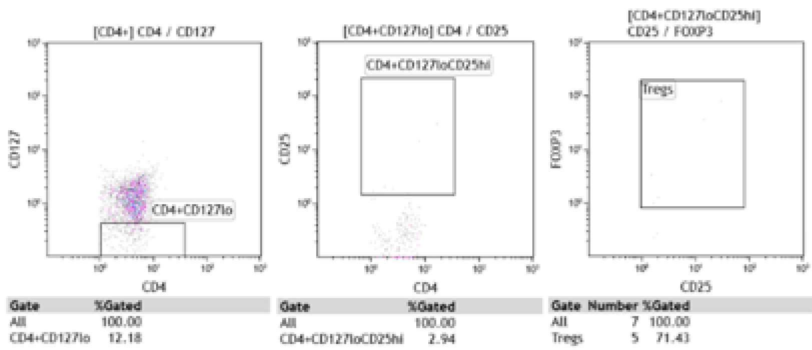



3A 3B 3C

**Figure 3A:** Alterations in Tregs in responders and non-responders. Non-responders had significantly increased Tregs at timepoint 0 compared to HDs (p=0.02) and responders (p=0.03). Figure 3B: No significant alterations in Tregs between the sequential time points within non-responders; Tregs continued to be significantly increased compared to HDs at time points 1 (p=0.02) and 2 (p=0.03). Figure 3C: Tregs were increased early after treatment initiation in the responders' group; at timepoint 2, Tregs were significantly increased compared to HDs (p=0.02).

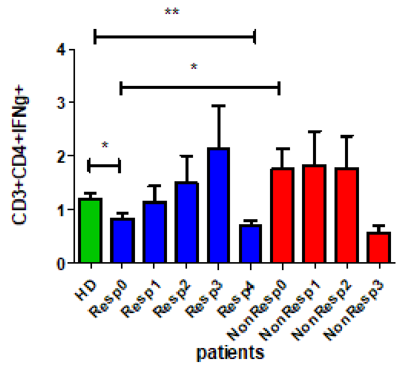



3D

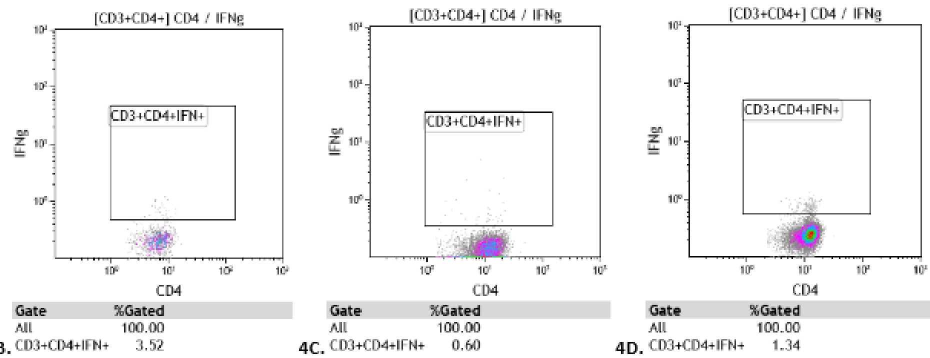



3E

**Figure 3D, 3E:** Tregs at timepoint 0 expressed as CD4+CD127loCD25hiFOXP3+ in one non-responder and one responder patient respectively.

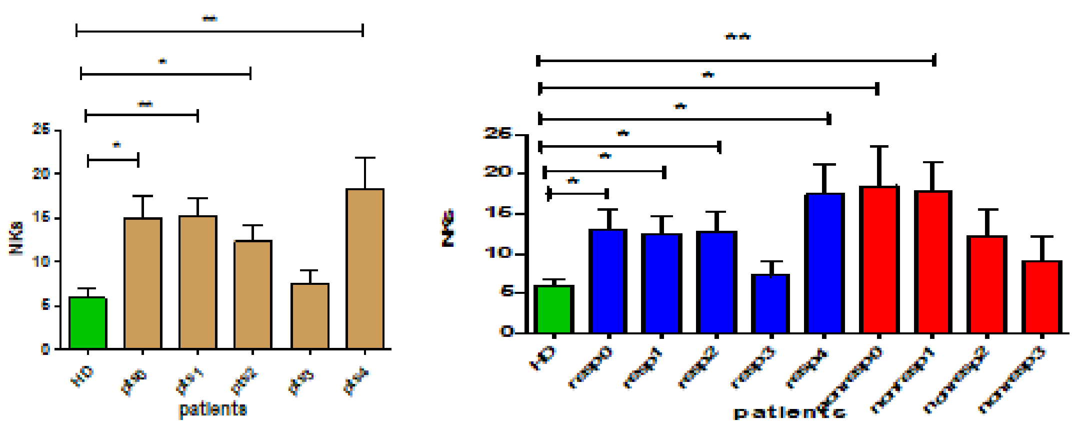



4A.




4B. 4C. 4D.

**Figure 4A:** Responders had significantly decreased CD3+CD4+IFNγ+ lymphocytes at baseline compared to HD (p=0.04) and non-responders (p=0.02). After an initial post-treatment numerical increase, responders significantly decreased CD3+CD4+IFNγ+ lymphocytes six months after treatment compared to HD (p=0.007). **Figure 4B, 4C,4D:** CD3+CD4+IFNγ+ lymphocytes at timepoint 0 in one non-responder patient, one responder patient and one HD respectively.




5A 5B

**Figure 5A:** All CPI-treated patients (pts) had significantly increased NK cells compared to HD at timepoints 0 (p=0.04), 1 (p=0.005), 2 (p=0.04) and 4 (p=0.005). Figure 5B: Similar trend of alterations in NK cells in each patient group and comparable percentages of NK cells between responders and non-responders.

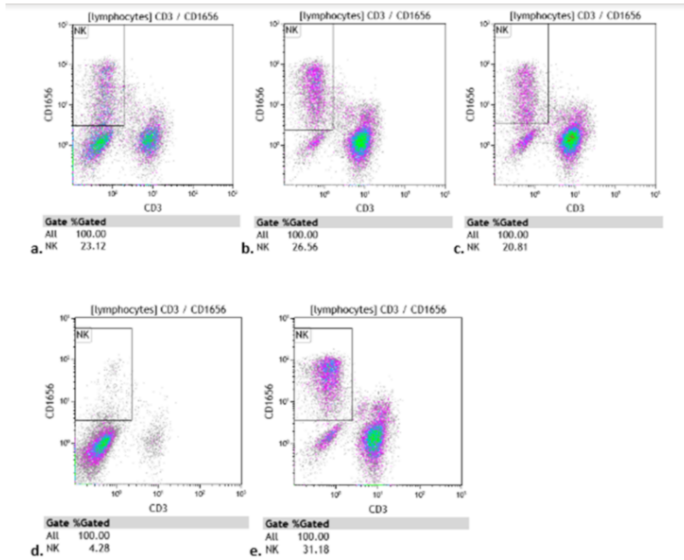



**Figure 5C:** sequential alterations in NK cells in one responder patient starting from timepoint 0 (graph a) to timepoint 4 (graph e).

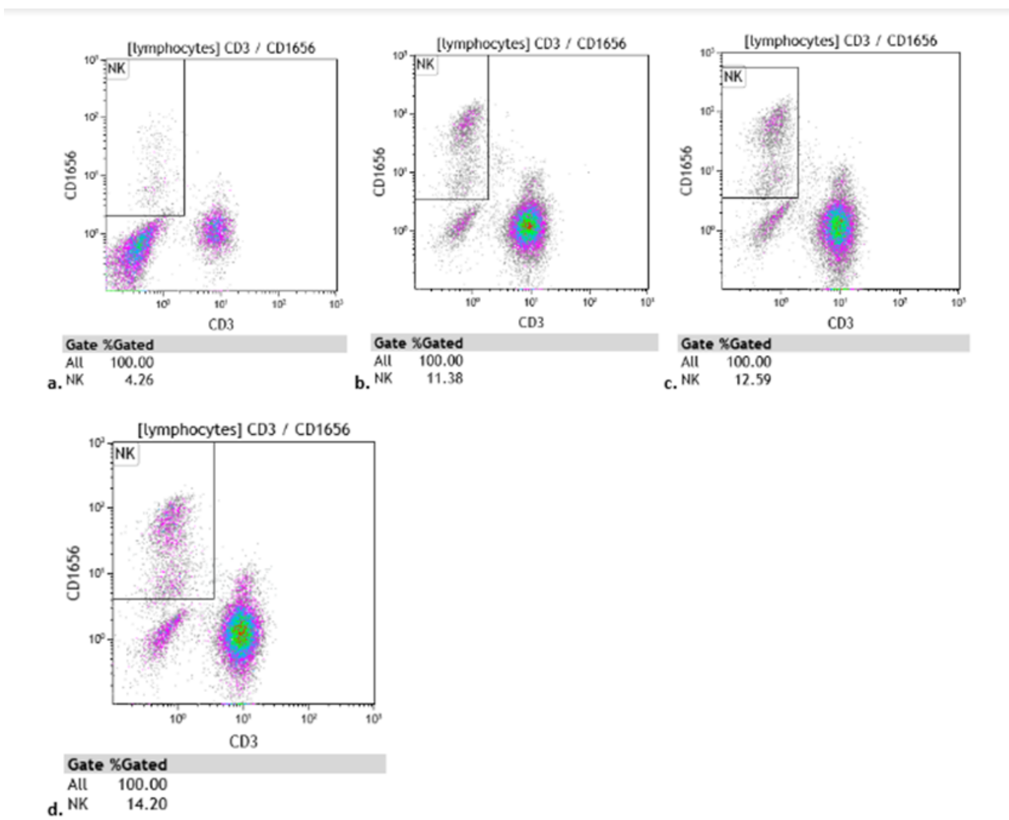



**Figure 5D:** sequential alterations in NK cells in one non-responder patient starting from timepoint 0 (graph a) to timepoint 3 (graph d).

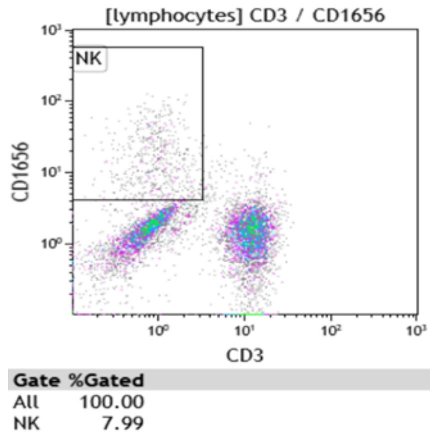



**Figure5E:** NK cells in one HD.

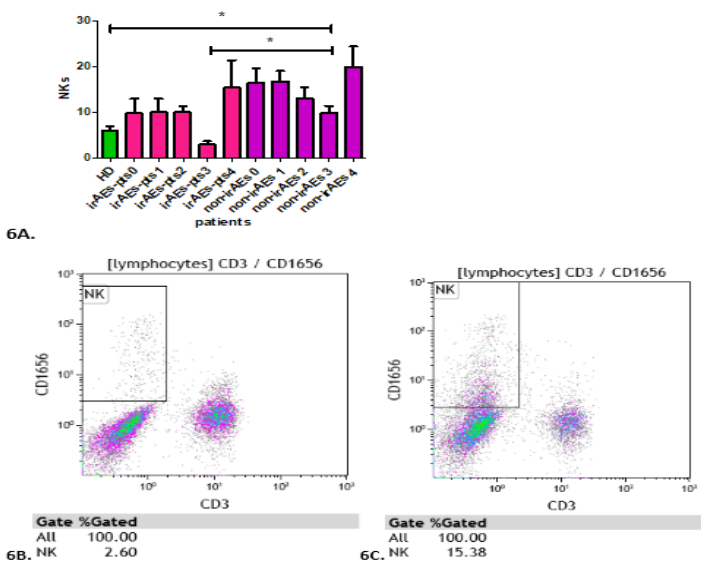



**Figure 6A:** At the timepoint of three months (tp3) patients who developed irAEs (irAEs-pts) (n=4) had significantly decreased NK cells compared to CPI-treated patients who did not develop autoimmune complications (non-irAEs pts) (p=0.01) and numerically decreased NKs compared to HDs. At this time point, NK percentages were significantly increased in the group of non-irAEs pts compared to HDs (p=0.04). 6B: NK cells from the patient with autoimmune pneumonitis at time point 3. 6C: NK cells from one patient with no irAEs at time point 3.

| Patient Number | Sex | Age At CPI Initiation | Type Of Malignancy/ Histology | Stage At CPI Initiation | CPI | Line Of Tx | Best Outcome On CPI | Timepoint Of Last Evaluation | irAEs |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 65 | NSCLC/ adenocarcinoma | IVB | nivolumab | 2 | PD† | tp2 | - |
| 2 | F | 47 | NSCLC/ adenocarcinoma | IIIC | nivolumab | 2 | PD | tp4 | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3 | M | 71 | NSCLC/ adenocarcinoma | IVA | nivolumab | 2 | PD† | tp2 | - |
| 4 | M | 70 | NSCLC/ adenocarcinoma | IVA | nivolumab | 2 | R | tp2 | - |
| 5 | M | 53 | NSCLC/ adenocarcinoma | IVA | nivolumab | 2 | PD | tp3 | - |
| 6 | F | 65 | NSCLC/ adenocarcinoma | IVB | nivolumab | 2 | R | tp4 | - |
| 7 | F | 62 | NSCLC/ adenocarcinoma | IVA | nivolumab | 2 | R | tp4 | thyroiditis |
| 8 | F | 64 | NSCLC/ adenocarcinoma | IVB | nivolumab | 2 | PD† | tp2 | - |
| 9 | M | 66 | NSCLC/ adenocarcinoma | IVB | nivolumab | 2 | PD† | tp1 | - |
| 10 | M | 76 | NSCLC/ adenocarcinoma | IVA | nivolumab | 2 | R | tp4 | thyroiditis |
| 11 | M | 64 | NSCLC/ adenocarcinoma | IIIC | nivolumab | 2 | PD | tp3 | - |
| 12 | M | 63 | NSCLC/ squamous cell carcinoma | IVA | nivolumab | 2 | R | tp4 | - |
| 13 | M | 80 | NSCLC/ squamous cell carcinoma | IVA | pembrolizumab | 1 | R | tp4 | colitis |
| 14 | F | 56 | NSCLC/ squamous cell carcinoma | IIIC | pembrolizumab | 1 | R | tp4 | - |
| 15 | M | 84 | NSCLC/ squamous cell carcinoma | IIIA | pembrolizumab | 1 | R | tp4 | - |
| 16 | M | 61 | invasive, high-grade UC | IVB | pembrolizumab | 2 | PD† | tp2 | - |
| 17 | M | 63 | invasive, high-grade UC | IVA | pembrolizumab | 2 | R | tp4 | - |
| 18 | M | 75 | invasive, high-grade UC | IVB | pembrolizumab | 2 | PD† | tp2 | - |
| 19 | M | 55 | invasive, high-grade UC | IV | pembrolizumab | 2 | non-evaluable† | tp3 | pneumonitis |
| 20 | F | 53 | RCC | IV | nivolumab | 2 | R | tp4 | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 21 | M | 60 | HNSCC | IVC | nivolumab | 2 | R | tp4 | - |
| CPI; checkpoint inhibitor, tp; time point, irAEs; immune-related adverse events, PD; progressive disease, R; response (complete response, partial response, stable disease), NSCLC; non-small cell lung carcinoma, UC; urothelial carcinoma, RCC; renal cell carcinoma, HNSCC; head and neck squamous cell carcinoma. Patients 8, 14, and 15 were excluded from the flow cytometric analysis due to non-evaluable blood samples at baseline. † : indicates patients who are deceased | | | | | | | | | |

**Table 1:** Patients characteristics and patients results.

| Target antigen | Clone | Isotype | Fluorochrome |
|---|---|---|---|
| CD4 | SFCI12T4D11 | IgG1 Mouse | ECD |
| CD3 | SK7 | Mouse IgG1, κ | PE-Cy™ 7 |
| CD25 | B1.49.9 | IgG2a Mouse | PE |
| CD127 | R34.34 | IgG1 Mouse | PC7 |
| FOXP3 | PCH101 | Rat / IgG2a, kappa | FITC |
| IL-17A | BL168 | Mouse IgG1, κ | PE |
| IFN-γ | 25723.11 | Mouse IgG2b | FITC |
| CD3/16/56 (antibody mix) | UCHT1/ 3G8/ N901 (NKH-1) | Mouse IgG1 | CD3-FITC/CD (16+56)-PE |

**Supplemental Table:** Flow cytometry antibodies used in all experiments/

## Discussion

In this prospective study, we included five consecutive time points of clinical evaluation and blood sampling, starting from CPI initiation and up to six months of treatment, to establish an easy and widely accessible marker for CPI response. This is one of the few studies to show T lymphocyte alterations at five serial time points. In the present work, we focused on circulating immune cells essential for anticancer immunity, namely total CD3+CD4+, CD3+CD4+ IFN-γ-producing lymphocytes, regulatory T cells, and NK lymphocytes. We also studied alterations in T helper 17 lymphocytes, which play a crucial role in the pathogenesis of many autoimmune conditions. Our aim was to correlate possible changes in immune cell subpopulations with the CPI-treatment response and the development of irAEs. CD4+ T cells are crucial mediators of immune responses, either effector or regulatory, owing to their ability to interact with immune cells and produce a variety of cytokines. An intact CD4+ T helper compartment gives rise to activated CD4+ helper lymphocytes, which represent the main source of IL-2. IL-2 is a potent stimulus for the proliferation and differentiation of lymphocytes, especially CD8+ cytotoxic and memory cells [34,35]. Recent data from melanoma patient's support that the non-responders to combination therapy with anti-CTLA4 and anti-PD-1 agents had lower CD4+/IL-2 gene signatures in pre-treatment biopsies and that the addition of IL-2 improved treatment responses ex vivo [36]. The potential implications of circulating total CD4+ lymphocytes in CPI-treatment responses have yet to be established. In this study, we showed that the baseline levels of CD3+CD4+ lymphocytes in the peripheral blood of non-responders were significantly lower than those in HDs (p=0.002), whereas responders and HDs had comparable levels of CD4+ T cells (p=0.09). Furthermore, immunotherapy had no significant impact on CD4+ lymphocytes in the non-responder group (F= 0.12, p= 0.89); CD4+ T cells within non-responders remained significantly decreased compared to HDs at time points 1, 2, and 3. In contrast, significant changes in CD4+ counts were noted in responders (F=7.023, p=0.0005); CD4+ counts increased significantly between 3 and 6 months. Our results are in accordance with previous studies which demonstrated that higher pre-treatment CD4+ levels and greater post-treatment increases in CD4+ levels are associated with better CPI responses [37–40], and with others showing CD4+ lymphocytes from CPI-treated non-responder patients to be less proliferative after antigen stimulation [41,42]. Current evidence from animal models and humans suggests that IL-17 may play an important role in tumorigenesis and tumour progression [43–50]. One possible explanation for the contribution of IL-17 to cancer development is via chronic inflammation that leads to the recruitment of neutrophils in the tumour microenvironment and the subsequent switch of neutrophil phenotype to myeloid-derived suppressor cells (MDSCs). MDSCs are currently known to suppress cytotoxic responses and produce angiogenic factors [43,51–53]. Reduced expression of Th17-associated genes at tumor sites has been correlated with longer progression-free survival rates in patients with colorectal cancer [54], whereas lower levels of Th17 in patients with pancreatic cancer have been associated with increased overall survival rates [55]. However, other studies have suggested a favourable contribution of the IL-17 pathway in anticancer immunity [56–58]. In the present study, we examined Th17 lymphocytes, which are the main source of IL-17. Non-responders had significantly increased pre-treatment Th17 compared to HDs (p=0.03) and responders (p=0.047), and despite an early post-treatment decrease, Th17 remained significantly increased compared to HDs at time point 1 (p=0.04). Although not statistically significant, Th17 levels within non-responders were numerically increased compared to HDs and responders at time points 2 and 3. In contrast, comparable levels of Th17 cells were observed between responders and HDs at all-time points. In accordance with our findings, Zhao et al. showed that lower levels of IL-17 after the second cycle of CPI treatment are associated with better clinical outcomes [59]. Besides their role in tumorigenesis, IL-17 and Th17 lymphocytes also play important roles in the pathogenesis of autoimmunity and inflammation [60–63]. In our study, in the small group of patients who developed irAEs, up to the 3rd month of CPI treatment, a numerical increase in Th17 percentages was noted until time point 3. In contrast, in CPI-treated patients who did not develop irAEs, a different alteration trend in Th17 lymphocytes was noted (a slight numerical decrease). Current evidence supports a positive association between higher Th17 percentages and soluble IL-17 with the risk of severe irAEs [64,65]. Additionally, the resolution of cutaneous toxicities secondary to PD-1 inhibition by IL-17 axis blockade has been reported [66], whereas in another study, IL-17 inhibition had a protective action against thyroid immune-related toxicities in patients receiving CPI [67]. Tregs can suppress effector T cell responses and facilitate, thus, malignant cells to evade of host's anticancer immunity [68–72]. Many studies have reported an increased prevalence of Tregs infiltrating tumor sites or circulating in the peripheral blood of patients with various malignancies [73–82]. Accumulating evidence suggests that increased levels of certain Treg subsets or total peripheral Tregs are associated with poor prognosis in patients with untreated cancer [83–85]. Bioinformatics analysis from patients with stomach adenocarcinoma suggests that higher Treg infiltration is correlated with lower survival rates [86]. In immunotherapy-treated patients, gene signature profiles of TIICs showed a negative correlation between infiltrating Tregs and CPI responses [87], whereas a high Treg-to-total infiltrating lymphocyte ratio has been associated with recurrence in patients with NSCLC in another study [88]. However, the potential role of circulating Tregs as predictive biomarkers in CPI immunotherapy remains unclear. Our results demonstrate that pre-treatment peripheral Tregs were significantly increased in non-responders compared to HDs (p=0.02) and responders (p=0.03), whereas baseline Tregs in responders were comparable to those in HDs (p=0.15). Kagamu et al. also showed higher percentages of pre-treatment Tregs within non-responders, while in another study lower baseline Tregs were correlated to better CPI responses [38,89]. Opposite results have been obtained in other studies, with significantly increased baseline Tregs characterizing CPI-responders [39,90,91]. However, it is important to note that only melanoma- and ipilimumab-treated patients were enrolled in one of these studies, whereas Tregs were not stained for intracellular FOXP3 in the other one. In addition, we found that the impact of immunotherapy on Tregs in non-responders was not statistically significant, with Tregs remaining almost unchanged and significantly increased compared to HDs at time points 1 and 2. In contrast, in the responder group, an increase was noted early after treatment initiation. This finding is consistent with literature data supporting that greater post-treatment increases in Tregs are associated with improved overall survival rates, progression-free survival rates, and treatment responses [39,92,93]. A CPI-induced decline in Treg immunosuppressive capacity could provide a possible explanation for Treg subpopulation expansion without reducing antitumor immunity in responders [94,74]. Polarization of T helper cells to the Th1 phenotype and the subsequent production of IFN-γ by the activated Th1 lymphocytes is essential for CD8+-mediated cytotoxic responses [95,96]. IFN-γ is also necessary for immune cells to migrate to the tumor microenvironment and may play an anti-angiogenic role [97]. Current evidence suggests that PD-1 inhibition enhances Th1 responses in terms of IFN-γ production in animals and humans [98–102]. It has also been shown that preserved IFN-γ-mediated gene signatures in tumour cells are associated with better CPI responses, whereas the loss of IFN-γ pathway genes is correlated with CPI resistance [103,104]. In the present work, it was shown that circulating IFN-γ-producing Th1 lymphocytes were significantly decreased in the responders at treatment initiation and after six months of treatment. A possible explanation for this finding is that besides activated Th1, other immune cells also produce IFN-γ, such as NKs and CD8+ [97]. Also, IFN-γ, besides its antitumor effect, may play a pro-tumorigenic role depending on the tumor microenvironment [105]. NK cells are crucial mediators of anticancer immune responses via either direct granzyme and performing mediated cytotoxicity against tumour cells or the production of effector cytokines, such as IFN-γ[96]. Our results indicated increased NK cells in both groups of responders and non-responders at all-time points, pre-and post-treatment, compared with HDs. It is important to note that NK cells were not significantly different between responders and non-responders, and CPI treatment did not significantly impact NK cells in either patient group. Hence, to our understanding, increased percentages of NK cells in the patient population studied are more likely to be an epiphenomenon of the underlying malignancy than of prognostic significance. Results from current literature regarding the NK cells in CPI-treated patients are controversial; according to recent reports higher baseline NK percentages or increases in NKs during treatment are related to better treatment responses, whereas decreased NK percentages are associated with poorer outcomes [38,39,106–110]. Current evidence also supports that phenotypically active NKs are increased in responders [111,112]. In contrast, few studies have demonstrated that CPI responders have lower baseline NKs [40,113]. However, our results did not confirm the prognostic value of NKs in CPI immunotherapy. NK cells in patients who developed irAEs (n=4) had a different alteration trend from that noted in non-irAEs patients; NK percentages in irAEs-patients were not significantly increased but comparable to those of HDs, whereas at timepoint 3, irAEs-patients had significantly decreased NK cells compared to the non-irAEs patients. However, the potential relationship between decreased pre- and early post-treatment NK cell levels and the risk of irAEs should be further validated.

**Conclusions**

Our study demonstrates that baseline flow cytometric analysis of certain peripheral T cell subpopulations may provide a useful and easily accessible tool for the stratification of patients more likely to respond to CPI treatment. Although only a small number of patients were included in the present study, the results are clear and can be used for further evaluation. A larger cohort will enable confirmation of the potential role of total CD3+CD4+, Th17, CD3+CD4+IFN-γ-producing, and regulatory T lymphocytes as prognostic biomarkers in anti-PD-1 immunotherapy at specific time points, as shown herein. The identification of easily accessible, cheap, and noninvasive tests to predict who will benefit the most from PD-1 inhibitors is needed for the best-personalized treatment options and minimization of the risk of adverse events.

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