



# Characterisation of clinical response and transcriptional profiling of proliferating CD8 T cells in the blood of cancer patients after PD-1 monotherapy or combination therapy

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## ABSTRACT

**Objective** Immune checkpoint inhibitors (ICI) that block the programmed cell death 1 (PD-1) pathway have shown promise with limited benefit. We and others have shown in small patient cohorts that an early proliferative CD8 T-cell response in the blood may be predictive of clinical response. However, these studies lack detailed analyses and comparisons between monotherapy and combination therapies.

**Methods and analysis** We analysed longitudinal blood samples from 103 patients with cancer who received  $\alpha$ PD-1 monotherapy or combined with anti-cytotoxic T lymphocyte-associated protein 4 ( $\alpha$ CTLA-4) or chemotherapy. Transcriptional analysis of CD8 T cells after the first treatment cycle with effector cells generated following yellow fever virus (YFV-17D) vaccine-induced infection was also compared.

**Results** An early proliferative (Ki-67<sup>+</sup>) CD8 T-cell response was observed after cycle 1 in 60 patients (58.3%). Patients with early-and-sustained proliferative responses (cycle 1 and beyond) had better clinical responses and survival than patients with an early-but-limited response ( $p=0.02$ ). The proliferating cells had an effector-like phenotype. The transcriptional profiles of the effector-like CD8 T cells were similar irrespective of treatment type or clinical response but distinct from that of YFV-specific effector CD8 T cells.

**Conclusions** Our data suggest that early proliferative CD8 T-cell response in the blood is predictive, and that an early-and-sustained proliferative response may further identify patients with prolonged survival. The ICI-induced effector-like CD8 T cells are transcriptionally distinct from highly functional YFV-specific cells, suggesting opportunities for improved T-cell effector function with combination therapies for better clinical outcome.

## INTRODUCTION

The expansion and reinvigoration of T cells by immune checkpoint inhibitors (ICI)

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ We and others have previously shown in small cohorts of patients that anti-programmed cell death 1 ( $\alpha$ PD-1) induced CD8 T cell proliferation in the blood is detected early within 2–3 weeks after the first infusion and early response correlates with clinical response.

## WHAT THIS STUDY ADDS

⇒ Our study is an extension of our previous study with a larger cohort where we confirm our previous findings. Here we further show that among early responders, patients who have a sustained proliferative CD8 response (beyond one cycle) have better clinical outcomes and survival compared with patients with limited CD8 response. We also show that immune checkpoint inhibitors (ICI)-induced effector-like CD8 T cells are not transcriptionally different between treatment modalities or clinical responses. Importantly, the effector-like cells generated after  $\alpha$ PD-1 therapy are transcriptionally and phenotypically different from highly functional effectors and memory cells generated after yellow fever vaccination.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our research shows that the transcriptional profile of CD8 effectors generated after ICI treatment leaves room for improvement and new combination therapies may be helpful in improving the quality of these CD8 effector cells.

such as antibodies against programmed cell death 1 (PD-1) and CTLA-4 has significantly impacted the clinical management of patients with cancer.<sup>1–7</sup> Immunological responses are

evident in up to two-thirds of patients with cancer who receive ICI.<sup>8,9</sup> However, the clinical response rates for patients treated with ICI alone or in combination with other therapies range between 20% and 50%.<sup>9–12</sup> Thus, there is a need to identify early predictive biomarkers to better assess clinical efficacy during treatment.

We have previously demonstrated that a burst of proliferative effector CD8 T cells is evident in the peripheral blood during the early treatment phase of PD-1 blockade and that this immunological response in the blood is a potential predictive biomarker for effective immunotherapy.<sup>8</sup> The proliferative CD8 T-cell response is not indiscriminate, as a minimal response is observed in Epstein-Barr virus-specific PD-1<sup>+</sup> cells. Other studies have reported similar findings showing the proliferation of PD-1<sup>+</sup> CD8 T cells in the blood as early as 1 week after the initial infusion of PD-1-targeted monotherapy in small cohorts of patients with melanoma and thymic tumours with evidence of the same T cell receptor (TCR) clonotypes present in the blood and tumour.<sup>9,13</sup>

However, the temporal patterns and predictive value of the proliferative CD8 T cells for patients who receive PD-1-targeted therapy in combination with other therapies such as chemotherapy and anti-CTLA-4 have not been determined. Additionally, the transcriptional profile of proliferating CD8 T cells induced by PD-1-targeted therapy in patients with cancer has not been directly compared with highly functional effector cells that are induced after acute viral infection in humans. In this study, we prospectively assessed the proliferating CD8 T cells in a larger cohort of 103 patients with 17 different cancer types. We compared the proliferative response and clinical outcomes in patients who received PD-1-targeted therapy alone or in combination with chemotherapy ( $\alpha$ PD-1/chemo) or anti-CTLA-4 ( $\alpha$ PD-1/ $\alpha$ CTLA-4). Furthermore, we evaluated the transcriptional profile of the proliferating PD-1<sup>+</sup> CD8 T cells from patients who received ICI and compared it to highly functional effector cells induced after vaccination with the live attenuated yellow fever virus vaccine (YFV-17D) that causes an acute infection.

## MATERIALS AND METHODS

### Study design and participants

Patients with cancer who received care at the Winship Cancer Institute at Emory University were consented after approval by an institutional review board. Study participants received PD-1-targeted therapy (pembrolizumab, nivolumab, atezolizumab or durvalumab) alone (68 patients) or in combination with chemotherapy (carboplatin and pemetrexed, 9 patients) or  $\alpha$ CTLA-4 therapy (ipilimumab or tremelimumab, 26 patients) every 2–4 weeks as part of standard care or as part of a clinical trial. Study participants (n=103) were enrolled in the study from April 2017 to September 2018. Baseline peripheral blood was drawn prior to the first treatment infusion on the same day. Post cycle treatment blood samples were collected between 2 and 6 weeks, immediately prior to

each subsequent treatment infusion. Clinical response to treatment was evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST) V.1.1 criteria on radiological restaging performed between 6 and 12 weeks after treatment initiation and review of oncology notes. Patients who had complete or partial response were grouped together as objective response. The clinical response data were obtained from the clinical notes in the electronic medical records of the patients and were reflective of the independent assessments of the treating oncologists and radiologists. The oncologists and radiologists did not have access to the proliferative data and were thus blinded to the immunological response. Baseline tumour burden was determined using the most recent imaging information prior to treatment initiation and estimated based on the RECIST V.1.1 criteria when available. Overall survival was assessed from the date of treatment initiation to the date of death or date of last visit/lost to follow-up at the conclusion of the study. Follow-up time ranged between 1 and 40 months. Immune-related adverse events were graded according to the American Society of Clinical Oncology guidelines.<sup>14</sup>

### Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated using BD vacutainer CPT tubes (BD Biosciences) according to the procedures described<sup>8</sup> and stained for markers of interest (CD3 (UCHT1), CD8 (RPTA-T8), CD4 (RPA-T4), FOXP3 (236A/E7), CD45RA (2H4LDH11LDB9), CCR7 (150503), Ki67 (B56), PD-1 (EH12.2H7), IgG4 (HP-6025), HLA-DR (1243), CD38 (HIT2), CD28 (CD28.2), TIM-3 (F38-2E2), and granzyme B (GB11)) after red blood cell lysis. The antibodies were purchased from BioLegend, eBiosciences or BD Biosciences. For patients who received  $\alpha$ PD-1 antibody as treatment (pembrolizumab or nivolumab), PD-1 expression was also detected using a biotinylated anti-IgG4 (Sigma) followed by a streptavidin-conjugated fluorophore (BioLegend). FOXP3 Fixation Kit (eBiosciences) was used for the intracellular staining of Ki67, FOXP3 and granzyme B. Near Infrared Dead Cell Stain Kit (Life Technology) was used to discriminate between live and dead cells from frozen PBMCs. Stained samples were acquired using the LSR II Flow Cytometer (BD Biosciences) and analysed using the FlowJo software (Tree Star). A proliferative (Ki67<sup>+</sup>) response was defined as a 1.5-fold or more increase from baseline Ki67 expression in the T cells.

### Cell sorting and RNA sequencing

CD38<sup>+</sup>HLA-DR<sup>+</sup>CD8<sup>+</sup> T cells from 13 patients with early proliferative responses were sorted from frozen PBMCs from post cycle 1 samples using an FACSria II (BD Biosciences) for bulk RNA sequencing analysis. Of these patients, eight had objective responses (complete or partial response; OR) or stable diseases (SD) and five progressed (PD) clinically. Nine of the patients received monotherapy (four clinical responders and five clinical non-responders) and four patients received  $\alpha$ PD-1/ $\alpha$ CTLA-4

(all clinical responders). Naïve ( $CCR7^+CD45RA^+$ ) CD8 T cells were also sorted from 9 of the 13 patients for comparison. RNA from the sorted cells was purified with the AllPrep DNA/RNA micro kit (QIAGEN) and sequenced on Illumina HiSeq platform. Following sequencing, reads were aligned to the human hg38 reference genome using HISAT2.<sup>15</sup> Expression counts were quantified using featureCounts function of the Subread package. Normalisation and differential expression analysis were done in R using the DESeq2 package.<sup>16</sup> RNA sequencing of naïve and YFV-tetramer<sup>+</sup> effector (days 14, 21 and 28) and long-term memory (>3 years) CD8 T cells was done as previously described.<sup>17</sup>

Transcriptomic expression analysis was performed in R using the edgeR package and the thresholds for identifying differentially expressed genes were set to false discovery rate (FDR) <0.05 and  $\log_2FC > 1$ . Raw counts were normalised by a model known as counts per million reads mapped for appropriate inter-sample comparisons. Principal component analysis (PCA) and heat maps of selected genes were generated with the R package 'ggbiplot' and 'pheatmap', respectively. For each comparison, we then performed Gene Set Enrichment Analysis (GSEA) to determine the overlap of differentially expressed genes by comparing them to various Molecular Signatures Database V.7.1. For comparison between patients with cancer and YFV vaccinees,<sup>17</sup> gene expression was normalised using corresponding naïve samples before further analysis.

### Statistical analysis

Categorical variables were summarised as frequency or percentages and evaluated using the  $\chi^2$  test or Fisher's exact test, where appropriate, with corresponding p values. Continuous variables were reported as median with range or mean with SD, and were evaluated using analysis of variance. Analysis of different T-cell subsets within the same sample was evaluated using the Wilcoxon test. The Kaplan-Meier method was used to generate survival curves which were then compared using the log-rank test. Statistical analyses were performed using the Prism software (GraphPad V.9.4.0) and SAS V.9.4 (SAS Institute, Cary, North Carolina, USA) and a significant value was set at less than 0.05. All tests were two-sided unless otherwise noted.

The Strengthening the Reporting of Observational Studies in Epidemiology cohort reporting guidelines were used.<sup>18</sup>

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

## RESULTS

### Patient characteristics and schematic diagram of PD-1-targeted treatment cycles

The clinicopathological characteristics of the patients are listed in online supplemental tables 1,2. Patients with

non-small cell lung cancer (NSCLC) comprised 48.5% (50/103 patients) of the study population, 8.7% (9/103 patients) had melanoma and 42.7% (44/103 patients) had other cancer types including urological, head and neck, hepatocellular and neuroendocrine malignancies. 68 patients (66%) received PD-1-targeted monotherapy and the rest received combination therapy (online supplemental table 1; therapy type and online supplemental table 2). Nine patients received  $\alpha$ PD-1/chemo and 26 patients received  $\alpha$ PD-1/ $\alpha$ CTLA-4. For T-cell analysis, blood was obtained from the patients at the indicated time points (figure 1A).

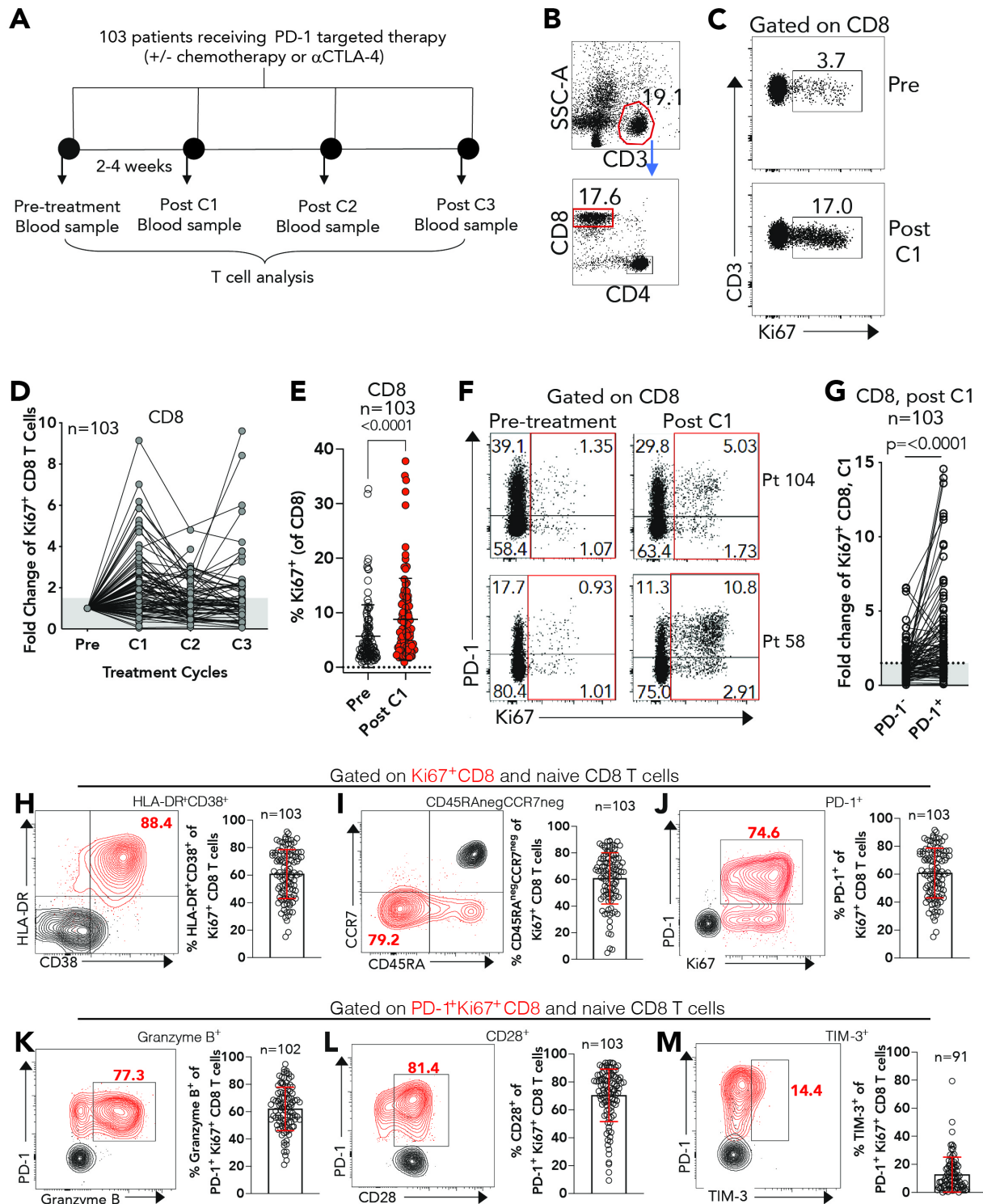
### Phenotype of proliferating T cells in the peripheral blood of patients receiving PD-1-targeted therapy

To evaluate the changes in the T-cell response in the blood after PD-1-targeted treatment, T cells were isolated from PBMCs at baseline and after each treatment cycle. The gating strategy for CD8 T cells is shown in figure 1B. The proportion of  $Ki67^+$  CD8 T cells in the blood varied between patients at baseline (0.9%–32.7%), but an expansion of these cells was evident for most of the patients after receiving PD-1-targeted therapy (figure 1C, D). The frequency of  $Ki67^+$  cells increased significantly after the first treatment cycle (post-C1) for most patients (figure 1D,E). Overall, 63 of 103 patients (61.2%) had a proliferative CD8 T-cell response of greater than or equal to a 1.5-fold increase in the frequency of  $Ki67^+$  cells at any time point over the course of three cycles of treatment. The major population of the proliferating CD8 T cells post-C1 was PD-1<sup>+</sup> (figure 1F,G) and these cells had an activated phenotype with most of the cells expressing human leukocyte antigen-DR isotype (HLA-DR) and CD38 (figure 1H). Most of the proliferating CD8 T cells also exhibited an effector memory-like ( $CD45RA^{neg}/CCR7^{neg}$ ) phenotype, and expressed PD-1 (figure 1I,J). Furthermore, most of the proliferating PD-1<sup>+</sup> CD8 cells expressed granzyme B (figure 1K) as well as co-stimulatory molecules such as CD28 (figure 1L). A small fraction of the PD-1<sup>+</sup> $Ki67^+$  CD8 T cells expressed inhibitory molecules like T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) (figure 1M). Increases in the proportions of  $Ki67^+CD4^+$ , total  $FOXP3^+CD4^+$  ( $T_{reg}$ ) and  $Ki67^+T_{reg}$  cells were also observed post-C1 (online supplemental figure 1); however, the largest fold increase was observed in the CD8 T-cell subset (2.3 vs 1.6 ( $CD4$ ) and 1.5 ( $T_{reg}$ )).

### Proliferative T-cell responses based on therapy type

We observed variation in the magnitude of the fold change in proliferating CD8 T cells irrespective of therapy type and there was no difference in the average fold change in  $Ki67^+$  CD8 T cells post-C1 (2.14 (monotherapy) vs 2.12 ( $\alpha$ PD-1/ $\alpha$ CTLA-4) vs 2.14 ( $\alpha$ PD-1/chemo); online supplemental figure 2A,B). Patients who received  $\alpha$ PD-1/chemo maintained on average,  $\geq 1.5$ -fold increase in  $Ki67^+$  cells over the treatment cycles. The average fold increase in proliferating CD8 T cells dropped to 1.4 and then





**Figure 1** Peripheral CD8 T-cell response and phenotype after PD-1-targeted therapy. (A) Schematic diagram of treatment, blood collection, and T-cell analysis from patients. (B) Gating strategy for CD8 T-cell analysis by flow cytometry. (C) Representative dot plots of the proportion of Ki67<sup>+</sup> of CD8 T cells before and after cycle 1 (post C1). (D) Fold change of Ki67<sup>+</sup> CD8 T cells before and after treatment cycles. Shaded area in the graphs indicates the 1.5-fold cut-off for an early proliferative response. (E) Summary plot of the percent Ki67<sup>+</sup> CD8 T cells before and after cycle 1. (F) Representative dot plots from two patients showing pretreatment and post-C1 proportion of Ki67 and PD-1 expression in CD8 T cells. (G) Fold change in Ki67<sup>+</sup> CD8 T cells in PD-1<sup>neg</sup> and PD-1<sup>+</sup> subsets for each patient after the first cycle of treatment. (H–J) Representative contour plots and summary graphs of CD38 and HLA-DR (H) CD45RA and CCR7 (I) and PD-1 (J) expression on Ki67<sup>+</sup> CD8 T cells (red) in comparison with naïve CD8 T cells (black). (K–M) Representative contour plots and summary graphs for granzyme B (K) co-stimulatory molecules (CD28 (L) and inhibitory molecule TIM-3 (M) expression on PD-1<sup>+</sup>Ki67<sup>+</sup> CD8 (red) and naïve (black) cells after C1. Bar graphs/horizontal lines and error bars represent mean and SD, respectively. P values represent Wilcoxon tests. PD-1, programmed cell death 1.

increased to 2.52 after cycles 2 and 3, respectively, in the  $\alpha$ PD-1/ $\alpha$ CTLA-4 cohort. In the PD-1-targeted monotherapy group, the fold change in Ki67<sup>+</sup> cells fell to 1.26 and 1.4 after cycles 2 and 3, respectively. The fold increase in proliferating cells was significantly higher in the PD-1<sup>+</sup> subset in the monotherapy and  $\alpha$ PD-1/ $\alpha$ CTLA-4 groups (online supplemental figure 2C). In general, there was minimal variation in the proportion of circulating T<sub>reg</sub> cells irrespective of treatment type (online supplemental figure 3). There was no reduction in the proportion of T<sub>reg</sub> cells and the proportion of proliferating T<sub>reg</sub> cells significantly increased post-C1 only in the monotherapy group.

### Clinical response and overall survival in patients treated with PD-1-targeted therapy

At radiological restaging and clinical review based on RECIST V.1.1 criteria, OR was achieved in 11 patients (10.6%), SD was observed in 53 patients (51.5%) and PD was noted in 32 patients (31.1%) (online supplemental table 3). Similar clinical responses were observed in patients irrespective of sex, age, smoking status, performance status, prior radiation status, tumour burden, tumour type or the type of therapy received. However, patients who received PD-1-targeted therapy as first-line therapy had better clinical responses, with 9 of 44 (20.4%) patients achieving OR in contrast to 1 of 44 patients who received PD-1-targeted therapy as second line or later line of therapy (online supplemental table 3; line of PD-1-targeted therapy). Patients who had grade 2–4 immune-related adverse events (irAEs) also had better clinical responses in comparison to patients who had grade 1 or no irAE (online supplemental table 3; irAEs). The fold change in proliferative CD8 T-cell response over the weeks of treatment for all study participants is presented by the clinical response in online supplemental figure 4.

Quantitative changes in tumour burden from baseline available for all the study participants are shown using waterfall plot (figure 2A). Patients with OR or SD after initiation of any modality of PD-1-targeted therapy had improved overall survival compared with patients with PD (figure 2B). The survival trends were similar among patients who received PD-1-targeted monotherapy alone (figure 2C). However, no significant differences in overall survival were observed among patients who received combination therapy based on clinical response (figure 2D).

### Early proliferative CD8 T-cell response in the peripheral blood is associated with clinical response

We previously ascertained that a proliferative CD8 T-cell response of  $\geq 1.5$ -fold above baseline Ki67<sup>+</sup> cells within 4–6 weeks after the beginning of treatment may be associated with improved clinical outcome.<sup>8</sup> To validate these findings, we categorised patients into early or delayed/no proliferative response groups based on the detection of  $\geq 1.5$ -fold increase in Ki67<sup>+</sup> CD8 T cells within 4 weeks post-C1 (C1; early proliferative response) or at

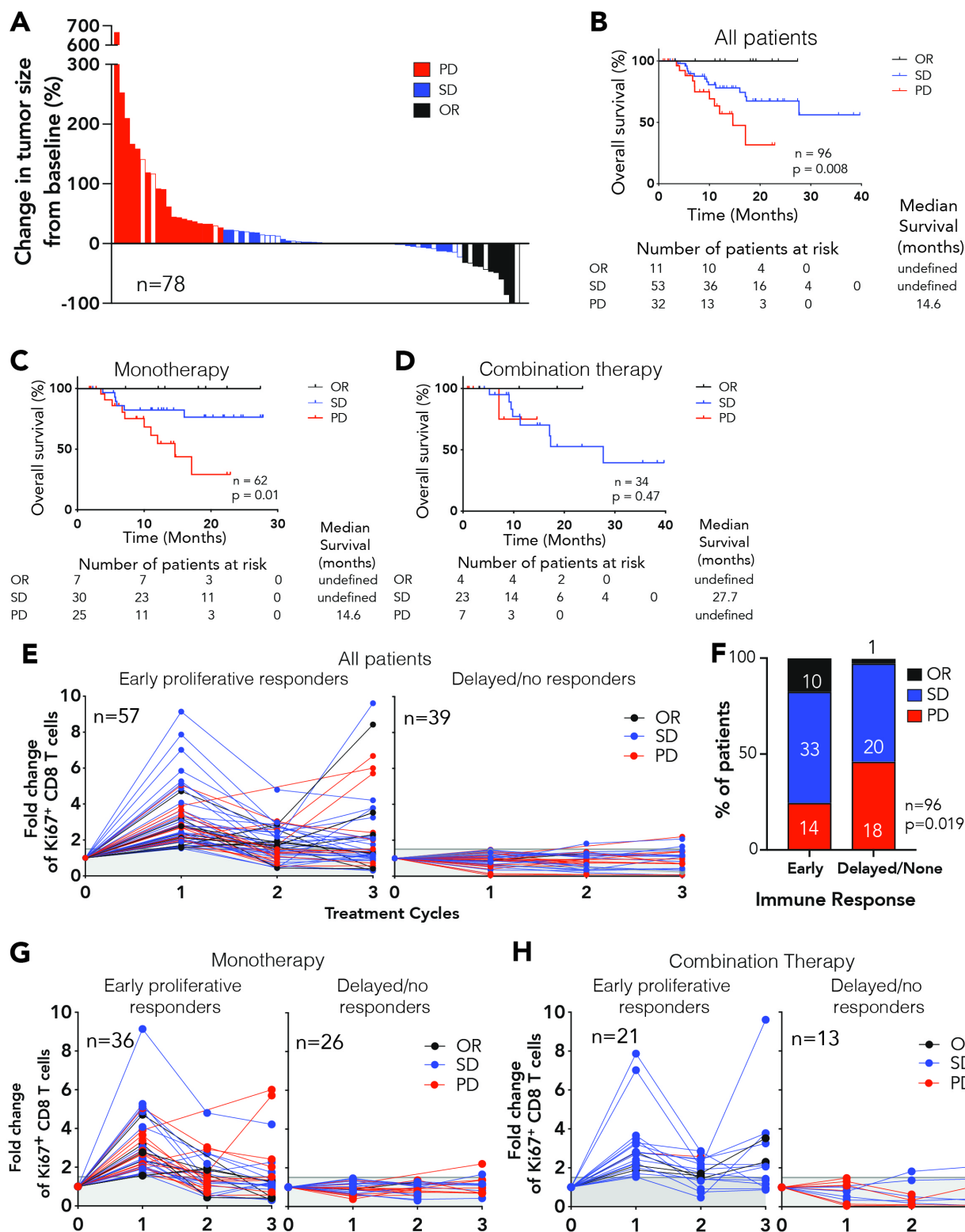
later time points (delayed/no proliferative response). 60 of 103 patients (58.3%) had an early proliferative response. Much less variation in the proportion of Ki67<sup>+</sup> cells was seen in patients with delayed/no proliferative response over the three cycles of treatment and most of them (>90%) did not reach the threshold for proliferative response at any time point during treatment. Radiological restaging or clinical review could not be obtained for seven patients (6.8%) due to symptomatic deterioration and were thus not included in the clinical response analyses.

Of the 96 patients with available clinical response data, an early proliferative CD8 T response was detected in 57 patients (59.4%) while 39 patients (40.6%) had a delayed/no response (figure 2E). As we reported before, early proliferative response was associated with clinical response (figure 2F). Ten (17.5%) patients with early proliferative CD8 T-cell response achieved OR in comparison with 1 patient (2.6%) in the delayed/no response group. 36 of 62 patients (58.1%) who received monotherapy and 21 of 34 patients (61.8%) who received combination therapy had an early proliferative response (figure 2G,H). Seven of nine patients (77.8%) who received  $\alpha$ PD-1/chemo had an early proliferative response and 14 of 26 patients (53.8%) who received  $\alpha$ PD-1/ $\alpha$ CTLA-4 had an early proliferative response (online supplemental figure 5A,B). In our patient cohort, no significant differences in clinical response were seen in patients based on tumour type or PD-1-targeted monotherapy (online supplemental table 4). However, a strong association was observed between early proliferative response and clinical response in patients who received combination therapy. Of the 25 patients who received  $\alpha$ PD-1/ $\alpha$ CTLA-4, 13 of 14 (92.9%) of the early proliferative responders achieved OR or SD (online supplemental figure 5C). All the patients who received  $\alpha$ PD-1/chemo achieved OR (two early proliferative responders) or SD (five early proliferative responders and two delayed/non-responders; online supplemental figure 5D). Among the early proliferative responders, clinical response was significantly associated with NSCLC, combination therapy, irAE and PD-1-targeted therapy as first-line treatment (online supplemental table 5).

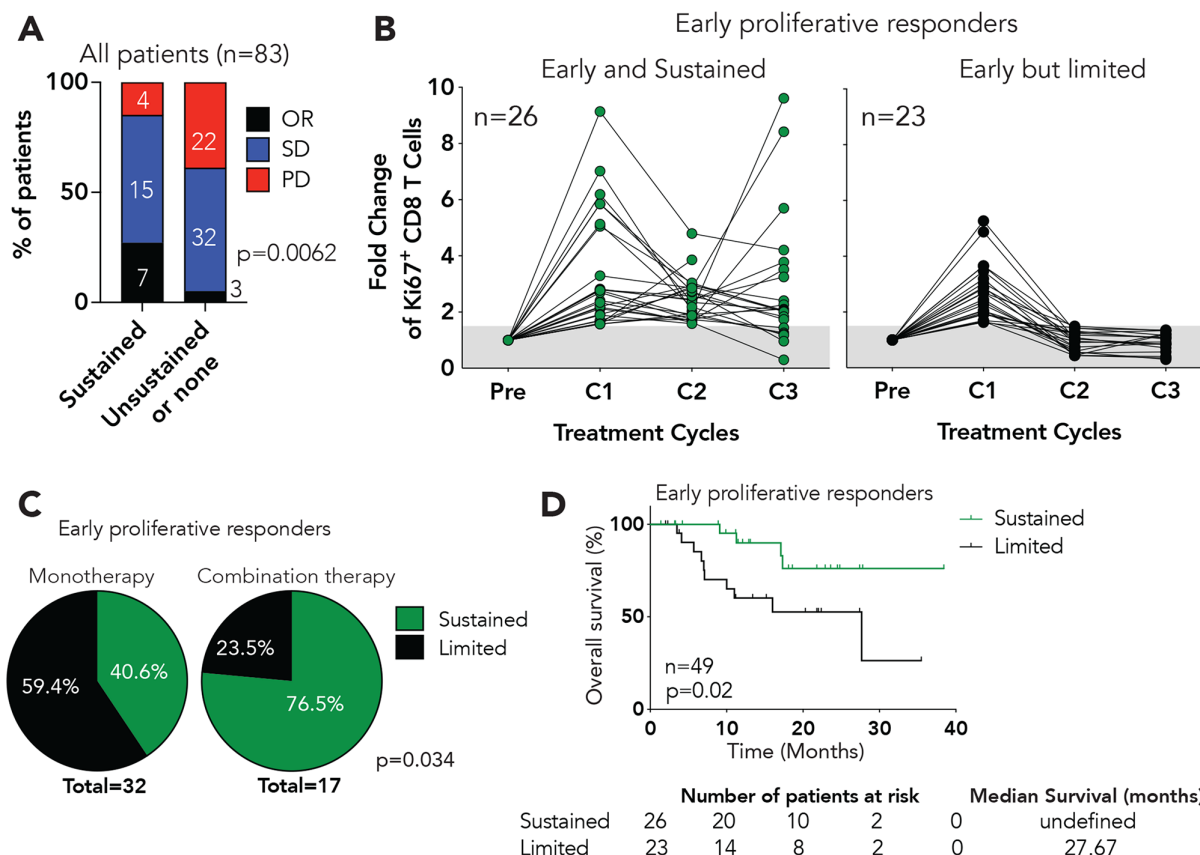
### Early-and-sustained proliferative response is associated with clinical response and overall survival

A subset of patients maintained a proliferative response of  $\geq 1.5$ -fold increase in Ki67<sup>+</sup> CD8 T cells over at least two consecutive cycles of treatment. Therefore, we evaluated the impact of sustained proliferative CD8 T-cell responses on the clinical outcome of the study participants. A complete set of blood samples from post-C1, post-C2 and post-C3 were available from 85 of 103 patients. 56 of 85 patients received PD-1-targeted monotherapy and 29 patients received combination therapy, of which 9 received  $\alpha$ PD-1/chemo and 20 received  $\alpha$ PD-1/ $\alpha$ CTLA-4.

27 of 85 patients (31.8%) maintained a  $\geq 1.5$ -fold increase in Ki67 over two cycles of treatment. Of those patients, 26 of 27 had sustained proliferative responses



**Figure 2** Association of early proliferative response with clinical response and overall survival. (A) Waterfall plots showing change in tumour size at the time of follow-up imaging (6–12 weeks) from baseline for all patients. Patients who received monotherapy are indicated with closed bars and those who received PD-1/chemo or PD-1/CTLA-4 are indicated with open bars. (B–D). Overall survival of all patients (B) patients who received monotherapy (C) and those who received combination therapy (D) based on clinical response. OR (objective response), SD (stable disease), PD (progressive disease). P values of log-rank test. (E) Spider plot showing fold change of Ki67<sup>+</sup> CD8 T cells after each cycle of treatment for early proliferative responders and delayed/no responders. (F) Proportion of patients with early or delayed/no proliferative response who had OR, SD, or PD (p value of  $\chi^2$  test). (G–H) Spider plots showing fold change of Ki67<sup>+</sup> CD8 T cells after each treatment cycle in patients who received monotherapy (G) or combination therapy (H). Shaded area in the graphs (E, G, H) indicates the 1.5-fold cut-off for early proliferative responses. PD-1, programmed cell death 1.



**Figure 3** Association between sustained proliferative response and clinical response and overall survival. (A) Clinical response of patients who had sustained vs unsustained or no proliferative response; p value of  $\chi^2$  test. (B) Fold change in Ki67<sup>+</sup> CD8 T cells from patients with early-and-sustained proliferative responses and patients with early-but-limited proliferative responses. Shaded area represents 1.5-fold cut-off for early proliferative responses. (C) Proportion of patients with early-and-sustained proliferative responses and those with early-but-limited responses among patients who received PD-1-targeted monotherapy, PD-1/chemo, or PD-1/CTLA-4. P value is of Fisher's exact test. (D) Overall survival of early-and-sustained proliferative responders and early-but-limited proliferative responders. P value of log-rank test. OR, objective response; PD, progressive disease; PD-1, programmed cell death 1; SD, stable disease.

between C1 and C2 while one patient had a sustained response over C2 and C3. 16 patients had sustained proliferative responses over all three cycles. Two of 85 patients had symptomatic deterioration and one of those patients had sustained proliferative responses. 26 of 83 patients (31.3%; excluding the 2 patients with symptomatic deterioration) maintained a  $\geq 1.5$ -fold increase in Ki67 expression over two consecutive cycles and this sustained response was also strongly associated with clinical response (figure 3A). This cohort of 26 patients included five who received  $\alpha$ PD-1/chemo, 9 who received  $\alpha$ PD-1/ $\alpha$ CTLA-4 and 12 who received PD-1-targeted monotherapy.

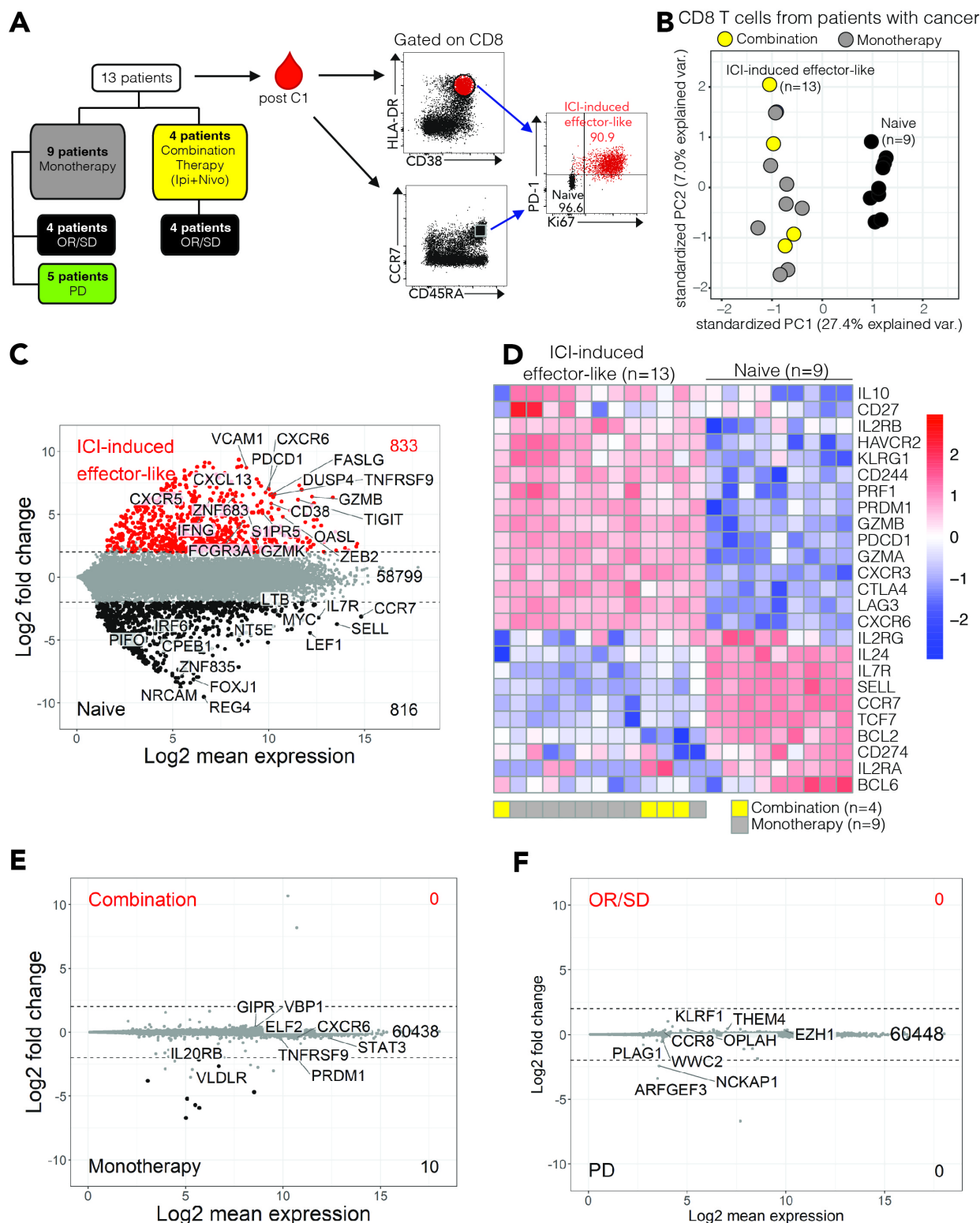
To further evaluate the associations between sustained proliferative responses with early proliferative responses and treatment type, we categorised patients with early proliferative responses into early-and-sustained ( $\geq 1.5$ -fold increase in Ki67 post-C1 and C2) and early-but-limited ( $\geq 1.5$ -fold increase in Ki67 post-C1 only) groups (figure 3B). More patients who received combination therapy had early-and-sustained proliferative responses than the monotherapy group (figure 3C). Majority of the patients who received  $\alpha$ PD-1/chemo (5/6; 83.3%)

and those who received  $\alpha$ PD-1/ $\alpha$ CTLA-4 (8/11; 72.7%) had sustained proliferative responses. In comparison with the subset of patients with an early-but-limited proliferative response, patients with an early-and-sustained proliferative response had significantly better overall survival (figure 3D).

#### Transcriptional profile of proliferating ICI-induced effector-like CD8 T cells from the peripheral blood of patients with cancer

RNA sequencing analysis was performed on bulk activated effector-like CD8 T cells sorted from the post-C1 blood samples of 13 early proliferative responders (figure 4A). Naïve cells were also sorted from 9 of the 13 patients for comparison. As expected, PCA showed that naïve cells from the patients clustered together and were distinct from the ICI-induced effector-like cells (figure 4B,C). Similar to what was observed by flow cytometric analysis, a heat map of selected immune marker genes in cells revealed a very distinct transcriptional profile of the effector-like cells in comparison to the naïve cells (figure 4D). The ICI-induced effector-like cells expressed higher levels of





**Figure 4** Transcriptional profile of ICI-induced effector-like CD8 T cells from the peripheral blood of patients with cancer. (A) Patient samples and sorting strategy for ICI-induced effector-like (HLA-DR<sup>+</sup>CD38<sup>+</sup>) and naïve (CD45RA<sup>+</sup>CCR7<sup>+</sup>) CD8 T cells from early proliferative responders for bulk RNA sequencing (after cycle 1). (B) Principal component analysis of the naïve and ICI-induced effector-like CD8 T cells. (C) MA plot displaying the log2 fold-change compared with log2 mean expression generated using a DESeq2 data set, with default log2 fold-change thresholds of -2 and 2 for naïve CD8 T cells versus ICI-induced CD8 T cells. (D) Heat-map of relative expression of selected immune-related genes in naïve and ICI-induced effector-like CD8 T cells from the peripheral blood of early proliferative responders. Scales of color represent Z-scores. (E–F) MA plot displaying the log2 fold-change compared with log2 mean expression for combination therapy versus monotherapy (E) and PD versus OR/SD responses (F). ICI, immune checkpoint inhibitors; Ipi, ipilimumab; Nivo, nivolumab; OR, objective response; PD, progressive disease; SD, stable disease.



effector molecules such as *GZMB*, *GZMA* and *PRF1* and inhibitory molecules such as *PDCD1*, *HAVCR2*, *CTLA-4*, *LAG3* and *CD244*. In contrast, the naïve cells expressed higher levels of *IL7R*, *SELL*, *TCF7*, *CCR7* and *IL2RA*.

### Effector-like CD8 T cells have similar transcriptional profiles irrespective of treatment type or clinical response

Comparative analysis between effector-like CD8 T cells from the PD-1-targeted monotherapy and  $\alpha$ PD-1/ $\alpha$ CTLA-4 groups yielded minimal differences in the number of differentially expressed genes (figure 4E). In fact, we observed only 117 genes differentially expressed between monotherapy and combination therapy. We also evaluated the transcriptional profile of patients who showed clinical response (clinical responders) from patients who progressed (non-responders). Of note, all the clinical non-responders received monotherapy. Similar to our observations for treatment type, there were minimal differences in differentially expressed genes between clinical responders and non-responders (figure 4F).

### Comparison of the transcriptional profiles of effector-like CD8 T cells induced by ICI in patients with cancer and highly functional effector cells induced by acute yellow fever infection in healthy volunteers

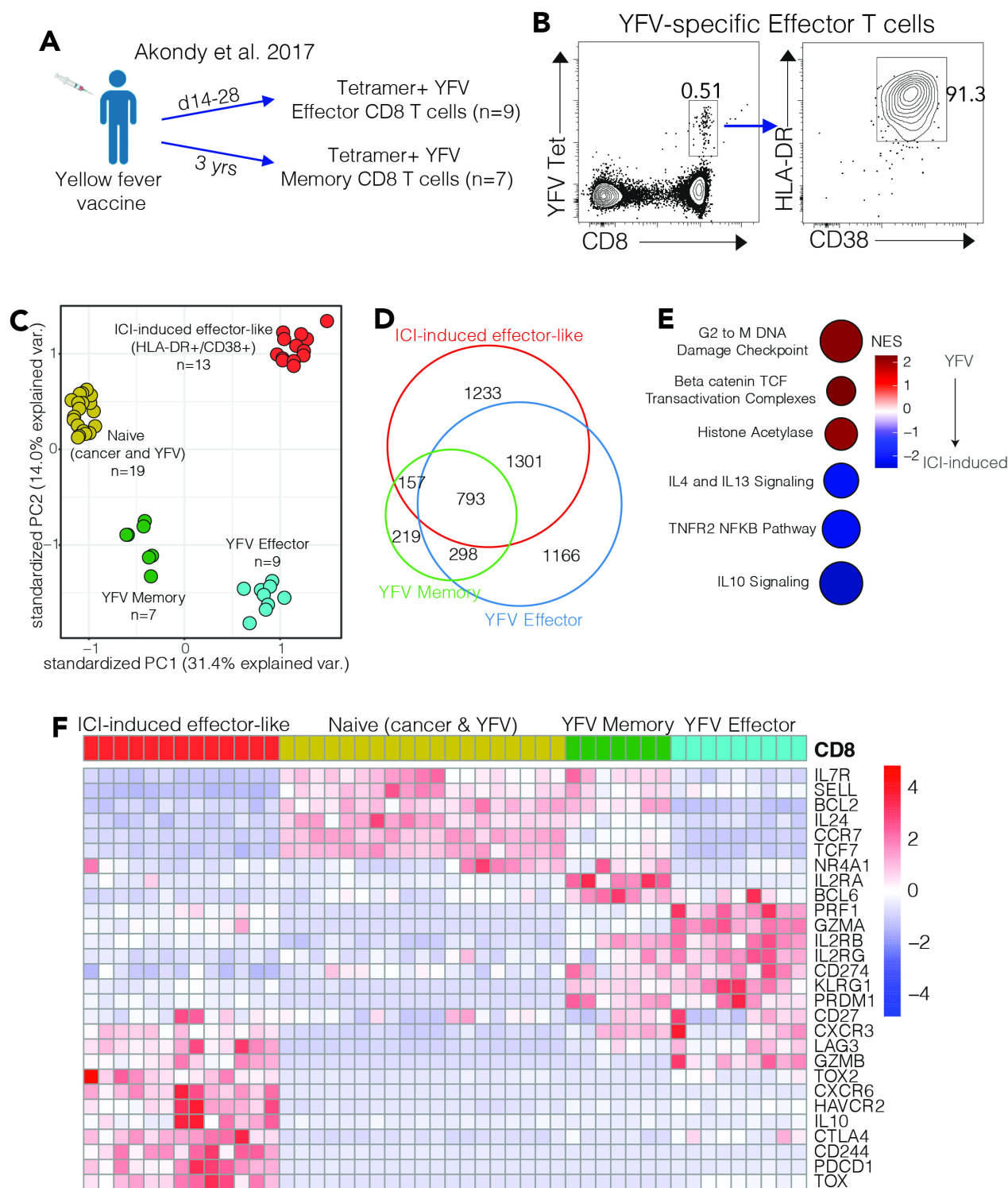
Blockade of the PD-1 pathway results in a proliferative burst and the generation of new effector cells from PD-1<sup>+</sup> stem-like progenitor CD8 T cells<sup>19</sup>. However, how the transcriptional profile of the new effector cells compares to that of highly functional effectors has not been evaluated. Effector YFV-specific CD8 T cells isolated between days 14 and 28 after immunisation are highly functional effectors that confer protective immunity.<sup>17</sup> Thus, we investigated whether the ICI-induced effector-like cells proliferating after cycle 1 were similar to effector cells generated after YFV. We compared the ICI-induced effector-like cells with conventional effector and memory cells that develop after the resolution of the acute viral infection (figure 5A,B). PCA showed that effector cells generated after YFV were quite different from the effector-like cells that were induced by ICIs in our cohort (figure 5C). Furthermore, memory cells derived from the YFV also clustered separately from naïve, YFV-induced effectors and the ICI-induced effector-like cells. There were approximately 2400 differentially-expressed genes (DEGs) between the ICI-induced effector-like cells (compared with naïve cells from patients with cancer) and the YFV effector cells (compared with naïve cells from YFV), and approximately 2000 DEGs were shared between the two effector states (figure 5D). Many immune-related genes were also differentially expressed. GSEA analysis focused on immune-related genes demonstrated upregulation of gene sets related to interleukin 10 (IL-10), IL-4 and IL-13 signalling, and nuclear factor kappa light chain enhancer of activated B cells (NFkB) pathway in the ICI-induced effector-like cells (figure 5E). In the YFV-specific effector cells, there was an enrichment for histone

acetylase, beta-catenin/T cell factor (TCF) transactivation complexes and DNA damage checkpoint genes. Effector markers such as *PRF1*, *GZMA*, *IL2RB*, *IL2RG*, *KLRG1* and *PRDM1* were more upregulated in effector YFV-specific cells than the ICI-induced effector-like cells (figure 5F). In contrast, more inhibitory markers such as *TOX*, *HAVCR2*, *CTLA4*, *CD244* and *PDCD1* were upregulated in the ICI-induced effector-like cells similar to the transcriptional profile of effector cells in other tumour and chronic viral models.<sup>19–23</sup> Some effector and inhibitory markers such as *GZMB* and *LAG3* were upregulated on both effector YFV-specific and ICI-induced effector-like cells. Additional differences in genes involved in transcriptional regulation (eg, *EOMES*, *PRDM1*, *LEF1* and *FOXPI*), kinases (eg, *MTOR*, *LCK*, *JAK2* and *PIK3CA*), cytokine expression (eg, *XCL1*, *XCL2*, *CXCL13*, *CXCL16*, *IL-10*) and G-protein receptors (eg, *NR4A3* and *SIPR4*) were observed between the ICI-induced effector-like and YFV-specific effector cells (online supplemental figure 6).

## DISCUSSION

Cancer immunotherapy using ICI is being rapidly integrated into the standard of care for patients with cancer. However, only a fraction of patients derives clinical benefit. Thus, there is a need to identify predictive biomarkers of response for patients receiving ICI. As we and others have shown, tumour-specific CD8 T cells are present in the blood<sup>9 24 25</sup> and T-cell responses to PD-1 blockade can be monitored by assessing changes in Ki67<sup>+</sup>PD-1<sup>+</sup> CD8 T cells in the blood over the course of treatment.<sup>8 9 24</sup> We previously determined that proliferating PD-1<sup>+</sup> CD8 T cells in the blood during PD-1-targeted monotherapy may be a useful tool in predicting clinical response in a small cohort of patients with NSCLC.<sup>8</sup> We report similar findings in this study with a larger cohort and multiple cancer types for PD-1-targeted monotherapy and extend our findings to  $\alpha$ PD-1/chemo and  $\alpha$ PD-1/ $\alpha$ CTLA-4 combination therapies. In addition, we now demonstrate an association between early proliferative response and overall survival. Furthermore, an early-and-sustained proliferative response appears to be a stronger predictor of clinical response and overall survival, suggesting that the administration of at least two cycles of PD-1-targeted therapy may be necessary for better prognostication of clinical outcomes. An in-depth analysis of the transcriptional profile of the proliferative cells in blood induced after monotherapy or  $\alpha$ PD-1/ $\alpha$ CTLA-4 showed that these cells had similar transcriptional profile irrespective of clinical outcome (clinical responders vs non-responders) or treatment type (monotherapy vs combination therapy). In contrast, these ICI-induced effector-like cells from patients were transcriptionally distinct from highly functional effector and memory T cells derived from YFV.

Analysis of the transcriptional gene signature of T cells provides important insights into functional states and strategies to improve function. To our knowledge, a comparison of the transcriptional profile of ICI-induced



**Figure 5** Comparison of transcriptional profiles between ICI-induced effector-like CD8 T cells and effector and memory CD8 T cells derived from YFV. (A–B) Schematic diagram of the isolation and sorting of effector and memory YFV-specific CD8 T cells (sorted by tetramer) after vaccination<sup>17</sup>. (C) Principal component analysis of ICI-induced effector-like CD8 T cells from early proliferative responders, effector and memory YFV-specific cells, and naïve CD8 T cells from patients with cancer and YFV. Gene expression in the ICI-induced effector-like and effector and memory YFV-specific CD8 T cells were normalised to the average of expression of naïve samples from the corresponding counterpart. (D) Venn diagram depicting the number of shared and differentially expressed genes expressed in ICI-induced effector-like CD8 T cells, effector YFV-specific and memory YFV-specific cells. Circle size represents negative log p value. (E) Gene Set Enrichment Analysis of the ICI-induced effector-like CD8 T cells from early proliferative responders and effector YFV-specific cells. NES - normalized enrichment score. (F) Heat-map of selected immune-related genes showing relative expression between naïve, ICI-induced effector-like, effector YFV-specific and memory YFV-specific cells. Scales of color represent Z-scores. ICI, immune checkpoint inhibitors; IL, interleukin; YFV, yellow fever virus.

effector-like CD8 T cells and effector and/or memory cells derived from vaccination or infection has not been done. In this study, we compare effector CD8 T cells from patients with cancer to highly functional effector and memory cells generated from YFV that are ideal effectors for a protective immune response.<sup>17</sup> Data from our transcriptional analyses show that the proliferating effector-like cells isolated from the blood of patients with cancer are distinct from virus-specific effector and memory cells isolated from the blood of healthy individuals after YFV. The effector-like cells that expand after PD-1 blockade express a number of inhibitory markers similar to existing effector cells present in the tumour microenvironment.<sup>20–22</sup> Thus, while there is a positive association between the early proliferative response and clinical outcome, our transcriptional comparison of ICI-induced effector-like cells and YFV effectors indicates that the quality of the ICI-effector-like cells could be improved to enhance clinical efficacy. Our recent preclinical studies have shown that PD-1-targeted therapy in combination with the cytokine IL-2 modifies the transcriptional profile of differentiating effector cells that results in the accumulation of transcriptionally and epigenetically distinct effectors resembling highly functional effectors.<sup>23–26</sup> These findings provide a framework to design more effective combinatorial approaches for cancer immunotherapy.

In summary, early-and-sustained proliferative CD8 T cells in the blood serve as a predictive biomarker for clinical response and survival for patients with cancer. Combination therapy also confers better clinical outcomes than PD-1-targeted monotherapy. Thus, combinatorial approaches that direct CD8 T-cell differentiation towards highly functional effectors may further enhance the efficacy of immunotherapy for patients with cancer.

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