Immune awakening revealed by peripheral T cell dynamics after one cycle of immunotherapy

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Our understanding of how checkpoint inhibitors (CPIs) affect T cell evolution is incomplete, limiting our ability to achieve full clinical benefit from these drugs. Here, we analyzed peripheral T cell populations after one cycle of CPI treatment and identified a dynamic awakening of the immune system, as revealed by T cell evolution in response to treatment. We sequenced T cell receptors in plasma cell-free DNA and peripheral blood mononuclear cells and performed phenotypic analysis of peripheral T cell subsets from patients with metastatic melanoma treated with CPIs. We found that early peripheral T cell turnover and T cell receptor repertoire dynamics identified which patients would respond to treatment. Additionally, the expansion of a subset of immune effector peripheral T cells we call TIE cells correlated with response. These events are prognostic and occur within 3 weeks of starting immunotherapy, raising the potential for monitoring patients’ responses by using minimally invasive liquid biopsies.

Checkpoint inhibitor (CPI) drugs awaken the immune system so that it attacks tumors. CPIs have revolutionized cancer care and, over the past decade, have contributed to a fourfold improvement in the survival of patients with metastatic melanoma. Despite these remarkable advances, our understanding of T cell evolution under the selective pressure of CPI is still incomplete and this limits our ability to derive full clinical benefit from these drugs. Consequently, most patients with advanced-stage melanoma still die of the disease. Sharing features with responses to infectious diseases, tumor control by the immune system requires coordination between systemic and intratumoral immunity, and although several studies have investigated intratumoral responses to immunotherapy, few have focused on how CPIs affect the peripheral immune system, or whether changes in peripheral T cells are associated with patient responses.

We hypothesized that because immune responses to tumors mirror normal defensive responses to pathogens, it would be possible to study patient responses to CPI by monitoring peripheral T cell evolution during treatment. TCRs are generated by error-prone recombination of the TCR locus, creating the enormous diversity needed for effective immune function. This process is ~80% efficient, so most peripheral T cells carry only productive TCR sequences. However, in ~20% of peripheral T cells, the first attempts at TCR locus rearrangement failed due to acquisition of stop codons or because the protein-coding region was out of frame, so these T cells carry both productive and non-productive TCR sequences. The complementarity-determining region 3 (CDR3) of the TCR in particular is highly variable and the sequences are unique to individual T cell clones, so both the productive and non-productive TCR sequences serve as ‘fingerprints’ for individual T cell clones.

We posited that by sequencing peripheral T cell CDR3 regions, we could track T cell responses to CPI, and because dying cells release their DNA into the circulation, we could also sequence CDR3 regions in cell-free DNA (cfDNA) in the blood to monitor T cell turnover in patients receiving CPIs. We found an increase in productive TCR sequences in the plasma cfDNA of patients who responded to CPIs, and this correlated with response. These events were accompanied by evolution of the peripheral T cell repertoire in a manner that mimicked changes induced by antiviral vaccines. The dynamics of T cell turnover revealed by the cfDNA analysis also correlated with expansion of a specific subset of cytotoxic memory effector peripheral T cells we call immune effector cells (or TIE cells). Importantly, TIE cell expansion after one cycle of CPIs enabled us to anticipate which patients would go on to respond to treatment. Our data reveal an awakening of the immune system that occurs within 3 weeks of initiating CPI treatment and anticipates clinical response to first-line therapy. These changes are dynamic and quantifiable.
and can be monitored with minimally invasive liquid biopsies—features that could be used to identify which patients will benefit from CPIs early during their treatment, allowing the delivery of more precise treatment planning.

**Results**

**Immunotherapy does not alter thymic output.** First, we examined the effects of CPIs on thymic function. We used fluorescence-activated cell sorting (FACS; Extended Data Fig. 2) to quantify the ETEs (CD3⁺CD45RA⁻CD45RO⁻CCR7⁻CD27⁺CD31⁺ T cells⁴) in peripheral blood mononuclear cells (PBMCs) from 50 patients with metastatic melanoma (patients 1–50) receiving first-line anti-programmed cell death protein 1 (anti-PD1) or anti-PD1/anti-cytotoxic T lymphocyte-associated protein 4 (anti-CTLA4) treatment (Extended Data Fig. 2i). As expected,⁵ we observed an age-related decrease in ETE levels in pre-treatment (t₀) patient blood (Fig. 1a), but we also found that one cycle of CPIs did not affect ETE levels measured at week 3 (P₀ = 0.274; Fig. 1b). Next, we examined the TCR excision circle (TREC) in the peripheral T cells of 16 of our patients (1, 10–13, 22, 24–27, 30, 42 and 51–54). The TREC—a by-product of TCR locus rearrangements—is a non-replicating episome that is diluted when T cells divide⁶ (Extended Data Fig. 1a–d). We found that the TREC-to-genome ratio in T cells was not affected by CPIs (P₀ = 0.129; Fig. 1c).

**CPIs induce TCR repertoire divergence in peripheral T cells.** The observations above indicate that CPIs did not affect thymic output in patients with melanoma, so to monitor how CPIs affected post-thymic T cell evolution, we analyzed the TCR in peripheral PBMCs and melanoma metastases. For patient 12, we obtained a fresh tumor biopsy at t₀, as well as whole blood at t₀ and after the first cycle of CPI at week 3. Using ImReP,¹ we identified 16 unique CDR3 DNA sequences from the biopsy and found that six of these were also present in the PBMCs and cfDNA (Fig. 1d and Fig. 2). Thus, about one-third of the sequences in the tumor were also in the periphery: four in pre-treatment PBMCs (sequences CDR3₁DNA₁, CDR3₂DNA₁, CDR3₃DNA₁ and CDR3₅DNA₁), three in week-3 PBMCs (CDR3₁DNA₂, CDR3₂DNA₂ and CDR3₃DNA₂) and one in week-3 cfDNA (CDR3₅DNA) (Fig. 2). Intriguingly, CDR3₁DNA₂, CDR3₂DNA₁ and CDR3₂DNA₂ both encoded TCR CDR3₃DNA₂ (Fig. 2), suggesting convergence by these TCRs on dominant tumor antigens. We also analyzed CDR3 sequences in 18 paired PBMCs and tumor-infiltrating lymphocytes (TILs) from a published melanoma cohort.⁸ As an example, at t₀, patient 1 presented 123,981 unique CDR3 sequences in bulk PBMCs, 21,052 in TILs and 3,741 shared sequences (Fig. 1e), and comparable patterns were seen in the other 27 patients (Supplementary Table 2). Intriguingly, the numbers of unique PBMC/cfDNA-shared CDR3 sequences increased after one cycle of CPIs (Fig. 3b), so we investigated how CPIs affected the immune-recognition landscape in these pools. In t₀ blood from patient 27, we observed 14,112 unique CDR3 sequences in bulk PBMCs, 844 in cfDNA and 193 shared sequences (Fig. 3a). Comparable patterns were seen in the other 27 patients (Supplementary Table 2). Clinically, critical changes to the PBMC/cfDNA-shared pool decreased significantly in patients who achieved disease control at week 12, but not in patients with progressive disease (Fig. 3d), suggesting there is repertoire divergence in the T cells that turnover in responding patients.

CPIs induce peripheral T cell turnover. Next, we compared the CDR3 clonal relatedness in PBMCs and cfDNA of 28 CPI-treated patients with metastatic melanoma (11–27 and 29–39). In t₀ blood from patient 27, we observed 14,112 unique CDR3 sequences in bulk PBMCs, 844 in cfDNA and 193 shared sequences (Fig. 3a). Comparable patterns were seen in the other 27 patients (Supplementary Table 2). Clinically, critical changes to the PBMC/cfDNA-shared pool decreased significantly in patients who achieved disease control at week 12, but not in patients with progressive disease (Fig. 3d), suggesting there is repertoire divergence in the T cells that turnover in responding patients.

The cfDNA CDR3 sequences come from T cell turnover in the thymus and periphery and contain both productive and non-productive sequences (Extended Data Fig. 1). We used ImmunoSeq to quantify productive (reading frame intact) and non-productive (out-of-frame or stop codon) CDR3 sequences and to calculate a rearrangement efficiency score (RES; productive/(productive + non-productive)). In healthy donors, the PBMC RES was ~0.8, as expected,¹ but in cfDNA, the RES was 0.44 (P < 0.001; Fig. 3c), presumably from non-productive TCR sequences released by T cells failing β selection in the thymus. The t₀ PBMC and cfDNA RES (RESᵣ) values were similar in healthy donors and patients (Fig. 3e), suggesting that melanoma does not overtly affect the

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**Fig. 1 | CPI treatment induced peripheral TCR repertoire divergence.** a. Graph showing ETEs in pre-treated patients’ blood (% ETEs relative to total naïve T cells; determined by FACS) relative to age (P₀ = 0.002; linear regression, R² = −0.17; n = 50). b. Levels of ETE in paired patient samples pre-treatment (t₀) and at week 3 (W3) of CPI treatment (P₀ = 0.274; n = 50). c. The TREC concentration relative to genomic DNA was measured by ddPCR in sorted CD3⁺ peripheral T cells at t₀ (median: 0.5×10⁻³) and week 3 (median: 0.1×10⁻²) (P₀ = 0.129; n = 17). d. TIL CDR3 sequences also present in peripheral PBMC DNA and cfDNA for one patient at t₀ and week 3 (see Fig. 2 for specific DNA sequences); tot, total. e. Venn diagram showing unique predicted productive CDR3 sequences in PBMCs and TILs for patient 1 at t₀ (Supplementary Table 1)¹. Numbers show unique nucleotide sequence counts for PBMC-private (pink), TIL-private (brown) and tePBMC pools (intersection; orange). f. Clonal relatedness (the proportion of amino acid sequences that were related by a maximum edit distance of 3) for CDR3 in the PBMC-private, tePBMC and TIL-private pools at t₀. **P = 0.003; ***P < 0.0001 (n = 18; median = 0.4×10⁻⁴ for PBMC-private; 0.2×10⁻¹ for tePBMC; and 0.4×10⁻¹ for TIL-private). g. Clonal relatedness (maximum edit distance = 3 amino acids) for CDR3 sequence in PBMC TCR pools at t₀ and week 3. Comparisons were made between the clonal relatedness of PBMC-private TCRs of patients with progressive disease (PD; orange; n = 11; median = 4.3×10⁻⁵ and 5.6×10⁻⁵, respectively) or disease control (DC; green; n = 7; median = 4.0×10⁻⁴ and 8.0×10⁻⁵, respectively) after 12 weeks of treatment (P = 0.413 and P = 0.999, respectively) and between the clonal relatedness of tePBMC TCRs of patients with progressive disease (n = 11; median = 0.0002 and 0.0008, respectively) or disease control (n = 7; median = 0.0017 and 0.0007, respectively) (P = 0.638 and P = 0.031, respectively). All statistical comparisons in b, c, f and g were made by two-sided Wilcoxon test. NS, not significant. Each dot represents one patient, lines represent median values, error bars show s.d., connecting lines show paired samples and n values represent the number of patients.
efficiency of T cell rearrangements in the thymus and also that it does not affect bulk T cell turnover in the periphery. We therefore compared the RES in PBMCs and cfDNA at $t_0$ and week 3 to generate difference scores ($\Delta W_3$RES) and measure how CPIs affect T cell rearrangement and turnover during the first cycle of immunotherapy. The PBMC $\Delta W_3$RES measured changes in TCR rearrangement efficiency and was ~0 irrespective of whether the patients responded or not (Fig. 3f). Thus, CPI treatment did not affect TCR rearrangement efficiency in the thymus, meaning that the cfDNA $\Delta W_3$RES measured changes in the peripheral T cell turnover alone. Notably, the cfDNA $\Delta W_3$RES was ~0 in patients with progressive disease but rose to 0.09 ($P=0.037$) in patients who achieved disease control at 12 weeks (Fig. 3f). Thus, CPI increased peripheral T cell turnover in responding patients, but not in non-responding patients.

CPIs stimulate expansion of specific peripheral T cell subsets. Our data reveal that there is dynamic TCR repertoire reorganization during T cell expansion/contraction in responding patients, so we...
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Among patients with disease control from patients with progressive disease (Extended Data Fig. 4a). Accordingly, when we analyzed published CyTOF data1, we confirmed TIE to be a distinct T cell subset in PBMCs of patients with metastatic melanoma (Extended Data Fig. 4b,c), but consistent with our findings, the TIE levels at week 12 did not distinguish patients with disease control from patients with progressive disease (Extended Data Fig. 4d). Thus, changes at week 3 were prognostic for melanoma responses to CPI, but were not more prognostic by week 9 or week 12, showing the dynamic nature of these responses, and consistent with a previous study showing that the peak of immune activation is at week 3 (refs. 7,8).

We note that the week-3 TIE expansion identified patients who achieved disease control early during treatment with superior accuracy to the week-3 peripheral T lymphocyte invigoration-to-tumor burden ratio (Ki67/TB), where the accuracy was 0.64 (16/24 patients with Ki67/TB > 1.94 had an objective response compared with 3/17 patients with Ki67/TB < 1.94)7. We also note that the week-3 TIE expansion also had greater accuracy than programmed death-ligand 1 (PD-L1) staining in pre-treatment melanoma biopsies where the accuracy in a phase III clinical trial was 0.67 (78/148 patients with PD-L1+ biopsy had an objective response compared with 89/270 patients with PD-L1− biopsy)36.

Fig. 2 | TIL CDR3 sequences also present in peripheral PBMCs and cfDNA for patient 12 at t0 and week 3. Displayed are the DNA sequences (CDR3DNA1–4) and their paired predicted protein sequences (CDR3prot1–4) for the TCRs that were identified in the pre-treatment tumor biopsy and also in the periphery, either in t0 or week-3 PBMCs or in week-3 cfDNA. For the proteins, the different CDR3 sequences are color coded, with CDR3prot1 in red, CDR3prot2 in purple and CDR3prot4 in blue. The black text is the flanking TCR protein sequence. The red underlined bases in CDR3DNA1.1 and CDR3DNA1.2 highlight that CDR3DNA1.1 and CDR3DNA1.2 encode the same protein. Similarly, the red underlined base in CDR3DNA2.2 highlights that CDR3DNA2.1 and CDR3DNA2.2 encode the same protein. *CDR3 sequence count in the biopsy. °CDR3 sequence frequency in the sample.

Table 2 | Clonal expansions in TIE subsets of patients with minor responses. The number of TCRs detected in TIE subsets at t0 and week 3 is provided for patients with progressive disease (W3RES > 0.90; area under the curve (AUC) at week 3). The frequency of clonal expansions is provided for individual TCRs detected in TIE subsets at week 3 (AUC > 0.8) in patients with minor responses (W3RES > 0.90; AUC = 0.82). The frequency of clonal expansions is provided for individual TCRs detected in TIE subsets at week 3 (AUC > 0.8) in patients with minor responses (W3RES > 0.90; AUC = 0.82).

| Peripheral sample | Sequence | CDR3DNA1.1 | CDR3DNA1.2 | CDR3DNA2.1 | CDR3DNA2.2 |
|-------------------|----------|-------------|-------------|-------------|-------------|
| t0 PBMCs          | S H P S P  | 10          | 0.0016176   | 0.0000176   | 0.00162     |
|                   | Q T G Y F  | 1           | 8.56 × 10^−5 | 10          | 1           |
|                   | P B T R L  | 1           | 8.56 × 10^−5 | 1           | 1           |
|                   | T R L     | 1           | 8.56 × 10^−5 | 1           | 1           |
|                   | Q T G Y F  | 1           | 8.56 × 10^−5 | 1           | 1           |
|                   | P B T R L  | 1           | 8.56 × 10^−5 | 1           | 1           |
|                   | T R L     | 1           | 8.56 × 10^−5 | 1           | 1           |

To study cytotoxic T cell turnover, we sequenced the TCR in t0 and week-3 purified CD8+ peripheral memory and naïve T cells from three patients with progressive disease and three patients with disease control (12, 16–19 and 29). More than other peripheral T cell subsets, the TIE cells had the highest similarity to cfDNA sequences (Fig. 4b) and presented the highest clonality and consistent with a previous study showing that the peak of immune activation is at week 3 (refs. 7,10).

Fig. 1k) and have been shown to be associated with response to infections21,22. A late response could not be measured, but from day 40 we observed a steady decline in this patient's NRAS expression in the TCR DNA27 (Fig. 6c), revealing that consistent with the observed expansion in TIE cells, the patient achieved a biochemical response (Fig. 6c). An increase of >0.8% in the TIE ratio relative to all CD8+ memory T cells at week 3 was associated with increased overall survival and segregated disease control (including late responders) from patients with progressive disease, with a sensitivity of 0.94 and a specificity of 0.79 (accuracy = 0.87; area under the curve = 0.85) (Fig. 6d). The hazard ratio for patients without week-3 TIE expansion was 3.7 (95% confidence interval: 1.12–11.9; P = 0.032) (Fig. 6e). We confirmed these observations in an independent cohort of 20 CPI-treated patients (31–50; Fig. 6f,g), with a sensitivity of 0.82 and a specificity of 1 (accuracy = 0.90; area under the curve = 0.92). By week 9, TIE cells no longer discriminated patients with disease control from patients with progressive disease (Extended Data Fig. 4a).

Next, we analyzed PBMCs from 30 CPI-treated patients with metastatic melanoma from our cohort (1–30) and show that the TIE cells expanded at week 3 in patients who achieved disease control, including late responders, but not patients with progressive disease (P = 0.0007; Fig. 6a), irrespective of the therapy protocol (P = 0.200; Fig. 6b). Notably, despite a week-3 TIE expansion of >20%, the week-12 computed tomography scan revealed that patient 20 was progressing (Fig. 6a). Unfortunately, the patient died of complications.
Fig. 3 | CPI treatment induced peripheral T cell turnover. a, Venn diagram showing unique predicted productive CDR3 sequences in PBMC (pink), PBMC/cfDNA-shared (intersection; purple) and cfDNA pools (blue) for patient 27 at t₀ (Supplementary Table 2). b, Total number of CDR3 clones at t₀ (pink) and week 3 (purple) in the PBMC/cfDNA-shared pool (P = 0.010; two-sided Wilcoxon test). c, Clonal relatedness (maximum edit distance = 3 amino acids) for CDR3 in the PBMC-private, PBMC/cfDNA-shared and cfDNA-private pools at t₀. ****P < 0.0001 for both comparisons (two-sided Wilcoxon test; median = 0.3 × 10⁻³ for PBMC-private; 0.01 for cfDNA-private; and 0.06 for PBMC/cfDNA-shared). d, Clonal relatedness of CDR3 sequence in the PBMC/cfDNA-shared pool at t₀ and week 3 for patients with progressive disease (n = 12; orange) or disease control (n = 16; green) at week 12. For patients with progressive disease versus those with disease control, P = 0.623 at t₀ (median = 0.04 and 0.08, respectively) and *P = 0.026 at week 3 (median = 0.06 and 0.03, respectively) (both two-sided Mann–Whitney U-test). For t₀ versus week 3, P = 0.733 for patients with progressive disease and *P = 0.039 for patients with disease control (both two-sided Wilcoxon test). e, Pre-treatment TCR RES (RES₀) of rearranged CDR3 in healthy donors (HD) and patients on CPI treatment, in PBMC DNA and cfDNA. For healthy donors versus patients, P = 0.445 for PBMC DNA (median = 0.83 and 0.81, respectively; n = 77 replicates from 4 healthy donors; n = 29 patients) and P = 0.09 for cfDNA (median = 0.44 and 0.62, respectively; n = 3 healthy donors; n = 28 patients) (both two-sided Mann–Whitney U-test). For HD versus patients, P = 0.0445 for PBMC DNA (median = 0.83 and 0.81, respectively; n = 77 replicates from 4 healthy donors; n = 29 patients) and P = 0.09 for cfDNA (median = 0.44 and 0.62, respectively; n = 3 healthy donors; n = 28 patients) (both two-sided Mann–Whitney U-test). For PBMC DNA versus cfDNA, P < 0.0001 for healthy donors (two-sided Mann–Whitney U-test) and P < 0.0001 for patients (two-sided Wilcoxon test). f, ΔRES according to response group at week 12. **P = 0.008 (two-sided Wilcoxon test; median = 0.001 and 0.08, respectively, for PBMC and cfDNA). *P = 0.037 (two-sided Mann–Whitney U-test; median = 0.02 and 0.08, respectively, for progressive disease and disease control). The total number of patients with melanoma was 28. Each dot represents one patient, error bars show s.d., connecting lines show paired samples and horizontal lines represent median values.
Next, we analyzed published single-cell RNA expression and protein sequencing (REAP-Seq) data from healthy donors and found that TIE cells have the additional surface phenotype CD69^+/CD155^−/CD40^med/high^CD154^med/high^CD357^med/high^ and a distinct transcriptome signature including immune activation genes (cluster 9 in Extended Data Fig. 5a,b and Supplementary Table 4). Our analysis of these data also showed that TIE cells expanded from healthy donor CD8^+ naive PBMCs following in vitro stimulation, and that they expressed genes associated with immune effector function (Extended Data Fig. 5c,d). Using FACS analysis of five patients' PBMCs (1, 24, 29, 42 and 54), we observed a trend for TIE reinvigoration (increased Ki67 expression) after one cycle of CPI treatment (Extended Data Fig. 6a), although the limited sample size could not support robust conclusions. Note that the week-3 TIE expansion was not associated with toxicity, but expansion of a separate T regulatory (Treg) subset characterized by the surface phenotype CD3^+CD4^+CD8^+CD25^+CD127^−/low^ (ref. 28) correlated with toxicity grade (Fig. 7a,b).

CPIs induce peripheral T cell repertoire rearrangements. Our findings revealed intriguing parallels between immune responses to infection and CPI treatment, and we hypothesized that immune responses to CPIs mirror the defense against pathogens. To test this, we compared T cell repertoire rearrangements in people receiving vaccination or CPIs. Using published data (ref. 29,30), we calculated T cell clonality (measures clone dominance) and diversity (indicates heterogeneity) and note that 1–2 weeks after antiviral vaccines were administered, healthy donor TCR repertoires presented bifurcated

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**Fig. 4 | Identification of TIE cells.** a, Correlation between TIE cell abundance (ΔW3 TIE) and cfDNA ΔW3 RES (P = 0.001; linear regression, R² = 0.34; n = 28 patients). The dotted line is the linear regression line. b, Similarity matrix of TCR sequences in cfDNA and peripheral CD8^+ T cell subsets in six patients. Median similarity scores for central memory (CM) cells, TIe cells, naïve cells and ETEs at t₀ were 0.026, 0.045, 0.004 and 0.003, respectively (P = 0.0013; Friedman analysis of variance; Friedman statistic = 15.64). Median similarity scores at week 3 were 0.043, 0.136, 0 and 0.003, respectively (P < 0.0001; Friedman analysis of variance; Friedman statistic = 23.05). The naïve subset was not assessed for patient 16 at either time point. c, Clonality (Gini coefficient) in peripheral CD8^+ T cell subsets. The median TIE subset clonality relative to the other subsets was 0.46 at t₀ and 0.61 at week 3 (after the first cycle of CPI) in six matched patient samples (*P = 0.0006 and *P = 0.0002, respectively; Friedman analysis of variance; Friedman statistics = 12.6 and 13.08, respectively). Again, the naïve subset not assessed in patient 16. Horizontal lines show median values and error bars represent s.d. The small sample size did not allow for comparison between responders (patients 16–18) and those whose disease progressed (patients 12, 19 and 29). d, Graph showing the frequency of pre-treatment TIL CDR3 sequences in sorted peripheral CD8^+ T cell subsets of patient 12 at t₀ and week 3. Data points in a and c represent individual patients.
reorganization with either increased clonality or increased diversity (Fig. 8a). Next, we compared clonality (Δc, clonality) and diversity (Δw, diversity) in t0 and week-3 PBMCs from 17 CPI-treated patients with metastatic melanoma (11–27). In patients who went on to achieve disease control at week 12, we observed bifurcated reorganization of the TCR repertoire, with substantially increased clonality or diversity, whereas no such response occurred in patients with progressive disease (Fig. 8b). Using our training cohort, we developed a linear discriminant analysis (LDA) algorithm that at week 3 classified patients according to response (assessed at week 12) with an accuracy of 0.9 (specificity = 1; sensitivity = 0.8). We validated these findings in an independent cohort of 27 patients with advanced melanoma34, and again found a bifurcated TCR repertoire reorganization in patients with disease control, but not patients with progressive disease (Fig. 8c). Our LDA accuracy for response prediction was 0.77 in this validation cohort.

Discussion

We examined how the selective pressure of a single cycle of CPIs affects peripheral T cell evolution in patients with previously untreated metastatic melanoma. We found that CPIs induced immune awakening that was revealed by increased levels of productive CD3 sequences released into the blood, and dynamic changes in the TCR repertoire. CPIs did not affect thymic output but did induce peripheral T cell turnover, and this correlated with the expansion of a CD8+ cytotoxic memory effector subset that was CCR7−CD27+. This subset of lymphocytes is involved in cytotoxic response to infections31,32 and we have now established that they are also associated with CPI responses. Immune effector cells are the cells of the immune system that support antitumor immune surveillance33, and since our data identified a specific T cell subset involved in this network, we called them TIE cells.

It was recently shown that following CPI treatment, the expansion of tumor-infiltrating T cell clones did not come from pre-existing TILs34, but rather from novel clonotypes, most probably in the peripheral compartment. Those observations are consistent with a model in which the tumor is an open compartment with active cross-talk with the peripheral immune system; accordingly, in responding patients, we observed a significant early T cell repertoire rearrangement in the fraction of TILs circulating in the blood, which we call tePBMCs.

Clonotype modulation by checkpoint blockade has been described previously (largely in the tumor microenvironment35–37), but we determined that the pattern of peripheral turnover and overall repertoire rearrangement of T cells in blood identify the patients with an effective immune awakening who will go on to respond to CPI treatment. It has also been shown in animal models that anti-CTLA4 and anti-PD1 drugs induce distinct cellular reactions38. That we did not observe significant differences between single-agent and anti-PD1/CTLA4 combined therapy supports that our observations reflect the final effects needed for tumor elimination, and importantly we showed that these changes could be detected in the periphery. Although we could not determine whether the T cell clones driving these changes were melanoma specific, or whether this reflected a general, off-target immune activation, our results nonetheless contribute to our understanding of the dynamics of immune system evolution after one cycle of CPI treatment. That these responses also occur with infection could limit specificity in the CPI setting, necessitating further kinetic analysis and clinical validation, but our results have established that these responses...
could provide tractable tools for the delivery of precision immunotherapy. Moreover, our hypothesis-generating results contribute to improved understanding of immune system biology and could have broader implications beyond the oncoimmunology field.

Note that CCR7 and CD27 were downregulated in TIE cells and that phenotype has been associated with differentiated effector T cell release from lymph nodes to the periphery\(^9\)\(^{12}\). Also, in line with previous observations that in vitro stimulation of CD8\(^+\) T cells induced downregulation of CD27 and CCR7 (refs. \(^43\),\(^44\)), our analysis of previous data showed that TIE cells expanded following in vitro stimulation. Our data also showed that the expansion of these cells after one cycle of CPI identified the patients for whom therapy overcame melanoma-induced immune suppression. Within the scope of this study, we have not analyzed the antitumor reactivity of TIE cells, but our data show that peripheral TIE clones infiltrated melanoma and represent an abundant fraction of tumor-infiltrating lymphocytes with high repertoire clonality. The TIE cells were also in active turnover. The relatively small size of our sample could limit the generalization of our results, but both TIE cell and TCR peripheral repertoire reorganization could identify which patients will benefit from CPI treatment with greater accuracy than standard biopsy PD-L1 staining or Ki67/TB. Future research will investigate the antitumor cytotoxicity and specificity of these cells, and their potential for clinical development.

In summary, here we identified a peripheral blood early immune signature characterized by significant rearrangements of the peripheral repertoire reorganization could identify which patients will benefit from CPI treatment with greater accuracy than standard biopsy PD-L1 staining or Ki67/TB. Future research will investigate the antitumor cytotoxicity and specificity of these cells, and their potential for clinical development.
and Armaria et al.18; two-sided linear regression analysis, \( P = 0.0001 \); according to toxicity at any time between 2 weeks and 6 months (12 weeks of immunotherapy; green: patients who achieved disease control after 12 weeks of immunotherapy). Dotted lines are linear regression lines. \( n = 16 \) patients at week 12 of anti-PD1-based treatment. each dot represents one healthy donor or patient sample (orange: patients who progressed after CD3\(_\text{CD8}^+\), Changes in \( \Delta b_0 \) and \( \Delta b_a \), Clonality (Gini coefficient) and diversity (Rényi index; \( \alpha = 1 \)) in peripheral T cells from \( t_0 \) to weeks 1 and 2 in healthy donors who received antiviral vaccination (\( n = 25 \) healthy donor samples). \( \Delta b_a \) and \( \Delta b_0 \), Diversity (Rényi index; \( \alpha = 1 \)) in peripheral T cells from \( t_0 \) to week 3 in the validation cohort (The Christie NHS Foundation Trust, Huang et al.7 and Armaria et al.18; \( n = 12, n = 4 \) and \( n = 11 \) patients, respectively) of patients with advanced melanoma who progressed (\( n = 11 \) patients) or responded (\( n = 16 \) patients) at week 12 of anti-PD1-based treatment. Each dot represents one healthy donor or patient sample (orange: patients who progressed after 12 weeks of immunotherapy; green: patients who achieved disease control after 12 weeks of immunotherapy). Dotted lines are linear regression lines.

Fig. 7 | Expansion of a peripheral regulatory T cell subset associated with toxicity. a. Graph showing \( \Delta_{T_{\text{reg}}} \) in patients without (median = 1.6; \( n = 33 \)) or with greater than or equal to grade 3 (G3) toxicity (median = 3.72; \( n = 17 \)). \( P = 0.347 \) (two-sided Mann–Whitney U-test). Lines show median values, error bars represent s.d. and data points represent individual patients (orange: progressive disease; green: disease control; triangles: single-agent anti-PD1; squares: combination ipilimumab + nivolumab). b. Expansion of CD3\(^+\)CD4\(^+\)CD8\(^+\)CD25\(^+\)CD127\(^-\)/\(^{-}\)/\(^{-}\) T\(_{\text{reg}}\) cells (Extended Data Fig. 2g) at week 3 (\( \Delta_{T_{\text{reg}}} \)) according to toxicity at any time between 2 weeks and 6 months (\( P < 0.0001 \); \( n = 50 \); two-sided linear regression analysis, \( R^2 = 0.29 \)). The dotted line is the linear regression line. In \( a \) and \( b \), \( n \) represents the number of patients.

Fig. 8 | TCR repertoire evolution after immune stimulation. a. Changes in CDR3 clonality (\( \Delta_{\text{CDR3}^{\text{clonality}}} \); Gini coefficient) and diversity (\( \Delta_{\text{CDR3}^{\text{diversity}}} \); Rényi index; \( \alpha = 1 \)) in peripheral T cells from \( t_0 \) to weeks 1 and 2 in healthy donors who received antiviral vaccination (\( n = 25 \) healthy donor samples). b, Changes in CDR3 clonality (\( \Delta_{\text{CDR3}^{\text{clonality}}} \); Gini coefficient) and diversity (\( \Delta_{\text{CDR3}^{\text{diversity}}} \); Rényi index; \( \alpha = 1 \)) in peripheral T cells from \( t_0 \) to week 3 in the training cohort (The Christie NHS Foundation Trust) of patients with advanced melanoma receiving first-line anti-PD1-based immunotherapy (\( n = 17 \) patients) who progressed (\( n = 9 \) patients) or responded (\( n = 8 \) patients) at week 12. c. Changes in CDR3 clonality (\( \Delta_{\text{CDR3}^{\text{clonality}}} \); Gini coefficient) and \( \Delta_{\text{CDR3}^{\text{diversity}}} \) (Rényi index; \( \alpha = 1 \)) in peripheral T cells from \( t_0 \) to week 3 in the validation cohort (The Christie NHS Foundation Trust, Huang et al.7 and Armaria et al.18; \( n = 12, n = 4 \) and \( n = 11 \) patients, respectively) of patients with advanced melanoma who progressed (\( n = 11 \) patients) or responded (\( n = 16 \) patients) at week 12 of anti-PD1-based treatment. Each dot represents one healthy donor or patient sample (orange: patients who progressed after 12 weeks of immunotherapy; green: patients who achieved disease control after 12 weeks of immunotherapy). Dotted lines are linear regression lines.

Critically, they provide a potentially tractable tool to identify which patients will benefit from CPIs early during treatment. This could help clinicians to stratify their patients more effectively to thereby improve personalization of therapeutic planning.

Methods

Patient samples. Blood samples from patients and healthy donors were collected under Manchester Cancer Research Centre (MCRC) Biobank ethics application 07/H1003/161+5 with written informed consent from the patients at The Christie NHS Foundation Trust. The study was approved by MCRC Biobank Access Committee application 13_RIMA_01. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki and good clinical practice guidelines. A total of 54 patients with metastatic melanoma, treated with either pembrolizumab or nivolumab plus ipilimumab as first-line therapy, as per the guidelines. A total of 54 patients with metastatic melanoma, treated with either pembrolizumab or nivolumab plus ipilimumab as first-line therapy, as per the guidelines. A total of 54 patients with metastatic melanoma, treated with either pembrolizumab or nivolumab plus ipilimumab as first-line therapy, as per the guidelines. 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PBM and plasma extraction. PBMcs were isolated from blood samples using Lymphoprep (STEMCELL Technologies) and SepMate tubes (STEMCELL Technologies) as per the manufacturer’s instructions. Red cell lysis was performed with RBC Lysis Buffer (BioLegend) as per the manufacturer’s instructions. The DNA of PBMcs and sorted CD3\(^+\) T cell subsets was extracted using QIAamp DNA Blood Mini kits (Qiagen) as per the manufacturer’s instructions.

cDNA analyses. Extraction and quantification of cDNA was carried out as described previously46 for patients 11–27 and 29–39.
FACS analysis. Following isolation, PBMCs from patients 1–50 were kept at 4°C in phosphate buffered saline plus 2% fetal bovine serum (FBS) and analyzed within 24 h. PBMCs were suspended in FACS buffer (phosphate buffered saline containing 2% FBS, 2 mM EDTA and 0.02% sodium azide) plus 0.1% Pluronic F-68 (BD Biosciences) and Human TruStain FCx (BioLegend) as per the manufacturer’s instructions, and incubated at room temperature for 40 min with Tm and Tnaive panels of fluorochrome-labeled antibodies. The Tm panel consisted of CD3 (1:100; catalog number 317336), CD4 (1:100; catalog number 317438), CD8a (1:100; catalog number 329937), CD27 (1:200; catalog number 303610), and CD127 (1:40; catalog number 351304), whereas the Tnaive panel consisted of CD3 (1:100; catalog number 317337), CD4 (1:100; catalog number 317438), CD45RA (1:40; catalog number 309006), CD45RA (1:100; catalog number 304130), CD45RO (1:200; catalog number 304228), CD31 (1:40; catalog number 303118), CD27 (1:200; catalog number 356410) (all from BioLegend) and CCR7 (1:20; catalog number 560765; BD Pharmingen) and LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific) was added to the final suspension to exclude dead cells. Stained PBMCs were washed once at 300 g for 7 min in FACS buffer and analyzed using LSR II, LSRSortera, Aria II or Aria III (Special Order Research Product) (BD Biosciences) cytometers and FlowJo software (version 10; Tree Star). CD8+ T cell subsets were determined using an Aria II (1:40; catalog number 304130) and TREC-plasmid. 

For Tnaive gating (performed for patients 1, 24, 29, 42 and 54), PBMCs previously frozen in FBS + 10% dimethyl sulfoxide were thawed in cold RPMI and washed twice. Then, PBMCs were suspended in FACS buffer and Human TruStain FCx (BioLegend), as per the manufacturer’s instructions, and incubated at room temperature for 30 min with antibodies and CD3 fluorescein isothiocyanate (FITC) (catalog number 317337), CD3 (1:100; catalog number 317438), CD4 (1:40; catalog number 309006), CD45RA (1:100; catalog number 304130), CD45RO (1:200; catalog number 304228), CD27 (1:200; catalog number 356410), PDI (1:40; catalog number 329939) (all from BioLegend), CCR7 (1:20; catalog number 560765; BD Pharmingen) and LIVE/DEAD Fixable Blue Dead Cell Stain (Thermo Fisher Scientific) were then fixed and permeabilized with Cytoperm kit (BD, catalog number 554714) according to the manufacturer’s instructions, and stained for Ki67 (1:20; catalog number 350507; BioLegend) for 30 min at room temperature. Stained cells were resuspended in FACS buffer and analyzed using the LSRSortera (BD Biosciences) cytometer and FlowJo software (version 10; Tree Star).

In patients 1, 10, 11, 12, 23, 24, 25, 26, 27, 30, 42 and 51–54, an aliquot of PBMCs was frozen in FBS + 10% dimethyl sulfoxide immediately after separation and then thawed and stained with CD3 fluorescent antibody as above; CD3+ cells were sorted with an Aria III (BD Biosciences) and used for TREC quantification. CD27+ cells were quantified as the percentage of CD27+ TCCR- cells in the CD3+CD45RA-CD45RO+ gate (Extended Data Extended Data Fig. 1). The gating strategy is shown in Extended Data Fig. 1.

TREC. TREC analysis was performed using frozen PBMCs from patients 1, 10, 11, 12, 23, 24, 25, 26, 27, 30, 42 and 51–54. TREC quantification was performed with droplet digital PCR (ddPCR) using a custom TREC assay (TREC forward primer 5′-CACATGCCCTTTCAACATGC-3′ at a final concentration of 450 nM, TREC reverse primer 5′-GGCCAGCTGAGGTTATTG-3′ at a final concentration of 450 nM and HEX-Black Hole probe 5′-ACACCTCTGTTTGTGTAAGGGCCT-3′ at a final concentration of 300 nM) and 10 ng of PBMC DNA. PCR amplification was performed with Quick-Load Taq 2X Master Mix (NEB) on a Mastercycler Nexus Gradient thermal cycler (Eppendorf). Cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 68 °C for 5 min. The resulting amplicon was purified using the QIAquick PCR Purification Kit (Qiagen), then cloned into the pGEM-T Easy plasmid (Promega) and transformed into competent Escherichia coli strain JM109 cells prepared using the Mix & Go E. coli Transformation Kit (Zymo Research). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen), and Sanger sequencing using the T7 5′-TAATACGACTCACTATAGGG-3′ and SP6 5′-TTATTTGAGCTCATATAG-3′ primers was used to perform correct insert identity. The final plasmid was designated TREC primer.

RNA sequencing. RNA was extracted from a pre-treatment human fresh frozen tumor sample for one patient with available tissue (patient 12) using an AllPrep DNA/RNA kit (Qiagen) according to the manufacturer’s instructions. Indexed poly(A) libraries were prepared using 200 ng total RNA and 14 cycles of amplification with the Agilent SureSelect Strand-Specific RNA Library Preparation Kit for Illumina Sequencing (Agilent; G9691B). Libraries were quantified by quantitative PCR using the KAPA Library Quantification Kit for Illumina platforms (Kapa Biosystems; KK4873). Pair-end 100-base pair sequencing was carried out by clustering 15 pM of pooled libraries on the cBot and sequenced on the Illumina HiSeq 2500 in high output mode using TruSeq SBS Version 3 chemistry (Illumina). After removing adapters using Cutadapt (version 1.14) and trimming poor-quality base calls using Trimmomatic (version 0.36), the reads were aligned to GRCh37 release 75 using STAR (version 2.5.1) aligner44.

TCR analysis. TCR sequences were inferred from RNA sequencing data from one patient for whom we had a frozen pre-treatment metastasis biopsy sample using ImReD50 and ImmunoSEQ TCRB Assay kit (Adaptive Biotechnologies) was used to amplify and sequence TCR sequences in cDNA and the DNA of PBMCs as per the manufacturer’s instructions. We loaded the same DNA input for all PBMC (350 ng; patients 11–39) and cDNA samples (40 ng; patients 11–27 and 29–39), while for the sorted T cell subsets (for patients 12, 16, 17, 18, 19 and 29), we loaded all of the DNA extracted from the sorted cells. A metafile is available with each single sample and anonymous patient information from https://gitlab.com/CRUK-mi/tcell-immune-awakening. Pooled libraries were quantified by quantitative PCR using the KAPA Library Quantification Kit for Illumina platforms (Kapa Biosystems; KK4873). Sequencing was carried out by clustering 0.6–1.1 pM of pooled libraries on the Illumina Nova SEQ400 according to the TREC+ protocol for Adaptive Biotechnologies. Healthy donor PBMC TCR control data were downloaded from the immune ACCESS repository immunoSEQ (Adaptive Biotechnologies; https://doi.org/10.21417/ADPT2017TR). TCR sequencing data were analyzed using immunoSEQ Analyzer (Adaptive Biotechnologies) and the R LympoSeq package (version 3.4.1; R Foundation for Statistical Computing). Matched paired pre-treatment metastasis biopsy samples with matched time points from patients with locally advanced melanoma were downloaded from the referenced accession list to Amaria et al.18 TCR sequencing data were downloaded from the European Genome-phenome Archive (EGA) with the identifier EGAS00001003178 (EGA study accession dataset EGAD00001001680) and the patient clinical history metadata file was downloaded using the identifier EGAS00001004352. Consecutive patients 11–27 from The Christie NHS Foundation Trust constituted the training cohort. The external validation data were pooled from an independent cohort of patients from The Christie NHS Foundation Trust (patients 28–39), a cohort of patients with metastatic melanoma from Huang et al.17 (PBMC CD38+ plus PBMC CD38−emergent populations from patients 12288, 13471, 14746 and 14835; TCR sequencing data were made available by the authors) and the cohort of patients with locally advanced treatment-naive melanoma from the referenced accession list to Amaria et al.18 (patients 1, 2, 4, 5, 6, 7, 8, 10, 11, 13 and 15; TCR sequencing data were downloaded from EGAS00001003178 (EGA study accession dataset EGAD00001001680); the patient clinical history metadata file was downloaded using the identifier EGAS00001004352). The Gini coefficient was used as a measure of clonality and calculated using the function clonality from the LympoSeq R package. Clonal relatedness was calculated by setting an edit distance of 3, using the clonalRelatedness LympoSeq function, and similarity was assessed by means of the Bhattacharyya coefficient using the bhattacharyyaMatrix function that was calculated using the R package compareClones52,59. Time-point pairwise analysis for each individual patient LDA coefficient (of linear discriminants L1D1: [ΔRenyi index] = 5.5; L2D2: [ΔGini coefficient] = 261.3) and validation to calculate balanced accuracy were performed using the packages MASS and caret in R (version 3.4.1, R Foundation for Statistical Computing). For further details, refer to the extended R scripts available on Gitlab (https://gitlab.com/cruk-mi/tcell-immune-awakening).
define the T_h cluster (Setllidet command). The cell differential expression analysis in the T_h cells from PBMCs versus after in vitro stimulation was performed with the function FindMarkers in the combined Seurat object (RunCCA). The antibody–derived tag data Seurat matrices were imported in Cytobank to analyze the differential representation of the CD8+ subsets under different experimental conditions (Extended Data Fig. 5c).

Statistics and reproducibility. Unless otherwise stated, all statistical tests were two-sided. The statistical differences between two groups for numerical variables were assessed using a two-tailed Mann–Whitney U-test (unpaired comparisons) or Wilcoxon test (paired comparisons). The statistical differences between multiple, paired measures were assessed using the Friedman test. Delta values were calculated as the difference between week-3 and t0 values. The statistical differences of categorical variables between groups were assessed using chi-squared or Fisher's exact tests, according to group dimensions. Correlation between continuous variables was assessed with Spearman's test (independent variables) or linear regression (dependent variables). Kaplan–Meier plots with the log-rank test (3-week landmark analysis) were used to analyze survival data. Univariate Cox regression was used to calculate the hazard of death. P < 0.05 was considered significant. Cox–Snell residuals were used to verify the proportional hazard hypothesis (P = 0.141, with P > 0.05 confirming the hypothesis). Sample size calculation was performed using G*Power software,98 using the effect size and standard deviation. For the comparison of ΔRES in PBMCs versus cDNA in patients with disease -recurrence, the sample size was n = 14 for α = 0.05 and 1 - β = 0.8. For the T cell subset analyses for the T_h cells in patients with progression versus disease control, the total sample size was n = 32 for α = 0.05 and 1 - β = 0.8. No data were excluded from the analyses. The investigators were blinded during the experiments and outcome assessment was performed after the experiments. Analyses were performed with GraphPad Prism version 7 (GraphPad Software) or R (version 3.4.1; R Foundation for Statistical Computing).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Sample metadata files and custom scripts are available from GitLab (https://gitlab.com/cruk-mi/tcell-immune-awakening). The data from all TCR sequencing performed in this study are deposited in the immuneACCESS repository immunoS Browse (https://doi.org/10.21417/SV2020M). The RNA sequencing data for patient 12 can be downloaded from EGA (accession code EGAS00001004043). TCR sequencing data for matched pre-treatment and week-3 melanoma biopsy and PBMC samples of patients with locally advanced melanoma were re-analyzed here were downloaded from referenced accession EGAS00001001378 (EGA study accession dataset EGA200010001606). TCR sequencing data of matched pre-treatment and week-3 PBMCs of patients with melanoma from Huang et al. were re-analyzed here were made available by the authors. TCR sequencing data of matched pre-treatment and week-3 PBMCs for the cohort of patients with locally advanced treatment-naïve melanoma from referenced accession Amaria et al. were re-analyzed here were downloaded from the EGA (accession code EGAD00001001606) and the patient clinical history metadata file was downloaded from the EGA via accession dataset EGAD00001004352. PBMC and biopsy CyTOF data from Krieg et al. and Greenplate et al. were re-analyzed here were downloaded from referenced accessions https://flowrepository.org/experiments/1124 and http://flowrepository.org/ al/FR-FCM-2ZMC, respectively. PBMC REAP-Seq data from Peterson et al. re-analyzed here were downloaded from referenced Gene Expression Omnibus accession GSE100501. The authors confirm that for approved reasons (UK Data Protection Act 2018), some access restrictions apply to the data containing patient medical records (specifically, dates of birth). Source data for Figs. 1–7 and Extended Data Figs. 3–6 are provided with the paper. Additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability
ImmuNoSEQ Analyzer (Adaptive Biotechnologies), FlowJo (version 10; Tree Star), Cytobank,10 Trimmomatic (version 0.36)11 and STAR (version 2.5.1)12 aligner are published or commercial codes and software programs. Diversity was calculated using the S<sub>5</sub> index (σ = 1) as per Spreafico et al.13. Differential marker expression analysis was performed on CyTOF data using the custom workflow described by Nowicka et al.14. Custom R scripts are available from GitLab (https://gitlab.com/cruk-mi/tcell-immune-awakening).

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Author contributions
S.V. and R.M conceived of and designed the study. S.V., N.D. and R.M. developed the methodology. S.V., E.G., P.A.M., A.R., P.M., J.B., J.T., G.G., A.G., S.M., Z.S., F.B., J.W., N.D., P.C.L. and R.M. acquired the data (managed the patients, provided facilities, provided bioinformatics supervision, performed the experiments and so on). S.V., P.A.M., N.D. and R.M. analyzed and interpreted the data (for example, they performed statistical analysis, biostatistics and computational analysis). S.V. and A.B. prepared the figures. S.V., N.D. and R.M. wrote the manuscript with input and final approval from all authors.

Competing interests
R.M. is a consultant for Pfizer and has a drug discovery program with Basilea Pharmaceutica. P.L. serves as a paid advisor/speaker for Bristol-Myers Squibb, Merck Sharp and Dohme, Roche, Novartis, Amgen, Pierre Fabre, Nektar and MelaGenix. P.L. receives research support from Bristol-Myers Squibb. A.G. received honoraria and consultancy fees from Bristol-Myers Squibb and Novartis. The other authors declare no competing interests.

Additional information
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Supplementary information is available for this paper at https://doi.org/10.1038/s43018-019-0022-x.

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Extended Data Fig. 1 | Schematic summarizing T cell maturation and life-cycle. a Pro-T cells undergo sequential somatic recombination of their T cell Receptor β (TCR) loci in attempts to generate functional TCR with unique CDR3 antigen binding regions. Cells that fail to generate a functional TCRβ at the first attempt can recombine their second TCR allele, but cells which fail to produce a functional TCR at the end of the process (crossed red box) are eliminated (β-selection) and their DNA, which encodes the CDR3 unique regions, enters the blood as circulating cell-free DNA (cfDNA). Surviving cells retain the T cell receptor excision circle (TREC) generated during TCR locus rearrangement as an episome in the nucleus. The TREC does not replicate so is diluted during subsequent cell divisions. b T cells with a functional TCR undergo positive and negative selection (± selection) for HLA and self-antigen recognition. The CDR3 DNA from T cells eliminated during this step is released into the blood. c Naive T cells enter the circulation as early thymic emigrants (eTe). d T cells primed by antigen presenting cells (APC) in the lymphatic system undergo clonal expansion, which dilutes the TREC amongst the daughter cells. e T cell homeostasis is maintained by subsequent contraction (turnover cycles), releasing further CDR3 DNA into the blood.
Extended Data Fig. 2 | Gating strategy for the identification of T cell subsets in peripheral blood of melanoma patients. Multiparametric fluorescence activated cell sorting analysis using the indicated gates. a Lymphocyte gate on side scatter/forward scatter; b single cell gate to exclude doublets; c live gating to exclude dead cells from subsequent gates; d CD3+ gate for T cells; e,f CD4+ and CD8+ gates for “helper” and “killer” T cell subsets, CD8 was detected with a PE-Cy7 labelled antibody for the Treg panel (e) and with a FITC labelled antibody for the T maturation panel (f); g CD4+/CD25+/CD127−/low regulatory T cells (Treg); h naive (top left) and memory (bottom right) gates total T cells; i ETE (top) and CD31+ naive (bottom) gates for naive T cells; j naive (top left) and memory (bottom right) gates for CD8+ T cells; k CD8+ memory T cell subsets, the left bottom subset (CCR7−/CD27−) represents the Treg cells.
**Extended Data Fig. 3** | Clonal relatedness in tumor infiltrating T cells and PBMC. a Clonal relatedness changes in PBMC-private and TIL-private TCR pools; comparison of week 3 (W3) CDR3 clonal relatedness in patients with progressive disease (PD, n=11 patients) and disease control at week 12 (DC, n=7 patients) in the PBMC-private (P=0.724, median=0.6x10^{-6} and 0.6x10^{-6}, respectively; two-sided Mann-Whitney U test) and TIL-private pools (P=0.246, median= 0.5x10^{-4} and 0.8x10^{-5}, respectively; two-sided Mann-Whitney U test). Dot represents one patient; green indicates DC; orange indicates PD; error bar is standard deviation.
Extended Data Fig. 4 | Identification of TIE in CPI-treated patient PBMC. a Comparison of differential abundance of TIE in CD8+ memory T cells in the PBMC of The Christie NHS Foundation Trust patients with best response progressive disease (PD, orange, n=14) and disease control (DC, green, n=16) at T0 (n=30, light shade) and week 9 (W9; n=10, dark shade; PD, n=4, DC, n=6). Differences over time were not significant for PD (median=15.2 and 35.5; P=0.375; two-sided Wilcoxon test) or DC (median=7.9 and 24; P=0.219; two-sided Wilcoxon test); PD vs DC patient values did not differ at T0 (P=0.275; two-sided Mann-Whitney U test) or W9 (P=0.762; two-sided Mann-Whitney U test). b Distributions of marker intensities of the T cell surface markers in the 20 cell populations (clusters) for PBMC from a published cohort3 (n=20 patients). Cluster 5 was identified as the TIE subset. Blue densities are calculated over all the cells and serve as a reference and red densities represent marker expression for cells in a given cluster. Arrows highlight the TIE subset. c T-stochastic neighbor embedding of single cell profiles (dots) performed in an external cohort3 using the T cell surface markers CD3, CD4, CD8, CD45RA, CD45RO, CCR7 and CD27; different colors are attributed by clustering. Arrow highlights the TIE subset. d Comparison of the differential abundance of the TIE cluster in the PBMC from a published cohort3 of patients with PD (orange, n=9) or DC (green, n=11) at pre-treatment (light shade, n=20; PD, n=9; DC, n=11) and at week 12 (W12, dark shade, n=20) on treatment with pembrolizumab or nivolumab in the external cohort. Horizontal bars indicate the differences over time for the PD (median at T0=5.9 and W12=9.1; P=0.164; two-sided Wilcoxon test) or DC patients (median at T0=3.8 and W12=3.3; P=0.831; two-sided Wilcoxon test), and difference in the two response groups at T0 or W12 (P=0.37 and P=0.201, respectively; two-sided Mann-Whitney U test). Light and dark orange indicate PD for T0 and W9-W12, respectively, light and dark green indicate DC for T0 and W9-W12, respectively; n represents patients; ns means not significant P values; error bars are standard deviation.
Extended Data Fig. 5 | Characterization of TIE in PBMC. Analysis of published cohort of PBMC single cell data from reference #27. a Violin plots of the expression level of selected phenotypic and transcriptomic features of the clusters identifying peripheral T cell subsets (n=7488 single cells), the cluster with TIE phenotype is indicated in red; the plots represent the density probability, the area shapes reflect the data distribution; horizontal lines represent the minima and maxima values; central dots represent the medians. Overall minima, mean and maxima values: surface CD3=0, 0.3785, 4.1396; surface CD8a=0, 0.96327, 6.21476; surface CD45RA=0, 0.8161, 4.8508; surface CD45RO=0, 0.6628, 4.6468; surface CD197/CCR7=0, 0.8961, 5.7975; surface CD69=0, 0.5219, 4.2200; surface CD279=0, 0.09787, 3.84886; surface CD25=0, 0.08853, 4.00428; surface CD155=0, 0.4850, 4.6679; surface CD40=0, 0.6003, 5.5083; surface CD154=0, 0.4062, 3.8159; surface CD357=0, 0.1193, 4.0316; LGALS2=0, 0.561, 6.089; TyROBP=0, 1.337, 6.662; FCN1=0, 1.290, 6.789; CST3=0, 1.404, 6.504; LST1=0, 1.042, 6.097; LYZ=0, 1.775, 6.859. b T-SNE plot showing the clusters identified by means of the antibody derived tags (ADT) targeted to surface markers (n=7488 single cells); the black arrow indicates the cluster with TIE phenotype. c Plot showing the proportion of cells with the TIE phenotype from the same published cohort after standard in vitro culture (CTRL, n=3 sorted healthy donor peripheral blood CD8+ naïve T cell samples in standard culture) or following stimulation with anti-CD3/anti-CD27 Dynabeads® (STIM, n=3 sorted healthy donor peripheral blood CD8+ naïve T cell samples after stimulation) (P=0.0267, two-sided paired t test, two degrees of freedom) and d Volcano plot representing the transcriptomic differential expression of the cells with the TIE phenotype in PBMC presented in a (n=7488 single cells) or expanded from naïve CD8+ T cells from the experiment presented in c (n=12217 single cells; two-sided Wilcoxon test with Bonferroni correction for multiple comparisons).
Extended Data Fig. 6 | Expression of Ki-67 and PD-1 in peripheral T<sub>e</sub> cells before and after 1 cycle of CPI. a Expression of Ki67 and PD1 in the T<sub>e</sub> subset as measured by FACS in n=5 frozen samples of PBMC from The Christie NHS Foundation Trust metastatic melanoma patients treated with CPI, at pre-treatment (T0) and after 1 cycle of CPI (W3); horizontal line indicates median; error bar indicates standard deviation. The small sample size did not allow statistical comparison of the outcome groups.
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Software and code

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Data collection

The external validation anti-PD1 data were pooled from the cohorts of patients from AC Huang et al. Nature 2017 (CD38+ plus CD38- merged populations) and RN Amaria et al. Nat Med 2018 for which TCR sequencing was available from baseline and pre-second cycle blood, and who had had no previous cytotoxic treatment 2 weeks prior to CPI or previous immunotherapy (IFN/L2) or targeted therapy; these data were downloaded from Adaptive ImmunoSeq ANALYSER repository and EGA repository. Differential marker expression analysis was performed on CyTOF (cytometry by time-of-flight mass spectrometry) data from Krieg et al. Nature Medicine 2018, downloaded from a publicly available repository. Differential proteomic and RNA expression analysis was performed on REAP-Seq data from VM Peterson et al. Nat Biotechnol 2017; these data were downloaded from a publicly available repository.

Data analysis

Analyses were performed with GraphPad Prism version 7 (GraphPad Software, La Jolla California USA) or R (with relevant packages, v. 3.4.1, The R Foundation for Statistical Computing, Vienna, Austria), ImmunoSEQ® ANALYZER (Adaptive Biotechnologies, Seattle, WA, USA), Flowjo (v.10, Tree Star Inc., Ashland, OR, USA), Cytobank, Triemmatic (v0.36) and STAR (v2.5.1) aligner are published or commercial codes and softwares used for some of the analyses. Diversity was calculated using Renyi index (β=1) as per Spreafico et al.

Differential marker expression analysis was performed on CyTOF data was performed using the custom workflow described in Nowicka et al... Custom R scripts are available in GitLab (https://gitlab.com/cruk-mi/tcell-immune-awakening).

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Sample metadata file, and patient metadata data files are available as online Extended Data and from Gitlab (https://gitlab.com/cruk-mi/tcell-immune-awakening). All TCR sequencing data used to perform this study is deposited in ImmunoSEQ® Immune ACCESS repository (https://clients.adaptivebiotech.com/immuneaccess). The RNA-Seq data for patient #12 can be downloaded from EGA (accession code EGAS00001004043). Immune ACCESS repository. RNA-Seq data (patient #12) will be deposited in a publicly available repository (GO). The authors confirm that, for approved reasons, some access restrictions apply to data containing patient medical records. Anonymised cytometry data will be available upon request to the corresponding authors.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: Sample size calculation was performed using G*Power software (Erdfelder, Faul, & Buchner, 1996), using the effect size and standard deviation. For the comparison of delta-RES in peripheral blood mononuclear cells vs cfDNA in patients with disease response sample size was 7+7 for alpha=0.05 and 1-beta=0.8. For the linear discriminant analysis we used the power and sample size calculation for linear regression with 2 covariates and effect size f^2=0.55, and total sample size was calculated =17 for alpha=0.05 and 1-beta=0.8. For the T cell subset analyses for the immune-effector T cells in patients with progression vs disease control sample size was 32 for alpha=0.05 and 1-beta=0.8.

Data exclusions: Patients with insufficient material to perform analyses were excluded.

Replication: Experiments could not be replicated because they were performed with patient-derived samples.

Randomization: Randomization was not relevant for this study because it’s a longitudinal, not interventional biomarker study.

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Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ✕   | Antibodies           |
| ✕   | Eukaryotic cell lines|
|     | Palaeontology         |
|     | Animals and other organisms|
|     | Human research participants|
|     | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq               |
|     | Flow cytometry         |
|     | MRI-based neuroimaging |

Antibodies

Antibodies used

| Antibodies used |
|-----------------|
| Treg panel:     |
| CD3 (1:100, PerCP-Cy5.5, BioLegend, clone OKT3, 1:100, cat 317337, lot B257385, B231920), |
| CD4 (1:100, BV605, BioLegend, clone OKT4, 1:100, cat 317438, lot B239151, B249670, B234836), |
| CD8a (1:40, PE-Cy7, BioLegend, clone HIT8a, 1:40, cat 300914, lot B226514), |
| CD25 (1:10, APC, BioLegend, 1:10, clone BC96, cat 302610, lot B258747), |
| CD127 (1:40, PE, BioLegend, clone A01905, cat 351304, lot B230365); |
Validation

All antibodies are commercially available and were used only for applications validated by the manufacturers. The manufacturer provided QC certificates. Relevant citations about all the specific antibodies used include:

Treg panel:
CD3 (BioLegend)
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CD4 (BioLegend)
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CD4 [BioLegend]
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CD45RO [BioLegend]
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CD31 [BioLegend]
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Teinvigoration panel:
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CD4 (Biologic)
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Human research participants

Policy information about studies involving human research participants

Population characteristics
Blood samples from patients and healthy donors were collected under the Manchester Cancer Research Centre (MCRC) Biobank ethics application #07/H1003/161+5 with written informed consent from the patients at The Christie NHS Foundation Trust. The study was approved by MCRC Biobank Access Committee application 13_RIVA_01. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki and good clinical practice guidelines. A total of 50 patients with metastatic melanoma, treated with either pembrolizumab or nivolumab plus ipilimumab as first-line therapy as per standard of care were included in the study (Extended Data Table 1). Inclusion criteria were the diagnosis of metastatic melanoma, no previous systemic oncological treatment in the neoadjuvant, adjuvant or metastatic setting for melanoma or other cancers, no concomitant therapy with immunosuppressant drugs at enrolment and no synchronous other active malignancies. A total of 54 patients from The Christie NHS Foundation Trust with metastatic melanoma, treated with either pembrolizumab or nivolumab plus ipilimumab as first-line therapy as per standard of care were included in the study. Median age of the patients was 69 years (range 35–85), male patients were 60%, female 40%; BRAF mutated melanoma (V600 mutation) were 70%; BRAF wild type melanomas were 30%; stage was inoperable III/M1a=20%, M1b=26%, M1c/M1d=54%.

Recruitment
Patient recruitment occurred during outpatient clinical practice visits, every potential candidate was screened according to the inclusion and exclusion criteria described above in “Population characteristics”. We note an enrichment in BRAF wild type melanoma patients in our cohort, reflecting the decision of clinicians to treat some BRAF mutated melanoma patients with first line BRAF inhibitors rather than immunotherapy. Patients who become too unwell after treatment start dropped out from the study because they could not have week 3 bloods collected. Although this was a rare event, it might lead to exclusion of patients with worst prognosis from the study.

Ethics oversight
Manchester Cancer Research Centre (MCRC), Biobank ethics application #07/H1003/161+5

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☐ All plots are contour plots with outliers or pseudocolor plots.
☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Peripheral blood mononuclear cells were isolated from patient blood samples using lymphoprep (STEMCELL Technologies, Cambridge, UK) and SepMate tubes (STEMCELL Technologies, Cambridge, UK) as per manufacturer’s instructions. Red cell lysis was performed with RBC Lysis Buffer (Biolegend, San Diego, CA, USA) as per manufacturer’s instruction.

Instrument
Peripheral blood mononuclear cells were analysed using LSR II, LSRFortessa or Aria III (Special Order Research Product) (BD Biosciences) cytometers.
| Software | FlowJo software (v.10, Tree Star Inc., Ashland, OR, USA) |
|-----------------|--------------------------------------------------------|
| Cell population abundance | The abundance of sorted CD3+ cells and CD8+ T cell subsets was determined by ARIA III Diva software purity check and was consistently above 98%. |

| Gating strategy | First, the peripheral blood mononuclear cells are gated on the FSC/SSC lymphocyte population, then doublets are excluded on FSC- A/SSC-A panel. Live cells are then gated on the “negative” population for Live/dead blue stain. T cells are identified as CD3+ cells. CD4+ and CD8+ populations are gated from the CD3+ cells. Then, CD3+/CD4+/CD8-/CD25+/-CD127- low identified the regulatory T cell subset described in the study. CD3+/CD4-/CD8+/CD45RA-/CD45ROhigh/CD27-/CCR7- cells were defined as immune-effector T cells. Ki-67 and PD1 were measured in the immune-effector T cell gate. Early thymic emigrant were defined as CD3+/CD45RA+/CD45RO-/CCR7+/CD2+/-CD31+ T cells. Boundaries between “positive” and “negative” cells are positioned according to the “full-minus-one” staining acquisition results. |

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.