Clonal replacement of tumor-specific T cells following PD-1 blockade

Kathryn E. Yost1,14, Ansuman T. Satpathy1,2,3,14*, Daniel K. Wells3, Yanyan Qi1, Chunlin Wang4, Robin Kageyama3, Katherine L. McNamara5,6,7, Jeffrey M. Granja1,6,8, Kavita Y. Sarin9, Rynane A. Brown2,9, Rohit K. Gupta10, Christina Curtis5,6,7, Samantha L. Bucktrout3, Mark M. Davis3,11,12,13, Anne Lynn S. Chang9* and Howard Y. Chang1,3,6,9,13*

Immunotherapies that block inhibitory checkpoint receptors on T cells have transformed the clinical care of patients with cancer1. However, whether the T cell response to checkpoint blockade relies on reinvigoration of pre-existing tumor-infiltrating lymphocytes or on recruitment of novel T cells remains unclear2–4. Here we performed paired single-cell RNA and T cell receptor sequencing on 79,046 cells from site-matched tumors from patients with basal or squamous cell carcinoma before and after anti-PD-1 therapy. Tracking T cell receptor clones and transcriptional phenotypes revealed coupling of tumor recognition, clonal expansion and T cell dysfunction marked by clonal expansion of CD8+CD39+ T cells, which co-expressed markers of chronic T cell activation and exhaustion. However, the expansion of T cell clones did not derive from pre-existing tumor-infiltrating T lymphocytes; instead, the expanded clones consisted of novel clonotypes that had not previously been observed in the same tumor. Clonal replacement of T cells was preferentially observed in exhausted CD8+ T cells and evident in patients with basal or squamous cell carcinoma. These results demonstrate that pre-existing tumor-specific T cells may have limited reinvigoration capacity, and that the T cell response to checkpoint blockade derives from a distinct repertoire of T cell clones that may have just recently entered the tumor.

We generated droplet-based 5’ single-cell RNA-sequencing (scRNA-seq) and T cell receptor-sequencing (TCR-seq) libraries from 11 patients with advanced basal cell carcinoma (BCC) before and after anti-PD-1 treatment in site-matched primary tumors (Fig. 1a, Supplementary Table 1, Methods). CD3 immunohistochemistry and whole-exome sequencing supported an immunological response to checkpoint blockade, including increased CD3+ T cell infiltration (Fig. 1b) and mutational loss following treatment, which affected both clonal and subclonal mutations and neoepitopes, suggestive of tumor immunoediting2 (Fig. 1c, Extended Data Fig. 1a–c, Supplementary Tables 1–3).

In total, we obtained scRNA-seq profiles from 53,030 malignant, immune and stromal cells with paired TCR sequences in 28,371 out of 33,106 T cells (86%; Fig. 1d,e, Extended Data Fig. 1d, Methods). We identified 19 cell clusters based on scRNA-seq profiles, including two malignant clusters, six T cell clusters, four stromal clusters, three myeloid clusters, three B cell clusters and one natural killer (NK) cell cluster. Immune cell classifications were consistent with the surface markers that were used to isolate cells and bulk RNA-seq from reference populations1 (Fig. 1c, Extended Data Fig. 2a–d). Notably, immune cells from different patients clustered together, indicating consistency in the tumor microenvironment (TME) of immune cell types across patients. Single-cell estimations of copy number variation (CNV) revealed patient-specific CNVs only in malignant cells, which were consistent with CNVs detected by whole-exome sequencing and previously described CNVs in BCC (Fig. 1f, Extended Data Fig. 3a, Methods).

Reclustering of 3,548 malignant cells revealed clustering by patient and BCC subtype (Fig. 1g,h), indicating significant inter-tumoral heterogeneity as has been observed in other cancers10. We identified a shared malignant gene expression program that included EPCAM, BCAM and TP63, which are known markers of BCC10–12 (Extended Data Fig. 3b). We identified 577 genes that were differentially expressed between the tumors of patients—including Ras signaling genes—which suggests aberrant activation of squamous cell pathways in BCC, as has previously been reported11.

Scoring of malignant cells for enrichment of expression signatures of BCC and squamous cell carcinoma (SCC) from bulk RNA-seq13,14 revealed a differentiation continuum with basal signature enrichment in nodular BCCs and squamous signature enrichment in infiltrative and metatypical BCCs (Fig. 1i). Taken together, these results demonstrate that gene expression in BCC is driven by patient-specific malignant pathways, but largely does not influence immune cell phenotypes in the TME.

We next focused on tumor-infiltrating lymphocytes (TILs) to understand the clonal T cell response to checkpoint blockade. First, we reclustered 33,106 TILs and identified 9 distinct T cell clusters containing cells from multiple patients and pre- and post-treatment time points (Methods, Fig. 2a,b, Extended Data Fig. 4a–d). CD4+ clusters included regulatory T cells (Treg) cells, follicular helper
Fig. 1 | Characterization of the BCC TME pre- and post-PD-1 blockade by scRNA-seq. a, Workflow for sample processing and scRNA-seq analysis of advanced BCC samples collected pre- and post-PD-1 blockade. Graphics courtesy of the Parker Institute for Cancer Immunotherapy. NA, not applicable. b, Immunohistochemistry staining of CD3, showing CD3+ cells in representative BCC tumors before and after PD-1 blockade. Tumor boundaries are denoted with dashed lines. Scale bars, 100 μm. Immunohistochemistry was performed once for each sample (n = 16 samples). c, Bar plot of neoepitope burden pre- and post-treatment based on exome sequencing. Variants were classified as predicted neoepitopes if the peptide was found to bind to the MHC allele with a binding strength of less than 500 nM and its wild-type cognate peptide bound to the same allele with a binding strength of greater than 500 nM. d, Uniform manifold approximation and projection (UMAP) of all tumor-resident cells before and after therapy for all 11 patients with BCC. Clusters denoted by color are labeled with inferred cell types, which include two malignant clusters, two CD4+ clusters, three CD8+ T cell clusters, three CD8+ T cell clusters, proliferating (prolif) T cells, endothelial cells, melanocytes, myofibroblasts, cancer-associated fibroblasts (CAFs), dendritic cells (DCs), macrophages, plasmacytoid dendritic cells (pDCs), three B cell clusters and one NK cell cluster. e, UMAP of tumor-resident cells colored by patient identity (top left), combined analysis of TIL phenotypes and clone dynamics (bottom left), UMAP of malignant cells colored by enrichment of BCC and SCC gene signatures (top; signatures were obtained from previously published studies14,15). Malignant cells ordered based on the difference between BCC and SCC signatures (bottom). The clinical diagnosis (left) and clinical subtype (right). f, Inferred CNV profiles based on scRNA-seq data. Non-immune, non-malignant cells (fibroblasts and endothelial cells, n = 2,122) were used as normal reference for CNV inference of malignant cells (n = 3,548). g, Representative examples of hematoxylin and eosin staining of different BCC subtypes. Scale bars, 100 μm. Hematoxylin and eosin staining was performed once for each sample (n = 9 samples). h, UMAP of malignant cells colored by patient (left) and clinical subtype (right). i, UMAP of malignant cells colored by enrichment of BCC and SCC gene signatures (top; signatures were obtained from previously published studies14,15). Malignant cells ordered based on the difference between BCC and SCC signatures (bottom). The clinical diagnosis associated with each cell and expression of signature-associated genes are indicated.

T (T_{\text{m}}) cells and T helper 17 cells (T_{\text{h}}17) cells. CD8+ clusters included naive cells, memory cells, effector memory cells, activated cells, chronically activated/exhausted cells—which are hereafter referred to as exhausted cells—and intermediate exhausted/activated cells, which co-expressed activation- and exhaustion-associated genes. Notably, we observed an increased frequency of T_{\text{h}}1 cells...
Fig. 2 | Exhausted CD8⁺ tumor-infiltrating T cells are clonally expanded and express markers of tumor specificity. **a.** UMAP of tumor-infiltrating T cells present in BCC samples pre- and post-PD-1 blockade. Clusters are denoted by color labeled with inferred cell types (left). The UMAP was also colored by patient (top right) and anti-PD-1 treatment status (bottom right). **b.** Heat map of differentially expressed genes (rows) between cells belonging to different T cell subsets (columns). Specific genes that are associated with different T cell clusters are highlighted. Bars at the top of the heat map indicate the number of cells, post-therapy enrichment and number of patients in each cluster. **c.** Diffusion map of naive, memory, activated and exhausted CD8⁺ T cells using the first two diffusion components (left). Cells are colored based on cluster identities as in **a.** Cells are also colored by diffusion pseudotime and treatment status (top right). Average expression of selected core activation and exhaustion genes is quantified along diffusion components 1 and 2 (bottom right). **d.** Co-expression analysis of differentially expressed genes (n = 146 genes) between activated, exhausted and activated/exhausted CD8⁺ T cells (n = 5,454 cells). Inset shows the core exhaustion module identified by hierarchical clustering, with canonical exhaustion genes highlighted. **e.** Diffusion map of CD8⁺ T cell subsets colored by clone size (left) and box plot of Gini indices for each CD8⁺ T cell cluster calculated for each patient (right), showing significant clonal expansion within exhausted CD8⁺ T cells (n = number of patients, one-tailed unpaired Student’s t-test, relative to base mean; for all box plots: box center line, median; box limits, upper and lower quartiles; box whiskers, 1.5× the interquartile range). Exhausted refers to both exhausted and exhausted/activated clusters. **f.** Activation score (based on expression of top 50 genes most correlated with IFNG expression) versus exhaustion score (based on expression of top 50 genes most correlated with HAVCR2 expression) for all CD8⁺ T cells (n = 17,561), colored by expression levels of indicated genes. **g.** Activation score versus exhaustion score enrichment for TCR clones with more than one cell (n = 6,422) based on average activation and exhaustion scores of individual cells belonging to that clone, colored by the most frequently assigned phenotype for cells belonging to that clone, and size based on clone size (top right) or cell cycle score (bottom right).
cells and activated, exhausted and exhausted/activated CD8+ T cells post-treatment, supporting reports that have suggested that PD-1 blockade primarily affects CD8+ T cells\textsuperscript{1,7} (Fig. 2b).

We used diffusion maps to visualize the relationship between CD8+ T cell clusters and order cells in pseudotime\textsuperscript{18} (Methods, Fig. 2c). The first diffusion component separated activated and exhausted cells and was highly correlated with T cell exhaustion genes, including *PDCD1* and *HAVCR2*, whereas the second diffusion component separated naive and memory cells from activated and exhausted cells and was highly correlated with T cell activation genes, including *IFNG* and *TNF* (Fig. 2c, Extended Data Fig. 5a,b). We used co-expression analysis to identify a core T cell exhaustion signature in the context of checkpoint blockade, which included known exhaustion markers (*HAVCR2*, *TIGIT*), tissue-resident memory T cell (T<sub>RAM</sub>) markers (*ITGAE*, *CXCR6*)\textsuperscript{15,20} and markers of tumor-reactive CD8+ TILs, including *CD39* (which is encoded by *ENTPD1*)\textsuperscript{11–24} (Fig. 2d). These results suggest that exhausted CD8+ TILs increase after PD-1 blockade and express gene signatures of chronic activation, T cell dysfunction and tumor reactivity.

As tumor antigen-specific CD8+ T cells expand clonally during a productive immune response, we analyzed single-cell TCR sequences to identify clonally expanded cells as an indicator of tumor specificity. We grouped cells by T<sub>RA</sub> (which encodes TCRα and T<sub>RB</sub> (which encodes TCRβ) sequences and noted large clone sizes in exhausted T cells compared to other CD8+ clusters (Fig. 2e). We measured clonality using the Gini index and observed significantly higher clonality in exhausted T cells compared to all other CD8+ T cells (Fig. 2e). Analysis of CD4+ T cells demonstrated increased clonality in T<sub>H</sub> cells post-treatment, which in one patient was accompanied by an increase in B cells that expressed germinal center markers (Extended Data Fig. 5c–g).

To examine the link between clonal expansion and exhaustion, we scored all CD8+ cells for activation and exhaustion signatures based on the top 50 genes correlated with *IFNG* and *HAVCR2* expression, respectively (Methods, Fig. 2f). We found that T cells with a high exhaustion signature exhibited gene expression patterns associated with tumor reactivity, including *CD39* (encoded by *ENTPD1*) and *CD103* (encoded by *ITGAE*) expression, and absence of *KLRG1* expression\textsuperscript{21–24}. To characterize individual clones, we averaged exhaustion and activation scores for all cells in a clone and observed high exhaustion gene signatures in the largest clones (Fig. 2g). Exhausted clones also exhibited a high proliferation signature, as has previously been reported for melanoma TILs\textsuperscript{17} (Methods, Fig. 2g). We next analyzed lineage relationships between T cell phenotypes and clonotypes. Globally, we found that cells grouped by clonotype were more likely to share a common phenotype and were more correlated in gene expression than randomly grouped cells, in line with previous studies\textsuperscript{25–27} (Fig. 3a–c, Extended Data Fig. 6a–c). We used GLIPH (grouping of lymphocyte interactions by paratope hotspots) to identify ‘TCR specificity groups’—clusters of distinct TCR sequences that likely recognize common antigens through shared motifs in the CDR3 sequence\textsuperscript{22}. T cells that expressed distinct TCRs within a specificity group were more likely to share a common phenotype and were more correlated in gene expression, compared to randomly grouped clones (Fig. 3a–c, Extended Data Fig. 6b,d). These results suggest that clonally expanded TILs are highly correlated in cellular phenotype and that PD-1 blockade does not promote phenotype instability within a clone. Moreover, specificity group analysis suggests that antigen specificity also contributes to T cell fate.

We investigated whether divergent phenotypes within clones could inform lineage transitions between T cell phenotypes. We aggregated all clonotypes in a given cluster (the primary phenotype) and measured the fraction shared with another cluster (the secondary phenotype) (Fig. 3d). Broadly, we noted significant overlaps between CD8+ T cell phenotypes, including memory and activated T cells, suggesting that there are common transitions between activation states. We detected minimal clonotype sharing between exhausted and effector cells, supporting a strict bifurcation between these phenotypes\textsuperscript{21}. CD4+ T cell clones were largely restricted to single phenotypes, suggesting that there is limited plasticity between CD4+ cell states. We also observed a non-random distribution of phenotypes of individual clones within specificity groups, suggesting that specific T cell phenotypes may result from different TCR signal strength thresholds (Extended Data Fig. 6e). We noted overlaps between CD8+ and CD4+ phenotypes within specificity groups, such as specificity groups containing CD8+ exhausted and CD4+ T<sub>reg</sub> and T<sub>H</sub> clones, suggesting that CD4+ and CD8+ TILs that respond to the same antigen may arise from distinct clonotypes.

To track clonal cell fates after PD-1 blockade, we matched clonotypes between treatment time points based on TCR sequences and compared the primary phenotypes at each time point for all matched clones (Fig. 3e, Extended Data Fig. 7a,b). We observed stability among CD4+ clusters and frequent transitions among CD8+ clusters, similar to clonotype sharing at individual time points.
Although we observed frequent transitions between memory and effector to activated states, pre-treatment exhausted clones did not transition to non-exhausted phenotypes post-treatment, suggesting that exhausted TILs have limited capacity for phenotype transition after PD-1 blockade.

Previous studies have identified stem-like T cells that express the transcription factor \( TCF7 \) that proliferate after PD-1 blockade.\(^{17,29-32} \) To investigate whether similar cells existed in our dataset, we scored CD8\(^+\) cells for exhaustion or \( TCF7^+ \) stem-like signatures (Fig. 3f). We observed a small population of cells (28% of exhausted cells, 1.5% of CD8\(^+\) T cells) with high expression of both \( TCF7^+ \) and exhaustion signatures. We found that for both memory and exhausted phenotypes, persistent clones had a significantly higher \( TCF7^+ \) signature pre-treatment compared to clones that contracted (Fig. 3f). However, this analysis was limited to only two exhausted clones that significantly expanded, prompting us to identify 10 exhausted clones that increased in frequency after therapy but that had been previously excluded due to low clone size and limited expansion (Fig. 3g). The majority of these clones remained exhausted, although those with a high \( TCF7^+ \) signature pre-treatment expanded more substantially. Nevertheless, only a small fraction (10.3%) of post-treatment exhausted clones were derived from
clones that contained TCF7+ cells, suggesting that post-treatment exhausted clones may be derived from additional sources (Fig. 3b).

As few pre-existing exhausted T cells showed expansion after therapy, we investigated how clone abundance changed globally following treatment by comparing pre- and post-treatment frequencies of each clone (Fig. 4a). We identified significantly expanded clones after therapy, many of which were not detected before treatment (68% of significantly expanded clones). Integration of scRNA-seq data revealed strikingly different persistence patterns for each phenotype. Namely, post-treatment exhausted clones were significantly enriched for novel clonotypes; on average, 84% of exhausted clones were derived from novel clonotypes for each patient, compared to only 40% of naive, activated, memory or effector memory clones (Fig. 4a,b). Across all patients, 55% of post-treatment exhausted clones containing at least five cells were derived from novel clonotypes, compared to 20% of memory clones (Fig. 4c). We next asked how expansion of novel clones, a phenomenon we termed clonal replacement, contributed to exhausted T cell frequency in each patient (Fig. 4d, Extended Data Fig. 8a). We found that 7 out of 11 patients had an increased exhausted CD8+ T cell frequency following treatment and, in 6 out of 7 patients, the majority were derived from novel clonotypes (Fig. 4d). Interestingly, post-treatment exhausted clones were enriched in novel TCR specificity groups, suggesting that novel clones may represent new antigen specificities (Extended Data Fig. 8b).

To increase sensitivity to detect rare clonotypes, we performed bulk TCR-seq on the remaining biopsy material obtained from samples of 8 out of 11 patients (Methods, Fig. 4e). Similar to single-cell TCR-seq analysis, we observed a substantial number of novel expanded clones that were either not expanded (<5 cells) or undetected pre-treatment. Compared to all other CD8+ phenotypes, exhausted cells had a higher proportion of significantly expanded clones following treatment, and the majority of expanded clones were derived from novel clonotypes, which was consistently observed across patients with expanded exhausted cells after therapy (Fig. 4f, Extended Data Fig. 8c). To address whether this resulted from sampling bias, we performed bulk TCR-seq on site-matched samples from one patient twice before therapy and twice after therapy at approximately two-month intervals (Extended Data Fig. 8d). We only observed clonal replacement of exhausted clones when comparing pre- to post-treatment samples, suggesting that TCR dynamics of exhausted cells were mainly influenced by PD-1 blockade, not the timing or location of the tumor biopsy.

To determine whether novel clonally expanded TILs could be detected in peripheral blood, we performed bulk TCR-seq on 10 blood samples from 5 patients. Overall, 41% of TIL TRB clonotypes could also be detected in blood, but they only represented 6% of blood clonotype diversity (Fig. 4g, Extended Data Fig. 9a). Importantly, blood clonotypes represented all TIL phenotypes, including novel exhausted TILs (Extended Data Fig. 9a,b). Overall, 35.5% of novel exhausted TIL clonotypes were detected in peripheral blood post-treatment and, notably, 11.8% of novel exhausted TIL clonotypes were detected in peripheral blood pre-treatment, despite being undetectable by deep TCR-seq in the tumor pre-treatment (Fig. 4g). We compared clonotype enrichment in the tumor over peripheral blood by comparing clonotype frequency in each location. We noted a significant increase in the enrichment of exhausted clones and specificity groups relative to other phenotypes post-treatment, suggesting that there is preferential expansion and retention in the tumor, supporting the notion that these clones are tumor specific (Fig. 4h, Extended Data Fig. 9c). These results suggest that it may be feasible to monitor the clonal tumor-specific T cell response to checkpoint blockade in the blood and that novel TIL clones may be recruited from peripheral sources.

Finally, we asked whether clonal replacement of exhausted cells could be observed in a different cancer type. We generated scRNA-seq and scTCR-seq profiles of 26,016 TILs obtained from serial tumor biopsies of 4 patients with SCC who were treated with anti-PD-1 therapy (Fig. 4i, Extended Data Fig. 10a). SCC samples were obtained on average 31 days after treatment, enabling the analysis of TIL dynamics relatively early after treatment. We first confirmed our findings regarding TIL phenotypes—including clonal expansion of exhausted CD8+ T cells that expressed tumor-specificity markers, including CD39+/CD73+ (Extended Data Fig. 10b–d)—and TIL clonotype dynamics, including the stability of clone phenotypes (Extended Data Fig. 10e–j). We observed that a considerable proportion of exhausted cells detected post-treatment were derived from novel clonotypes (Fig. 4j). Integration of scRNA-seq data with bulk TCR-seq confirmed clonal replacement preferentially following PD-1 blockade.

**Fig. 4 | Clonal replacement of exhausted CD8+ T cells following PD-1 blockade.** a. Scatterplots comparing TRB clone frequencies pre- and post-treatment measured by scRNA-seq and scTCR-seq for all patients with BCC (n=11 patients). Clones that were significantly expanded or contracted post-treatment based on a Fisher exact test (P < 0.05) are highlighted on the left. Clones for which the majority of cells exhibit an exhausted CD8+ phenotype (middle, red) or a memory CD8+ phenotype (right, blue) are highlighted. In this and subsequent panels, exhausted refers to both exhausted and exhausted/activated clusters. b. Box plot of the fraction of novel clones detected by scRNA-seq and scTCR-seq within each cluster following treatment (n=number of patients, two-tailed unpaired Student’s t-test). Clones with only one cell detected and cells from su003 with no clonotype overlap between time points were excluded. c. Lorenz curve of TRB clone frequencies based on scRNA-seq and scTCR-seq for exhausted CD8+ T cell clones (left) and memory CD8+ T cell clones (middle) with at least five cells, colored by presence of each clone before treatment. Proportion of novel clones in each phenotype is quantified on the right. d. Fraction of exhausted clones out of total T cells detected by scRNA-seq and scTCR-seq for each patient, separated by treatment status. Cells belonging to novel clones detected post-treatment are highlighted. e. Scatterplots comparing TRB clone frequencies pre- and post-treatment measured by bulk TCR-seq (n=8 patients). Clones that were significantly expanded or contracted post-treatment based on a binomial test (two-sided, Bonferroni-corrected, P < 0.01) are highlighted on the left, with expanded clones further separated based on their detection pre-treatment. Clones for which the majority of cells share an exhausted CD8+ phenotype based on scRNA-seq (middle, red) or a memory CD8+ phenotype (right, blue) are highlighted. f. Bar plot of fraction of clones with significant expansion post-treatment based on bulk TCR-seq, separated by phenotype and colored by replacement status. g. Overlap between TRB clones in peripheral blood and tumor-infiltrating T cells detected by bulk TCR-seq (n=5 patients, left). Fraction of TIL clones detected in peripheral blood, separated by sample (top right). Fraction of novel exhausted TIL clones detected in peripheral blood mononuclear cells (PBMCs), separated by treatment status (bottom right). h. Violin plot of clone enrichment (tumor frequency/PBMC frequency) detected by bulk TCR-seq, separated by phenotype and treatment status (data from five patients, n=number of clones, one-tailed unpaired Student’s t-test). i. Characteristics of SCC samples treated with anti-PD-1 (left) and UMAP of tumor-infiltrating T cells present in SCC samples pre- and post-PD-1 blockade (right). Clusters denoted by color are labeled with inferred cell types. Graphics courtesy of the Parker Institute for Cancer Immunotherapy. j. Fraction of exhausted cells out of total T cells detected by scRNA-seq and scTCR-seq for each patient, separated by treatment status. Novel clones detected post-treatment are highlighted. Sample su010-S was derived from an SCC lesion from patient su010 who presented with both BCC and SCC lesions. k. Bar plot of fraction of clones with significant expansion based on bulk TCR-seq post-treatment, separated by phenotype and colored by replacement status.
in exhausted T cells compared to other phenotypes; overall 50% of expanded exhausted clones were derived from novel clonotypes compared to only 29% of other expanded CD8+ clones (Fig. 4k, Extended Data Fig. 10). Notably, we observed limited expansion of pre-existing clones at early time points in SCC, similar to later time points in BCC.

Here, we performed single-cell profiling of clinical tumor biopsies, including the integration of TCR clonotype and scRNA-seq
phenotype, which revealed that clonally expanded cells were highly enriched in exhausted CD8+ T cells and expressed markers of tumor specificity, including CD39 and CD10323,24, and that the clonal repertoire of exhausted CD8+ T cells was largely replaced by novel clones after therapy compared to other phenotypes. These results suggest that chronic activation and exhaustion of pre-existing TILs limits their re-invigoration after checkpoint blockade1, and that the T cell response to immunotherapy derives from a distinct repertoire of tumor-specific T cell clones.

Clonal replacement of tumor-specific T cells is consistent with several previous findings in the context of PD-1 blockade, including limited reinvigoration of exhausted T cells due to broad epigenetic remodeling4,6, proliferation of CXCR5+CD8+ T cells in lymphoid organs but not other tissues19 and loss of anti-tumor T cell responses following chemical inhibition of T cell migration6. Importantly, our study did not identify the source of novel T cell clones and clones could derive from tumor-extrinsic sources, including lymphoid organs, or rare unexpanded clones within the TME or tumor periphery. Although our bulk TCR-seq suggests the first possibility, further work will be required to identify the source of novel T cell clones and their influence on clinical response. Importantly, both possibilities are compatible with the potential derivation of these cells from a TCF7+ precursor population19,20,32.

Furthermore, expansion of novel TCR clones and specificity groups following PD-1 blockade, coupled with neoeptope loss, suggests that novel T cell clones may initiate a distinct immunodomiing wave7. The antigen identities recognized by each wave require further investigation, perhaps using high-throughput tumor-specificity assays20,22. Finally, our results suggest that improved checkpoint blockade activity in immune-infiltrated (that is, immunologically ‘hot’) compared to immune-desert (that is, immunologically ‘cold’) tumors may result from an intrinsic ability to constantly attract new T cells8, rather than reactivation of pre-existing TILs. In summary, this study reveals insights into the clonal T cell response to checkpoint blockade in human cancer, which has important implications for the design of checkpoint blockade immunotherapies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41591-019-0522-3.

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Author contributions
K.E.Y., A.T.S., A.L.S.C. and H.Y.C. conceived the project. K.E.Y., A.T.S., Y.Q., R.K.G., R.A.B. and K.Y.S. performed experiments. K.E.Y., A.T.S., D.K.W., R.K., C.W., K.M., J.M.G., R.A.B. and K.Y.S. analyzed data. S.L.B., C.C., M.M.D., A.L.S.C. and H.Y.C. guided data analysis. K.E.Y., A.T.S. and H.Y.C. wrote the manuscript with input from all authors.

Competing interests
H.Y.C. is a co-founder of Accent Therapeutics, Pretzel Therapeutics, and is an advisor for 10x Genomics, Arsenal Biosciences, and Spring Discovery. A.L.S.C. was an advisory board member and clinical investigator for studies sponsored by Merck, Regeneron, Novartis, Galderma and Genentech Roche. A.T.S. and D.K.W. are advisors for Immunai.

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Correspondence and requests for materials should be addressed to A.T.S., A.L.S.C. or H.Y.C.

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Methods

Human subjects. This study was approved by the Stanford University Administrative Panels on Human Subjects in Medical Research, and written informed consent was obtained from all participants. All patients had histologically proven advanced or metastatic RCC or SCC and were not suitable candidates for surgical resection. Exclusion criteria included previous exposure to checkpoint blockade agents and systemic immunosuppressive use, treatment with radiotherapy or other anti-cancer agents within 4 weeks of first biopsy. Patients were treated with 200 mg pembrolizumab every 3 weeks or 350 mg cemiplimab every 2 weeks. A subset of patients received ongoing treatment with 150 mg visnidegib daily (Supplementary Table 1). Response was assessed by RECIST version 1.1[12].

Sample collection and processing. Fresh biopsies were collected from the primary tumor site. A portion of the tumor was stored in RNAlater for whole-exome sequencing and bulk TCR-seq. The remaining tissue was processed for scRNA-seq.

Hematolxyn and eosin staining and immunohistochemistry. For hematoxylin and eosin staining, formalin-fixed, paraffin-embedded tissues were cut at 4 μm. Hematoxylin and eosin staining and immunohistochemistry. Sequencing and bulk TCR-seq. The remaining tissue was processed for scRNA-seq.

Fresh biopsies were collected from the primary tumor site. A portion of the tumor was stored in RNAlater for whole-exome sequencing and bulk TCR-seq. The remaining tissue was processed for scRNA-seq. For sample su010-S, we identified peri-tumoral T cells were isolated and used for scRNA-seq. Preparation of scRNA-seq libraries. The scRNA-seq and scTCR-seq libraries were prepared using the 10x Single Cell Immune Profiling Solution Kit, according to the manufacturer’s instructions. In brief, FACs-sorted cells were washed once with PBS containing 0.04% bovine serum albumin (BSA) and resuspended in PBS containing 0.04% BSA to a final concentration of 100–800 cells per μl as determined by hemacytometer. Cells were captured in droplets at a targeted cell recovery of 500–7,000 cells, resulting in estimated multiplet rates of 0.4–5%.

Following reverse transcription and cell barcoding in droplets, emulsions were broken and cDNA purified using Dynabeads MyOne SILANE followed by PCR amplification (98 °C for 45 s; 13–18 cycles of 98 °C for 20 s, 67 °C for 30 s, 72 °C for 2 s; 1 min). Amplified cDNA was then used for both 5’ gene expression library construction and TCR enrichment. For gene expression library construction, 2–50 ng of amplified cDNA was fragmented and end-repaired, double-sided size-selected with SPRIselect beads, PCR amplified with sample indexing primers (98 °C for 45 s; 14–16 cycles of 98 °C for 20 s, 54 °C for 30 s, 72 °C for 2 s; 1 min), and double-sided size-selected with SPRIselect beads. For TCR library construction, TCR transcripts were enriched from 2 μl of amplified cDNA by PCR (primer sets 1 and 2; 98 °C for 45 s; 10 cycles of 98 °C for 20 s, 67 °C for 30 s, 72 °C for 2 s; 1 min). Following TCR enrichment, 5–50 ng of enriched PCR product was fragmented and end-repaired, size-selected with SPRIselect beads, PCR amplified with sample-indexing primers (98 °C for 45 s; 9 cycles of 98 °C for 20 s, 54 °C for 30 s, 72 °C for 2 s; 1 min) and size-selected with SPRIselect beads.

Sequencing. The scRNA-seq libraries were sequenced on an Illumina NextSeq or HiSeq 4000 to a minimum sequencing depth of 25,000 reads per cell using read lengths of 26 bp read 1, 8 bp p7 index, 96 bp read 2. The single-cell TCR libraries were sequenced on an Illumina NextSeq or HiSeq 4000 to a minimum sequencing depth of 5,000 reads per cell using read lengths of 150 bp read 1, 8 bp i7 index, 150 bp read 2.

Data processing of exome libraries. Whole-exome sequencing was preprocessed using a standard GATK approach. In brief, both tumor and normal samples were aligned to GRC38 with bwa-mem and further processed to remove duplicates and to recalibrate base quality scores. All processing was performed in FireCloud.

Mutation calling and neoepitope prediction. Small somatic variants were identified using Mutect2 and further annotated with the GATK. Somatic copy number variants were identified using the GATK best-practices pipeline. Variants were visualized using GenomeBrowse (version 1.14.2). HLA typing was performed on the germline whole-exome sequenced sample using xHLA. Neoepitopes were identified using pVAC-seq and a peptide–MHC pair was considered a neoepitope if the peptide was found to bind to the MHC allele with less than 500 nM binding strength and the wild-type cognate peptide bound to the same allele with a binding strength of greater than 500 nM. We also examined the expression of the neoepitopes filters in Extended Data Fig. 1b, including mutant-peptide binding strength that were less than 500 or 50 nM and observed similar trends across patients and treatment conditions.

Tumor clonal composition analysis. For the clonal evolution analysis, somatic single-nucleotide variants were called using Mutect 1.1.7 and the variant assurance pipeline for filtering and rescuing. The variant assurance pipeline filters for formalin-fixed, paraffin-embedded artefacts as well as for other artefacts.
and also leverages sequencing data from related samples to salvage false-negative hits that would otherwise occur owing to limits of the variant caller. Variant allele frequencies were calculated for the detected and rescued variants by dividing the number of reads that contain the variant by the total number of reads at that position. For each sample, mutations covered by less than 20 reads in any sample were removed, as were mutations for which the alternate allele was not supported by at least 4 reads in at least 1 sample.TitanCNVA was used to define local copy number and purity of the tumor samples. Observed variant allele frequencies were adjusted for local copy number and purity using the CHAT framework to generate estimates of the cancer cell fractions for each mutation in each sample. Samples from patient su002 were excluded from further clonal evolution analysis because the samples had a purity of <15% (as inferred by TitanCNVA) in both the pre-treatment and post-treatment samples, reducing the accuracy of imputed values of the cancer cell fractions. Next, we used PyClone to define mutational clusters and assess clonal evolution, with clusternumber of principal components, k, adjusted for cell cycle effects and did not regress out cell cycle scores. Variable genes were called on each sample independently based on average expression above, with the following modifications. For malignant-cell and T-cell-specific clustering in BCC samples, we isolated the Gene Ontology biological process term ‘cellular response to heat’.

Cell cluster annotation. Clusters were annotated based on expression of known marker genes, including CD3G, CD3D, CD3E, CD2 (T cells), CD8A, GZMA (CD8+ T cells), CD4, FOXP3 (CD4+ T cells and Treg cells), KLRC1, KLRC2 (NK cells), CD19, CD79A (B cells), SLAMF7, IGKC (plasma cells), FCGR2A, CSF1R (macrophages), FLT3 (dendritic cells), CLEC4F (plasmacytoid dendritic cells), COL1A2 (fibroblasts), MCM4, MYLK (myofibroblasts), FAP, PDPP (cancer-associated fibroblasts), EPCAM, TP63 (malignant cells), PECAM1, VWF (endothelial cells), PMEL and MLANA (melanocytes). Clusters were also confirmed by identifying differentially expressed marker genes for each cluster and comparing to known cell-type-specific marker genes. Finally, we downloaded bulk RNA-seq count data from sorted immune cell populations from a previously published study and compared bulk gene expression to pseudo-bulk expression profiles from single-cell clusters. UMI counts were summed for all cells in each cluster to generate pseudo-bulk profiles. Gene counts from aggregated single-cell and pseudo-bulk data were then used to construct consensus gene expression profiles from bulk RNA-seq data.

Data processing of single-cell TCR-seq libraries. TCR reads were aligned to the GRCh38 reference genome and consensus TCR annotation was performed using cellranger vdx (10x Genomics, version 2.1.0). TCR sequences were aligned to a minimum depth of 5,000 reads per cell, with a final average of 15,341 reads per cell. On average, 12,335 reads mapped to either the TRA or TRB loci in each cell. TCR annotation was performed using the R package RSeq v.4 Ultra Low Input RNA Kit (Clontech) with 2 ng of input RNA. Sequencing data were then used to determine clonal evolution of specific clonotypes detected, an average of 1.84 cells were assigned to each clonotype, 5,291 sequences and two TRB sequences and two TRA sequences. We detected an average of 1,863 unique clonotypes on average in each patient (range 151–4,081). Of 27,956 total clonotypes detected, an average of 1.84 cells were assigned to each clonotype, 5,291 clonotypes consisted of more than one cell, and clonotype sizes ranged from 1 cell to 564 cells.

GLIPH analysis. To identify TCR specificity groups, GLIPH analysis was carried out as described previously. GLIPH clusters TCRs based on two similarity indexes: (1) global similarity, that is, the CDR3 sequences differ by up to one amino acid; and (2) local similarity, that is, two TCRs contain a common CDR3 motif of two, three or four amino acids (enriched over random sub-sampling of unselected repositories). We performed GLIPH with the following modifications: (1) for clusters based on global similarity, CDR3b fragments within the same cluster are required to be different by one amino acid at most, and this difference must be at the same amino acid location in all fragments within the cluster; and (2) for clusters based on local motifs, the starting positions of motifs of the same cluster within CDR3b fragments must be within three amino acids to be considered.

Single-cell CNV detection. Single-cell CNVs were detected using HoneyBAGGER. The log-transformed UMI counts were used as input, after removing genes with the highest expression lower than 0.1 normalized counts (7,189 genes passed the filter, 75–753 genes per chromosome). Non-immune, non-malignant cells were used as a normal reference, including fibroblasts, endothelial cells and melanocytes (n = 2,122). CNVs were detected based on the average gene expression in sliding windows across each chromosome (n = 101 genes per window) relative to average expression in normal reference cells. CNV profiles of malignant and reference cells were visualized with z-score limits of −0.6 and 0.6.

Generation and data processing of bulk RNA-seq libraries. For bulk CD4+ T cell subset RNA-seq, cdNA library construction was performed using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech) with 2 ng of input RNA. Sequencing libraries were then prepared using the 10x DNA Lib Prep kit. Sequencing libraries, quantified using the Qubit dsDNA HS Kit (Thermo Fisher Scientific) and pooled in equimolar ratios. Final pooled libraries were sequenced on an Illumina HiSeq 2500 with paired-end 50-bp read lengths. Paired-end RNA-seq libraries from BCC tumors, cutaneous SCC tumors and T cell subsets (and this study) were aligned to the GRCh38 reference genome using STAR (version 2.6.1a) following adapter trimming by cutadapt (version 1.17). Uniquely mapped reads were counted with featureCounts (version 1.6.2) using Ensembl GRCh38 GTF transcript annotations. Differential expression analysis was performed using DESeq2 to identify cell-type-specific expression programs (version 1.18.1). Gene expression signature scoring. Individual cells were scored for bulk RNA-seq expression programs derived from bulk RNA-seq data as follows. Raw UMI counts were used as input into the AUCell package to score each cell for gene set enrichment based on AUC scores to correct for gene dropout and library size differences. After building a gene expression ranking for each cell, the gene set
enrichment was calculated for each cell using the area under the recovery curve using default parameters.

Activation and exhaustion signatures were derived by identifying variable genes across all CD8+ T cells using the FindVariableGenes function in Seurat with an average expression cut-off of 0.05 and dispersion cut-off of 0.5. The Pearson correlation between reference genes IFNG (activation signature) and HAVCR2 (exhaustion signature) and all variable genes across all CD8+ T cells was computed using scaled expression values. Exhaustion and activation signature genes consisted of the top 50 genes with the highest correlation with the reference genes IFNG and HAVCR2. The \( \text{TCF7l}^{+} \) stem-like signature was obtained from previously published processed data \(^6\). Individual cells were scored for enrichment of gene signatures using the function AddModuleScore in Seurat. Cell cycle scoring was performed as previously described \(^7\). In brief, cells were scored for enrichment of cell-cycle-associated genes using the CellCycleScoring function in Seurat.

**Diffusion map and pseudotime analysis.** Single cells from BCC samples assigned to CD8+ T cell clusters were used for diffusion map and pseudotime analysis. Differentially expressed genes were used to recalculate principal components. Data were then exported to Scmapy (version 1.2.2) \(^8\) for diffusion map and pseudotime analysis. Data was preprocessed by computing a neighborhood graph using 40 neighbors, the first 20 principal components. The first 3 components of the diffusion map were then computed. A randomly selected naive T cell was used as the root cell for diffusion pseudotime computation using the first 3 diffusion components and a minimum group size of 10.

**Sources for bulk RNA-seq data.** Reference bulk RNA-seq from sorted immune populations were obtained from Gene Expression Omnibus (GEO) (GSE118165). Reference bulk RNA-seq data from CD8+ T cells were obtained from GEO (GSE113590). Reference bulk RNA-seq data from BCCs were obtained from GEO (GSE58377). Reference bulk RNA-seq data from SCCs were obtained from the ArrayExpress database (E-MTAB-5678).

**Statistical analysis.** The statistical methods used for each analysis are described within the figure legends and in the Reporting Summary linked to this article.

**Reporting Summary.** Further information on research design is available in the Life Sciences Reporting Summary linked to this article.

**Data availability**

All ensemble and sRNA-seq data have been deposited in GEO and are available under accession number GSE123814. Exome-sequencing data have been deposited in the Sequence Read Archive (SRA) and are available under accession number PRJNA533341. Bulk TCR-seq data can be accessed through the ImmuneACCESS database of Adaptive Biotechnologies (https://doi.org/10.21417/KY2019NM; https://clients.adaptivebiotech.com/pub/ynet-2019-natmed/). All other relevant data are available from the corresponding authors upon reasonable request.

**Code availability**

All custom code used in this work is available from the corresponding authors upon reasonable request.
Extended Data Fig. 1 | see figure caption on next page.
Extended Data Fig. 1 | Mutational landscape of BCC tumors following PD-1 blockade. Related to Fig. 1. a, Summary of mutation burden, potential driver mutations and mutation frequencies detected in whole-exome sequencing data. Potential driver mutations were selected based on frequently mutated genes in BCCs that have been identified previously. Del, deletion; Ins, insertion. b, Bar plots of nonsynonymous mutation burdens before and after treatment detected by exome sequencing (top) and predicted neoepitope burden using only the predicted binding strength of the mutant peptide, for peptides with a binding strength of less than 500 nM (bottom left) or less than 50 nM (right). c, Changes in the composition of clonal mutations detected in exome sequencing data following treatment, with persistent mutation clusters in gray, mutation clusters decreasing in cellular prevalence following treatment in blue or green and mutation clusters increasing in cellular prevalence following treatment in red. For clonal composition analysis, variant allele information from matched pre- and post-treatment tumor samples was leveraged to rescue shared low-frequency variants that did not pass standard variant filtering (Methods). Bar plots of the ratio of predicted neoepitopes to nonsynonymous mutations in each mutation cluster (right), with two novel tumor subclones emerging after treatment that were devoid of predicted neoepitopes. Predicted neoepitopes were based on binding strength of less than 500 nM for the mutant peptide and greater than 500 nM for the corresponding wild-type peptide (as in Fig. 1c). d, Representative flow cytometry staining of dissociated BCC cells. Similar results were obtained for each sorted sample (including SCC samples, n = 32). Cells were stained for expression of the indicated markers, and two-color histograms are shown for cells pre-gated as indicated by the arrows and above each diagram. Numbers represent the percentage of cells within the indicated gate. Bottom panels demonstrate cell size differences between tumor and stromal cells, immune cells (non-T cells) and T cells.
Extended Data Fig. 2 | see figure caption on next page.
Extended Data Fig. 2 | Characterization of cell types present in BCC TME. Related to Fig. 1. a, Heat map of differentially expressed genes (rows) between cells belonging to each cell-type cluster (columns). All malignant cells were treated as one cluster. b, Correlation between aggregated expression profiles from immune-cell-type clusters identified in BCC TME and bulk RNA-seq profiles from sorted reference populations (data were obtained from a previously published study6, n = 1–4 biologically independent samples from different donors). c, UMAP of all BCC TME cells colored by cell-type-specific markers. d, Bar plots indicating relative proportions of markers used for sorting that were detected in each cluster (excluding cells that were not sorted using any markers), proportions of cells for which a TCR sequence was detected in each cluster, relative proportions of each non-malignant cell type detected per patient, relative proportions of cells from each patient detected in each cluster, and proportions of cells detected pre- and post-treatment in each cluster.
Extended Data Fig. 3 | see figure caption on next page.
Extended Data Fig. 3 | Copy number alterations and gene expression of individual BCC tumors. Related to Fig. 1. a, Inferred CNV profiles for malignant cells separated by patient based on scRNA-seq (scCNV) and whole-exome sequencing (WES). Dashed line indicates a potential subclone identified by scCNV highlighted for su005. For all patients, pre- and post-treatment malignant cells were analyzed together and exhibited similar CNV profiles, with the exception of su006. For su006, differences between time points were apparent in CNV profiles obtained from both scRNA-seq and exome sequencing, analogous to the changes in mutation composition identified in Extended Data Fig. 1c. b, Heat map of differentially expressed genes (rows, $n = 577$) across malignant BCC cells ($n = 3,548$) aggregated by patient (columns, $n = 8$). Cut-offs for differential expression were adjusted $P < 0.01$ (Bonferroni-corrected, two-tailed Wilcoxon rank-sum test), log-transformed average fold change $> 0.3$ and difference in fraction of positive cells $> 0.3$. Core BCC genes that are differentially expressed between all malignant cells and other TME cells are shown in the top cluster. Genes differentially expressed between patients are shown in the bottom clusters. Specific genes associated with cancer-associated pathways are highlighted.
Extended Data Fig. 4 | Characterization of T cell subtypes present in BCC TME. Related to Fig. 2. a, Enrichment of bulk T cell subtype signatures for each T cell cluster identified in the BCC TME. T cell subtype signatures were derived from bulk datasets (from this study and a previously published study21, n = 3–7 biologically independent samples from different donors) and single T cells from a BCC dataset were scored for signature enrichment. Heat maps represent the z-scored average signature enrichment for each cluster. b, Heat map of Pearson correlation between T cell clusters based on first 20 principal components used for clustering (n = 33,106 cells). c, UMAP of all T cells colored according to marker gene expression. d, UMAP of all T cells separated by patient and colored according to anti-PD-1 treatment status.
Extended Data Fig. 5 | see figure caption on next page.
Extended Data Fig. 5 | Characterization of diffusion map trajectories and increase in T_{FH} cell clonality accompanied by B cell expansion. Related to Fig. 2. a, Violin plots of cell coordinates in diffusion components 1 and 2 separated by cluster identity (left, middle). Violin plot of pseudotime values separated by cluster identity (right). n = number of cells. b, Heat map of expression of genes with highest correlation with diffusion components 1 and 2 (rows) across cells belonging to each cell-type cluster (columns). c, Box plot of Gini indices for each CD4+ T cell cluster separated by time point, showing clonal expansion of T_{FH} cells after treatment. Each point represents a patient with more than 10 cells belonging to a cluster at that time point; the size is proportional to the number of cells. d, UMAP of all cells detected for patient su001 colored by treatment time point (left) and relative proportions of each immune cell type (right), showing increased frequency of B cells after treatment. e, UMAP of T cells detected for patient su001 colored by treatment time point (left) and relative proportions of CD4+ phenotype (right), showing increased frequency of T_{FH} cells after treatment. f, Bar plot of percentage AICDA+ B cells, separated by patient. g, Hematoxylin and eosin (H&E) staining of the post-treatment BCC tumor of patient su001 demonstrating islands of BCC in sclerotic stroma with a peripheral cuff of dense lymphoid tissue. Scale bars, 400 μm (top) and 100 μm (bottom). Hematoxylin and eosin staining was performed once for each sample.
Extended Data Fig. 6 | see figure caption on next page.
Extended Data Fig. 6 | Correlations between TCR clonotypes or TCR specificity groups and scRNA-seq phenotypes. Related to Fig. 3. 

**a**, Distributions of the proportion of cells within each clone (≥3 cells) that share a common cluster identity, separated by patient (for patients with >3 clones with ≥3 cells), compared to randomly selected and size-matched groups of T cells (n = number of clones, two-tailed unpaired Student’s t-test).

**b**, Distribution of the proportion of CD4+ cells (left) and CD8+ cells (right) within each clone or TRB clones within each TCR specificity group (≥3 cells) that share a common cluster identity, separated by treatment time point, compared to randomly selected and size-matched groups of T cells from the same sample (left, n = number of clones, two-tailed unpaired Student’s t-test).

**c**, Bar plot of T cell cluster assignments for all clones with more than 10 cells, separated by patient and treatment status (top: pre-treatment, bottom: post-treatment).

**d**, Bar plot of T cell cluster assignments for the largest 10 TCR specificity groups, separated by TRB clone and treatment status (top, pre-treatment; bottom, post-treatment). Conserved motifs between TRB clones identified by GLIPH highlighted in red. Representative TRB sequences shown for TCR specificity groups with more than four unique clonotypes.

**e**, Heat map of the fraction of TCR specificity groups with clones belonging to a given primary phenotype (rows) that also contain clones belonging to a secondary phenotype (columns).
Extended Data Fig. 7 | Details of clone transitions. Related to Fig. 3. a, Heat map of TRB clonotype overlap between all samples, indicating correct pairing of samples and a significant number of overlapping clones between time points within individual patients with the exception of one pair with limited cell numbers and no clonotype overlap (su003). b, Bar plot of T cell cluster assignments for matched TRB clones between time points for the top 60 clones with at least 3 cells per time point. Related to Fig. 3e.
Extended Data Fig. 8 | see figure caption on next page.
Extended Data Fig. 8 | Clonal expansion in tumor and peripheral blood detected by bulk TCR-seq. Related to Fig. 4. a, Scatterplots comparing TRB clone frequencies pre- and post-treatment measured by scRNA-seq and TCR-seq, separated by patient. Clones for which the majority of cells share an exhausted CD8⁺ phenotype (red) or a memory CD8⁺ phenotype (blue) are highlighted. Patient su003 without clonotype overlap between time points was excluded. In this and subsequent panels, exhausted refers to both exhausted and exhausted/activated clusters. b, Box plot of the fraction of novel TCR specificity groups within each cluster after treatment for TCR specificity groups that contain at least two distinct TRB sequences and at least three cells, separated by patient (n = number of patients). c, Bar plot of the fraction of clones with significant expansion post-treatment based on bulk TCR-seq, separated by patient and phenotype and colored according to replacement status. d, Scatterplots comparing TRB clone frequencies between time points measured by bulk TCR-seq for sequential time points in patient su001; clones for which the majority of cells share an exhausted CD8⁺ phenotype (red) or a memory CD8⁺ phenotype (blue) are highlighted. Novel clones that emerged between time points are highlighted in dark red and were detected only in pre- and post-treatment comparisons, but not in comparisons between pre-treatment time points, suggesting that replacement is primarily a result of PD-1 blockade rather than time between sampling.
Extended Data Fig. 9 | TCR overlap between peripheral blood and tumor detected by bulk TCR-seq. Related to Fig. 4. a, Pie chart of the percentage of TRB clones detected in peripheral blood that were also detected in the tumor, expanded to show the distribution of phenotypes in the tumor, as well as the fraction of exhausted clones detected in peripheral blood, colored according to replacement status in the tumor. In this and subsequent panels, the exhausted category includes both exhausted and exhausted/activated clusters. b, Bar plot of the percentage of peripheral T cells that match tumor-infiltrating TRB clones with exhausted phenotypes post-treatment as detected by scRNA-seq. c, Violin plot of TCR specificity group enrichment (tumor frequency/PBMC frequency) detected by bulk TCR-seq, separated by phenotype and treatment status (n = number of TCR specificity groups, two-tailed unpaired Student’s t-test).
Extended Data Fig. 10 | see figure caption on next page.
Extended Data Fig. 10 | Clonal replacement analysis in SCC TILs following PD-1 blockade. Related to Fig. 4. a, UMAP of tumor-infiltrating T cells present in SCC samples pre- and post-PD-1 blockade colored by patient (top) and anti-PD-1 treatment status (bottom). b, Heat map of correlation between averaged RNA expression between BCC and SCC T cell clusters. Ex/Act, exhausted/activated. c, Box plot of Gini indices for each CD8+ T cell cluster calculated for each patient (n = number of patients). In this and subsequent panels, exhausted refers to both exhausted and exhausted/activated clusters, unless otherwise noted. d, Abundance of the top 12 exhausted clones in sample su010-S identified by unsupervised clustering compared to the abundance of the same clones in sorted CD8+CD39+ T cells, colored by assigned phenotype. e, Distribution of the proportion of cells within each clone or TRB clones within each TCR specificity group (≥3 cells) that share a common cluster identity, separated according to treatment time point, compared to randomly selected and size-matched groups of T cells from the same sample (left, n = number of TRB clones or TCR specificity groups, two-tailed unpaired Student’s t-test). f, Heat map of the fraction of clonotypes belonging to a given primary phenotype cluster (rows) that are shared with other secondary phenotype clusters (columns). g, Heat map of all observed phenotype transitions for matched clones during PD-1 blockade for clones with at least three cells for each time point. h, TCF7+/stem-like score versus exhaustion score for all CD8+ T cells, colored according to gene expression (left). TCF7+/stem-like score versus exhaustion score for exhausted cells and cells of other phenotypes belonging to primarily exhausted clones, colored according to phenotype (top right). Violin plot of TCF7+/stem-like score for exhausted cells and cells of other phenotypes belonging to primarily exhausted clones, demonstrating that the highest TCF7+/stem-like score is observed in cells with an exhausted phenotype (bottom right, n = number of cells). i, Violin plot of TCF7+/stem-like score for memory and exhausted cells separated by change in clone abundance after treatment (left, n = number of cells, two-tailed unpaired Student’s t-test). Clones were defined as expanded or contracted if they significantly changed in abundance by a Fisher exact test (P < 0.05 and fold change > 0.5) and persistent if they did not significantly change in abundance and at least one cell was detected at each time point. j, Scatterplots comparing TRB clone frequencies pre- and post-treatment measured by bulk TCR-seq for SCC patients (n = 3). Clones that were significantly expanded or contracted post-treatment based on a binomial test (two-sided, Bonferroni-corrected P < 0.01) are highlighted on the left. Clones for which the majority of cells share an exhausted CD8+ phenotype (middle, red) or a memory CD8+ phenotype (right, blue) are also highlighted.
Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement.
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- The statistical test(s) used AND whether they are one- or two-sided.
- A description of all covariates tested.
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons.
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals).
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted.
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings.
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes.
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated.

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code.

Data collection

- Single cell RNA-seq reads were aligned to the GRCh38 reference genome and quantified using cellranger count (10X Genomics, version 2.1.0). TCR reads were aligned to the GRCh38 reference genome and consensus TCR annotation was performed using cellranger vdj (10X Genomics, version 2.1.0). Bulk RNA-seq data from basal cell carcinoma tumors, cutaneous squamous cell carcinoma tumors and T cell subsets were aligned to the GRCh38 reference genome using STAR (version 2.6.1a) following adapter trimming by cutadapt (version 1.17). Uniquely-mapped reads were counted using featureCounts (version 1.6.2) using Ensembl GRCh38 GTF transcript annotations.
- Whole exome sequencing was preprocessed using GATK (version 4.0, us.gcr.io/broad-gatk/gatk:4.0.4.0). Briefly, both tumor and normal samples were aligned to GRCh37 using bwa-mem and further processed to remove duplicates and recalibrate base quality scores.
Single cell RNA-seq data was analyzed using Seurat (version 2.3.4). Single cell CNVs were detected using HoneyBADGER (not versioned, https://github.com/JEFworks/HoneyBADGER). Bulk RNA-seq data was analyzed using DESeq2 (version 1.18.1). Single cells were scored for expression signatures using AUCell (version 3.8). Diffusion map and pseudotime analysis was performed using Scanpy (version 1.2.2). TCR specificity groups were identified using GLIPH (https://github.com/immunoengineer/gliph) with the following modifications: 1) for clusters based on global similarity, CDR3b fragments within the same cluster are required to be at most one amino acid different, and this difference must be at the same amino acid location in all fragments within the cluster, and 2) for clusters based on local motifs, the starting positions of motifs of the same cluster within CDR3b fragments must be within 3 amino acids to be considered. For exome data analysis, small somatic variants were identified using Mutect2 and further annotated with the GATK (version 4.0). Somatic copy number variants were identified using the GATK best practices pipeline. HLA typing was performed on the germline whole exome sample using xHLA (not versioned, https://github.com/humanlongevity/HLA). Neoantigens were identified using pVAC-seq (not versioned, https://github.com/griffithlab/pVACtools). For the clonal evolution analysis, somatic single-nucleotide variants (SNVs) were called using Mutect 1.1.7 and the variant assurance pipeline (VAP, not versioned, https://github.com/cancersysbio/VAP) for filtering and rescuing. TitanCNA (version 1.8.0) was utilized to define local copy number and purity of the tumor samples. PyClone (version 0.13.0) was used to define mutational clusters and assess changes in cluster frequencies across treatment. Bulk TCR sequencing data was processed using the ImmuneSEQ Analyzer (version 3.0, Adaptive Biotechnologies). HLA Typing analysis was performed using MIA FORA FLEX v3.0 software (Immucor, Inc.).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

All ensemble and single-cell RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) under accession GSE123814. Exome sequencing data has been deposited in the Sequence Read Archive (SRA) under accession PRJNA533341. Bulk TCR sequencing data have been deposited in Adaptive Biotechnologies’ ImmuneACCESS database (doi:10.21417/KY2019NM; https://clients.adaptivebiotech.com/pub/yost-2019-natmed).

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

Sample size
No statistical method was used to determine the patient sample size. The number of patient samples included in this study was primarily determined by the availability of samples obtained in a proof-of-concept clinical trial for PD-1 blockade in BCC. However, the sample size in this study are consistent with prior single-cell studies in cancer immunotherapy, and the samples include patients with either a clinical response or progression during anti-PD1 treatment. For single-cell sample size per patient, we attempted to obtain greater than 1,000 paired scRNA/TCR cells per sample. This benchmark was based on published estimates of the clonal frequency of tumor-specific TILs in tumors and on preliminary flow cytometry measurements of T cell frequency in each sample.

Data exclusions
No data were excluded from analysis.

Replication
Single cell sequencing data from patients were not suitable for replication but findings related to T cell clonotype frequencies were confirmed using bulk TCR sequencing from the same sample.

Randomization
No randomization was used for patient samples, since all patients received PD-1 blockade, and no control (no therapy) samples were analyzed in this study.

Blinding
No blinding was used for patient identity, since all patients received PD-1 blockade, and no control (no therapy) samples were analyzed in this study.
Antibodies

Antibodies used included anti-human-CD45 conjugated to V500 (clone HI30, cat. no. 560779, lot no. 7172744, BD Biosciences), anti-human-CD3 conjugated to FITC (clone OKT3, cat. no. 11-0037-41, lot no. 2007722, Invitrogen), anti-human-CD8 conjugated to Pacific Blue (clone 3B5, cat. no. MHCD0828, lot no. 1964935, Invitrogen), anti-human-CD39 conjugated to APC (clone A1, cat. no. 328210, lot no. B268898, BioLegend), anti-human-PD-1 conjugated to APC/Cy7 (clone EH12.2H7, cat. no. 329921, lot no. B245235, BioLegend) and anti-human-HLA-DR conjugated to evolve 605 (clone LN3, cat. no. 83-9956-41, lot no. 1949784, Affymetrix-Ebioscience), anti-human-CD45RA conjugated to PERCP-Cy5.5 (clone HI100, cat. no. 304107, lot no. B213966, BioLegend), anti-human-CD127 conjugated to Brilliant Violet 510 (clone A019D5, cat. no. 351331, lot no. B197159, BioLegend), anti-human-CD4 conjugated to APC/Cy7 (clone OKT4, cat. no. 317417, lot no. 329921, BioLegend), anti-human-CCR6 conjugated to PE (clone G034E3, cat. no. 353409, lot no. B203239, BioLegend), anti-human-CD25 conjugated to FITC (clone BC96, cat. no. 302603, lot no. B168869, BioLegend), anti-human-CXCR3 conjugated to Brilliant Violet 421 (clone G025H7, cat. no. 353715, lot no. B206003, BioLegend), anti-human-CXCR5 conjugated to Alexa-Fluor-647 (clone RF8B2, cat. no. 558113, lot no. 5302688, BD Pharmingen), and anti-human-CD3E conjugated to Pacific Blue (clone UCHT1, cat. no. 558117, lot no. 4341657, BD Biosciences). All antibodies were used at a 1:200 dilution, with the exception of anti-CD45 and anti-HLA-DR antibodies which were used at a 1:100 dilution.

Validation

All antibodies were validated by the manufacturer directly in human peripheral blood mononuclear cells, and antibody-specific staining was compared to isotype and no staining control samples.

Human research participants

Policy information about studies involving human research participants

Population characteristics

Covariant-relevant population characteristics, including prior treatment regimens and histological subtype are provided in Supplementary Table 1. Subjects included both males and females, aged 50-88.

Recruitment

All recruited patients had histologically-proven advanced or metastatic BCC or SCC and were not good candidates for surgical resection. Exclusion criteria included prior exposure to checkpoint blockade agents, use of systemic immunosuppressants within 4 weeks of first biopsy, or treatment with radiotherapy or any other anticancer treatment within 4 weeks of first biopsy. Potential selection biases include the demographic characteristics of Stanford Hospital patients, which may differ from other institutions. It is unlikely, though possible, that changes in these characteristics will significantly influence the presented results in the manuscript.

Ethics oversight

This study was approved by the Stanford University Administrative Panels on Human Subjects in Medical Research, and written informed consent was obtained from all participants.

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

Fresh tumor biopsies were minced and digested in 5 mL digestion media (DMEM/F12 media + 250 ug/mL Liberase TL + 200 U/mL DNAse I) in a C-tube using the gentleMACS Octo system at 37°C for 3 hours at 20 rpm. Following digestion, 50 mL of 500 mM EDTA was added and sample collected by centrifugation at 300g for 5 minutes. The cell suspension was then passed through a 70 um filter and pelleted by centrifugation at 300g at 4°C for 10 minutes. Cells were then resuspended in 1 mL of RPMI media and cryopreserved in FBS supplemented with 10% DMSO until further processing.
All samples were sorted using a FACSAria II.

Flow cytometry data was analyzed using Flowjo.

Post-sort purities were assessed by flow cytometry and confirmed to be > 95%. Post-sort purities were also validated by single cell RNA-sequencing data.

Cells were first gated on FSC/SSC, then gated to exclude doublets based on FSC-A/FSC-W. Dead cells were excluded based on propidium iodide staining. Cells were categorized as peri-tumoral T cells (CD45+CD3+), other peri-tumoral lymphocytes (CD45 +CD3-) and tumor/stromal cells (CD45-CD3-). An example of the gating strategy used is shown in Supplementary Figure 1C. For sample su010-S, we additionally isolated CD8+CD39+ peri-tumoral T cells (CD45+ CD3+ CD8+ CD39+). For bulk RNA-seq datasets, CD4+ T helper cells were sorted as naive T cells (CD4+CD25-CD45RA+), Treg (CD4+CD25+IL7Rlo), Th1 (CD4+CD25-IL7RhiCD45RA- CCR3+CCR6+), Th2 (CD4+CD25-IL7RhiCD45RA-CCR3-CCR6+), Th17 (CD4+CD25-IL7RhiCD45RA-CCR3-CCR6+), Th1-17 (CD4 +CD25-IL7RhiCD45RA-CCR3+CCR6+), and Th subsets (CXCR5+ counterparts of each).

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