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Single-cell RNA-sequencing reveals predictive features of response to pembrolizumab in Sézary syndrome

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ABSTRACT

The PD-1 inhibitor pembrolizumab is effective in treating Sézary syndrome, a leukemic variant of cutaneous T-cell lymphoma. Our purpose was to investigate the effects of pembrolizumab on healthy and malignant T cells in Sézary syndrome and to discover characteristics that predict pembrolizumab response. Samples were analyzed before and after 3 weeks of pembrolizumab treatment using single-cell RNA-sequencing of 118,961 peripheral blood T cells isolated from six Sézary syndrome patients. T-cell receptor clonotyping, bulk RNA-seq signatures, and whole-exome data were integrated to classify malignant T-cells and their underlying subclonal heterogeneity. We found that responses to pembrolizumab were associated with lower *KIR3DL2* expression within Sézary T cells. Pembrolizumab modulated Sézary cell gene expression of T-cell activation associated genes. The CD8 effector populations included clonally expanded populations with a strong cytotoxic profile. Expansions of CD8 terminal effector and CD8 effector memory T-cell populations were observed in responding patients after treatment. We observed intrapatient Sézary cell heterogeneity including subclonal segregation of a coding mutation and copy number variation. Our study reveals differential effects of pembrolizumab in both malignant and healthy T cells. These data support further study of *KIR3DL2* expression and CD8 immune populations as predictive biomarkers of pembrolizumab response in Sézary syndrome.

Introduction

Immune checkpoint blockade immunotherapy has been shown to produce favorable outcomes in a variety of cancers. Pembrolizumab is a monoclonal antibody against PD-1, an immune checkpoint molecule that induces T-cell exhaustion. Blockade of PD-1 can switch exhausted T-cells to a more invigorated state, in which they can recognize and eliminate cancer cells[1].

Sézary syndrome is a leukemic subtype of cutaneous T-cell lymphoma typically derived from CD4 central memory T-cells.[2] Pembrolizumab was associated with a 27% overall response rate in patients with Sézary syndrome, and these responses appeared to be especially deep and durable.[3] Thus, biomarkers of response to pembrolizumab in patients with Sézary syndrome are needed to select the subset of patients who are most likely to experience long-lasting responses. The two currently proposed biomarkers of response are not readily translatable because they are either rare and limited to mycosis fungoides such as PD-L1 structural variants[4] or require complex spatial analysis from multiplexed imaging data.[5]

A major obstacle in relating potential biomarkers to clinical outcomes is accurately distinguishing the malignant CD4 + T-cells of Sézary syndrome from other similar CD4 cell populations. This is of particular importance in assessing the response to PD-1 inhibition, where the target PD-1 is not only expressed on the immune cells of interest but also by the Sézary cells themselves.[6] Another key challenge in biomarker identification is the underlying tumor heterogeneity of Sézary syndrome. Sequencing of the clonal T-cell receptor (TCR) represents the most specific method to identify the malignant T-cells. Single-cell RNA-sequencing (scRNA-seq) paired with single-cell sequencing of the TCR enables both the assessment of the clonal heterogeneity of Sézary syndrome and the discrimination of malignant from normal T-cells.

In this study, we sought to identify peripheral blood biomarkers of response to pembrolizumab and to decipher the differential effects of pembrolizumab on healthy-T cells versus Sézary cells.

We conducted scRNA-seq with paired TCR sequencing on 118,961 T-cells isolated from three Sézary syndrome patients that responded to pembrolizumab treatment and three patients that failed to respond. We analyzed transcriptomic and inferred genomic features, such as mutations and copy number variations, to better understand the immunological, transcriptional, and genomic characteristics that underlie pembrolizumab response.

Materials and methods

Human subjects and sample collection

Cryopreserved peripheral blood mononuclear cells were collected as part of a phase II clinical trial of pembrolizumab

treatment for mycosis fungoides and Sézary syndrome as previously described (NCT02243579).[3] For each patient, PBMCs were obtained before the first dose of pembrolizumab (pretreatment, Pre), and three weeks later, prior to administration of the second dose of pembrolizumab (Cycle 2, C02). Additional samples from later timepoints were analyzed for two of the patients. The best clinical response was assessed by consensus global response criteria[7] according to the trial protocol. Patients that had either complete or partial response in the clinical trial were classified as "responder" in this study, and patients that had either progressive disease or stable disease were classified as "non-responder." Written informed consent was obtained from all enrolled patients in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board at Stanford University.

T-cell isolation and scRNA-seq

Cryopreserved PBMCs were thawed through sequential dilution. Live T cells were then immediately isolated using the Human Pan T cell Isolation Kit (Miltenyi Biotec) followed by Dead Cell Removal Kit (Miltenyi Biotec). Cells were resuspended into CryoStor® CS10 freezing medium (STEMCELL Technologies) and transferred into a cold (4°C) Nalgene® Mr. Frosty® Freezing Container (ThermoFisher Scientific) which was immediately placed into −80°C freezer for four to 24 hours before long-term storage in liquid nitrogen. ScRNA-seq libraries were generated from the cryopreserved T cells using the Chromium Next GEM Single Cell 5' v2 chemistry (10x Genomics) according to manufacturer's instructions by MedGenome Inc. (Foster City, CA, USA). TCR sequencing was performed from the barcoded cDNA using the Chromium Single Cell Human TCR Amplification kit following the manufacturer's instructions by MedGenome Inc. (Foster City, CA, USA). Libraries were sequenced on a NovaSeq 6000 (Illumina), and the generation of FASTQ files from BCL file using Cell Ranger (10x Genomics) mkfastq function.

Computational analyses of scRNA-seq

Detailed descriptions of computational analyses are provided in the Supplementary methods. Alignment and read counts were performed using Cell Ranger 4.0.0 (10x Genomics). All subsequent analyses were performed using Seurat 3.1.4[8] with R 3.6.1 unless otherwise specified. Each dataset was normalized, and cells were assigned using thresholds for the number of features and percentage of counts mapping to mitochondrial or ribosomal genes. All 15 datasets were integrated and scaled using the IntegrateData and ScaleData functions. In total, 118,961 cells passed all quality control metrics and were included in the analyses.

Sézary cells were purified via fluorescent-activated cell sorting based on their CD4 and CD26 expression from cryopreserved PBMCs collected prior to pembrolizumab treatment. Total RNA was extracted. RNA-seq libraries were made using SMARTer stranded total RNA-seq Kit v2, pico input kit (Takara) and sequenced on HiSeq 4000 (Illumina). These bulk RNA-seq data were used to build personalized reference transcriptional profiles of Sézary cells for each patient. Bulk RNA-seq data from NCBI SRP125125[9] was used to build reference transcriptional profiles of all other T-cell subtypes. The normalized count matrices from the bulk RNA-seq data were normalized and log-transformed to yield a personalized transcriptional profile, which was integrated into a previously defined dataset of 29 cell populations.[9] The SingleR[10] package was used to assign a T-cell subtype identity using this individualized reference transcriptional profile.

Differential gene expression analyses were performed with the FindMarkers function between comparator populations. To avoid identification of genes driven by one sample or patient, significant genes were identified through pairwise sample-to-sample analysis, rather than analyzing merged data from comparator groups. A statistical overrepresentation test was performed using the PANTHER Pathways annotation data set (PANTHER 16.0[11]) for the differentially expressed genes from Pre to C02 for each patient. Fisher's Exact test was performed, and pathways with a False Discovery Rate of less than 0.05 were considered statistically significant.

Pseudotime analysis was performed with TSCAN 2.0.0[12] in R 4.0.2. Each top-clone cell (at Pre and C02 for each patient) was assigned a pseudotime position (in a trajectory from Pre to C02) based on its UMAP location and to find/visualize dynamically expressed genes along the pseudotime trajectory.

For each dataset, the CellCycleScoring function was used to calculate for each cell an S phase score and a G2M phase score based on expression of S phase and G2M phase markers and also to assign each cell a cell cycle phase.

A list of 454 genes with an increased expression in CD8 naive cells as compared to CD8 effector memory T cells was used to create an effector score.[13] A list of 16 genes defining a cytotoxicity signature was used to create a cytotoxicity score.[14] For each dataset, the AddModuleScore function was then used to calculate the effector score and cytotoxicity score for each cell based on its expression of the genes in these lists, respectively.

Single nucleotide variants analysis

A list of somatic variants were determined from prior exome sequencing for all patients except Pt6.[3] For each patient's Pre-dataset, cb_sniffer[15] with Python 3.8.5 was used to detect UMIs and cell barcodes with coverage overlying variant genomic positions. Variants were filtered for those with above 10,000 total UMIs detected at the position of the variant. Cells with at least one variant of UMI were assigned as containing the mutation. If no variant UMIs were present then they were assigned as wild type for the mutation if there were six or more reference UMIs. Cells with five or fewer reference UMIs and no variant UMIs were categorized as "unknown".

Copy number variation analysis

InferCNV 1.7.1 in R 4.0.2 was used to computationally infer the copy number variation for each patient's top-clone cells from pretreatment time points. The cells with TRB CDR3s other than those of the top or second top clone were used as a reference to establish a baseline upon which copy number variations of the top-clone cells were calculated.

Flow cytometry

T-cells from the pre-treatment timepoint for each patient were purified as above and viably frozen. Cells were then thawed and stained with a panel including antibodies against CD3, CD4, CD8, PD1, CD39, and KIR3DL2, as well as 7-AAD for viability. Specific antibody clones are listed in Supplementary Methods. Data were collected on a BD™ LSR II or FACSymphony A5 flow cytometer. Live Sézary cells were individually gated by the co-expression of CD4 and CD3, and lack of expression by CD26 and CD8. Analysis was performed with Cytobank 9.1.

Results

scRNA-seq clustering and identification of Sézary cells

To determine the differential effects of pembrolizumab on malignant and healthy T-cells and to find features that predict response to pembrolizumab, we analyzed peripheral blood T-cells from three patients with response to pembrolizumab and three patients with no response (either progressive disease or stable disease). scRNA-seq and single-cell TCR sequencing was performed on a total of 118,961 T-cells isolated from 15 samples collected before and during pembrolizumab treatment (Figure 1(a,b)). We analyzed the transcriptome of these cells, as well as inferred various genomic features such as mutations and copy number variations from the scRNA-seq data (Figure 1a), to gain a better understanding of the immunological, genomic, and transcriptomic features that underlie pembrolizumab response.

Although TCR clonotyping has traditionally been considered the gold standard to identify malignant T-cells in cutaneous T-cell lymphomas (CTCL), recent observations have suggested that the intratumoral heterogeneity of CTCL may include multiple TCR clonotypes.[16,17] In addition to a clear
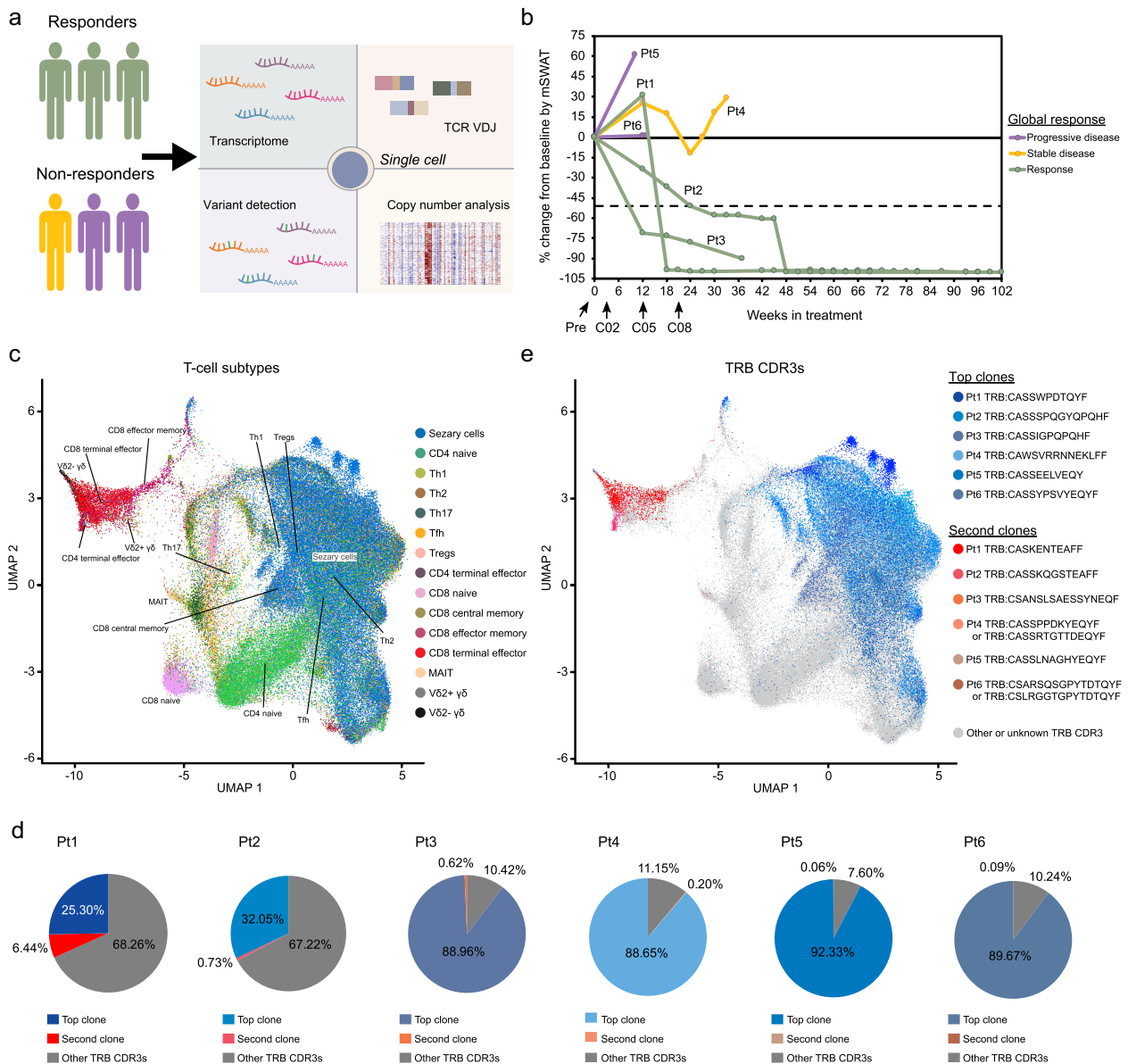

Figure 1. Experiment schematic and clustering of cells. a). Experiment schematic. b). Skin response for each patient as measured by modified Severity Weighted Assessment Tool (mSWAT). c). Uniform Manifold Approximation and Projection (UMAP) of all cells in the analysis, colored by computationally inferred T-cell subtype. d). Frequency of the two most common TRB clonotypes in each patient inclusive of both Pre and C02 timepoints. e). UMAP of all cells in the analysis, colored by TRB clonotype. Top clones (in shades of blue) and second most common (in shades of red) for each patient are highlighted in color.

dominant TCR clonotype, we noted multiple high-frequency TCR clonotypes in several samples as high as 6.4% of all T-cells (Figure 1d), raising the possibility of multiple Sézary clonotypes. Therefore, to distinguish malignant T-cells from normal T-cells, we incorporated both transcriptomic and TCR clonotypic data to ensure accurate assignment of cell type.

Because of the marked interpatient heterogeneity of Sézary syndrome,[18–20] we used bulk RNA-seq data on immunophenotypically sorted Sézary cells to determine personalized reference transcriptomes for each patient's Sézary disease and merged these with previously defined references for 29 immune cell types.[9] We then computationally assigned each cell to one of the reference cell types (Figure 1c). We related the resulting cell classification to the two most frequent TRB CDR3 clonotypes for each patient.

For all patients, cells with the most common TRB CDR3 clonotype ("top clone") were classified as Sézary cells (Figure 1e, Figure 1c). In contrast, cells with the second most common TRB CDR3 clonotype ("second clone") had a distinct transcriptomic profile. Thus, it appears that the multiple high-frequency TCR clones observed in these patients were due to the expansion of nonmalignant T-cells.

Sézary gene expression associates with response to pembrolizumab

Since the top clone invariably corresponded with Sézary cells, we used the TRB CDR3 to identify Sézary cells for further analysis. Sézary cell markers were consistent with previous studies (Figure 2a).[21–23] We next analyzed pretreatment samples to identify the genes differentially expressed within Sézary cells between responding and non-responding patients (Figure 2b, Supplementary Table S1). We found three genes consistently differentially expressed between every responding and non-responding patient (Supplementary Table S2). Responses to pembrolizumab were associated with lower expression of *KIR3DL2* and higher expression of *KIF5B* and *HSB17B11* within the Sézary cell population. When assessed by flow cytometry, the association between response and
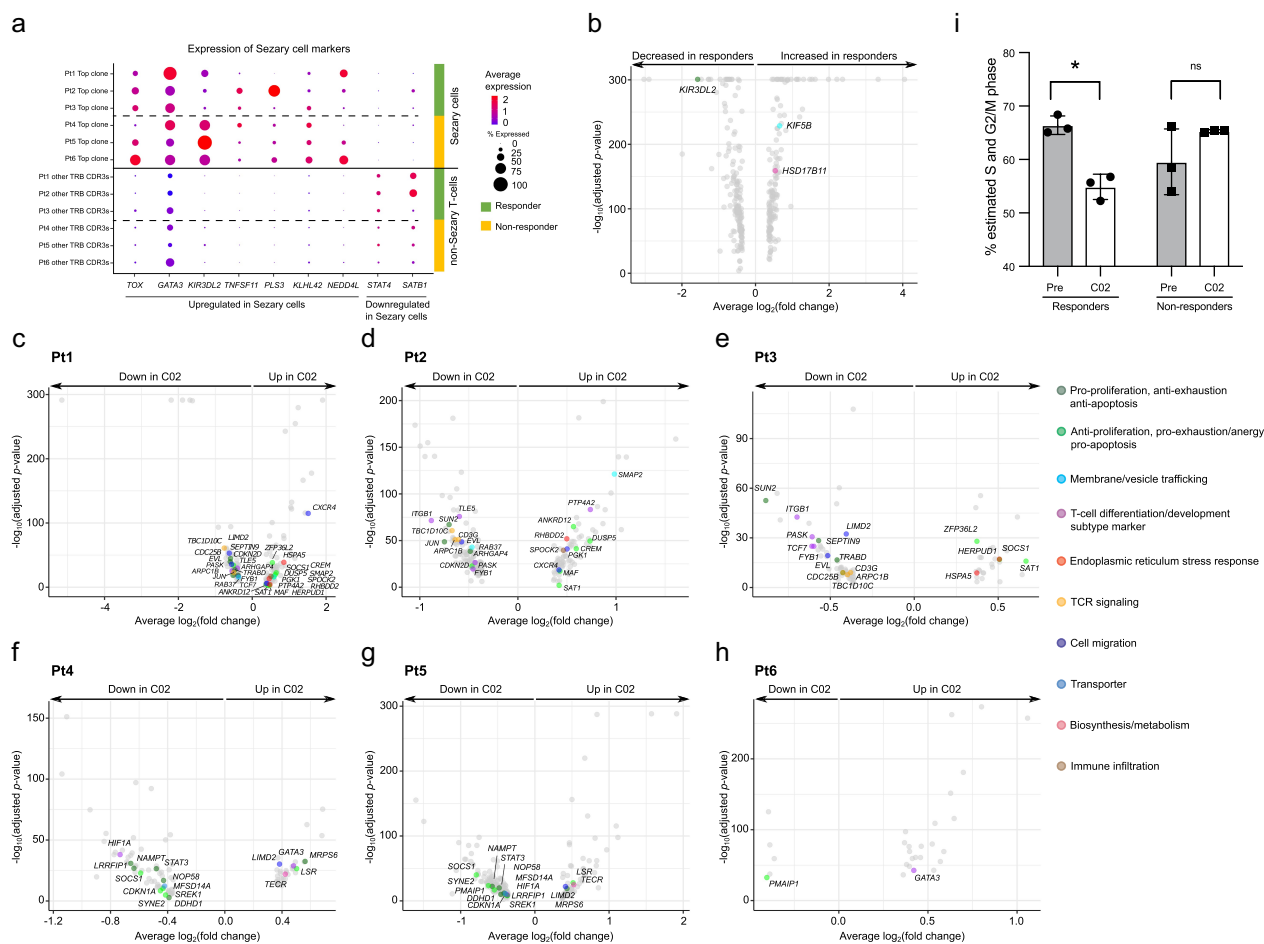

Figure 2. Sézary cell (defined by TCR clonotype) characteristics. a). Expression of known Sézary cell markers are shown for the Sézary (top) and non-Sézary (bottom) cells. Dot color depicts average expression and dot size depicts the percentage of cells with detectable expression within the cell population b). Differential gene expression comparing responders and non-responders. Sézary cells from the pretreatment timepoint were analyzed. Genes that were differentially expressed in the same direction in all individual non-responder to responder pairwise comparisons for the Pre datasets are highlighted. c-h). Differential gene expression within the Sézary cell population comparing Pre to C02. Genes that were significantly differentially expressed in the same direction in either multiple responders or non-responders are highlighted. i). Percentage of Sézary cells that were computationally determined to be in either the G2M or S phase of the cell cycle. * $p < .05$; ns, not significant.

KIR3DL2 expression showed a similar trend, with the exception of Pt 4, whose Sézary cells had low KIR3DL2 expression by flow cytometry but high *KIR3DL2* by scRNA-seq. The difference in KIR3DL2 expression by flow cytometry was not significantly different between responding and non-responding patients (Supplementary Fig. 1).

Effects of pembrolizumab treatment on Sézary cells

Because these Sézary cells are known to have varying expression of PD-1,[3] we sought to determine the effects of pembrolizumab treatment on the Sézary cell population. We compared samples obtained prior to treatment and to those collected three weeks after the first dose of pembrolizumab.

Pembrolizumab treatment modulated expression of genes associated with T-cell activation and apoptosis signaling within the Sézary cell population (Figure 2c-h, Supplementary Tables S3-S4). Pembrolizumab significantly increased *SOCS1* expression in the Sézary cells of two responding patients but significantly decreased *SOCS1* expression in two non-responding patients. In all responding patients, pembrolizumab treatment was associated with a decrease in expression of the actin cytoskeletal genes *FYB1, TBC1D10C*, and *ARPC1B*. Additionally, in the Sézary cells of responders after pembrolizumab treatment, we observed a decreased expression of *TCF7*, which has been shown to be required for self-renewal of both CD8 and CD4 T-cells.[24,25] A pseudotime analysis of the Sézary cells further validated that many of these differentially expressed genes were also dynamically changed in the same direction along a pseudotime trajectory from pretreatment to 3 weeks after the first dose (Supplementary Fig. 2, Supplementary Table S5).

These effects of pembrolizumab on the Sézary cell population were durable in the responders. For one of the responders, the modulation of T-cell activation was still observed in Sézary cells 3.5 years after the initiation of treatment (Supplementary Table S6).

Treatment of T-cell malignancies with immune checkpoint inhibitors presents a theoretic risk of disease hyperprogression through release of T-cell inhibition. Examples of hyperprogression with PD-1 treatment have been observed in adult T-cell leukemia and peripheral T-cell lymphomas[26,27] and stimulation of leukemic CTCL with nivolumab increases malignant T-cell proliferation *ex vivo*.[28] To further evaluate the effect of pembrolizumab on Sézary cell proliferation, alterations in the cell cycle after three weeks of treatment were computationally inferred for Sézary cells. The percentage of Sézary cells in the more proliferative phases of the cell cycle (G2M and S) decreased significantly for responders while no significant change was observed for the non-responders (Figure 2i).

Effects of pembrolizumab treatment on expanded cytotoxic clonotypic cells

We next explored the phenotype of the high frequency, non-malignant T-cell clones by analyzing the second most common T-cell clonotype in each patient. Differential gene expression comparison to other T-cell clonotypes revealed that the expanded second clones were associated with markers of cytotoxic lineage (Figure 3a, Supplementary Table S7). Together the clustering of the second clones with CD8 terminal effector T cells on the UMAP projection (Figure 1e, Figure 1c), these results indicated that the second-clone cells were most likely cytotoxic CD8 terminal effector T cells.

We noted increased expression of cytotoxicity markers in the second clones of responding patients as compared to non-responders (Figure 3a). To further investigate the effector and cytotoxicity characteristics of the second clone, an effector score and a cytotoxicity score were calculated for each cell based on previously defined gene signatures.[13,14] For all patients, the second clone appeared to have higher effector and cytotoxicity scores than cells with other TRB CDR3s (Figure 3b, Figure 3c). Both the cytotoxicity and effector score of the second clones appeared to be higher in responding patients as compared to those in non-responding patients (Figure 3b, Figure 3c).

Proliferation of CD8 T-cells has been associated with response to PD-1 inhibition in other malignancies.[29] Therefore, we tested whether pembrolizumab differentially affected the expansion of CD8 populations in responders versus non-responders. We compared the change in frequencies for computationally inferred T-cell subtypes for each patient after 3 weeks of pembrolizumab treatment. Pembrolizumab treatment yielded a significantly greater expansion of the CD8 effector population (terminal effector and effector memory) in responders as compared to non-responders (Figure 3d).

In two of the responders, the effects of pembrolizumab on the CD8 effector population appeared durable. At timepoints further than 3 weeks after the first dose, both the second clone and the CD8 effector population remained in higher abundance than pretreatment, and the second clone cells retained their phenotype with high cytotoxicity and effector scores (Supplementary Fig. 3).

Intrapatient Sézary cell heterogeneity through integrated single cell analysis of single nucleotide variant and copy number alterations

Sézary syndrome has been shown to have variable genomic intrapatient heterogeneity[17,30] typically with multiple identified subclones. Because intratumoral genomic heterogeneity has been correlated with clinical response to immunotherapy in other malignancies,[31] we leveraged this dataset to explore clonal heterogeneity at a single cell level through integration of TCR clonotype, subclonal copy number alterations, and single nucleotide variants.

We searched within our scRNA-seq data for patient-specific single nucleotide variants (SNV) determined through bulk exome sequencing.[3] The detection of these variants by scRNA-seq is strongly dependent on both gene expression and the cDNA position of the variant. Therefore, our analysis was restricted to five variants associated with three patients (Pts 1, 2, and 5). For all mutations, cells expressing the variant were almost exclusively limited to the Sézary TRB clonotype (Figure 4a-d). The small percentages of variant-expressing cells identified as possessing a non-Sezary TRB clonotype are within the margin of

error of this variant detection approach and therefore are favored to be unlikely to be truly expressing the somatic variant (Supplementary Fig. 4). Sézary cells displayed heterogeneity for some variants (Figure 4d). For the responding Pt2, the Sézary cells were more clonal with respect to the analyzed variants comparing to the non-responding Pt5 (Figure 4e).

Next, we explored clonal heterogeneity through inferred copy number variants (CNVs) within the Sézary cell populations. Clonal CNVs were present in all patients, including clonal aberrations in chr17 (5 of 6 patients, Figure 5b-f), which is one of the most common copy number alteration in Sézary syndrome.[32,33] Additionally, at least one subclonal CNV was identified in each patient. These subclones typically comprised only a small fraction of the total Sézary population. No remarkable difference in copy number variation was observed between responders and non-responders (Figure 5a-f).

To further elucidate the clonal architecture, we integrated cell-specific single nucleotide variants with the inferred copy number variants. We focused on Pt5 where the Sézary cells demonstrated a subclonal RHOA^{G17V} mutation as well as subclonal CNV variants. We found that copy gains in chr4 and

chr8 and the RHOA^{G17V} variant appeared to be mutually exclusive and present in independent subclones (Figure 5g-h).

Finally, we explored whether resistance to pembrolizumab was due to selection of specific resistant subclones. However, we found no significant change in the subclonal composition of Sézary cells when comparing pretreatment samples with those collected after three weeks of pembrolizumab treatment based on the limited number of assessable SNVs and CNVs (data not shown).

Discussion

Distinguishing malignant from nonmalignant T-cells in CTCL poses a major challenge in identifying potential biomarkers of response to immunotherapy. High-dimensional analysis such as mass cytometry or highly multiplexed imaging can more reliably identify malignant T-cells, but still may be imperfect in resolving CTCL cells from similar CD4 T-cell subsets. We used single-cell analysis with TCR sequencing to better resolve tumor-intrinsic and tumor-extrinsic features. Three Sézary cell-intrinsic markers were discovered through this approach. High expression of *HSD17B11* and *KIF5B* was associated with
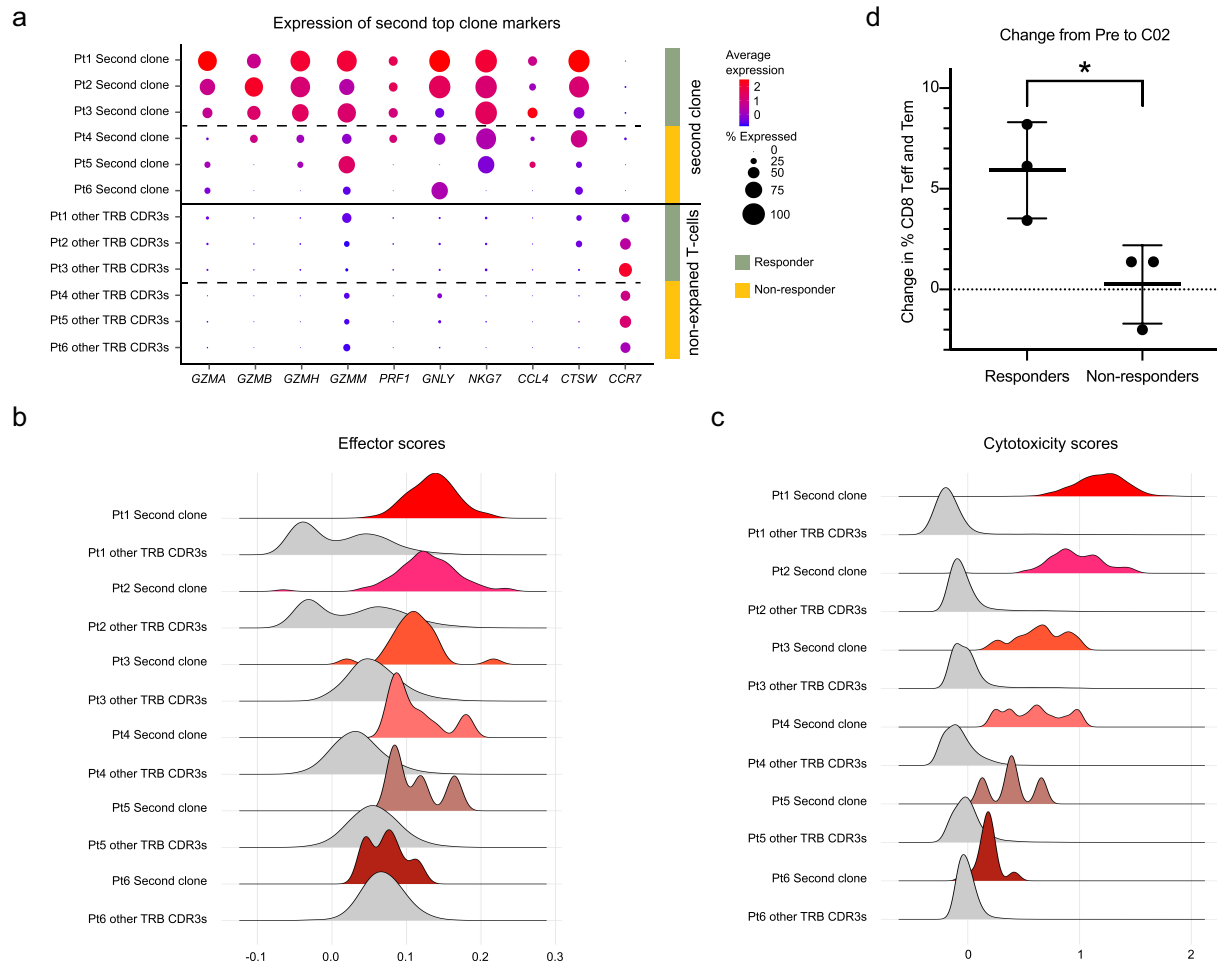

Figure 3. Characterization of expanded clones (cells with the second most common TRB CDR3) from each patient. a). Expression of cytotoxicity and effector T-cell are shown for the second clone (top) and all other non-Sézary T-cells (bottom). b). Effector scores of the second clone and cells with other TRB CDR3s in each patient. c). Cytotoxicity scores of the second clone and cells with other TRB CDR3s in each patient. For (a-c) analyses were performed on cells from both Pre and C02 timepoints combined. d). Change from Pre to C02 in CD8 terminal effector or CD8 effector memory T cells as a percentage of non-Sézary T-cells in responders and non-responders. *: *p* < .05, Teff: terminal effector T cell, Tem: effector memory T cell.
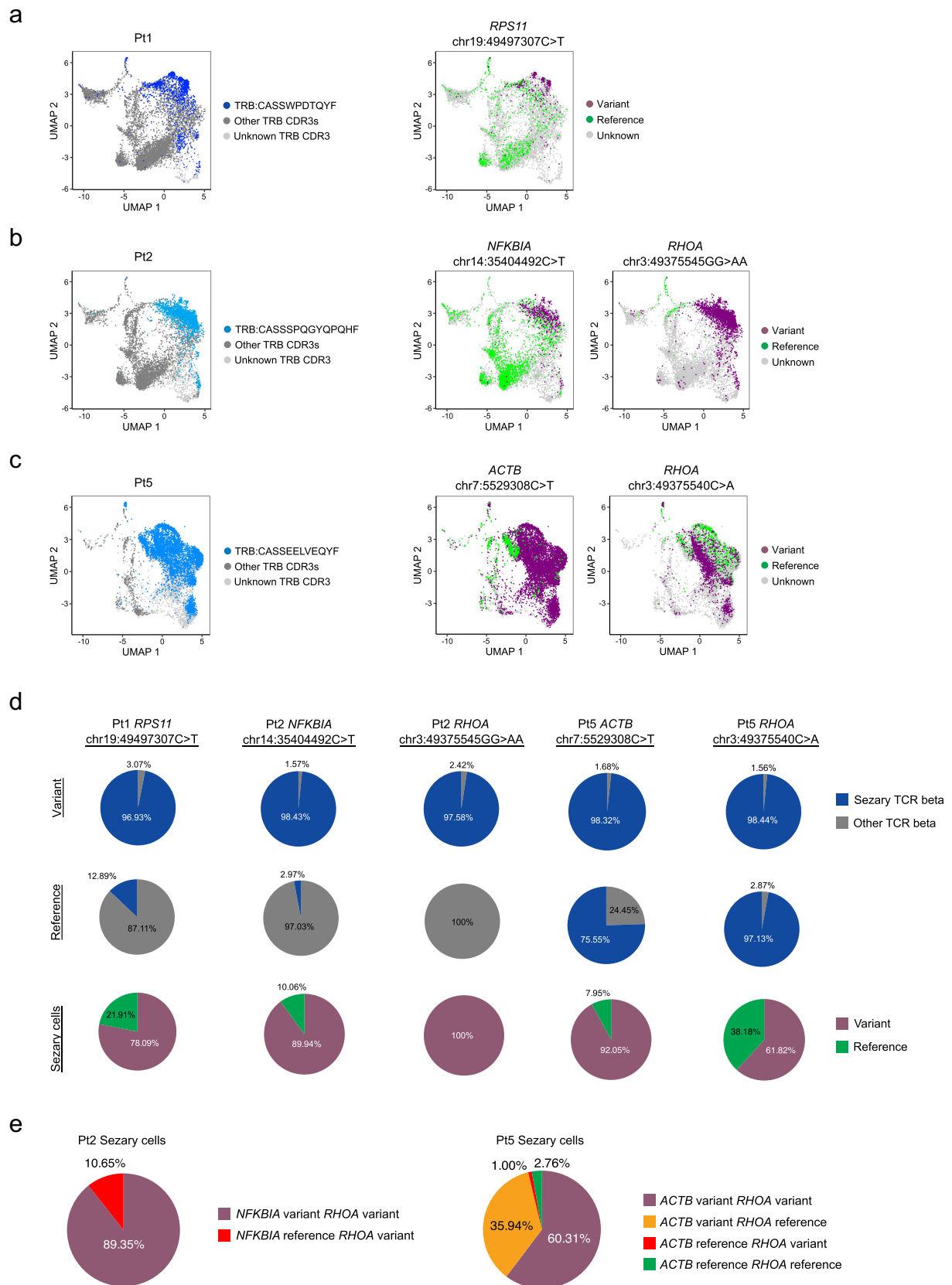
Figure 4. Heterogeneity of Sézary population through detection of single nucleotide variants. a-c). UMAP visualization highlighting the Sézary clonotype for three patients (left) and patient-specific somatic variants (right). Cells are labeled as containing the variant if there was at least one variant read detected. Cells with six or more non-variant reads and no variant reads are labeled as reference. d). For each of the mutations shown in (a-c), The percentage of variant or reference cells identified as Sézary or non-Sézary by TRB clonotype is shown (top and middle rows). The percentage of Sézary cells expressing either the variant or reference sequence is also shown (bottom row). e For the two patients with multiple somatic variants detected, the percentage of Sézary cells with one or both variants is shown.
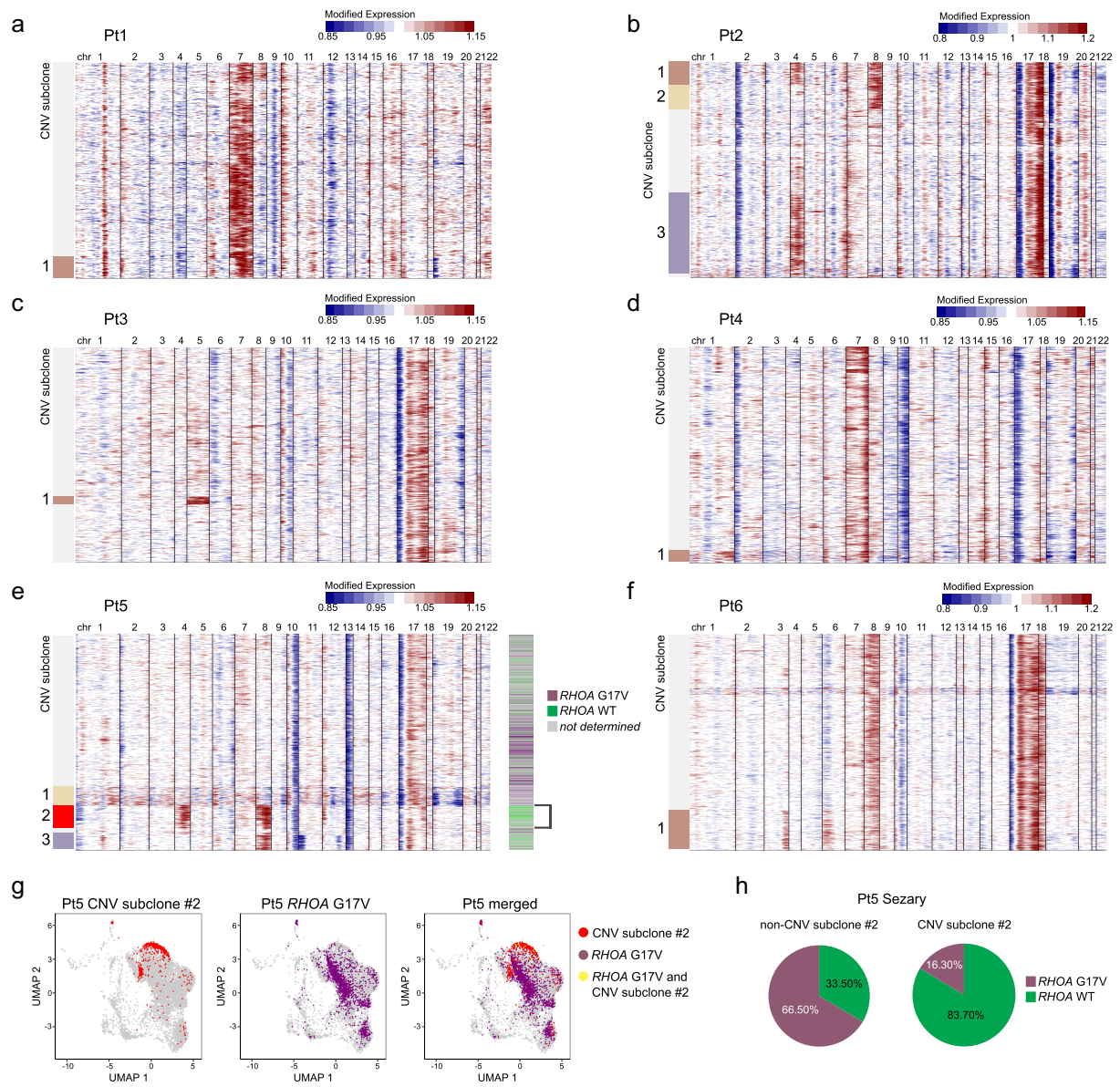
Figure 5. Heterogeneity of the Sézary population through copy number analysis. a-f). Computationally inferred copy number variation heatmap of the Sézary clones prior to treatment for each patient with each row representing a Sézary cell. Subclones indicate subsets of cells with unique copy number variations. In panel e (right) the genotype of the RHOA[G17V] for each cell is shown with the bracket corresponding to cells belonging to CNV subclone 2. g). UMAP plots of the pretreatment sample for Pt5 highlighting cells belonging to CNV subclone 2 (left), expressing the RHOA[G17V] variant (middle) and merging both characteristics (right). h). Percentage of RHOA[G17V] variant in Pt5 Sézary cells with or without the CNV of subclone 2. CNV: copy number variation, WT: wild-type.

responding patient. The short-chain alcohol dehydrogenase gene *HSD17B11* has previously been identified as a serologic tumor antigen in CTCL with 4 out of 6 patients demonstrating serologic reactivity against the antigen.[34] *KIR3DL2* has consistently been found to be overexpressed in Sézary cells.[35] *KIR3DL2*, according to previous studies, helps Sézary cells avoid activation-induced cell death.[36,37] Expressions of *KIR3DL2* and *PD-1* have been proposed to define three subclasses of Sézary syndrome with distinct skin immune microenvironments including higher numbers of reactive CD8 + T-cells in the setting of low *KIR3DL2* expression.[38] Since low expression of *KIR3DL2* was associated with response, our results raise the possibility that treatment with anti-KIR3DL2 treatment such as lacutamuab[39] could synergize with pembrolizumab therapy.

Although the mechanism of action of pembrolizumab relates to its effects on healthy immune cells, we discovered tumor-intrinsic effects of pembrolizumab on the malignant Sézary cells, including altered expression of TCR signaling pathway genes. Additionally, the expression of genes involved in T-cell differentiation such as *GATA3* and *TCF7* was also significantly changed after treatment with pembrolizumab. *TCF7* has been associated with T-cell "stemness."[40] Therefore, it is possible that by decreasing *TCF7* expression, pembrolizumab may inhibit the ability of Sézary cells to continue their renewal in responding patients. The changes in the cell cycle distribution and *TCF7* expression of Sézary cells after pembrolizumab treatment suggest that the response to therapy may at least in part be due to intrinsic effects of pembrolizumab on Sézary cells.

Inhibition of the PD-1/PD-L1 axis in PD-1 expressing T-cell lymphomas carries a theoretic risk of triggering hyperprogression through release of PD-1 inhibition.[41,42] Evidence for hyperprogression has been reported in *ex vivo* studies as well as in cases of PD-1 expressing T-cell malignancies.[27,43] The two completed trials of PD-1 treatment in cutaneous T-cell lymphoma, including the associated trial of this study, have not reported cases of hyperprogression.[3,44] While we did not observe evidence for accelerated Sézary T-cell growth or proliferation in this study, we cannot exclude the possibility of a proliferative effect of pembrolizumab due to the small number of patients and focus on early treatment timepoints.

Our data suggest that effector CD8 populations also play a role in mediating the response to pembrolizumab. While tumor infiltrating CD8 + T-cells have consistently been implicated in response to immune checkpoint therapies in solid malignancies,[45] no correlation was seen between cutaneous CD8 T-cells and response to pembrolizumab in this trial.[3,5] However, in analyzing circulating lymphocytes, we found peripheral blood CD8 terminal effector and CD8 effector memory T-cell populations expanded in responding patients upon pembrolizumab treatment. Furthermore, we found a marked clonal expansion of CD8 terminal effector cells in patients with Sézary syndrome, reaching a frequency of >6% of all peripheral blood T-cells in one patient with a long-term response to pembrolizumab. This population shared a strong cytotoxic profile that mirrors those seen in clonally expanded peripheral blood CD8 populations in metastatic melanoma.[46] The clonal populations were largest in the three responding patients in our cohort and clonal size of expanded CD8 + T-cell clones has been shown to predict the response to immune checkpoint blockade in metastatic melanoma.[46,47]

Finally, by integrating prior genomic data, we were able to investigate the clonal architecture of these Sézary patients at a single cell level. Recent studies have suggested that CTCL may generate multiple malignant TCR clonotypes. By relating transcriptomes, TCR sequence, copy number variants and single nucleotide variants at the single cell level, we found no evidence of multiple malignant TCR clonotypes. Our data nevertheless did confirm the marked intratumor heterogeneity that has been observed in multiple complementary studies.[17,18,20,30,48] We further demonstrate the first example of branched evolution in CTCL involving subclonal segregation of a single nucleotide mutation (RHOA G17V) and a copy number variant (gain of chr 4 and 8). We found no significant changes in the subclonal composition in these patients after pembrolizumab treatment. However, due to technical limitations of scRNA-seq data, our analysis was limited to only five variants from three patients, and it is likely that one cycle of treatment is too short of a period to detect changes in the clonal heterogeneity. Future methodological advancements in inferring genomic variations from scRNA-seq data will be needed to better track somatic variants and assess clonal evolution in response to checkpoint therapy.

In conclusion, our study shows that responses to pembrolizumab in Sézary syndrome are associated with tumor-intrinsic features and with characteristics of circulating CD8 T cell populations. The expression of the CTCL marker *KIR3DL2* deserves particular consideration as a potential biomarker of response in future studies of immune checkpoint therapy in CTCL. Single cell-based analysis with paired TCR sequencing is an effective strategy to dissect tumor-intrinsic and extrinsic effects of immune therapies in CTCL.

Disclosure statement

The authors report there are no competing interests to declare.

Data availability statement

Data are available via dbGaP: phs002933.v1.p1.

Author contributions

Concept and design: T.S. and M.S.K. Development of methodology: T.S. and M.S.K. Acquisition of data: T.S., G.E.D., A.C.K., N.R., S.P.F., Y.K., M.S.K. Analysis and interpretation of data: T.S., G.E.D., A.C.K. and M.S.K. Writing, review, and/or revision of manuscript: T.S. and M.S.K. with input from all authors.

Ethical approval and informed consent

This study was approved by the Stanford University Institutional Review Board (IRB). A written informed consent was obtained from all patients who participated in this study in accordance with the Declaration of Helsinki.

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