Evaluating T-cell cross-reactivity between tumors and immune-related adverse events with TCR sequencing: pitfalls in interpretations of functional relevance

Tricia Cottrell,1,2,3 Jiajia Zhang,2,4,5 Boyang Zhang,6 Genevieve J Kaunitz,7 Poromendro Burman,2,4,5 Hok-Yee Chan,2,4,5 Franco Verde,8 Jody E Hooper,1 Hans Hammers,4,9 Mohamad E Allal,2,4 Hongkai Ji,9 Janis Taube,1,2,4,10 Kellie N Smith2,4,5,10

ABSTRACT
T-cell receptor sequencing (TCRseq) enables tracking of T-cell clonotypes recognizing the same antigen over time and across biological compartments. TCRseq has been used to test if cross-reactive antitumor T cells are responsible for development of immune-related adverse events (irAEs) following immune checkpoint blockade. Prior studies have interpreted T-cell clones shared among the tumor and irAE as evidence supporting this, but interpretations of these findings are challenging, given the constraints of TCRseq. Here we capitalize on a rare opportunity to understand the impact of potential confounders, such as sample size, tissue compartment, and collection batch/timepoint, on the relative proportion of shared T-cell clones between an irAE and tumor specimens. TCRseq was performed on tumor-involved and -uninvolved tissues, including an irAE, that were obtained throughout disease progression and at the time of rapid autopsy from a patient with renal cell carcinoma treated with programmed death-1 (PD-1) blockade. Our analyses show significant effects of these confounders on our ability to understand T-cell receptor overlap, and we present mitigation strategies and study design recommendations to reduce these errors. Implementation of these strategies will enable more rigorous TCRseq-based studies of immune responses in human tissues, particularly as they relate to antitumor T-cell cross-reactivity in irAEs following checkpoint blockade.

INTRODUCTION
PD(L)-1 checkpoint blockade is complicated by the development of immune-related adverse events (irAEs) in 5%–20% of treated patients.1 Severe irAEs have been reported in up to 10% of patients and can result in hospitalization, interruption or discontinuation of therapy, and rarely, death.1 Notably, irAEs increase in prevalence and severity with combination immunotherapy, an approach likely required to improve disappointingly low response rates.2 Prediction, prevention, and treatment of irAEs will require delineation of etiological mechanisms. One hypothesis is that irAEs are the result of cross-reactivity of an antigen-specific antitumor immune response. Supporting this hypothesis, improvements in response rates and survival in patients who develop irAEs have been observed in studies across tumor types.3–6 T-cell repertoire profiling with T-cell receptor sequencing (TCRseq) enables tracking of individual T-cell clones recognizing the same antigen.7 Multiple case series have identified shared T-cell clones in tumor and irAE tissues, thereby providing a foundation for shared antigen specificity between the tumor and irAE.8–10 Unfortunately, these types of analyses may be subject to several sources of confounding that are rarely considered and are often difficult to address in the context of human immune-oncology.

The current study evaluates notable sources of confounding in the analysis of T-cell receptor (TCR) repertoire overlap between tumor-involved and irAE specimens in a patient who developed a refractory irAE (dermatitis) while receiving PD-1 blockade for metastatic renal cell carcinoma (RCC). We demonstrate how these analytical pitfalls could lead to erroneous interpretation of TCRseq data obtained from distinct biological compartments and time-points within the same patient. These factors have previously been considered as potential confounders in large-scale genomic datasets11 but have not been evaluated or implemented in TCR studies.

CASE REPORT
A woman in her early 70s underwent radical nephrectomy for clear cell RCC followed by
systemic therapy, including anti-PD-1. The patient’s clinical course, including development of an irAE in the form of a lichenoid dermatitis (LD),\textsuperscript{12–16} and biospecimen collection are shown in figure 1. The immune-related LD (irAE) persisted despite two treatment breaks and systemic prednisone (figure 1). Anti-PD-1 therapy was discontinued because of the severity of the irAE. Progressing metastases in the small bowel and a new brain metastasis were confirmed by biopsy. The patient died approximately 2 months after cessation of anti-PD-1 therapy and a rapid autopsy was performed. Findings included metastatic RCC involving the brain (specimen T\textsubscript{M1}), jejunum (specimen T\textsubscript{M2}), and mesentery (specimen T\textsubscript{M3}). Of the three mediastinal lymph nodes (LN) sampled, one was histologically unremarkable (LN1); one showed multiple large fibrotic nodules (LN2); and one showed multiple small subcapsular fibrotic nodules (LN3). An inflamed seborrheic keratosis (benign skin lesion) was sampled from the skin (SK) as well as uninvolved normal tissues from the left kidney and normal small bowel (NSB).

To test for possible T-cell cross-reactivity among the tumor and irAE (figure 1D), TCR V\textbeta CDR3 sequencing was performed on all specimens (online supplemental table S1).\textsuperscript{17 18} The irAE shared 147 unique clonotypes (4.7\%) with the pretreatment primary tumor (T\textsubscript{p}) and 118 unique clonotypes (3.7\%) with jejunal metastasis (T\textsubscript{M2}) (online supplemental figure S1A,B). In total, 127 unique T-cell clones present in the irAE (4.0\%) were also found in at least one tumor specimen and absent in all healthy, non-lymphoid specimens (online supplemental figure S1C). We next tested if library size (the total number of productive sequencing reads) influences T-cell repertoire overlap among specimens. The number of clones shared with a given specimen was highly correlated with library size, illustrated for the irAE (Spearman’s rho, R=0.7, p=0.031; online supplemental figure S1D, left) and LN2 (R=0.78, p=0.012; online supplemental figure S1D, right). Random subsampling weighted by clonal abundance within each specimen was used to equalize library sizes to eliminate this confounding (online supplemental figure S1E).\textsuperscript{7 11} Not surprisingly, a strong correlation was observed between library size and the number of unique clonotypes (R=0.93, p=2.2e\textsuperscript{-16}; online supplemental figure S2). The degree of clonal sharing was also correlated with the number of unique clonotypes in each specimen (R=0.85 and p=0.0035 for the irAE, and R=0.92 and p=0.00047 for LN2; online supplemental figure S3A). Using weighted downsampling,\textsuperscript{7 11} we normalized specimens to the same library size, which eliminated the correlation between the number of unique clonotypes in a specimen and clonal sharing (online supplemental figure S3B). Therefore, we used weighted downsampling for the remainder of our analyses.

Tumor-specific T cells can be detected in paired uninvolved tissue, even when the normal tissue is collected 10–15 cm from the tumor itself.\textsuperscript{19–21} Likewise, clonotype sharing analyses could be confounded by bypassing viral-specific T cells. We performed a reanalysis of a previously published functional assay\textsuperscript{20 21} and found that viral-specific T-cell clones showed notable clonotype sharing across multiple tissue compartments, including the tumor, in a patient with non-small cell lung cancer. A similar pattern was previously observed with neoantigen-specific T-cell clones,\textsuperscript{20 21} suggesting that T cells can traffic across tumor involved/uninvolved compartments regardless of the presence of antigen (online supplemental figure S4A,B). Indeed, after mapping the TCRs from our present study to a public TCR database with annotated antigen specificity (vdjdb, https://vdjdb.cdr3.net/), we found an Epstein-Barr Virus (EBV)-specific clone that was detected in tumor-involved tissues from our study participant (online supplemental figure S4C–D). This emphasizes that clonotype sharing alone is not necessarily associated with biological relevance. Additional abundance measurement and antigen specificity analyses are warranted to assist further interpretation. The SK and LN outliers in online supplemental figure S1D (red arrows) also indicate increased T-cell repertoire sharing between specimens from the same tissue compartment, even when collected at different locations and timepoints (ie, the irAE and SK). *Tissue compartment confounding*—a greater degree of T-cell repertoire overlap between specimens collected from the same tissue site—is illustrated with pairwise comparisons in online supplemental figure S5A,B. While T-cell repertoire sharing is reduced in samples from different tissue compartments, shared clones are still detected between seemingly unrelated specimens. These could reflect circulating clones at the time of tissue collection and are not necessarily reflective of biologically meaningful clonal sharing, (ie, *batch effect confounding*). We used the Morisita Overlap Index (MI), which incorporates relative clonal abundance and is not influenced by library size in our dataset (online supplemental figure S6A), to calculate the overlap between the irAE and all other specimens. The relative clonal sharing among all specimens is illustrated in a chord diagram (online supplemental figure S6B), in which the width of the bands is proportional to the MI values. The highest MI was observed between specimens collected from the same batch and from the same tissue compartment (online supplemental figure S6C). These population-level comparisons likely capture a combination of biological and batch effects, which cannot be distinguished in this dataset.

We next tested for evidence of cross-reactive T cells in the tumor and irAE while accounting for the sources of confounding identified previously (figure 1E). Within the limitations of specimen availability, tissue compartment and batch effect were considered in selecting comparator specimens for meaningful analyses. First, the T-cell repertoire overlap between the primary tumor and the metastases was quantified using MI, which demonstrated a significantly higher overlap among the progressing metastases relative to the mediastinal LNs and normal tissues (p=0.044; figure 1F,G). A chord diagram highlights population-level sharing among all tumor specimens
Figure 1  Clinical course and assessment of TCR repertoire overlap among tumor specimens and the irAE. (A) Timeline shows the clinical course from RCC resection to autopsy. Therapies included pazopanib (yellow bar), nivolumab (green bar), and prednisone (blue bars), with treatment dates shown below the bars. Radiographical assessments (gray boxes) included mediastinal metastases with partial response to nivolumab followed by PD in the bowel and brain. Tissue specimens collected (blue boxes) included the resected RCC (photomicrograph shown), biopsies of the immune-related lichenoid dermatitis (irAE), and multiple specimens collected at the time of rapid autopsy (see online supplemental table S1). The anatomical sites of the collected specimens are illustrated, including the primary renal tumor (black diamond), two sites of irAE (black pentagons), mediastinal LNs at the site of tumor regression (green circles) and progressing lesions in the jejunum, mesentery, and brain (red circles). (B) Intravenous contrast-enhanced CT of the chest demonstrated right lower paratracheal adenopathy which resolves after nivolumab treatment (red arrow). (C) A photograph (left) and photomicrograph (right) of the irAE, the latter showing the brisk lichenoid lymphocytic infiltrate and necrotic keratinocytes. (D) Diagram illustrating two hypotheses for the development of irAEs following immune checkpoint blockade: (i) the cross-reactivity hypothesis proposed that T cells activated as part of the antitumor immune response cross-react at the site of the irAE; this would be supported by detection of overlapping TCR repertoire signatures between the tumor and irAE; (ii) in contrast, if the irAE and antitumor immune responses were independent of each other, it was unlikely significant TCR repertoire overlap between the two sites would be detected. All photomicrographs (Continued)
at ×400 magnification, scale bars 50 um. (E) Proposed approaches to maximize interpretive value and minimize confounding in TCR sequencing data analysis, including (i) the MI for quantifying global TCR repertoire sharing among multiple specimens in a sample size-independent manner, (ii) proportional downsampling for library size normalization to enable relative interpretations of clonal sharing, and (iii) normalization to relative clonal abundance in each specimen to assess relative sharing among the most abundant T-cell clones across multiple specimens. (F) The Morita index demonstrates that the three metastatic lesions consistently showed a greater degree of sharing with the Tp (MI median 0.014, range 0.012–0.088) relative to the normal control tissues (NSB MI 0.004, NK MI 0.003). (G) Chord diagram illustrates TCR repertoire overlap among the Tp and multiple post-treatment progressing metastases (red). Non-tumor specimens are shown in gray. (H) Following library size normalization, a metastasis (TM2, left), LN (LN2, middle), and NSB (right) were evaluated for T-cell repertoire overlap with the primary tumor (TP). Clonal expansion of clones shared with TM2 is suggested by 9.1% of shared TP clonotypes representing 27.1% of TP reads. Subsampled comparisons are indicated (*) and the 95% CIs for shared TP clones were 5.2% to 6.8% for LN2 (middle, representing 4.9%–15.8% total TP reads) and 3.3% to 4.3% for NSB (right, representing 3.5%–14.7% total TP reads). (I) The Morita Index demonstrates an overall low degree of TCR repertoire between the irAE and the other specimens, although the relative sharing with two of the three metastatic lesions (MI median 0.02, range 0.0003–0.026) and the three regression site LNs (MI median 0.025, range 0.021–0.027) was higher than with the normal tissues (NSB MI 0.0004, NK MI 0.003). (J) Chord diagram highlighting sharing with the irAE as assessed by the MI in red (sharing among other specimens shown in gray for reference). (K) Following library size normalization, pairwise quantification of shared clones shows that while 4.7% of irAE clones were shared with the primary tumor and a regression site LN (LN2, 95% CI 4.6% to 5.9% irAE clones), the shared clones were not expanded in the irAE (representing 4.5% and 4.7%, 95% CI 4.7% to 6.4% of the total irAE reads, respectively). Sharing between the irAE and NSB shown for comparison, 1.6% shared clones (95% CI 1.3% to 2.2%) represent 1.8% total irAE reads (95% CI 1.3% to 2.2%). Subsampled comparisons are indicated (*). (L) The GLIPH2 clustering algorithm was used to detect and quantify potential specificity clusters based on TCR CDR3 sequencing information. Motif clusters were included in downstream analysis if there were ≥3 unique CDR3s, ≥10 reads for each CDR3, a βv score <0.05, and a length score of <0.05. The barplot shows the clonal abundance of the significantly enriched ‘SSQD’ motif in the dermatitis and respective clonal abundance in other tissue compartments. GLIPH2, grouping lymphocyte interactions by paratope hotspots 2; irAE, immune-related adverse event; LN, lymph node; MI, Moritsa Overlap Index; NK, normal tissues from the left kidney; NSB, normal small bowel; PD, progressive disease; RCC, renal cell carcinoma; SK, skin; TCR, T-cell receptor; Tp, pretreatment primary tumor.

(figure 1G), with the greatest TCR repertoire overlap observed between the metastases from the brain (T/M1) and mesentery (T/M3, MI 0.47). Although batch effect is a potential confounder, this degree of overlap is not observed with the small bowel metastasis (T/M2, MI 0.03 and 0.04 with T/M1 and T/M3, respectively), also collected at autopsy. Since the primary tumor and metastases were collected at different time points and from different tissue sites, batch and tissue compartment effects could not be confounders in this analysis. We recognize that a small sample size limits interpretation of the aforementioned findings, given that T/M1 and T/M3 have the smallest library sizes (online supplemental table S1), even though they satisfied our criteria for inclusion. Consequently, we focused on the metastasis with the largest library size (T/M3, 6945 reads) for additional analyses.

Following library size normalization, 9.1% of unique primary tumor clonotypes are shared with T/M2 relative to 5.8% (95% CI 5.2% to 6.8%) and 3.7% (95% CI 3.3% to 4.3%) of T/M1 clonotypes shared with LN2 and NSB, respectively (figure 1H). Clonal expansion of shared clones in the primary tumor was greatest for those shared with T/M3, with shared clones representing 27.1% of total primary tumor reads. The TCR repertoire overlap between the primary tumor and LN2 may suggest an antitumor signature in the mediastinal LNs, a site of radiographical tumor regression. The same approach was used to evaluate TCR repertoire overlap between the irAE and tumor specimens. The irAE repertoire was most similar to T/M1, T/M3, and the regression site LNs (figure 1I), with intermediate overlap with Tp and the least overlap with the normal control specimens. Although the relative degree of overlap with the irAE potentially suggest a biologically relevant pattern, the magnitude of population-level TCR repertoire sharing is quite small relative to values observed among other specimens (figure 1J).

Specimen libraries were then normalized to allow direct pairwise comparison of clonal sharing with the irAE. Sharing between the irAE and Tp (largest library size of 12,588 reads) was compared with LN2 and NSB. A similar degree of irAE clonotype sharing was observed in Tp and LN2, which was greater than that observed for NSB (figure 1K). There was no evidence of clonal proliferation in the irAE or Tp (figure 1K). Finally, we evaluated the most abundant clonotypes in each specimen for overlap with the irAE, given that prior studies have implicated the highest-frequency intratumor clonotypes in mediating antitumor immunity.22 There was no enrichment of irAE-shared clones in the tumor relative to the non-tumor specimens (online supplemental figure S7).

Based on observations that antigen specificity may be determined by limited contact sites in the TCR CDR3, we applied the grouping lymphocyte interactions by paratope hotspots 2 (GLIPH2) algorithm23 24 to identify and cluster TCR sequences into possible antigen specificity groups. In order to be included in downstream analyses, clusters had to contain ≥3 unique CDR3s, ≥10 reads for each CDR3, a variable gene beta (vb) score of <0.05, and a length score <0.05. One cluster with significant enrichment in the irAE was identified. Notably, the primary tumor (3.6%) had the highest abundance of T-cell clones in the ‘SSQD’ CDR3 motif cluster (figure 1L), followed by the irAE (2.4%), which were both higher than representation of this motif in non-diseased TCR repertoires.
from four healthy donors (range: 0.03%–1.67%, online supplemental figure S8). Though the human leukocyte antigen (HLA) information is unknown, three of the four healthy donors had common clonotypes shared with the patient in our study, indicating that at least one HLA allele was shared among them. By querying additional published skin/tumor-reactive TCR data, the specific motif SSQD was reported in a T-cell clone recognizing an epitope derived from Maspin, which functions as a tumor suppressor gene in epithelial cells. Collectively, this indicates that, though analyses of the total TCR repertoire and a subset of high abundance clones do not show a signature of enriched sharing between the irAE and tumor specimens relative to non-tumor specimens at the clonotype level, more ‘antigen-driven’ approaches may be useful to identify potential specificity clusters, especially when coupled with functional assays to confirm antigen specificity and cross-reactivity between irAEs and tumors.

**DISCUSSION**

As immune checkpoint blocking agents become first-line and second-line therapies for a growing number of tumor types, we are faced with an increasing number of diverse irAEs that may develop during or after treatment. The association of cutaneous irAEs with clinical benefit in some patients suggests that there may be a common antigen that may underlie both durable antitumor responses and clinically significant irAEs. It is conceivable that T cells with a common TCR could mediate both tumor regression and irAE development and progression, as has been evidenced by prior studies evaluating clonal overlap of TCR clonotypes between tumor and irAE tissues and that expansion of peripheral blood T cell clones prior to irAE onset positively correlates with irAE severity during checkpoint blockade treatment.

The large number and circulating nature of T cells predispose these studies to detecting false positive signals, that is, detection of differential or statistically significant clonal overlap that is not necessarily of pathogenic relevance. Biological differences exacerbate this issue, including variation in T-cell numbers and clonality in different tissue types. In addition, due to differences in sampling, clonotype detection can be limited, particularly for rare/low-frequency clonotypes. The analysis pitfalls and mitigation strategies identified in this study are summarized in table 1, and we present considerations for prospective specimen collection in online supplemental figure S9. Many of these factors are already considered as a standard part of large-scale genomic analyses, but they are not yet routinely applied to immune receptor sequencing datasets and, to date, no studies have demonstrated the differential outcomes when these important sources of confounding are not acknowledged. Strengths of this study include the rare opportunity to analyze the TCR repertoire in the same patient across time, tissue compartments, and disease states, and the ability to compare with published tumor-reactive/skin-reactive TCRs and non-irAE skin TCRs. We recognize that we are limited in our ability to comprehensively dissect all potential sources of confounding owing to limited sample availability. Lastly, the data-driven recommendations made in this study highlight the scientific value of rapid autopsy to answer complex questions using human tissue specimens.

**METHODS**

**Case selection**

Specimens from the underlying primary tumor and/or metastatic site and from skin affected by the cutaneous irAE were collected from the Johns Hopkins Hospital surgical pathology archives and the Rapid Autopsy program and Franklin Square Hospital. Overall patient response to anti-PD-1 therapy was classified according to Response Evaluation Criteria in Solid Tumors V1.1.

**TCRseq and bioinformatic analysis**

DNA extraction from formalin-fixed paraffin-embedded (FFPE)-preserved tumor and skin biopsy specimens was performed using the DNeasy Blood and Tissue Kit (Qiagen). The TCR-B locus was amplified and sequenced using the ImmunoSEQ assay (Adaptive Biotechnologies). Non-productive TCR CDR3 sequences (premature stop or frameshift), sequences with amino acid length less than 7, and sequences not starting with ‘C’ or ending with ‘F/W’ were excluded from the final analyses. Specimens with at least 1000 reads were included in the final analysis. To focus on T cells recognizing the same antigen, we analyzed amino acid clonotypes exclusively.

The degree of clonality for each specimen was assessed by the productive clonality matrix, which is defined as 1-Pielou’s evenness. Values near one represent samples with one or a few predominant clones (monoclonal or oligoclonal samples), whereas values near 0 represent a polyclonal population.

A random subsampling approach weighted by clonal abundance was used to equalize library sizes for relative comparisons of TCR repertoire overlap. For subsampling, each clonotype at amino acid level was treated as a sample and specimens were randomly sampled with replacement and weighted by clonal abundance (or frequency) until the total read count equaled that of the comparator library. To account for subsampling variation, the procedure was repeated 100 times and the 95% CIs for all subsampled comparisons are reported.

The degree of T cell clone overlap at the species level was evaluated using the Morisita overlap index. This measurement accounts for differences in library size and diversity per specimen, values near one the species occur in the same proportion in both samples, whereas values near 0 implies the two samples do not overlap in terms of species. Clonotypic sharing at the individual clone level was assessed in pairwise biological compartments before and after normalization to the same library size. Clones that were copresented in any of the compartment pairs are defined as shared clones. Based on the clonal frequency...
distributions (online supplementary figure 4), we assessed the top 40 clones in each specimen for overlap with the irAE repertoire. GLIPH2 was used for antigen-specific clustering. The motif significantly-enriched in the irAE was queried in a published dataset of tumor-reactive/skin-reactive TCRs in lung cancer and in a dataset of dermal/epidermal TCRs from healthy donors.

**Statistical analysis**

Statistical analysis was performed using R software. The Mann Whitney U test was used for comparison of 2-group data. For analysis of >2 group data, Kruskal-Wallis was used. Spearman’s ρ correlation was used to determine correlation significance. TCR preprocessing was performed using the tcR package. Chord diagram was performed using the circlize package. p<0.05 was considered significant.

**Data and code availability**

Bulk TCR Vβ sequencing data generated by Adaptive Biotechnologies ImmuneACCESS repository at DOI: 10.21417/TRCJZ2021JITC. The code to perform down-sampling of the TCR repertoire to the same library size and relevant figures are available online (https://github.com/BKI-immuno/dermatitis/).

**Author affiliations**

1Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
2Bloomberg~Kimmel Institute for Cancer Immunotherapy, Baltimore, MD, USA
3Queen’s Cancer Research Institute at Queens University, Kingston, Ontario, Canada
4Sidney Kimmel Comprehensive Cancer Center, Baltimore, Maryland, USA
5Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA
6Department of Biostatistics, Johns Hopkins University, Baltimore, Maryland, USA
7Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
8Department of Radiology, Johns Hopkins School of Medicine, Baltimore, MD, USA
9Harold C. Simmons Comprehensive Cancer Center, Dallas, TX, USA
10The Mark Foundation Center for Advanced Genomics and Imaging, Baltimore, MD, USA

**Twitter** Hongkai Ji @jihk99 and Kellie N Smith @SmithImmunology

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### Table 1 Mitigating pitfalls and approaches for interpretation of TCRseq data

| Potential confounders | Pitfall | Mitigation |
|-----------------------|---------|------------|
| Batch effect          | Circulating T-cell clones may be ‘shared’ by multiple specimens collected at the same time point. | Control normal tissue(s) collected at the same timepoint can be used to identify these background clones. |
| Blood                 | During active immune responses,* both relevant and non-relevant clones circulate in blood. | Functional assays enable identification of disease-relevant clones (vs batch background). |
| Tissue compartment effect | Specimens from the same organ share tissue resident T cells, including antitumor clones. | Clonotype sharing with ‘paired’ normal tissue does not preclude biological relevance. Measurements such as abundance and antigen specificity (antigen-driven clustering/functional assays) are needed for further discernment. |
| Library size variation | Increased read count→more clones sampled→a larger proportion of shared clones | Analyses must correct for sample size variation (eg, Morisita Overlap Index, normalization, etc) |
| LNs/lymphoid-rich tissues | Increased probability of repertoire overlap given large, diverse T-cell populations | Avoid analysis of background lymphoid tissue in LN metastases; interpret LN data with caution. |
| Interpretation        | Definition | Approach |
| Clonal abundance (relative read count) | Relative proportion of sequencing reads for a unique clonotype (surrogate for clonal proliferation) | Assess for signals of clonal proliferation in relevant tissues to suggest functional relevance.† |
| Low abundance         | Meaningful threshold for exclusion of ‘background’ clones has not been rigorously defined | Exclude specimens with <1000 reads. Sample size informs interpretation of low-read clones. |
| High abundance        | Increasing abundance suggests clonal proliferation (and antigen exposure) in a given tissue,* | Proliferation of shared clones in disease-relevant tissues supports potential mechanistic overlap. |
| TCR repertoire sharing | Mechanistic interpretations of TCR repertoire overlap are limited by several confounders. | Multispecimen analyses, antigen-driven clustering (such as GLIPH2), and functional assays maximize interpretability of TCRseq data. |

*During tumor killing (early in treatment) or active autoimmunity (immune-related adverse events).
†Assuming systemic clonal proliferation (batch effect) has been excluded.

GLIPH2, grouping lymphocyte interactions by paratope hotspots; LN, lymph node; TCR, T-cell receptor; TCRseq, T-cell receptor sequencing.
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Contributors  TC, GJK, and H-YC conceived of and conducted the experiments. KNS and JT oversaw the clinical care of the patient and led the specimen acquisition. All authors contributed to and edited the manuscript.

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Patient consent for publication  Not required.

Ethics approval  This study was approved by the institutional review board (IRB) at Johns Hopkins University (JHU) and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. The patient described in this study provided written informed consent as approved by the IRB of JHU.

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Supplementary Data

Evaluating T cell cross-reactivity between tumors and immune-related adverse events with TCR sequencing: pitfalls in interpretations of functional relevance

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| ID  | Description             | Location       | Time from diagnosis | Procedure | Treatment status   | Total TCR Reads* | Productive Rearrangements* | Total Included Reads** | # Nucleotide Clones | # Amino Acid Clones |
|-----|-------------------------|----------------|---------------------|-----------|--------------------|------------------|--------------------------|------------------------|-------------------|--------------------|
| Tp  | Primary tumor           | Right kidney   | 0                   | Resection | Pretreatment       | 12637            | 4195                     | 12588                  | 3908              | 3843               |
| irAE| Lichenoid dermatitis (irAE) | Right calf skin | 9 months           | Biopsy    | On therapy         | 4194             | 1034                     | 4147                   | 3184              | 3159               |
| Tm1 | Metastasis              | Brain          | 28 months          | Autopsy   | Discontinued       | 1011             | 797                      | 1008                   | 777               | 775                |
| Tm2 | Metastasis              | Jejunum        | 28 months          | Autopsy   | Discontinued       | 6969             | 3493                     | 6945                   | 3315              | 3279               |
| Tm3 | Metastasis              | Mesentery      | 28 months          | Autopsy   | Discontinued       | 1364             | 1109                     | 1360                   | 1074              | 1070               |
| LN1 | Mediastinal lymph node  | Paratracheal   | 28 months          | Autopsy   | Discontinued       | 12061            | 10999                    | 12001                  | 10680             | 10568              |
| LN2 | Mediastinal lymph node  | Carinal        | 28 months          | Autopsy   | Discontinued       | 34166            | 29881                    | 34058                  | 29229             | 28861              |
| LN3 | Mediastinal lymph node  | Mediastinal    | 28 months          | Autopsy   | Discontinued       | 15895            | 14353                    | 15842                  | 13901             | 13764              |
| NK  | Normal                  | Left kidney    | 28 months          | Autopsy   | Discontinued       | 1387             | 976                      | 1381                   | 949               | 944                |
| NSB | Normal                  | Small bowel    | 28 months          | Autopsy   | Discontinued       | 36454            | 6702                     | 30397                  | 6229              | 6096               |
| SK  | Inflamed skin lesion    | Skin           | 28 months          | Autopsy   | Discontinued       | 10522            | 5770                     | 10493                  | 5529              | 5470               |

*From Adaptive Biotechnologies

**Following additional pre-processing to remove non-productive reads
Figure S1. Library size confounding in hypothesis testing with TCR sequencing data. (A) Pairwise comparison demonstrates overlapping T cell clones present in the irAE and primary tumor (4.7% of irAE clones). To focus on T cells recognizing the same antigen, amino acid clonotypes were analyzed exclusively. Each dot represents a unique TCR amino acid sequence, with non-shared clones (gray dots) plotted along the axis of the corresponding specimen and shared clones (red dots) plotted centrally as a function of frequency in each specimen. The number of total TCR sequencing reads is listed for each specimen (n). (B) Pairwise comparison demonstrates overlapping T cell clones present in the irAE and one of the progressing metastases (Tm2) at the time of autopsy (3.7% of irAE clones). (C) Across all specimens, 127 T cell clones were identified in the irAE and at least one tumor specimen(s) and absent in the non-lymphoid normal tissues. (D) Library size confounding is illustrated by the correlation between library size and the number of shared T cell clones across multiple specimens, illustrated for both the irAE (left) and a lymph node (LN2, right). Notable outliers include specimens from the same tissue site (the seborrheic keratosis and lymph node specimens for the irAE and LN, respectively, red arrows), which show greater sharing than predicted by library size alone. In contrast, the NSB is an outlier that shows less sharing than expected based on the library size for both the irAE and LN2. (E) When the library sizes are normalized by down-sampling to the same read count (n=1008, with sharing plotted as a function of the original read counts), this positive correlation is eliminated for both the irAE (left) and LN2 (right).
Figure S2. Specimen clonality and the correlation between library size and the number of unique clonotypes. (A) Productive clonality as determined by using the productive amino acid (AA) sequence of the CDR3. (B) Correlation of library size and total number of clonotypes. The blue line indicates the robust linear regression line (fitted using R function ‘rlm’ from ‘MASS’ package based on M estimator), and the gray area indicates the upper and lower boundaries of the 95% confidence interval.
Figure S3. Effects of library size normalization. (A) T cell repertoire sharing is highly correlated with the clonal diversity of the specimens, shown for the irAE (left) and LN2 (right). (B) Normalizing for the number of clones in each specimen removes this correlation.
Figure S4. Clonal tracking of antigen-specific T cells. Clonal tracking of CMV, EBV, and flu (CEF)-specific clones (A) and neoantigen-specific clones (B) across compartments in a non-small cell lung cancer patient treated with anti-PD1 (assay published previously in Forde PM, et al, *N Engl J Med*, 2018 and Danilova et al, *Cancer Immunol Res*, 2018). (C) and (D) Clonal tracking of a EBV-specific clone in tumor involved tissues in the patient from our study. The EBV-specific clone CATGTGDSNQPQHF, highlighted in blue, was reported by two independent studies to be EBV-specific in vDJDB database. Clones shared in two specimens were colored in red; non-shared clones were colored as grey.
**Figure S5**

(A) Tissue compartment confounding in hypothesis testing with TCR sequencing data. Following library size normalization (down-sampled specimen reads listed as $n_d$), significant T cell repertoire overlap can also be identified by comparing samples from the same tissue compartment, shown for two lymph nodes (LN1 and LN2, top left) and two small bowel specimens (NSB and $T_{M2}$, top right). The percent of clonotypes shared is shown for each comparison. Subsampled comparisons are indicated (*) and the 95% CI for shared clones between NSB and $T_{M2}$ is 24-26%. (B) In contrast, less overlap is seen between specimens from distinct tissue compartments, as shown for LN2 and NSB (bottom left) and skin (SK) and $T_{M2}$ (bottom right). The percent of clonotypes shared is shown for each comparison. Subsampled comparisons are indicated (*) and the 95% CI for shared clones between NSB and LN2 is 0.7-0.9%. No confidence interval is calculated for comparison of non-down-sampled specimens (e.g., SK and $T_{M2}$).
Figure S6. Batch effect confounding in hypothesis testing with TCR sequencing data. (A) The Morisita overlap index is calculated between the irAE and all other specimens and plotted as a function of library size (total reads). There is no significant correlation observed between the MI and library size. (B) The chord diagram illustrates the large number of T cell clones shared among specimens collected at the same timepoint. The width of the bands corresponds to the Morisita Index between the two specimens. Red bands illustrate sharing between specimens from the same tissue compartment and blue bands show sharing across different tissue compartments. The two specimens collected at different timepoints (i.e., different batches, irAE and Tp) are shown in gray. (C) The individual pairwise MI calculations are shown for all specimens included in the analysis. Specimens are color coded by batch of collection and tissue compartment to highlight batch and tissue compartment confounding, with the highest MI observed between specimens collected in the same batch and from the same tissue compartment (red).
Figure S7. Overlap of the irAE T cell repertoire with highly abundant clones in tumor vs. non-tumor specimens. The number of clones shared with the irAE (y-axis) is shown for the top x clones (x-axis) for each specimen “group”. Tumor tissues are indicated with a solid line and non-tumor tissues are indicated with a dotted line. No increase in clonal overlap with the irAE is observed when comparing the most abundant clones in tumor and non-tumor specimens. Sample size is normalized in this analysis by including discrete numbers of unique clones from each specimen (i.e. the top 5, 10, 20, 30 and 40 unique clones).
Figure S8. Cumulative abundance of dermal/epidermal clonotypes with the “SSQD” motif in skin from four healthy individuals. Samples with at least 1000 reads were included in the analysis. * individuals having at least one shared clonotypes with the irAE patient in our cohort.
Figure S9. Recommendations for study design and analysis strategies for TCR sequencing-based assessment of cross-reactivity. Appropriate specimen collection enables negative selection of background (batch-effect) clones and positive selection of disease-related clones to enrich for functionally relevant TCR repertoire signatures. It is optimal to collect specimens used for signature enrichment (e.g., tumor-normal or tumor-tumor specimens) from different tissue sites of origin to minimize tissue compartment confounding. Given the practical limitations likely restricting such a specimen collection protocol, the integration of antigen-driven clustering and functional assays to identify tumor reactive T cell clones is a more robust approach for identifying disease relevant T cell signatures for the analysis of multi-specimen TCR sequencing data.