

Single-nucleus multimodal spatial transcriptomics reveals spatial colocalization of neoantigen-expressing tumor cells and cognate T cells

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Abstract

Improved methods to identify therapeutically relevant tumor neoantigens and their cognate T cells would aid the development of precision medicines for cancer. Here, we developed Slide-GoTags, a droplet-based single-nucleus spatial transcriptomics approach that characterizes neoantigen-specific immunity by integrating targeted transcript genotyping and T cell receptor (TCR) sequencing with single-nucleus RNA-sequencing from the same slice of frozen tissue. Application of Slide-GoTags to mouse and human tumors revealed colocalization of clonally expanded, neoantigen-specific T cells with tumor cells expressing their cognate neoantigen. We also identified distinct spatial immune landscapes shaped by anti-PD-1 or anti-CTLA-4 blockade in mouse colorectal tumors. Across human tumor types, Slide-GoTags detected TCR-neoantigen interactions through spatial proximity and identified an enrichment of interferon-driven immunogenicity niches in immunologically ‘hot’ tumors compared to ‘cold’ tumors. These niches harbored three T cell clonotypes that colocalized with genotyped neoantigens, highlighting a spatially organized anti-tumor immune response. Collectively, Slide-GoTags establishes a framework for *in situ* mapping of T cell-tumor interactions directly from patient tissue.

Main

Tumor neoantigens, derived from nonsynonymous somatic mutations^{1,2}, enable precise T cell targeting of malignant cells and have emerged as promising candidates for personalized immunotherapies. Advances in genomic sequencing technologies and epitope binding prediction algorithms have expanded the repertoire of candidate tumor neoepitopes^{3,4}. Their consistent detection and functional validation, however, remain limited. Direct confirmation of their presentation by tumor cells can be achieved by HLA ligandome analysis of malignant cells, but powerful mass spectrometry-based detection methods have been challenging due to the typically low tumor expression of neoantigens. Likewise, the presence of clonally expanded neoantigen-specific T cells would provide further evidence of the immunogenicity of such epitopes. But, since the majority of tumor-infiltrating T cells are bystander cells rather than tumor-specific⁵, it has been generally difficult to detect these critical cell populations using conventional approaches.

Recent advances in spatial transcriptomic technologies have enabled integration of tumor transcriptional data with spatially resolved immune dynamics⁶⁻⁹. Several approaches have extended this framework to spatial TCR profiling, including Slide-TCR-seq and other targeted enrichment-based methods that capture transcripts on positionally barcoded surfaces¹⁰⁻¹⁴. However, accurate single-cell assignment remains limited by surface feature resolution and lateral transcript diffusion¹⁵. Moreover, reliance on 3' poly(A) capture-based enrichment of TCR transcripts can reduce sensitivity for V(D)J detection^{12-14,16}. Image based sequencing approaches preserve tissue morphology, provide high spatial resolution, and have been used to map tumor clones, integrate bulk RNA derived TCR clonality with spatial analysis, or perform targeted TCR capture using predefined probe panels¹⁷⁻¹⁹. Thus, existing spatial genomic methods remain limited in their ability to adequately characterize neoantigen-specific immunity.

Directly deciphering the interaction dynamics between neoantigen-specific T cells and the evolving tumor microenvironment (TME) can reveal innate or acquired resistance mechanisms driven by therapeutic pressure and guide strategies for durable tumor control. We hypothesized that understanding the spatial relationships between tumor-specific T cells and antigen-expressing tumor cells could provide critical insights into the interactions governing anti-tumor immune responses. To this end, we developed Slide-GoTags, a droplet-based, single-nucleus spatial transcriptomics platform designed to capture the spatial distribution of diverse T cell states and clonotypes at single-cell resolution, while simultaneously resolving patterns of tumor neoantigen expression and interactions within the TME. Using this approach, we mapped neoantigen-expressing tumor cells and their cognate T cell clonotypes across mouse and human tumors, revealing antigen-specific interactions associated with distinct T cell states and interferon-rich niches.

Results

Demonstration of genotyping and TCR recovery in spatially mapped snRNA-seq

We sought to integrate the single cell resolution spatial transcriptomics method Slide-tags⁹ with targeted cDNA genotyping and T cell receptor (TCR) amplification from the same slice of fresh frozen tissue (**Fig. 1a**). We therefore applied spatial barcoding to nuclei from an intact 20 μ m fresh frozen tissue section on a 5.5 mm² array. Following nuclei isolation, gene expression profiling was performed using the 10x Genomics 5' droplet-based platform. To simultaneously capture somatic mutations and TCR sequences, we incorporated custom target-specific primers during reverse transcription (RT) to enrich for defined mutation loci and TCR CDR3 regions, respectively. These targeted loci were subsequently amplified from the cDNA library using RNase H-dependent (rh)

PCR²⁰, thereby enhancing primer specificity and sensitivity of transcript detection (Supplementary Table 1). The enriched amplicons were further subjected to long-read sequencing, enabling direct recovery of mutations and full-length V(D)J TCR sequences while facilitating reconstruction of long amplicons that are challenging to assemble from short-read sequencing^{10,11,21}. We then applied our computational processing pipeline (Extended Data Fig. 1a and Methods) to linked amplicon mutational status ('genotype'), TCR sequences, and nuclei spatial coordinates to single-nucleus gene expression profiles using shared cell barcodes.

To evaluate our ability to spatially map transcript genotypes, TCR sequences, and gene expression at single-nucleus resolution, we applied Slide-GoTags to an immunogenic murine colorectal cancer model, MC38²²⁻²⁴, which was engineered to express the well-defined epitope of ovalbumin SIINFEKL, which is recognized by antigen-specific OT-I T cells. We subcutaneously injected mice with a mixture of MC38-SIINFEKL and MC38-WT cells, followed by adoptive transfer of OT-I T cells on day 2 and tumor harvest on day 9 (Fig. 1b and Methods). Slide-GoTags analysis yielded 2,938 high-quality nuclei (Extended Data Fig. 1b and Supplementary Table 2), with spatial assignments for 1,957 (67%) nuclei. The spatially mapped structure closely mirrored the tumor architecture, as determined by H&E (Fig. 1c,d). Among 185 T cells annotated by gene expression, Slide-GoTags captured 23 α - and 29 β -TCR unpaired chains, representing a 2.6- and 3.6-fold increase, respectively, relative to clonotypes detected from the same sample using 10x Genomics Chromium single-cell V(D)J sequencing. The OT-I clone was detected by both platforms. A similar enrichment was observed in a human melanoma tumor sample, with 11.8-fold and 13.6-fold increases in TCR α and TCR β detection, respectively (Extended Data Fig. 1c,d

and **Methods**). As expected, the OT-I TCR was the dominant clonotype, representing 64% of all T cells with a detectable α - or β -TCR (**Fig. 1e**).

Detection of SIINFEKL in both gene expression and genotyping libraries enabled assessment of genotyping efficiency. SIINFEKL transcripts were identified in 70 tumor cells in the gene expression library, with targeted genotyping successfully detecting SIINFEKL exclusively in 61% of these tumor cells, corresponding to 2.1% of all spatially mapped nuclei (**Fig. 1f,g** and **Extended Data Fig. 1e,f**). Immunofluorescence staining of CD45.2⁺ OT-I T cells and SIINFEKL peptide presented by H-2K^b in the same tumor confirmed the presence of SIINFEKL antigen presenting cells (0.2% of all DAPI⁺ cells; **Fig. 1 h,i**).

Capture of SIINFEKL and of TCR α or β chains, was similar across replicates from two independent sections (n=2; **Extended data Fig. 1g**). Genotyping assignments showed strong concordance with gene expression-based detection ($\phi = 0.8$, F1=0.76); therefore, only cells in which the genotyped mutation co-occurred with gene-specific expression were retained and subsequently spatially mapped alongside TCR-expressing T cells (**Extended Data Fig. 1h,i** and **Supplementary Table 3**). Altogether, Slide-GoTags achieves high-throughput spatial mapping of the key cellular and molecular components involved in anti-tumor T cell responses, including T cell clonotypes and their cognate antigens expressed on tumor cells.

Neoantigen-specific tumor-immune interactions following treatment with immune checkpoint blockade

Immune checkpoint blockade (ICB), including anti-PD-1 and anti-CTLA-4 therapy, drives distinct T cell responses in tumors²⁵, including unique T cell activation states and effector phenotypes, and has been associated with shifts in immune and stromal cell composition within the TME²⁶. Still,

the spatial distribution of neoantigen expressing-tumor cells, their cognate T cell clonotypes, and the interactions shaping the broader TME following ICB remain incompletely understood. We therefore applied Slide-GoTags to MC38 tumor-bearing mice treated with anti-CTLA-4 or anti-PD-1 therapy (n=3 per group; **Fig. 2a**). Among 25,992 profiled nuclei that clustered into tumor and immune populations, 15,250 (60%) were spatially mapped (**Fig. 2b,c** and **Extended Data Fig. 2a**).

Across treated tumors and an untreated control, we genotyped seven known MC38 neoantigens²³ (**Extended Data Fig. 2b-c**; **Extended Data Fig. 3** and **Supplementary Table 4**). The genotyping rate was highest for the ribosomal protein L18 (*Rpl18*) mutation p.Q125R, an immunodominant MC38 neoantigen²³. We genotyped 76% of all expressed *Rpl18* transcripts (median unique molecules per genotyped cell = 3) and assigned expressed *Rpl18* p.Q125R genotypes to 66% of tumor cells. To identify *Rpl18* p.Q125R-specific CDR3 sequences, we performed bulk TCR-sequencing of tetramer-sorted spleen-derived T cells from independently vaccinated mice immunized with the *Rpl18* p.Q125R peptide (**Methods**). These tetramer-derived TCR sequences were used as a reference to annotate antigen specificity among Slide-GoTags-identified clonotypes. Using this approach, 11% of all Slide-GoTags TCRs were assigned to *Rpl18* p.Q125R-specific α and β TCR CDR3 sequences ('*Rpl18*-specific'; **Fig. 2d-e**). A trend of greater T cell infiltration was observed following anti-PD-1 treatment ($P = 0.1$, mean 5.6%) compared to anti-CTLA-4 administration (0.9%) or to lack of treatment (0.6%; **Extended Data Fig. 2d**). 117 TCR α or β clonotypes mapped to 520 T cells, with the most abundant clonotype found exclusively in the anti-PD-1 treated mouse (92 T cells), corresponding to an *Rpl18*-TCR (TCR α CDR3 = CAVSMEGGSSNAKLTF, TCR β CDR3 = CASSRTGGNQDTQYF; **Extended Data Fig. 2e** and **Supplementary Table 5**).

Given that cytotoxic T cells recognize antigen-expressing target cells via transient cell-cell interactions^{27,28}, we hypothesized that neoantigen-specific T cells would colocalize with tumor cells expressing their cognate antigen when presented by tumor cells. To test this, we applied the normalized mixing score (NMS) with permutation testing²⁹ and confirmed spatial proximity between *Rpl18* p.Q125R neoantigen-expressing tumor cells and cognate T cell clonotypes in two independent anti-PD-1 treated mice (Anti-PD1_A: NMS = 2.07, $P = 0.04$; Anti-PD1_B: NMS = 0.88, $P = 0.012$; **Supplementary Table 6**). By contrast, this spatial proximity was not observed for other non-*Rpl18* recognizing T cell clonotypes (Anti-PD1_A: NMS = 0.71, $P = 0.75$; Anti-PD1_B: NMS = 0.41, $P = 0.09$) or *Rpl18*-specific T cells relative to *Rpl18* wildtype (WT) expressing tumor cells in the anti-PD-1 treated mice (**Fig. 2f** and **Supplementary Figure 1a,b**).

Rpl18-specific T cell clones were enriched for effector³⁰ ($P_{\text{adj}} = 0.03$) and exhaustion-associated states³¹ ($P_{\text{adj}} = 1.8 \times 10^{-3}$) compared to other clonotypes in the anti-CTLA4 treated mice. This transcriptional profile was consistent with an antigen-driven CD8⁺ T cell response³²⁻³⁴. Among non-*Rpl18* clonotypes, anti-PD1 treatment was associated with an effector phenotype ($P_{\text{adj}} = 0.0011$), whereas anti-CTLA4 treatment was associated with a central memory phenotype, as previously reported ($P_{\text{adj}} = 0.03$; **Fig. 2g** and **Supplementary Table 7**). To further define the local functional state of *Rpl18*-specific T cells, we examined their proximity to *Rpl18* p.Q125R expressing tumor cells. *Rpl18*-specific T cells positioned near their cognate antigen exhibited higher CD8⁺ proliferative scores compared to the non-specific T cells ($P = 0.01$ versus $P = 0.84$, respectively; **Fig. 2h** and **Supplementary Fig. 1c**). Concordantly, *Rpl18*-specific T cells displayed enrichment of metabolic and proliferative pathways (**Fig. 2i** and **Supplementary Table 8**). These observations prompted us to ask whether such antigen-associated functional states are dependent

upon the level of neoantigen expression. We therefore introduced the *Rpl18* p.Q125R mutation into the natively *Rpl18*-wild-type MC38K line under the control of either the PGK promoter (moderate expression) or the CMV promoter (high expression; **Supplementary Fig. 1d** and **Methods**)³⁵. CMV-driven high *Rpl18* p.Q125R expression in tumor cells resulted in an increased frequency of exhaustion detected in *Rpl18* tetramer⁺ CD8⁺ T cells compared to PGK-driven expression ($P = 0.031$; **Fig. 2k** and **Supplementary Fig. 1e**). Thus, the transcriptional state of *Rpl18*-specific T cells is shaped by both proximity to cognate neoantigen-expressing tumor cells and the level of neoantigen expression.

Beyond changes in T cell populations, fibroblasts were present at a higher proportion in the anti-PD-1 treated mice compared to the anti-CTLA-4 treated mice (27.9% vs. 9.9%; **Extended Data Fig. 2f**) Subtype analysis using previously defined gene signatures³⁶ revealed a marked enrichment of antigen-presenting cancer-associated fibroblasts (apCAFs), characterized by high MHC class II and CD74 expression, relative to myofibroblastic cancer-associated fibroblasts (myCAFs ; $P = 0.001$), with a similar trend relative to inflammatory cancer-associated fibroblasts (iCAFs, $P = 0.006$; **Extended Data Fig. 2g** and **Supplementary Table 7**). Immunofluorescence staining of tumor sections from six mice per treatment group using previously defined apCAF markers CD74 and S100A4^{36,37}, confirmed the presence of apCAFs and revealed that T cells proximal to apCAFs were enriched in the anti-PD-1 treated mice compared to the anti-CTLA-4 treated and untreated control mice ($P = 0.04$, $P = 0.02$, respectively; **Extended Data Fig. 2h,i** and **Supplementary Fig. 1f-h**).

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 are reservoirs of stem-like tumor-specific T cells in renal cell carcinoma. *Nature*, accepted in
 principle., ‘*NSUN5*-specific’; **Supplementary Table 11,12**).

Four tissue sections were processed via Slide-GoTags analysis (two serial pairs, A-B and C-D
 sampled from opposing regions of the tumor; **Fig. 3a**), profiling 27,518 nuclei (**Extended Data**
Fig. 4a), of which 14,914 (54%) were spatially mapped (**Extended Data Fig. 4b**). Comparing
 immune cell abundance amongst the tissue sections, we observed the most marked enrichment of
 T, NK, and myeloid cells in section A (**Extended Data Fig. 4c**) with T cells exhibiting the greatest
 spatial autocorrelation (Moran’s $I = 0.07$, $k = 50$ nearest neighbors, $P = 1 \times 10^{-3}$; **Supplementary**
Fig. 1i,j), suggesting a focal tumor immune response within this section.

Across all four sections, we could assign *NSUN5* p.Q18K mutation status in 8% of all *NSUN5*
 expressing nuclei from tumor cells (66 mutant tumor cells, median unique molecules per cell = 1;
Extended Data Fig. 4d-f). For T cells, 345 TCR- α and 485 TCR- β unpaired sequences were
 identified. *NSUN5* p.Q18K mutant tumor cells were detected across all four sections, with *NSUN5*-
 specific TCR α or TCR β clonotypes significantly enriched in section A showing a 5.8- to 6.4-fold
 increase relative to sections B and C ($P = 0.03$ and 4×10^{-3} , respectively) and a non-significant
 two-fold increase compared to section D ($P = 0.11$; **Fig. 3b**, **Extended Data Fig. 4g-j** and
Extended Data Fig. 5a-b). *NSUN5* p.Q18K-specific and tumor-specific T cell clones exhibited
 higher transcription-defined exhaustion levels than clonotypes with unknown specificity,

reflecting the strong association between expression of T cell exhaustion markers and antigen-driven activation⁴⁷ ($P < 1.13 \times 10^{-6}$; **Extended Data Fig. 5c**). To directly evaluate platform sensitivity, we profiled the same tumor sample (RCC_1) using Slide-TCR-seq, a 3'-based spatial transcriptomic platform with cell types inferred by Robust Cell Type Decomposition (RCTD⁴⁸). Compared to Slide-GoTags, the percentage of T cells with detectable TCR α/β sequences was markedly reduced using Slide-TCR-seq across *NSUN5*-specific, tumor-specific and other clonotypes ($P_{\text{adj}} < 2 \times 10^{-15}$; **Extended Data Fig. 5d-f**). Moreover, only a single tumor cell harboring the *NSUN5* p.Q18K mutation was detected (**Extended Data Fig. 5g**). These findings support the suitability of Slide-GoTags for spatial detection of antigen specificity in tumor tissues.

We observed colocalization of *NSUN5*-specific T cells with *NSUN5* p.Q18K expressing tumor cells from section A, (NMS = 2.26, $P < 1 \times 10^{-3}$; **Fig. 3c**, **Extended Data Fig. 6a** and **Supplementary Table 13**). The interaction distance likely reflects biologic and cytoarchitectural differences such as tissue density, and immune cell infiltration that shape spatial proximity between T cells and target cells *in situ* (**Supplementary Table 2**). Such colocalization with *NSUN5*-specific T cells was not detected with *NSUN5*-WT tumor cells (**Extended Data Fig. 6b**). Notably, *NSUN5*-specific T cells were in close proximity to *NSUN5*-MUT tumor cells and exhibited an elevated chemokine/chemokine-receptor gene signature ($P = 0.03$; **Extended Data Fig. 6c**), consistent with the upregulation of chemokine receptors following TCR stimulation^{49,50}.

To further dissect the potential immunologic factors shaping the observed tissue organization of section A, we defined areas enriched for this neoantigen-driven tumor-immune engagement using kernel density estimation (KDE) of *NSUN5* p.Q18K-expressing tumor cells and their cognate T

cells. The area of overlap between high-density tumor and T cells was defined as ‘Region 1’, while the surrounding area was defined as ‘Region 2’ (**Fig. 3d-e**, **Extended Data Fig. 6d** and **Extended Data Fig. 7a** and **Supplementary Table 14**). In assessing T cell functional states, we observed that Region 1 T cells displayed a greater exhaustion score compared to those in Region 2 (**Extended Data Fig. 7b** and **Supplementary Table 7**), consistent with previous reports of a strong correlation between T cell avidity and phenotype in tumor-specific T cells^{47,51}. Moreover, higher avidity *NSUN5*-specific clonotypes were enriched in Region 1 ($EC_{50} < 80$ pg/ml, determined by peptide titration measuring TCR-mediated activation; odds ratio (OR) = 5.40, $P = 5.82 \times 10^{-3}$; **Fig. 3f** and **Supplementary Table 7,12**) and exhibited greater cytotoxicity score compared to low avidity *NSUN5*-specific clonotypes (**Extended Data Fig. 7c**), suggesting a potential role of T cell avidity in shaping underlying spatial distribution of tumor-specific clonotypes.

While T cell functional state is a key driver of anti-tumor immunity, the local tumor cell state, which is shaped by the local TME, also influences susceptibility to immune-mediated control⁵²⁻⁵⁵. To explore this, we compared gene expression profiles of malignant cells between Region 1, characterized by spatial colocalization of a neoantigen and its cognate TCR, and Region 2. Notably, tumor cells in Region 1 showed significant enrichment of both individual genes and pathway-level signatures associated with IFN- γ signaling, including HLA class I and II molecules and key mediators of interferon response (**Extended Data Fig. 7d-e** and **Supplementary Table 15,16**). Building on this finding, and previous studies suggesting a role for interferon-stimulated gene (ISG) expression in malignant cells associated with productive anti-tumor immunity⁵⁵, we examined whether pan-cancer IFN-response metaprograms (MP17/18) were heterogeneously

expressed across the ccRCC tumor sections in relation to T cells and their neoantigen targets. Consistent with the colocalization of *NSUN5*-specific clonotypes and neoantigen-expressing tumor cells, malignant cells in section A displayed marked enrichment of pan-cancer IFN-response metaprograms MP17/18 (Moran's $I = 0.19$, $P = 9.9 \times 10^{-3}$; **Extended Data Fig. 7f,g** and **Supplementary Table 7**), most prominently in Region 1, as independently supported by Slide-seq profiling of the same RCC_1 tumor section (Pearson $r = 0.61$, $P < 1 \times 10^{-3}$; **Fig. 3g** and **Extended Data Fig. 7h-j**). Nearest-neighbor analysis further demonstrated that *NSUN5*-specific clonotypes were preferentially surrounded by tumor cells residing within the MP17 region when compared to tumor specific clonotypes ($P = 5.2 \times 10^{-3}$; **Supplementary Fig. 1i-n**). In this region, T cells exhibited elevated IFN- γ expression, while myeloid and endothelial cells showed upregulation of HLA genes and ISGs, supporting an interferon-mediated interplay between T cells, tumor cells, and the surrounding cells in the microenvironment (**Extended Data Fig. 7k** and **Supplementary Table 17,18**).

To further dissect the tumor-immune crosstalk underlying the neoantigen-driven immune response present in section A, we examined spatially contextualized predicted receptor-ligand interactions⁵⁶. This analysis revealed well-characterized interactions along several axes of tumor-immune communication and regulation (**Fig. 3h** and **Supplementary Table 19**). These included T cell recruitment mediated by dendritic cell (DC) derived *CXCL9* via *CXCR3* on T cells ($P = 0.01$), an interaction linked to antitumor immunity and tumor control^{57,58}, and T cell inhibitory interactions, including *BTLA* on T cells and *TNFRSF14* on anti-inflammatory M2-like macrophages ($P = 6.52 \times 10^{-6}$)⁵⁹. These interactions were all notably enriched in Region 1 (**Extended Data Fig. 7l**, **Extended Data Fig. 8** and **Supplementary Table 20**). Together, through

the analysis of a known tumor neoantigen and a collection of TCRs recognizing this neoantigen, we establish that Slide-GoTags accurately detects TCR-neoantigen interactions through spatial proximity. Our analysis further reveals a spatially localized interaction network characterized by malignant cells expressing immunogenic neoantigens, recruitment and activation of cognate IFN- γ -secreting clonotypes, and induction of IFN response within tumor cells and the TME, thereby sustaining an immunogenic tumor niche.

Interferon-driven immunogenicity niches harbor TCR-neoantigen interactions

Applying Slide-GoTags to an extended spectrum of human tumors (n=9), including additional ccRCC (3 tumors; RCC_2, RCC_2_met, RCC_3), glioblastoma (2 tumors; GB; GB_1,GB_2), melanoma (2 tumors; MEL_1, MEL_2), and ovarian cancer (2 tumors: OV_1, OV_2; **Fig. 4a**, **Supplementary Fig. 2,3**, **Supplementary Table 10** and **Supplementary Table 21**), we profiled 130,866 nuclei (**Fig. 4b** and **Extended Data Fig. 9a**), spatially mapping 60% of them (78,651; **Supplementary Table 22-24**). Somatic mutations were selected for genotyping on a patient-specific basis according to predefined criteria (**Supplementary Table 1,4,10** and **Methods**), including predicted binding of translated peptide sequence to patient HLA class I (i.e., predicted neoantigen), proximity of the variant to the transcript 5' end (<1000 bp), and high gene expression in tumor based on bulk or previously generated single-cell RNA-seq. Based on these criteria, 59 nonsynonymous and indel mutations with the potential to generate neoantigens were selected for targeted genotyping across all samples (median 6 variants per sample; range 1-10), drawn from a broader pool of candidate variants (median 91 per sample). Ultimately, we identified 1,856 malignant cells expressing at least one predicted neoantigen transcript and 1,053 T cells expressing

a TCR α or β chain (paired TCR sequences; **Fig. 4c, Supplementary Figure. 4-7 and Supplementary Table 25**).

Since the interactions with a known TCR-neoantigen pair in RCC_1 were enriched within an IFN-driven ‘immunogenicity niche’, we postulated that malignant cells expressing such ISGs within neighborhoods characterized by high T cell density would likely harbor anti-tumor immune synapses with tumor-specific T cell clonotypes. We therefore classified our collection of tumors based on presence of immunogenicity niches, defined by the detection of spatial overlap between regions of T cell density and malignant cell density, weighted by MP17 score (**Methods**). By comparing the relative abundance of this overlap across samples, we identified immunogenicity niche^{high} (melanoma, ccRCC), and immunogenicity niche^{low} (GB, ovarian cancer) clusters (**Fig. 4d-e, Extended Data Fig. 9b and Supplementary Fig. 8-11**), highlighting an IFN- γ mediated immune response in T cell-enriched tumor regions. Immunogenicity niches were characterized by increased frequencies of exhausted CD8⁺ T cells (CD8⁺ T Ex) and antigen presenting cells in ccRCC and melanoma specimens (**Extended Data Fig. 9c**; OR = 2.16 and 4.43, $P = 0.01$ and 0.03, respectively). The T cells exhibited elevated expression of antigen presentation, IFN response, and activation gene sets (**Extended Data Fig. 9d,e and Supplementary Table 26,27**). Examination of the CD8⁺ T cell phenotypic landscape across tumor types using established gene sets (**Supplementary Table 7**), revealed enrichment of a progenitor exhausted phenotype ($P_{\text{adj}} = 4 \times 10^{-4}$; **Extended Data Fig. 9f**).

These progenitor-like CD8⁺ T cells were preferentially localized in neighborhoods enriched for DCs and exhibited specific interactions with antigen presenting cells, notably co-stimulatory

engagement via *CD6* ($P_{\text{adj}} = 7.23 \times 10^{-9}$, OR = 1.38; **Extended Data Fig. 9g**, **Extended Data Fig. 10a** and **Supplementary Table 28**). While prior studies have linked spatially organized immune niches, such as tertiary lymphoid structures (TLS) to T cell stemness and local antigen presentation within the TME^{60,61}, we observed enriched spatial overlap of TLS-like regions with MP17-expressing tumor cells in three of nine tumors with detectable MP17 signature (Mel_1, RCC_1, RCC_2; **Extended Data Fig. 10b,c** and **Supplementary Fig. 12,13**), suggesting that additional mechanisms likely contribute to the immunogenicity niche formation.

These niches were also enriched for expanded TCR clonotypes (TRA or TRB clonotypes present in at least 3 cells, TRB $P=0.02$; **Fig. 4f**). Colocalization analysis across all tumors identified ten TCR α or β chains associated with neoantigen-expressing tumor cells, two of which were validated by TCR deconvolution testing (**Supplementary Fig. 14** and **Supplementary Table 29**).

Three of these ten TRA or TRB chains were significantly enriched within the immunogenicity niche (**Fig. 4g**), including one chain validated by TCR deconvolution testing (RCC_1: TRA CAMREDRDDKIIF, *NSUN5* neoantigen) and two chains identified only by spatial colocalization (RCC_3: TRA CALRPGRGGSNYKLTF with the *TRAPPC9* neoantigen; RCC_3: TRB CASSVDSGGGYNEQFF with the *TRAPPC9* and *YPEL3* neoantigens), supporting a potential role of these regions in antigen specific anti-tumor immunity. In support of the contribution of IFN- γ rich niches in promoting antigen specific T cell recruitment, we observed increased antigen specific infiltration of T cells in MC38-SIINFEKL tumors following intratumoral IFN- γ treatment and adoptive transfer of OT-1 T cells compared to contralateral tumors lacking IFN- γ treatment ($P = 0.03$; **Fig. 4h,i**).

To further test for regulatory tumor-immune interactions that characterize these immunogenic cellular neighborhoods, we performed an immunogenicity-niche enrichment analysis of spatially

predicted receptor-ligand interactions across all tumors. Shared immune-resistance interactions were enriched in immunogenicity-rich regions, consistent with immunological selective pressure. Notably, tumor expressed *FAM3C* interacting with *CLEC2D* on T cells was enriched in GB and melanoma samples, suggesting a possible immune evasion mechanism⁶² ($P_{\text{adj}} < 0.05$; **Extended Data Fig. 10d** and **Supplementary Table 30,31**).

Collectively, the cellular composition, functional states, and interactions characterizing immunogenicity niches support their potential role in harboring and maintaining anti-tumor immune responses. Moreover, interferon-driven immunogenicity niches appear to foster neoantigen-specific T cell responses, which in turn drive the emergence of shared immune resistance features across tumor types, shaping the TME.

Tumor cells bearing putative immunogenic neopeptides associate with distinct local immune niches

Distinct tumor mutant subclones may shape the local immune microenvironment through generation of mutant peptides that differ in their potential for HLA presentation and T cell recognition. To evaluate this, we classified genotyped mutant tumor cells based on whether their mutations were predicted by NetMHCpan⁶³ to generate strong or weak HLA-I binders (defined as %Rank<0.5 and 0.5<%Rank<2, respectively; **Fig. 5a** and **Supplementary Table 32**).

In total, across the 9 tumors, we identified 32 strong and 17 weak predicted HLA-I binders, classified according to their highest predicted binding category across at least one HLA-I allele. For each binder group, non-overlapping high-density regions of mutant tumor cells were defined by KDE as areas above the 95th percentile (**Fig. 5b**, **Supplementary Table 33** and **Methods**).

Strong binder regions were enriched for CD8 T cells ($P_{\text{adj}} = 3.7 \times 10^{-4}$; OR = 1.63), whereas weak binder regions showed increased representation of anti-inflammatory M2-like macrophages ($P_{\text{adj}} = 1.1 \times 10^{-5}$; OR = 0.59), which may inhibit productive anti-tumor T cell responses⁶⁴ and NK cells ($P_{\text{adj}} = 4.4 \times 10^{-2}$, OR = 0.38; **Fig. 5c** and **Supplementary Fig. 15a**). Thus, distinct local immune microenvironments appear to be associated with HLA-I binding strength of putative presented neoepitopes.

We further detected differences in the phenotypic state of the CD8 T cells between these regions. CD8 T cells in weak binder regions were enriched for effector memory programs ($P_{\text{adj}} = 4.34 \times 10^{-12}$; **Fig. 5d** and **Supplementary Fig. 15b**), whereas those in the strong binder regions were enriched for an exhaustion/stress associated signature ($P_{\text{adj}} = 0.003$), consistent with antigen recognition driven activation⁴⁷. To further assess the relationship between CD8 T cells and predicted neoantigen binders beyond regional enrichment, we examined CD8 T cell state in proximity to mutant tumor cells. Strong binder mutations proximal to CD8 T cells were associated with a higher exhaustion score ($P = 0.045$; **Fig. 5e**), supportive of the notion that these neoantigens may be more effective at engaging local T cells. Together, these data link capacity of tumor clones to present putative neoepitopes with the local immune composition and functional state of the surrounding microenvironment.

DISCUSSION

Although T cell based immunotherapies have transformed clinical outcomes across a variety of solid tumors^{65,66}, durable tumor control remains elusive in many cases due to adaptive immune

458 escape and the immunosuppressive TME. Single cell sequencing approaches have provided critical
459 insights into tumor transcriptional heterogeneity and the immune functional states associated with
460 therapeutic outcome; however, these approaches are only beginning to adequately capture the
461 cellular interactions influencing tumor control, and the spatial architecture of heterogeneous cell
462 populations.

463 Slide-GoTags provides a means for addressing this gap by capturing full-length cDNA from the
464 entire transcriptome, enabling unbiased detection of both TCRs and tumor variants from the same
465 sample. By applying Slide-GoTags to detect expressed neoantigens across diverse solid tumors,
466 we gained several insights regarding the spatial organization of neoantigen-directed immunity,
467 thereby highlighting the utility of such a multimodal spatial approach. First, application of Slide-
468 GoTags to murine and human tumors revealed spatial colocalization between antigen-specific T
469 cell clonotypes and neoantigen-expressing tumor cells, which we validated on the basis of
470 detection of known neoantigen-TCR pairs. These colocalized interactions exhibited enrichment
471 for T cell exhaustion and chemokine signaling signatures, thereby linking spatial proximity
472 between neoantigen-expressing tumor cells and tumor-infiltrating lymphocytes with functional
473 markers of antigen-specific T cells and supporting the notion that such interactions impact the
474 cellular composition of the TME. Second, across human tumor types, we identified IFN-driven
475 immunogenicity niches characterized by local expansion of clonotypes exhibiting activated or
476 progenitor states. Across the tumors analyzed, we observed a relative enrichment of these niches
477 in immunologically ‘hot’ tumors, including melanoma and ccRCC, compared to ‘cold’ tumors
478 (i.e., GB and ovarian cancer). Notably, we identified three TCR-neoantigen pairs within these
479 immunogenicity niches, emphasizing the relevance of these niches as focal sites of tumor specific
480 immune engagement. These findings suggest that these regions may function as key sites of

productive anti-tumor T cell activity. Further elucidation of the underlying molecular and cellular mechanisms driving niche formation could expand opportunities to harness their therapeutic potential.

In addition to dissecting the spatial organization of neoantigen-directed immunity, this spatial genotyping approach helps address a longstanding challenge in cancer immunotherapy: deconvoluting TCR specificity for cognate antigens. Although both *in silico* and experimental approaches exist for the identification of TCR-neoepitope pairs, these strategies are often constrained by limited sample availability, labor-intensive protocols and variable predictive accuracy. Notably, no method to date has incorporated information regarding the spatial proximity between T cells and their target tumor cells, a key feature of TCR engagement during the formation of an immunological synapse and critical in driving T cell activation and shaping anti-tumor responses^{66,67}. We reasoned that spatial proximity between TCRs and their potential cognate antigens could provide an additional layer of information to help identify clonotypes specific for selected neoantigens. Indeed, we observed specific colocalization of previously deorphanized TCRs with their cognate antigens in both mouse and human tumors. We anticipate that such spatial colocalization approaches could be integrated with computational methods to prioritize candidate TCRs for downstream *in vitro* validation

While Slide-GoTags enables multimodal spatial resolution of T cell specificity combined with targeted genotyping, several technical limitations remain. As with other cDNA-based capture approaches, genotyping efficiency is influenced by target transcript abundance and the proximity of mutations to the transcript's 5' end, potentially hindering detection of lowly expressed or 3'

proximal neoantigens. Transcript dropout events may further reduce genotyping fidelity and can lead to the failure to detect one allele in heterozygous cells, resulting in genotype misassignment. Additionally, discrepancies can arise between RNA-based genotyping and genomic allele frequencies as a consequence of transcriptional regulation, epigenetic modifications, allelic imbalance, or post-transcriptional modifications⁶⁸⁻⁷⁰. The current implementation of Slide-GoTags, optimized for single-nucleus profiling of frozen tissues, may limit sensitivity due to loss of cytoplasmic mRNA and may therefore miss low abundance transcripts, including rare T cell clonotypes, thereby limiting complete characterization of the TCR repertoire. In addition, variable nuclei recovery across samples, influenced by sample quality and tissue processing, may further reduce assay sensitivity, affect cell type representation and potentially limit detection of spatial interactions. Future advances in single-cell spatial technologies may help improve capture fidelity. Finally, although spatial colocalization serves as a valuable proxy for inferring TCR specificity, it may be influenced by co-expression of multiple neoantigens by a single tumor clone, multiple TCR clonotypes proximal to the same tumor cell, or stacking of nuclei due to tissue thickness. Consequently, spatial associations may in some cases reflect regional cell density rather than direct cell-cell contact. Nonetheless, Slide-GoTags expands the toolkit for investigating TCR-tumor interactions, providing a spatially informed framework that can be integrated with orthogonal validation and discovery strategies.

Broadening the use of Slide-GoTags across diverse cancer types, immune-related diseases, and longitudinal samples will deepen insights into spatially organized immune responses and antigen-specific T cell activity. Studies in larger patient cohorts may help clarify how T cell avidity influences clinical response. This framework paves the way for spatially informed immunotherapy

strategies, with implications for the identification of tumor-reactive TCRs, mapping mechanisms of resistance, and guiding the design of personalized T cell therapies.

Funding

This work was supported in part by the NIH/NCI (CA276865), the Endeavor Award from the Mark Foundation for Cancer Research (F.C., C.J.W) and the Dana-Farber/Harvard Cancer Center Kidney SPORE (2P50CA101942-16) (T.K.C, C.J.W) and the Dana-Farber/Harvard Cancer Center Program (5P30CA006516-56). A.N. discloses support from the Leukemia & Lymphoma Society Cancer Development Program Fellowship (5648-24). A.S. discloses support from the Wellcome Trust Early Career Award (227000/Z/23/Z). J.Y.G. discloses support from the National Institutes of Health National Cancer Institute Award F30CA298302 and National Institute of General Medical Sciences Awards T32GM007753 and T32GM144273. C.R.O. discloses support from the NIH/NCI fellowship K00CA245819 as well as the Helen Gurley Brown Foundation and Dana-Farber Cancer Institute. A.S. discloses support from the Novo Nordisk Foundation (NNF22OC0071681) as well as the LEO foundation (LF18500). T.K.C. is the Kohlberg Chair at Harvard Medical School, and discloses support from the Trust Family, Michael Brigham, the Pan Mass Challenge, the Hinda and Arthur Marcus Fund, and Loker Pinard Funds for Kidney Cancer Research at DFCI. L.R.O. discloses support from the Novo Nordisk Foundation (NNF22OC0071681) as well as the LEO foundation (LF18500). S.S. discloses support from the NCI Harvard Cancer Consortium (HCC) Kidney Cancer Program (P30 29 CA06516), HCC Kidney Cancer SPORE (P50-CA101942-12), 1R01CA266424, and DOD CDMRP (KC230269P1) grants. D.A.B. discloses support from the Department of Defense Early Career Investigator Award (KCRP AKCI-ECI, W81XWH-20-1-0882), the Louis Goodman and Alfred

Gilman Yale Scholar Fund, the NIH/NCI (1R37CA279822-01), and the Yale Cancer Center (NIH/NCI P30CA016359). G.O. discloses support from the Claudia Adams Barr Program for Innovative Cancer Research, by Young Investigator Award of Melanoma Research Alliance and by the American Cancer Society. C.J.W. is a member of the Parker Institute for Cancer Immunotherapy at DFCI and discloses support from in part, by the Parker Institute for Cancer Immunotherapy. C.J.W. is also the Lavine Family Chair for Preventative Cancer Therapies at DFCI. The remaining authors declare no relevant funding.

Acknowledgments

We thank the N. Hacohen and F. Chen laboratories for constructive discussions; A. Sharp for providing MC38-SIINFEKL cells.

Contributions

A.N., A.S., J.Y.G., and C.J.W. conceived the study. A.N., A.S. and J.Y.G., developed the Slide-GoTags protocol and performed experiments, with help from J.A.W., A.J.C.R., G.J.M., W.S.L., M.V.O., S.L., E.L. and K.J.L. A.N., A.S. and J.Y.G. performed data analysis with help from Q.G., A.Son, L.R.O, C.T. and B.S. G.J.M. and V.K. synthesized the barcoded beads. M.C.S., D.A.B., P.A.O., T.K.C., D.A.R., and C.H.Y. provided human tissue biopsies. G.R.D.O. and S.S. performed assessment of tertiary lymphoid structures. C.R.-O. and L.P. performed mouse experiments. K.H. assisted with immunofluorescence imaging. A.B.A. and G.O. performed TCR reconstruction and specificity screening from RCC samples. A.N., A.S., J.Y.G., N.H., F.C., and C.J.W. wrote the manuscript with contributions from all authors.

Competing Interests

D.A.R. has received personal fees for consulting and advisory board activities from AnHeart Pharmaceuticals, Aptitude Health, BlueRock Therapeutics LP, CeCaVa GmbH & Co.KG, Chimeric Therapeutics, Elsevier, F. Hoffmann-La Roche, Genenta Science, Inovio, Insightec,

577 Janssen, Johnson & Johnson Pharma, Jupiter Life Sciences Consulting, LLC, Kintara, Kiyatec,
 578 Lumanity, Menarini Stemline, Miltenyi Biomedicine GmbH, Neuvogen, Novocure, Paradigm
 579 Medical Communications, Putnam Inizio Associates, LLC, Servier, Sumitomo Dainippon Pharma
 580 Oncology, Triangle Insights Group, Vivacitas Oncology, Inc., and WebMD. He has also received
 581 research support from Agenus, Ashvattha Therapeutics, Boehringer Ingelheim, Bristol Myers
 582 Squibb, Corbus Pharma, EMD Serono, Enterome, Invios, Medicenna Therapeutics, Mogling Bio,
 583 NeoTX Ltd., Numiera Therapeutics, Sapience Therapeutics, SphereBio, and Vaccinex. N.H. holds
 584 equity in and advises Danger Bio/Related Sciences, serves on advisory boards of Repertoire
 585 Immune Medicines and CytoReason, holds patents licensed to BioNTech, and receives research
 586 funding from Bristol Myers Squibb and Calico Life Sciences. T.K.C. reports grants, personal fees
 587 and non-financial support from Roche and Genentech during the conduct of the study; and reports
 588 institutional and/or personal, paid and/or unpaid support for research, advisory boards,
 589 consultancy, and/or honoraria from Alkermes, AstraZeneca, Aravive, Aveo, Bayer, Bristol Myers-
 590 Squibb, Calithera, Circle Pharma, Eisai, EMD Serono, Exelixis, GlaxoSmithKline, Gilead, IQVA,
 591 Infinity, Ipsen, Jansen, Kanaph, Lilly, Merck, Nikang, Nuscan, Novartis, Oncohost, Pfizer, Roche,
 592 Sanofi/Aventis, Scholar Rock, Surface Oncology, Takeda, Tempest, Up-To-Date, CME events
 593 (Peerview, OncLive, MJH and others), outside the submitted work. Institutional patents filed on
 594 molecular alterations and immunotherapy response/toxicity, and ctDNA. Equity: Tempest, Pionyr,
 595 Osel, Precede Bio, CureResponse, InnDura Therapeutics, Primium. Committees: NCCN, GU
 596 Steering Committee, ASCO/ESMO, ACCRU, KidneyCan, ODAC. Mentored several non-United
 597 States citizens on research projects with potential funding (in part) from non-United States
 598 sources/Foreign Components. S.S. declares receiving commercial research grants from Bristol-
 599 Myers Squibb, AstraZeneca, Exelixis, Merck, NiKang Therapeutics, Arsenal Biosciences; is a

consultant/advisory board member for Merck, AstraZeneca, Bristol Myers Squibb, NextPoint Therapeutics, AACR, and NCI; receives royalties from Biogenex; and mentored several non-US citizens on research projects with potential funding (in part) from non-US sources/Foreign Components. P.A.O. declares research funding from and/or has advised Agenus, Arcturus, Amgen, Armo BioSciences, Array, AstraZeneca/MedImmune, Bristol-Meyers Squibb, Celldex, CytomX, Evaxion, Immunetune, Imunon, LGChem, Merck, Neon Therapeutics (now BioNTechUS), Novartis, Pharmajet, Phio, Pfizer, Oncorus, Roche/Genentech, Servier, Xencor. D.A.B. reports share options in Elephas, advisory board, consulting or personal fees from Cancer Expert Now, Adnovate Strategies, MDedge, CancerNetwork, Catenion, OncLive, Cello Health BioConsulting, PWW Consulting, Haymarket Medical Network, Aptitude Health, ASCO Post and Harborside, Targeted Oncology, Merck, Pfizer, MedScape, Accolade 2nd MD, DLA Piper, Dechert, AbbVie, Compugen, Link Cell Therapies, Scholar Rock, NeoMorph, Nimbus, Exelixis, AVEO, Eisai, Daiichi Sankyo, and Elephas; and research support from Exelixis and AstraZeneca, outside of the submitted work. F.C. is an academic founder of Curio Bioscience and Doppler Bioscience and a scientific advisor to Amber Bio; his interests were reviewed and managed by the Broad Institute in accordance with its conflict-of-interest policies. A.N., J.Y.G., A.S., and C.J.W. are listed as inventors on a patent application related to the work. C.J.W. holds equity in BioNTech, receives research funding from Pharmacyclics, and serves on the advisory boards of Repertoire Immune Medicines, Aethon Therapeutics, Nature's Toolbox, and Adventris. The other authors declare no competing interests.

Figure legends

Fig. 1: Slide-GoTags for genotyping of spatially mapped snRNA-seq. **a**, Schematic of the Slide-GoTags workflow. Spatially barcoded nuclei from a 20 μ m fresh-frozen tissue section were profiled using 10x Genomics droplet-based snRNA-seq as per the Slide-tags protocol³. The cDNA library was used for whole-transcriptome amplification, as well as TCR amplification and genotyping of mutations. **b**, Schematic of the syngeneic tumor model. A single mouse was implanted subcutaneously (s.c.) with 1×10^6 MC38 cells composed of a mixture of wild-type and SIINFEKL-expressing MC38 cells. Two days later, the mouse received 2×10^5 CD8⁺ OT-I T cells via intravenous (i.v.) injection. Tumors were harvested on day 9 post-implantation. **c**, Serial section of the MC38 tumor stained with H&E. This staining was performed once on the section shown. **d**, Spatial mapping of snRNA-seq profiles, colored by cell type. **e**, Bar plot showing the frequency of TCR α and β CDR3 sequences in snRNA-seq T cells. **f**, Proportion of tumor cells genotyped for SIINFEKL, based on SIINFEKL expression. **g**, Spatial mapping of tumor cells (light blue), SIINFEKL-genotyped tumor cells (blue), OT-I T cells expressing CDR3 TRA/TRB (red), and other T cells with non-OT-I CDR3 sequences (pink). **h,i** Immunofluorescent staining showing CD45.2⁺ OT-I T cells (red) and SIINFEKL-H-2K^b positive cells (light blue) in the same tumor. This staining was performed once on the section shown.

Fig. 2: Spatial profiling of neoantigen-specific immunity during ICB. **a**, Schematic of the syngeneic tumor model. MC38 cells (2.5×10^5) were subcutaneously implanted into mice, which were either untreated or administered intraperitoneal injections of anti-PD-1 or anti-CTLA-4 antibodies on days 7 and 11. Tumors were harvested on day 12 post-implantation. Each treatment condition included $n = 3$ per group. **b,c**, Spatial mapping of tumors from anti-PD-1 (b) and anti-CTLA-4 treated (c) mice, colored by cell type. **d,e**, Spatial mapping of *Rpl18* p.Q125R genotyped

645 tumor nuclei and T cells with detected TCRs in anti-PD-1 (d) and anti-CTLA-4 treated (e) tumors,
 646 colored by annotation: *Rpl18* wild-type (WT, light blue), *Rpl18*-mutant (MUT, dark blue), where
 647 MUT indicates the presence of at least one mutated genotype per nucleus. T cells with *Rpl18*
 648 p.Q125R-specific CDR3 TRA/TRB (red), and other TCR clonotypes (pink). **f**, Spatial
 649 colocalization analysis using the Normalized Mixing Score (NMS) between *Rpl18*-MUT or *Rpl18*-
 650 WT tumor cells and T cells expressing *Rpl18* p.Q125R specific TCR (red) or other TCR clonotypes
 651 (pink) in mouse tumor A treated with anti-PD-1. The gray shaded region denotes radii with
 652 significant colocalization, assessed using a one-sided permutation test ($P < 0.05$). **g**, Dot plot of
 653 CD8⁺ T gene signatures from mouse and human datasets^{30,31,47}. *Rpl18*-specific TCRs, were pooled
 654 across all treated mice and compared to other clonotypes within each treatment group. Statistical
 655 significance was determined using a two-sided Wilcoxon rank-sum test with Bonferroni
 656 correction. **h**, Nearest distance between *Rpl18*-MUT tumor cells and T cells with *Rpl18* p.Q125R
 657 specific TCR (red) or other TCR clonotypes (pink), correlated with the Oliveira 2021⁴⁷ CD8⁺ T
 658 proliferation module score in the anti-PD-1-treated mouse. The *P* value was calculated using linear
 659 regression to assess the significance of the association between *Rpl18* p.Q125R specific T cell
 660 distance to *Rpl18*-MUT tumor cells and the CD8⁺ T proliferation module score. **i**, Dot plot of
 661 significantly enriched Gene Ontology pathways among differentially expressed genes in *Rpl18*-
 662 specific T cells. *P* values were adjusted for multiple comparisons across pathways using the
 663 Benjamini-Hochberg method. **j**, Schematic of the syngeneic tumor model experiment, in which
 664 C57BL/6 mice were subcutaneously implanted with MC38-K (1×10^6) cells engineered to express
 665 *Rpl18* WT or the *Rpl18* p.Q125R mutant under PGK or CMV promoters ($n=6$ per group). **k**,
 666 Frequency of TIM-3⁺LAG-3⁺ cells among *Rpl18* tetramer⁺ CD8⁺ T cells, each dot represents an

independent mouse (n=6). Bars indicate mean $\pm$ s.e.m. Statistical significance was determined using a two-sided Wilcoxon rank-sum test.

Fig. 3: Colocalization of TCR-neoantigen pairs in human ccRCC. **a**, Schematic of Slide-GoTags analysis performed on four ccRCC sections from RCC_1. **b**, Spatial mapping of *NSUN5* p.Q18K genotyped tumor nuclei and T cells with detected TCRs, colored by annotation: *NSUN5*-WT (light blue), *NSUN5*-MUT (blue), T cells with *NSUN5* p.Q18K-specific CDR3 TRA/TRB (red), tumor-specific CDR3 TRA/TRB (orange), and other clonotypes (pink). Bottom, immunofluorescent staining of a serial section showing cell nuclei (DAPI, blue) and T cells (CD3, red). **c**, Spatial colocalization analysis using NMS between *NSUN5*-MUT or *NSUN5*-WT tumor cells and T cells expressing *NSUN5* p.Q18K specific TCRs (red), tumor-specific TCRs (orange), or other TCR clonotypes (pink). The gray shaded region denotes radii with significant colocalization, assessed using a one-sided permutation test ($P < 0.05$). **d**, Kernel Density Estimation (KDE) analysis defining tumor regions based on the overlap of *NSUN5*-MUT tumor cells and *NSUN5*-specific TCRs. **e**, Alluvial plot of TCR clonotype frequency across two regions, with *NSUN5*-specific TCRs (red), tumor-specific TCRs (orange), and other CDR3 sequences (gray). **f**, Spatial mapping of *NSUN5* p.Q18K -specific TCRs stratified by avidity: high ($EC_{50} > 80$ pg/ml, red) and low ($EC_{50} \leq 80$ pg/ml, brown). **g**, KDE analysis of the interferon and MHC II module score (MP17). **h**, Selected spatially co-occurring receptor-ligand interactions between specific sender-receiver cell type pairs. P values were adjusted for multiple comparisons across tested receptor-ligand pairs using the Benjamini-Hochberg method; FC, fold change. Panels b-h correspond to analyses from ccRCC_1 section A.

Fig. 4: Immunogenicity niches are associated with TCR-neoantigen colocalization. **a**, Schematic of human tumor types analyzed using Slide-GoTags, denoting treatment status, tumor

690 progression stage (primary or metastatic) and somatic mutations genotyped. **b**, UMAP of snRNA-
 691 seq profiles, colored by cell type annotation. **c**, UMAP of genotyped nuclei, showing WT (light
 692 blue) and MUT (blue), with T cells expressing CDR3 TRA/TRB (red). **d**, KDE analysis of T cell
 693 density, and tumor density overlaid with MP17, based on the 95th percentile, in representative
 694 sections. **e**, Heatmap of spatial overlap between T cell density and MP17-expressing tumor cells
 695 per specimen. Clustering based on spatial similarity; red, high overlap; blue, low overlap. Clusters
 696 are marked by dashed lines. **f**, Boxplots showing the proportion of expanded T cell clonotypes in
 697 immunogenicity niche-rich (red) and immunogenicity niche-poor (blue) regions. Expanded
 698 clonotypes were defined as TRA or TRB CDR3 sequences detected in at least two T cells within
 699 a sample. Each dot represents a paired sample (TRA, n=4; TRB, n=7). Boxplots show the median
 700 as the center line, the 25th to 75th percentiles as the lower and upper box limits, and whiskers
 701 extending furthest observed data point within 1.5x the interquartile range. Statistical significance
 702 was determined using a two-sided paired Wilcoxon test (TRA, $P=0.25$; TRB, $P=0.016$). **g**,
 703 Volcano plot of TRA and TRB CDR3 sequences enriched in immunogenicity niches, colored by
 704 tumor type. Data shown are from patient samples with spatially co-localized neoantigens and
 705 TCRs (**Supplementary Table 29**). Red: TCRs spatially colocalized with their genotyped
 706 neoantigen; blue: known virus-specific TCRs^{71,72}. **h**, Schematic of the experimental design.
 707 Bilateral subcutaneous MC38-SIINFEKL tumors were established in C57BL/6J CD45.1⁺ mice (n
 708 = 5). CD45.2⁺ OT-I T cells were adoptively transferred intravenously on day 2. Tumors were
 709 treated intratumorally (i.t.) with IFN- γ or PBS (5 μ g) on days 9, 11, 13, and 15, followed by tumor
 710 harvest on day 16. **i**, Quantification of OT-I T cell infiltration by immunofluorescence imaging.
 711 OT-I cells (CD45.2⁺) are shown as a percentage of total nuclei (DAPI). Each dot represents an

individual tumor; n=5 paired mice, bars indicate mean $\pm$ s.d. Statistical significance was assessed using a two-sided paired t-test ($P = 0.03$).

Fig. 5: Spatial organization of predicted HLA-I neoantigen binders in the tumor microenvironment. **a**, Distribution of predicted HLA-I neoantigen binders per gene across tumor types. Analyses were performed on tumors with more than one genotyped gene. Binding affinity was determined using NetMHCpan, and binding class was assigned according to the highest predicted binding category. **b**, Representative spatial maps of non-overlapping strong and weak MUT tumor regions. High-density strong (blue) and weak (gray) MUT tumor regions were defined separately using the 95th percentile of each KDE distribution, and non-overlapping regions were then identified. **c**, Cell type composition of non-overlapping regions enriched for strong versus weak binder MUT tumor cells across samples. **d**, Distribution of CD8 T module scores^{31,47} in strong versus weak MUT tumor regions, calculated using UCell⁷³. Statistical significance was determined using two-sided Wilcoxon rank-sum tests with Benjamini-Hochberg correction. **e**, Nearest distance to tumor cells harboring mutations predicted to generate strong or weak HLA-I binders, plotted against CD8⁺ T cell exhaustion (T-Ex)⁷⁴ module score across tumors. Lines show fitted linear regression models for strong-binder and weak-binder mutant tumor cells. Shaded bands indicate the 95% confidence interval around the fitted regression line. P values were calculated using linear regression to assess the association between proximity to neoantigen-expressing tumor cells and CD8⁺ T-Ex score.

Extended Data legends

Extended Data Fig. 1. Cell type assignment and gene expression in MC38-SIINFEKL mouse tumor. **a**, Schematic of the genotyping workflow combining the Nanoranger pipeline²¹, with

Slide-GoTags for variant detection and genotype assignment to single nuclei. **b**, UMAP of snRNA-seq profiles, colored by cell type annotation. **c**, Alluvial plot showing clonotype frequencies of TRA and TRB CDR3 sequences detected by 10x Genomics Chromium single-cell V(D)J kit and Slide-GoTags. Slide-GoTags showed higher sensitivity, detecting more unique clonotypes (orange; 6 TRA and 15 TRB) compared to 10x V(D)J kit (2 TRA and 3 TRB). Shared OT-I clones were consistently detected across both platforms. **d**, Alluvial plot as in panel c, showing clonotype frequencies detected by the 10x Genomics Chromium single-cell V(D)J kit and Slide-GoTags in a human melanoma tumor. **e**, Distribution of normalized SIINFEBL gene expression across cell types. **f**, UMAP of tumor cells (light blue), SIINFEBL-genotyped tumor cells (blue), OT-I T cells expressing CDR3 TRA/TRB (red), and other T cells with non-OT-I CDR3 sequences (pink). **g**, Bar plot showing detection rates of TRA, TRB, and SIINFEBL transcripts across two independent Slide-GoTags replicates generated from separate tissue sections. *P*-values were calculated using two-sided Fisher's exact test. **h**. Spatial mapping of a T cell module score. **i**, Spatial mapping of SIINFEBL gene expression.

Extended Data Fig. 2. Neoantigen genotyping in the MC38 mouse model treated with ICB.

a, UMAP of snRNA-seq profiles, colored by cell type annotation across all tumors. **b**, UMAP of *Rpl18* p.Q125R genotyped nuclei: *Rpl18*-WT (light blue) and *Rpl18*-MUT (blue), with T cells expressing *Rpl18* p.Q125R specific CDR3 TRA/TRB (red) or other TCR clonotypes (pink) across all tumors. **c**, UMAP of neoantigen-genotyped tumor nuclei from MC38 tumors, combining ICB-treated and untreated control mice, with quantification of WT and MUT nuclei. Individual genotype maps are shown for: *Adgpk9* p.R304M; *Atg9a* p.L299Q; *Cpnel* p.L299Q; *Tmem135* p.R421L; *Dpagt1* p.V213L; *Ndfip2* p.A238P. **d**, Bar plot of T cell frequencies across treatment conditions profiled by Slide-GoTags. Each dot represents one mouse; untreated, *n* = 1; anti-CTLA-

4, n = 3; anti-PD-1, n = 3. Bars indicate mean $\pm$ s.d. **e**, Bar plot of the top 30 most frequent TRA and TRB CDR3 detected by snRNA-seq. *Rpl18* p.Q125R specific TCRs are red; other clonotypes are pink. **f**, Frequency of cell types across the various treatment conditions. **g**, Fibroblast classified as Cancer-Associated Fibroblasts (CAFs) based on the highest-scoring CAF gene module⁷⁴ across the anti-PD1 treated mice. Dots indicate the mean score for each mouse (n=3). Statistical significance was assessed using one-sided paired t-tests. **h**, Immunofluorescent staining of tumor sections from untreated, anti-CTLA4 and anti-PD1 treated mice (n=6 mice per group) antibodies against S100A4 and CD74 (antigen-presenting CAFs ;apCAFs)^{36,37}, and CD3 (T cells). Each point represents the total number of T cells colocalized with apCAFs per mouse, summed across n=36 images per mouse. Boxes indicate median and interquartile range; whiskers denote range. Statistical significance was assessed using a two-sided Wilcoxon rank-sum test. **i**, Representative immunofluorescence image of an anti-PD-1 treated tumor showing nuclei (DAPI, blue), T cells (CD3, red), and dual expression of CD74 (green) and S100A4 (white) marking apCAFs. Staining was performed on n = 6 mice per group, as quantified in h. **j**, Selected spatially co-occurring receptor-ligand interactions between specific sender-receiver cell type pairs. P values were adjusted for multiple comparisons across tested receptor-ligand pairs using the Benjamini-Hochberg method.

Extended Data Fig. 3. Slide-GoTags spatial mapping of MC38 neoantigens during ICB.

Spatial mapping of tumor nuclei genotyped for *Adgpk9* p.R304M (green), *Atg9a* p.L299Q (pink), *Cpne1* p.L299Q (blue), *Ndfip2* p.A238P (purple), *Tmem135* p.R421L (yellow) and *Dpagt1* p.V213L (orange) across anti-PD1 and anti-CTLA4 treated MC38 mouse tumors.

Extended Data Fig. 4. Spatial profiling of NSUN5 mutations and cognate TCRs in ccRCC. a,

UMAP embedding of snRNA-seq profiles of ccRCC_1 sections A-D, colored by cell type

annotation. **b**, Spatial mapping of sections A-D, colored by cell type (as in **a**). **c**, Cell type frequency across RCC_1 sections colored by cell type (as in **a**). **d**, UMAP of *NSUN5* p.Q18K-genotyped nuclei across all four sections: *NSUN5*-WT (light blue) and *NSUN5*-MUT (blue), with T cells expressing *NSUN5* p.Q18K-specific CDR3 TRA/TRB (red), tumor-specific CDR3 TRA/TRB (orange), or other clonotype CDR3s (pink). **e**, Normalized *NSUN5* genotyping across cell types (WT, light blue; MUT, blue). **f**, Spatial mapping of *NSUN5* expression in RCC_1_A. **g**, Alluvial plot of TCR clonotype frequencies across sections A-D. *NSUN5*-specific TCRs are red, tumor-specific TCRs are orange, and the other TCRs are gray. **h-j**, Spatial mapping of *NSUN5* p.Q18K genotyped nuclei and T cells expressing TCRs, *NSUN5*-WT (light blue) and *NSUN5*-MUT (blue), with T cells expressing *NSUN5*- p.Q18K specific CDR3 TRA/TRB (red), tumor-specific CDR3 TRA/TRB (orange), or other clonotype CDR3s (pink).

Extended Data Fig. 5. TCR clonotype distribution, functional states, and platform comparison in ccRCC. **a,b**, Bar plot of top 30 TRA and TRB CDR3 frequencies in T cells from snRNA-seq across four sections of a ccRCC specimen. *NSUN5* p.Q18K-specific TCRs are red; Tumor specific TCRs are orange; other TCR clonotypes are pink. **c**, Exhaustion module score in T cells grouped by clonotype across sections A-D. Statistical significance was determined using a two-sided Wilcoxon rank-sum test comparing *NSUN5* TCRs ($P = 1.3 \times 10^{-6}$) or tumor-specific TCRs ($P = 9.73 \times 10^{-13}$) with other clonotypes. **d**, Proportion of T cells bearing *NSUN5* p.Q18K neoantigen-specific TCRs (red), tumor-specific TCRs (orange), or other clonotypes (pink), detected by Slide-GoTags (purple outline; section RCC_1_A) or Slide-TCR-seq (gray outline). Statistical significance was assessed using two-sided Fisher's exact tests with Benjamini-Hochberg correction for multiple comparisons (P_{adj} , adjusted P value). **e,f**, Percentage of T cells with detected TCR α (top) or TCR β (bottom) CDR3 sequences, colored by TCR category as in **d**.

g, Spatial distribution of genotyped tumor cells and TCR-bearing T cells profiled by Slide-TCR-seq.

Extended Data Fig. 6. Spatial analysis of *NSUN5*-mutant tumor cells and cognate T cells across ccRCC sections. **a**, Spatial colocalization using NMS between *NSUN5*-MUT or *NSUN5*-WT tumor cells and T cells expressing *NSUN5* p.Q18K-specific TCRs (red), tumor-specific TCRs (orange), or other TCRs (pink), in sections B-D. **b**, NMS colocalization of tumor cells grouped by *NSUN5* expression level. **c**, Nearest distance between *NSUN5*-MUT tumor cells and *NSUN5* TCR (red), tumor-specific TCRs (orange), or other TCRs (pink), and correlation with the chemokine/chemokine receptor module score in section A. The *P* value was calculated using linear regression to assess the significance of the association between *NSUN5* p.Q18K-specific T cell distance to *NSUN5* p.Q18K tumor cells and the chemokine/chemokine receptor module score. **d**, KDE analysis of *NSUN5* p.Q18K genotyped tumor nuclei and *NSUN5* p.Q18K specific TCRs, in section A.

Extended Data Fig. 7. IFN gene set enrichment in ccRCC. **a**, Proportion of spatially mapped tumor cells genotyped for *NSUN5*, comparing Region 1 and 2 in section A. **b**, Exhaustion module score in T cells stratified by spatial region in section A. Statistical significance was determined using a two-sided Wilcoxon rank-sum test ($P = 0.016$). **c**, Cytotoxicity module score in *NSUN5* TCR-expressing T cells stratified by TCR avidity. Statistical significance was determined using a two-sided Wilcoxon rank-sum test ($P = 0.004$). **d**, Volcano plot of differentially expressed genes between tumor Regions 1 and 2. Statistical significance was determined using a two-sided Wilcoxon rank-sum test with Bonferroni correction. **e**, Gene Ontology enrichment of differentially expressed genes in tumor cells enriched in region 1 section A (from **d**). *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg method. **f**, Heatmap of interferon and MHC

II module scores (MP17 and MP18), comparing Regions 1 and 2. **g**, Heatmap comparing interferon and MHC II module scores across the four ccRCC sections. **h**, RCC_1 tumor section profiled with Slide-seq showing the spatial distribution of the MP17 interferon and MHC II module score. **i-k**, KDE plots of gene expression: **h**, MHC class I genes in tumor cells; **i**, MHC class II genes in tumor cells; **j**, IFN- γ expression in T cells. **l**, Volcano plot of spatial interaction intensity scores for receptor-ligand pairs between regions in tumor cells. P values were adjusted for multiple comparisons across tested receptor-ligand pairs using the Benjamini-Hochberg method.

Extended Figure. 8. Spatially mapped receptor-ligand interaction in RCC_1. Spatial mapping of interaction intensity scores for selected receptor-ligand pairs shown in **Fig 3h**.

Extended Data Fig. 9. Pathways enriched in immunogenicity niches across tumor types. a, UMAP embedding of snRNA-seq T cell profiles from all Slide-tags samples, colored by cell type annotation. **b**, Heatmap of spatial overlap between T cell density and MP17-expressing tumor cells, grouped by tumor. Red, high overlap; blue, low overlap. **c**, Cell type frequency in immunogenicity-rich and poor regions across the tumor types. **d**, Volcano plot of differentially expressed genes in T cells from immunogenicity-rich vs. poor regions. Statistical significance was determined using a two-sided Wilcoxon rank-sum test with Bonferroni correction. **e**, Gene Ontology analysis of differentially expressed genes (as in **d**) in T cells from immunogenicity rich regions. P values were adjusted for multiple comparisons using the Benjamini-Hochberg method. **f**, Distribution of CD8⁺ gene signature score⁴⁷ in immune niche-rich versus immune niche-poor regions, calculated using Ucell method⁷³. Statistical significance was determined using two-sided Wilcoxon rank-sum tests with Bonferroni correction. **g**, Composition of neighboring cell types proximal to CD8⁺ T cell subpopulations within 200 μ m radius.

Extended Data Fig. 10. TLS-associated overlap of MP17 tumor cells and T cell dense regions.

a, Selected spatially co-occurring receptor-ligand interactions between specific sender-receiver cell type pairs per patient, highlighting interactions involving Progenitor-Exhausted (Progen_Ex) and Terminally Exhausted (Terminal_Ex) CD8⁺ T cells. P values were adjusted for multiple comparisons across tested receptor-ligand pairs using the Benjamini-Hochberg method. **b**, Representative immunofluorescent staining of tumor sections for CD3⁺ T cells (red), CD20⁺ B cells (green), and DAPI (blue), with TLS-like regions indicated by dashed boxes (top). KDE analysis of T and B cell densities from Slide-GoTags on a consecutive section (bottom). Spatial overlap of high-density T cell, B cell, and MP17 tumor cell regions identifies immune-tumor interaction niches (right). **c**, Scatter plot showing the percentage of T cell-B cell overlap area versus the percentage of T cell-MP17 tumor cell overlap area. Each point represents the mean across tumors from an individual patient. **d**, Volcano plot of receptor-ligand interaction intensity scores comparing immunogenicity rich and poor regions. P values were adjusted for multiple comparisons across tested receptor-ligand pairs using the Benjamini-Hochberg method.

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1067 **Methods**

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1069 **Sample information and processing**

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1071 **Human biospecimens**

1072 Human melanoma, ovarian cancer, ccRCC, and GB tissue samples were obtained from patients
 1073 enrolled in clinical trials approved by the Dana-Farber/Harvard Cancer Center Institutional Review
 1074 Board. Tissue samples were embedded in OCT, snap-frozen after surgery and stored at -80 °C.
 1075 Specimens included treatment-naïve, recurrent, post treatment and both primary and metastatic
 1076 disease sites. Additional information about patient clinical characteristics and human specimen
 1077 information can be found in **Supplementary Table 10**.

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1079 **Spatial analysis of murine tissues**

1080 Female wild-type C57BL/6J CD45.1 mice and C57BL/6-Tg(Tcr α Tcr β)1100Mjb/J (OT-I) CD45.2
 1081 mice (Jackson Laboratory) were housed in a 12-h light-12-h dark cycle with *ad libitum* access to
 1082 food and water at Dana-Farber Cancer Institute (DFCI), under DFCI Institutional Animal Care and
 1083 Use Committee (IACUC) protocol 17-014. For analysis of syngeneic tumors, MC38 and MC38-
 1084 SIINFEKL cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS). For
 1085 culture of MC38-SIINFEKL cells, medium was supplemented with 10 μ g/mL blasticidin. Cell
 1086 lines were confirmed to be negative for mycoplasma contamination prior to use *in vivo*. Mice were
 1087 injected subcutaneously on the right flank with 2.5×10^5 MC38 cells in 100 μ L sterile PBS. For
 1088 MC38/MC38-SIINFEKL mixed tumors, mice were injected with 100 μ L of a mixture of 75%
 1089 MC38-SIINFEKL cells and 25% MC38 cells, with a total cell number of 1×10^6 cells. Tumors were

measured three times weekly and did not exceed 2 cm in diameter. At 8-12 weeks of age the mice were euthanized. Mouse tumors were harvested, embedded in OCT compound, and flash-frozen for one minute in liquid nitrogen chilled isopentane and moved to -80°C for long term storage. For treatment with immune checkpoint therapy, mice were first implanted with MC38 tumors. On days 7 and 11 following tumor implant, mice were treated with 150µg anti-PD-1 antibody (BioLegend, clone 29F.1A12, 135248) or anti-CTLA-4 antibody (InvivoGen, mctla4-mab10-1) by intraperitoneal injection in 100 µL sterile PBS. Mice were euthanized and tumors were collected for analysis 12 days after tumor implant, and tissues were frozen for spatial profiling. For adoptive transfer experiments, mice were implanted with mixed MC38/MC38-SIINFEKL cells. Two days following implantation, CD8⁺ T cells were isolated from the spleens of naive OT-I mice using bead-based negative selection, and 20,000 T cells were injected into tumor-bearing recipient mice via intravenous injection in a volume of 100 µL sterile PBS. Mice were euthanized and tumors were collected 9 days following tumor implant; tissues were frozen for spatial profiling.

***Rpl18* p.Q125R TCRs**

Mice were first implanted with MC38 tumors, on days 4, 7, and 10 following tumor implant mice were treated with *Rpl18* vaccination consisting of 50 µg *Rpl18* peptide (Genscript) and 50 µg Hiltonol (Oncovir) in 200 µL sterile PBS given subcutaneously in the left flank. On day 13 after tumor implantation, mice were euthanized and spleens were isolated, mechanically dissociated, and filtered through a 70 µM filter. Red blood cells were lysed using ACK lysing buffer (Gibco, A10492-01), and *Rpl18* p.Q125R specific T cells were isolated by FACS-sorting of CD3⁺CD8⁺CD44⁺*Rpl18* tetramer⁺ staining. The *Rpl18* p.Q125R tetramer was provided by the NIH

1112 Tetramare Core Facility. TCR sequences were subsequently identified using bulk TCR
1113 sequencing.

1114

1115 **Murine Rpl18 overexpression model**

1116 MC38-Kerafast (MC38-K) cells, do not harbor the *Rpl18* p.Q125R mutation, were engineered to
1117 express *Rpl18* WT or the *Rpl18* p.Q125R mutant using pLenti-CMV-Blast-DEST (Addgene
1118 #17451) or pLenti-PGK-Blast-DEST (Addgene #19065). C57BL/6 mice were subcutaneously
1119 implanted with 1×10^6 tumor cells, and tumors were harvested on day 12. Tumors were
1120 mechanically dissociated and enzymatically digested in RPMI containing collagenase (C5138,
1121 Sigma-Aldrich, 0.5mg/mL) and DNase I (D5025, Sigma-Aldrich; 2 µg/ml) at 37 °C for 45 min to
1122 generate single-cell suspensions, followed by filtration through a 70 µm strainer. Cells were
1123 stained with Zombie Aqua fixable viability dye, followed by *Rpl18* tetramer staining (1:500, 30
1124 min on ice) and surface antibody staining, including TIM-3 (1:100, BV785, clone RMT3-23,
1125 BioLegend) and LAG-3 (1:100, BV650, clone C9B7W, BioLegend). Samples were acquired on a
1126 flow cytometer and analyzed using FlowJo software.

1127

1128 **Histologic processing of murine and human tissues**

1129 Serial 10 µm glass-mounted frozen tissue sections were equilibrated to room temperature. For
1130 hematoxylin & eosin (H&E) staining, sections were fixed in formalin, processed through a graded
1131 ethanol series, stained with H&E, dehydrated, and coverslipped. Bright-field images were taken
1132 using the Leica Aperio ScanScope AT Turbo under a 40x objective. For immunofluorescence
1133 staining, sections were fixed in 4% paraformaldehyde for 10 min at room temperature, followed
1134 by 3 washes in PBS. Samples were blocked with 5% normal goat serum, 0.2% Triton X-100, 0.1%

tween in PBS for 1 hour at room temperature. The slides were then placed in a humidity tray and stained with CD3 primary antibody (human samples: 1:100, DAKO, A0452, mouse samples: 1:100, Invitrogen, 17A2), CD20 (1:100, DAKO, clone L26, M0755), Alexa-Fluor 488 CD45.2 antibody (1:100, BioLegend, 109816), Alexa-Fluor 488 CD74 antibody (1:100, BioLegend, 151005), S100A4 (1:100, abcam, EPR14639(2)), SIINFEBL-H-2K^b (1:100, invitrogen, 25-D1.16), in PBS containing 1% normal goat serum overnight at 4 °C. Samples were washed in Phosphate-buffered saline containing Tween-20 (PBST) for 5 min 3 times and a secondary antibody was added for 1 hour at room temperature (1:200, Jackson ImmunoResearch, 711165152, and 715-175-150). After incubation, the samples were washed in PBST for 5 min 3 times, stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature, coverslipped with ProLong Diamond Antifade Mountant (Life Technologies, P36965). Images were taken using the Leica Thunder imager, under a x10 objective. For quantification analysis, images were taken using LSM980 microscope and ZEN Desk 3.4 software (Carl Zeiss GmbH).

Image segmentation and quantifications

Mouse sections were stained with S100A4, CD74 and CD3 antibodies and quantified with Cellpose⁷⁵ and Cellprofiler. A total of 36 images were analyzed per sample type. The masks were generated using Cellpose pre-trained model “cyto2” and a cell diameter of 120 pixels. CD3-positive cell masks were then examined post-segmentation by overlaying with the original image to achieve accurate segmentation of this cell population. A CD3-positive cell was considered proximate to an S100A4 and CD74 positive cell if the two cells were in direct contact. Each image contained approximately 30 segmented S100A4 & CD74 cells, with more than 99% of the cells included in the segmentation. The segmented masks and original images were then processed in

CellProfiler to study the cell distribution. For human samples, CD3 immunofluorescence staining was performed on tissue sections from the same sample, T cells and total nuclei were quantified as previously described using a cell diameter of 35 pixels. For larger tumors, regions of interest (ROIs) were selected to match the approximate area where Slide-GoTags was performed (pipelines provided in the Supplementary File).

Slide-tags 5' snRNA-seq library preparation

Slide-tags was performed as previously described, with the following modifications for compatibility with 5' snRNA-seq: capture sequence on spatial bead barcode oligonucleotides was replaced with the reverse complement of the 10x Genomics template switch oligonucleotide sequence (5'-CCCATATAAGAAA-3'). Briefly, fresh frozen tissues were cryo-sectioned to 20 µm on a Cryostat (CM1950, Leica) and placed onto a 5.5 mm square bead array. The spatial tagging procedure and nuclei preparation was performed as previously described, with 38.7 µl of counted nuclei was loaded into the 10x Genomics Chromium controller using the Chromium Next GEM Single Cell 5' Kit v2 (10x Genomics, PN-1000263) with Feature Barcode technology for Cell Surface Protein (10x Genomics, PN-1000541). A gene-specific primer spike-in was added during the RT step 1.1 (Guide RT primer at 0.12 µM final concentration, **Supplementary Table 1**) to increase capture and detection of the transcript of interest. At cDNA amplification step 2.2, another spike-in primer (additive primer) was added to increase the yield of the same transcript (**Supplementary Table 1**). For the spatial library, the Chromium Next GEM Single Cell 5' v2 Cell Surface Protein User Guide CG000330 Rev F was followed with small modifications. Spatial barcode indexing was performed similarly to step 6.1, but the Dual Index Plate TT Set A was used in place of the Dual Index Plate TN Set A. We performed 13 cycles of PCR according to step 6.1

conditions. A double-sided SPRI beads cleanup (0.6x then 1.2x) was conducted for post sample index PCR size selection. The snRNA-seq library and TCRseq library were generated based on the 10x user guide CG000330 Rev F. All the sequencing libraries were quantified using the High Sensitivity DNA Kit (Agilent, 5067-4626) with the Agilent 2100 Bioanalyzer, and normalized to 4nM before sequencing.

Slide-tags sequencing

We sequenced snRNA-seq and spatial barcode libraries on the Illumina NextSeq 1000 instrument using a P2 XLEAP-SBS 100 cycle kit (Illumina, 20100987) or using an Illumina NovaSeq 6000 system using the NovaSeq S4 flow cell with 28nt read 1, 90nt read 2, 10nt index 1, and 10nt index 2. Gene expression and spatial libraries were allocated 20,000 reads per cell and were sequenced to achieve a saturation approaching 80%.

Slide-tags data preprocessing

We used Cell Ranger⁷⁶ (v.7.1.0) mkfastq (10x Genomics) to generate demultiplexed FASTQ files from the raw sequencing reads. We aligned these reads to human GRCh38 or mouse mm10, for human and mouse experiments, respectively, and quantified gene counts as UMIs using Cell Ranger count (10x Genomics). For experiments involving MC38-SIINFEKL, we added the sequence for SIINFEKL-woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to the reference transcriptome. We used CellBender v.0.3.0 for background noise correction and cell calling⁷⁷, adjusting the learning rate when required from the default value of 1×10^{-4} after manual inspection of the learning curve. Spatial data processing was performed as previously described. The Seurat package (5.1.0) was used to perform unbiased clustering, for

individual datasets⁷⁸. The data were log-normalized using a scale factor of 10,000. Before clustering, datasets from the same sample were integrated using Harmony. Following integration, principal component analysis was performed using variable genes using recommended settings (*i.e.* top 2000 variable genes using variance stabilizing transformation)⁷⁸. Clusters were manually assigned based on differentially expressed genes using the FindAllMarkers function using default settings (using all genes that are detected in a minimum of 25% of cells in either of the two comparison sets as input, and log-transformed fold change of 0.25 as the threshold). Clusters representing low-quality cells, identified by criteria such as high mitochondrial gene expression, low RNA content, or high levels of ambient RNA contamination, were subsequently removed from further analysis. CAF heterogeneity was characterized by calculating module scores for three established subtypes, inflammatory CAFs (iCAFs), antigen-presenting CAFs (apCAFs), and myofibroblastic CAFs (myCAFs), applying Seurat's AddModuleScore function to annotated fibroblasts using published genes sets (**Supplementary Table 7**). Each fibroblast was assigned to the CAF subtype with the highest module score. In a similar manner, module scores were calculated for non-CD4⁺ T cell populations, based on published gene sets. Each non CD4 T cell was assigned to the subtype corresponding to the highest module score. For visualization of spatial plots, dot sizes were scaled for display using an average cell diameter estimated from consecutive H&E-stained tissue sections.

Selection of variants for genotyping

All analyzed tumor samples had previously undergone RNA-seq and matched WES of tumor and germline DNA^{79,80}. Variant selection for genotyping during Slide-GoTags involved a series of

considerations including those related to technical efficiency of genotyping based on variant characteristics and neoantigen prediction. Variants were selected based on the following criteria:

1. Predicted neoantigen:

Following conversion into peptide sequences, the translated neoantigens (nonsynonymous mutations, frameshift and indels) were tiled across 8-11 amino-acid lengths for HLA class I binding prediction. Antigen prediction was performed using HLAthena (v.0) and NetMHCpan (v.4) (eluted ligand rank), as previously described⁸¹.

2. Mutations in predicted cancer driver genes^{82,83}.

3. High expression in tumor cells, as indicated by snRNA-seq data, or high expression of the gene of interest from bulk RNA-seq data.

4. Preference for variants occurring within 1000 kb of the 5' end of the canonical transcript.

Targeted genotyping and TCR amplification

Following variant selection, rhPCR primers were designed with a 6-nucleotide 3' tail starting with a ribonucleotide and ending with a mismatch base, followed by a 3' spacer (/3SpC3/) to block polymerase extension. Biotin was added to the 5' end (/5Biosg/) for streptavidin capture. Primers were 18-24 nucleotides in length, with 40-60% GC content and a predicted melting temperature (T_m) of 55 °C (excluding the 3' tail) and were designed to avoid homopolymer runs greater than 3 G's, 3C's, 4 A's or 4 T's. Nested primers were positioned closer to the 5' end of the transcript, following similar design principles with a predicted T_m of 57°C. In PCR reaction 1: 10 ng of cDNA library was amplified with 1 µl of 10 µM AA0272 and 1 µl 10 µM bio-AA0273 primers using 10 µl 2x KAPA HiFi Uracil+ Kit (Roche, 07959052001). The following program was used on the thermal cycler: 1 cycle of 98°C for 3 min, 6 cycles of 98°C for 20 s, 65°C for 30 s, and

1249 72°C for 8 min, and then hold at 4°C. Then 65 µL of ProNex beads (1.3x, Promega, NG2002) were
 1250 added for clean-up following the manufacturer's instructions and eluted into 20 µL of ProNex
 1251 Elution Buffer. Biotinylated fragments were captured using 5 µl 10 mg/ml Dynabeads (Invitrogen,
 1252 60101) according to manufacturer instructions and resuspended in 10 µl TE pH 8.0 (Invitrogen,
 1253 AM9849). PCR reaction 2: 1 µl bead-bound PCR 1 product was amplified using rhPCR with
 1254 1.25 µl 20 µM rhCGA-venus primer, 2.5 µl 20 µM patient-specific primer or primers specific to
 1255 the TCR α - and β -chain constant regions (**Supplementary Table 1**), 2.5 µl 20x rhPCR buffer
 1256 (300 mM Tris-HCl pH 8.4, 500 mM KCl, 80 mM MgCl₂), 0.8 µl 25 mM dNTPs (Fisher Scientific,
 1257 FERR1121), 1.25 µl 20mU/µl RNase H2 (Integrated DNA Technologies, 11-02-12-01), 2 µl 5U/µl
 1258 Hot Start OneTaq DNA Polymerase (New England Biolabs, M0481L). The following program
 1259 was used on the thermal cycler: 1 cycle of 95°C for 5 min, 16 cycles of 96°C for 20 s, 60°C for 6
 1260 min, and 72°C for 4 min, followed by hold at 4°C. Then 65 µL of ProNex beads (1.3x) were added
 1261 for clean-up following the manufacturer's instructions and eluted into 50 µL of ProNex Elution
 1262 Buffer. Biotinylated fragments were captured using 12.5 µl 10 mg/ml Dynabeads according to
 1263 manufacturer instructions and resuspended in 10 µl TE pH 8.0. PCR reaction 3: 2 µl bead-bound
 1264 PCR 2 product was amplified with 25 µl Q5 Hot Start High-Fidelity 2x Master Mix (New England
 1265 Biolabs, M0492S), 1 µl of 20 µM CGAvenus.PS primer, 1 µl of 20 µM of sample-specific nested
 1266 primer. The following program was used on the thermal cycler: 1 cycle of 98°C for 5 min, 18
 1267 cycles of 98°C for 20 s, 62°C for 30 s, and 72°C for 4 min, followed by hold at 4°C. Then 65 µL
 1268 of ProNex beads (1.3x) were added for clean-up following the manufacturer's instructions and
 1269 eluted into 12 µL of ProNex Elution Buffer. Quantification of PCR products was performed using
 1270 TapeStation (Agilent, 5067-5366).

1271

Oxford Technologies sequencing of TCRs and targeted genotyping

Sequencing adapters were ligated using the SQK-LSK114 kit and the NEBNext Companion Module (New England Biolabs, E7180S) according to manufacturer instructions with 100 fmol input per sample. Sequencing was performed using R10.4 flow cells on a GridION (Oxford Nanopore Technologies). Basecalling was performed using the MinKNOW 24.02.16 with dorado (7.3.11)⁸⁴, guppy version 6.1.2 with the module dna_r10.4_e8.1_sup. An overview of the alignment, variant calling and assignment of genotype status to nuclei is provided in **Extended Data Fig. 1a**.

Bulk TCR sequencing

Bulk TCR sequencing was performed using five 30 μ M sections of each frozen sample, RNA was extracted using the RNeasy Micro kit (Qiagen, 74004) as per the manufacturer's protocol. α and β TCR repertoire analysis in bulk RNA samples was performed using an adapted rhTCRseq protocol published previously⁸⁵. Specifically, 10 ng bulk RNA was used per RT reaction, and four replicates were processed for each sample. Excess RT primers were eliminated by exonuclease digestion, and then rhPCR was completed based on the published protocol⁸⁵. Sequencing was performed on a NextSeq 1000 instrument using a P2 XLEAP-SBS 300cycle kit (Illumina, 20100985) according to the manufacturer's protocol with the following parameters: 248nt read 1, 48nt read 2, 8nt index 1, and 8nt index 2.

Alignment, variant calling and assignment of genotypes to single nuclei

Following basecalling of long-read data with dorado (7.3.1)⁸⁴, raw reads in the format of fastq files are processed using the first stage of nanoranger (<https://github.com/mehdiborji/nanoranger>) as

described previously. For calling of single nucleotide variants (`extract_mutation()`, `extract_indel()` and `extract_length_diff()`), reads mapping to the locus were processed using pysam by extracting the base for each read using the nanoranger script `perform_pileup.py`⁸⁶. We introduce several advances to variant calling to increase the genotyping fidelity. Firstly, we only retain barcode-UMI-transcript pairs with at least two or more supporting reads and where the reference and expected mutation genotype represent >95% of all variant calls⁸⁷. Secondly, by employing a network-based UMI correction to account for PCR and sequencing errors, we quantified the number of unique molecules genotyped per cell barcode⁸⁸. Thirdly, to address potential barcode swapping, we retain only barcode-UMI-transcripts pairs in our genotyping library that match barcode-UMI-transcripts pairs in our gene expression library. To recover molecules in the genotyping library which do not match molecules in our gene expression library, where the barcode-UMI pair are not assigned a different gene in our gene expression library, we recover molecules where the number of reads is greater than the local minimal read count of the bimodal distribution⁸⁸. Additionally, we remove assigned genotypes from a cell barcode if Cellbender removes all molecules for the given gene from the gene expression library for the specific cell barcode^{77,86}. Finally, cells were assigned mutated or wildtype status on a per-sample and per-gene basis, based on the number of mutated molecules assigned to each barcode (**Supplementary Note 1**).

Processing of single nucleus TCR data

For VDJ reconstruction the query sequence (V gene transcripts used as reference in the input) and 100 nucleotides in the 3' softclip of the alignment, which would cover the CDR3 region and J gene, were extracted. The “read1” record was generated by extracting the barcode region for each of the

alignments. For VDJ reconstruction, the read2 fastq was processed using MiXCR (v. 3.0.13) and a reference library of TRA and TRB v-transcripts from vdj.online (accessed 17th March 2024)⁸⁹. Following VDJ reconstruction, a network-based UMI correction was employed to allow for quantification of the number of unique molecules genotyped per cell barcode⁸⁸. High confidence TCR-sequences were then defined based on a threshold per chain following generation of the barcode rank knee plot. Where multiple chains were assigned to the same barcode, the chain-specific CDR3 sequence with the highest number of unique molecules defined the final assignment. Following the identification of high-confidence chains, TCR- α and TCR- β sequences were integrated at the single-nucleus level by matching unique barcodes.

Processing of bulk TCR data

Reads were demultiplexed using minibar with an edit distance of 2, method “3” and a basepair start and end sequence search length of 200. Demultiplexed reads were then processed using MiXCR (v.4.7.0) with the “generic-ont-with-umi” preset and species set to mouse.

Comparison of SIINFEKL gene expression and genotyping library

The phi coefficient was used to compare the genotyping of SIINFEKL in the gene expression and genotyping library with the number of cells genotyped, represented as a 2x2 contingency table.

Analysis of spatial cell interactions between TCR clonotypes and genotyped tumor cells

To ensure correct assignment of TCR of mutated genotypes to cell barcodes, we assigned TCR-sequences to T cells (as defined by the gene expression) and somatic genotypes to the tumor cells. To assess the degree of colocalization of each clonotype to genotyped tumor cell, we utilize the

NMS and a permutation-based neighborhood test at the scale of the whole tumor. The mixing score (MS) was defined as the number of immune-tumor interactions divided by the number of immune-immune interactions⁹⁰. Given that the MS is highly dependent on the total number of cells in the reference and target populations, a scale factor was added to normalize the MS by the total possible interactions between cells, creating the NMS

$$\text{Normalised mixing score} = \frac{n_{\text{ref to target interactions}} \times (n_{\text{ref}} - 1)}{2 \times n_{\text{ref to ref interactions}} \times n_{\text{target}}}$$

Where n_{ref} is the total number of reference cells and n_{target} is the total number of target cells. To assess the statistical significance of the observed normalized mixing score for a given set of target cells, reference cells and radius, we performed a permutation-based analysis. Specifically, we generated a null distribution of normalized mixing scores by randomly permuting all cell type labels while preserving spatial coordinates. This approach maintains the overall number of reference and target cells but breaks the spatial association between them, simulating the null hypothesis of no spatial relationship. For each permutation, the normalized mixing score was recalculated at the same radius using the permuted cell type labels. The P value was then calculated as the proportion of 1000 permuted normalized mixing scores that were greater than or equal to the observed normalized mixing score.

CNV analysis of snRNA-seq

SCEVAN⁹¹ was used to infer large-scale CNVs from gene expression count matrices with the subclones option set to TRUE and no normal provided. Final tumor cell annotations in human

samples were based on consensus calls of Leiden clustering of gene expression and SCEVAN CNV calls.

Distance analysis

To assess the functional state of T cell subpopulations in the TME, we calculated module scores for predefined gene sets and examined their relationship with spatial proximity to mutant cells. Module gene lists were extracted from a predefined reference (**Supplementary Table 7**), and only genes present in the dataset were included in the scoring process. The module score was calculated as the average expression of the set of genes of interest after subtraction by the average expression of a reference set of genes, randomly sampled for each binned expression value. The Euclidean distance between each T cell subpopulation and the nearest mutant cell was computed. To evaluate the association between proximity to -mutant cells and functional module scores, we performed linear regression for each module score against the nearest distance. Significant associations (P value < 0.05) were visualized using scatter plots with regression lines.

Spatial autocorrelation analysis using Moran's I

To assess the spatial autocorrelation of specific features within a sample, we calculated Moran's I , for selected categorical variables. For each sample, we constructed a binary indicator variable for each category of interest, assigning a value of 1 to cells expressing the feature and 0 to all others. To define spatial relationships between cells, we computed a distance-based weight matrix using a k-dimensional tree, selecting the 25 nearest neighbors for each cell. Moran's I was then computed for each category within each sample. The significance of spatial clustering was

Differential gene expression analyses were performed using FindMarkers from Seurat⁹² (v5.1.0) using parameters `min.pct = 0.1` and `log2fc.threshold = 0.25`. Gene Set Enrichment Analysis was performed using the `clusterProfiler` (v4.14.4) package in R with gene symbols converted to ENTREZ IDs. Gene Ontology enrichment analysis was conducted using the `gseGO` function with `org.Hs.eg.db` (v3.20.0) as the reference database, including all GO categories (`ont = "ALL"`).

Spatial receptor ligand prediction

To predict receptor-ligand interactions between cell type pairs, we computed a receptor-ligand score based on a spatial correlation index (SCI) as previously described. Briefly, the SCI, which is defined as:

$$SCI = \frac{\sum_i^N \sum_j^N w_{ij} r_i l_j}{\sum_i^N \sum_j^N w_{ij}}$$

The SCI measures spatial co-expression between N receiver cells expressing receptor r and M sender cells expressing ligand l , using `sctransform`-normalized counts. We defined the spatial weights matrix of dimensionality $N \times M$ as an adjacency matrix, denoting 1 for when sender cell i is between 50-200 $\mu\text{m}^{93,94}$ of receiver cell j and 0 otherwise. For each receptor-ligand pair, we calculated the log fold-change of the observed SCI relative to a null distribution and assessed statistical significance using the Benjamini-Hochberg procedure. This allowed comparison of interaction strengths across cell types without adjusting for differences in cell number.

Spatial contextualization of receptor-ligand interactions

To spatially resolve receptor-ligand interactions, we decomposed spatial correlation indices into cell-level Local Interaction Spatial Association (LISA) scores, as previously described. These scores quantify each cell's contribution to the overall spatial correlation for significant receptor-ligand pairs ($P_{adj} < 0.05$), and are defined as:

$$LISA = \frac{r_i \sum_{j=1}^M w_{ij} l_j}{\sum_{j=1}^M w_{ij}}$$

where r_i is receptor expression in receiver cell i , l_j is ligand expression in neighboring sender cell j , and w_{ij} represents the spatial adjacency matrix. LISA scores and SCTransformed receptor expression values were compared across spatial regions using the Wilcoxon rank-sum test, with P values adjusted by the Benjamini-Hochberg method.

To detect receptor-ligand interactions enriched in immunogenic niches across patient samples, we employed a linear mixed-effects model (LMER) to analyze LISA scores:

$$\log_2(\text{LISA_score}) \sim \text{region} + (1 \mid \text{sample_id})$$

where region (immunogenicity niche versus non-immunogenicity niche) was modeled as a fixed effect, and sample as a random effect, accounting for sample-specific variability. Statistical significance was assessed by applying the Benjamini-Hochberg correction to P-values from the LMER model.

TCR reactivity testing

1446 *NSUN5* p.Q18K specific TCR reactivity was assessed by CD137 upregulation following co-culture
 1447 with peptide-pulsed autologous Epstein-Barr virus transformed lymphoblastoid cell line (EBV-
 1448 LCL), with background subtraction using DMSO-pulsed controls. Mutant and wild-type peptides
 1449 were tested across a dose range (10^8 - 10^9 pg/mL); minimal epitopes (9-11 amino acids) were
 1450 predicted using HLAthena. Dose-response curves were normalized and EC_{50} values were
 1451 calculated.

1452 **Identification and quantification of immunogenicity niches**

1453 *Categorization of immunogenicity niches*

1454 We define immunogenicity niches, or regions of likely anti-tumor interactions between tumor
 1455 infiltrating lymphocytes and tumor cells, by evaluating the overlap of relative areas of T cell
 1456 aggregation with tumor cells aggregates of high expression of interferon-stimulated genes (ISGs),
 1457 such as HLA.

1458 To quantify spatial overlap of T cells and MP17-expressing tumor cells, we used KDE and overlap
 1459 metrics. First, MP17 expression scores were derived using a predefined signature of 50 genes using
 1460 the *score_genes* function in the scanpy package. KDE was calculated separately for T cells and
 1461 MP17-expressing tumor cells using *scipy.stats.gaussian_kde* with default bandwidth (Scott's rule)
 1462 on a spatial grid of 300×300 points.

1463 Tumor cell density was weighted by MP17 expression, with the MP17 scores first normalized
 1464 using min-max normalization within each sample. T cell density was scaled by the relative
 1465 frequency of T cells in each sample. Both density estimates were then independently normalized
 1466 to a [0,1] range within each sample:

$$\rho_{\text{norm}} = \frac{\rho - \rho_{\min}}{\rho_{\max} - \rho_{\min}}$$

where ρ is the original density estimate and $\rho_{\max}$ and $\rho_{\min}$ are the minimum and maximum density values within each individual sample.

Local thresholds were defined as the 95th percentile of each sample-specific normalized KDE. Areas of high T cell and MP17-expressing tumor cell density were defined as regions where density exceeded these local thresholds. Areas where both the T cell density and MP17-expressing tumor cell density simultaneously exceeded their respective local thresholds were defined as "immunogenicity niches".

Comparison of relative abundance of KDE overlap

Using the normalized density estimates, we calculated a metric to quantify the spatial overlap between T cells and MP17-expressing tumor cells across the entire sample area. Volume of Intersection (VI) was calculated for each sample as follows:

$$VI = \frac{1}{N} \sum_i \min(\rho_i^T, \rho_i^M)$$

where N is the number of pixels, and ρ_i^T and ρ_i^M are the density values for T cell and MP17-expressing tumor cells at pixel i, respectively.

Correlation Heatmap and Hierarchical Clustering

Using the VI metric from the spatial analysis, we generated correlation heatmaps by first converting the similarity of samples to a distance matrix. The similarity between samples i and j was calculated as:

$$\text{similarity}(i, j) = 1 - \frac{|value_i - value_j|}{\max(value_i, value_j)}$$

where $value_i = M_i$ and $value_j = M_j$, with M representing the VI metric for samples i and j . The similarity matrix was then converted to a distance matrix using:

$$\text{distance}(i, j) = 1 - \text{similarity}(i, j)$$

To prepare the data for hierarchical clustering, the square distance matrix was converted to a condensed vector form using `scipy's squareform` function. Hierarchical clustering was performed using Ward's method, which minimizes the variance within clusters by merging clusters that result in the smallest increase in within-cluster variance.

To account for technical replicates and spatial heterogeneity samples from the same tumor were combined. Specifically, we merged samples from the same tumor, and for each tumor, VI metrics were averaged across the component samples. The similarity matrix, hierarchical clustering, and visualization were then performed as described above, but using these tumor-level combined metrics instead of individual sample metrics.

For visualization, a custom magma-based colormap was used for the clustered correlation heatmap. Sample annotations for tumor types (GB, Melanoma, Ovarian, RCC) were indicated with color sidebars. Visualization was performed using Matplotlib (3.9.3) and seaborn (0.13.2). Statistical analysis was performed using SciPy (1.14.1), Scikit-learn (1.6.0), NumPy (2.0.2) and Pandas (2.2.3).

Immunogenicity niche TCR enrichment analysis

To assess enrichment of neoantigen-colocalized clonotypes within immunogenicity niches, we compared for each sample and for TRA and TRB chains separately, the relative frequency of a given clonotype in an immunogenicity niche to its frequency in non-niche regions. Comparisons for a given sample were carried out only when both compartments contained at least one T cell bearing the relevant TRA or TRB sequence. A pseudocount of 0.05 was added to clonotype counts and denominators for fold-change calculations, and significance was evaluated with two-sided Fisher exact tests on the raw counts. Only clonotypes with > 1 cell within a sample were displayed in **Fig. 4g**. Virus-specific TCRs were annotated using publicly available databases (TCRdb, VDJdb). Neoantigen-specific TCRs were defined based on experimental antigen specificity testing (**Supplementary Table 12**) and significant co-localization of neoantigen-genotyped tumor cells and TCR-expressing T cells (**Supplementary Table 29**).

MC38-SIINFEKL bilateral tumor model with IFN- γ treatment

Bilateral subcutaneous tumors were established in C57BL/6J CD45.1⁺ mice by injection of 100 μ L of a mixture containing 75% MC38-SIINFEKL cells and 25% MC38 cells on each flank. Two days following implantation, naive CD8⁺ T cells were isolated from the spleens of untreated OT-I mice using bead-based negative selection (Stemcell 19858), and 20,000 T cells were adoptively transferred intravenously into tumor-bearing recipient mice in 100 μ L sterile PBS. On days 9,11,13 and 15 tumors on one flank were treated intratumorally with recombinant murine IFN- γ (5 μ g per injection; PeproTech, cat. no. 315-05-50 μ g), while the contralateral tumor received PBS as a control. Tumors were harvested on day 16 for downstream analyses.

TLS-MP17 tumor region identification

To evaluate spatial overlap between TLS-like regions and MP17-expressing tumor cells, we performed KDE analysis for B cells, T cells and tumor cells in each sample. Tumor cell density was weighted by MP17 score and B cell density by B cell/TLS score, with both scores generated using `scanpy.tl.score_genes` and min-max normalized within each sample. High-density regions were defined using the 95th percentile of the normalized KDE in each sample. TLS-MP17 overlap was defined as regions where high-density B cell, T cell and MP17-weighted tumor cell signals coincided.

Strong-weak mutant binder tumor density

To define spatial regions enriched for strong- and weak- mutant binder tumor cells, tumor cells were classified within each sample according to mutations predicted by NetMHCpan to generate strong or weak HLA-I binders. KDE analysis was performed for strong-binder and weak-binder mutant tumor cells using `scipy.stats.gaussian_kde`. High-density regions for each group were defined by the 95th percentile of the sample-specific KDE. Strong-only and weak-only regions were defined as non-overlapping high-density regions for each group. Samples with fewer than five strong-binder or five weak-binder mutant tumor cells were excluded.

Data Availability

Slide-GoTags datasets have been deposited at the Broad Institute Single Cell Portal under the following accession numbers: [SCP3655](#) (MC38-SIINFEKL), [SCP3657](#)
[SCP3660](#)
[SCP3667](#) (Human tumors)⁹⁵.

1551 **Code Availability**

1552 Code for processing spatial sequencing libraries is available at GitHub

1553 (<https://github.com/amitsud/Slide-GoTags>)⁹⁶.

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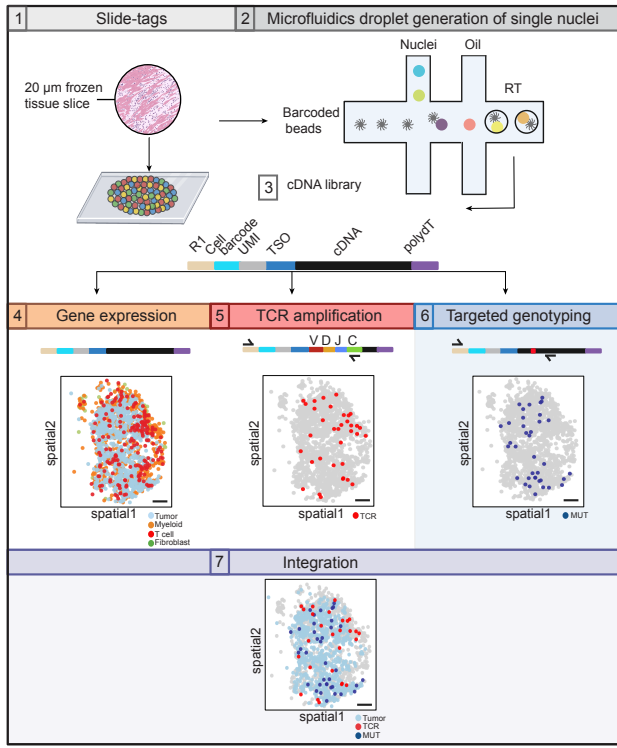
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1556 **Method Reference**

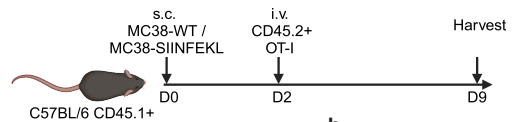
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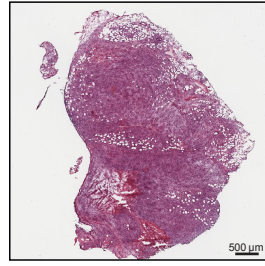
Fig. 1
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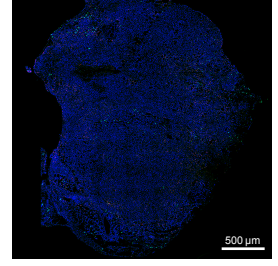
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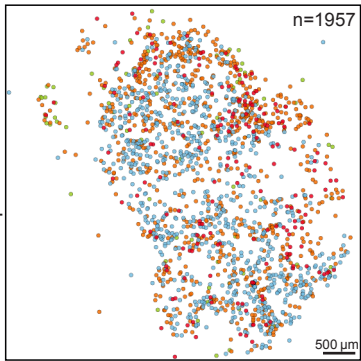
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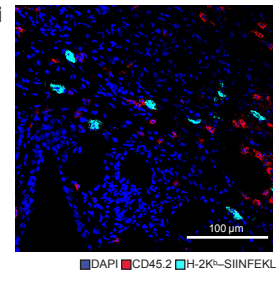
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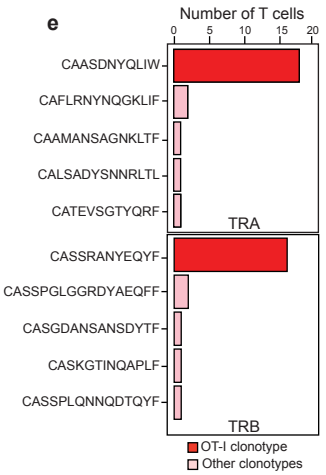
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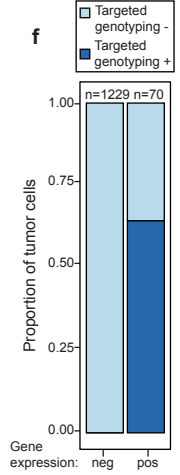
i



e



f



g

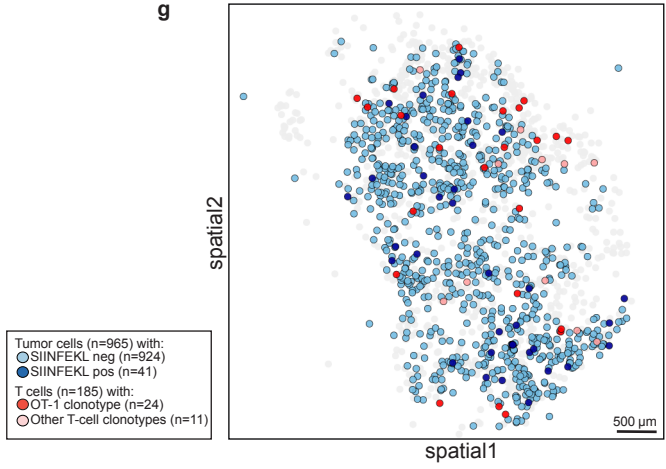


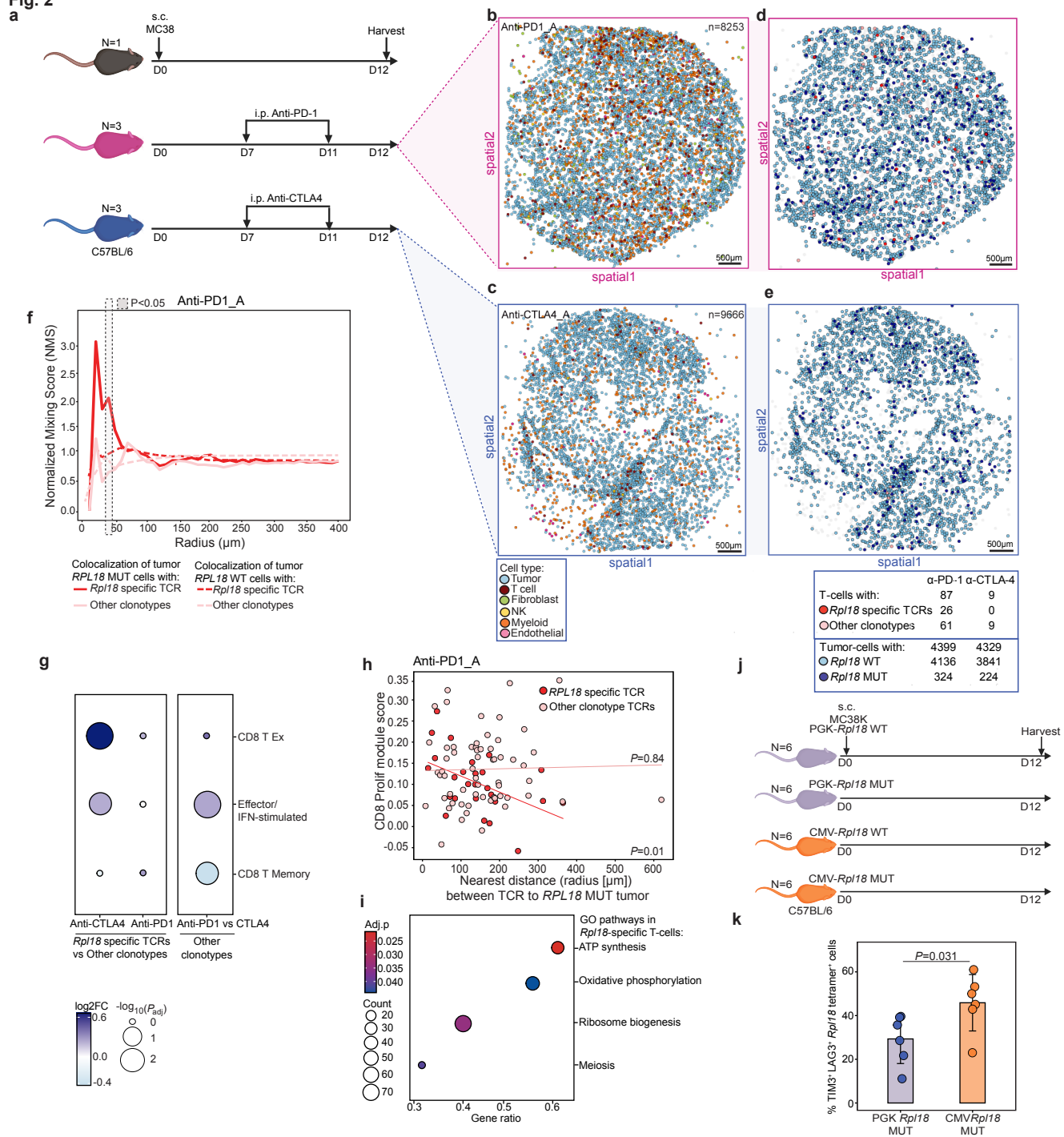
Fig. 2

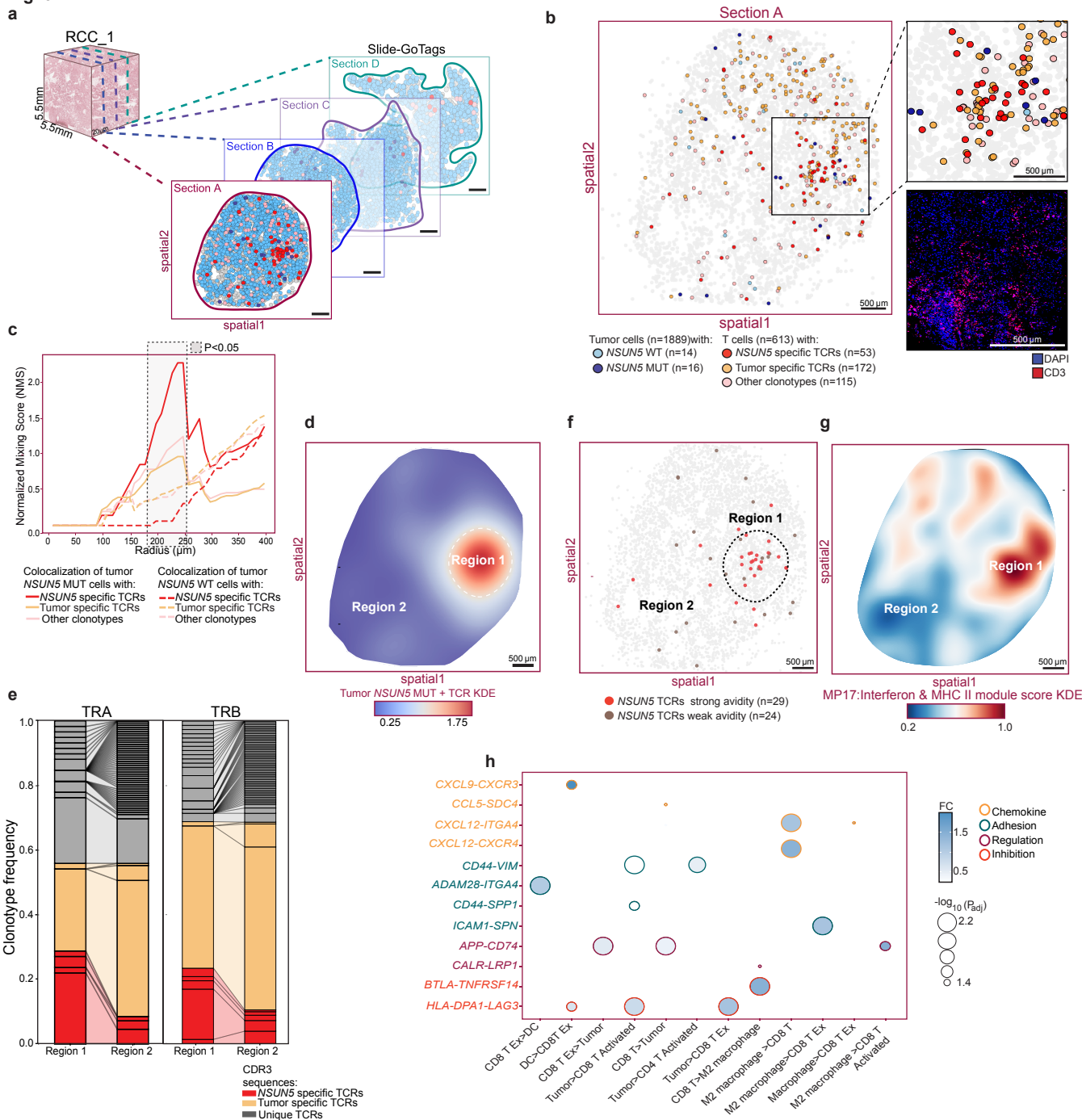
Fig. 3

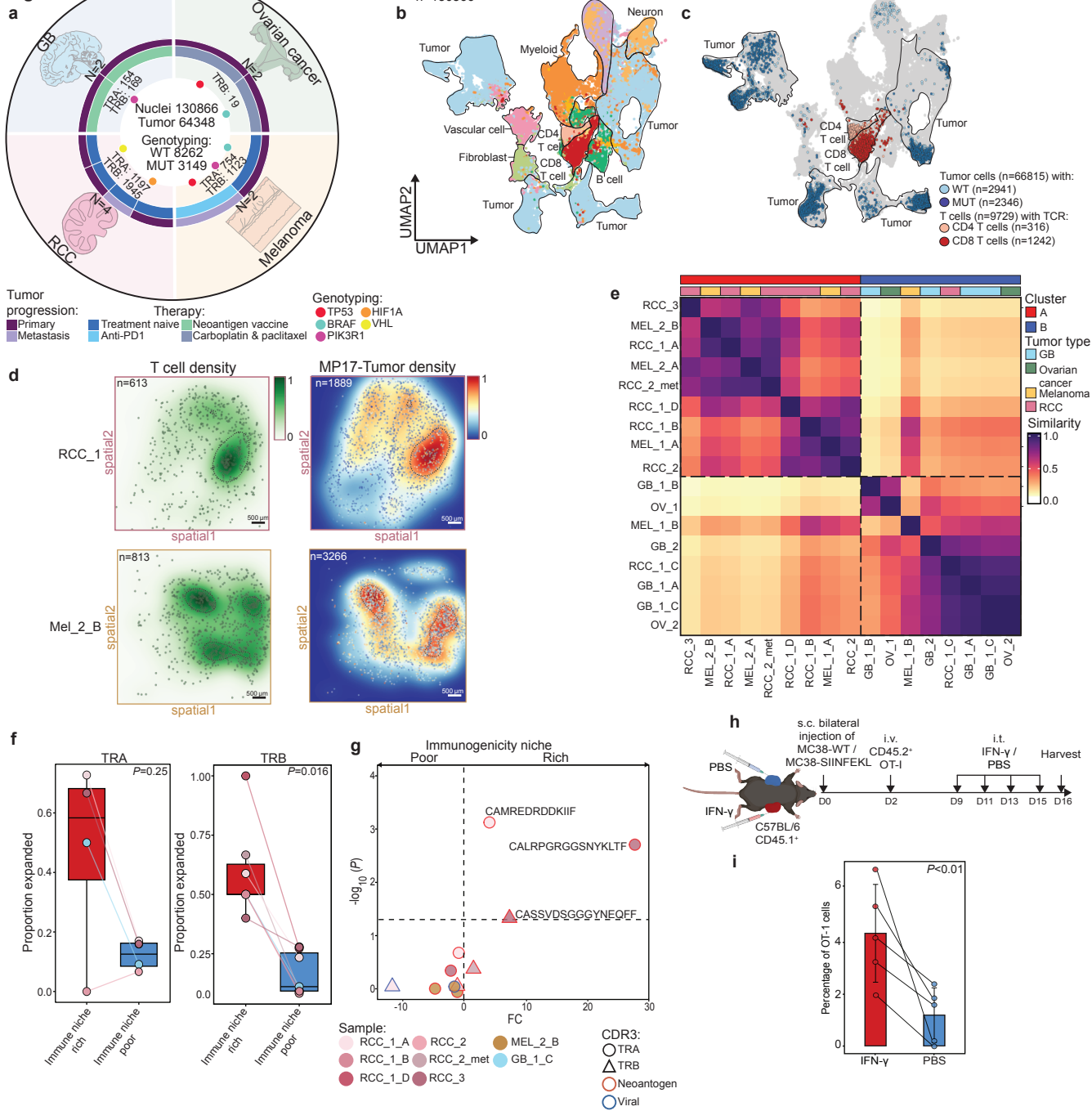
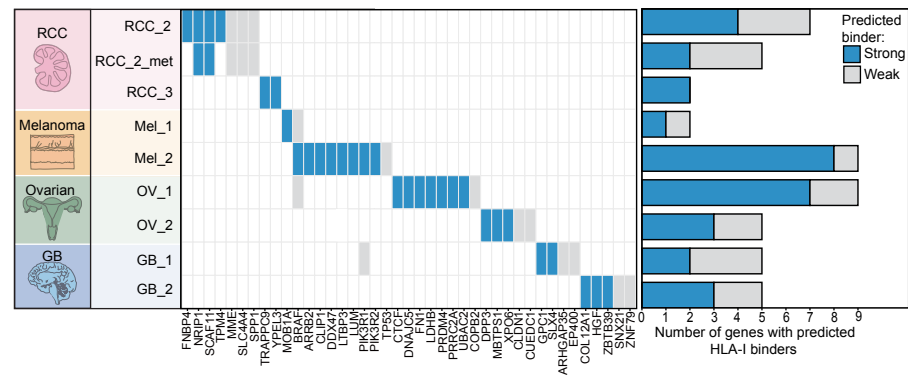
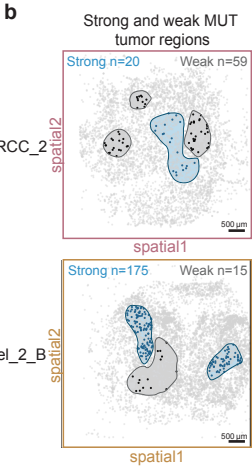
Fig. 4

Fig. 5

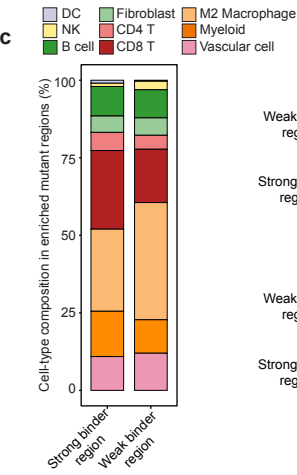
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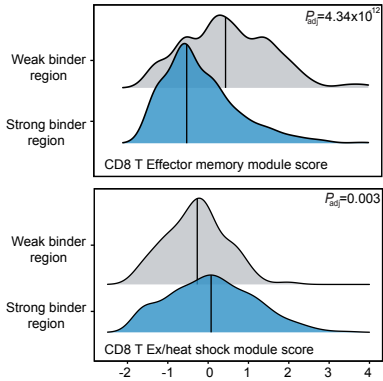
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