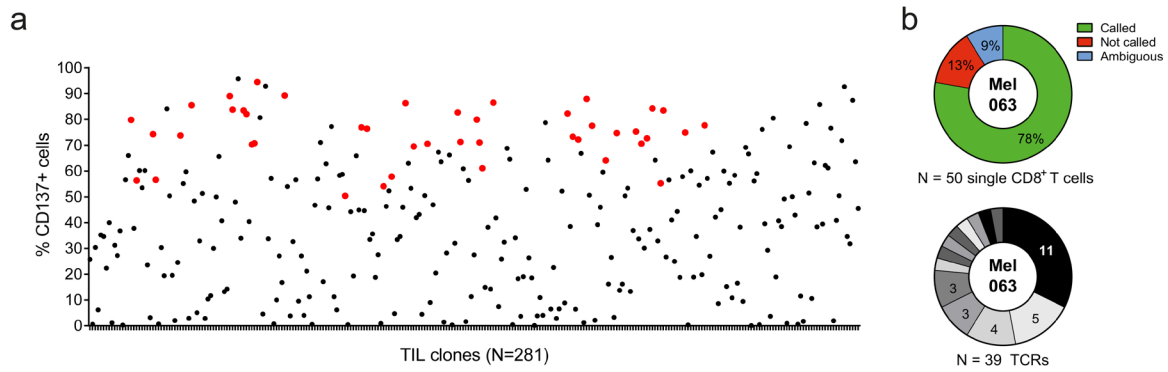


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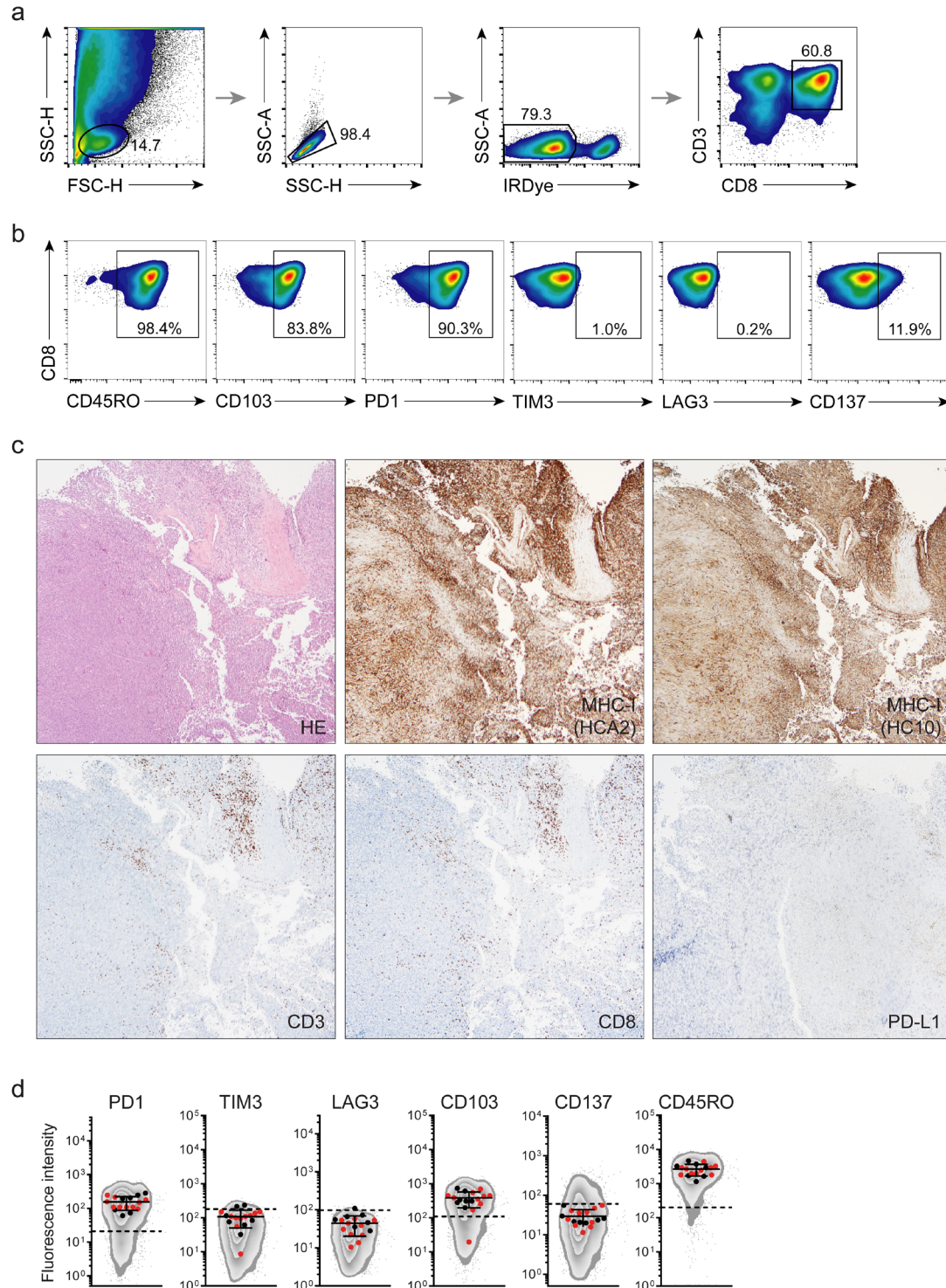
Low and variable tumor reactivity of the intratumoral TCR repertoire in human cancers

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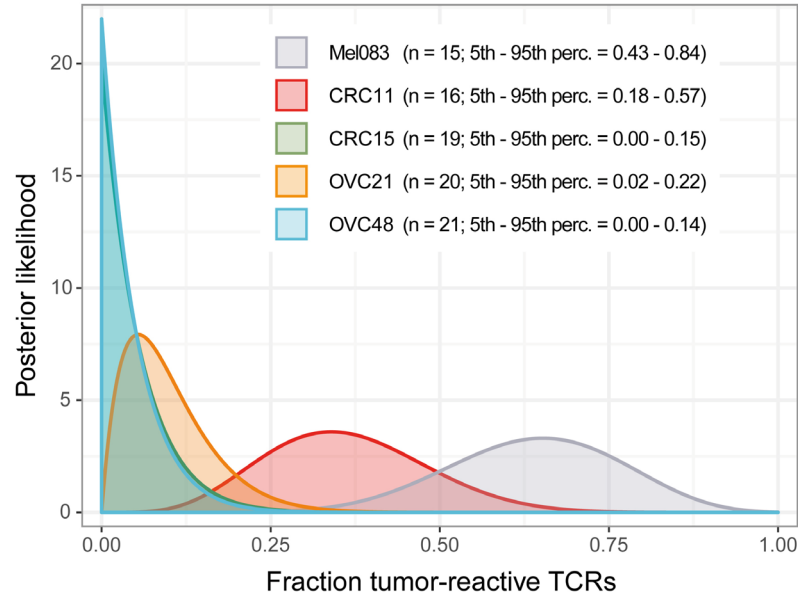


Supplementary Figure 1. Tumor-reactivity of CD8⁺ T cell clones derived from melanoma tumor Mel063. (A) *In vitro*-expanded CD8⁺ TIL clones from melanoma patient Mel063 were incubated with autologous tumor cells and tumor-reactivity was assessed by measuring CD137 expression using flow cytometry. Red dots indicate clones used for TCR sequencing. (B) TCR recovery rates (top) and clonality distribution (bottom) of identified TCRs. For recurring clonotypes, numbers indicate the occurrence of TCRs within the sampled repertoire.

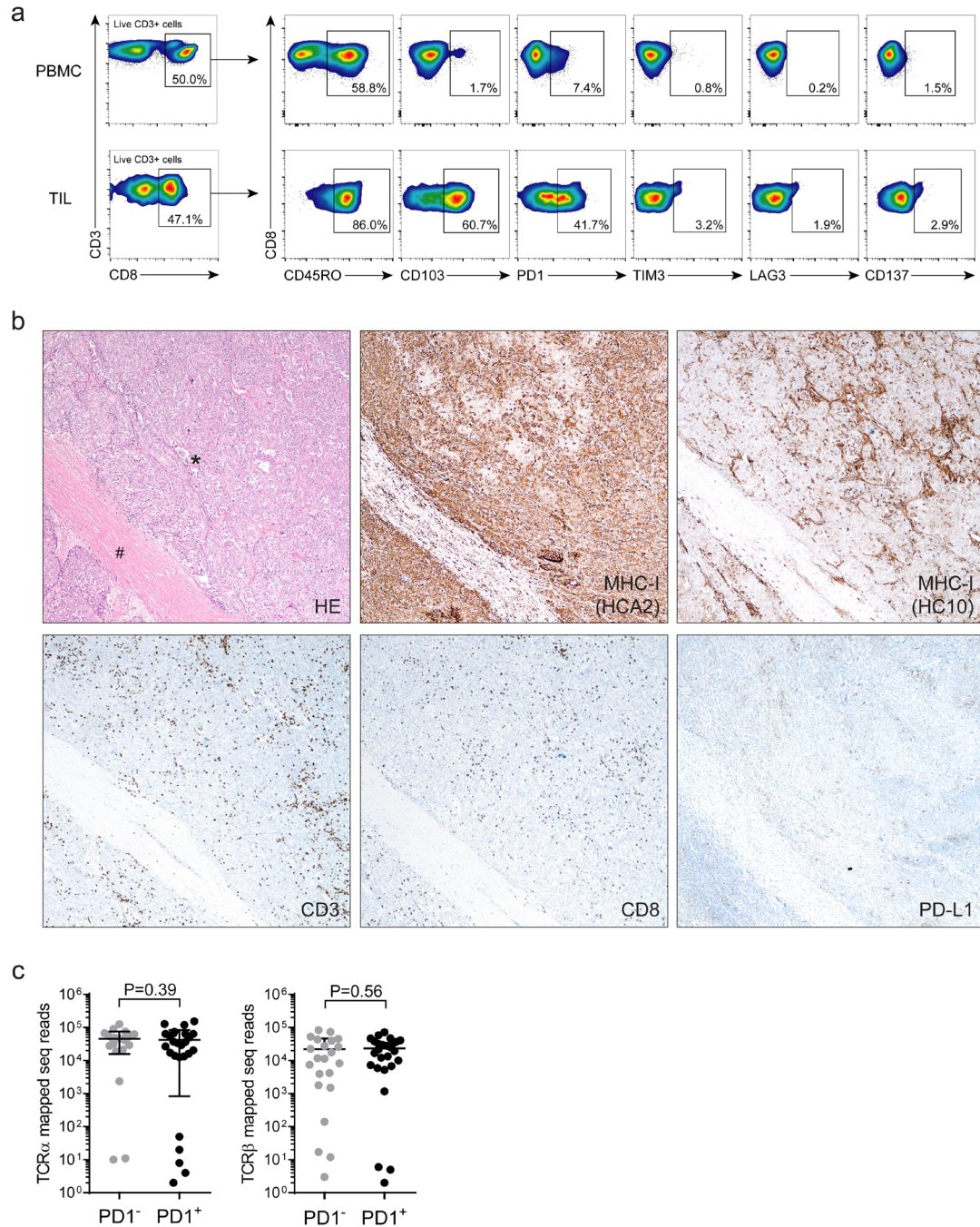


Supplementary Figure 2. CD8⁺ TIL phenotype and immunohistochemistry of tumor Mel083. (A) Gating strategy used for single cell sorting of intratumoral CD8⁺ T cells. (B) Tumor digest of patient Mel083 was stained with antibodies against indicated molecules and analyzed by flow cytometry. Data depict live CD3⁺CD8⁺ cells. (C) Excised tumor tissue was fixed and paraffin-embedded, and sequential slides were stained with antibodies against CD3, CD8, MHC class I, or PD-L1. (D) *In situ* phenotype of parental CD8⁺ TIL of which TCRs tested in Fig. 1e

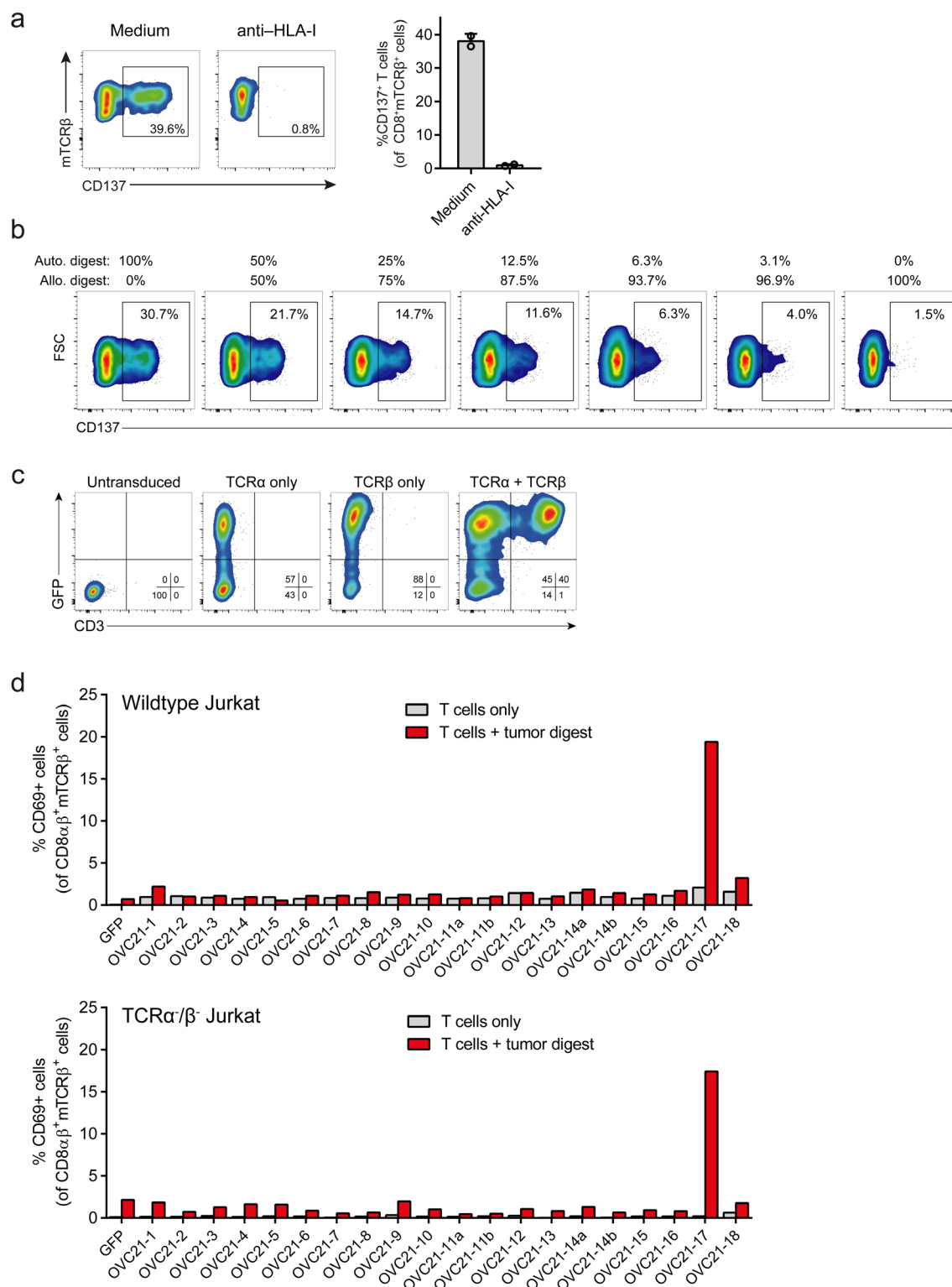
were isolated (n=19 independent cells). Grey clouds represent expression of indicated markers of all live CD3⁺CD8⁺ cells measured in the tumor sample. Dots depict data of individual TIL of which TCRs were functionally tested, with red dots corresponding to parental T cells from which tumor-reactive TCRs were isolated. Dashed lines indicate gates used in flow cytometric analysis. Means $\pm$ S.D. are indicated.



Supplementary Figure 3. Probability distributions of the fractions of tumor-reactive TCRs in patient samples. To quantify the generalizability of the observed fractions of tumor-reactive TCRs (0.60, 0.05, 0, 0.31 and 0 for patients Mel083, OVC21, OVC48, CRC11 and CRC15, respectively; see Figures 1-3) to the untested intratumoral T cell repertoire, the likelihood of TCR reactivity, given the observed data, was modelled for each patient individually using a Bayesian approach (see Methods). In this, we accounted for the 92% sensitivity of our platform (see Fig. 1). For patient Mel083, the fraction of tumor-reactive TCRs within the overall pool of CD8⁺ located with high probability to the range of 0.5-0.8, with the 5th and 95th percentiles of the likelihood distribution located at 0.43 and 0.84. For OVC and CRC patients, the fraction of reactive TCRs located with high probabilities in the range 0-0.2 for 3/4 patients, with the 95th percentiles of the distributions located at 0.22, 0.14, 0.57 and 0.15 for patients OVC21, OVC48, CRC11 and CRC15, respectively.

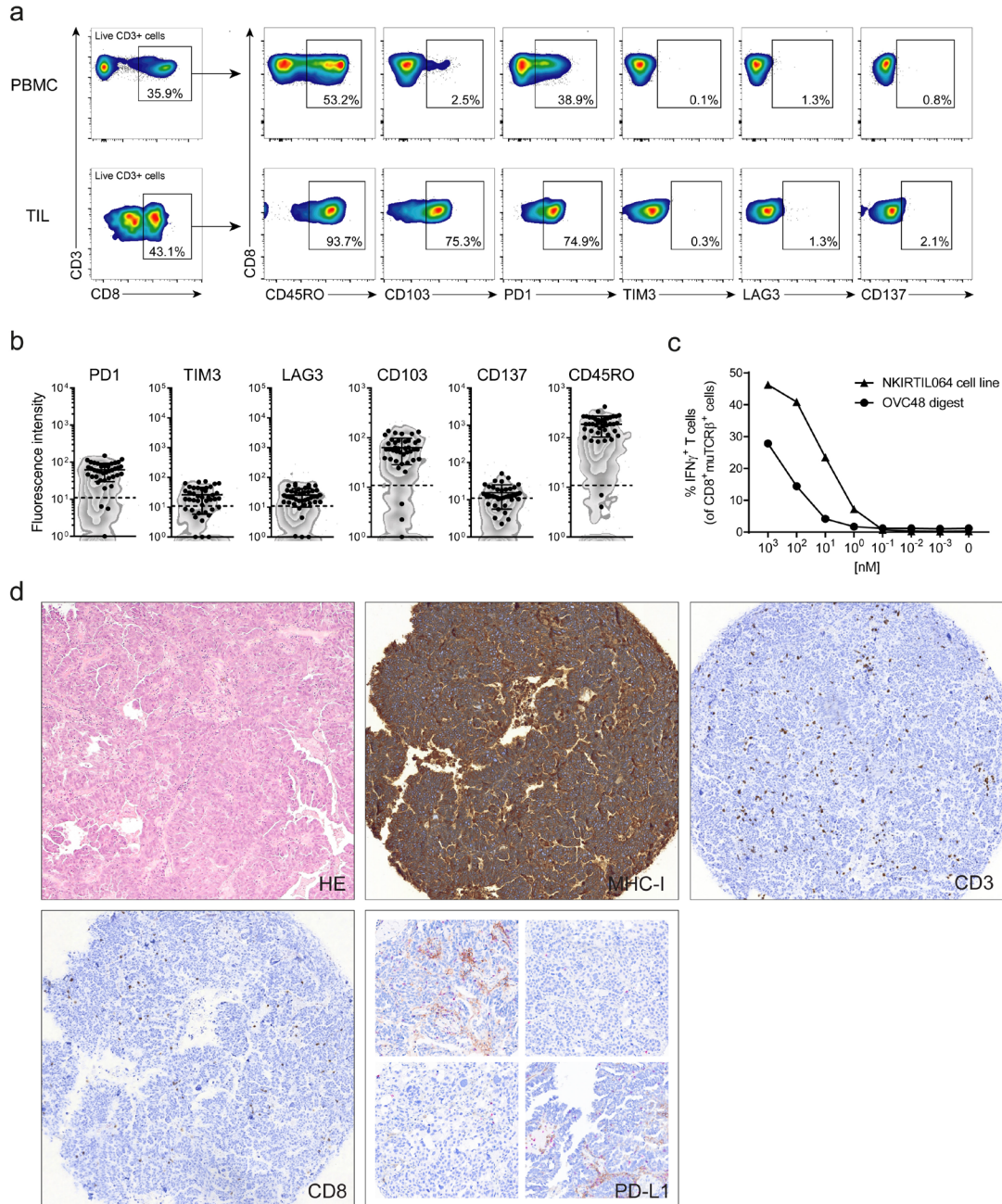


Supplementary Figure 4. CD8⁺ TIL phenotype and immunohistochemistry of tumor OVC21. (A) PBMC and TIL of patient OVC21 were stained as in Supplementary Fig. 2a and 2b. Samples were stained and measured in separate experiments. **(B)** Excised tumor tissue was treated and stained as in Supplementary Fig. 2c. Tumor parenchyma is indicated with an asterisk (*), tumor stroma with a pound sign (#). **(C)** Sequencing reads mapping to TCR α (left) and TCR β (right) chains of single samples were matched to the PD1 expression status of parental cells using the flow cytometric index data. Statistical significance of differences between PD1⁺ (n=26) and PD1⁻ (n=22) cells was analyzed using the two-tailed Mann-Whitney test, and P values are indicated. Medians are indicated, error bars represent interquartile ranges.



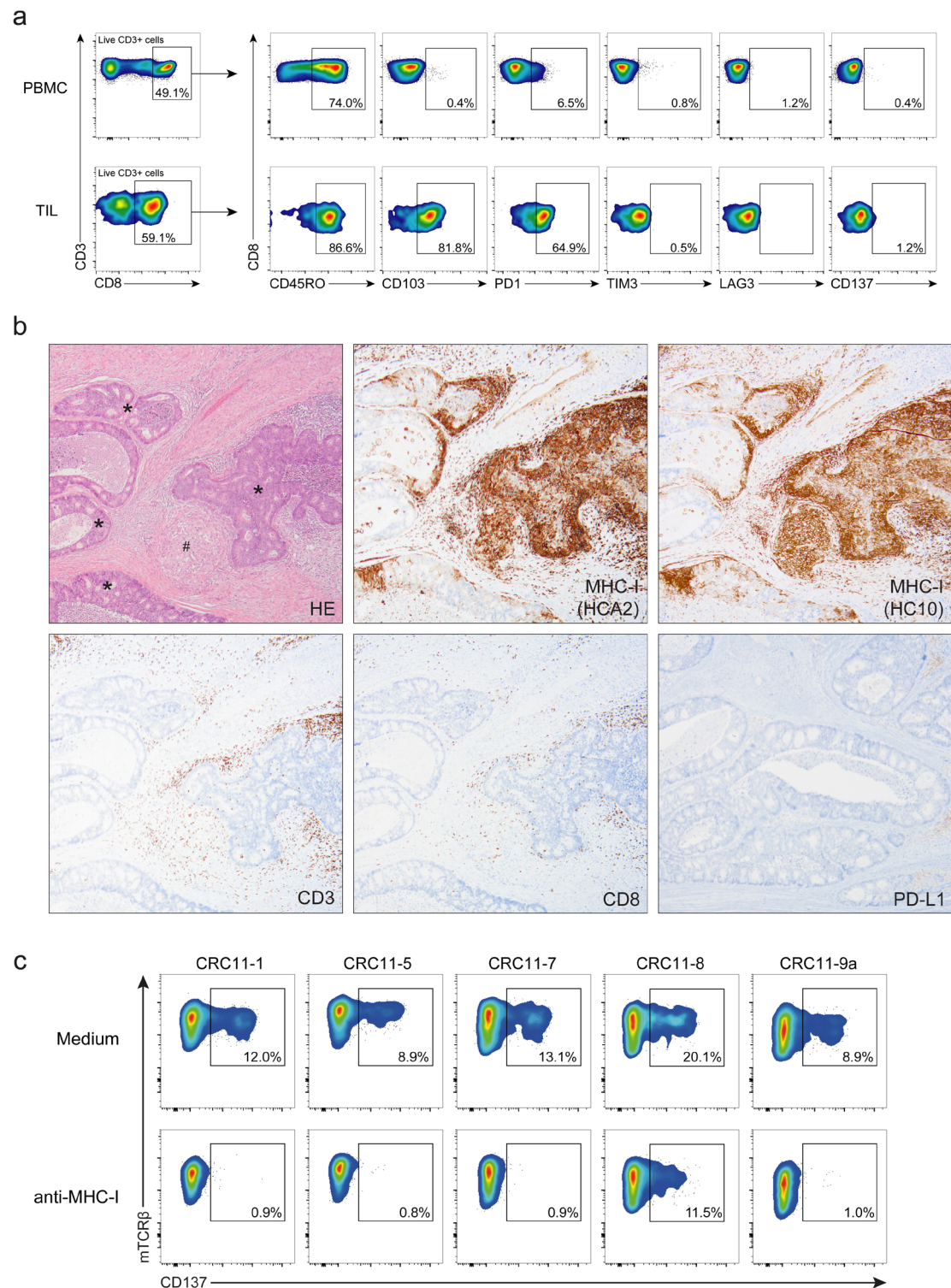
Supplementary Figure 5. MHC class I restriction of TCR OVC21-17, and sensitivity of detection of tumor-reactivity. (A) A patient-derived xenograft (PDX) line was generated from OVC21 tumor digest and cultured with OVC21-17 TCR-modified donor T cells in the presence or absence of an MHC class I-blocking antibody (clone W6/32). Expression of CD137 on TCR-

transduced T cells was measured by flow cytometry. Data depict representative flow cytometry plots (left) and mean CD137 levels of technical replicates (right; error bars indicate S.D.). **(B)** Donor T cells transduced with TCR OVC21-17 were cultured with different ratios of autologous OVC21 tumor digest and digest of an allogeneic ovarian tumor, and CD137 levels on T cells were assessed by flow cytometry. All data are gated on live CD8⁺mTCRβ⁺ cells. **(C)** A Jurkat T cell line deficient for both the TCRα and TCRβ chain was generated using CRISPRs/Cas9, and knockout of both the TCRα and the TCRβ chain in Jurkat cells was confirmed by successful restoration of CD3 expression after co-transduction with both a TCRα and a TCRβ chain, but not with either chain alone. A GFP reporter was included in both vectors to assess transduction efficiency. **(D)** To test whether the observed sparsity of tumor-reactivity among tested TCRs (Fig. 2) could be explained by competition between transgenic and endogenous TCRs for expression at the cell surface, both wildtype and TCRαβ⁻ Jurkats were transduced with CD8α and CD8β, sorted to purity based on CD8αβ expression, and transduced with indicated OVC21 TCRs. Cells were subsequently incubated with OVC21 tumor digest and surface expression of CD69 was measured by flow cytometry. As was observed when utilizing healthy donor T cells as recipient cells (Fig. 2), tumor reactivity was observed for OVC21-17 but not for any of the other TCRs, when either using TCR-proficient or TCR-deficient CD8αβ⁺ Jurkat cells as recipient cells. Data depicted in **(B)** and **(D)** are derived from single experiments.



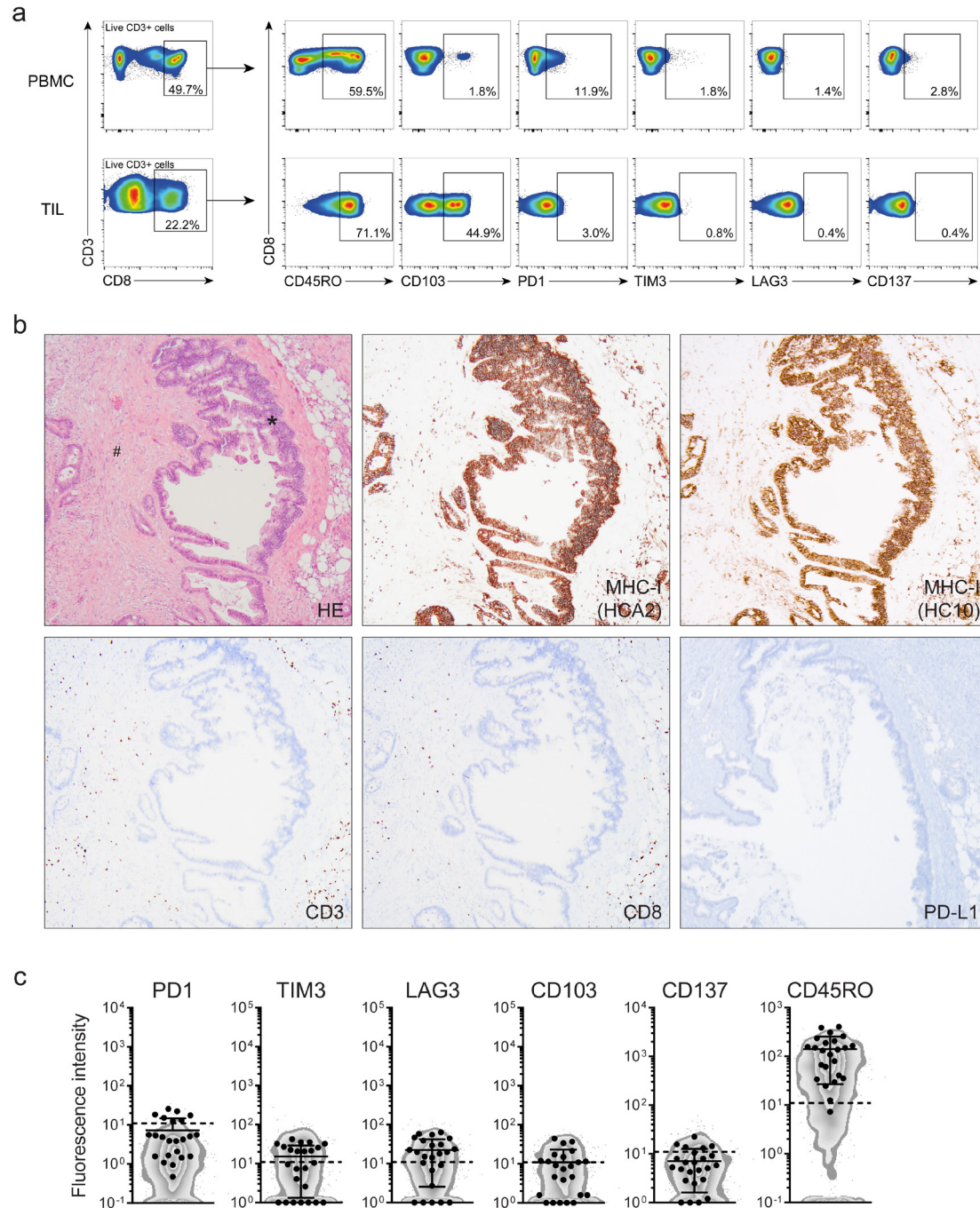
Supplementary Figure 6. CD8⁺ TIL phenotype, peptide-pulsing and immunohistochemistry of tumor OVC48. (A) PBMCs and TIL of patient OVC48 were stained as in Supplementary Fig. 2a and 2b. Samples were stained and measured in separate experiments. (B) Phenotype of parental CD8⁺ intratumoral T cells of patient OVC48 in which analyzed TCRs were detected (n=41 independent cells). Grey clouds represent expression of indicated markers of all live CD3⁺CD8⁺ cells measured in the tumor sample. Dots depict data of individual TIL of which TCRs were functionally tested. Dashed lines indicate gates used in flow cytometric analysis. Means $\pm$ S.D. are indicated. (C) OVC48 tumor digest and an HLA-A24:02⁺ cell line (NKIRTL064) were pulsed with the indicated concentrations of WT1₂₃₅₋₂₄₃ peptide, incubated with WT1 TCR-transduced donor T cells, and T cell activation was read out by analysis of IFN γ production. Data are derived from a single experiment. (D) Excised tumor tissue was treated and

stained as in Supplementary Fig. 2c. Multiple sections are shown for PD-L1 staining to reflect heterogeneity of PD-L1 expression within the tumor specimen.



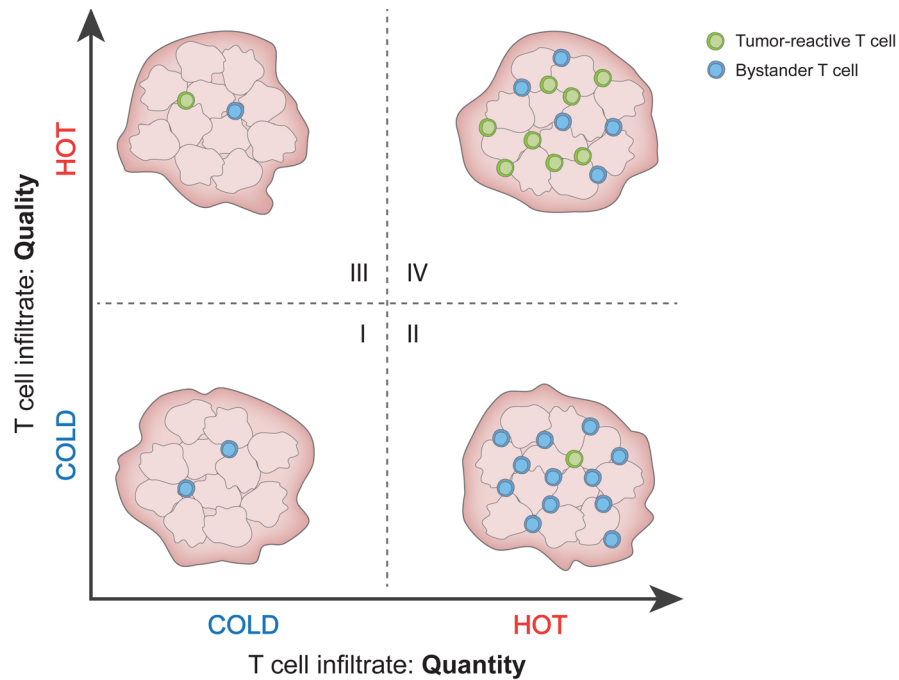
Supplementary Figure 7. CD8⁺ TIL phenotype and immunohistochemistry of tumor CRC11. (A) PBMCs and TIL of patient CRC11 were stained as in Supplementary Fig. 2a and 2b. Samples were stained and measured in separate experiments. (B) Excised tumor tissue was treated and stained as in Supplementary Fig. 2c. Colorectal cancer glands are indicated with an asterisk (*), the surrounding tumor stroma with a pound sign (#). (C) Donor T cells transduced

with tumor-reactive TCRs CRC11-1, -5, -7, -8 or 9a were incubated with autologous tumor organoids in the presence or absence of MHC class I-blocking antibody (clone W6/32). Data depict representative flow cytometry plots (gated on live CD8⁺mTCR β ⁺ cells) and are derived from a single experiment.

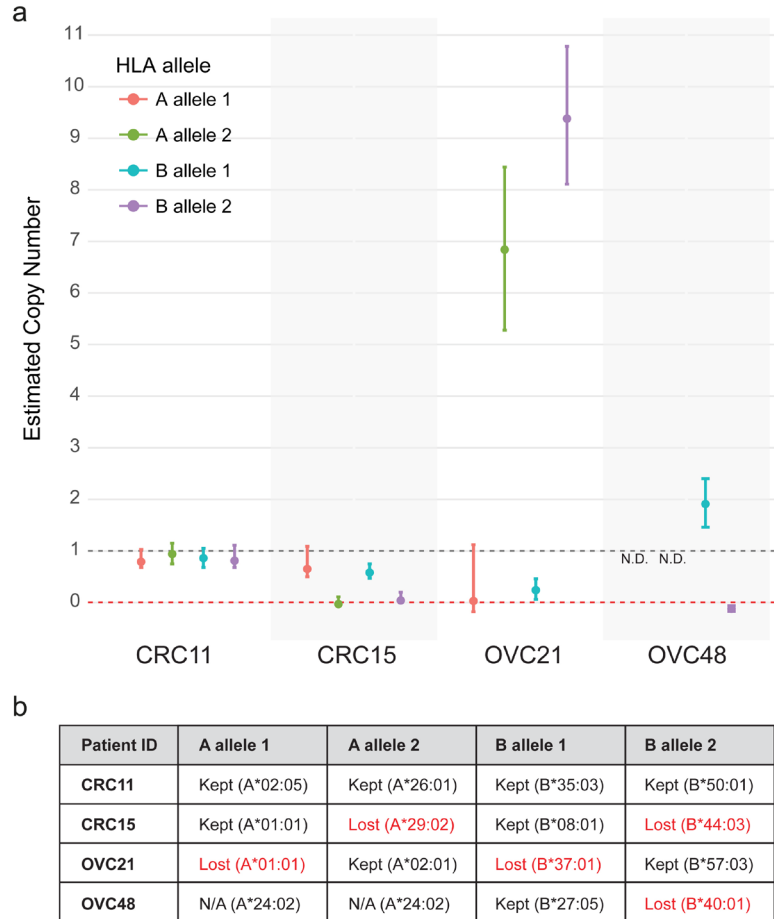


Supplementary Figure 8. CD8⁺ TIL phenotype and immunohistochemistry of tumor CRC15. (A) PBMCs and TIL of patient CRC15 were stained as in Supplementary Fig. 2a and 2b. Samples were stained and measured in separate experiments. (B) Excised tumor was treated and stained as in Supplementary Fig. 2c. Colorectal cancer glands are indicated with an asterisk (*), the surrounding tumor stroma with a pound sign (#). (C) Phenotype of parental CD8⁺ intratumoral T cells of patient CRC15 in which tested TCRs in Fig. 3 were detected (n=24 independent cells). Grey clouds represent expression of indicated markers of all live CD3⁺CD8⁺ cells measured in the tumor sample. Dots depict data of individual TIL of which TCRs were

functionally tested. Dashed lines indicate gates used in flow cytometric analysis. Means $\pm$ S.D. are indicated.



Supplementary Figure 9. Extending the concept of immunologically ‘hot’ and ‘cold’ tumors. Prior work has established that the number of infiltrating CD8⁺ T cells in human cancers has both prognostic and predictive value, leading to the concept of immunologically ‘hot’ and ‘cold’ tumors (horizontal axis). The current work demonstrates that such T cell infiltrates can have low and also variable reactivity to surrounding tumor tissue (vertical axis). Taking into account both the quantity and quality of tumor-infiltrating T cell pools, four distinct scenarios may occur: **(I)** Very few T cells have infiltrated the tumor, and of these only a minor fraction has the intrinsic potential to recognize adjacent tumor tissue; **(II)** Large numbers of T cells have infiltrated the tumor, but only a minority of these can contribute to tumor control. While designated a ‘hot’ tumor on the basis of the magnitude of the T cell infiltrate, the true anti-tumor potential of the infiltrated T cell pool is modest. **(III)** Very few T cells have infiltrated the tumor, but of these a substantial fraction has the intrinsic potential to recognize adjacent tumor tissue. **(IV)** Tumors that are ‘hot’ with respect to both the number of T cells and the intrinsic tumor-reactivity of these T cells. At present it is unclear whether T cell quantity and quality are to some extent correlated, or whether the two parameters are independent properties.



Supplementary Figure 10. Loss of HLA class I alleles in tumor samples. (A) HLA class I allele-specific copy number was inferred for all tumor samples from exome sequencing data using the LOHHLA software package. Dots represent HLA allele-specific copy number estimates; error bars indicate 95% confidence intervals. The dotted grey line indicates normal HLA haploidy (copy number = 1), while the dotted red line indicates HLA allele loss (copy number = 0). Note: the LOHHLA package does not allow inference of copy number estimates for homozygous HLA alleles, such as the HLA-A alleles of patient OVC48. (B) Summary of LOHHLA analysis.