## 1021 A PAN-CANCER SIGNATURE IDENTIFIES TUMOR-REACTING CD8+ TILS AND REVEALS THEIR FUNCTIONAL HETEROGENEITY

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**Background** Identification of $CD8^+$ tumor-reactive tumor-infiltrating lymphocytes (TILs) is a key focus for understanding TIL:tumor interactions in the tumor microenvironment and improving adoptive cell therapies. Current methods concentrate on the detection of TILs endowed with tumor-specific T cell receptors (TCRs) and largely overlook their functional profiles. In addition, analyses of actively tumor-reacting TIL function are often analyte limited and may not reflect the heterogeneity of TIL responses to stimulation. Therefore, we set out to identify tumor-reacting $CD8^+$ TILs and thoroughly characterize their functional profiles at the single-cell level pan-cancer.

**Methods** Minimally *in vitro* cultured TILs and matching autologous tumor cell lines (TCLs) were derived from metastatic melanoma (n=6), renal cell carcinoma (n=3), sarcoma (n=2), colorectal cancer (n=1), and ovarian cancer (n=1) patient samples. Bulk TILs were cocultured with their wildtype or a major histocompatibility complex I and II deficient control TCL, sorted for $CD8^+$ (fluorescence-activated cell sorting), and then underwent single-cell RNA sequencing (scRNAseq, 10xGenomics 5' v2/3' v3.1) to form a discovery dataset. Publicly available scRNAseq datasets (n=25, 12 histologies) of fresh tumor biopsies were obtained from online databases (Gene Expression Omnibus, Genome Sequence Archive, European Genome-phenome Archive) or the authors directly and merged into a singular validation dataset. Cell Ranger and Seurat were used for data processing and analysis.

**Results** Distinct clusters of tumor-reacting $CD8^+$ TILs could be easily bioinformatically identified using known reactivity markers. A tumor-reacting CD8+ TIL signature (TR8S) derived via comparison to non-reacting cells clearly differentiated tumor-reacting and non-reacting $CD8^+$ bulk TILs for all samples and histologies. Deeper characterization of $TR8S^{hi}$ cells revealed diverse sub-populations with distinct and heterogeneous functional profiles, many of which could be re-identified in the validation dataset. In particular, an $XCL1^{hi}XCL2^{hi}$ cluster and a separate $CCL3^{hi}CCL4^{hi}$ cluster dominated the reacting population. The majority of tumor-reacting $CD8^+$ TIL sub-populations were shared across tumor histologies.

**Conclusions** Our results indicate that the TR8S is an effective pan-cancer tool for identifying and characterizing actively tumor-reacting $CD8^+$ TILs. Furthermore, our data highlight the complexity of the actively tumor-reacting $CD8^+$ TIL population and that deeper investigation of their functional abilities is warranted. Specifically, the prevalence of distinct clusters may have predictive/prognostic strength and could direct future immunotherapies.

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**Ethics Approval** Fresh tumour material from patients with cancer was acquired via surgical resection or needle biopsy in the context of standard treatment or enrolment in clinical trials (Ethical approval references: H-20070020, H-19076238, Data Protection approval P-2021-303) at the National Center for Cancer Immune Therapy (CCIT-DK), Department of Oncology, Copenhagen University Hospital, Herlev, Denmark. All procedures were performed in compliance with the clinical protocols approved by the Ethics Committee of the Capital Region of Denmark and national regulations for biomedical research.

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