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 v2/v3) 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 hiXCL2 hi cluster and a separate CCL3 hiCCL4 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.