RESEARCH HIGHLIGHT

Decoding T cell state-specific regulomes in cancer

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Cell Research (2024) 0:1–2; https://doi.org/10.1038/s41422-024-00926-3

Tumor-infiltrating CD8 T cells comprise a spectrum of differentiation states associated with distinct functional, transcriptional, and epigenetic features; understanding the gene networks regulating each state holds the potential to redirect CD8 T cell functionality towards therapeutically relevant states. In a recent *Nature* paper, Zhou et al. performed a single-cell CRISPR knock-out screen of 180 transcription factors in tumor-infiltrating CD8 T cells, identifying factors that regulate cell state transitions and represent therapeutically actionable targets.

Tumor-infiltrating CD8 T cells are highly heterogenous, containing stem-like and effector-like cells that are important for therapeutic responses as well as exhausted cells that are ineffective at controlling tumor growth. A better understanding of the factors governing the differentiation of these diverse states is pivotal for the development of therapies that leverage CD8 T cells for cancer treatment, including adoptive T cell therapies and immune checkpoint blockade (ICB). Although multiplex flow cytometry and single-cell genomics together have provided in-depth resolution of these states, a comprehensive understanding of their underlying causal regulatory networks requires the use of high-throughput perturbation screens at single-cell resolution in vivo.

CRISPR-Cas9-based genome editing enables efficient and high-throughput targeting of genes in cells. CRISPR-based knock-out (KO) screens in primary CD8 T cells have been performed in vitro with pooled sgRNA libraries followed by subsequent in vivo validation studies of the selected genes. Although these studies have pinpointed important regulators of T cell proliferation, activation, and cytotoxicity, they neither allow tracking of the effect of each perturbation at single-cell resolution nor do they account for the complexity of the tumor microenvironment (TME) and its effects on T cell differentiation.[1–3] In vivo CRISPR-based KO screens have studied the effects of specific perturbations on CD8 T cells in the TME but have also relied on pooled sgRNA libraries and pre-defined readouts of CD8 T cell fitness, expansion, survival, or effect on tumor growth for target gene(s) selection, precluding unsupervised target discovery.[4–6] Zhou and co-authors addressed these issues by performing a large in vivo single-cell CRISPR (scCRISPR) KO screen using OTI T cell receptor (TCR) transgenic T cells that constitutively express the *Streptococcus Pyogenes* Cas9 (SpCas9) protein and recognize Ovalbumin (OVA) antigen, endowing the T cells with the ability to recognize OVA-expressing tumors.[7]

The authors activated Cas9 OTI T cells and transduced them with a scCRISPR KO retroviral library targeting 180 transcription factors (TFs) known to play a role in CD8 T cell differentiation (including 40 non-targeting controls), prior to their transfer into B16-OVA melanoma-bearing mice (Fig. 1a). Seven days post T cell transfer, tumor-infiltrating OTI T cells were isolated and profiled by single-cell RNA-sequencing (scRNA-seq) to assess the expression of each sgRNA and the transcriptome. The authors then used these data to define co-functional modules and downstream co-regulated gene programs. TF perturbations that resulted in similar regulatory effects, defined by gene expression changes upon perturbation with respect to the non-targeting controls, were grouped into nine co-functional modules (M1–M9). Six of the nine co-functional modules showed the strongest perturbation effect on four gene programs (effector, exhaustion, stemness, and proliferation), with some of these modules showing both positive and negative effects on multiple gene programs simultaneously. Focusing on these six modules, the authors built a gene regulatory network where for each module central hub TFs (i.e., defined based on the number of differentially expressed genes affected after each TF perturbation) were highlighted. Positive and negative interactions between TFs across and within modules were revealed and included previously known as well as newly identified TF interactions. To study state-specific transcriptional drivers, the authors annotated four T cell clusters: a precursor-exhausted state 1 ($T_{pex}1$), mostly comprised of stem-like cells; a precursor-exhausted state 2 ($T_{pex}2$) that resembles the previously described $Ly108^{+}CD69^{-}$ Tex^{prog2} population[8] that retains high proliferative capacity; a terminal exhausted state 1 ($T_{ex}1$) that resembles previously described effector populations[8,9]; and a terminal exhausted state 2 ($T_{ex}2$). Although the use of new terminology adds confusion to the already heterogenous nomenclature used throughout the literature, the subsets identified in the study align well with previously described CD8 T cell states.

Analyses of sgRNA depletion or enrichment in each cell state with respect to the others identified transcriptional activators or repressors for each state (Fig. 1b). IKAROS (encoded by *Ikzf1*) was found to promote the $T_{pex}1$-to-$T_{pex}2$ transition. Transfer of *Ikzf1* KO $T_{pex}1$ cells into B16-OVA melanoma-bearing mice resulted in the accumulation of $T_{pex}1$ cells with increased expression of stemness genes and decreased expression of multiple metabolic and mTORC-associated genes, ultimately compromising the efficacy of ICB. These results underscore the concept that genetic interventions that simply result in increased frequency of

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Published online: 19 January 2024

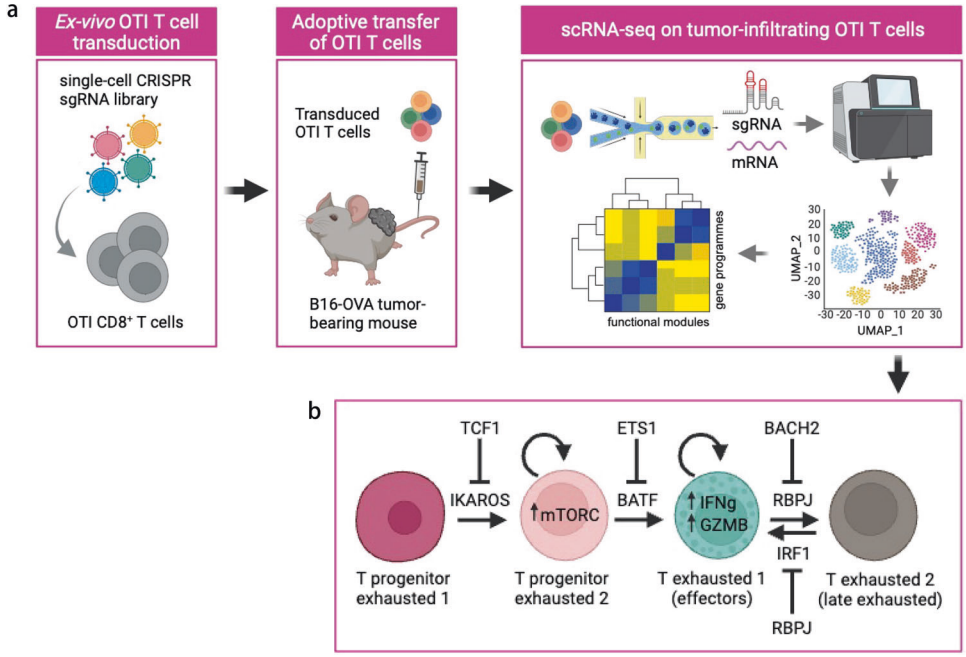

Fig. 1 In vivo scCRISPR KO screening of transcriptional regulators of tumor-specific CD8 T cells. a Cas9-expressing TCR (OTI) transgenic T cells are transduced with a scCRISPR sgRNA retroviral library targeting 180 TFs. Transduced OTI T cells are then adoptively transferred into B16-OVA melanoma-bearing mice. Tumor-infiltrating OTI T cells are then sorted and profiled by scRNA-seq. For each gene perturbation, the single-cell distribution is identified in the uniform manifold approximation and projection (UMAP) and compared with that of non-targeting control sgRNAs. Further, TF perturbations are clustered into co-functional modules based on their regulatory effects (positive and/or negative) on defined gene programs. **b** The three transcriptional axes (IKAROS-TCF1, ETS1-BATF, RBPJ-IRF1-BACH2) identified through the scCRISPR KO screen that regulate tumor-specific CD8 T cell states are depicted. Figure created with Biorender.

$T_{pex}1$ cells do not necessarily improve anti-tumor responses. Notably, analysis of *Ikzf1* KO $T_{pex}1$ cells identified TCF1 (encoded by *Tcf7*) as a functionally relevant target of IKAROS. Accordingly, co-targeting of *Tcf7* and *Ikzf1* reversed the accumulation of $T_{pex}1$ cells and rescued their transition into $T_{pex}2$ and T_{ex} cells. ETS1 was identified as a gatekeeper that negatively regulates the $T_{pex}2$-to-$T_{ex}1$ transition. Accordingly, *Ets1* KO resulted in expansion of $T_{ex}1$ effectors, improved anti-tumor responses and ICB efficacy. KO of both *Ets1* and *Batf* in OTI T cells abrogated this effect identifying BATF as a target of ETS1. Lastly, RBPJ was identified as a top positive regulator of the $T_{ex}1$-to-$T_{ex}2$ transition. Knocking out *Rbpj* in T_{ex} cells resulted in the accumulation of highly proliferative $T_{ex}1$ effectors, improved anti-tumor responses, and enhanced ICB efficacy. Mechanistically, RBPJ's effects on the $T_{ex}1$-to-$T_{ex}2$ transition were independent of Notch and relied on IRF1. Indeed, *Irf1* and *Rbpj* double KO in OTI T cells reversed the effects observed in RBPJ-deficient OTI T cells. Lastly, BACH2 was found to be necessary and sufficient to inhibit RBPJ expression. Therefore, scCRIPSR KO screens in primary mouse tumor-specific T cells of TFs relevant for CD8 T cell differentiation and function identified causal gene regulatory networks underlining CD8 T cell states in tumors.

The perturbation map provided by this study identifies actionable targets for tumor-specific T cell engineering. Future efforts should focus on perturbation screens that start from naïve CD8 T cells to capture early cell state transitions and on combining scCRISPR screens with spatial transcriptomics to study the cell-extrinsic effects of defined perturbations, as recently done for tumor cells.[10] Together, the implementation of scCRISPR screens to systematically investigate cell-intrinsic and -extrinsic gene effects holds great potential to improve cancer treatment.

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ADDITIONAL INFORMATION

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