WHITE PAPER

Accelerate immunotherapy research with CRISPRa and CRISPRi screens

Gene editing of primary immune cells to aid next-generation cellular immunotherapies

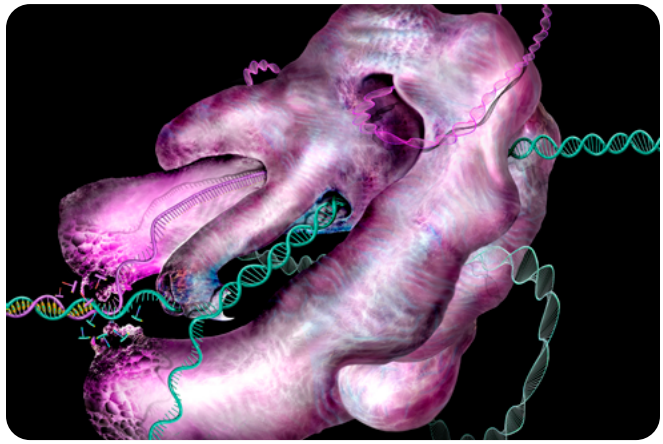
T-cell activation in response to cytokine production is critical for healthy immune function and is often dysregulated in autoimmunity, immunodeficiencies, and cancer. Gaining insights into how tumors manipulate this process is going to be key for developing effective cellular therapeutics. However, our current understanding of the pathways leading to cytokine production in humans predominantly originates from studies in transformed T-cell lines, which do not accurately recapitulate primary human cell biology.

Here we describe how loss- and gain-of-function studies within primary human T cells offer critical insights into the pathways required for cytokine regulation, which could help guide efforts to engineer the next generation of immunotherapies.

CRISPR activation and interference

Over the last decade, CRISPR genome editing has become a powerful and indispensable tool for gene function study in biological research. Recently, the CRISPR-Cas9 system has been adapted to generate two new technologies that modulate gene expression: CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi).

Instead of using Cas9 to cut the DNA, CRISPRa and CRISPRi require the sustained expression of endonuclease-dead Cas9 (dCas9), which contains mutations in two active endonuclease domains therefore causing loss of the cutting capability. Consequently, when coupled with a guide RNA



(gRNA), dCas9 still has the ability to target a specific DNA location but cannot introduce doublestrand breaks. In the CRISPRi system, dCas9 is fused to a transcriptional repressor leading to gene expression inhibition, while in the CRISPRa system dCas9 is fused to transcriptional activators enhancing expression of the target gene. This ability to control the transcript levels of endogenous genes allows researchers to define the relationship between a phenotype of interest and levels of gene expression.

Until recently, lentiviral delivery challenges have limited the application of CRISPRa and CRISPRi in primary cells at scale. However, researchers based at the Gladstone Institutes and UC San Francisco (UCSF) have overcome these limitations and successfully deployed CRISPRa/i in primary human T cells. The study is the first to use these systems at such a large scale in primary cells.[1] "Scalability is a huge plus with these systems," enthused co-first author of the paper Dr. Zachary Steinhart, who is a post-doctoral scholar at Gladstone-UCSF Institute of Genomic Immunology. "By working with primary immune cells, we can directly translate lessons learnt from these functional genomics experiments directly into cellular therapies using CRISPR-edited T cells."

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Genome-wide CRISPRa screens

First, the researchers needed to establish an effective process to continuously express the CRISPR machinery into primary cells. "This is challenging because the cells can only be cultured for a limited amount of time and harsh selection approaches for cells expressing the CRISPR machinery would not be feasible" explained co-first author Dr. Ralf Schmidt, who is also based at Gladstone-UCSF Institute of Genomic Immunology. "Consequently, we needed a really high efficiency in order to make this feasible for scaling up." After exploring several approaches, they developed an optimized high-titer lentiviral production protocol which enabled transduction efficiencies of up to 80 %. When they combined this with a second-generation CRISPRa synergistic activation mediator (SAM) system they observed a robust upregulation in target genes, including some that are not naturally expressed in T cells, which gave the researchers confidence to scale up their screen.

They then performed pooled genome-wide CRISPRa screens targeting more than 18,000 protein-coding genes with over 112,000 single-guide RNAs (sgRNAs). Fluorescence-activated cell sorting (FACS) was used to separate IL-2-producing CD4+ T cells and IFN-γ-producing CD8+ T cells into cytokine high and cytokine low bins. "IFN-γ and IL-2 are important in a variety of diseases, so we applied this sorting regime in order to gain insights into the regulation of fundamental immunological processes," said Dr. Schmidt.

Analysis of the IL-2 and IFN-γ CRISPRa screens revealed 444 and 471 hits, respectively, including 171 shared hits. "The detection of already well-established regulators confirmed our strategy," he said. For example, they observed that T-bet, which is known for promoting CD4+ T helper cell 1 (T_H1) differentiation, was a positive hit in the IFN-γ gamma screen. By contrast, GATA3, which promotes type II differentiation by antagonizing T-bet, downregulated IFN-γ. "Consequently, we were confident in the robustness of our screen and in the detection of previously unknown regulators."

Furthermore, the team selected 14 hits and performed arrayed CRISPRa experiments, validating the robustness of the screen. Dr. Schmidt noted that they mainly focused on positive regulators, as these might be of particular interest for the development of immune or cancer therapeutics.

Reciprocal CRISPRi screens and CRISPRa perturb-seq

Once the researchers had successfully established the CRISPRa screen, they deduced the method could easily be translated to the CRISPRi system by adapting their optimized lentiviral protocols. "Doing the reciprocal genome-wide loss-of-function screens with CRISPRi meant we could take this huge dataset and match up which regulators can be turned on or off and perturb cytokine production," said Dr. Steinhart. "What was really interesting was that some can be both. *VAV1* is a nice example of that, when you overexpress it you can increase cytokine production and when you repress it you can dampen it down. It's like a tunable master regulator."

One of the key observations was that the CRISPRa/i systems complemented each other well, which the team says is key for the discovery of cytokine regulators. For example, CRISPRi detected certain genes with critical regulatory functions, including some missed by CRISPRa, while CRISPRa identified genes that were missed by CRISPRi, potentially due to low expression levels of these genes or low activity of pathways these genes are part of.

In the final stage of their experiment the researchers developed a platform for pooled CRISPRa perturbations coupled with single-cell RNA-sequencing (scRNA-seq) readout (CRISPRa Perturb-seq) in primary human T cells. The aim was to characterize the molecular phenotypes of cytokine regulators. CRISPRa Perturb-seq was performed in 56,000 primary human T cells, targeting 70 hits and controls from their genome-wide CRISPRa cytokine screens. "This part of the study allowed us to deeply phenotype and really see what these genes are doing," said Dr. Steinhart. "For example, we can explore whether these genes shift cells towards therapeutically relevant states that we can then focus on in later studies."

Therapeutic potential

The researchers believe that the tools and technologies developed in their study will enable future screening approaches in primary human T cells and potentially other primary cell types. Furthermore, they believe their screening framework should be adaptable to other nonheritable editing applications of the CRISPR toolkit, expanding opportunities to investigate complex biological questions in primary cells.

The next focus for the team is to perform functional follow up of the positive regulators identified in their screens to determine whether they could be used to improve CAR T-cell function. “By co-introducing the CAR with regulators that reinforce T cell activation and cytokine production we hope to enhance the T cell effectiveness against cancer cells,” said Dr. Schmidt.

Reference

1. Schmidt R, Steinhart Z, Layeghi M, Freimer J, Bueno R, Nguyen V et al. CRISPR activation and interference screens decode stimulation responses in primary human T cells. Science. 2022;375(6580).

About the scientists

Zachary Steinhart, PhD

Dr. Steinhart is a postdoctoral fellow at the UCSF lab where he uses a variety of CRISPR/Cas9-based approaches to functionally dissect coding and noncoding determinants of T-cell function in cancer immunotherapy contexts within the Marson Lab. Born and raised in Canada, he completed his PhD in the lab of Stephane Angers at the University of Toronto where he studied functional genomics and CRISPR/Cas9 approaches to study cell signaling pathways in pancreatic and colorectal cancers. Dr. Steinhart has an active interest in using functional genomics approaches for the unbiased discovery of gene editing or druggable targets for improved cancer immunotherapies. When he is not in the lab he enjoys all sorts of outdoor activities, namely biking, skiing, and backpacking, or when the weather is bad, playing board games with friends.

Ralf Schmidt, MD

Dr. Schmidt is a postdoctoral fellow in the Marson lab at UCSF focusing on cancer immunology with the goal of identifying ways to engineer highly efficient cancer-fighting T cells. Within his research he uses CRISPR to enhance the killing capacity of T cells in the broader context of solid tumors. He first became interested in immunology when studying for his medical degree at the University of Vienna, which led him to conduct his medical thesis in Klaus Schmetterer’s Lab, where he dealt with fundamental questions of T-cell activation. Besides work, Dr Schmidt enjoys skiing and playing tennis from time to time.

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