Transcriptional Insights into target knockout-enhanced T cells: Assessing phenotypic states, clinical relevance and safety concerns

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Abstract

Recent FDA approvals of adoptive T cell therapies for solid tumors have demonstrated significant therapeutic potential, yet these therapies often suffer from low overall response rates, underscoring the need for further enhancements. Our in-house genetic screens at BioNTech have identified several lead T cell knock-out (KO) targets that could potentially improve the persistence and functionality of tumor-reactive T cells. These targets, aliased include GenT01, GenT02, GenT03 and GenT04, along with dual combination edits such as GenT01/GenT03 and GenT01/GenT02. Preliminary experimental validations have shown promising results, indicating improvements in both in vitro and in vivo settings. Additionally, computational analyses using public transcriptomic datasets on a subset of these single genetic modifications have uncovered edit-induced phenotypic changes in stemness, persistence, and functionality of edited cells. This thesis aims to comprehensively characterize these phenotypic changes in silico leveraging the publicly available target-KO derivedtranscriptomic read-outs as well as investigate gene programs

associated with T cell trafficking and tumor homing and assess preliminary target KO-associated safety concerns. The findings will in turn guide further mechanism-of-action studies and potentially some future safety evaluation experiments for advancing engineered T cell therapies to patients.

Background

The development of advanced T cell therapies, including the innovative BioNTech T cell products, represents a pivotal advancement in the treatment of solid tumors. Targeted genetic enhancement strategies have been preclinically validated to potentially increase the efficacy of adoptive T cell (ACT) therapies. These strategies are designed to combat the challenges of the harsh tumor microenvironment, namely the presence of immunosuppressive cells such as Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T-regs), hypoxic environment, tumor-mediated check on cytotoxic T cells (for instance, with PD1/PDL1 interaction), as well as inherent variability in T cell functionality across different patients. (Chmiel et.al., Cancer, 2023) By genetically engineering T cells to express specific cytokines, checkpoint inhibitors, or nutrient transporters, the survival, proliferation, and tumor-killing capabilities of these cells are enhanced under challenging conditions.

The utilization of synthetic biology tools has enabled precise modifications of T cell receptors (TCRs) and chimeric antigen receptors (CARs), allowing for the recognition and targeting of tumor-specific antigens while minimizing off-target effects. (Celichowski et.al, J Transl Med, 2023). This precision not only increases the safety profile of T cell therapies but

also enhances their therapeutic efficacy by minimizing antigen escape within the tumor. Since there are multiple KO previously published and evaluated in the clinic, so target perturbation becomes a potential strategy for mitigating the ACT limitations. Comprehensive genetic screens and target validation efforts from BioNTech have pinpointed several key T cell genes—such as (aliased) GenT01, GenT02, GenT03, and GenT04 — as promising targets for genetic knockout (KO). These KOs performed alone or in combinations such as GenT01/ GenT03 and GenT01/GenT02, have shown potential in enhancing the persistence and functionality of tumor-reactive T cells. These perturbations have the potential to enhance the key attributes of T cells (Chi et al., Front. Immunol., 2023), including:

1. Stemness and persistence: promoting the stem-like properties of T cells, enabling them to maintain their anti-tumor activity for extended periods of time;
2. Functional activation: optimizing the activation status of T cells, ensuring they are primed to mount a robust immune response; and
3. Resistance to exhaustion: mitigating the effects of T cell exhaustion, which is a state of functional impairment that would limit the effectiveness of anti-tumor immunity.

To elucidate the roles of these gene KOs further, this study leverages RNA-seq readouts from public omics data sources to investigate the following KO-induced cellular effects on key biological questions:

i. **T cell Stemness and Functionality**: The investigation aims to determine if any target KOs improve the T cell stemness and/or functionality phenotypes based on RNA-seq readouts, focusing on transcriptional signatures associated with T cell durability and regenerative capacity.

ii. **Mechanism of Action (MoA) - Apoptosis and/or Tumor Trafficking Induction**: GenT03 edited T cells were demonstrated to work in vitro but that did not translate into anti-tumor efficacy in vivo. Experimental analysis revealed that there were no GenT03 cells in the tumor or in the periphery after cell transfer. Therefore, this work aimed to test out two hypothesis: the impact of target KO on 1. cell trafficking by looking at transcriptional changes in cytokines, chemokines, and their receptors associated, and 2. cell apoptosis.

iii. **Clinical Relevance**: The relationship between the response induced by target KOs and the transcriptomic profiles of responders in a T cell therapy clinical trial is investigated.

iv. **Safety Assessment**: The potential risks of target KO, including oncogenic transformation, are evaluated.

Overall, this study strives to investigate the effectiveness and safety for adaptive T cell therapies with target KOs to improve treatment outcomes for patients with solid tumors by employing computational assessments alongside experimental validations. The goal is to harness the power of transcriptomic data to predict and enhance the safety and efficacy profiles of genetically modified T cells in clinical settings, providing a detailed interrogation

of transcriptional and cellular states that aids in the design of potent T cell therapies and addresses pivotal safety concerns crucial for regulatory approval and clinical adoption.

Materials and Methods

Transcriptomic Data Acquisition

To augment our experimental data, comprehensive computational analyses were performed on transcriptomic data derived from genetically edited T cells, which had undergone modifications including GenT01, GenT02, GenT03, and GenT04 knockouts. The data, sourced from various external studies and were summarized in the table below **(Table 1).** These external studies capture transcriptomic readouts at multiple time points post-editing, utilizing high-throughput RNA sequencing and/or single-cell RNA sequencing induced by specific gene KOs in diverse cell models.

Target	KO contrast of interest	Cell Model	Harvest timepoint	Number of Samples	Datatype
GenT01	GenT01 KO CAR Ts versus unedited CAR Ts	Primary human CD8+ CAR T cells	~Wk 4	1 healthy donor, 2+ replicates per condition	Bulk RNA-seq
GenT03	GenT03 KO CD4s versus Ctrl KO CD4s	Primary human CD4+ T cells	Day 6 post electroporation	~3 healthy donors	Bulk RNA-seq
GenT02	GenT02 KO OT-1s versus unedited Input OT-1s	OT-1 murine TILs in B16-Ova tumor bearing mice	7 days post OT-1 transfer	4 mice per group	Bulk RNA-seq
GenT04	GenT04 KO in OT-1s versus NTC KO in OT-1s versus Pos. ctrl. KO in OT-1s	OT-1 murine TILs in B16-Ova tumor bearing mice	7 days post OT-1 transfer	~3 mice in each KO group	Single cell RNA-seq

Table 1. Overview of external target perturbation studies used to assess gene knockout effects with T cell Models. The table details the experimental impacts of each specific gene knockout effects with different T cell model, describing the target gene (masked to

anonymize the targets), the KO contrast of interest, the cell model used, the time point of sample harvest, and the number of samples, as well as the type of RNA-seq data collected.

Differential expression analysis (Bulk RNA-seq and scRNA-seq)

- *Bulk RNA-seq Analysis*

For bulk RNA-seq, the gene-level count data was extracted from different target KOs studies. The DESeq2 package (v.1.46.0) was used for differential expression analysis between the target KO vs. control/wild type conditions. Raw read counts were normalized for library size and dispersion estimates were calculated. Genes with low read counts were filtered out. The Wald test was used to identify differentially expressed genes between conditions, with an adjusted p-value cutoff of 0.05 to control the false discovery rate.

- *scRNA-seq Analysis*

For scRNA-seq, the Use the single cell analysis R package, Seurat (v.5.1.0) for data (Stuart et al., 2019) was used for data processing and analysis. Raw feature-barcode matrices generated by 10x Genomics Cell Ranger pipeline were read into R. For quality control and filtering out low quality cells, we select cells expressing more than 500 genes and fewer than 6000 genes, as well as fewer than 15% mitochondrial genes.

The filtered count matrix was normalized using the "LogNormalize" method which normalizes gene expression by dividing each cell's gene counts by its total expression and then multiplies by a scale factor of 10^4, and log-transforms the result. Variable features (highly variable genes) were identified using the "vst" method. Linear dimensional reduction

was performed using PCA on the variable features. Significant PCs were determined using elbow plots and jackstraw resampling test.

Cells were clustered using a graph-based method implemented in Seurat's FindNeighbors and FindClusters functions. Cell clusters were visualized using UMAP (Uniform Manifold Approximation and Projection) on significant PCs.

To identify differentially expressed genes between clusters or groups of interest, a pseudo-bulk approach was used. Cells were grouped by sample (e.g. conditions, harvest timepoints) and cluster. The count data was then aggregated (summed) for each sample-cluster group to create a “pseudo-bulk” count matrix. This matrix was used as input to DESeq2, following the same steps as described for the bulk RNA-seq data, to identify differentially expressed genes between the target KOs vs. Wildtype (WT) conditions for each cluster. An adjusted p-value cutoff of 0.05 was used to determine significant genes.

Mouse-to-human gene name conversion approach

To reconcile the inconsistencies between the mouse and human gene names used in the various omics datasets (Table 1), ortholog mapping was performed using the biomaRt package (v.2.62.0) in R/Bioconductor (Durinck et.al., Nature Protocols, 2009; Durinck et.al., Bioinformatics, 2005). This allowed for the conversion of gene identifiers from the mouse OT-1 T cell model to their human equivalents, enabling integrated analysis across all four datasets. The biomaRt package provides a robust and efficient method for querying the Ensembl database and retrieving orthologous gene information across multiple species.

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed using the gseapy package (v.1.1.4) in Python to further investigate the biological significance of the differentially expressed genes (Fang et.al., Bioinformatics, 2022). The analysis utilized previously curated gene set signatures from external literatures, focusing on those related to T cell persistence/stemness, activation, and dysfunction/exhaustion/terminal differentiation (Table 2; Figure 1). The pre-ranked list of genes, based on their log2 fold changes, was used as input for the GSEA to evaluate the enrichment of these gene sets among the upregulated and downregulated genes in each knockout condition.

The gseapy package provides a Python implementation of the GSEA algorithm, allowing for seamless integration with the differential expression analysis pipeline. By running the GSEA with the pre-ranked gene list, where the ranking is based on how strongly each gene expression is correlated with the phenotypic classic being compared (KO vs. WT), so that the ranking metric reflects the magnitude of each gene's correlation with the phenotype. The enrichment scores and associated p-values were calculated for each gene set, enabling the identification of significantly enriched pathways.

Visualizations of the GSEA and DEG results, including enrichment plots, boxplots and heatmaps, were generated using the matplotlib (v.3.10,1) and Marsilea (v.0.4.6) libraries in Python. These visualizations facilitate the interpretation of the GSEA results and highlight the key biological pathways affected by the gene knockouts.

Signature type	Description	Example genes	Study
Stemness and persistence related	CD39- CD69- T cells associated with complete cancer regression and TIL persistence vs CD39+CD69+ in a TIL-ACT with ~50 patients.	*TCF7, LEF1, LYAR, TSAN32*	Krishna et. al, 2020
	General CD4+ resting naïve cell / Tcm enriched vs activated T cells from 4 healthy donors.	*LEF1, ATM, SELL, KLF2*	Szabo et. al., 2019
	Persistent vs non-persistent CD8 clones (against KRAS G12D antigen in a colon cancer partial responder patient)	*IL7R, ITGB1, KLF2, ZNF683*	Lu et. al., 2019
Activation related	T cell activation signature for Flu and CMV antigens	*TNFRSF9, IFNG, ZBED2*	Fuchs et.al, 2019
	General Proliferation associated genes upregulated in activated CD3+ vs resting T cells across tissue sites in healthy donors	*ILF, IL2, NME1, CENPV*	Szabo et. al., 2019
	Activated T cell population	-	Asaf et. al. (Internal)
Terminal differentiation/ Dysfunction/exhaustion associated	A terminally differentiated CD39-positive state (CD39+CD69+) associated with poor TIL persistence in a TIL-ACT with ~50 patients.	*CD40LG, GINS2, FEN1, IFNG*	Krishna et. al., 2020
	In vitro model of CAR T cell dysfunction recapitulates defined characteristics of T cell exhaustion and identifies hallmarks of CAR T cell dysfunction.	*HAVCR2, RGS16, LAYN, SRGAP3*	Good et. al., 2020
	CD4 and CD8 exhausted T cell populations	-	Zhao et. al. (Internal)

Table 2. Specific T cell signatures identified across studies to assess the transcriptional impact of target KOs. The table lists specific T cell signatures categorized by stemness/persistence, activation, and terminal differentiation/dysfunction/exhaustion, collated from various studies to assess the transcriptional effects of target KOs. Descriptions on contrasting cell populations comparing each specific signature and example genes are provided, along with the reference studies. BioNTech ("internal") gene signatures were also used.

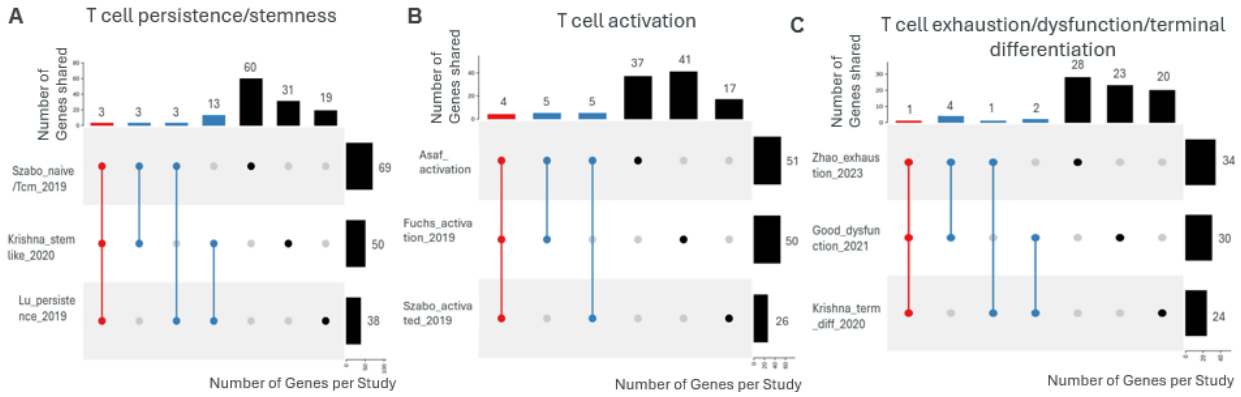


Figure 1. Overlap analysis in Gene set signatures on T cell phenotypes of interest across studies highlights most phenotypic signatures represent study-specific gene sets. The Upset plot visualizes the intersections and unique occurrences of gene sets associated with **(A)** T cell stemness/persistence, **(B)** T cell activation and **(C)** T cell terminal differentiation/dysfunction/exhaustion across multiple studies. The horizontal bars indicate the total number of genes identified in each category per study, while the vertical bar and the

connected points in line indicate the total number of common genes shared between studies. The color coding further indicates the level of overlap: red lines connect gene sets shared across three studies, blue lines connect gene sets shared across two studies, while black dots represent gene sets unique to individual studies.

Death/apoptosis related pathways enrichment

The GSEA analysis not only incorporates the curated gene set signatures from external literatures, but also includes the gene sets gathered from human Molecular Signatures Database (Human MsigDB collections), particularly the focus is paid to the cell death and apoptosis pathways.

Tumor trafficking genes expression assessment

The differentially expressed gene lists (DEGs), resulted from the differential expression analysis, were then specifically employed to investigate the expression dynamics of gene profiles with known markers of T cell migration to tumor or sites of inflammation, chemokine and/or ligand receptors (Kohli et. al., Nat. CGT, 2021; Kendirli et. al., Nat Neuro., 2023; Jin et. al., Elsevier, 2021), in various target KOs condition compared to the control condition.

Clinically Relevant gene expression assessment

The analysis extended beyond transcriptomic profiling to include assessments of the clinical implications with the observed genetic perturbations. The impact of gene KOs is further evaluated, by overlapping gene expression profiles with the clinical outcomes from a

study with publicly accessible clinical trial data (*Haradhvala et. al., Nature, 2022*). A previous study investigating BATF3 over-expressed (OE) perturbation has utilized this clinical data to discover the OE effects with the response profiles (MuCutheon et.al., Nat. Gen., 2023).The gene expressions from previously described DEGs were overlapped against the DEGs from the clinical signatures, in particular the responders vs. non-responders among refractory B cell lymphoma patients treated with CD19 CAR T cells (Tisa-cel). The aim was to identify genes whose expression significantly differs between these two groups, providing insights into the better single edit or combination of target KO with a greater therapeutic potential.

Safety Relevant-T cell lymphoma gene expression assessment

The safety analysis focused on the potential oncogenic risks associated with different target KOs. Previously, genes associated with T cell lymphoma have been identified, so this analysis investigates the expression of these genes in T cells that have undergone various genetic KOs. (*Dai et. al., PNAS, 2022*)

Results

GSEA reveals differential impacts of Target KOs on T cell functionalities

- *GenT01 KO promotes stem-like, persistence associated T cell states*

GSEA of CD8+ T cells following GenT01 KO revealed a significant enrichment of gene expression profiles associated with stem-like and persistence states **(Figure 2A).** This finding aligns with previous internal and external findings that GenT01 KO enhances CAR T

cell stemness and proliferation in a TCF-7 dependent manner. The enrichment of these gene sets suggests that GenT01 plays a crucial role in regulating the balance between T cell stemness and differentiation.

- *GenT03 KO enhances activation of T cells*

In CD4+ T cells, the GenT03 KO results in an enhancement of activation-association gene expression **(Figure 2B)**. This suggests that GenT03 plays a critical role in regulating the balance between T cell activation and exhaustion. This finding is also supported by the role of GenT03, it acts as a negative regulator of effector T cell functions, sosuppressing the activation of NF-kB signaling pathway would potentially prevent the uncontrolled inflammation response which may lead to autoimmunity (identified from GSEA, but not shown here).

- *GenT02 KO modulates some stemness and some terminal differentiation/ exhaustion in T cells*

GSEA of OT-1 T cells with GenT02 KO shows a complex modulation of pathway enrichments. It appears to upregulate certain stemness and persistence associated genes, while also downregulating some terminal differentiation associated genes . **(Figure 2C)** This result is further supported by other studies that GenT02 deficiency leads to improved CAR T cell persistence while increasing the formation of immunity-promoting “precursor exhausted” T cells.

“Even though the gene signatures analyzed did not show significant activation or suppression at the FDR cutoff of 0.01, a closer look at some specific pathways show

alignment with published findings. For example, in *Szabo_naïve/Tcm* (central memory) pathway, which is relevant with T cell persistence, we found the normalized enrichment score (NES) is 1.46 , with known markers associated with stemness/persistence, like LEF1, are enriched, as pointed out in the enrichment plot. (**Figure 2E**) LEF1 has pivotal roles in sustaining the functionality of mature CD8+ T cells, as reported in published literature saying it works with TCF-1 as an essential transcription factor that regulates the mature CD8+ T cell responses. GenT02 KO has been shown to improve long-term CAR-T cell persistence and function by increasing the expression of TCF1 and the formation of $T_{PEX.}$ Similarly, in *Krishna_term_diff* (terminal differentiation) pathway, the NES is 1.04, with the marker genes, such as CD39. **(Figure 2F)** CD39, also known as ENTPD1, is a well-known gene marker that characterizes CD8+ T cell dysfunction and exhaustion. Hence, this finding also aligns with the discovery from the paper demonstrates that GenT02 induces a "long-term effector memory" like phenotype. (Note that, the authors from that study curated "long-term effector memory" using a different set of gene signatures.)

- *GenT04 KO does not provide insights into specific pathways or gene programs*

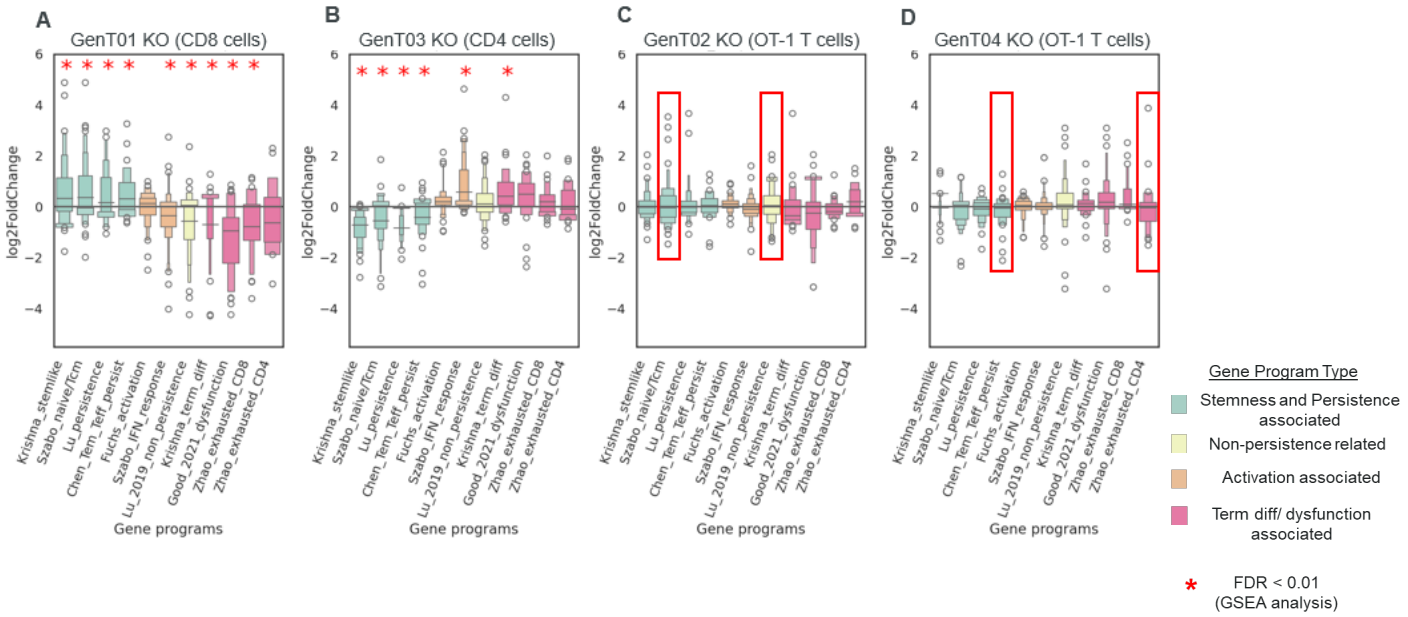
GenT04 KO in OT-1 T cells exhibited an overall downregulating trend in persistence/stemness, activation, and terminal differentiation/dysfunction pathways. However, these gene programs did not reach statistical significance based on the FDR threshold of 0.01 in the GSEA boxplot **(Figure 2D).**

When examining specific pathways, such as *Chen_Tem_Teff_persist* (T effector memory and T effector cells persistence) and *Zhao_exhausted_CD4* (CD4 T cell exhaustion), the p-

values and FDRs did not show statistical significance. With the bulk RNA-seq data, the results for GSEA with GenT04 are unclear.

In summary, the collective results from these KOs indicate distinct roles for each gene in modulating T cell behavior, which could have significant implications for designing more effective T cell-based therapies. By tailoring gene edits, it might be possible to enhance specific T cell properties that are critical for the success of adoptive cell transfer therapies in cancer treatment.

Notice that the data extracted for the GenT04 KO was obtained from an external study with single-cell RNA sequencing (scRNA-seq), which differs from the bulk RNA sequencing data used for the other conditions. We further investigate the differentially expressed genes (DEGs) from the scRNA-seq data using the pseudo-bulk approach.



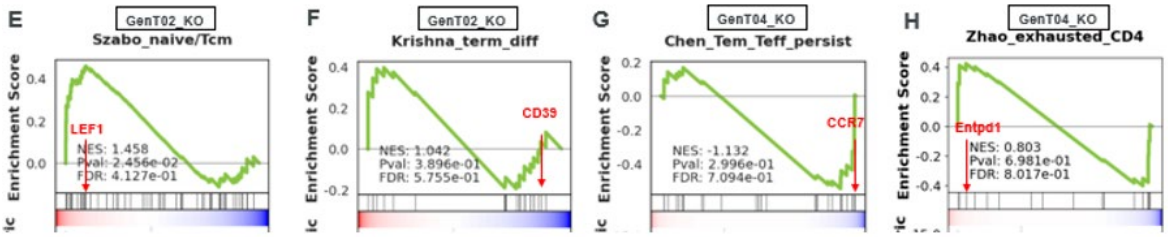


Figure 2. GSEA reveals differential impacts of target gene knockouts on T cell functionalities. **(A-D)** Boxplots showing the normalized enrichment scores (NES) of gene sets associated with stemness and persistence (green), non-persistence-related states (grey), activation (purple), and terminal differentiation/dysfunction (blue) in CD8+ T cells with GenT01 KO (A), CD4+ cells with GenT03 KO (B), and OT-1 T cells with GenT02 KO (C) or GenT04 KO (D). Gene sets with FDR < 0.01 are indicated by asterisks. **(E-H)** Representative GSEA enrichment plots depicting specific gene sets of interest for each target KO condition: *Szabo_naive/Tcm* set for GenT02 KO (E), *Krishna_term_diff* set for GenT02 KO (F), *Chen_Tem_Teff_persist* set for GenT04 KO (G), and *Zhao_exhausted_CD4* set for GenT04 KO (H). NES, normalized enrichment score; Gene ranks represent the ordering of genes in KO vs. WT condition based on LFC values. Heatmaps below the enrichment plots display the relative expression of the top-ranked genes from each gene set.

Phenotypic characterization of GenT04 KO edited OT-1 T cells using single-cell transcriptomics data

Single-cell RNA sequencing was employed to comprehensively assess the impact of GenT04 knockout (KO) on the phenotypic landscape of T cells, with a specific focus on their exhaustion profiles and cytotoxic potential. The analysis was performed on GenT04 knocked-out OT-1 T cells collected from spleen and tumor sites of recipient B16-Ova tumor bearing mice. These recipient mice had ex-vivo edited T cells transferred from syngeneic donor mice. The primary objective in analyzing this dataset was to elucidate the alterations in T cell phenotypes post-transfer and evaluate the influence of GenT04 KO on exhaustion and cytotoxic activity.

Upon analysis at Day 7 post-transfer, the single-cell transcriptomic data revealed distinct clustering of the edited OT-1 T cells, segregating them from the recipient mouse T

cells **(Figure 3A).** This separation facilitated accurate identification and analysis of the genetically modified cells. Further refinement of the data identified five T cell sub-populations based on their gene expression profiles, including T cells with stem-like features (Slamf6+), intermediate/proliferative (Gzmb+, Mk67+), and effector/terminal phenotypes (Gzmb+ Mk67-) **(Figure 3B).**

Marker gene analysis across these T cell sub-population clusters revealed varying expression levels of key genes such as Ccna2, Slamf6, Mcm2, Mk67, Gzmb, Ifng, Havcr2, and Lag3. These marker genes indicating differences in cell cycle activity, stemness, and exhaustion provide further validations for the sub-population characterization **(Figure 3C).** Notably, cells with the GenT04 KO in Tex_eff/term (effector-like and terminally differentiated exhausted T cell population, as shown in the green cluster from Figure 3B) exhibited lower leves of exhaustion markers and higher levels of cytotoxic markers like Gzmb, suggesting enhanced cytotoxic capacity without substantial exhaustion. Specifically, the expression levels of Gzmb were significantly elevated in GenT04 KO T cells compared to control groups **(Figure 3D),** which align with the existing findings of the original paper and supporting the hypothesis that GenT04 KO, within the effector-like and terminally differentiated exhausted T population, may augment cytotoxic functionalities by shown by increase expression of Gzmb.

To quantitatively assess exhaustion profiles for this specific cluster, a module score approach, implemented in the Seurat package (with default settings), was utilized, where lower scores corresponded to reduced exhaustion. GenT04 KO T cells, within the effector-like and terminally differentiated exhausted T population, demonstrated lower exhaustion

module scores compared to controls, indicating a less exhausted state post-edit (Figure 3E). This observation was a novel discovery.

The analysis of specific CD8 T cell exhaustion genes, including Pdcd1, Tigit, Tox, and Lag3, further revealed differential expression patterns. In GenT04 KO cells, there was a general trend towards reduced expression of these exhaustion markers, which was consistent with the observed lower exhaustion module scores **(Figure 3F).**

In conclusion, the single-cell transcriptomic analysis provides compelling evidence for the role of GenT04 in shaping the phenotypic landscape of T cells post-genetic modification. The data suggests that GenT04 KO can potentially enhance cytotoxic potential while attenuating exhaustion, offering new avenues for improving the efficacy of T cell-based immunotherapies. Further validation and mechanistic studies are warranted to fully elucidate the molecular underpinnings of these observations and translate them into clinical applications.

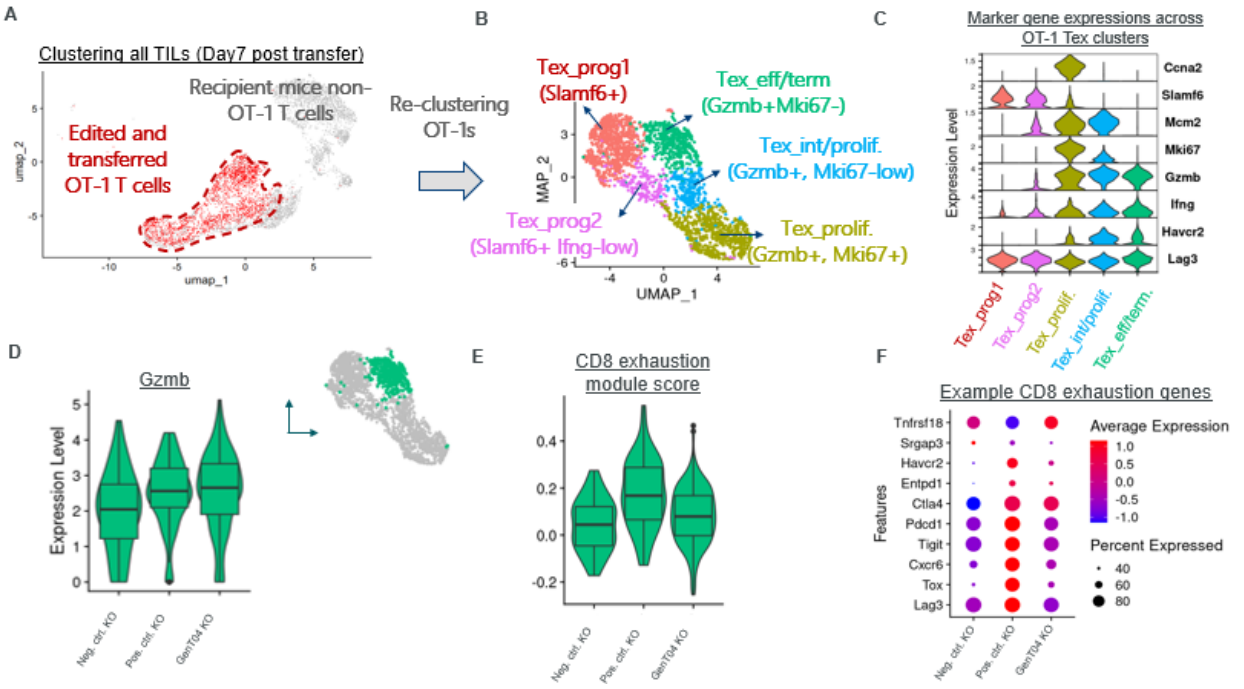


Figure 3. GenT04 knockout enhances cytotoxic potential and reduces exhaustion in tumor-infiltrating T cells. (A) UMAP plot depicting the clustering of all T cells isolated from tumor-bearing mice 7 days post-transfer, with edited and transferred OT-1 cells highlighted. **(B)** Re-clustering of the OT-1 subset reveals distinct T cell populations based on their gene expression profiles. **(C)** Heatmap displaying the relative expression of marker genes across the identified OT-1 T cell clusters. **(D)** Violin plots comparing the expression levels of Gzmb, a key cytotoxicity gene, between GenT04 KO and control T cells, indicating increased cytotoxic potential in the GenT04 KO group. **(E)** Module scores for CD8 exhaustion signature genes, demonstrating reduced exhaustion in GenT04 KO T cells compared to control. (F) Dot plot analysis of representative exhaustion-related genes, showcasing the impact of GenT04 knockout on attenuating the expression of these markers in tumor-infiltrating T cells.

GenT03 KO is a "double-edged sword"

The divergence between in vitro and in vivo outcomes for certain targets was particularly striking in the functional studies and became a major challenge for target KO selection, reported thus far. While in vitro 3D spheroid assays demonstrated effective killing of GenT03 KO cells upon multiple T cell rechallenges, this advantage did not translate to improved tumor control in vivo. The in vivo studies showed comparable tumor growth

trajectories between GenT03 KO and control conditions across multiple timepoints post-inoculation.

Then, we want to investigate potential mechanisms that underlies the discrepancy between the in vitro cytotoxicity and in vivo tumor killing efficacy observed with GenT03 knockout (KO) T cells. Since the cells may be dying in vivo before being able to accumulate at the tumor site, we first examined the impact of target gene KOs on apoptosis pathways. In particular, the GSEA was performed using the *Hallmark Apoptosis* gene set to assess enrichment of apoptosis-related genes in each KO condition **(Figure 4).**

We observed that GenT03 KO demonstrated a positive enrichment score of approximately 1.60 at the leading edge, suggesting upregulation of cell death and apoptosis associated gene programs, though the FDR and p value indicates a lack of statistical significance at the FDR 0.05 cutoff **(Figure 4C).** This enrichment pattern was specific to GenT03, as other genetic perturbations (GenT01, GenT02, and GenT04) showed negative enrichment scores.

In summary, GenT03 perturbation revealed a positive enrichment of apoptosis-related pathway, suggesting a potential role in cell death regulation. While GenT03 KO activates NFKB signaling and provides survival benefit, it can also potentially induce cell death. It is yet unknown if this is in fact what happens in vivo, so these findings raise important questions about how the TME might involve and influence the apoptotic pathways potentially driven by GenT03 deletion.

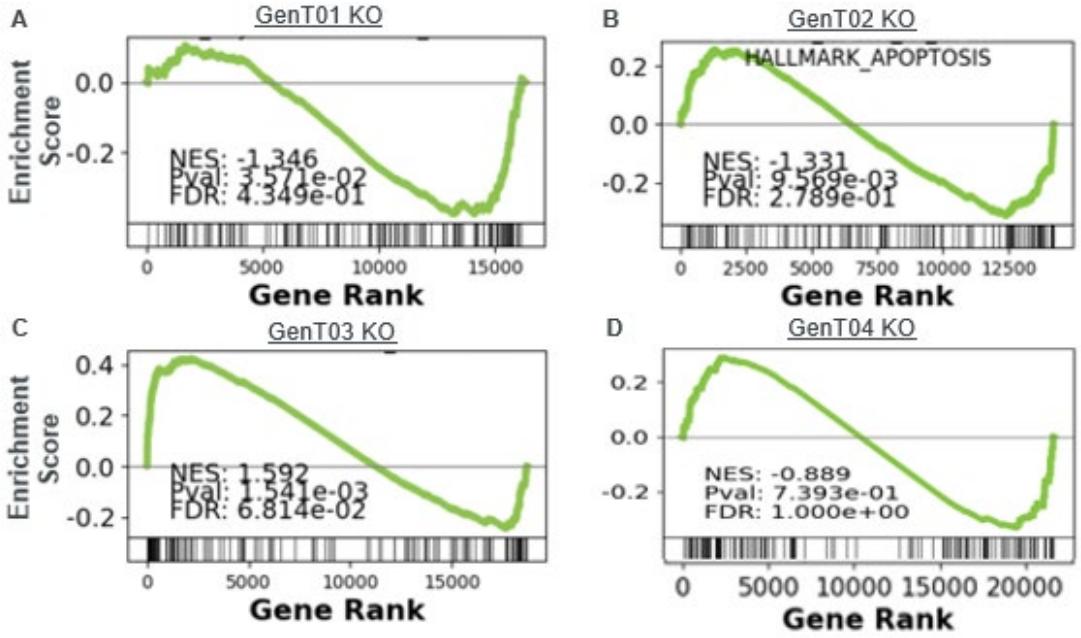


Figure 4. GSEA Enrichment Plots for the Hallmark Apoptosis Pathway in Target Gene Knockouts. The figure presents gene set enrichment analysis (GSEA) results for the Hallmark Apoptosis pathway in four different target gene knockouts (KOs): **(A)** GenT01 **(B)** GenT02 **(C)** GenT03 **(D)** GenT04. Each plot displays the enrichment score (ES) curve, which represents the degree to which the gene set is overrepresented at the top or bottom of the ranked gene list. The normalized enrichment score (NES), p-value, and false discovery rate (FDR) are provided for each analysis.

GenT03 perturbation disrupts T Cell trafficking pathways

Apart from apoptosis, it is also possible that cells in vivo are hampered to reach and infiltrate tumor sites during trafficking process. Therefore, the analysis of chemokine expression patterns was carried out, and it revealed that GenT03 knockout significantly impacts T cell migration-associated pathways **(Figure 5)**. Differential expression analysis demonstrated substantial alterations in key chemokines and trafficking molecules when comparing GenT03 KO to wild-type conditions, as visualized in the heatmap.

Most notably, GenT03 KO led to significant downregulation of several crucial T cell trafficking mediators **(Table 1)**. CCR6, characterized as a "peripheral tissue trafficking marker," showed a marked reduction in expression. This finding is particularly relevant given CCR6's validated role in facilitating T cell migration and improving tumor clearance in xenograft models (Jin et al., Elsevier, 2021). Similarly, the chemo-attractant CCL5, which exhibits strong correlation with CD8+ T cell infiltration across multiple cancer types (Kohli et al., Nat. CGT, 2021), was also downregulated in GenT03 KO cells.

The impact extended to the CXCL9/10/11 signaling axis, with decreased expression of these CXCR3-binding chemokines in GenT03 KO cells. This reduction could be particularly consequential as these chemokines are known to be critical for T cell infiltration into tumors (Kohli et al., Nat. CGT, 2021). Furthermore, TBX21, which directly regulates CXCR3 expression and influences T-bet+ Treg cell accumulation at sites of TH1-mediated inflammation (Koch et al., Nat Immunol. 2009), showed reduced expression in the GenT03 KO condition.

Additional analysis revealed downregulation of TSLP, a cytokine that induces the release of T cell-attracting chemokines from monocytes and DCs. Studies have shown that TSLP exposure leads to CCL17 and CCL22 production by CD11c+ dendritic cells, which can attract Th2-type CD4+ T cells (Ziegler et al., Curr. Imm., 2010).

These findings provide two potential mechanistic explanations for the previously observed discrepancy between in vitro and in vivo efficacy of GenT03 KO. While GenT03

deletion may lead to cell death of edited T cells, or it might also hinder the ability for edited T cells to infiltrate tumor sites in vivo.

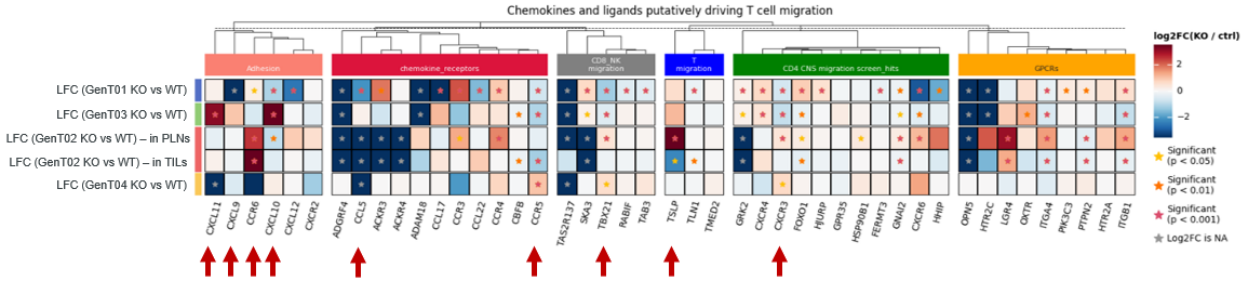


Figure 5. Differential expression analysis of chemokines and ligands associated with T cell migration across genetic perturbations. Heatmap showing log2 fold change (LFC) comparisons between knockout (KO) and wild-type (WT) conditions for key chemokines and migration-related genes. Red arrows indicate molecules of particular interest that have established roles in T cell trafficking and tumor clearance, including CCR6 (peripheral tissue trafficking), CCL5 (chemo-attractant), TSLP (T cell-attracting chemokine inducer), CXCL9/10/11 (CXCR3 ligands), and TBX21 (CXCR3 transcriptional regulator). Color scale represents log2 fold change values, with red indicating upregulation and blue indicating downregulation. Statistical significance is denoted by asterisks (* [yellow] $p < 0.05$, * [orange] $p < 0.01$, * [red] $p < 0.001$), and missingness of the gene is indicated by asterisks (* [grey] NA).

Molecule	T cell / target relevance	Validation/PoC	Reference
CCR6	CCR6 labeled as "Peripheral tissue trafficking marker"	Shown to help with T cell migration in vitro and improve tumor clearance in vivo in a xenograft model	*Jin et. al., Elsevier., 2021*
CCL5/ CCR5	CCL5 binds to CCR5 and acts as a chemo-attractant.	"CCL5 and CXCL9 had the highest correlation with CD8+ T cell infiltration across different cancer types"	*Kohli et. al., Nat. CGT, 2021*
TSLP	Cytokine that induces release of T cell-attracting chemokines from monocytes and DCs.	"Studies using human CD11c⁺ dendritic cells showed that these cells produced CCL17 and CCL22 following exposure to TSLP, chemokines capable of attracting Th2-type CD4⁺ T cells "	UniProt, *Ziegler et. al., Curr. Imm., 2010*
CXCL9/ 10/11 & CXCR3	CXCL9/10/11 bind to CXCR3 receptor in T cells.	"CCL5 and CXCL9 had the highest correlation with CD8+ T cell infiltration across different cancer types"	*Kohli et. al., Nat. CGT, 2021*
TBX21	TBX21 directly regulates the transcription of the CXCR3 gene, which is the primary receptor for CXCL9/10/11 chemokine	"T-bet promoted expression of the chemokine receptor CXCR3 on Treg cells, and T-bet+ Treg cells accumulated at sites of TH1-mediated inflammation."	Koch et.al., Nat Immunol. 2009

Table 3. Key T cell migration and trafficking molecules identified in the chemokine analysis. Summary of selected molecules including their functional relevance to T cell biology and target interactions, validated mechanisms of action, and supporting literature.

The validation/proof-concept (PoC) column provides experimental evidence demonstrating each molecule's role in T cell trafficking and tumor response, with citations to peer-reviewed publications documenting these findings.

GenT01 and GenT03 KOs overlap with clinical CAR T cell response signatures

In order to bridge the gap between preclinical findings and potential therapeutic applications, we investigated whether our genetic perturbations could recapitulate transcriptional patterns associated with favorable clinical outcomes in CAR T cell therapy. This analysis was particularly relevant given the ongoing challenges in predicting and improving patient responses to cellular immunotherapy in refractory B cell lymphoma.

To evaluate the clinical relevance of our genetic perturbations, we analyzed the transcriptional overlap between our knockout-induced changes and response signatures from CD19 CAR T cell therapy trials in B cell lymphoma patients. We leveraged data from a clinical study of 13 refractory B cell lymphoma patients treated with tisa-cel (Haradhvala et al., Nature, 2022), which identified 147 differentially expressed genes between responders and non-responders.

The comparative analysis revealed distinct patterns of overlap with clinical response signatures across different genetic perturbations. Notably, GenT03 knockout showed positive correlation with clinical response patterns, upregulating approximately 9% (7 out of 79) of responder-associated genes. More strikingly, GenT01 knockout demonstrated substantial suppression of non-responder associated genes, downregulating approximately 31% (21 out of 68) of genes linked to poor clinical outcomes.

The magnitude of these effects was particularly noteworthy when compared to another therapeutic approach with a different gene target. Recent work by a study (McCutcheon, Nat. Gen., 2023) demonstrated that BATF3 overexpression resulted in a 20% increase in response-associated genes and a 35% decrease in non-responder genes. While GenT03's impact on responder-associated genes was modest by comparison, GenT01's suppression of non-responder genes approached the efficacy seen with BATF3 overexpression.

Our obsertations suggest potential therapeutic relevance for our genetic perturbations, particularly GenT01, in improving CAR T cell therapy outcomes. The comparable effectiveness of GenT01 knockout to BATF3 overexpression in suppressing non-responder associated genes indicates promising clinical applications, potentially achieving by combing two genetic modification that resulted in dual-effect edited T cells.

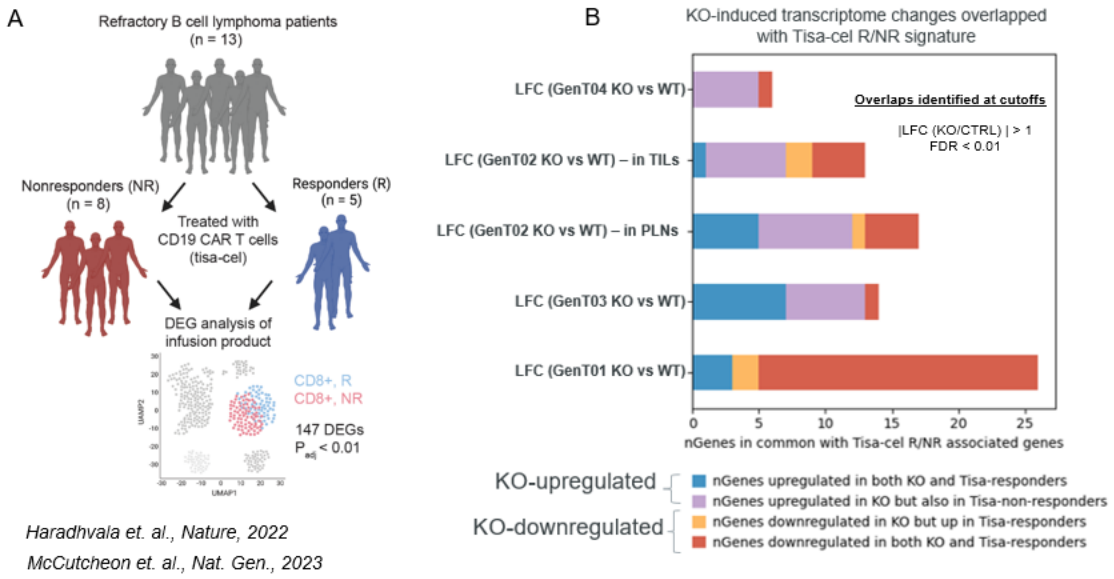


Figure 6: Transcriptional overlap between genetic perturbations and clinical CAR T cell response signatures. (A) Schematic of differential gene expression analysis from a clinical trial (n=13) of CD19 CAR T cell therapy (tisa-cel) in refractory B cell lymphoma patients,

comparing responders (R, n=5) and non-responders (NR, n=8). Analysis identified 147 differentially expressed genes (DEGs) between R and NR groups (P adj < 0.01). (Haradhvala et, al., Nature, 2022) **(B)** Comparative analysis of transcriptional changes induced by different gene knockouts (KOs) versus wild-type (WT), showing overlap with tisa-cel responder/non-responder (R/NR) gene signatures. Bar segments represent the number of genes showing concordant or discordant regulation between KO conditions and clinical response patterns (|LFC| > 1, FDR < 0.01).

Safety Assessment of Target Gene Knockouts Reveals Limited Activation of Lymphoma-Associated Pathways

Given the therapeutic potential of genetic modifications in T cell therapy, assessing the oncogenic risk of target gene knockouts is crucial for clinical translation. This safety evaluation is particularly important as T cell lymphomas represent a significant concern in cellular therapy development, and understanding the potential oncogenic effects of genetic perturbations is essential for patient safety and regulatory compliance.

To evaluate the oncogenic potential of our genetic perturbations, we analyzed the expression patterns of established T cell lymphoma-associated genes across different knockout conditions. Using a gene set curated from recent lymphoma studies (Dai et al., PNAS, 2022), we assessed whether our target gene knockouts might inadvertently activate oncogenic programs.

The comprehensive analysis revealed that target gene knockouts generally maintained stable expression of lymphoma-associated genes, with limited evidence of oncogenic pathway activation. Most lymphoma-associated genes showed either unchanged or decreased expression across knockout conditions, suggesting a favorable safety profile for these genetic modifications.

However, we observed modest upregulation of two genes of potential concern: LYL1 and LMO2. While this finding warrants attention, contextual analysis from previous studies suggests that elevated expression of these genes may reflect early T cell progenitor states rather than necessarily indicating oncogenic transformation on differentiated cells (Dai et al., PNAS, 2022).

Several important caveats should be considered when interpreting these results. First, the oncogenic potential of individual gene knockout often requires complex interaction networks rather than single gene dysregulation. Second, our analysis primarily captured acute expression changes from the public datasets, and long-term expression dynamics remain to be characterized. Finally, the oncogenic implications of these expression patterns may vary depending on T cell state and developmental stage.

Overall, these findings provide initial safety insights for our genetic perturbation approach, though additional experimental validation will be essential to confirm the long-term safety profile. Future studies should focus on extended time-course analyses and evaluation of combinatorial effects in different T cell subtypes.

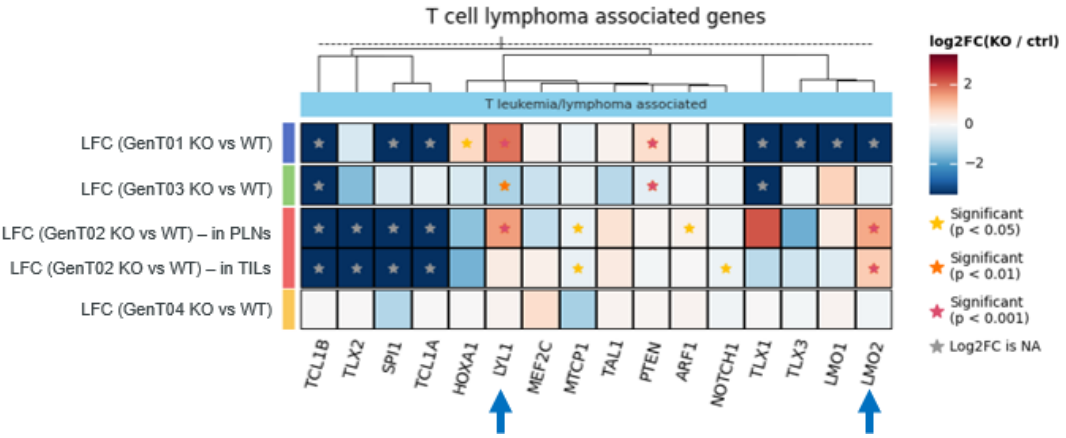


Figure 7. Impact of genetic perturbations on T cell lymphoma-associated gene expression. Heatmap showing differential expression analysis of T cell lymphoma-associated genes (Dai et al., PNAS, 2022) across various knockout conditions compared to wild-type controls. Log2 fold changes (LFC) are represented by the color scale, with red indicating upregulation and blue indicating downregulation. Statistical significance is denoted by asterisks (* [yellow] $p < 0.05$, * [orange] $p < 0.01$, * [red] $p < 0.001$), missingness of the gene is indicated by asterisks (* [grey] NA), and blue arrows highlight genes of particular interest (LYL1 and LMO2).

Conclusion

The in-depth transcriptional analysis of individual T cell KO reveals valuable insights into their impacts on T cell functionalities as well as therapeutic potential. By leveraging external transcriptomic data from diverse T cell models, this study has demonstrated the following observations: GenT01 knockout promotes a stemness and persistence-associated T cell state, while GenT03 knockout enhances T cell activation and suppresses terminal differentiation and exhaustion. Additionally, GenT02 knockout appears to upregulate some stemness associated genes, while also appears to suppress some terminal differentiation and exhaustion associated genes, whereas GenT04 knockout shows limited impact on T cell phenotypes with this approach.

Incorporating scRNA-seq data adds another layer of granularity to the analysis, revealing that GenT04 knockout tumor-infiltrating lymphocytes (TILs) in effector like and terminally differentiated exhaustion subtype exhibit reduced exhaustion and enhanced cytotoxic potential compared to control cells. This finding highlights the benefits of employing both bulk RNA and scRNA sequencing data, which can help us gain a more comprehensive understanding of gene expression patterns and cell-level heterogeneity within a sample.

However, the study also mentioned potential challenges associated with GenT03 KO. Despite enhancing in vitro tumor killing, GenT03 KO cells did not demonstrate improved in vivo efficacy, possibly due to the disruption of key T cell trafficking pathways and potential cell death induction. We observed an enrichment of apoptosis pathways as well as downregulation of critical chemokines, such as CCR6, CCL5, and CXCR3 ligands. These two findings could both potentially impair the ability of edited T cells to migrate to tumors, giving us the possible explanations for the discrepancy between in vitro and in vivo results.

In addition, the overlap between GenT01 and GenT03 KOs with the clinical CAR T cell response signatures is a key finding to the ACT clinical relevance. GenT01 KO suppresses non-responder genes, while GenT03 promotes responder genes, suggesting their potential to improve CAR T cell therapy outcomes.

From a safety perspective, the target KOs investigated in this study generally did not activate concerning levels of T cell lymphoma-associated genes. However, the modest upregulation of LYL1 and LMO2 observed in some conditions warrants further long-term evaluation to fully characterize the oncogenic potential of these genetic modifications.

In summary, this study provides key insights to guide the target gene selection of T cell edits for the ACT therapeutic development. GenT01's impacts on stemness and persistence, and GenT03's potential in vivo limitations, as well as the effects of other targets, all need further evaluation and discussion with more transcriptomic data. Afterall, this study could enable targeted T cell therapies with better efficacy and safety for solid tumor treatments.

Discussion

Strengths and Limitations

One of the key strengths of this study is the comprehensive approach in assessing the effects of target KOs by integrating transcriptomic data from external literatures. The utilization of both bulk and single-cell RNA-sequencing techniques provides a thorough understanding from the sample level as well as cell level.

Furthermore, the identification of gene edits overlapping with the clinical response, such as GenT01, which is associated with favorable patient responses in CAR T cell therapy trials, also strengthens the confidence in the potential for different target KOs to improve therapeutic outcomes in clinical settings.

However, it is also important to acknowledge several limitations of this study. Firstly, many of the cross-knockout comparisons inherently assess the effects of gene KOs in different T cell models and harvest time points. To better delineate the KO-specific differences, future studies should prioritize more controlled comparisons, where T cells are subjected to matched culture conditions, stimulation protocols, and time points. This approach would provide a cleaner readout of the effects attributable to each individual gene knockout.

Additionally, the clinical relevance analysis in this study relies on a single dataset from a CAR T cell therapy trial. To further solidify the clinical implications of the findings, it would be beneficial to expand the analysis to include additional datasets from CAR T, tumor-infiltrating lymphocyte (TIL), and T cell receptor-engineered T cell (TCR-T) studies. This

expansion would provide a more robust assessment of an edit's potential to generate clinically successful T cell products.

Lastly, the safety analysis presented in this study primarily focuses on evaluating acute gene expression changes associated with T cell lymphoma. To thoroughly characterize the oncogenic potential of these gene knockouts, long-term follow-up studies, especially in the context of combination edits, will be essential. Incorporating tumorigenicity studies in rodent models could further help mitigate safety concerns prior to clinical translation.

Future Directions

The insights gained from this thesis open up several promising avenues for future research. Firstly, conducting controlled comparisons of the effects of different gene knockouts on T cells internally, with matched culture conditions, stimulation protocols, and harvest time points. This approach would enable more precise delineation of the target KO impacts.

What's more, expanding the gene set enrichment analysis (GSEA) to include dual-knockout conditions, such as GenT01/GenT03 and GenT01/GenT02, could unveil potential synergistic or combinatorial effects that may not be apparent from the analysis of individual gene knockouts alone.

In addition, we could also incorporate additional clinical trial datasets, could be from CAR T, TIL, and TCR-T, etc. This would provide a more comprehensive assessment of clinical relevance associated with target KOs.

Moreover, extending the safety assessment to include long-term follow-up of edited T cells, particularly in the context of dual-knockout conditions, will be crucial for thoroughly evaluating the oncogenic risks associated with these genetic modifications.

Finally, the observed discrepancy between the in vitro and in vivo efficacy of GenT03 knockout T cells requires further investigation. Detailed in vivo studies assessing tumor infiltration, in situ apoptosis, and dynamic chemokine receptor expression in GenT03 knockout T cells could help elucidate the biological mechanisms underlying this difference in performance. These insights could inform the development of combination editing strategies or conditioning regimens aimed at rescuing the trafficking defect and enhancing the therapeutic potential of GenT03-edited T cells.

By integrating the transcriptomic interrogation presented in this thesis with the proposed functional validation and mechanistic studies, we aim to improve the efficacy and effectiveness of targeted T cell therapies. This approach holds great promise for developing T cell products with enhanced efficacy and safety profiles, ultimately benefiting solid tumor patients with more promising and personalized treatment.

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