**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 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 have uncovered edit-induced phenotypic changes in stemness, persistence, and functionality of edited cells. This thesis aims to comprehensively characterize a subset of these single genetic modifications using the transcriptomic read-outs 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 therapies. These strategies are designed to combat the challenges of the harsh tumor microenvironment, which include immunosuppressive cells such as Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T-regs), hypoxia, immune checkpoints like PD1/PDL-1, as well as inherent variability in T cell functionality across different patients. 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. This precision not only increases the safety profile of T cell therapies but also enhances their therapeutic efficacy by reducing antigen escape variants within the tumor. Comprehensive genetic screens and target validation efforts from BioNTech have pinpointed several key T cell genes—such as 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. This approach builds on findings from external studies that illustrate the critical roles of these genes in T cell phenotype signatures critical to clinical outcomes, namely T cell stemness and persistence, functional activation, and exhaustion.

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

1. **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 that indicate T cell longevity and regenerative capacity.
2. **Mechanism of Action (MoA) - Apoptosis and/or Tumor Trafficking Induction**: The impacts of target KOs on cytokines, chemokines, and their receptors associated with tumor homing are explored. The potential of target KO to induce mechanisms such as apoptosis or enhance immune cell trafficking to the tumor microenvironment is examined.
3. **Clinical Relevance**: Understanding the implications of gene KOs on cytokine and chemokine profiles that could influence tumor microenvironment dynamics, and the therapeutic response is critical.
4. **Safety Assessment**: The association of target KOs with oncogenic genes potentially leading to T cell transformation or lymphoma is assessed.

Overall, this study strives to establish more effective adaptive T cell therapies 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 captured transcriptomic readouts at multiple time points post-editing, utilizing high-throughput RNA sequencing and/or single-cell RNA sequencing to delineate the cellular phenotypes induced by specific gene KOs in diverse cell models.

*Gene-set signatures Analysis*

To better understand the impact of specific gene edits on T cell properties, the study examined gene set signatures related to T cell persistence/stemness, activation, as well as dysfunction/exhaustion/terminal differentiation phenotypes. This involved analyzing how genes in these categories were differentially expressed across different experimental setups and genetic backgrounds from a list of public curated literatures. **(Table 2)** Using Upset plots, a visualization technique for intersecting gene sets, the analysis provided a detailed look at the common and/or unique genes across multiple studies. **(Figure 1)** Many of these signatures have also proved associated with improved clinical response externally. For instance, previous studies showed that GenT01-deficient CAR-T cells initially showed a slower or delayed response to cancer cells, but over time, they demonstrated significantly better long-term persistence compared to the other CAR-T cells. Given that the study would provide with differentially expressed genes corresponding to the persistence phenotype vs. the wild type, then we will extract the genes and compile it up to the persistence related gene sets.

*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. 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 Seurat package (v.5.1.0) was used for data processing and analysis. Raw feature-barcode matrices generated by 10x Genomics Cell Ranger pipeline were read into R. Low quality cells and genes were filtered out based on the number of detected genes per cell, percentage of mitochondrial genes, and number of counts per gene.

The filtered count matrix was normalized using the "LogNormalize" method which normalizes gene expression measurements for each cell by total expression, multiplies by a scale factor, 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, harverst 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 conditions for each cluster. An adjusted p-value cutoff of 0.05 was used to determine significant gen

*Mouse-to-human gene name conversion.*

To reconcile the inconsistencies between the mouse and human gene names used in the various omics datasets, ortholog mapping was performed using the biomaRt package (v.2.62.0) in R/Bioconductor. 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. The analysis utilized previously curated gene set signatures, focusing on those related to T cell persistence/stemness, activation, and dysfunction/exhaustion/terminal differentiation. 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, 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.

*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*

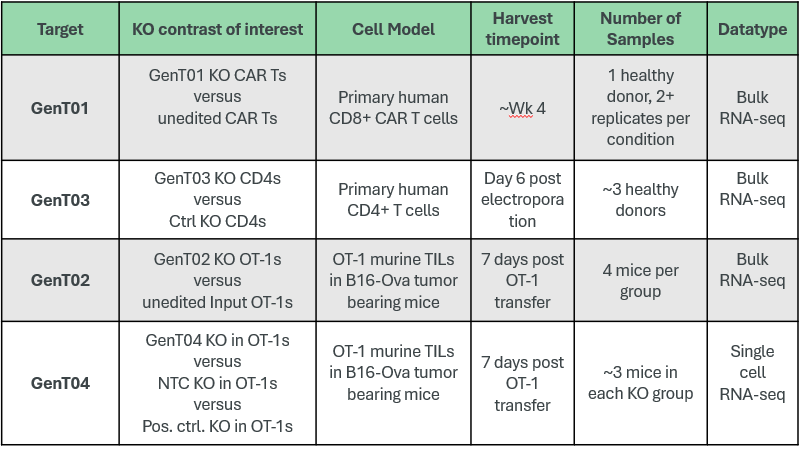
The differentially expressed gene lists (DEGs), resulted from the differential expression analysis, were then specifically employed to investigate the expression dynamics of gene profiles with known markers of T cell migration to tumor or sites of inflammation, chemokine and/or ligand receptors, in various target KOs condition compared to the control condition.

*Clinically Relevant gene expression assessment*

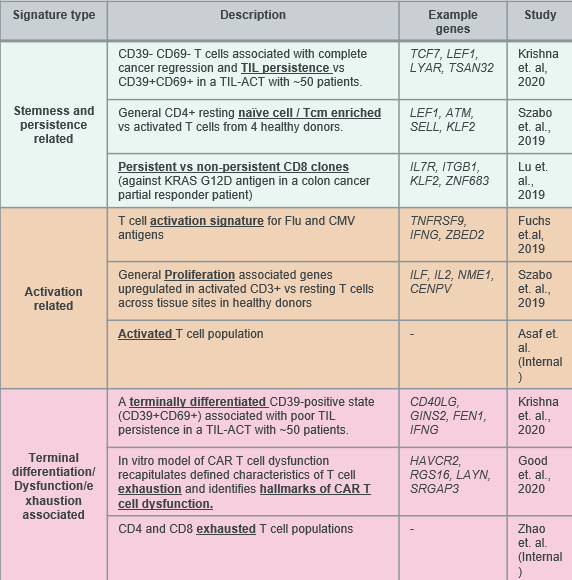
The analysis extended beyond transcriptomic profiling to include assessments of the clinical implications with the observed genetic perturbations. The impact of gene KOs on the T cells’ abilities migrating to tumors and their persistence within the hostile tumor microenvironment is further evaluated, by correlating gene expression profiles with the clinical outcomes from a study with clinical trial data. (*Haradhvala et. al., Nature, 2022*) The gene expressions from previously described DEGs overlapped with 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. By utilizing data from Dai’s study, which identifies genes associated with T cell lymphoma, this analysis investigates the expression of these genes in T cells that have undergone various genetic KOs. (*Dai et. al., PNAS, 2022*)

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**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.

**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, as well as 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.

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**C**

**A**

**B**

**Figure 1. Analysis of Overlap in Gene set signatures on T cell phenotypes of interest across studies.** 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 as well as 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.

**Results**

*GSEA reveals differential impacts of Target KOs on T cell functinoalities*

* *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 studies demonstrating 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 and suppresses terminal differentiation in T cells*

In CD4+ T cells, the GenT03 KO results in an enhancement of activation-association gene expression and a suppression of genes related to terminal differentiation as well as dysfunction and exhaustion**. (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 as a negative regulator of effector T cell functions, by suppressing the activation of NF-kB signaling pathway which potentially prevents the uncontrolled inflammation response that could lead to autoimmunity.

* *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-associated and persistence pathways, while also downregulating some terminal differentiation pathways. **(Figure 2C)** This result is further supported by other studies that GenT02 deficiency leads to CAR T cell persistence enhancement while increasing the formation of “precursor exhausted” T cells which are immunity-promoting.

Though not shown significance by FDR < 0.01 in the GSEA boxplot, the enrichment plots for some specific pathway can assist the 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 and 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 early published literature saying it works with TCF-1 as essential transcription factors that regulate the mature CD8+ T cell responses. GenT02 KO from another study demonstrated that it could improve long-term CAR-T cell persistence and function by increasing the expression of TCF1 and the formation of TPEX.  Similarly, in Krishna\_term\_diff (terminal differentiation) pathway, the NES is 1.04, with the marker genes, such as CD39, shown in positive gene ranking. **(Figure 2F)** CD39, also known as ENTPD1, is a well-known gene marker that characterizes CD8+ T cell dysfunction and exhaustion. What’s more, this finding also aligns with the discovery from the paper demonstrates that GenT02 induces a “long-term effector memory” like phenotype. However, 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*

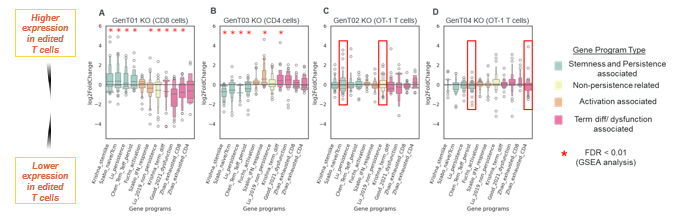
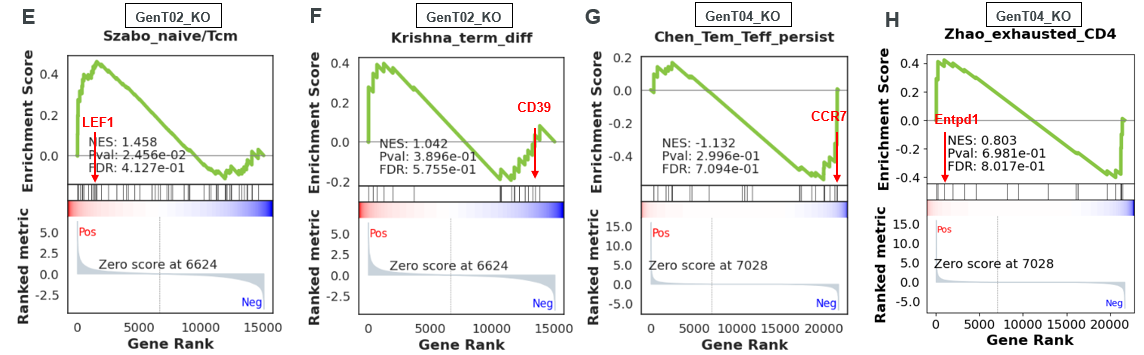
GenT04 KO in OT-1 T cells exhibited an overall downregulating trend in persistence/stemness, activation, and terminal differentiation/dysfunction pathways. However, these gene programs did not reach statistical significance based on the FDR threshold of 0.01 in the GSEA boxplot **(Figure 2D).**

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. Nevertheless, it was observed that CCR7, a known marker, was negatively ranked in the persistence-associated gene program, while Entpd1 was positively ranked in the exhaustion-associated gene program (Figure 2H). This suggests that GenT04 may have some impact on promoting T cell persistence and suppressing certain T cell exhaustion functionalities, although further investigation is needed to confirm these effects.

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.

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. To identify differentially expressed genes (DEGs) from the scRNA-seq data, a pseudo-bulk approach was employed. This method involves aggregating the gene expression counts from individual cells within each sample or condition to create a "pseudo-bulk" sample, which can then be analyzed using traditional bulk RNA-seq differential expression tools.

However, the pseudo-bulk approach may potentially exclude cells with low gene expression counts, leading to a possible loss of information about certain genes. This loss of information could limit the comprehensive understanding of the effects of GenT04 KO on T cell phenotypes. To mitigate this issue and gain a more complete picture of the gene expression changes induced by GenT04 KO, additional analyses on scRNA data specifically were performed. These analyses aimed to capture the full spectrum of gene expression alterations caused by GenT04 KO, including genes that may have been overlooked due to the limitations of 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; Pos, positively correlated; Neg, negatively correlated. Ranking metric scores reflect the correlation of each gene with the phenotype. 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 T cells derived from OT-1 mice, which had undergone genetic editing followed by transfer into recipient mice. The primary objective 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 multiple T cell subpopulations 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 clusters revealed varying expression levels of key genes such as Ccna2, Slamf6, Mcm2, Mk67, Gzmb, Ifng, Havcr2, and Lag3, indicating differences in cell cycle activity, stemness, and exhaustion (Figure 3C). These marker genes were manually curated and selected from the previous gene set signatures that cross-referenced with a wide range of external literatures. The genes selected represented the common genes that were shared from multiple studies. Notably, cells with the GenT04 KO exhibited lower levels 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 2D),** which align with the existing findings of the original paper (where the extracted data from) and supporting the hypothesis that GenT04 KO may augment cytotoxic functionalities by shown by increase expression of Gzmb while mitigating extensive T cell exhaustion.

To quantitatively assess exhaustion profiles, a module score approach was utilized, where lower scores corresponded to reduced exhaustion. GenT04 KO T cells demonstrated lower exhaustion module scores compared to controls, indicating a less exhausted state post-edit (Figure 2E). 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 2F).** These findings are in agreement with the other studies that demonstrate that modulating exhaustion-related genes can enhance the efficacy of adoptive T cell therapies.

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.

A diagram of a dna molecule

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**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 was particularly striking in the functional studies and became a major challenge for target KO selection, reported from the previous paper and experiments. While in vitro 3D spheroid assays demonstrated effective killing of GenT03 KO cells upon multiple T cell rechallenges, as indicated by decreased green fluorescence intensity, 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.

To investigate potential mechanisms underlying the discrepancy between the in vitro cytotoxicity and in vivo tumor killing efficacy observed with GenT03 knockout (KO) T cells, 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 **(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.

**A graph of gene rank

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**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*

Analysis of chemokine expression patterns 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 a potential mechanistic explanation for the previously observed discrepancy between in vitro and in vivo efficacy of GenT03 KO. While GenT03 deletion may enhance tumor cell susceptibility to T cell-mediated killing in vitro (as demonstrated by the apoptosis pathway enrichment), the concurrent disruption of T cell trafficking signals could impair effective T cell migration to tumors in vivo. This dual effect may explain why the enhanced killing observed in vitro does not translate to improved tumor control in vivo.

A table with text on it

Description automatically generated**A screenshot of a computer

Description automatically generatedFigure 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).

**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 established therapeutic approaches. 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 through combination approaches incorporating multiple genetic modifications.

A close-up of a chart

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**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 (Dai et al., PNAS, 2022).

Several important caveats should be considered when interpreting these results. First, the oncogenic potential of individual genes often requires complex interaction networks rather than single gene dysregulation. Second, our analysis primarily captured acute expression changes, 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.

**A diagram of a cell lymphoma

Description automatically generated**

**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), and blue arrows highlight genes of particular interest (LYL1 and LMO2).

**Conclusion**

The comprehensive transcriptional analysis of individual T cell knockout targets conducted in this study has yielded valuable insights into their distinct phenotypic effects and therapeutic potential. By leveraging external target perturbation transcriptomic data from diverse T cell models, this study has demonstrated that 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 modulate aspects of both stemness/persistence and terminal differentiation/exhaustion, whereas GenT04 knockout shows limited impact on T cell persistence, activation, or exhaustion pathways.

The incorporation of scRNA-seq data has provided an additional layer of granularity to the analysis, revealing that GenT04 knockout tumor-infiltrating lymphocytes (TILs) exhibit reduced exhaustion and enhanced cytotoxic potential compared to control cells. This finding underscores the value of employing high-resolution transcriptomic techniques to uncover subtle but potentially impactful changes in T cell phenotypes induced by genetic perturbations.

However, the analysis also uncovered potential challenges associated with GenT03 knockout. Despite enhancing in vitro tumor killing, GenT03 knockout cells did not demonstrate improved in vivo efficacy, possibly due to the disruption of key T cell trafficking pathways and potential cell death induction. The observed enrichment of apoptosis pathways and downregulation of critical chemokines, such as CCR6, CCL5, and CXCR3 ligands, in GenT03 knockout cells suggests an impaired ability to migrate to tumors, which could explain the discrepancy between in vitro and in vivo results. This finding highlights the importance of evaluating the multifaceted impact of genetic perturbations on T cell function, extending beyond their direct effects on cytotoxicity.

One of the most promising findings of this study is the overlap between GenT01 and GenT03 knockouts and clinical CAR T cell response signatures. The substantial suppression of non-responder genes by GenT01 knockout and the upregulation of responder genes by GenT03 knockout indicate their potential to improve patient outcomes in the context of CAR T cell therapy. These results underscore the translational relevance of the transcriptional interrogation approach employed in this study and its ability to identify genetic modifications that could enhance the clinical success of T cell-based therapies.

From a safety perspective, the target knockouts 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. This finding emphasizes the need for rigorous safety assessments that extend beyond acute gene expression changes to ensure the long-term safety of genetically engineered T cell therapies.

In summary, this transcriptional interrogation provides crucial preclinical insights that can guide the selection and prioritization of individual and combination T cell edits for further therapeutic development. While highlighting the promising effects of targets like GenT01 in promoting stemness and persistence, the study also uncovers potential limitations of other targets, such as GenT03, which may require careful consideration and the development of mitigation strategies in future translational studies. By integrating these findings with the proposed functional validation and mechanistic studies, this work lays the foundation for advancing the next generation of targeted T cell therapies with enhanced efficacy and safety profiles for solid tumor patients in need.

**Discussion**

*Strengths and Limitations*

One of the key strengths of this study lies in its comprehensive approach to assessing the effects of individual gene knockouts on T cell phenotypes by integrating transcriptomic data from diverse T cell models, genetic perturbation target, and harvest time points. The utilization of both bulk and single-cell RNA-sequencing techniques provides a multi-faceted perspective on the edit-induced changes, enabling a more thorough understanding of the cellular processes affected by each knockout.

Furthermore, the study's effort to benchmark the findings against established clinical response signatures enhances its translational relevance. The identification of gene edits, such as GenT01, that closely mimic the gene expression patterns associated with favorable patient responses in CAR T cell therapy trials, strengthens the confidence in their potential to improve therapeutic outcomes in clinical settings.

However, it is important to acknowledge several limitations of this study. Firstly, many of the cross-knockout comparisons inherently assess the effects of gene knockouts in T cells at different states and time points. To better delineate the knockout-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 subjected to matched culture conditions, stimulation protocols, and time points would enable a more precise delineation of the edit-specific effects on T cell functionality. This approach would help identify the key cellular processes and pathways uniquely affected by each gene knockout, facilitating the selection of optimal gene targets for T cell engineering.

Secondly, 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. This exploration could highlight the most promising editing strategies for enhancing T cell function and inform the development of next-generation T cell therapies.

Thirdly, broadening the clinical benchmarking analysis by leveraging additional datasets from CAR T, TIL, and TCR-T clinical trials would provide a more comprehensive assessment of an edit's potential to generate clinically successful T cell products. Integrating these findings with prognostic biomarker data could further refine the understanding of the clinical impact of specific gene knockouts and guide the prioritization of gene targets for translational studies.

Fourthly, 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. Conducting tumorigenicity studies in rodent models could provide additional evidence to mitigate safety concerns and support the clinical translation of these gene-edited T cell therapies.

Finally, the observed discrepancy between the in vitro and in vivo efficacy of GenT03 knockout T cells warrants 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 can refine and advance the next generation 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 in need of more effective treatment options.

**References (APA)**

1. Dai et al., Nat.. Biot. 2023
2. Friemer et. al, Nat. Gen, 2022
3. Wei et al., Nat., 2019
4. Schlabach et al.,JCI, 2024
5. Krishna; et. al., Science, 2020
6. Szabo et. al., Nat. Comm., 2019
7. Lu et. al., CIR AACR, 2019
8. Chen et. al., Can. Disc., 2021
9. Fuchs et. al., Front. Imm., 2019
10. Haradhvala et. al., Nature, 2022
11. McCutcheon et. al., Nat. Gen., 2023
12. Kohli et. al., Nat. CGT, 2021
13. Kendirli et. al., Nat Neuro. 2023
14. Jin et. al., Elsevier., 2021
15. Chiffelle et. al., Immunity, 2024
16. Dai et. al., PNAS, 2022
17. UMass Kelliher Lab studies