Identification of IL-4 as a Regulator of CAR T-Cell Exhaustion Using Functional Genomics and Correlates of the Zuma-1 Clinical Trial

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Study Objectives

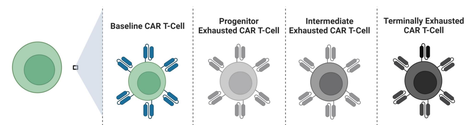
While chimeric antigen receptor (CAR) T-cell therapy targeting CD19 (CART19) has shown remarkable overall response rates in the treatment of hematological cancers, the durable responses are low[1]. One proposed mechanism of resistance includes T-cell exhaustion[2]. As such, we utilized the following four independent approaches to investigate the epigenetic regulation of exhaustion:

1. **RNA and ATAC sequencing on baseline and exhausted healthy donor CART19-28ζ cells**
2. **RNA and ATAC sequencing on pre-infusion patient-derived CART19 cells from responders and non-responders in the Zuma-1 clinical trial**
3. **A genome-wide CRISPR knockout screen with healthy donor CART19-28ζ cells that have undergone our *in vitro* exhaustion assay**
4. **Functional validation studies for lead exhaustion driver genes**

Relevant Background

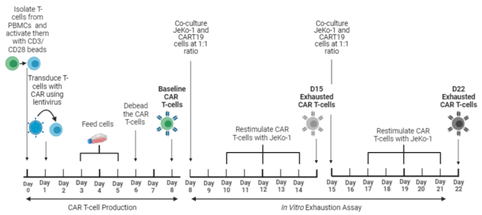
T-cell exhaustion is an acquired state of dysfunction that is characterized by[3]:

- Decreased proliferation.
- Loss of $CD4^+$ T-cells.
- Increased expression of inhibitory receptors.
- Reduced production of effector cytokines such as IL-2 and TNF-α.

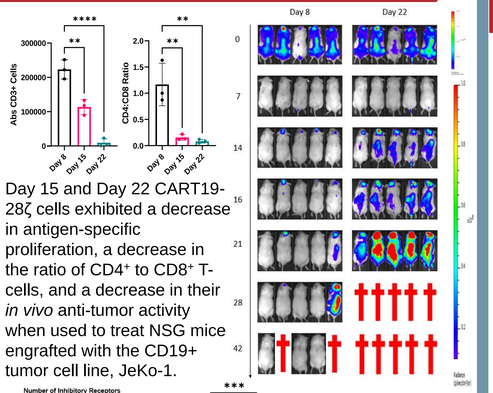


	Baseline CAR T-Cell	Progenitor Exhausted CAR T-Cell	Intermediate Exhausted CAR T-Cell	Terminally Exhausted CAR T-Cell
Proliferation	+++	++	+	+/-
CD4/CD8 Ratio	++	-	-	-
Expression of Inhibitory Receptors	-	+	++	+++
Cytokine Production	+++	+	+/-	+/-

In Vitro Model For Exhaustion



Validation Of Exhaustion Model

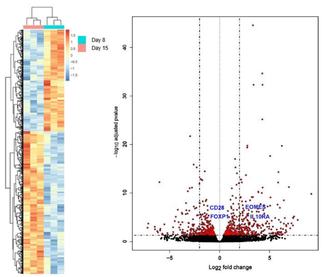


Day 15 and Day 22 CART19-28ζ cells exhibited a decrease in antigen-specific proliferation, a decrease in the ratio of $CD4^+$ to $CD8^+$ T-cells, and a decrease in their *in vivo* anti-tumor activity when used to treat NSG mice engrafted with the CD19+ tumor cell line, JeKo-1.

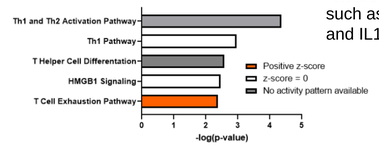
Day 15 and Day 22 CART19-28ζ cells also displayed signs of exhaustion such as an increase in the expression of inhibitory receptors (PD-1, TIM-3, CTLA-4, and LAG-3) and a decrease in the production of IL-2 and TNF-α after stimulating them for four hours with JeKo-1 cells at a 1:5 ratio.

These schematics depict results from three biological replicates. (*p<0.05, **p<0.01, ***p<0.001)

1) Epigenetic and Transcriptomic Landscape of Exhaustion



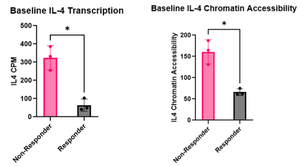
Comparing RNA sequencing of Day 8 to Day 15 samples, we observed the development of a distinct transcriptomic profile that is characterized by an upregulation in known exhaustion-related genes such as EOMES and IL10RA.



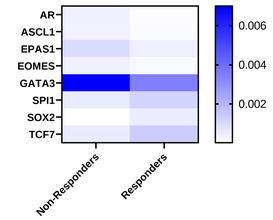
Top Upstream Regulators		
Name	**p-value**	**Molecule Type**
IL-2	**2.13E-10**	**Cytokine**
IL-15	**1.25E-09**	**Cytokine**
Tcf7*	**1.57E-07**	**Transcription Regulator**
ITK	**8.41E-07**	**Kinase**
IL-4	**5.05E-06**	**Cytokine**

Ingenuity pathway analysis of the genes that are both differentially expressed and accessible when comparing Day 8 to Day 15 cells showed an enrichment in the T-cell exhaustion pathway and identified IL-2, Tcf-7, and IL-4 as potential upstream regulators.

2) Clinical Trial Correlates to CAR T-Cell Dysfunction

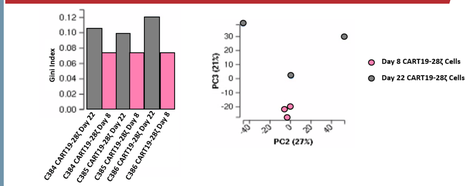


After performing RNA and ATAC sequencing on baseline Axi-cel products from 3 responders and 3 non-responders in the Zuma-1 clinical trial, only two genes are both upregulated and more accessible in the baseline non-responder products, IL-4 and HLA-DQB1.



To determine the differential role of transcription factors in baseline Axi-cel products from responders and non-responders, we used a previously established multi-omics data analysis framework, Taiji[4], to reveal an enrichment in GATA3 and EOMES in patient non-responders.

3) Identifying Key Genes in the Development of Exhaustion with a Genome-Wide CRISPR Screen

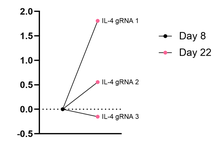


Using our *in vitro* model for exhaustion, we completed a genome-wide CRISPR screen with three healthy donor T-cells. As seen with the gini index for each sample, positive selection of gRNAs occurred by Day 22. In addition, PCA analysis showed clustering based on sample timepoint as opposed to biological replicates.

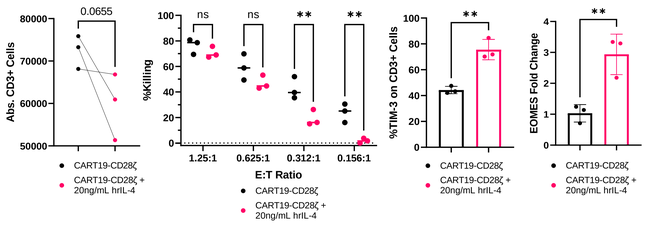
	P-Value
Regulation of JAK-STAT Cascade	**1.22E-5**
Nucleoside Transport	**5.17E-5**
Regulation of Interferon-Gamma-Mediated Signaling Pathway	**7.14E-5**
Negative Regulation of Interleukin-2 Mediated Signaling Pathway	**1.08E-4**
Negative Regulation of Interleukin-4-Mediated Signaling Pathway	**1.08E-04**

To investigate the pathways that are enriched through positive selection, we performed gene ontology enrichment analysis. This revealed an enrichment in genes involved in the IL-2 and IL-4 pathways. In particular, we saw an enrichment in 2/3 gRNAs targeting IL-4 in Day 22 samples as compared to Day 8 samples.

Average Fold Change of IL-4 gRNAs



4) Validation Of IL-4 as a Regulator Of CAR T-cell Dysfunction



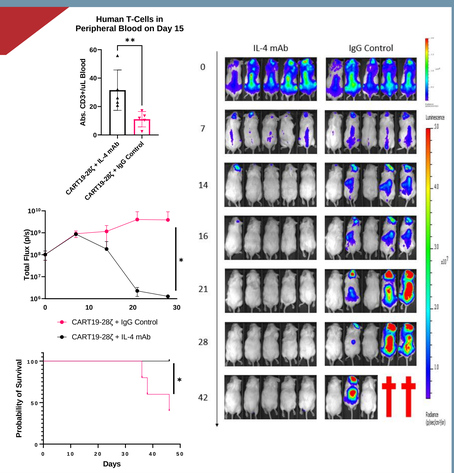
To validate IL-4 induced CAR T-cell dysfunction *in vitro*, we treated CART19-28ζ cells with either diluent or 20ng/mL human recombinant IL-4 (hrIL-4). Treatment with hrIL-4:

- Reduced proliferative ability and cytotoxicity
- Increased the expression of the inhibitory receptor, TIM-3, and the transcription of the known exhaustion-related transcription factor, EOMES, by Day 15.

These schematics depict results from three biological replicates. (*p<0.05, **p<0.01, ***p<0.001)

To evaluate if IL-4 inhibition in combination with CART19-28ζ cell therapy can prevent the development of exhaustion and therefore improve overall efficacy, we treated Luciferase+ JeKo-1 xenograft NSG mice with either Day 8 CART19-28ζ cells + 10 mg/kg IL-4 monoclonal antibody (mAb) or with Day 8 CART19-28ζ cells + 10 mg/kg IgG Control. IL-4 inhibition with mAb treatment resulted in:

- Increased *in* vivo CAR T-cell proliferation
- Decreased tumor flux
- Increased overall survival



Conclusions and Innovations

While investigating the development of CAR T-cell exhaustion, our lab has:

- **Developed an *in vitro* model for exhaustion.**
- **Identified IL-4 as a key regulatory gene in CAR T-cell dysfunction through three independent approaches.**
- **Showed the induction of CAR T-cell dysfunction *in vitro* when CAR T-cells were treated with hrIL-4.**
- **Improved the antitumor activity of CART19-28ζ cells *in vivo* through a combination treatment with an IL-4 mAb.**

These findings not only suggest a new role for IL-4 in CAR T-cell dysfunction, but they also could provide a translatable approach to improve the durable response to CART19 cell therapy

References and Acknowledgements

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