AWARD NUMBER: W81XWH-19-1-0761

TITLE: Transcription Control of Exhaustion Program in CAR T-Cell Therapy

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CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center
Houston, TX

REPORT DATE: December 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2022	Final	01Sep2019-31Aug2022

4. TITLE AND SUBTITLE

Transcription Control of Exhaustion Program in CAR T-Cell Therapy

5a. CONTRACT NUMBER
W81XWH-19-1-0761

5b. GRANT NUMBER
GRANT12713485

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The proposed research plan directly addresses the urgent need for novel therapies by developing the next generation of targeted T cells with chimeric antigen receptors (CARs) for use in therapy of pancreas cancer. The significance of our new strategy aimed at optimizing CAR T cell therapy is emphasized by the lack of clinical success in solid tumors. This project layout the strategy of "armoring" CAR-T cells with genetic modifications in transcriptional "master regulators" that control T cell exhaustion and/or longevity to generate a long-lasting response against the tumor. Utilizing a clinically relevant immune-competent endogenous mouse model of pancreatic cancer, we will assess the effect of enhanced "armored" CAR-T cells on the tumor and how their function will persist compared with unmodified CAR-T. Defining factors that would allow CAR T cells to be genetically "reprogrammed" to maintain an active state is critical for successful use of CAR T cells for patients with pancreatic cancer who currently lack other treatment options. The core discovery driving methods and technical innovations are mutually synergistic and thus will efficiently spearhead my research and provide collaborative opportunities thus enhance prospects of my research and career development.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	19	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers for which no effective therapy has been identified. Our proposed research plan directly addresses the urgent need for novel therapies by developing the next generation of targeted T cells with chimeric antigen receptors (CARs) for use in therapy of PDAC. CAR-T cells show activity in a number of pilot clinical trials and they have significant potential for therapy of many cancers that are currently incurable however, are largely ineffective in PDAC due to T cell exhaustion. Our overarching premise is that CAR-T cell exhaustion, which limits function in PDAC, is regulated by specific changes in gene expression, and that those changes can be prevented by modulating the transcription factors that drive the changes First, we will identify and validate transcriptional "master" regulators" of CAR-T cell exhaustion. Our preliminary computational analyses identified genetic signature of over 700 genes specifically regulated in exhausted T cells, including 50 transcription factors (TFs), 13 of which show significant correlation with biomarkers of exhaustion. We will test the identified TFs by modulating their expression in CAR-T cells and analysis of cancer killing properties. Next, we will decipher the function of the identified TFs in driving the specific CAR-T cell exhaustion phenotype. Analysis of hallmarks of exhaustion and RNA-seq to determine the effect of our modifications on expression of exhaustion signature genes will be used to define potentially non-overlapping and/or synergistic functions of each identified TF. Finally, we will rigorously evaluate the potency, safety and limitations of synthetically enhanced CAR-T cells in pre-clinical mouse models of PDAC. Based on our experience and preliminary data we are confident that the proposed research will yield significantly improved CAR-T cells that could be rapidly translated to the clinic. Together, these experiments will identify molecular mechanisms that currently limit the use of CAR-T cells for PDAC therapy.

2. KEYWORDS:

Pancreatic cancer, chimeric antigen receptors

3. ACCOMPLISHMENTS:

Several genes that were highly expressed in pancreatic cancer have been proposed as immunological targets for PDAC. By immunostainings, we initially selected Mesothelin (MSLN) as the most universally and strongly expressed in PDAC and cancer cell lines but absent in normal pancreas. This observation was further confirmed by reanalyzing single-cell RNA-seq dataset of human PDAC patients (**Fig. 1**). MSLN was the only gene that was highly enriched in malignant epithelial cells but not any other cell types.

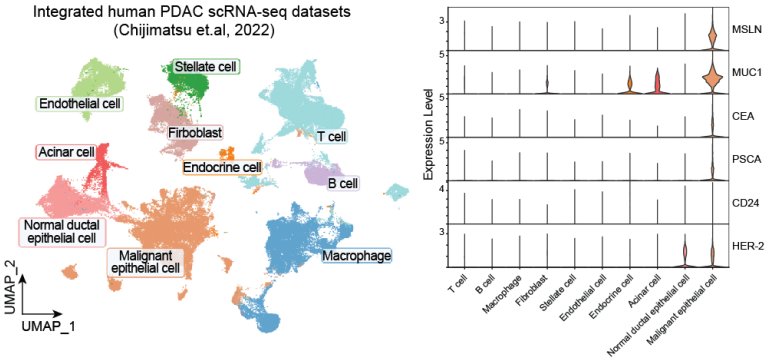

Figure 1. Expression of common CAR T targets in human pancreatic cancer patients (single-

Specific Aim 1 -- To test the effect of TF genetic modification on CAR-T efficacy

Major Task 1 *– Generate and validate mouse and human chimeric antigen receptor expressing T-cells targeting human and mouse MSLN expressing cancer cells.*

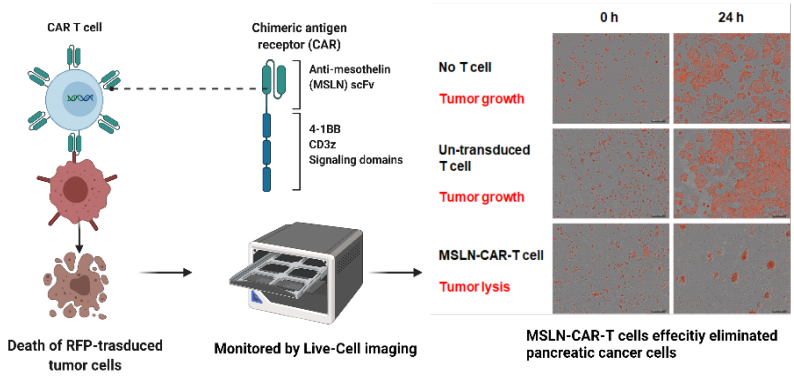

Figure 2. Live-cell imaging of pancreatic cancer killing mediated by MSLN-CAR-T cells.

We've successfully generated a CAR-T construct that is composed of an anti-mesothelin (MSLN) scfv, 4-1BB costimulatory domain and CD3z signaling domain. This CAR can recognize both human and mouse mesothelin even when the antigen is low, owing to its high affinity. When transduced into primary T cells, the CAR could lyse MLSN-positive pancreatic cells (**Fig. 2**). The fact that a control CAR recognizing CD19 did not lead to cancer cell killing, together with the observation that the MSLN-CAR-T cells didn't target MSLN-knockout cells, confirmed the effectiveness and specificity of our MSLN-CAR design (**Fig. 3**).

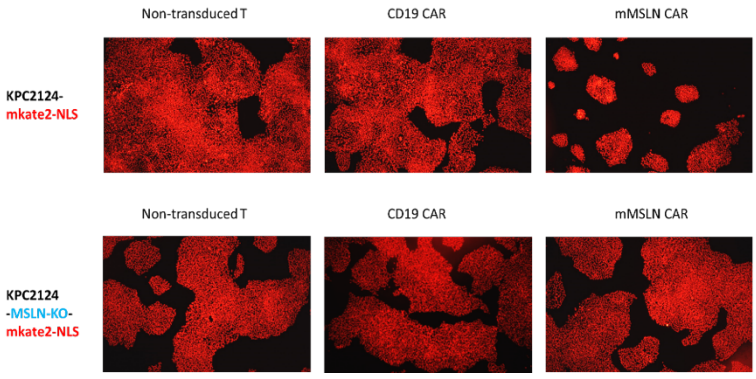

Figure 3. Validation of the specificity of MSLN-CAR-T cells using MSLN-knockout pancreatic cancer cells.

Major Task 2 *– Generation of CAR T cells with overexpression or downregulation of the identified TFs.*

We employed a novel approach to suppress transcription factor PRDM1, a potential master regulator of T cell exhaustion based on our computational analysis, by introducing a dominant-negative PRDM1 (DN-PRDM1) to compete with the endogenous protein. Luciferase reporter assay confirmed that the DN-PRDM1 was able to attenuate the reporter suppression caused by wild-type PRDM1 (**Fig. 4**).

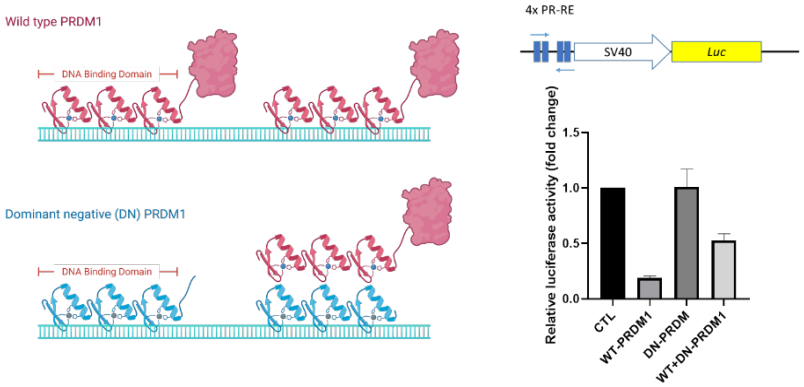

Figure 4. Development and validation of a dominant-negative PRDM1 to suppress the transcriptional activity of endogenous PRDM1

Meanwhile, we developed a strategy to co-delivery the MSLN CAR module and shRNAs targeting another essential transcription factor IRF8 governing the T cell exhaustion program. IRF8 was successfully depleted in human primary CAR-T cells (**Fig. 5**).

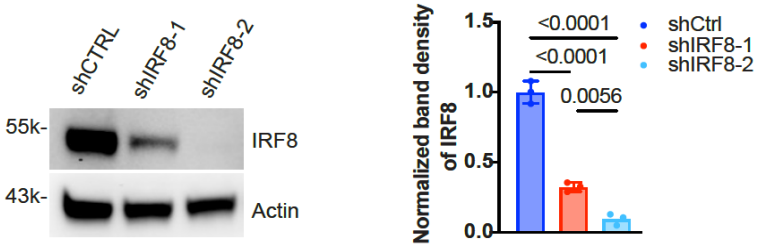

Figure 5. Efficient IRF8 knock down in human primary CAR T cells

Specific Aim 2 – To test the effect of TF genetic modification on CAR-T exhaustion phenotype

To test the performance of TF genetic modification on CAR-T exhaustion phenotype in a human primary T cell setting, we established an *ex vivo* CAR-T exhaustion model similarly as reported previously (Nature, 2019). In brief, a GD2-CAR that induces tonic signaling was introduced into human primary T cells, separately with a control CD19-CAR (without tonic signaling). The tonic signaling mimics repeated stimulation that leads to T cell exhaustion. Compared with CD19-CAR-T cells, GD2-CAR-T cells developed exhaustion features including elevated expression of immune checkpoints PD-1, LAG-3 and TIM-3 (**Fig. 6**).

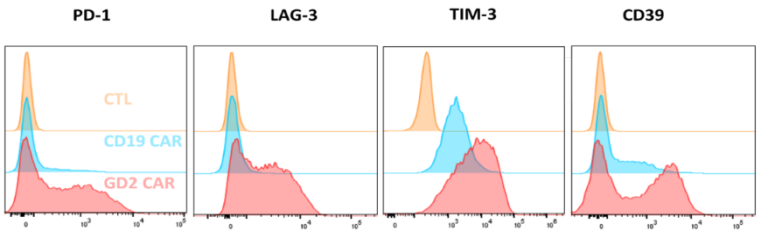

Figure 6. GD2-CAR-T cells shows signs of T cell exhaustion.

Major Task 3- *Measure levels of effector cytokines secretion*

We next took advantage of the human GD2-CAR-T exhaustion model to study the effect of transcription factor editing in human primary T cells. T cell exhaustion is characterized by diminished or markedly decreased cytokines production after stimulation. We examined the ability to secrete interferon gamma (IFNg) and IL-2 in control or DN-PRDM1-bearing GD2-CAR-T cells. As expected, GD-CAR-T cells showed deficiencies in the production of both IFNg and IL-2, compared with the control CD19-CAR-T cells. DN-PRDM1 effectively rescued IFNg and IL-2 production in GD2-CAR-T cells (**Fig. 7**), suggesting a reversal of T cell exhaustion. Using a similar system, we also examined the secretion of those effector cytokines in IRF8-knockdown CAR-T cells. Two different IRF8-targeting shRNAs consistently elevated the levels of IL-2, TNF-a and IFN-g (**Fig. 8**). Since T cell exhaustion is also featured by the compromised self-renew capacity, we also monitored cell growth changes between CAR T cells carrying control (shGFP) or IRF8 (shCtrl) shRNAs. We noticed that the CAR-positive fraction elevated gradually when IRF8 got knockdown, while knocking down an irrelevant protein (GFP) caused minimal effects. Notably, when challenged with tumor cells, only IRF8-knockdown cells showed a sustained ability to proliferate, suggesting a superior anti-tumor capacity (**Fig. 9**).

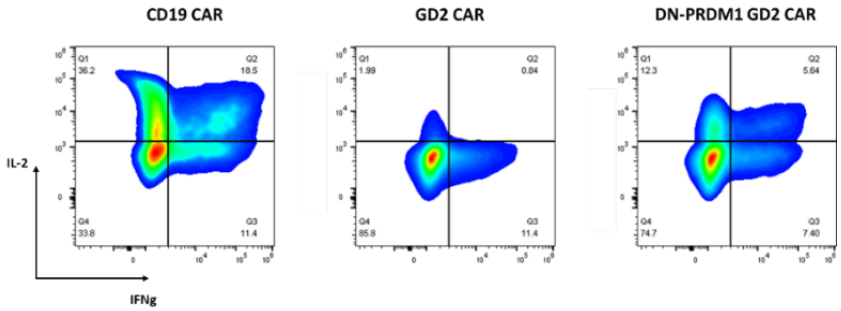

Figure 7. DN-PRDM1 restores cytokines secretion in human CAR T cells

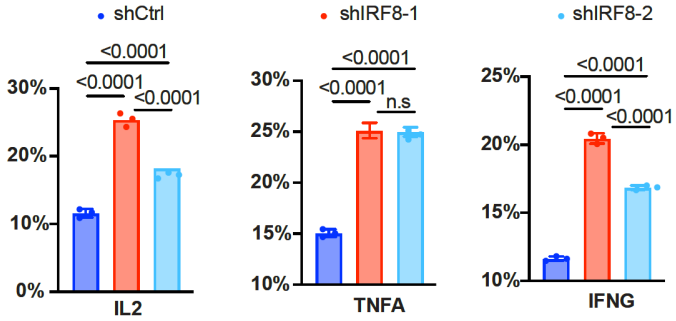

Figure 8. IRF8 knockdown attenuates cytokines diminishing during CAR T exhaustion

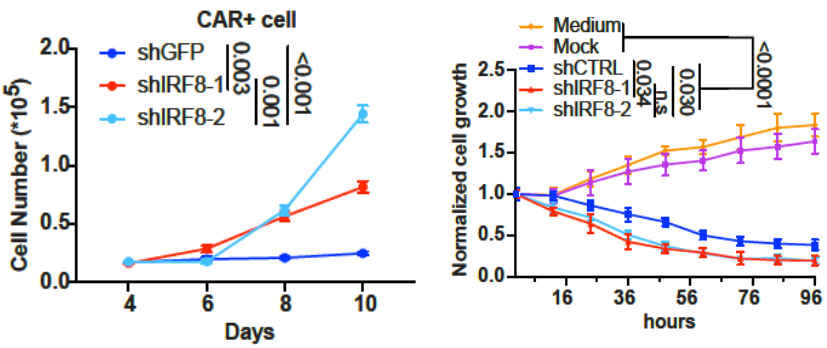

Figure 9. IRF8 knockdown attenuates cytokines diminishing during CAR T

Major Task 4- *Quantify expression of inhibitory immune checkpoint receptors in modified CAR T cells*

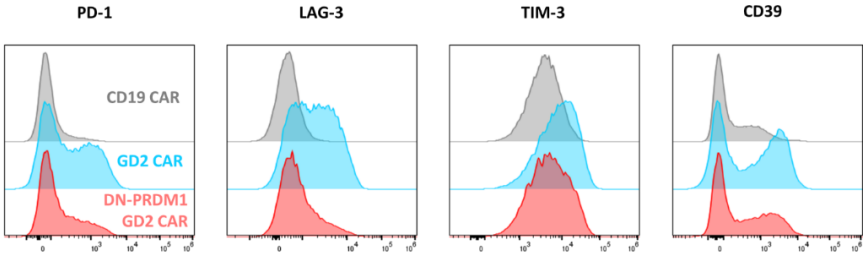

Figure 10. DN-PRDM1 reduces immune checkpoints expression in CAR-T cells

We further profiled CAR-T cells carrying DN-PRDM1 or shIRF8 by immune checkpoint receptor staining using flow cytometry. Compared with control GD2-CAR-T cells, DN-PRDM1-GD2-CAR-T cells and shIRF8-GD2-CAR-T cells exhibited drastically reduced expression of PD-1, LAG-3, TIM-3 and CD39, demonstrating action of exhaustion-prevention by DN-PRDM1 (**Figs. 10 and 11**).

Major Task 5- *Evaluate biomarkers of cytolytic activity and T cell activation and exhaustion markers*

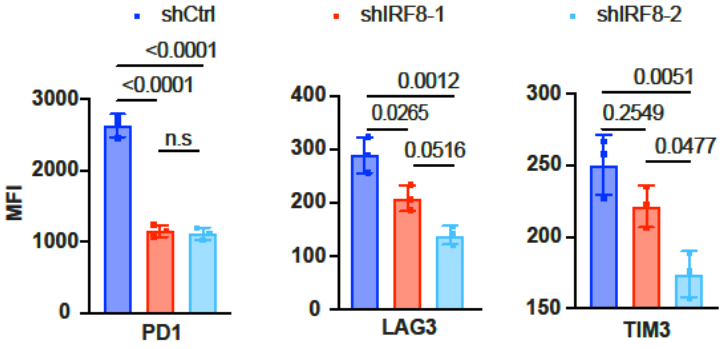

Figure 11. IRF8 knockdown reduces immune checkpoints expression in CAR-T cells

To eliminate the possibility that modifying the transcriptional factors suppressed T cell exhaustion by indirect mechanism, such as affecting the CAR expression level, we introduced a protein degradation system (dTAG) to conduct targeted DN-PRDM1 degradation. After adding the dTAG ligand, the dTAG-DN-PRDM1 protein underwent quick degradation in human T cells (**Fig. 12**). Degradation of DN-PRDM1 did not change the expression level of the GD2 CAR, while it led to much higher expression of immune checkpoints compared with CAR-T cells carrying persistent DN-PRDM1, providing direct evidence that DN-PRDM1 could directly inhibit T cell exhaustion (**Fig. 13**).

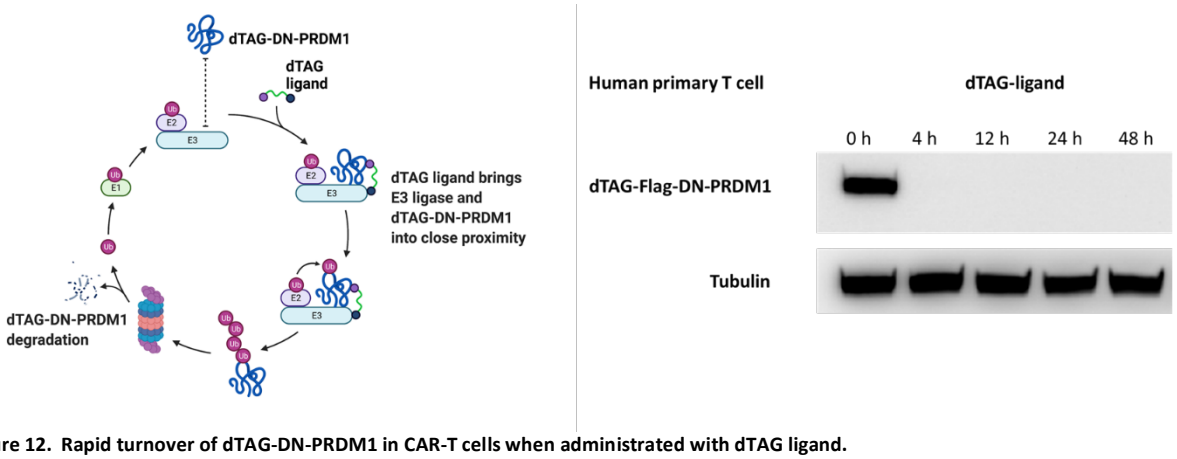

Figure 12. Rapid turnover of dTAG-DN-PRDM1 in CAR-T cells when administrated with dTAG ligand.

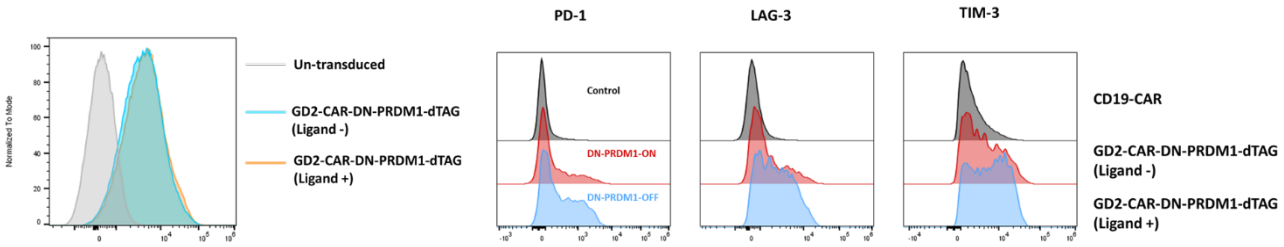

Figure 13. The dTAG system enables fair comparison between DN-PRDM1-ON and DN-PRDM1-OFF CAR-T cells, further supporting the exhaustion-prevention role of DN-PRDM1.

Major Task 6 - *Evaluate global gene expression in modified CAR T cell by RNA-seq*

To assess genome-wide transcriptional changes between PRDM1-deficient and control cells, single-cell RNA-seq was performed on GD2-CAR T cells carrying the dTAG-DN-PRDM1, treated with or without the dTAG ligand (**Fig. 14**), which enables direct comparison of the transcriptomes in the presence or absence of DN-PRDM1. Gene set enrichment analysis (GSEA) revealed enrichment of naive/memory-associated genes and downregulation effector-related genes when DN-PRDM1 was present in the CAR T cells (**Fig. 15**). These data strongly suggest that DN-PRDM1 was associated with a less differentiated T cell state.

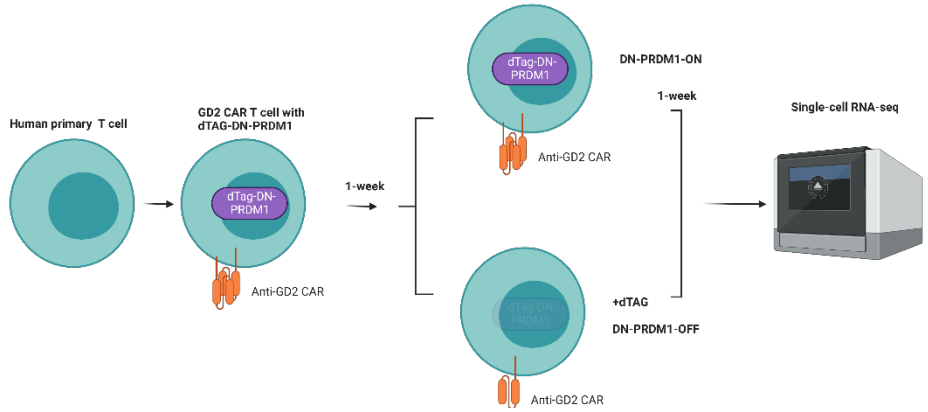

Figure 14. Schematics of single-cell RNA-seq of DN-PRDM1-ON/OFF CAR T cells

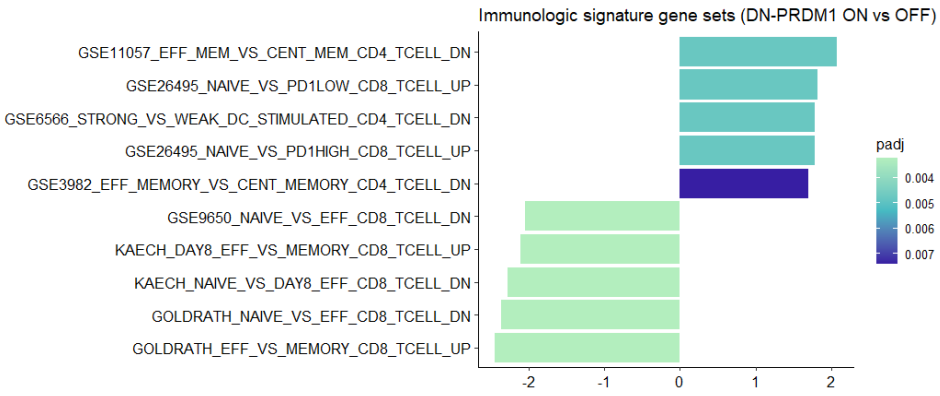

Figure 15. Schematics of single-cell RNA-seq of DN-PRDM1-ON/OFF CAR T cells

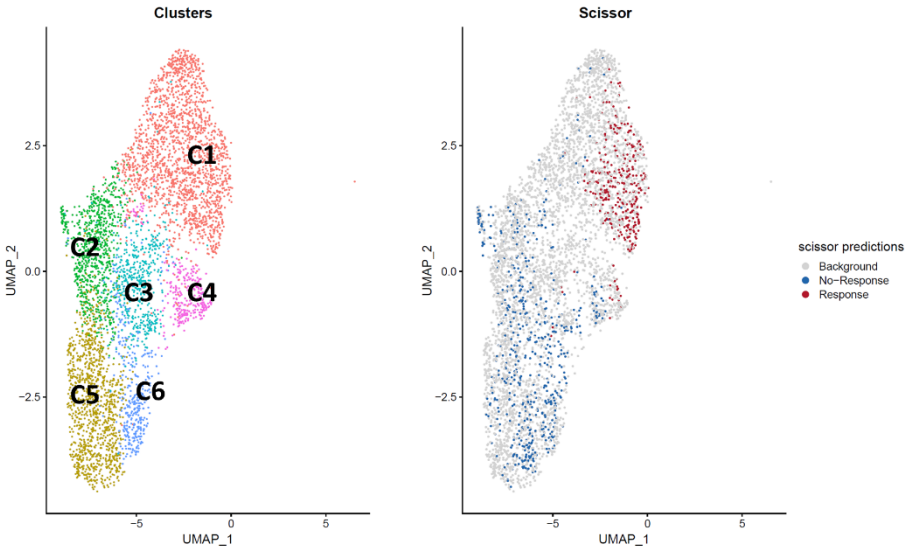

Figure 16. Transcriptome-based UMAP clustering of single-cell expression profiles (left) and the Scissor predicted response (right).

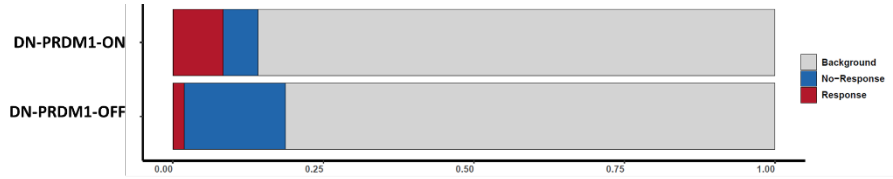

Figure 17. The proportion of CAR T cells associated with the Scissor-predicted clinical response

UMAP dimensional reduction followed by unsupervised clustering revealed that cells representing DN-PRDM1 ON/OFF modalities clustered into 6 distinct transcriptome profiles. Next, to associate the ex vivo CAR T cell transcriptional states with clinical outcomes, we applied a recently developed computational algorithm Scissor (Nat. Biotechnol, 2021) and tumor-infiltrating lymphocytes (TILs) scRNA-seq dataset from melanoma patients treated with PD-1 checkpoint therapy (Cell, 2018) as validated clinical benchmarks. Different cell clusters showed a diverse distribution of responding and non-responding potentials (**Fig. 16**). In line with the previous findings, When DN-PRDM1 was on, more responding and fewer non-responding cells were observed (**Fig. 17**). All the results imply that applying DN-PRDM1 could mimic a functional "PRDM1-low" status and may improve the efficacy of T cell therapy in cancer patients.

Specific Aim 3 – To test the effect of TF genetic modification on CAR-T exhaustion and potency in pre- clinical models of PDAC

Major Task 7 – *Quantify markers of exhaustion in modified CAR-T infiltrating PDAC tumors*

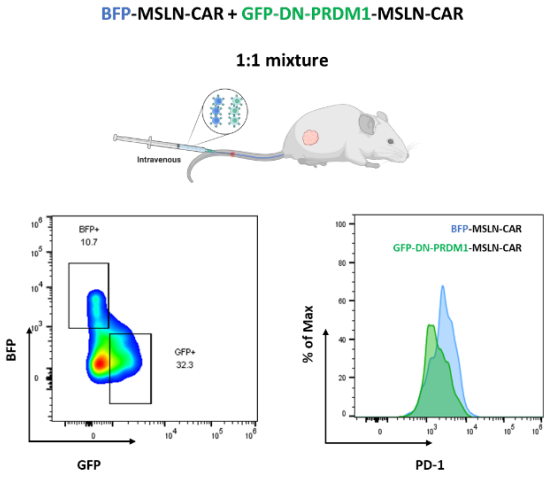

Figure 18. Competition *in vivo* between control MSLN-CAR and DN-PRDM1-MSLN-CAR show superior expansion of MSLN-CAR-T cells and reduced expression of immune checkpoint PD-1.

To enable a head-to-head comparison of control and DN-PRDM1 CAR-T cells *in vivo*, we designed a competition assay by introducing a BFP and a GFP into these two CAR-T groups. The transduced cells were mixed at 1:1 ratio and injected into NSG mice carrying subcutaneous xenografts of pancreatic cancer cells. TILs isolated from the tumors were subjected to flow cytometry. A higher fraction of GFP$^+$ DN-PRDM1-CAR-T cells compared with BFP-CAR-T cells was observed. The GFP$^+$ cells also exhibited a lower expression level of PD-1, implying the DN-PRDM1 CAR-T cells were less exhausted *in vivo* (**Fig. 18**).

Major Task 8 – *Quantify PDAC tumor killing and survival benefit of modified CAR-T*

NSG mice carrying human PDAC tumors were treated with conventional MSLN-CAR-T cells or DN-PRDM1-MSLN-CAR-T cells. CAR-T cells with DN-PRDM1 led to significant tumor suppression and survival extension (**Fig. 19**).

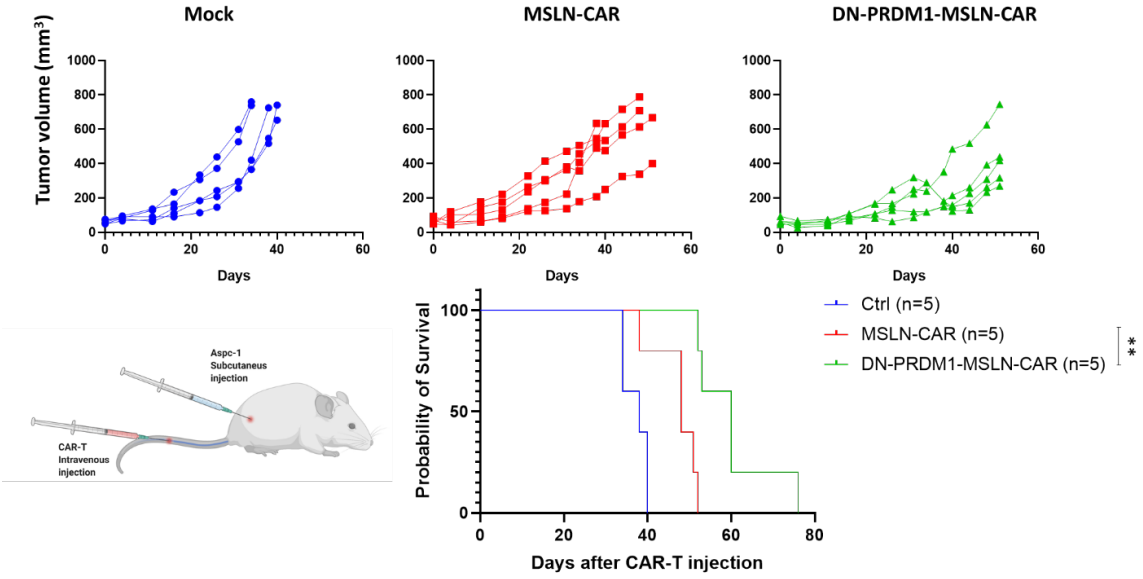

Figure 19. DN-PRDM1-CAR-T cells suppress tumor growth and improves survival in a subcutaneous xenograft model of

The strong beneficial phenotype of CAR T cells by inhibiting PRDM1 also promoted us to seek alternative methods to manipulate the expression of PRDM1. As reported previously BRD4 (an epigenetic regulator) occupies the super-enhancer region of PRDM1 (Cell, 2013). We therefore tested if BRD4 inhibitor JQ1 could suppress the expression of PRDM1 in human CAR T cells. CAR T cells treated with control or JQ1 were subjected to single-cell RNA-seq. Notably, CAR T cells treated with JQ1 had lower expression of PRDM1 and higher expression of immune checkpoints (PDCD1, LAG3, HAVCR2, ENTPD1, TIGIT, CTLA4) than the mock-treated groups (**Fig. 20**). The results highlighted the potential of using small molecules to target exhaustion-related transcription factors for enhancing CAR T performance.

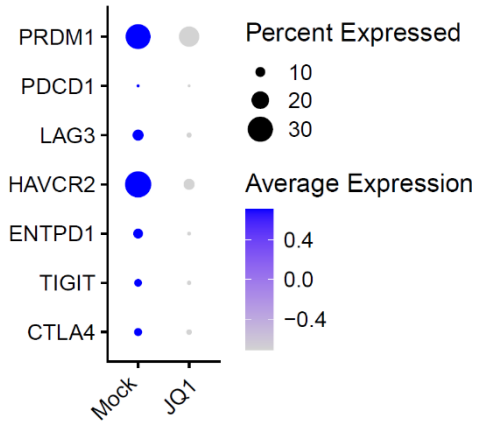

Figure 20. Expression analysis of PRDM1 and immune checkpoint molecules in CAR T cells treated with JQ1. Percentage of cells expressing indicated genes and relative levels of expression are shown.

In summary, we established anti-mesothelin CAR T platforms to study transcription control of T cell exhaustion programs in PDAC. Computational and experimental analyses identified **PRDM1 and IRF8 as key transcription factors promoting T-cell exhaustion**. Deletion of these TFs, either by overexpression of inhibitory dominant-negative domains, or by introducing shRNAs, endowed CAR T cells with improved resistance to exhaustion and superior anti-tumor efficacy.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

(1) *Wang Z, Hausmann S, Lyu R, Li TM, Lofgren SM, Flores NM, Fuentes ME, Caporicci M, Yang Z, Meiners MJ, Cheek MA, Howard SA, Zhang L, Elias JE, Kim MP, Maitra A, Wang H, Bassik MC, Keogh MC, Sage J, Gozani O,* ***Mazur PK****. SETD5-Coordinated Chromatin Reprogramming Regulates Adaptive Resistance to Targeted Pancreatic Cancer Therapy.* ***Cancer Cell.*** *2020 Jun 8;37(6):834-849.e13. doi: 10.1016/j.ccell.2020.04.014. Epub 2020 May 21. PMID: 32442403.*

Acknowledgement of federal support: YES

(2) *Sengupta D, Zeng L, Li Y, Hausmann S, Ghosh D, Yuan G, Nguyen TN, Lyu R, Caporicci M, Morales Benitez A, Coles GL, Kharchenko V, Czaban I, Azhibek D, Fischle W, Jaremko M, Wistuba II, Sage J, Jaremko L, Li W,* ***Mazur PK#, Gozani O#.*** *NSD2 dimethylation at H3K36 promotes lung adenocarcinoma pathogenesis.* ***Mol. Cell, 2021****. #corresponding authors*

Acknowledgement of federal support: YES

(3) *Yuan G, Flores NM, Hausmann S, Lofgren SM, Kharchenko V, Angulo-Ibanez M, Sengupta D, Lu X, Czaban I, Azhibek D, Vicent S, Fischle W, Jaremko M, Fang B, Wistuba II, Chua KF, Roth JA, Minna JD, Shao NY, Jaremko L,* ***Mazur PK#, Gozani O****#. Elevated NSD3 histone methylation activity drives squamous cell lung cancer.* ***Nature. 2021****. #corresponding authors*

Acknowledgement of federal support: YES

(4) *Lukinovic V, Hausmann S, Roth GS, Oyeniran C, Ahmad T, Tsao N, Brickner JR, Casanova AG, Chuffart F, Morales Benitez A, Vayr J, Rodell R, Tardif M, Jansen PWTC, Coute Y, Vermeulen M, Hainaut P,* ***Mazur PK#****, Mosammaparast N#, Reynoird N#. SMYD3 impedes small cell lung cancer sensitivity to alkylation damage through RNF113A methylation-phosphorylation crosstalk.* ***Cancer Discovery****, 2022. #corresponding authors*

Acknowledgement of federal support: YES

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Pawel Mazur*
Project Role:	*PI*
Researcher Identifier:	*ORCID ID: 0000-0002-5820-8344*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Mazur has performed experimental work and data analysis.*
Funding Support:	*This award*

Name:	*Jibo Wu*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier:	*Employee ID: 00080203*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Wu has contributed to experimental work and animal maintenance.*
Funding Support:	*This award*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New grants awarded during funding period:

R01 CA236118
Gozani/Mazur (co-PI)
03/01/2019 – 02/28/2024
Role of the METTL13 lysine methyltransferase in signaling and cancer

R01 CA236949
Mazur (PI)
04/01/2019 – 03/31/2024
Role of SETD5 in chromatin regulation and tumorigenesis

R01 CA266280
Wang/Mazur (co-PI)
10/01/2022 – 09/30/2027
Tumor cell lineage state diversity and composition in gastric cancer progression and therapy resistance

CPRIT RP220391
Mazur (PI)
06/01/2022 – 05/30/2025
Identification of oncogenic mechanisms driving lung squamous cell carcinoma

Describe partner organizations

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: