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14. ABSTRACT

My research focuses on developing Chimeric Antigen Receptor (CAR) T cells for adoptive immunotherapy. The goal of the research is to enhance the efficacy of CAR T therapy by developing potent CAR T cells. Another goal of this research is to shorten the CAR T manufacturing period to increase the availability of this therapy in resource constraint health care settings, as well those patients with rapidly progressive disease. CAR T cells are generated by transducing activated T cells with lentiviral vectors and expanding their progeny over 9-14 days. T cells progressively differentiate over time. Transducing non-activated T cells with CAR will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment, and in vivo activity. The purpose of this work is to determine the optimal T cell subset and corresponding optimal costimulatory domain for CAR when it is expressed in quiescent T cells. The scope of this research is that CAR T cells generated by transducing quiescent T cells will preserve the intrinsic stem-like properties of naïve and memory T cells. We found that interplay of co-stimulation and metabolic engineering provides context-specific benefits which can be emphasized in distinct tumor environments. We also demonstrated that by blocking IFN1, we can increase the transduction efficiency and potency of nonactivated T cells.

15. SUBJECT TERMS
None listed.

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Introduction

My research focuses on developing Chimeric Antigen Receptor (CAR) T cells for adoptive immunotherapy. The goal of the research is to enhance the efficacy of CAR T therapy by developing potent CAR T cells. Another goal of this research is to shorten the CAR T manufacturing period to increase the availability of this therapy in resource constraint health care settings, as well those patients with rapidly progressive disease. CAR T cells are generated by transducing activated T cells with lentiviral vectors and expanding their progeny over 9-14 days. T cells progressively differentiate over time. Transducing quiescent T cells with CAR will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment, and in vivo activity. The purpose of this work is to determine the optimal T cell subset and corresponding optimal costimulatory domain for CAR when it is expressed in quiescent T cells. The scope of this research is that CAR T cells generated by transducing quiescent T cells will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment, and in vivo activity. The purpose of this work is to determine the optimal T cell subset and corresponding optimal costimulatory domain for CAR when it is expressed in quiescent T cells. The scope of this research is that CAR T cells generated by transducing quiescent T cells will preserve the intrinsic stem-like properties of naïve and memory T cells.

Body

Our preliminary data indicated that genetically engineering CAR T cells with GOT1 enhances several aspects of metabolic fitness. CAR co-stimulation is a crucial factor in persistence, anti-tumor function, and metabolism[1-3]. Previous studies showed that the metabolic state of CAR T cells is dynamically regulated by the choice of intracellular costimulatory domain[1]. Metabolic state is an important parameter that is associated with, and regulates differentiation[1]. 4-1BB signaling supports the generation of mitochondrial enriched memory CAR T cells with improved durable efficacy in blood-based malignancies.

While CARs expressing CD28 signaling domains confer efficient cytolysis on demand, our clinical evidence shows their persistence is limited to 3 months following infusion[2]. In contrast, CARs with 4-1BB signaling domain persist more than 10 years[4]. Here, we further hypothesize that expressing GOT1 in non-activated 28ζ CAR T cells will improve their mitochondrial function (which supports durability) without any loss of effector competence.

In this experiment, T cells were infected with a CD19 specific 4-1BBζ CAR, using a standard method (activating and expanding the cells for 9 days). Motivated by these findings, we developed a novel solid tumor model using SY5Y cells engineered to express the CD19 target antigen.

Key Research Accomplishments

1. Comparison of the proliferation, cytolytic effector function, and metabolism of CD19-specific CAR T cells that are armed with GOT1, compared to GFP control, in the context of hypoxia and normoxia.
2. Improving CAR transduction efficiency by blocking IFN 1 production
3. Evaluating the efficacy of nonactivated CART19 with sustained exposure to anti-IFN1 by construction of lentiviral transfer plasmids encoding both CAR19 and secreted the protein binding secreting in Nalm6 mouse models of Leukemia.

To test the combined impact of costimulatory domain and GOT1 co-expression on non-activated CAR T function, quiescent T cells was transduced with a panel of humanized CD19-specific CARs engineered with different costimulatory domains (CD28 versus 4-1BB) with or without expressing GOT1 (**Fig. 1**). These constructs were designed previously using a self-cleaving T2A sequence to enable equimolar levels of constitutively expressed CAR19 and GOT1 from a single transcript (CAR19-GOT1), as previously described[5]. CAR19-GFP for each specific costimulatory domain, as well as donor-matched non-transduced (NTD) was used as control.

Following transduction, proliferation was assayed after in response to CAR stimulation using the CD19 expressing K562 cell line (K562-19), in hypoxia and normoxia (**Fig. 2**). CAR T cell cytolytic ability was assessed in real-time by eSIGHT RTCA Impedance technology, using SY5Y cells expressing CD19 (SY5Y-19) as target cells. eSIGHT analysis transforms cellular impedance into a "cell index" value reflecting cell number, size, and substrate attachment (**Fig. 3**). SY5Y-19 cells was seeded at 50,000 cells/well. After 24 hr, 100,000 effector cells (CART19-GOT1, CART19-GFP or NTD control) with either 4-1BB or CD28 costimulatory domain, was added.

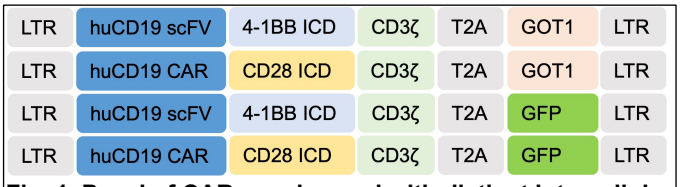

Fig. 1. Panel of CARs engineered with distinct intracellular signaling and gene co-expression.

To assess the metabolic profile of non-activated CAR T cells expressing GOT1 with either 4-1BB or CD28 signaling domain following CAR activation, rates of oxygen consumption (OCR) and corresponding extracellular acidification rates (ECAR) was measured at baseline and in response to pharmacologic inhibitors (**Fig. 4**).

After restimulation at hypoxia, and subsequent transfer to normoxia, we found that 28Z CAR T cells expressing GOT1 had superior proliferative capacity (**Fig. 2**). This doesn't support killing per se, as in an X-CELLigence-based killing assay, standard CAR T cells (BBZ co-stimulatory domain have enhanced cytotoxic activity relative to other groups (**Fig 3**). With respect to mitochondrial function, BBZ GOT-1 cells have superior oxidative capacity, as assessed by baseline and maximal (FCCP-stimulated) OCR in Seahorse assays (**Fig 4**). Collectively, these findings show that the interplay of co-stimulation and metabolic engineering provides context-specific benefits which can be emphasized in distinct tumor environments.

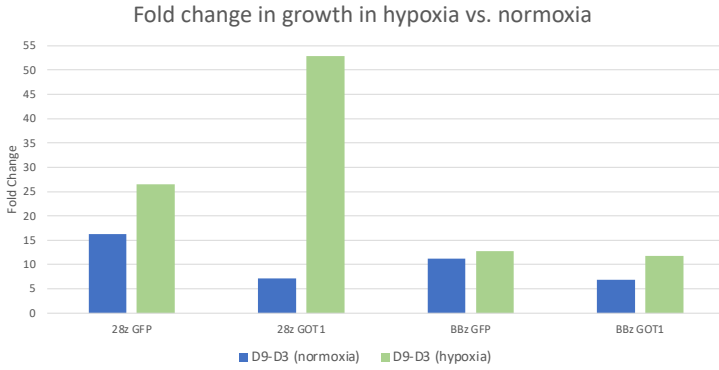

Fig. 2. T cells were transduced with lentivirus vector encoding CART119-GOT1 or CART119-GFP, with bbz and 28z costimulatory domains. The proliferative capacity of CAR T cells were analyzed in normoxia and hypoxia, after exposure to target cells encoding CD19 (k562-19). 28z-GOT1 CAR-T cells show superior proliferative capacity despite restimulation in hypoxic conditions.

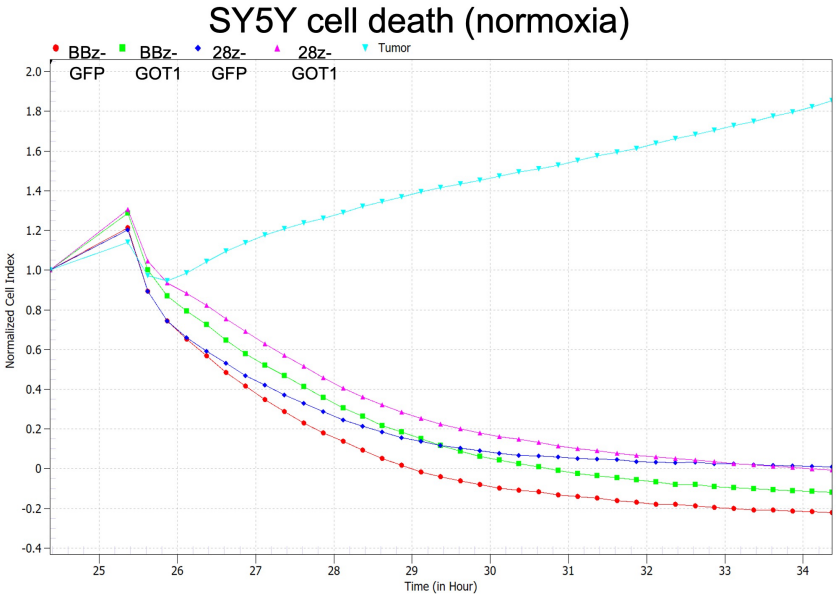

Fig. 3. T cells were transduced with CART119-GOT1 or CART119-GFP, with bbz and 28z costimulatory domain. CAR T cell cytolytic function was assessed by eSIGHT RTCA technology that transforms cellular impedance into a "cell index" value reflecting cell number, size and substrate attachment.

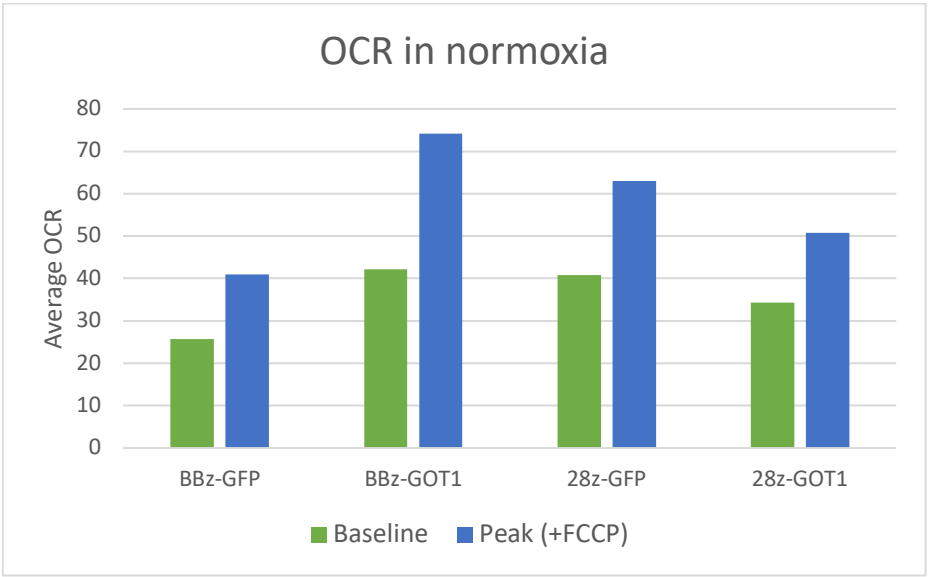

Fig. 4. T cells were transduced with lentivirus vector encoding CART119-GOT1 or CART119-GFP as a control. The metabolic parameters of 28z and BBz CAR T cells were analyzed by seahorse assay. The oxygen consumption rates (OCR) of CAR T cells during log-phase expansion (7 days following CAR stimulation) were measured under basal metabolic conditions and in response to mitochondrial inhibitors.

We demonstrated that MSCV-based gamma-retroviral vector and lentiviral vectors exhibit different efficiencies of transduction across T cells subsets (**Table 1**). In order to further enhance the transduction of freshly-isolated, quiescent primary human T cells for adoptive immunotherapy, I used an approach to Pre-treat non-activated T-

cells with the Vaccinia virus-encoded type I interferon-binding protein. We demonstrate that the lentiviral vector provokes an innate immune response in non-activated T-cells, with concomitant production of IFN-α, that leads to reduced transduction efficiency (**Fig. 5**). I tested the transduction efficiency of nonactivated T cells, using different concentration of the proteins. I showed that using this approach resulted in 7.2-fold greater CAR T transduction efficiency (**Fig. 6**).

I also evaluated the effects of sustained exposure to anti-IFN1 by construction of lentiviral transfer plasmids encoding both a CAR and secreted the protein binding. The anti IFN1-secreting CAR T-cells showed greater transduction efficiency as well as enriched central memory differentiation (**Fig. 7**).

Table 1: % CAR transduction across T cell subsets

	Tnaïve	Tcm	Tem	Tte
Lentiviral	48.7	35.5	21	5.4
Retroviral	57.8	67.7	41.3	23.6

The long-term engraftment and anti-tumor function of anti IFN-treated CAR T-cells was assessed using NOD-SCID γc−/− (NSG) mice bearing Nalm6 tumor xenografts. Mice aged 6-10 weeks were injected via the tail vein with 2 × 106 Nalm6 cells expressing click beetle green luciferase and enhanced green fluorescent protein (eGFP). Non-activated CAR T-cells were treated with anti-IFN protein or vehicle, and transduced with CAR19-T2A-GFP or CAR19-T2A-antiIFN (**Fig. 8A**). Four days after tumor injection, the mice were injected via the tail vein with CAR T cells. Tumor bioluminescence was measured periodically with the Xenogen IVIS Spectrum system. While mice treated with CAR-negative non-transduced cells showed progressive tumor growth, all CAR treatment arms showed prolonged tumor control without significant differences between groups at 50 days (**Fig. 8B)**. CAR T-cell levels in the peripheral blood measured at day 16 and 32 by flow cytometry demonstrated continued persistence without significant differences between groups (**Fig. 8C**).

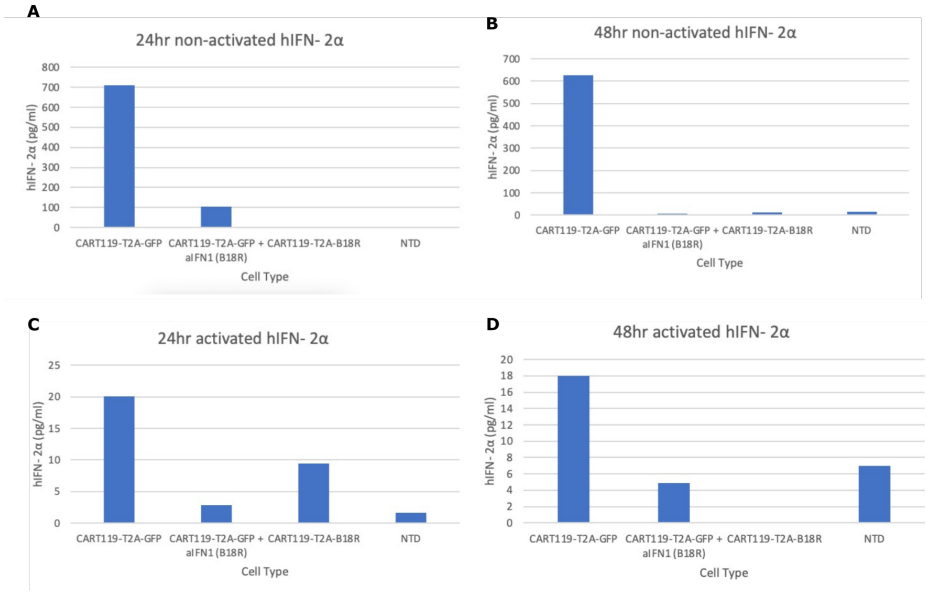

Fig. 5. a, Human T-cells were isolated from healthy donors, cultured overnight in media containing IL-7 and IL-15, treated with anti-IFN or vehicle, and transduced with lentiviral vectors encoding CAR19-T2A-GFP or CAR19-T2A-anti-IFN or left non-transduced (NTD) as indicated. At 24 h post-transduction, the cells were centrifuged and IFN-α concentration in the media was determined by ELISA. **b**, Conditions as in **a** except media collected at 48 h post-transduction. **c**, Freshly isolated human T-cells were activated by anti-CD3/CD28 Dynabeads overnight and then transduced with lentiviral vectors or left NTD as above. At 24 h post-transduction, IFN-α concentration in the media was determined by ELISA as above. **d**, Conditions as in **c** except media collected at 48 h post-transduction.

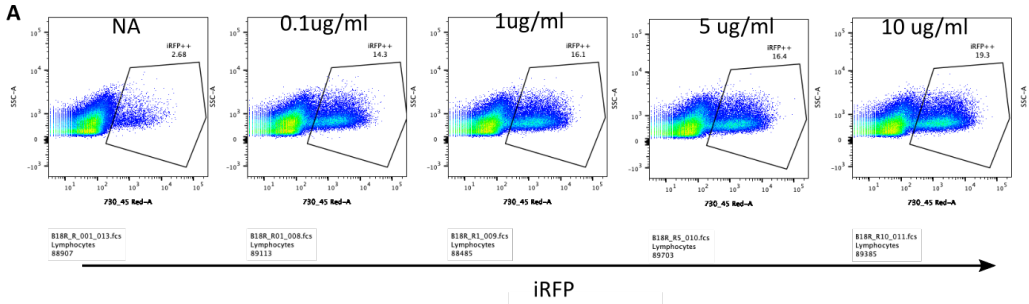

Fig. 6. a, Freshly isolated human T-cells were cultured overnight in media containing IL-7 and IL-15. Anti-IFN1 or vehicle was then added to the media, and the cells were incubated for one hour at room temperature. The cells were resuspended in complete media and then transduced with lentiviral vector encoding iRFP at an MOI of 5. Expression of iRFP was evaluated 96 h after transduction by flow cytometry.

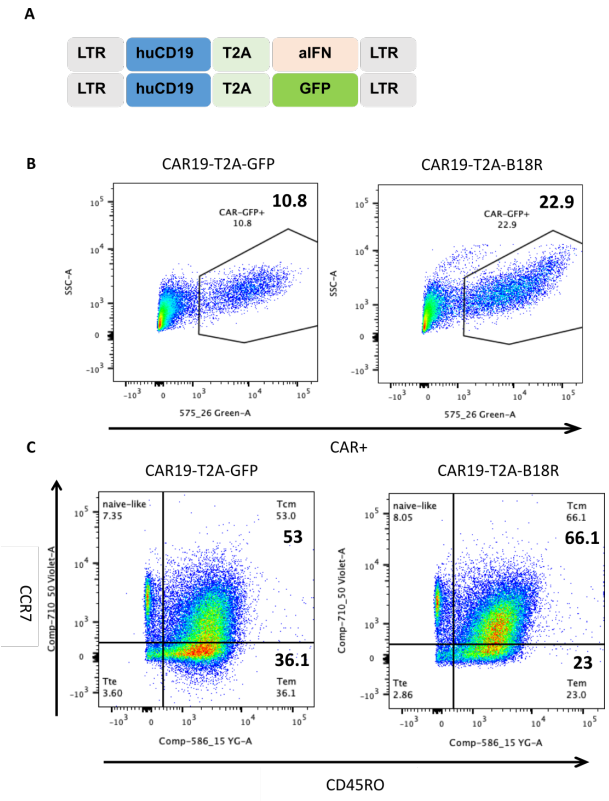

Fig. 7. a, Lentiviral transfer plasmids were developed that encode an anti-human CD19 CAR linked by a T2A self-cleaving peptide with either EGFP or anti-IFN1. **b**, Non-activated T-cells were prepared from freshly isolated human T-cells transduced with CAR19-T2A-GFP or CAR19-T2A-anti-IFN1. After 5 days the cells were stimulated using irradiated K562 cells. At day 10, the cells were stained using anti-CAR19 idiotype and CAR expression was evaluated by flow cytometry. **c**, Cells were prepared as in **b** and stimulated twice at 5 day intervals with irradiated K562 cells. At day 15, the cells were stained with anti-CD45RO and anti-CCR7 antibodies and samples were evaluated by flow cytometry. Data are representative of multiple experiments with three independent donors.

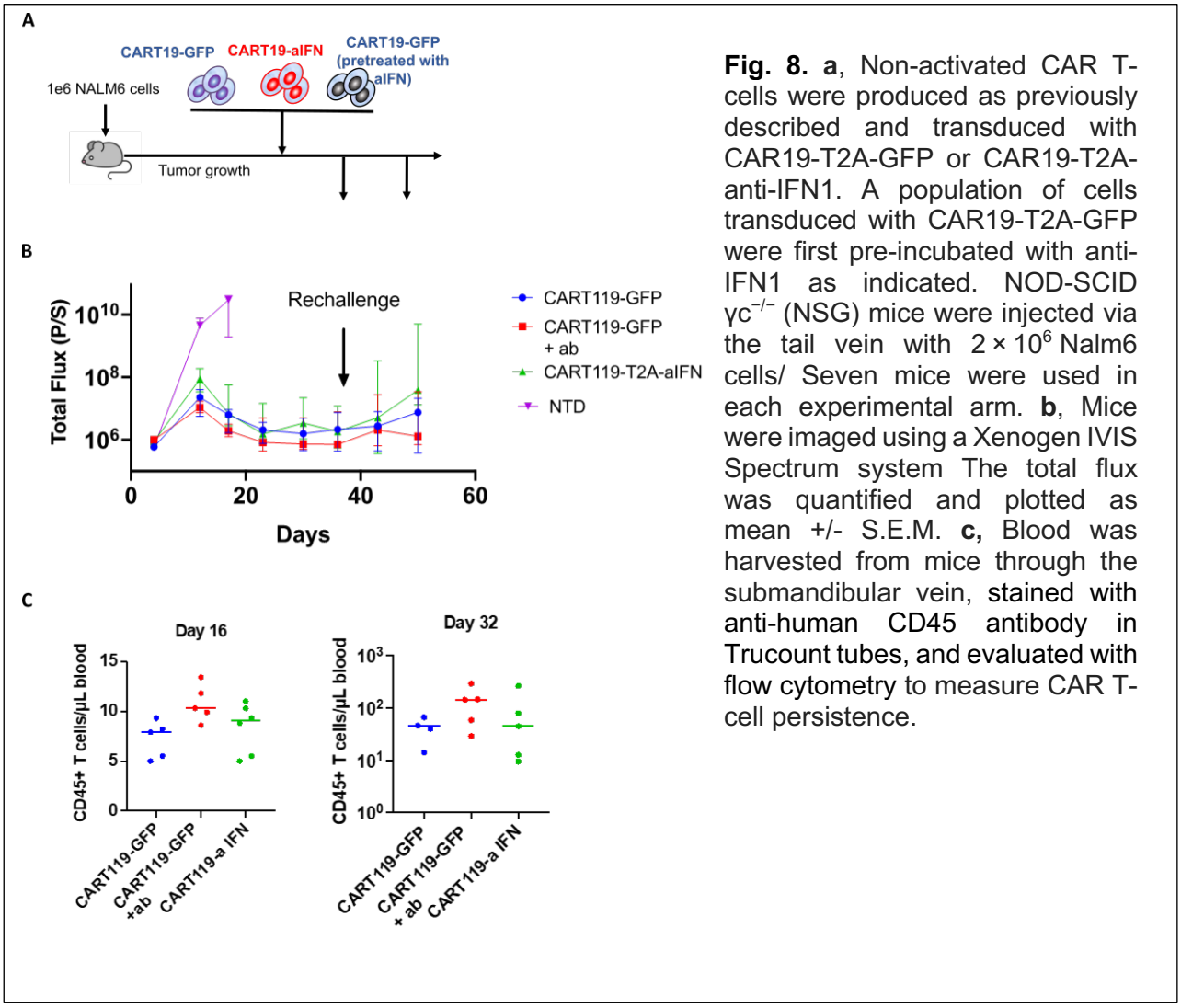

Fig. 8. a, Non-activated CAR T-cells were produced as previously described and transduced with CAR19-T2A-GFP or CAR19-T2A-anti-IFN1. A population of cells transduced with CAR19-T2A-GFP were first pre-incubated with anti-IFN1 as indicated. NOD-SCID $\gamma c^{-/-}$ (NSG) mice were injected via the tail vein with 2×10^6 Nalm6 cells/ Seven mice were used in each experimental arm. **b**, Mice were imaged using a Xenogen IVIS Spectrum system The total flux was quantified and plotted as mean +/- S.E.M. **c,** Blood was harvested from mice through the submandibular vein, stained with anti-human CD45 antibody in Trucount tubes, and evaluated with flow cytometry to measure CAR T-cell persistence.

Reportable Outcomes

I had several invited talks to present the results from my work listed below:

Quiescent CAR T Cells: Novel Strategies for Enhanced Cancer Immunotherapies, Immuno-Oncology Translational Network (IOTN) Translational Cellular Therapy Working Group Meeting	Jun	2023
CAR Engineered Tumor-Fighting T cells, National Institute of Standards and Technology (NIST), MD	Feb	2023
Exploring the Therapeutic Potential of Quiescent CAR T Cells in Cancer Immunotherapy, Baylor College of Medicine, Dallas, Tx	Jul	2023
Rewiring the Immune System: How CAR T cells Fight Cancer, Villanova University, Philadelphia, PA	Mar	2023
Non-Activated CAR T Cells for Cancer Immunotherapy, Norwegian Society for Immunology (NSI) Rising stars	May	2023

Non-Activated CAR T Cells for Cancer Immunotherapy, World Oncology Cell Therapy Congress, Boston, MA	Apr	2023
Engineering next generation CAR T cells with enhanced efficacy, Arsenal Bio, San Francisco, CA	Dec	2022

Below is the list of publication resulting from the work in the reporting period:

Computational model of CAR T-cell immunotherapy dissects and predicts leukemia patient responses at remission, resistance, and relapse	Journal for Immunotherapy of Cancer	December
Metabolic and epigenetic orchestration of (CAR) T cell fate and function	Cancer Letters	October

We also submitted an abstract to ASH 2023 based on the second part of the results from current work, titled as below:

Type I Interferon Blockade Enhances Transduction Efficiency and Efficacy of Non-Activated CAR T Cells

Joseph Durgin, MD[1,2*], Ashwin Sannecy[1*], Andre Kelly[1*], Roddy S O'Connor, PhD[1*] and **Saba Ghassemi, PhD**[1]

[1]Center for Cellular Immunotherapies, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA; [2]Department of Dermatology, University of Michigan, Ann Arbor, MI

Conclusion

As CAR therapy evolves, it will be important to unlock the inherent potencies associated with different T cell subsets. Expressing CD28-based CAR in effector cells may overcome the lack of CD28 ligand in the tumor environment and drive effector function “on demand”. Simultaneously, expressing 4-1BBζ CAR in Tcm may accentuate their ability to give rise to long-lasting progeny. T cell subset composition will influence the kinetics of T cell engraftment and accumulation of CAR T cells within the tumor compartment.

Our results suggest that inhibiting type I IFNs has specific effects on non-activated T-cells compared to Dynabead-activated T-cells that are exposed to lentivirus. We observe that non-activated T-cells produce IFN-α in response to lentiviral transduction, while Dynabead-activated T-cells have no detectable IFN-α response. This result is concordant with the known phenomenon that activated T-cells are easily transduced by lentiviral vectors whereas quiescent T-cells are resistant to infection.15 Mechanistically, anti-CD3 and CD28 mediated activation likely counteracts the T-cell’s anti-lentiviral restriction factors at multiple levels.

Blocking IFN 1, therefore, may have specific utility in the paradigm of non-activated CAR T-cell production, given that non-activated cells have a robust IFN-α mediated response to lentiviral vectors that limits CAR T manufacturing yield.

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