Precision-targeted epigenome editing enhances CAR T functional profiles and anti-tumor activity

Lucas Thompson[2], Akiko Seki[1], Alexandra Croft[2], Nathaniel Chartrand[2], Tyler Klann[1], Jason Dean[2], Andrew Hill[2], Nicole DeNamur[2], Britta Jones[1], Emi Tokuda[2], Veronica Gough[1], Malathi Chellappan[1], Stuart Sundseth[1], Nathaniel Lambert[2] and Blythe D Sather[2]

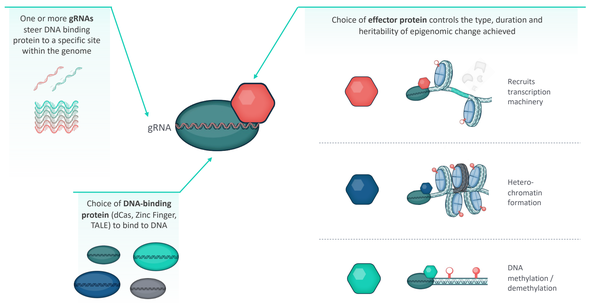
Tune Therapeutics, [1]300 Morris St, Suite 320, Durham NC 27701 & [2]1930 Boren Ave, Floor 5, Seattle WA 98101.

Abstract

Cell-based immunotherapy with gene-modified T cells with a synthetic Chimeric Antigen Receptor (CAR T) has become a successful clinical treatment paradigm for hematological malignancies, though effectiveness in solid tumors has been elusive. Clinical experience in the heme setting has helped to define some mechanisms of disease relapse with initial generation of CAR T cells, revealing important roles for cellular persistence and functional potency for mediating durable clinical responses. In the solid tumor setting, achieving clinical success has proven to be more difficult, with functional persistence of CAR T cells being hampered by sustained antigen exposure and suppressive features of the tumor microenvironment (TME) driving T cell functional exhaustion. Current preclinical data demonstrating enhanced CAR T cell activity - either by transgenic gene overexpression or gene knock-out(s) to modulate molecular pathways – suggest that engineering of T cells beyond CAR may have beneficial clinical effects that could overcome treatment hurdles in solid tumor settings. However, these approaches result in permanent or non-physiological molecular alterations of cells that may not be ideal for the inherent biology or plasticity of T cells. Additionally, it is likely that modulation of multiple molecular networks is necessary to overcome biological barriers needed to achieve clinical benefit against solid tumors, presenting a significant safety risk for engineering approaches that rely on breaking the DNA strand.

Targeted repression or activation of individual genes has been shown using modified gene-editing molecules such as enzymatically-dead Cas9 (dCas) linked to protein domains that activate or inhibit gene transcription via recruitment molecular complexes, without physical disruption of the DNA sequence. Here we show for the first time transiently-delivered dCas epi editing constructs mediating activation or repression of key target genes for improving CAR T cell function. Electroporation of T cells with dCas-epi editor mRNA and gRNAs during CAR T cell production results in temporary expression of the targeted epi editor molecule, followed by a durable modulation of target genes, which is maintained through cryopreservation and functional assays, both in vitro and in vivo. CAR T cells treated with the epi editor and target-specific guide RNA demonstrated markedly improved attributes associated with functional persistence, including elevated expression of key effector cytokines (IL2, IFNg, TNF), enhanced proliferation, and sustained serial target cell killing. Using a subcutaneous xenograft model of Her2-expressing human non-small cell lung cancer cell line NCI-H1975, Her2-specific human CAR T cells treated with dCas-epi editor mRNA and gRNAs exhibited superior tumor control and survival of engrafted mice, with enhanced pharmacokinetics of the CAR T cells in the blood. **Furthermore, we demonstrated that the transiently-delivered dCas-epi editor mRNA and gRNAs can be multiplexed to achieve compounded impacts on functional qualities of the CAR T cells. These results demonstrate a novel approach to safely and effectively epigenetically modulate T cells in a precisely targeted manner to achieve improved outcomes in clinically relevant models of CAR T cell function.**

TEMPO™ Platform: Durable and Specific Epi-Editing for Activation and Repression of Gene Targets



Rapid Discovery of Lead Epi-editors of T Cell Function using Pooled and Arrayed Screens

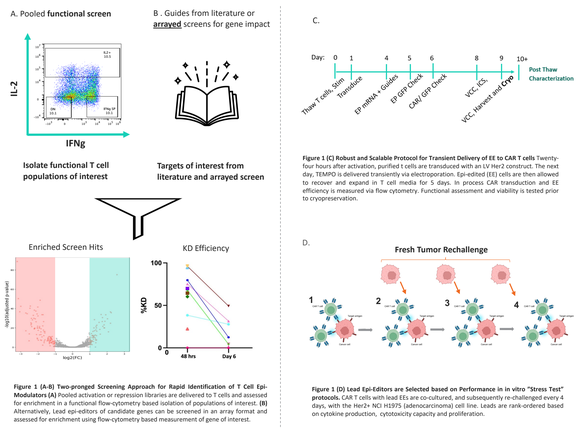


Figure 1 (A-B) Two-pronged Screening Approach for Rapid Identification of T Cell Epi-Modulators (A) Pooled activation or repression libraries are delivered to T cells and assessed for enrichment in a functional flow-cytometry based isolation of populations of interest. **(B)** Alternatively, Lead epi-editors of candidate genes can be screened in an array format and assessed for enrichment using flow-cytometry based measurement of gene of interest.

Figure 1 (C) Robust and Scalable Protocol for Transient Delivery of EE to CAR T cells Twenty-four hours after activation, purified t cells are transduced with an LV Her2 construct. The next day, TEMPO is delivered transiently via electroporation. Epi-edited (EE) cells are then allowed to recover and expand in T cell media for 5 days. In process CAR transduction and EE efficiency is measured via flow cytometry. Functional assessment and viability is tested prior to cryopreservation.

Figure 1 (D) Lead Epi-Editors are Selected based on Performance in in vitro "Stress Test" protocols. CAR T cells with lead EEs are co-cultured, and subsequently re-challenged every 4 days, with the Her2+ NCI H1975 (adenocarcinoma) cell line. Leads are rank-ordered based on cytokine production, cytotoxicity capacity and proliferation.

Abstract

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Targeted repression or activation of individual genes has been shown using modified gene-editing molecules such as enzymatically-dead Cas9 (dCas) linked to protein domains that activate or inhibit gene transcription via recruitment molecular complexes, without physical disruption of the DNA sequence. Here we show for the first time transiently-delivered dCas epi editing constructs mediating activation or repression of key target genes for improving CAR T cell function. Electroporation of T cells with dCas-epi editor mRNA and gRNAs during CAR T cell production results in temporary expression of the targeted epi editor molecule, followed by a durable modulation of target genes, which is maintained through cryopreservation and functional assays, both in vitro and in vivo. CAR T cells treated with the epi editor and target-specific guide RNA demonstrated markedly improved attributes associated with functional persistence, including elevated expression of key effector cytokines (IL2, IFNg, TNF), enhanced proliferation, and sustained serial target cell killing. Using a subcutaneous xenograft model of Her2-expressing human non-small cell lung cancer cell line NCI-H1975, Her2-specific human CAR T cells treated with dCas-epi editor mRNA and gRNAs exhibited superior tumor control and survival of engrafted mice, with enhanced pharmacokinetics of the CAR T cells in the blood. **Furthermore, we demonstrated that the transiently-delivered dCas-epi editor mRNA and gRNAs can be multiplexed to achieve compounded impacts on functional qualities of the CAR T cells. These results demonstrate a novel approach to safely and effectively epigenetically modulate T cells in a precisely targeted manner to achieve improved outcomes in clinically relevant models of CAR T cell function.**

Epi-Editing Improves CAR T cell Function In Vitro

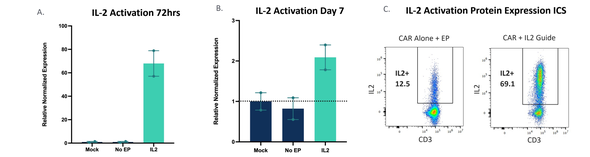


Figure 2 (A and B) Increased activation of IL-2 after transient delivery of TEMPO An EE targeting the IL-2 gene was transiently delivered to Her2 CAR T cells (n=2). **(A)** IL-2 expression, as measured by RT-qPCR, was increased in EE cells at 72 hours and 7 days post-electroporation, as compared to mock (no CAR) and non-electroporated CAR T cells. **(B)** Intracellular cytokine staining revealed a marked increase in IL-2 production in EE CAR T cells after tumor cell co-culture, as compared to non-EE control.

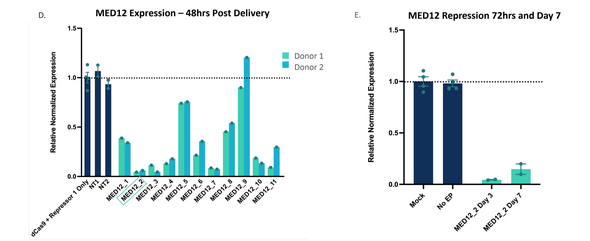


Figure 2 (D and E). TEMPO successfully knocks down MED12 gene expression (D) The top 11 guides from an arrayed screen for repression of MED12 were transiently delivered to Her2 CAR T cells (n=2) and gene expression was measured at 48 hours post electroporation. The 6 guides with the most statistically significant repression of MED12 were further characterized for modulation and function. **(E)** In a separate experiment, EE with guide MED12_2 was transiently delivered to Her2 CAR T cells (n=2) and gene expression was measured by RT-qPCR at 72 hrs and 7 days post-EP. Gene modulation was still present at day 7, demonstrating a robust and durable repression of the MED12 gene (Ref 1).

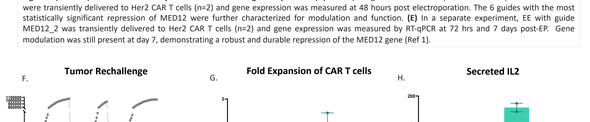


Figure 2 (F-H) Transient activation of IL-2 and repression of MED12, respectively, increases tumor-specific cytotoxicity, CAR T cell fold expansion and IL-2 production. CAR T cells with either EE targeting IL-2 or MED12 were stress tested via a co-culture rechallenge protocol and assessed for CAR-specific cytotoxicity, fold expansion, and secreted IL-2. **(F)** EE IL-2 and MED12 CAR T cells demonstrated superior tumor killing after rechallenge, as compared to the CAR alone condition. **(G)** Rechallenge of CAR T cells with Her2+ tumor cell lines and measurement of fold expansion (N=2). Measurable increase in fold expansion of EE IL-2 and MED12 CAR T cells after rechallenge, as compared to CAR alone. **(H)** Additionally, CAR T cells were rechallenged and secreted IL-2 was measured. As observed with cytotoxicity and fold expansion, EE CAR T cells produced more IL-2 than the non-EE CAR control.

TEMPO Improves CAR T Cell Function In Vivo

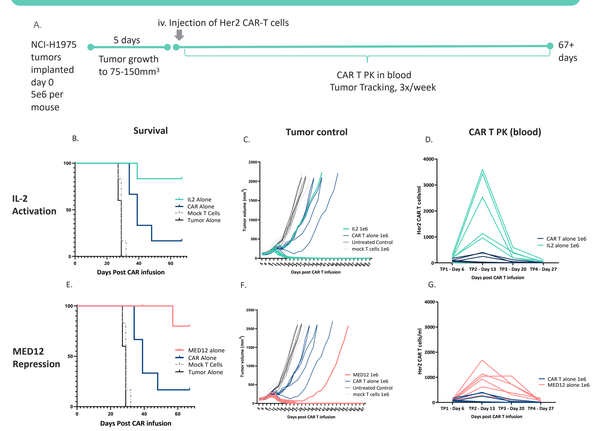


Figure 3 (A-G) EE CAR T cells demonstrate superior tumor clearance capabilities (A) Schematic of Her2+ in vivo efficacy study. Her2+ NCI-H1975 cells were implanted subcutaneously into the flank of NSG-MHC I/II DKO mice. Five days post implant, 1e6 Her2 CAR+ T cells were injected intravenously into the tail vein. T **(B, E)** Survival data of mice treated with IL-2 EE CAR T or MED12 EE CAR T, CAR T, mock (non-transduced) or tumor alone. Mice that received EE IL-2 or MED12 CAR T cells had a significant survival advantage over those that received non-EE CAR T cells (5/6 mice vs 0/6 mice at day 67) . **(C, F)** Mouse tumor volume was measured every 2-3 days after CAR T cell transfusion. Rapid and sustained tumor volume decrease for individual mice that received EE IL-2 or MED12 CAR T cells, as compared to CAR and tumor alone **(C,F)**, and Her2 CAR T cell expansion in the blood **(D,G)** were measured over 67 days for both IL2 and MED12 epi edited CAR T cells arms compared to controls of CAR alone, mock non-transduced T cells, and tumor alone.

Multiplexing EE Candidates Improves In Vitro T Cell Function

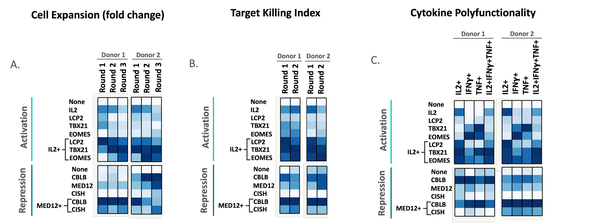


Figure 4. Multiplexing of activation and repression leads improves CAR T cell function. Fold CAR T cell expansion during production and after multiple rounds of co-cultures with Her2+ NCI-H1975 cells, average of two donors. **(A).** EE CAR T cell were functional during second and third rounds of CAR T co-culture with antigen-expressing target tumor cells, target cell killing index = area under the curve (AUC) of kill curves normalized to mock T cells (mock AUC- AUC EE)/ mock AUC **(B).** Polyfunctional cytokine expression among EE CAR T cells after exposure to antigen-expressing target cells, normalized to CAR control within donor, performed on Day 9 post EP **(C).** Lead activating combinations (IL-2+LCP2/TBX21, EOMES) and repressors (MED12+CBLB) showed superior performance across multiple read outs, as compared to single EE alone.

Multiplex EE is Achievable at Clinical Scale

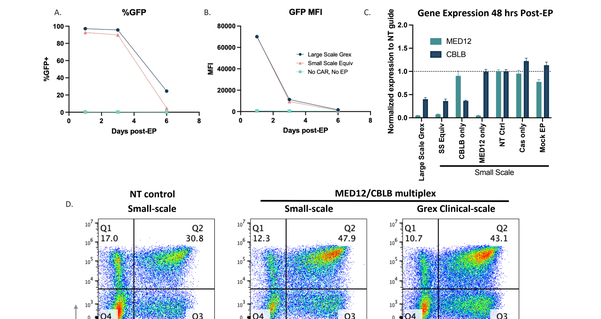


Figure 5 (A-D) Increase in double positive cytokine production in multiplexed EE CAR T cells produced at a clinically relevant scale To assess scalability of transient delivery of EE, CAR T cells from a healthy donor were split and electroporated using either a small or large scale EP unit with a multiplexed cocktail of MED12 and CBLB EE repressors. Cells were then cultured in a large scale G-Rex®100M or a G-Rex®24 Well Plate following our standard culturing protocol. **(A,B)** In process assessment of electroporation efficiency was measured using GFP percentile and MFI as surrogate measures of mRNA transfection. **(C)** Gene expression of MED12 and CBLB was measured at 48 hrs post EP using RT-qPCR. There was a measurable decrease in gene expression of MED12 and CBLB when EEs were multiplexed, demonstrating the ability to modulate two targets with a single transient delivery of EE cocktail. **(D)** EE CAR T cells were harvested at Day 7 post- activation, cryopreserved, thawed and placed into a co-culture with NCI H1975. Multiplexing of MED12 and CBLB increased the presence of double positive (IL2+IFN-Y+) antigen-specific cytokine secreting cells, as compared to a non-targeting EE control. Similar results were observed between cultures, suggesting transient delivery of EE is robust across protocols

Alternative Effectors Improve MED12 Repression and Enhance Durability

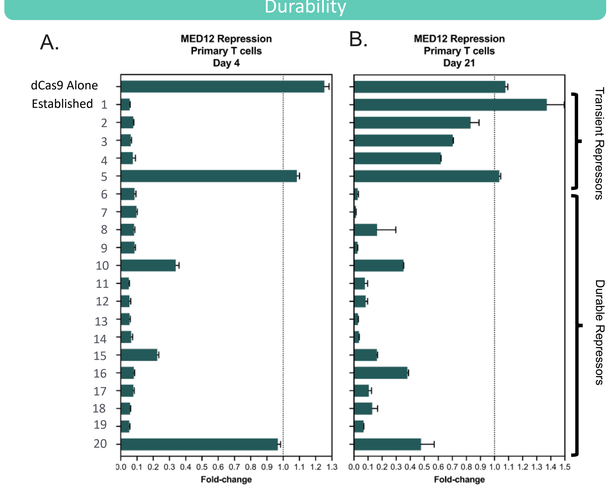


Figure 6. Multiple alternative effectors show enhanced and durable repression of MED12 T cells from a healthy donor were electroporated (n=2) with mRNA encoding dCas9 alone or dCas9 fused to a panel of repressor domains, in addition to a non-targeting gRNA or a gRNA targeting MED12. RNA was extracted, cDNA synthesized, and MED12 transcript levels were quantified by RT-qPCR at days 4 (A) and 21 (B) post-EP. The fold-change in MED12 expression for each effector was calculated relative to the corresponding effector with non-targeting gRNA. Most editors achieved robust repression at an early timepoint, including our in-house established repressor **(A)**. Some effector designs that achieved initial repression did not result in sustained at Day 21, while other designs achieved more durable repression above the transient repressors throughout the experiment **(B).** These data highlight that epigenetic-modulation with TEMPO can be tuned to increase durability through alternative effectors of repression.

Further Enhanced Durability and T Cell Function with Alternative Repressor for MED12

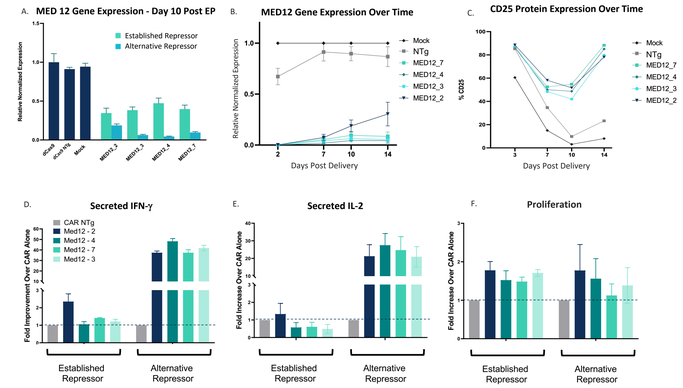


Figure 7. Improved MED12 gene repression and enhanced function of CAR T cells with an alternative repressor. Lead guides for MED12 were combined with the established or alternative repressor transiently delivered to Her2 CAR T cells generated from healthy donors (n=4). RNA was extracted, cDNA synthesized, and RT-qPCR was run post electroporation comparing MED12 gene expression at Day 10 **(A)** and over a time course **(B)** with gene knockdown of MED12 normalized to CAR T cells alone. The alternative repressor decreased the level of MED12 expression across all lead guides with durable modulation observed out to Day 14. **(C)** Flow cytometric analyses of CD25 (IL2RA) as a surrogate of MED12 repression over time. EE of MED12 increased CD25 expression over time, as compared to non-targeting or no EE. Stress test assays were designed with epi-edited Her2 CAR T cells that were co-cultured and rechallenged against Her2 positive NCI H1975 tumor cells (1:5 E:T ratio) every 4 days with up to two re-challenges. MSD for secreted cytokine IFN-γ **(D)** and IL2 **(E)** and was performed to detect cytokine production for each condition 24hours post co-culture after the second serial restimulation. **(F)** CAR T cell proliferation was measured after stim 2 via live cell counts of CAR positive T cells and was normalized to CAR alone samples. Delivery of MED12 EE with an alternative repressor demonstrated increased cytokine production with similar proliferation, as compared to our established repressor.

Further Enhanced Durability and T Cell Function with Alternative Repressor for TGFBR2

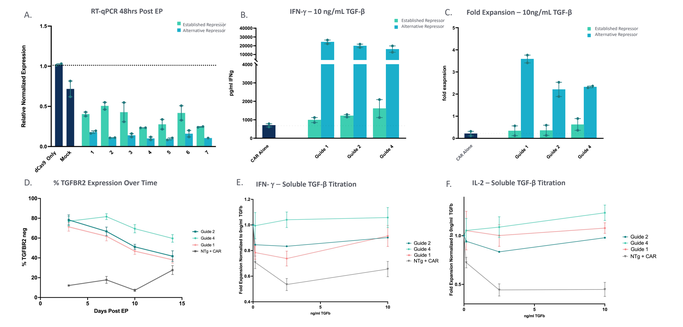
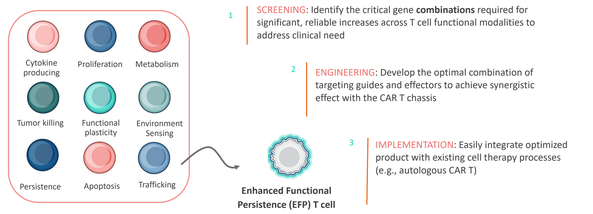


Figure 8. Improved TGFBR2 gene repression and enhanced function of CAR T cells with alternative repressor in the presence of inhibitory TGF-β. Lead guides for TGFBR2 were combined with the established or alternative repressor transiently and delivered to Her2 CAR T cells generated from healthy donors (n=2). RNA was extracted, cDNA synthesized, and RT-qPCR was run post-EP. **(A)** Gene expression post-EP with gene knockdown of TGFBR2 normalized to CAR T cells alone. As observed with MED12, EE with the alternative repressor showed an increase in modulation of TGFBR, as compared to the established repressor. Stress test assays were performed as previously described with the addition of soluble TGF-β. **(B)** Secreted IFN-Y levels and **(C)** proliferation, as measured via live cell counts of CAR+ T cells, 24 hours after second restimulation with Her2+tumor cells in the presence of 10ng/mL of exogenous recombinant human TGF-β **(D)** T cell TGFBR2 protein expression was measured via flow cytometry for 14 days post EP. T cells were activated with plate bound CD3/CD28 in the presence of a titrating amount of exogenous TGFb starting at 10ng/ml, 96hours post co-culture secreted cytokine was measure for IFN-γ **(E)** and IL2 **(F)** 96 hrs post co-culture. These data suggest that EE of TGFBR2 with a durable repressor may overcome the TGF-β driven immunosuppressive environment of the TME.

From Screening to Implementation: TEMPO Enables Rapid Identification and Advancement of Epi-Modulators for T Cell Function



- Transient delivery of epi-editors into CAR T cells imparted sustained improved function in vitro and in vivo
- Epi modulation can be multiplexed to enhance function of CAR T cells
- Epi-editors can be tuned for precise target modulation and associated functional outcomes

REFERENCES

1. Freitas, KA et al. Science. 2022 Nov 11;378