Genome-scale *in vivo* CRISPR/Cas9 T cell screen unveils targets enabling rational design of engineered Tumor Infiltrating Lymphocyte (eTIL™) therapies for solid tumors

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Abstract

Adoptive cell therapy (ACT) with *ex vivo* expanded tumor infiltrating lymphocytes (TIL) offers a potentially curative treatment for refractory solid tumors. However, the immunosuppressive tumor microenvironment (TME) limits the effectiveness of TIL therapy. To systematically identify targets that have the potential to improve T-cell function for ACT, we conducted two large-scale CRISPR/Cas9 functional screens using a novel Immune-CRISPRomics® platform.

The first screen was genome-wide and employed primary T cells i*n vivo,* assessing T cell infiltration into tumors (sgRNA guide enrichment). Notably, this screen identified clinically active molecules, such as PD-1, and identified multiple targets, including a target referred to as CT-1, that enhanced anti-tumor T-cell function superior to PD-1. The potential of CT-1 for ACT was validated in multiple syngeneic tumor models by adoptive transfer of CT-1-edited TCR-Tg CD8 T cells into tumor bearing mice, which showed robust efficacy in both PD-1 sensitive and insensitive models in the absence of any conditioning regimens. CT-1 edited TCR-Tg CD8 T cells established durable anti-tumor memory, with edited CD8 T cells characterized by enhanced accumulation and a T central memory phenotype.

The second screen employed human TIL and assessed the impact of gene inactivation on *in vitro* human TIL expansion under standard manufacturing conditions. This screen identified CT-1 as a top target, indicating that CT-1 inactivation enhances both anti-tumor activity and cell expansion. Potent and selective sgRNAs targeting CT-1 were identified, and the *in vitro* functional profile of CT-1 edited CRISPR/Cas9-engineered TIL (eTIL™) characterized, with increased production of pro-inflammatory cytokines observed. Together, these data demonstrate that the Immune-CRISPRomics® platform enables comprehensive target identification and validation of targets for the development of CRISPR/Cas9 engineered TIL (eTIL™) as a next generation adoptive cell therapy.

Methods

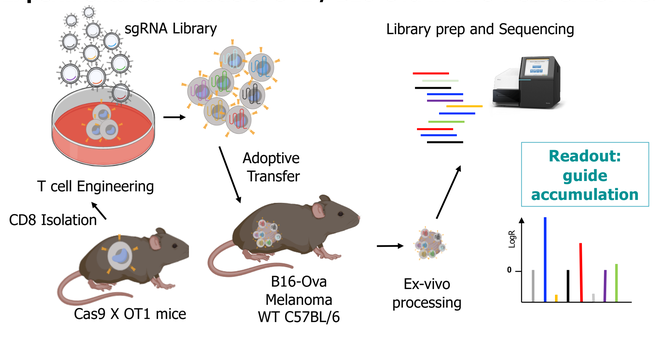
In vivo T-cell screen : CD8 T cells were isolated from OT1 x Cas9 Transgenic mice, transduced with an sgRNA library and adoptively transferred into B16-OVA tumor bearing mice. After in vivo expansion, tumors were collected, gDNA was isolated and sgRNA accumulation was evaluated by NGS.

In vitro TIL screen : Human melanoma TILs were expanded by culture with human recombinant IL-2. After expansion, TIL were transduced with an sgRNA library and subsequently transfected with mRNA encoding Cas9. Engineered TILs were expanded in a standard REP containing irradiated PBMCs, anti-CD3, and IL-2 for 14 days. After expansion gDNA was isolated and sgRNA accumulation was determined by NGS

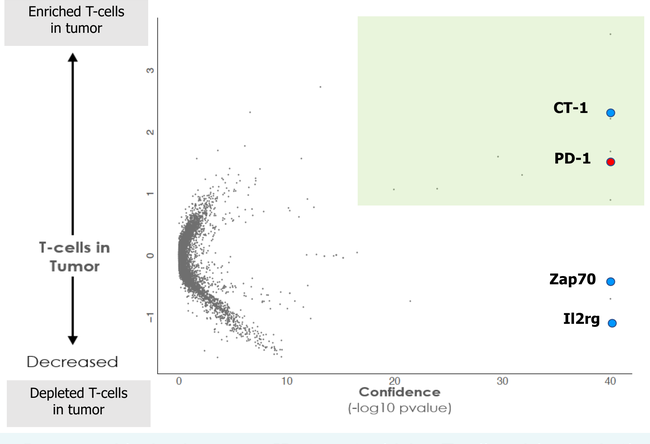
In vivo Target validation : B16-Ova melanoma or gp100 CRC cancer cells were injected s.c. into C57BL/6J mice. Mice were randomized for tumor volume (approx. mean 100mm^3) and either control, PD-1, or CT-1 edited OT1 or PMEL T cells were injected i.v.. Tumor volume and body weights were measured twice a week. For B16-Ova tumor re-challenge study, mice with complete tumor response 76 days following CT-1 treatment were re-injected with B16-Ova tumors and monitored for tumor regrowth. Peripheral blood draws were collected at baseline, and multiple timepoints post tumor re-challenge to assess the phenotype of adoptively transferred CT-1 edited OT-1 cells (fig 3). Relative potency of CT-1 vs control edited T cells was evaluated in the B16-Ova model at cell concentrations ranging from 0.41 to 41x10^6 cells. Day 21 post cell transfer, blood was collected from the highest dose groups and OT-1 T cells measured for expansion(fig4).

Figure 1: A genome-wide CRISPR/Cas9 *in vivo* T- cell screen identifies CT-1 as a top target

Experimental schematic of OT1 / B16-Ova in vivo T cell CRISPR screen



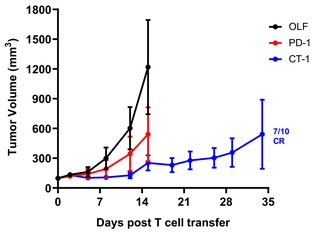
Genome-Scale in vivo Screen Identifies Novel T cell Targets



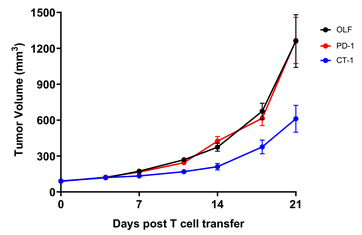
- Genome-wide *in vivo* screen IDs targets driving T cell enrichment in the tumor
- PD-1 serves as a positive control, CT-1 demonstrates activity greater than PD-1
- Screen passes multiple QC checks, including recovery of >90% sgRNAs in library, depletion of T cell essential genes (Zap70, Il2rg), and expression of hits in T cells

Figure 2: Adoptively transferred CT-1 edited T cells demonstrate robust efficacy across syngeneic models

PD-1 sensitive B16-Ova mouse melanoma model



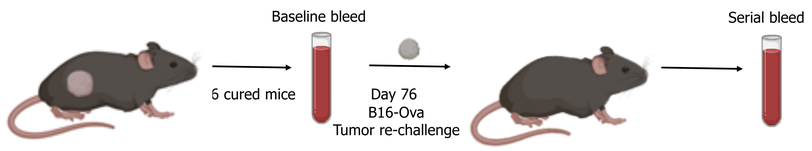
PD-1 insensitive gp100 mouse colon model



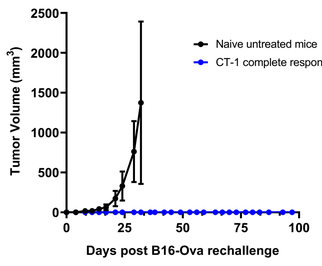
- Adoptive transfer of CT-1 edited OT1 or PMEL T cells into mice bearing established 100mm3 B16-Ova or gp100 mouse colon tumors demonstrate efficacy greater than PD-1-edited T cells
- No lymphodepletion or other conditioning regimens used

Figure 3: Cured mice reject tumors upon re-challenge, with CT-1 edited T cells persisting long-term as Tcm

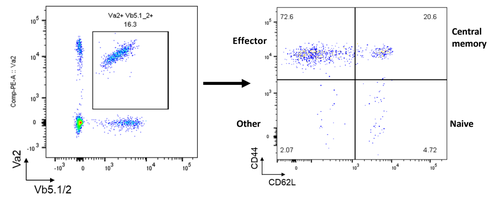
Experimental schematic of B16-Ova tumor re-challenge study



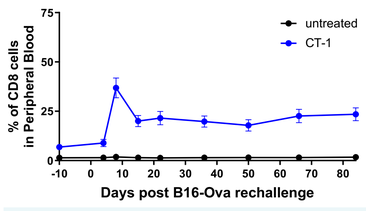
Cured mice reject B16-Ova re-challenge



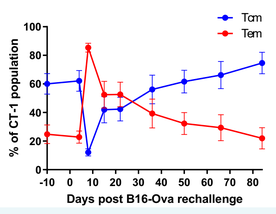
CT-1 edited OT1s are detectible in blood following re-challenge



CT-1 edited OT1s persist

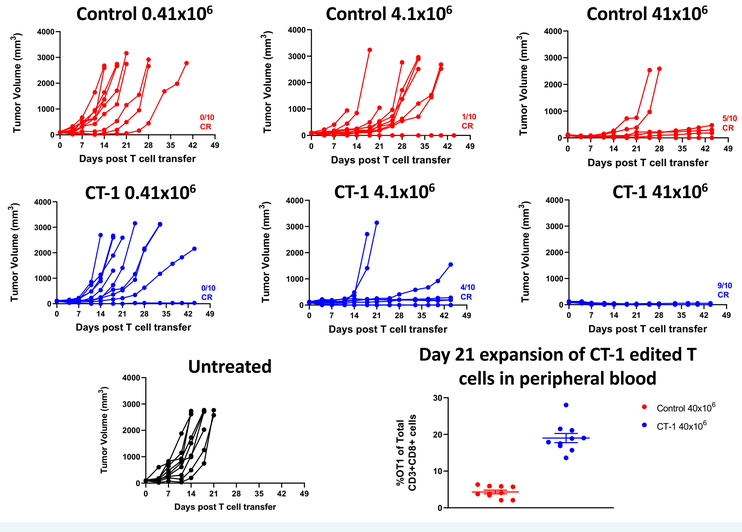


Dynamic Tcm/Tem balance of CT-1 edited OT1s at steady-state and following re-challenge



- B16-Ova mice cured post transfer of CT-1 edited OT1 T cells do not re-establish tumors following re-challenge
- CT-1 edited OT1 cells in peripheral blood display a high central memory phenotype prior to re-challenge, an effector phenotype at day 8 post tumor re-challenge, and progressive differentiation back to a Tcm state

Figure 4: CT-1 edited T cells are 10x more potent than control T cells, and accumulate 5x greater in peripheral blood



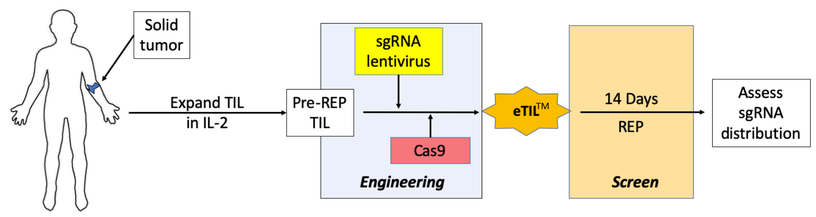
Untreated

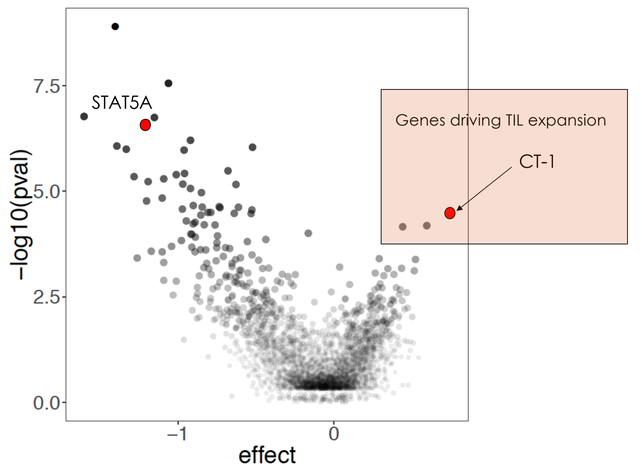
Day 21 expansion of CT-1 edited T cells in peripheral blood

- CT-1 edited T cells have 10x potency in the B16-Ova model compared to control T cells
- At 21 days post adoptive transfer, CT-1 edited T cells are found at a higher frequency in peripheral blood compared to control T cells

Figure 5: CT-1 is the top hit in a TIL manufacturing CRISPR screen

TIL CRISPR screen schematic:

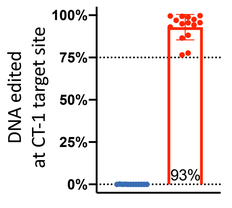




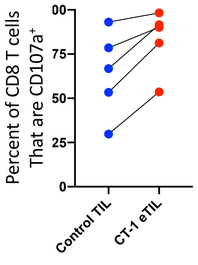
- A pooled CRISPR screen identified the CT-1 gene as the top hit for targets that enhance TIL expansion during manufacturing
- T cell relevant dropouts, including STAT5A, observed in screen, demonstrating screen quality

Figure 6: CT-1 edited human TIL demonstrate enhanced cytokine production and ability to degranulate

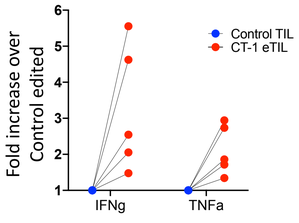
Amplicon sequencing of CT-1 target site



Degranulation (CD107a staining)



Cytokine secretion upon stimulation



5 unique donors, lines connect TILs from the same donor

- KSQ's manufacturing protocol leads to ~93% of TIL being edited at the CT-1 locus
- CT-1 edited TIL demonstrate:
  - Increased ability to degranulate in response to PMA/Ionomycin (4hr)
  - Increased IFNy and TNFa secretion upon restimulation with anti-CD3 antibody. Cytokine production measured by MSD, and normalized to control edited TIL.

Conclusions

- **Murine in vivo T cell and human in vitro TIL CRISPR screening identified CT-1 as a top target with potential to increase efficacy of TIL adoptive cell therapy**
- **CT-1 edited T cells exhibit increased and durable anti-tumor potential associated with memory formation in murine syngeneic models**
- **CT-1 edited human eTIL demonstrate enhanced inflammatory properties *in vitro***