Systematic identification of potent guide RNAs with minimal off-target activity for the CRISPR/Cas9 engineering of KSQ-001, an engineered Tumor Infiltrating Lymphocyte (eTIL™)

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Abstract

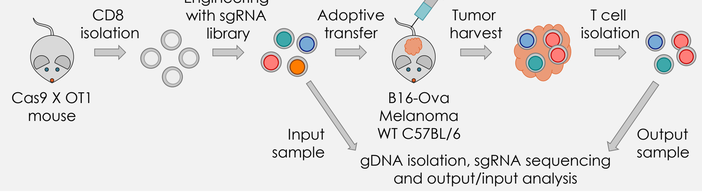
Adoptive cell therapy (ACT) with *ex vivo* expanded Tumor Infiltrating Lymphocytes (TIL) offers a potentially transformative treatment for refractory solid tumors. However, the immunosuppressive tumor microenvironment (TME) limits the effectiveness of TIL therapy. To identify gene targets capable of enhancing anti-tumor T cell function, we performed a genome-wide CRISPR screen in T cells *in vivo*. We discovered and subsequently validated CT-1 as a top target to improve T cell function for ACT. KSQ-001 is an engineered TIL (eTIL™) therapy with the CT-1 gene inactivated by CRISPR/Cas9. KSQ-001 is under development as an autologous ACT for treatment-refractory solid tumors. We describe herein the identification of potent and selective sgRNAs used to target the CT-1 gene during the manufacture of human TIL into KSQ-001.

To identify sgRNAs suited to engineer KSQ-001, we systematically evaluated all potential SpCas9 CT-1 sgRNAs for potency and selectivity. We rank-ordered sgRNA potency by screening sgRNA tiling libraries targeting CT-1 using a functional read-out in primary human T cells. Top hits were independently validated by assessing editing efficiency at the genomic cut-site and by performing CT-1-dependent functional assays in primary human T cells. To identify selective sgRNAs able to potently inactivate CT-1 with minimal off-target edits, an *in silico* approach was paired with unbiased experimental mapping of off-target cut sites in primary T cells using GUIDE-Seq. Identified CT-1 sgRNA off-target sites were then verified using targeted amplicon sequencing and target capture technology, with CT-1 sgRNAs further triaged. Using these assays, we identified a sgRNA targeting CT-1 with an editing efficiency at or above 90% in human TIL that translated to robust pathway modulation and possessed minimal off-target edits.

Together, these data demonstrate the discovery of a potent and selective CT-1 sgRNA that will be used for the manufacture of KSQ-001eTIL™ in the clinic.

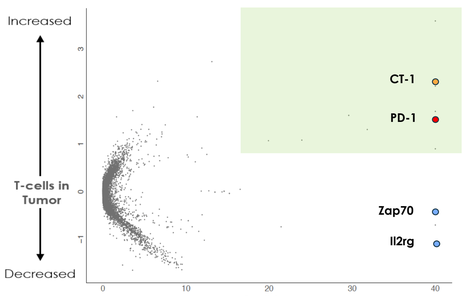
Identification and validation of CT-1 as top target for enhancement of anti-tumor T cell function

1 Screen design



- An *in vivo* genome-wide CRISPR screen in T cells was performed to identify targets enhancing T cell anti-tumor function.
- Screen QC checks included recovery of >90% sgRNAs contained in the library, depletion of T cell essential genes, and expression of identified hits in T cells.

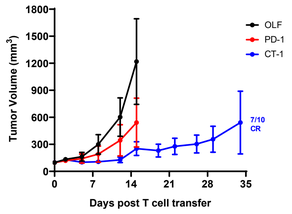
2 Genome-wide *in vivo* CRISPR T cell screen identifies CT-1 as novel target



- CT-1 was identified as a novel T cell target that demonstrated greater T cell enrichment in the tumor than PD-1.
- T cell essential genes Zap70, and IL2rg were depleted.

3 *In vivo* CT-1 target validation

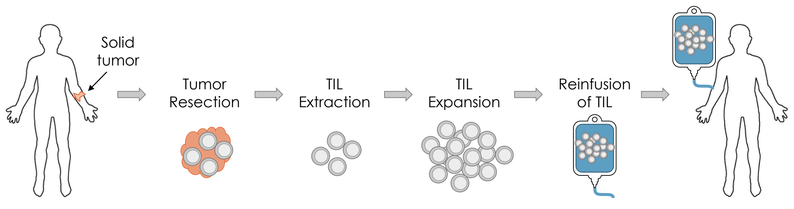
PD-1 sensitive B16-Ova mouse melanoma model



Adoptive transfer of CT-1 edited OT1 T cells into mice bearing B16-Ova melanoma tumors demonstrated greater efficacy than PD-1-edited T cells.

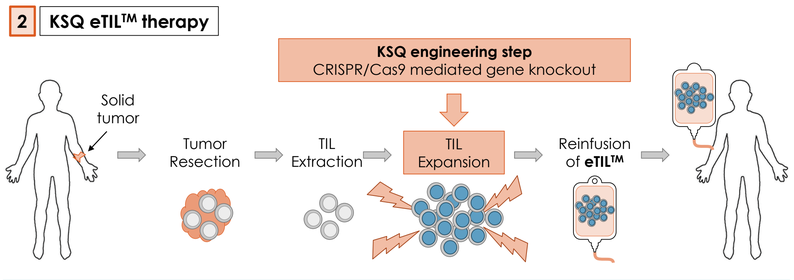
eTIL™ are *ex vivo* expanded, gene-edited TIL for the treatment of refractory solid tumors

1 Traditional TIL therapy



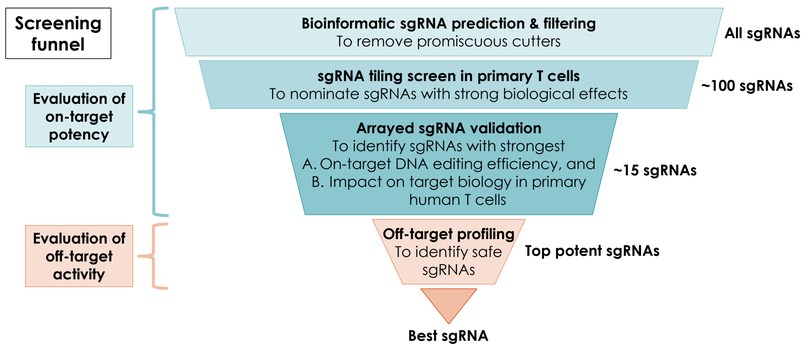
- TIL are grown from resected tumor biopsies and expanded *ex vivo* prior to re-infusion to the patient.
- Poor engraftment or an immunosuppressive TME can limit the effectiveness of TIL therapy.

2 KSQ eTIL™ therapy



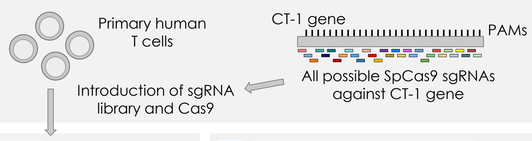
- Anti-tumor TIL function can potentially be enhanced by inactivating select targets during *ex vivo* TIL expansion.
- KSQ-001 is an engineered TIL (eTIL™) therapy in which the CT-1 gene is inactivated by CRISPR/Cas9-mediated gene editing.
- The sgRNA selection process was designed to maximize on-target editing and minimize off-target cutting activity.

Systematic evaluation of CT-1 sgRNAs for on-target potency and selectivity

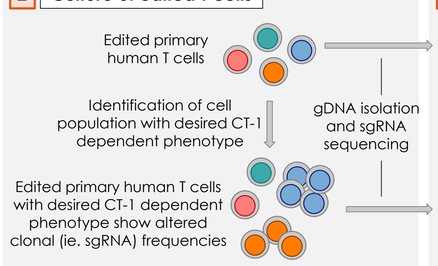


CRISPR tiling screen in primary human T cells for nomination of potent CT-1 sgRNAs

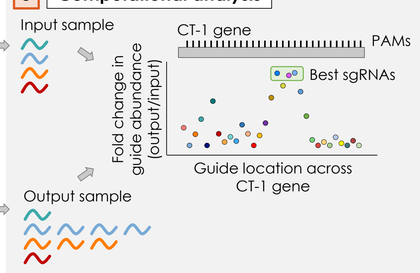
1 T cell engineering with CT-1 tiling sgRNA library



2 Culture of edited T cells



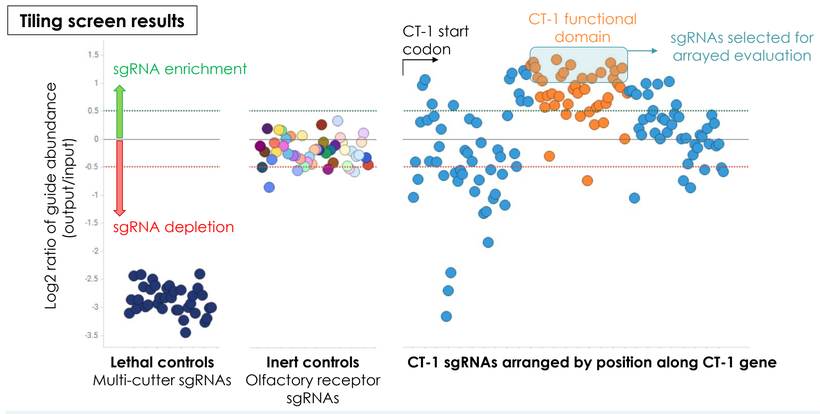
3 Computational analysis



- A lentiviral CT-1 tiling sgRNA library was created which contained all SpCas9-compatible CT-1 sgRNAs except those bioinformatically predicted to target more than 2 sites in the genome
- This tiling library was transduced into primary human T cells and screened for sgRNA enrichment in T cells displaying the desired CT-1 dependent phenotype.

sgRNAs targeting the CT-1 functional domain lead to strongest enhancement of T cell proliferation

Tiling screen results



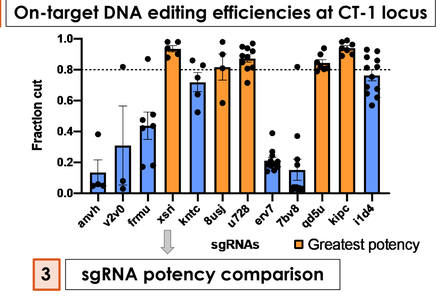
- Performance of CT-1 sgRNAs in this phenotypic screen depends on 1) DNA cutting efficiency, and 2) the nature of the resulting indels.
- Indels leading to a loss-of-function CT-1 variant have greater impact on T cell phenotype.
- sgRNAs targeting the CT-1 functional domain achieved greatest sgRNA enrichment, reflective of indels formed in this domain having a larger impact on CT-1 function.
- Pairing sgRNA performance in this screen with computationally predicted sgRNA selectivity, a subset of sgRNAs against the CT-1 functional domain were chosen for follow up validation studies.

Arrayed testing of CT-1 functional domain sgRNAs for on-target potency in primary human T cells

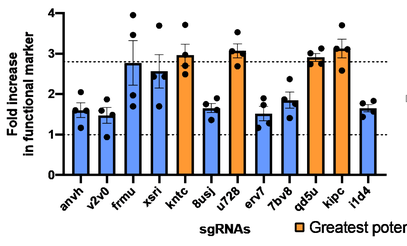
Potency of selected CT-1 sgRNAs was compared by evaluating

1. DNA editing efficiencies (assessed by targeted Amplicon Sequencing of the respective sgRNA cut sites), and
2. Fold increases in a functional marker of CT-1 inhibition (independent of the phenotypic read out used in the tiling screen). Marker levels observed with an inert control sgRNA served as a reference (set to 1).

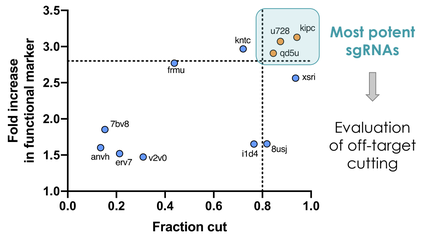
1 On-target DNA editing efficiencies at CT-1 locus



2 CT-1 dependent functional assay

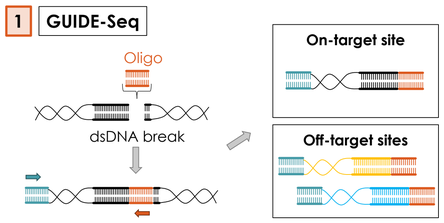


3 sgRNA potency comparison



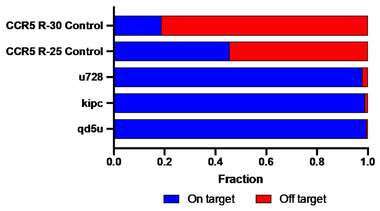
Off-target profiling in primary human T cells identifies selective CT-1 sgRNA for clinical use

1 GUIDE-Seq



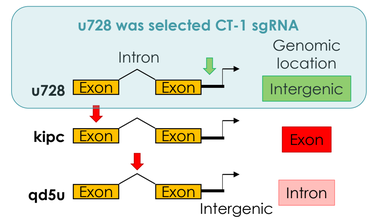
- Off-target editing of potent CT-1 sgRNAs was profiled by GUIDE-Seq
- Inclusion of a blunt-end dsDNA oligo during gene editing allows PCR amplification of break point adjacent DNA.
- NGS analysis of the resulting amplicons yields on-target vs off-target cutting frequencies and reveals off-target identities.

2 Frequency of on-target vs off-target cutting



- Two sgRNAs targeting CCR5 with previously reported high off-target frequencies served as positive controls (Cradick *et al.* (2013) Nucleic Acids Research).
- The evaluated CT-1 sgRNAs showed very low frequencies of off-target integration (~2% or less).

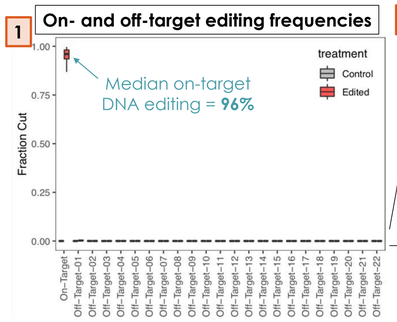
3 Genomic location of top off-target site



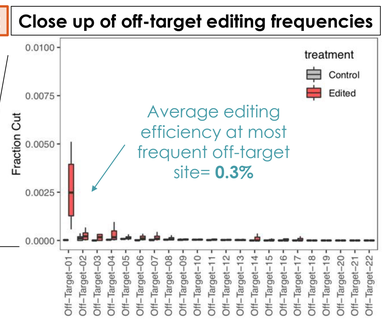
- The genomic location of off-target sites allowed further sgRNA differentiation, as we were careful to exclude sgRNAs with off-target sites in coding regions of the genome.
- The most frequent off-target site detected with the u728 guide resides in an intergenic region of the genome, making this the preferential sgRNA for clinical use.

CT-1_u728 sgRNA potency and specificity was confirmed in primary human TIL

1 On- and off-target editing frequencies



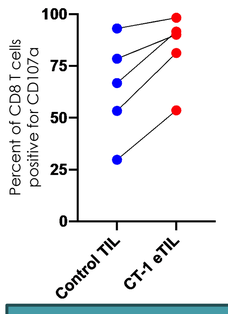
2 Close up of off-target editing frequencies



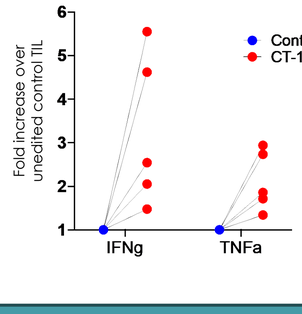
- GUIDE-Seq had shown overall off-target editing for sgRNA CT-1_u728 to be low, but identified 22 different potential off-target loci. (Notably, none in coding regions.)
- Amplicon Sequencing was used to evaluate editing efficiencies at the on-target and all 22 potential off-target sites in primary human melanoma TIL. Unedited TIL served as a control.
- With KSQ's optimized eTIL™ engineering protocol, CT-1_u728 achieved a median of 96% on-target DNA editing in primary human TIL.
- Off-target-01 was detectable at low levels (0.3%), while the majority of the remaining sites did not reach editing efficiencies above the NGS error rate.
- CT-1_u728 presents the desired profile for clinical use with high on-target gene editing, and minimal off-target cutting activity impacting no other coding regions of the genome.

CT-1_u278 sgRNA induces cytokine secretion and degranulation in primary human eTIL™ product

1 Degranulation (CD107a staining)



2 Cytokine secretion upon stimulation



CT-1 edited TIL demonstrated increased ability to degranulate in response to PMA/Ionomycin as well as increased IFNg and TNFa secretion upon re-stimulation with anti-CD3 antibody. Cytokine production (measured by MSD) was normalized to unedited control TIL. Data for five unique donors is shown, lines connect TIL from the same donor.

Conclusions

- **TIL therapy has shown promise for the treatment of solid tumors but the numbers of complete responses remain limited**
- **Using our CRISPRomics® platform we identified CT-1 as a top target for the enhancement of anti-tumor T cell function for ACT**
- **eTIL™ are promising therapeutics for treatment-refractory solid tumors, with KSQ-001 being the lead CT-1-edited eTIL™**
- **To enable clinical application of CT-1 editing, a systematic sgRNA screening funnel was established that identified potent and selective sgRNAs against CT-1**
- **The CT-1_u728 sgRNA together with our internally optimized editing protocol provides robust and consistent editing (>90%) of the CT-1 gene in primary human melanoma TIL, with minimal off-target editing at other sites**
- **These data support clinical evaluation of KSQ-001 eTIL™ as a therapy for treatment-refractory solid tumors**