A Versatile, Non-Viral Gene Editing Method for Directing Specificity and Enhancing Function of T Cells

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Introduction

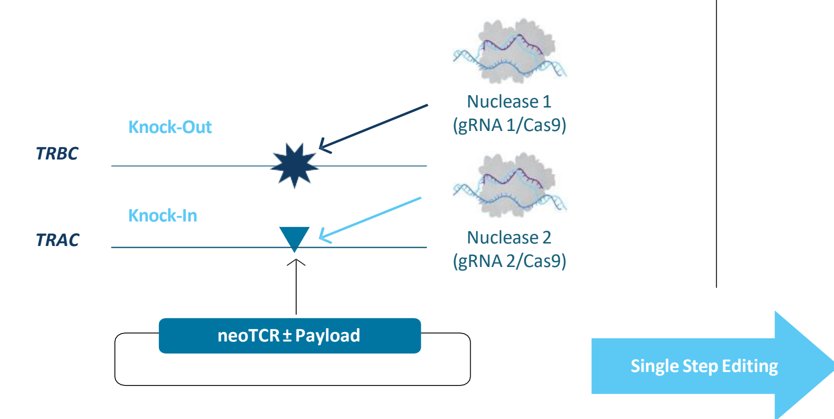
PACT Pharma has developed a robust single-step, targeted, non-viral method (PACT^NV™) for the manufacturing of personalized adoptive cells therapies for the treatment of solid cancers (NCT03970382). For this, we insert and express a neoepitope-specific T cell receptor (neoTCR) from the endogenous locus while simultaneously abolishing the expression of endogenous TCR.

The diversity of tumor types and challenges that can be presented to the immune system suggest that engineered immune cells may be more effective against a subset of tumors when manufactured with additional modifications. To this end, we have developed multiple non-viral gene editing methods to disrupt genes, express additional transgenes, and knockdown RNA transcript via shRNA.

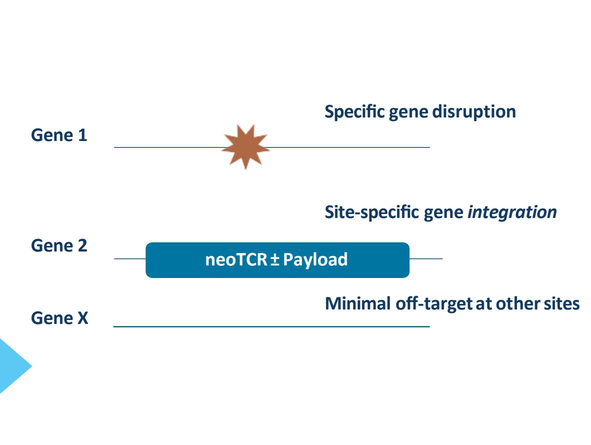
Here, we demonstrate the generation of a T cell product expressing a neoTCR, without the endogenous TCR, and with the additional deletion of the TGFBR2 gene. Second, we have designed a shRNA expression construct that can efficiently knock down transcript levels of chosen gene targets in cells expressing the neoTCR transgene without the generation of any additional double-strand breaks. Lastly, we demonstrate a non-viral knock-in strategy for expressing additional gene products under the control of a variety of promoters allowing the expression at various levels as well as at different cell states.

Methods

Process: Precision Editing



Result: Specific Genomic Modifications



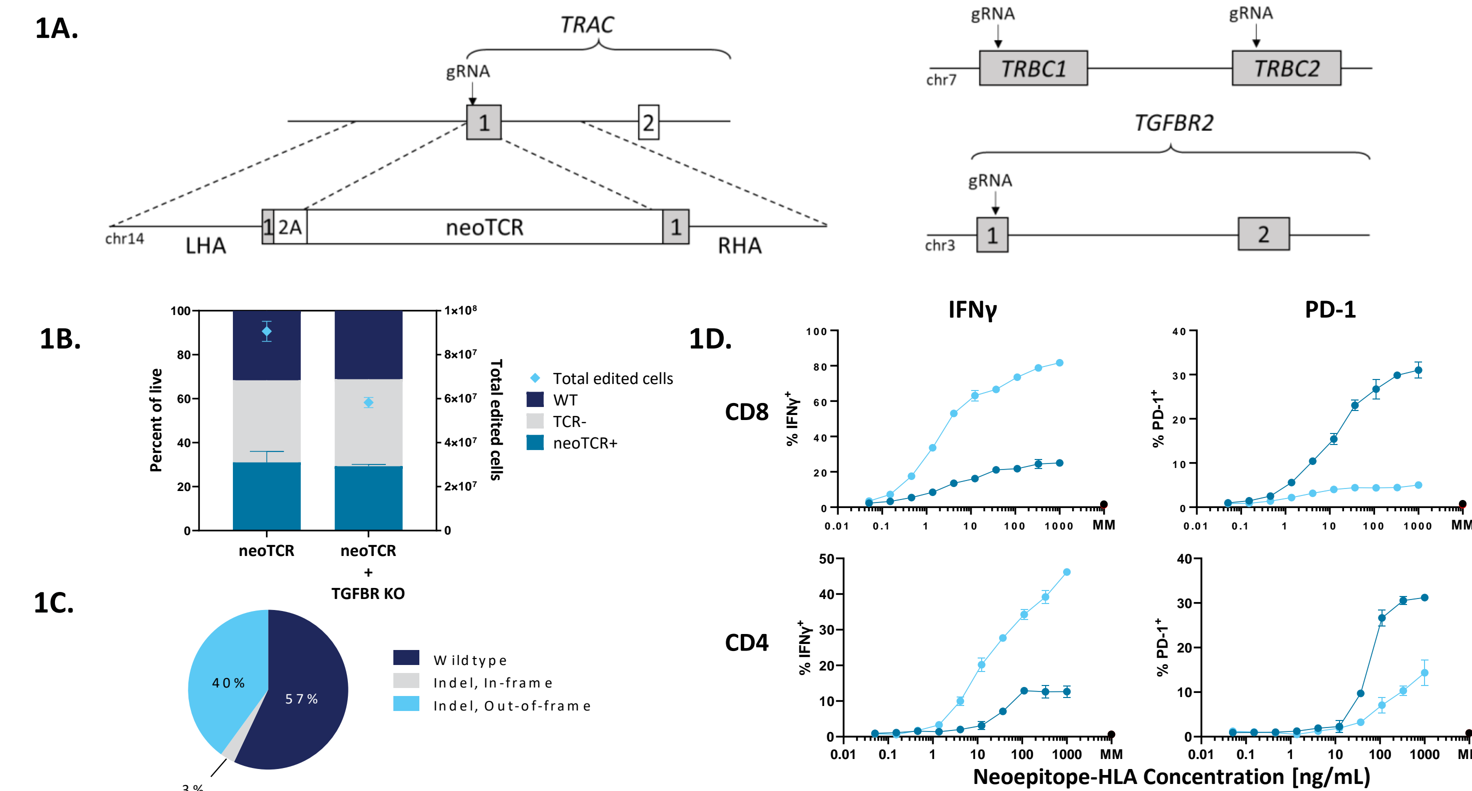
In a single-step process, T cells are edited at the *TRAC* and *TRBC* locus by two RNA-guided nucleases targeting each loci, knocking out endogenous TCR expression. A homology directed repair template encoding a neoTCR and an optional additional payload is then integrated into the *TRAC* locus by homologous recombination. As a result, the neoTCR is expressed and regulated naturally by the endogenous promoter and expression of the endogenous TCR is abolished. The additional payload integrated at the *TRAC* locus can also be expressed using the endogenous *TRAC* promoter or be driven by an exogenous promoter.

Conclusions

- The PACT^NV™ single-step, non-viral precision genome engineering technology is highly versatile with the ability to knock-out, knock-down, knock-in, and precisely regulate additional genes in a single step.
- We demonstrate the ability to generate neoTCR T cells with *TGFBR2* knock-out, greatly increasing the functionality of the cell product.
- neoTCR T cells were generated with PACT^NV technology to knock-down expression of multiple genes without any associated double-stranded break.
- Using PACT^NV technology, we demonstrate the capability to express at various strengths, a gene product induced by T cell activation in neoTCR T cells.
- The PACT^NV technology has the potential to expand the applicability of T cell drug products and can be applied to other cellular therapies.

Results

Figure 1. Engineering neoTCR Expressing, TGFBR2 Knock-out T Cells Using PACT^NV Technology



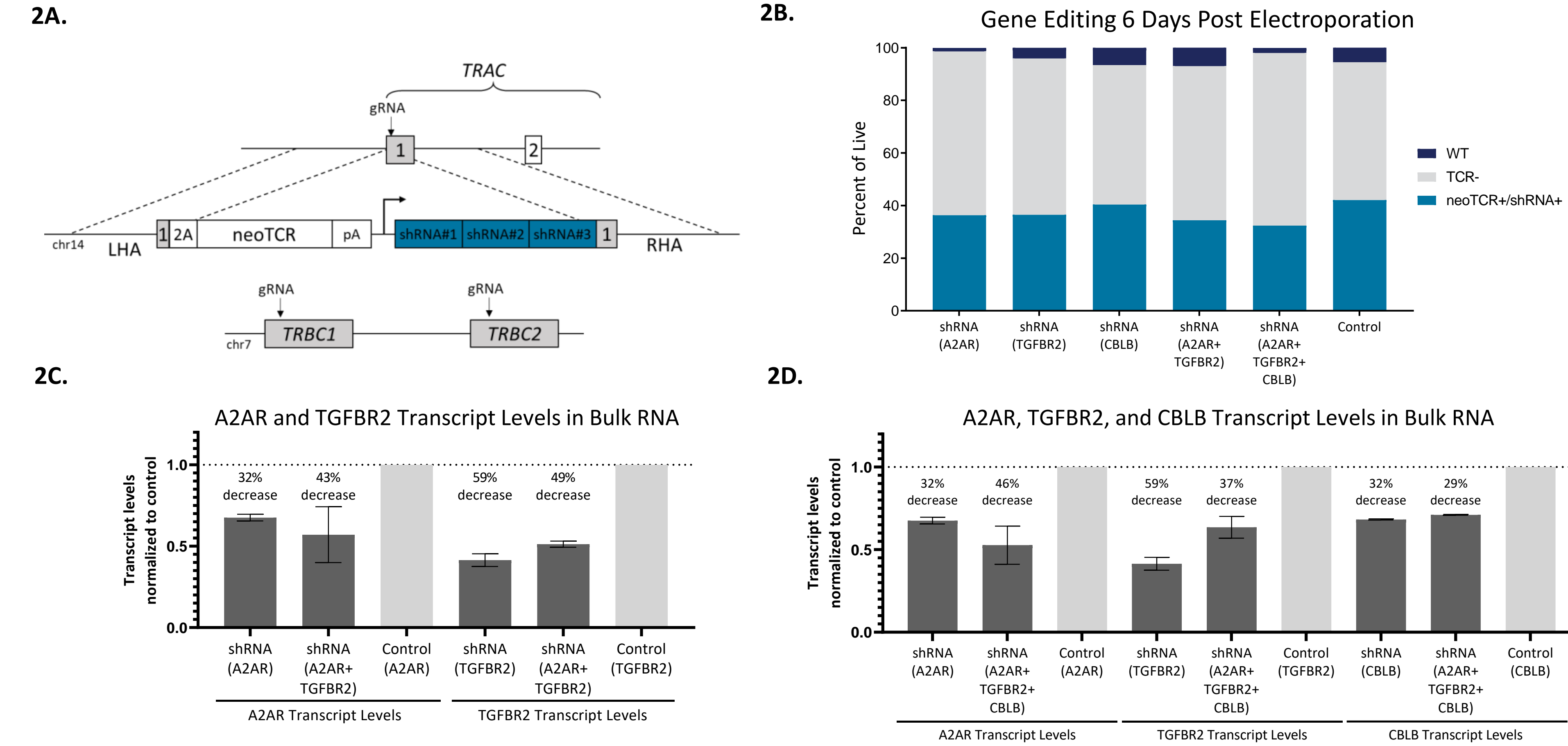
1A. *Primary human T cells were precision engineered to express neoTCR while abolishing expression of the endogenous TCR and the TGFβ receptor type 2 (TGFBR2). Site-specific nucleases targeting the first exon of TRAC, the first exons of TRBC1 and TRBC2, and the first exon of TGFBR2 were transfected along with a homology directed repair template containing the sequences for the 2A peptide, neoTCR, and left and right homology arms (LHA/RHA). The editing at all three loci was performed in a single step using PACT^NV technology.*

1B. *neoTCR expression and knock-out of the endogenous TCR in engineered T cells was measured using fluorescently-labeled neoepitope-HLA multimer and flow cytometry. Expression of the neoTCR was observed in over 30% of cells in this experiment and efficient knock-out of the endogenous TCR was observed. Total edit cells were calculated using the neoTCR+ percentage and total cell counts.*

1C. *Genomic DNA was harvested from engineered cells and the TGFBR2 locus was PCR amplified. Amplicons were Sanger sequenced and analyzed with ICE software to detect indels. Efficient disruption of the TGFBR2 gene was observed as indels in 43% of alleles, with a majority of indels (40/43%) generating out-of-frame mutations.*

1D. *T cells engineered to express the neoTCR (dark blue lines) or neoTCR with TGFBR2 knock-out (light blue lines) were stimulated using increasing concentrations of matched neoepitope-HLA and TGFβ. T cells with TGFBR2 knock-out were protected from the inhibitory effects of TGFβ. Reduced levels of PD-1 were also observed in TGFBR2 knock-out cells suggesting a reduction in exhaustion. Cytokine production and PD-1 surface expression was normalized to levels observed when cells were stimulated with mismatched neoepitope-HLA multimer (MM).*

Figure 2. Engineering neoTCR Expressing, shRNA Knock-down T cells Using PACT^NV Technology



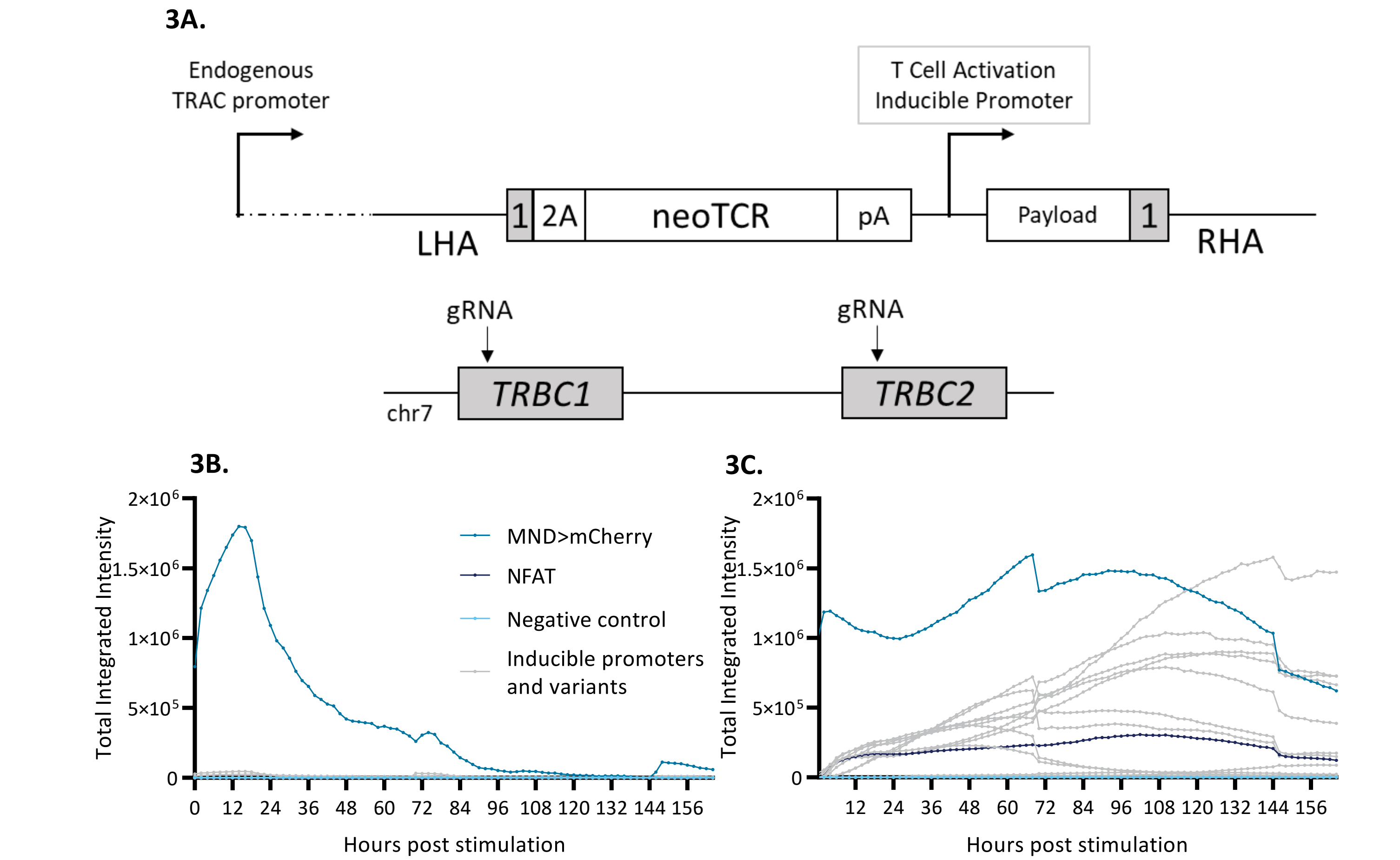
2A. *neoTCR+ T cells were generated using PACT^NV technology and engineered to express one, two, or three short hairpin RNAs (shRNAs) to knock-down the expression of various targets. The shRNA scaffolds were expressed under the control of a promoter introduced downstream of the neoTCR. neoTCR transcription was terminated using an exogenous polyadenylation signal (pA). As before, the expression of the endogenous TCR was abolished by knocking out expression of the TCR beta chain.*

2B. *neoTCR expression and knock-out of the endogenous TCR in engineered T cells was measured using fluorescently-labeled neoepitope-HLA multimer flow cytometry. In this experiment, robust expression of neoTCR and neoTCR+shRNA was observed 4 days post-electroporation (light blue bars).*

2C. *Total RNA from edited and unedited cells were extracted from PACT^NV engineered cells. Expression of A2AR and TGFBR2 was measured using ddPCR. Robust knock-down of shRNA target transcripts was observed in the bulk population when knocking down one (A2AR) or two (A2AR and TGFBR2) transcripts.*

2D. *Total RNA from edited and unedited cells were extracted from PACT^NV engineered cells. Expression of A2AR,TGFBR2, and CBLB was measured using ddPCR. Robust knock-down of shRNA target transcripts was observed in the bulk population when knocking down one (A2AR), two (A2AR and TGFBR2), or three (A2AR, TGFBR2, CBLB) transcripts.*

Figure 3. Generation of neoTCR+ T cells Expressing Payload Induced by T Cell Activation



3A. *To express a T cell activation induced payload in neoTCR-expressing T cells, inducible promoters were designed and introduced downstream of the neoTCR. The neoTCR remains naturally regulated by the endogenous TCR alpha chain promoter. neoTCR transcription was terminated using an exogenous polyadenylation signal (pA). As before, the expression of the endogenous TCR was abolished by knocking out expression of the TCR beta chain.*

3B and 3C. *T cells engineered to express neoTCR and an inducible payload, in this case, the fluorescent protein mCherry, were plated with mismatched peptide-HLA multimer (**3B**) and cognate peptide-multimer (**3C**). As a positive control, neoTCR T cells were engineered with a constitutive promoter (MND) driving the expression of mCherry. Fourteen inducible promoter variants were tested against a previously reported inducible promoter driven by the NFAT transcription factor. A negative control line was generated without an inducible promoter or mCherry. Cells were incubated at 37C for 164 hours and monitored by Incucyte time-lapse microscopy. Images were analyzed and total integrated intensity was plotted over time.*