Hotspot mutations in *KRAS* and *TP53* targeted by TCR-T cells genetically modified with the Sleeping Beauty transposon/transposase system

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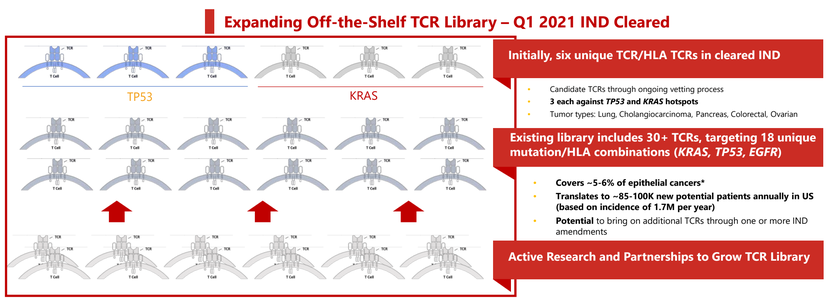
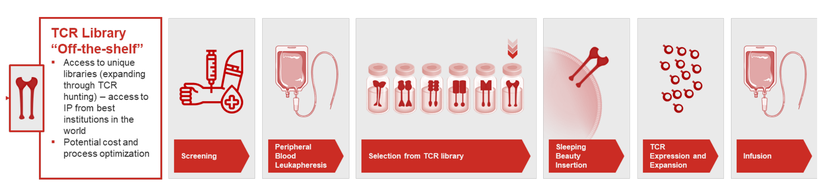
Submitted Abstract

Mutations in critical genes for cell survival and proliferation, *e.g., KRAS* and *TP53*, are found as clonal events in multiple tumor types of unrelated people likely due to their importance to the malignant phenotype. T cells recognize products of mutated genes, termed neoantigens, because they are expressed in the tumor but not in the normal tissues; thus, neoantigens are foreign entities from an immunological perspective. T-cell receptors (TCRs) with specificity to the neoantigen in the context of human leukocyte antigen (HLA) on the tumor cell surface can be isolated from the neoantigen-reactive T cell and potentially used for genetically-modified adoptive immunotherapy for any patient with matching mutation and HLA. The purpose of this study was to evaluate the ability of the non-viral *Sleeping Beauty* transposon/transposase gene transfer system to re-direct the specificity of T cells towards p53 and KRAS neoantigens and to characterize the resultant engineered TCR-T cell populations for specificity and function. Genes encoding the alpha and beta chains of p53 or KRAS neoantigen-specific TCRs were linked by a 2A ribosomal slip site linker and cloned into the clinical *Sleeping Beauty* transposon. These transposons were co-electroporated into donor peripheral blood leukocytes (PBL) with a DNA plasmid encoding the SB11 transposase and expanded *in vitro*. Logarithmic proliferation was observed and resulted in large numbers of highly pure TCR-T cells(>80% by introduced TCR expression). The TCR-T cells upregulated 41BB on the T-cell surface and secreted interferon-γ in response to antigen presenting cells with the appropriate HLA molecule pulsed with KRAS or p53 neoantigen peptides but not the cognate wild type peptide, confirming re-directed specificity to mutated *KRAS* or *TP53* genes, respectively. Similarly, TCR-engineered T cells demonstrated cytolysis of tumor cells expressing the neoantigen and appropriate HLA restriction, suggesting that adoptive transfer of these TCR-T cells could mediate anti-tumor responses. In all, we demonstrated that multiple TCRs with unique specificities targeting recurrent p53 and KRAS substitutions in frequent HLA haplotypes could be stably expressed using *Sleeping Beauty* transposition to re-direct peripheral blood T cells towards tumor cells. Translation of these TCR-T cells into adoptive immunotherapy could result in safe and effective treatments for any cancer patient with matching HLA and *KRAS* or *TP53* hotspot mutations.

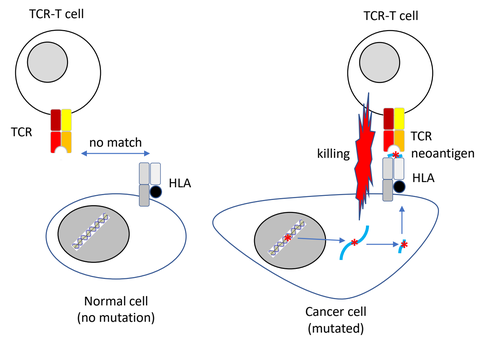
Library TCR-T cells targeting shared hotspot mutations



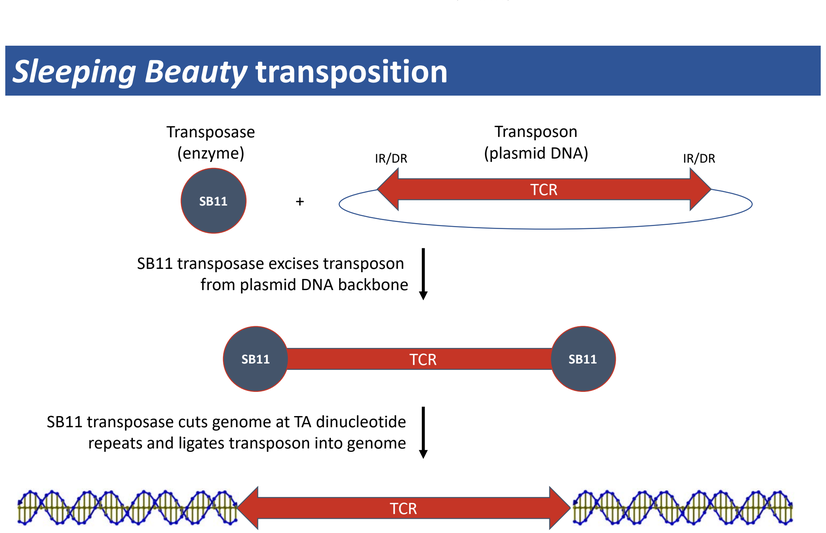
*HLA (http://www.allelefrequencies.net/) and COSMIC (https://cancer.sanger.ac.uk/cosmic) databases.



Neoantigens at tumor-specific targets of TCR-T cells

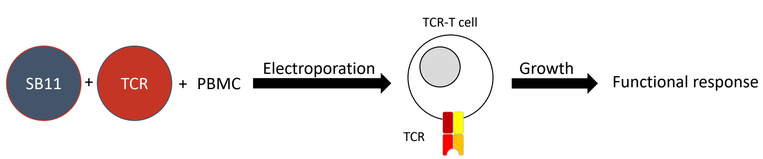


Sleeping Beauty transposition

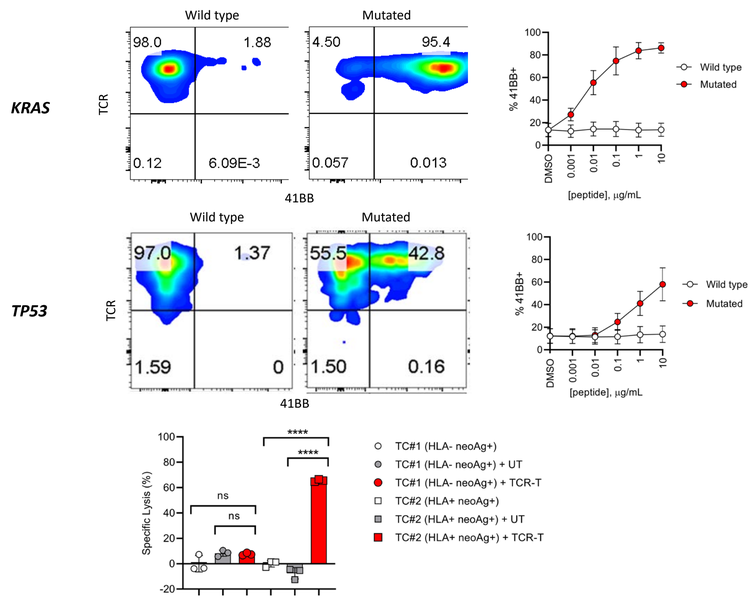


- SB11 transposase is transiently expressed (non-integrated)
- Stable expression of TCR is driven by internal promoter within the transposon cassette.
- Non-viral expression system capable of genetically modifying primary cells, *e.g.,* T cells.

Non-viral *Sleeping Beauty* transposition to generate TCR-T cells in peripheral blood T cells



TCR-T cells specifically recognize neoantigens and kill unmodified epithelial cancer cells



- TCR-T cells were co-cultured with antigen presenting cells pulsed with KRAS (top) or p53 (bottom) peptides either in wild type or mutated variants.
- Expression of T-cell activation was measured by up-regulation of 41BB on the TCR-T cell surface.
- Dose response to the mutated, but not the wild type, peptides was observed for both KRAS and p53 neoantigens, demonstrating that TCR-T cells were specific and did not recognize the germline sequences and are, therefore, unlikely to recognize normal tissues.
- Tumor cells expressing the hotspot mutation and either an irrelevant HLA (TC#1; HLA-neoAg+) or relevant HLA (TC#2; HLA+neoAg+) were co-cultured with no T cells (open shapes), open repertoire untransfected T cells (gray shapes) or TCR-T cells (red shapes). Tumor killing was evaluated by CellTiter-Glo assay which evaluates viable cells relative to control wells and was used to calculate relative specific lysis.
- Specific recognition of the tumor cells with matching HLA and mutation, but not tumor cells lacking the HLA restriction, by TCR-T cells and not untransfected T cells showed that specific tumor killing could occur through this approach.

Conclusions and future directions

- *Sleeping Beauty* transposition was effective in expressing neoantigen-specific TCRs in donor peripheral blood T cells to generate TCR-T cells.
- TCR-T cells were specific for KRAS and p53 neoantigens and could directly kill unmodified tumor cell lines with endogenous expression of HLA and hotspot mutation.
- Translation of these TCR-T cells for clinical use is promising and could be an "off-the-shelf" reagent used for the treatment of cancer for anyone with matching HLA and hotspot mutation.
- A Ziopharm-sponsored Phase I/2 clinical trial has been cleared by the FDA and will evaluate the ability of a library of autologous KRAS and p53 neoantigen-specific TCR-T cells to eliminate relapsed/refractory tumors in adult patients with a number of cancer types including lung cancer, gynecological cancers, colorectal cancer, pancreatic cancer and cholangiocarcinoma.