Development of multi-targeting immunomodulatory SeekR™ RNA therapeutics to overcome resistance to PD-1 blockade

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Abstract

T-cell exhaustion describes the dysfunctional state of effector T cells in the tumor microenvironment that results from chronic and prolonged exposure to antigen. It also represents an important mechanism of clinical resistance to immune checkpoint blockade. Recently, the transcription factor NR4A1 has been shown to be upregulated in tumor-specific T cells and plays a role in driving exhaustion during chronic antigen stimulation of T cells. Pro-oncogenic activities of NR4A1 have long been observed in various solid tumors including colorectal and breast cancers, and co-expression of NR4A1 with known immune checkpoint markers such as PD-L1 have also been observed in various cancer cell lines. These findings have identified NR4A1 as an important target for overcoming resistance to cancer immunotherapy. We hypothesize that multi-targeting to simultaneously block PD-1 signaling and silence NR4A1 gene expression can reverse T-cell exhaustion and expand the clinical benefit of PD-1 blockade in immune therapy resistant solid tumors. Using our SeekR™ RNA therapeutics platform, we combined aptamer binders to PD-1 with siRNA against NR4A1, and VHL and evaluated their ability to reverse T-cell exhaustion and re-activate T-cell based anticancer immunity in solid tumors. Specifically, we created SeekR™ therapeutic RNA molecules with dual flanking RNA aptamers binders to PD-1 and other checkpoint inhibitors, connected by a double stranded bridge that encodes for siRNA silencers that target multiple immunomodulatory genes, such as NR4A1. The novel anti-PD-1 RNA aptamer component serve to: A) direct and deliver the SeekR™ RNA oligo therapeutics to exhausted T-cells that express PD-1 receptors; and B) internalize the siRNA component into the T-cells. The siRNA components of the SeekR™ serve to silence NR4A1 which lead to reversal of the cytotoxic T-cell exhaustion following chronic in-vitro stimulation. These results demonstrate that dual targeting of PD-1 and NR4A1 has the potential to reverse T-cell exhaustion and the potential to overcome cancer immunotherapy resistance in the clinic.

Background

- During chronic infections and cancer, sustained antigen stimulation leads to a dysfunctional exhausted state with T cells displaying impaired effector functions.
- Severely exhausted T cells undergo apoptosis and are eliminated leading to a significant decline in antigen-specific T cells.
- Blockade of co-inhibitory receptors such as PD1 can reactivate impaired cytotoxic T cells, however these cells are differentially responsive.
- Exhausted T cells in the tumor microenvironment express multiple inhibitory receptors and single agent ICB therapy can only partially overcome suppressive signals which leads to the development of resistance to therapy.
- The expression levels of TOX[1,2], NR4a[3,4] and VHL[5] regulate expression of co-inhibitory receptors on T cells and are negatively correlated with response rates to PD1 blockade therapy.
- We are establishing the SeekR™ platform to facilitate targeted delivery of therapeutic siRNA for reactivating exhausted T cells.
- Differential binding cell-SELEX is used to identify novel, optimized aptamers for use in developing the SeekR™ platform.

T Cell Exhaustion in Chronic Infection and Cancer

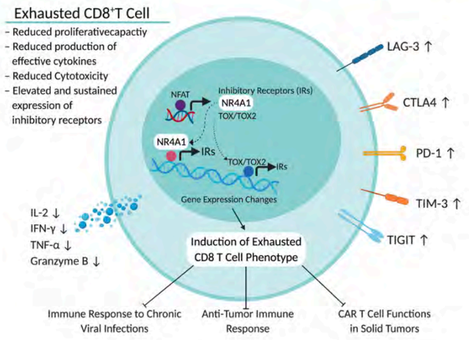

Figure 1. T Cell exhaustion is mainly characterized by a progressive loss of effector functions such as production of effector cytokines and cytotoxic activities. Exhausted T cells also have a reduced proliferative capacity, a sustained upregulation of multiple co-inhibitory receptors and display a unique transcriptional and epigenetic signature.

Cell-SELEX

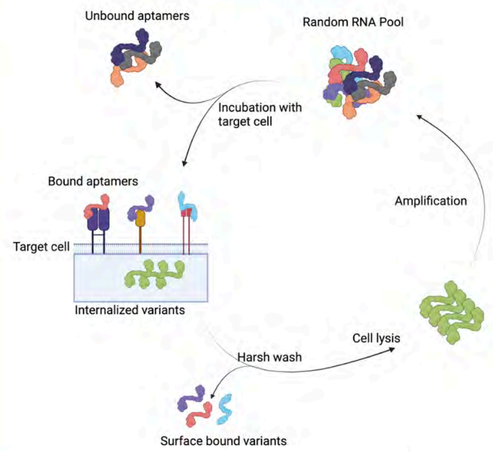

Figure 2. Cell systemic evolution of ligands by exponential enrichment (SELEX) has been used to generate aptamers against a variety of targets using live cells. A random RNA library is incubated with target cells and the unbound and surface bound aptamers are removed by extensive washing under harsh conditions. The internalized variants are then recovered by cell lysis and enriched by amplification through subsequent rounds of selection. Aptamers with strong binding and internalization properties can be used to better facilitate intracellular delivery of siRNA molecules.

In vitro T cell exhaustion

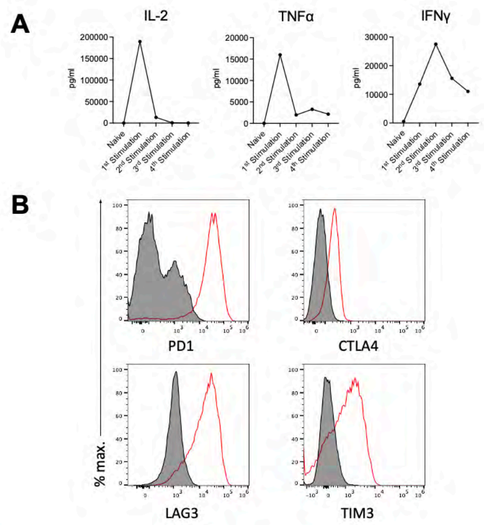

Figure 3. CD8 T cells were isolated from human PBMC and used to generate exhausted T cells by persistent activation through repeat stimulation with anti-CD3/CD28 beads every 2 days. **A)** ELISA shows an initial high secretion followed by a decreased production of the cytokines IL2, TNFα and IFNγ after each round of stimulation. **B)** Flow cytometry after the fourth stimulation (red line) show increased expression of the co-inhibitory receptors PD1, CTLA4, LAG3 and TIM3 compared to unstimulated cells (gray shade).

Validation of siRNA targets for T cell exhaustion

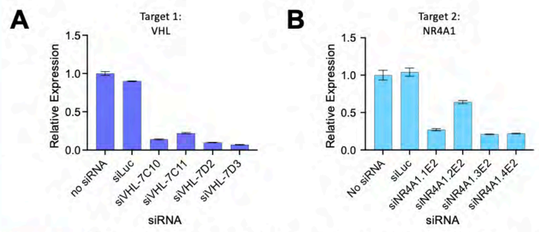

Figure 4. siRNA-mediated gene silencing was validated by transfection of cancer cell lines. **A)** HCT116 cells were transfected with control siRNA siLuc and VHL-targeting siRNAs (7C10, 7C11, 7D2, 7D3) or **B)** SKBR3 cells were transfected with siLuc and NR4A1-targeting (1E2, 2E2, 3E2, 4E2) siRNAs. Expression analysis was performed after 24 hours.

T cell immunomodulatory SeekR™

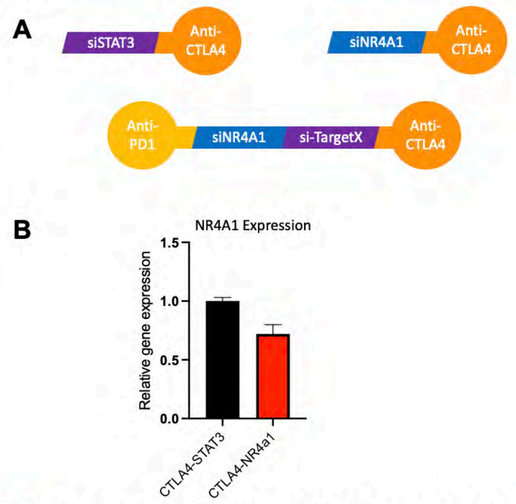

Figure 5. **A)** Schematic depicting monomeric (top) and a bivalent (bottom) T-cell directed SeekR™ for modulating exhausted T cells. **B)** Human PBMC was stimulated with PMA/Ionomycin for 4 hours before treatment with a monovalent CTLA4 SeekR™ containing either STAT3 or NR4A1 siRNA at 300nM concentration. Analysis for the expression of NR4A1 was performed at 48 hours after treatment by qPCR for RNA expression.

Conclusions

- We have generated CD8 T cells exhibiting an exhausted phenotype by persistent stimulation *in vitro*.
- We have generated aptamers with enhanced internalization properties, targeting a variety of immune checkpoint inhibitors through cell SELEX.
- We have identified siRNA sequences that effectively silences expression of target genes.
- SeekR™ constructs using CTLA4 aptamers and the NR4A1 siRNA can induce silencing *in vitro*.

Future Directions

- We are utilizing the SeekR™ platform to reactivate exhausted T cells and promote their survival in the tumor microenvironment by blocking immune checkpoint inhibitors or delivering therapeutic siRNA into these cells.
- We are developing multitargeting SeekR™ molecules with novel high-performing aptamers to other cell membrane proteins for improved targeted delivery.

References

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