A REGULATED CYTOKINE ENGINEERING STRATEGY TO AUGMENT IN VITRO AND IN VIVO PERSISTENCE OF ALLOGENEIC IPSC DERIVED CD8 T-CELLS

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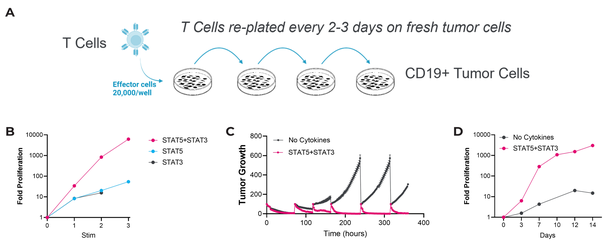
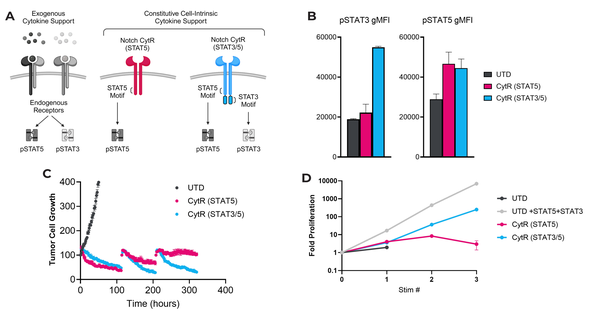
Figure 1: iPSC-derived CD8+ CAR-T cells require STAT3 and STAT5 support for long-term persistence and cytotoxicity.



(A) Schematic of in vitro serial tumor re-challenge assay. **(B)** Addition of exogenous cytokines providing both STAT5 and STAT3 support enable sustained proliferation of iPSC-derived CD8+ CAR-T cells. In contrast, the addition of only STAT5 support or STAT3 support is not sufficient to sustain proliferation of cells through 3 serial tumor challenges. **(C)** In the presence of STAT5 and STAT3 support, iPSC-derived CD8+ CAR-T cells control tumor target cells over a duration of two weeks, while in the absence of cytokines cells are unable to completely clear tumors. **(D)** In the absence of cytokine support, iPSC-derived CD8+ CAR-T cells fail to proliferate over a duration of two weeks, while in the presence of cytokines that drive STAT5 and STAT3 signaling the cells demonstrate robust proliferation.

Figure 2: An engineered cytokine receptor provides constitutive, cell-intrinsic STAT3 and STAT5 signaling to augment iPSC-derived CD8+ T cell proliferation and effector function.



(A) Notch's engineered cytokine receptor designs activate STAT5 or both STAT3 and STAT5 in a constitutive, cell-intrinsic manner. **(B)** Primary T cells transduced with CytR LVV induce pSTAT3 and pSTAT5. **(C)** iPSC-derived CD8+ CAR-T cells alone or transduced with a STAT5-only CytR design are unable to sustain tumor control in a serial re-challenge assay, whereas transduction with the chimeric STAT3/5 CytR design enables tumor control over repeated challenges. **(D)** Similarly, only transduction with the STAT3/5 CytR design enables iPSC-derived CD8+ CAR-T cell proliferation in the absence of exogenous cytokines. iPSC-derived CD8+ T cells alone or those transduced with a STAT5-only CytR design fail to proliferate over the course of a serial re-challenge assay.

Figure 3: Regulating engineered cytokine receptor through differentiation is critical for T-lineage commitment.

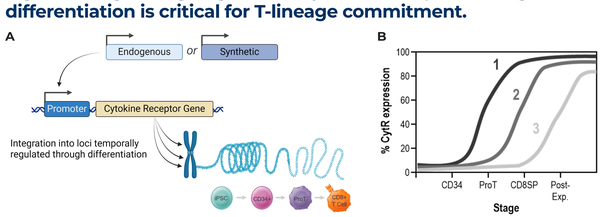
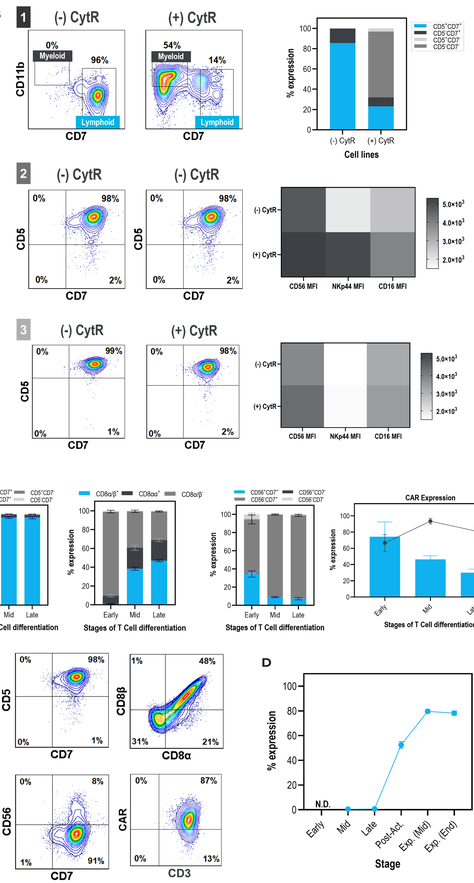
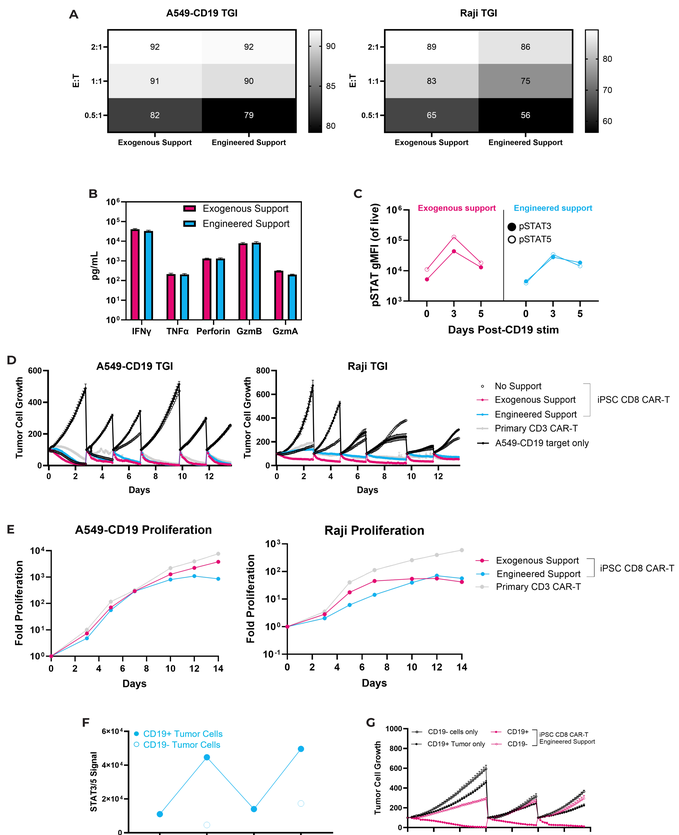


Figure 3 (CONT'D)



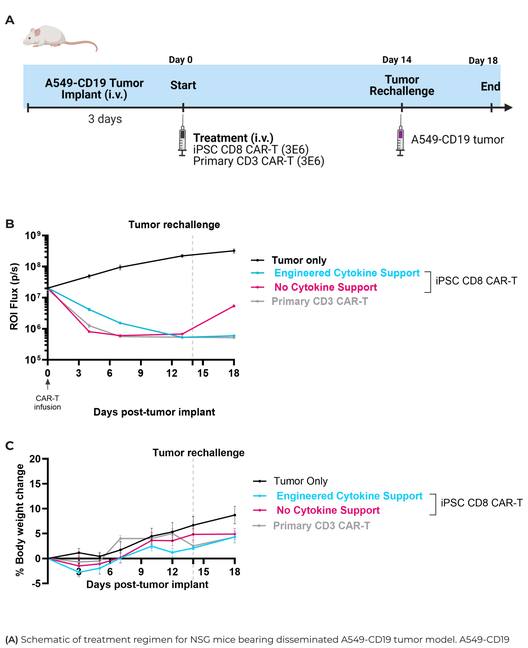
(A) Stringent regulated expression of the engineered cytokine receptor is critical for preserving the phenotype of cells through differentiation. Ideally, engineered cytokine receptor expression is repressed through differentiation and upregulated upon CD8SP activation. Regulation of cytokine receptor expression is achieved through the combination of promoter choice and site of gene integration. The engineered cytokine receptor gene cassette was integrated into the site of a gene with the desired expression profile with a synthetic, inducible promoter or coupled to the endogenous promoter. **(B)** Premature cytokine receptor (CytR) expression during differentiation results in suboptimal T-lineage commitment and emergence of off-target populations. CytR expression at the CD34 HPC stage leads to aberrant myeloid (CD11b)/lymphoid (CD7) lineage fate decisions, compromising subsequent T-cell progenitor specification and commitment stages of differentiation (1). Upregulation of CytR at intermediate developmental stages restores proper lymphoid commitment during T cell differentiation. However, expression of alternative innate lymphoid lineage markers (i.e. CD56, NKp44, CD16, etc.) is observed and can derail the course of downstream αβ T cell lineage commitment and maturation (2). Temporal regulation of CytR expression during later stages of T cell development is an essential step for the establishment of proper cell-fate decisions and generation of CD8αβ T cells in vitro (3). **(C)** iPSC-derived CD34+ HPCs differentiated to CD5+CD7+ lymphoid-progenitor cells are matured into CD8αβ+ T cells using Notch's proprietary, bead-based differentiation system. Assessment of key T-lineage markers (CD7, CD5, CD56, CD8α, CD8β) and CAR expression was performed throughout T cell differentiation using flow cytometry (n=2 biological replicates). Representative flow cytometry plots of iPSC-derived T cells. **(D)** Stage-specific induction of engineered cytokine receptor expression during T cell expansion. Engineered CytR surface expression was routinely monitored across multiple timepoints during T cell differentiation (Early, Mid, Late) and expansion (Post-Activation, Mid and End) using flow cytometry (n=2 biological replicates).

Figure 4: iPSC-derived CD8+ CAR-T cells harboring the engineered cytokine receptor demonstrate sustained effector function, long-term persistence, and antigen-dependent expression *in vitro*.



(A) In a single-stim assay iPSC-derived CD8+ CAR-T cells with engineered cytokine support exhibit comparable tumor growth inhibition to cells that receive optimal exogenous cytokine support against A549-CD19 and Raji target cells across multiple effector to target ratios. Tumor growth inhibition (TGI) after a 5-day coculture at multiple E:Ts was determined by Incucyte-based monitoring of target-cell fluorescence **(B)** Optimal cytokine secretion from iPSC-derived CD8+ CAR-T cells was also comparable between exogenous and engineered cytokine support measured at 48 hours after CD19 antigen stimulation, as measured by MSD **(C)** The engineered cytokine receptor design activates both STAT3 and STAT5 similar to exogenous cytokine support as measured by intracellular cytometry. **(D)** In a serial tumor challenge assay, iPSC-derived CD8+ CAR-T cells engineered with Notch's cytokine receptor exhibit comparable tumor cell control relative to cells with the optimal exogenous cytokine cocktail and primary blood-derived CD3 CAR-T cells. In contrast, iPSC-derived CD8+ CAR-T cells lacking cytokine support are unable to control target cells over the duration of the assay. **(E)** iPSC-derived CD8+ CAR-T cells with engineered cytokine support also demonstrate sustained proliferation over the duration of the serial challenge assay, like that of cells supported with exogenous cytokines or primary CD3 T cells. **(F)** iPSC-derived CD8+ CAR-T cells exhibit antigen-dependent cytokine receptor expression. Following initial tumor cell challenge, cytokine receptor expression is upregulated. Following tumor cell clearance, cytokine receptor expression returns to near baseline levels. Cytokine receptor expression remains low until T cells are rechallenged with tumor cells. Upon co-culture with CD19(-) target cells, cytokine receptor expression is not upregulated beyond baseline levels. **(G)** Consistent with antigen-dependent cytokine receptor expression, iPSC-derived CD8+ CAR-T cells do not demonstrate cytolytic activity when co-cultured with CD19(-) target cells and cytotoxicity is specific to CD19(+) target cells in culture.

Figure 5: iPSC-derived CD8+ CAR-T cells harboring the engineered cytokine receptor induce complete tumor clearance comparable to primary CD3 CAR-T cells *in vivo*.



(A) Schematic of treatment regimen for NSG mice bearing disseminated A549-CD19 tumor model. A549-CD19 cells co-expressing luciferase were implanted into 6–7-week-old NSG mice through lateral tail vein injection, three days before first CAR T injections. A single dose of 3x10^6 iPSC-derived CD8+ CAR-T cells with regulated cytokine support was administered intravenously. A single dose of 3x10^6 primary CD3+ CAR-Ts was used as a positive control. A single maximum dose of 1x10^7 of iPSC-derived CD8+ CAR-T without cytokine support was administered as a control. On Day 14, all treatment groups were rechallenged with additional A549-CD19 cells injected through the lateral tail vein.

(B) Tumor burden was monitored bi-weekly by bioluminescence imaging using IVIS Lumina III through intraperitoneal injection of D-luciferin substrate in sterile Phosphate-buffered saline. Quantification of tumor burden as region of interest (ROI) flux values are shown, with each dot representing mean of the treatment cohorts and error bars represent SEM.

(C) Percent change in body weight was monitored throughout study for assessment of acute toxicity.

SUMMARY AND CONCLUSIONS

- iPSC-derived CD8+ CAR-T cells require both STAT5 and STAT3 for long-term cytotoxicity and proliferation. We have demonstrated that an engineered cytokine receptor can provide cell-intrinsic support, eliminating the need for toxic and costly exogenous cytokines and opening an opportunity to eliminate lymphodepleting chemotherapy.
- Leveraging genomic sites that are regulated through T cell differentiation combined with context-specific regulatory sequences to regulate cytokine receptor expression is critical to ensure appropriate T-lineage commitment.
- Engineered iPSC-derived CD8+ T cells demonstrate tumor growth inhibition and persistence in the absence of exogenous cytokines with efficacy comparable to primary CD3+ T cells.
- These findings support the development of scalable, potent, uniform, and persistent allogeneic CAR-T cell therapies for the treatment of oncology and autoimmune patients.