

Background

The treatment of some patients with hematological malignancies has been transformed by chimeric antigen receptor T (CAR-T) cells. However, a large clinical need remains for more effective CAR-T therapies and to expand their use to broader patient groups. The exogenous administration of IL-2 or IL-21 were shown to enhance the engraftment, persistence, and functionality of CAR-Ts in preclinical models¹⁻⁵. However, clinical use of such cytokines with cell therapies is limited using current molecules due to their pleiotropic nature that can result in toxicity and undesired effects on endogenous immune cells, in addition to their beneficial effects on CAR-T cells⁶⁻¹⁰. To overcome this challenge, we applied our *cis*-targeting technology to develop CAR-T specific IL-2 and IL-21 molecules that selectively activate CAR-Ts and exhibit minimal activity on non-CAR cells.

Cis-targeting of CAR-T cells

Description of *cis*-targeting (Figure 1). Untargeted cytokine therapies confer effects both on CAR-T cells and endogenous cytokine-receptor expressing cells, limiting cytokine benefit to CAR-T cells by acting as a sink, driving toxicity, or inducing opposing effects (left). *Cis*-targeted cytokines selectively support CAR-T cells without activating endogenous cells (right). To generate an optimal *cis*-targeted cytokine, the cytokine potency must be reduced to decrease signaling to its cognate receptor (Figure 2). The activity is selectively rescued via avidity provided by a targeting domain that recognizes a cell-specific antigen.

Figure 1: Overview of *cis*-targeting

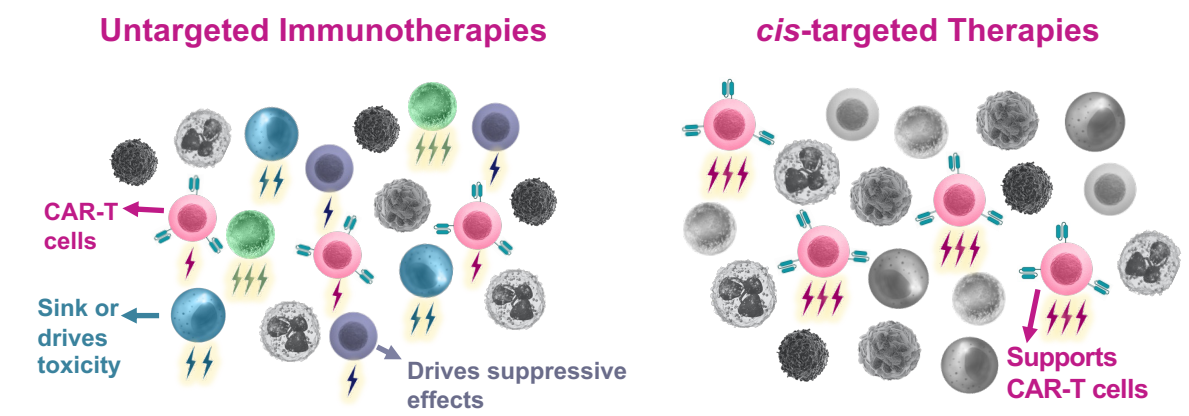


Figure 2: Generating a *cis*-targeted Cytokine

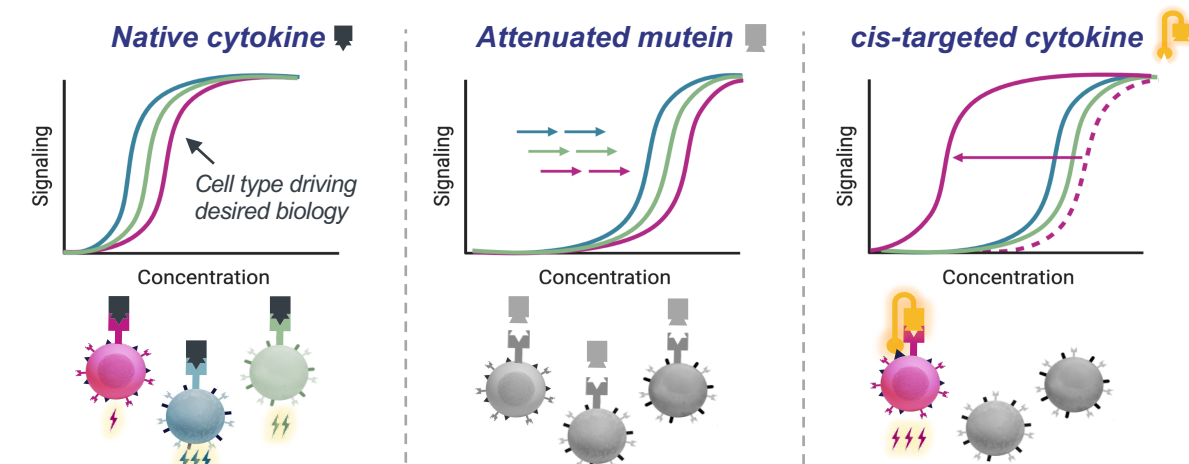
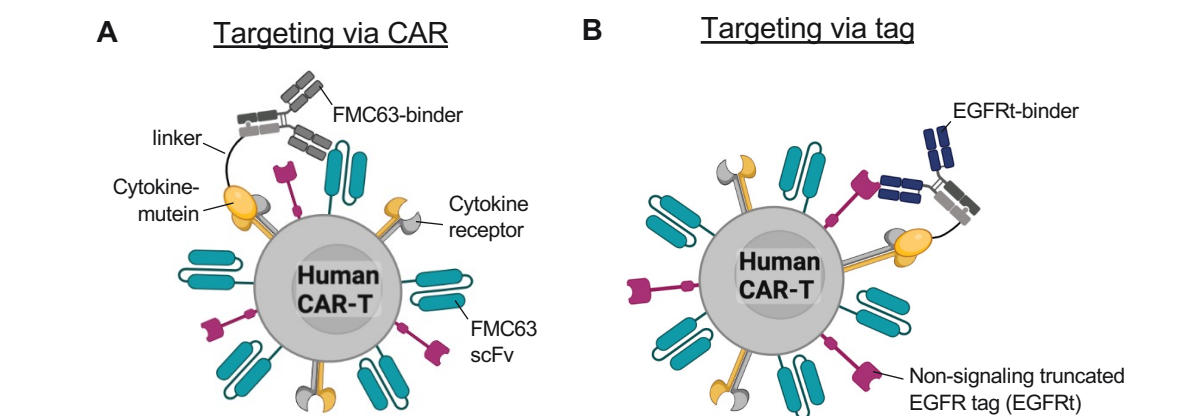


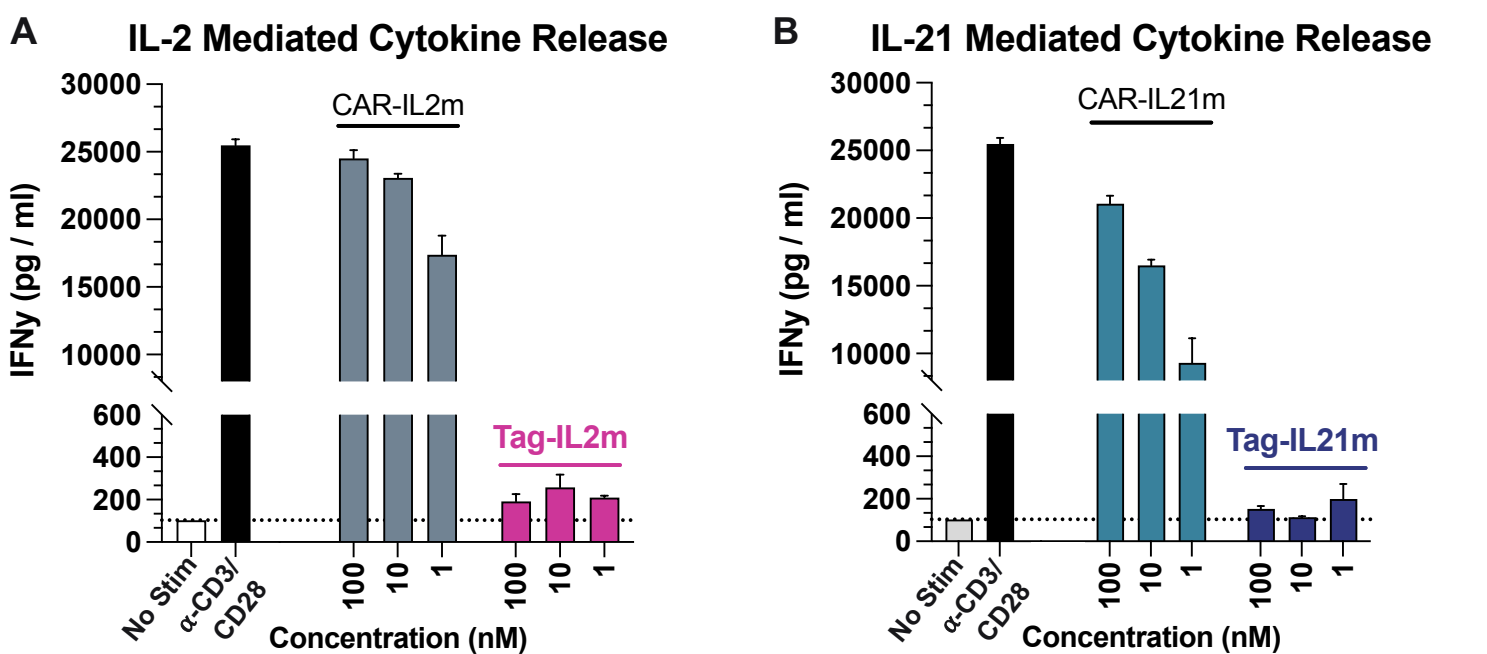
Figure 3: CAR-T Selective Cytokine Molecular Designs



Cis-targeting was used to design molecules with CAR-T cell-selective cytokine signaling. Targeting is achieved either via binding to the CAR directly (A) or a marker co-expressed on the surface of CAR-Ts (e.g. EGFRt, B).

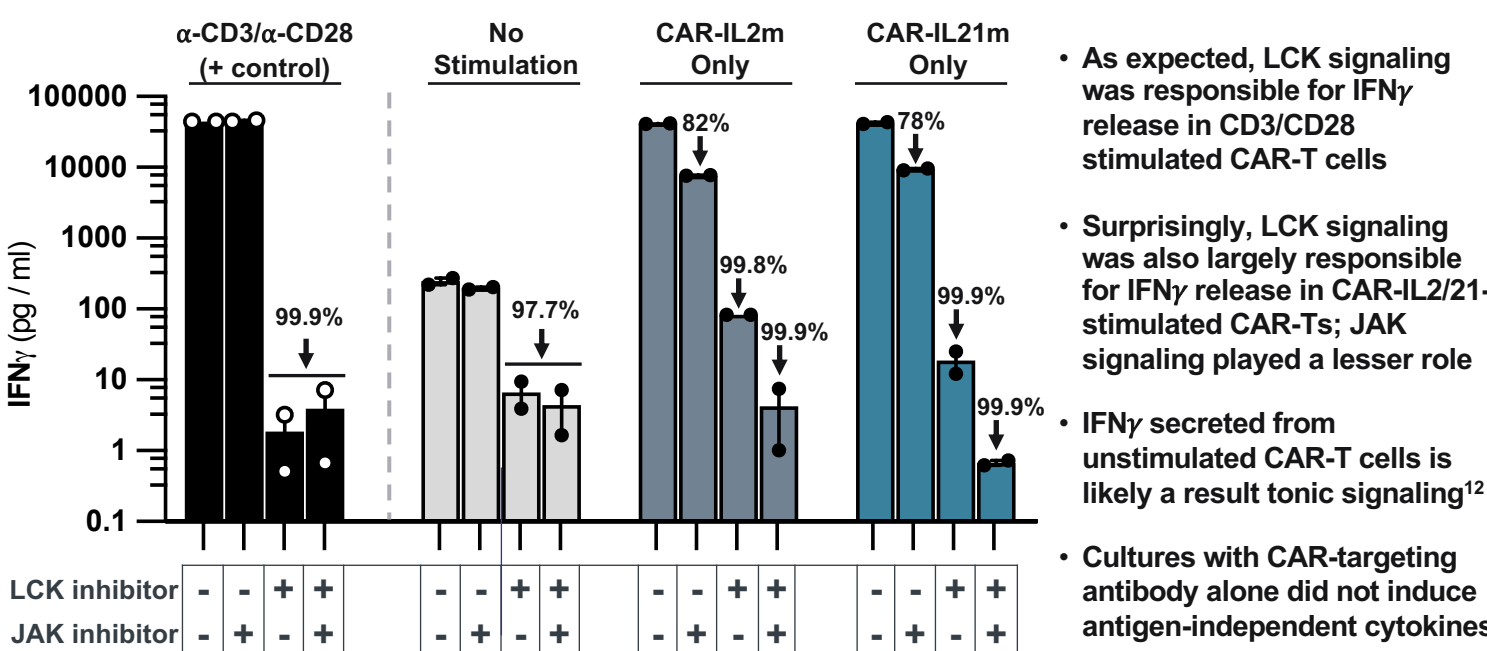
Results

Figure 4: Cytokines Targeted Directly to CAR Induce Antigen-Independent Cytokine Release Whereas Tag Targeting Avoids This Liability



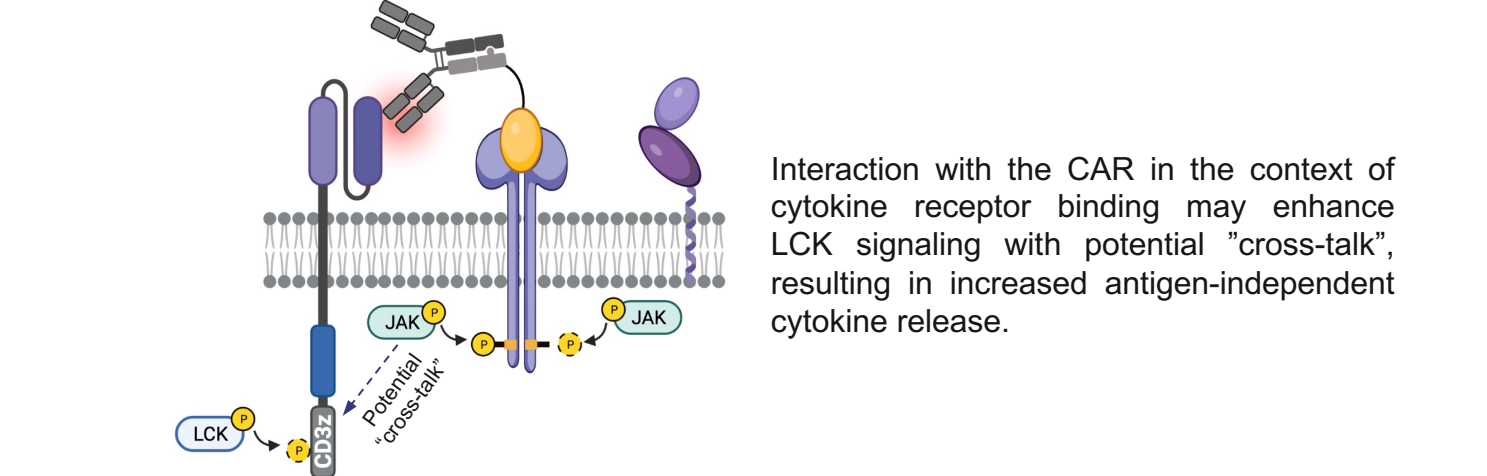
Human CD19 (FMC63) CAR-T cells that co-express an EGFRt-tag were cultured with targeted cytokine fusion molecules. *Cis*-targeted IL2 (A) or IL21 (B) mutants directed by a non-agonizing, CD19 non-blocking¹¹ FMC63 binder induce substantial release of antigen-independent IFN γ compared to low levels by an EGFR molecule binder (POC: panitumumab) after 72 hours of culture. CAR-T cells cultured in media only (grey bar and dotted line) or CAR-T cells stimulated with α -CD3/CD28 beads (black bar).

Figure 5: CAR Targeting May Drive Antigen-Independent Cytokine Release by Enhanced LCK Signaling



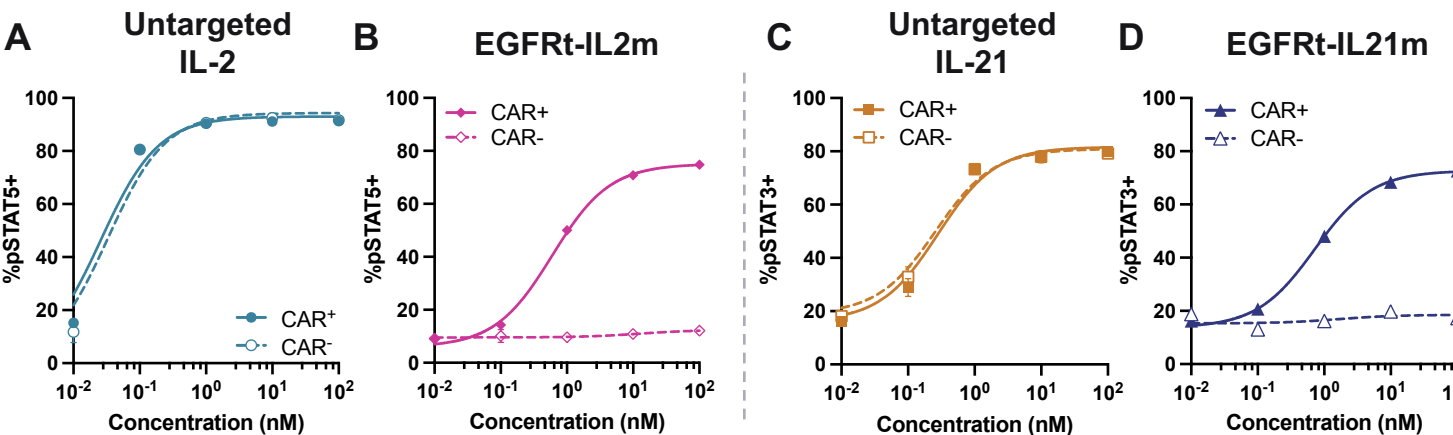
Human CD19 CAR-T cells were cultured in the presence of LCK and/or JAK inhibitors with *cis*-targeted cytokines delivered by fusions to non-agonizing FMC63 binders for 72 hours. CAR-T cells cultured in media only (grey bars) or CAR-T cells stimulated with α -CD3/CD28 beads (black bars); LCK inhibitor (dasatinib) was used at 100 nM; JAK inhibitor used at 500 nM.

Figure 6: Potential Model of Antigen-Independent Cytokine Release in CAR-T Cells with CAR-Targeted Cytokines



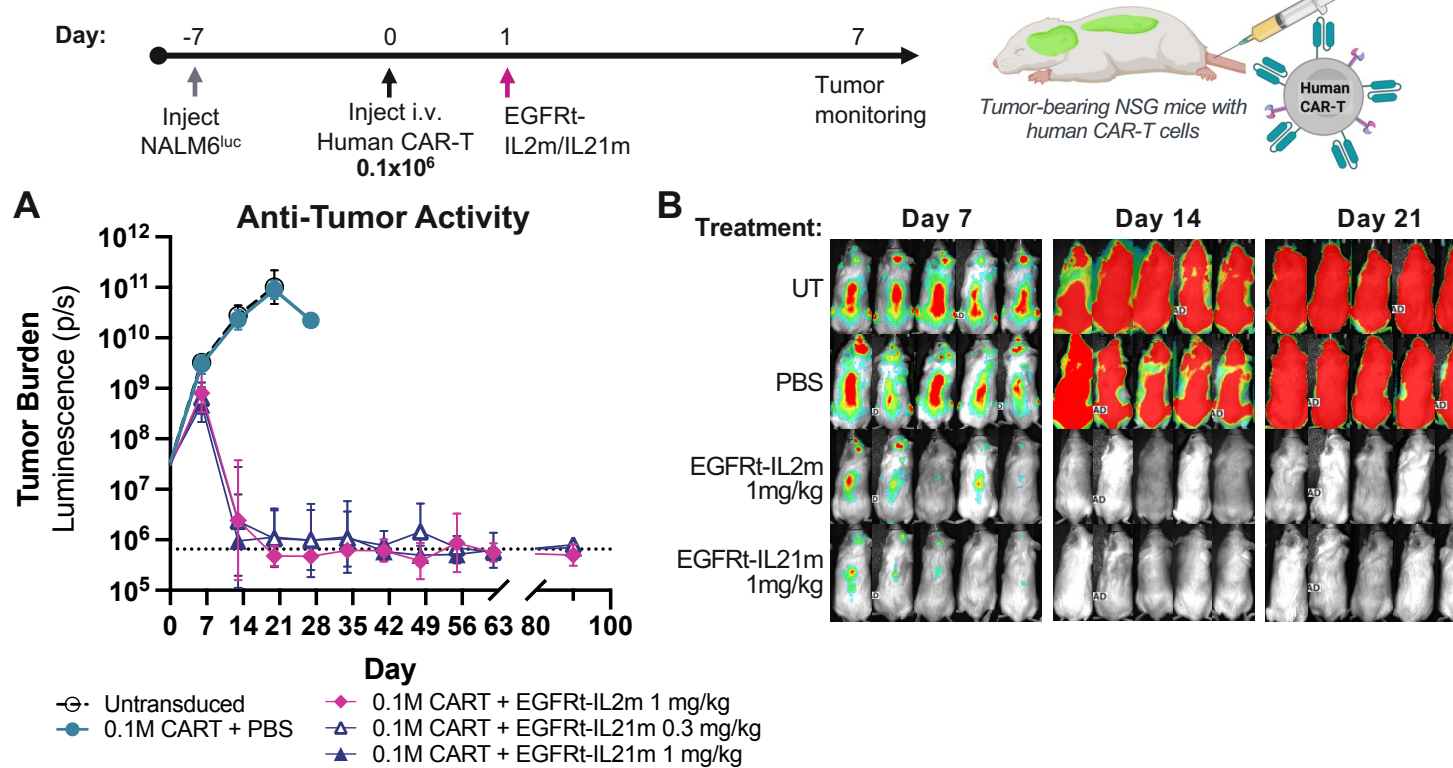
Schematic depicting the binding of a fusion molecule consisting of a non-agonizing CAR-binding antibody and a cytokine mutin to their targets.

Figure 7: EGFRt-targeted Cytokine Molecules are Highly Selective for CAR T cells



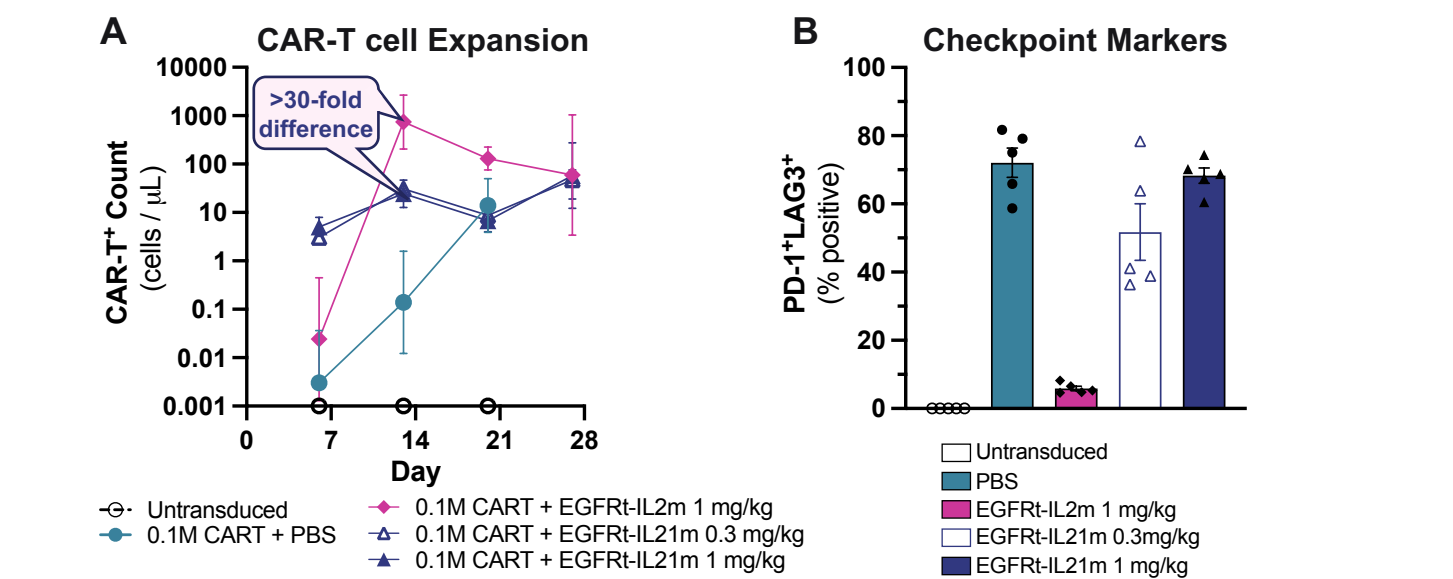
Induction of pSTAT5 signaling by non-selective Fc-fusion molecules, untargeted human IL-2 cytokine (A) and untargeted IL21 (C), versus targeted IL2 fusion molecules (B & D) in human CAR-T cells. *Cis*-targeted IL2 (B) and IL21 (D) to CAR T cells directed by an EGFR binder (POC: panitumumab) exhibit high selectivity (>500 fold) for CAR⁺ T cells over non-CAR cells.

Figure 8: A Single Dose of EGFRt-targeted IL2 or IL21 Converts Suboptimal Dose of CAR-T Cells to Complete Response in Preclinical Model of Leukemia



A single dose of EGFRt-IL2 or EGFRt-IL21 conferred complete regression of tumors with no recurrence over 90 days (A). Bioluminescence imaging of indicated groups (B). Recipients of untransduced T cells (UT) or CAR-T cells + PBS exemplify the aggressive nature of the NALM-6 model. Administration of EGFRt-IL2 or EGFRt-IL21 substantially augmented the efficacy of the suboptimal, non-curative CAR-T cell dose enabling 5/5 complete responses. Dashed line indicates baseline luminescence.

Figure 9: Distinct CAR-T cell Expansion and Phenotype by Targeted-IL2 and -IL21 Indicate Differential Mechanisms Despite Providing Similar Anti-tumor Activity



Peripheral blood CAR-T cell analysis of Figure 7. CAR-T cell numbers (A) revealed EGFRt-IL21 induced early expansion and sustained elevation of CAR-Ts (purple triangles). EGFRt-IL2 drove higher CAR-T counts later (Day 14) with contraction (pink diamonds) following tumor clearance. Analysis of checkpoint markers on day 20 (B) showed EGFRt-IL2 reduced the frequency of PD-1⁺LAG3⁺ CAR-Ts, while EGFRt-IL21 did not alter expected checkpoint marker expression upon tumor recognition.

Conclusions

- The modularity of *cis*-targeting enables delivery of multiple cytokines without extensive re-engineering of payloads.
- Cytokines targeted directly to the CAR induce substantial release of antigen-independent IFN γ cytokine by CAR-T cells.
 - This potential safety issue can be circumvented by targeting an exogenous clinically validated EGFRt-tag co-expressed by CAR-T cells with a properly designed attenuated cytokine.
- CAR Targeting of cytokines may drive antigen-independent cytokine release by enhancing LCK signaling.
 - LCK inhibition resulted in a complete abrogation of antigen-independent IFN γ release (decrease of 99.9% compared to untreated) by CAR-IL2m and CAR-IL21m.
 - Despite JAK inhibition, substantial levels of IFN γ were still observed.
- Cis*-targeted delivery of cytokines via tag selectively activates CAR-T⁺ cells over CAR-T⁻ cells in vitro and in vivo.
 - Targeted-cytokine constructs targeting EGFRt selectively induce pSTAT activity in CAR-T cells by >500-fold over non-CAR cells.
- Differential mechanisms are responsible for *cis*-targeted IL2 and IL21 mediated anti-tumor activity, despite both conferring complete tumor regression in a preclinical model of leukemia.
 - An early and sustained expansion of CAR-T cells is evident in EGFRt-IL21m groups, compared to a >30-fold expansion of CAR-Ts by EGFRt-IL2m at a later time point.
 - Peripheral CAR-T cells in recipients treated with EGFRt-IL2m exhibit a substantially lower frequency of double positive checkpoint markers.
 - CAR-T cells of EGFRt-IL21m treated recipients did not alter expected checkpoint marker expression despite providing CR 5/5.

Methods

- Human T cells were isolated from the peripheral blood of healthy donors and transduced with FMC63-scFv 41BB CD3 ζ (CD19 CAR) construct. Transduction efficiency was directed to ~50% allowing for an even mix of CAR-T⁺ and CAR-T⁻ cells for use in assays.
- Cytokine release assays were performed by seeding 5x10⁴ human CAR-T cells into 96 well plates and culturing in X-VIVO 15 media supplemented with 5% human AB serum with the indicated conditions for 72 hours. Experiments with LCK inhibitor (dasatinib 100 nM) and/or JAK inhibitor (500 nM) were treated at time of addition of targeted cytokine. Supernatants were collected and frozen at -80°C until cytokine analysis by MSD examining IFN γ , TNF α , IL-5, MIP1 α , IL-6 and analyzed using the MSD Discovery Workbench software version 4.0.
- Human phospho-STAT5 (pSTAT5) assays were performed by incubating FMC63 CAR-T cells with the indicated concentrations of cytokine molecules for 25 minutes. Cells were placed on ice, surface stained, washed, and then fixed using 4% PFA. Cells were then permeabilized with methanol, stained intracellularly, and analyzed by flow cytometry.
- Tumor efficacy studies were performed by injecting 1x10⁶ NALM-6^{luc+1/gfp+} cells intravenously (IV) via tail vein into NSG mice. After six days, tumor engraftment was analyzed by bioluminescence imaging (BLI) and mice were randomized into treatment groups. The designated number of FMC63 CAR-T cells were injected IV and the indicated treatments were administered one day later using the dosing schedule as described. Tumor volume was assessed by BLI and peripheral blood was collected weekly.

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