*Ortho*CARs: Engineered IL-2/IL-2Rβ orthogonal pairs that selectively enhance CAR T cell function *in vivo*

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ABSTRACT

CAR T cell therapy (CAR) has demonstrated remarkable clinical efficacy in hematological malignancies. However, barriers such as poor T cell effector function, lack of proliferation, and limited persistence prevent CARs from reaching their full curative potential. IL-2 is a potent stimulator of T cell proliferation, survival, and cytotoxic function, making it an attractive cytokine to support CARs. However, therapeutic use of IL-2 is limited by systemic toxicity due its promiscuous activation of undesired immune cell populations.

To facilitate selective expansion and activation of CARs we have developed a human orthogonal ligand/receptor system consisting of a pegylated IL-2 mutein (STK-009) that does not significantly activate the wild type IL-2 receptor and a mutated IL-2 Receptor Beta (hoRb) that is fully activated by STK-009, but does not respond to the native IL-2 ligand. This system enables *in vivo* IL-2 signaling in CARs engineered to express hoRb while avoiding signaling bystander T cells and NK cells. Here, we demonstrate the ability of the STK-009/hoRb receptor pair to selectively enhance the anti-tumor efficacy of hoRb expressing CD19 CARs (SYNCAR-001) in preclinical lymphoma mouse models at will. We also demonstrate, in a non-human primates (NHP), that STK-009 is selective for hoRb expressing cells.

SYNCAR-001 + STK-009 can lead to complete responses in subcutaneous Raji mouse models, even with SYNCAR-001 dosed at sub-efficacious levels (400,000 CAR-Ts per mouse). Subcutaneous dosing of STK-009 expands SYNCAR-001 cells systemically and drives infiltration of SYNCAR-001 into tumors. UMAP analysis of STK-009 treated SYNCAR-001 cells reveal a small $PD1^{+}LAG3^{+}$ subpopulation during tumor rejection which disappeared once tumors are controlled. The majority of T cells showed no exhaustion markers. Further, an $IL\text{-}7R^{+}$ population arises and remains after tumor control, indicating long term memory development. Nanostring transcriptome analysis confirms upregulation of IL-7R and other critical genes involved in cytotoxic activity and T cell memory persistence in SYNCAR-001 cells when treated with STK-009. These data demonstrate that STK-009 treatment expands SYNCAR-001 cells and also delivers a gene signature indicative of enhanced T cell fitness and activity.

Subcutaneous administration of STK-009 in NHP shows no evidence of toxicity or physiological IL-2 mediated activity on immune cells, including Teffs, Tregs, and NK cells. Pharmacokinetic analysis of STK-009 shows stable exposure with minimal clearance, demonstrating the selectivity of STK-009.

These findings validate an orthogonal platform that selectively drives potent T cell effector functions of engineered cells without the toxicities mediated by NK cells or non-tumor specific T cells associated with high dose IL-2 therapy. These results demonstrate the ability of this orthogonal platform to improve the efficacy and durability of CAR T cell therapies.

Orthogonal human IL-2/IL-2Rβ pair for selective CAR T enhancement *in vivo*

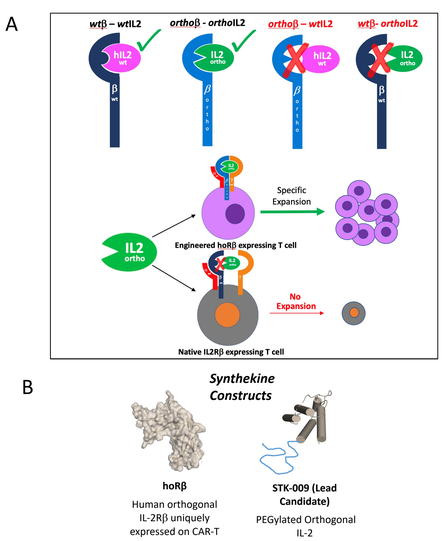


Figure 1. Orthogonal IL-2/IL-2Rβ schema. (A) Wild type (WT) and *ortho*IL-2Rβ receptors (hoRβ) exhibit significant preference for their cognate ligand, WT and *ortho*IL-2, respectively. Therefore, engineered T cells expressing the *ortho* receptor will respond selectively to *ortho*IL-2, thereby allowing specific expansion and enhancement of engineered T cell activity. **(B)** Synthekine constructs. hoRβ can be engineered into any payload for expression in a desired target cell. STK-009 is a pegylated orthogonal IL-2 for *subcutaneous* administration.

RESULTS

Proof of concept αCD19 orthoCAR: SYNCAR-001

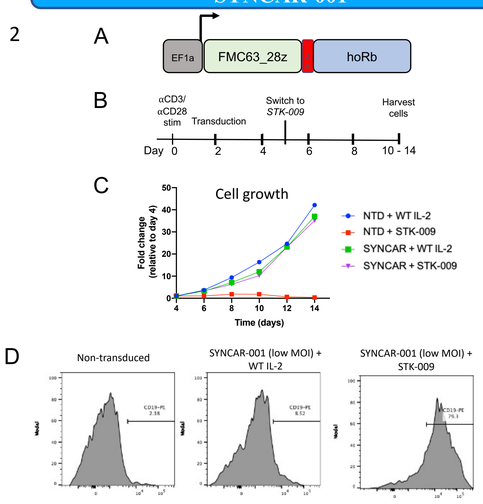


Figure 2. SYNCAR-001 construct and manufacturing (A) Lentiviral construct containing the anti-CD19 FMC63 scFv, CD28 transmembrane and co-stimulatory domain, followed by CD3zeta. The cleavage peptide T2A and hoRβ immediately follow the CAR construct and are expressed as a single mRNA **(B)** SYNCAR-001 T cell manufacturing schema. **(C)** Cell growth during SYNCAR-001 manufacturing. **(D)** Flow cytometric analysis of FMC63 expression was performed via recombinant human biotinylated Fc-CD19/streptavidin-PE.

STK-009 does not induce IL-2 mediated toxicity in non-human primates

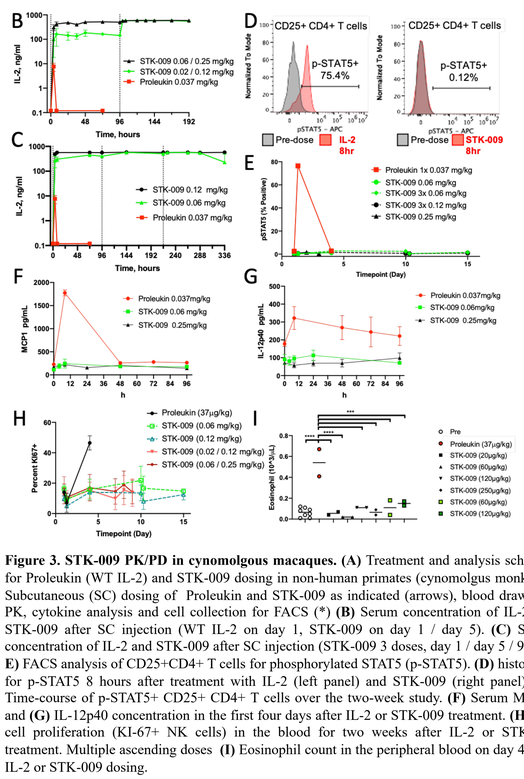
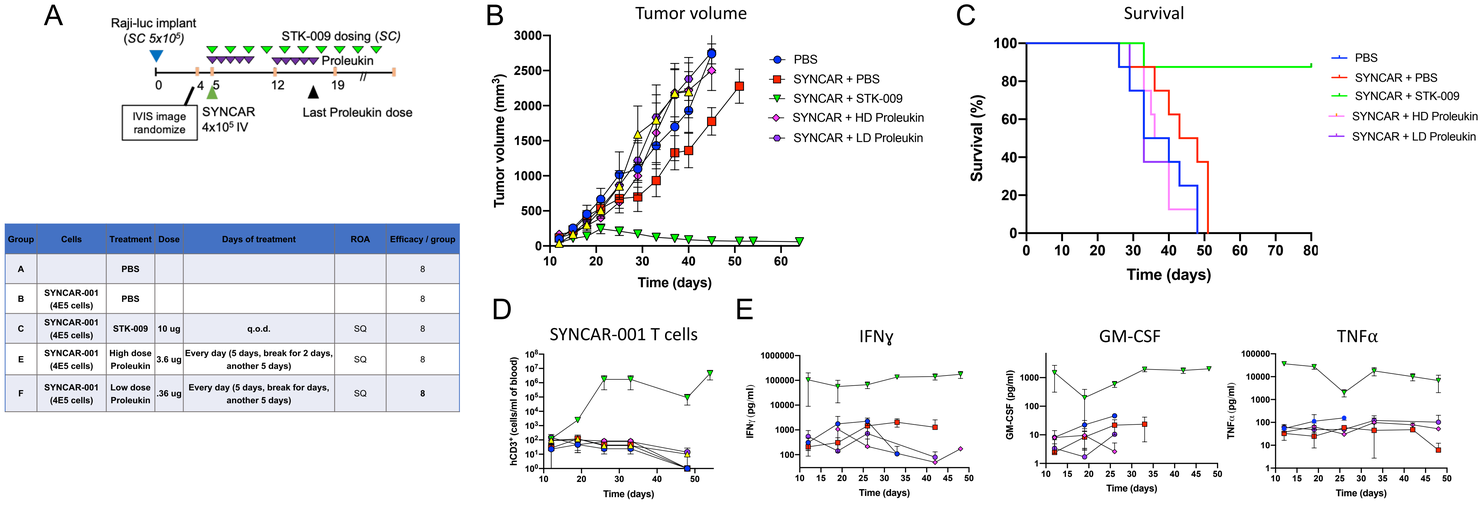


Figure 3. STK-009 PK/PD in cynomolgous macaques. (A) Treatment and analysis schedule for Proleukin (WT IL-2) and STK-009 dosing in non-human primates (cynomolgus monkeys). Subcutaneous (SC) dosing of Proleukin and STK-009 as indicated (arrows), blood draws for PK, cytokine analysis and cell collection for FACS (*) **(B)** Serum concentration of IL-2 and STK-009 after SC injection (WT IL-2 on day 1, STK-009 on day 1 / day 5). **(C)** Serum concentration of IL-2 and STK-009 after SC injection (STK-009 3 doses, day 1 / day 5 / 9). **(D, E)** FACS analysis of CD25+CD4+ T cells for phosphorylated STAT5 (p-STAT5). **(D)** histogram for p-STAT5 8 hours after treatment with IL-2 (left panel) and STK-009 (right panel). **(E)** Time-course of p-STAT5+ CD25+ CD4+ T cells over the two-week study. **(F)** Serum MCP-1 and **(G)** IL-12p40 concentration in the first four days after IL-2 or STK-009 treatment. **(H)** NK cell proliferation (KI-67+ NK cells) in the blood for two weeks after IL-2 or STK-009 treatment. Multiple ascending doses **(I)** Eosinophil count in the peripheral blood on day 4 after IL-2 or STK-009 dosing.

STK-009 but not high dose Proleukin induces tumor responses in combination with SYNCAR-001 in a CAR refractory subcutaneous lymphoma model



Group	Cells	Treatment	Dose	Days of treatment	ROA	Efficacy / group
A		PBS				8
B	SYNCAR-001 (4E5 cells)	PBS				8
C	SYNCAR-001 (4E5 cells)	STK-009	10 ug	q.o.d.	SQ	8
E	SYNCAR-001 (4E5 cells)	High dose Proleukin	3.6 ug	Every day (5 days, break for 2 days, another 5 days)	SQ	8
F	SYNCAR-001 (4E5 cells)	Low dose Proleukin	.36 ug	Every day (5 days, break for days, another 5 days)	SQ	8

Figure 4. STK-009 + SYNCAR-001 in a stress test mouse model of solid lymphoma. (A) Treatment schedule for STK-009 or Proleukin in combination with SYNCAR or high dose SYNCAR in solid Raji lymphoma model. **(B)** Tumor efficacy of SYNCAR alone or in combination with STK-009 or Proleukin. **(C)** Survival of mice with SC Raji lymphoma model over the course of the study. **(D)** SYNCAR T cell expansion in the blood in Raji lymphoma model. **(E)** ELISAs for human T cell derived cytokines in the serum of mice with Raji tumors over the course of the study. Color scheme and symbols for treatment groups identical throughout the figure.

Longitudinal UMAP analysis of SYNCAR-001 + STK-009 T cells from an in vivo SQ Raji model reveals a transient increase in $CD25^{hi}$ $PD\text{-}1^{hi}$ $LAG\text{-}3^{hi}$ cells and a stable increase of $IL\text{-}7R^{hi}$ cells over time

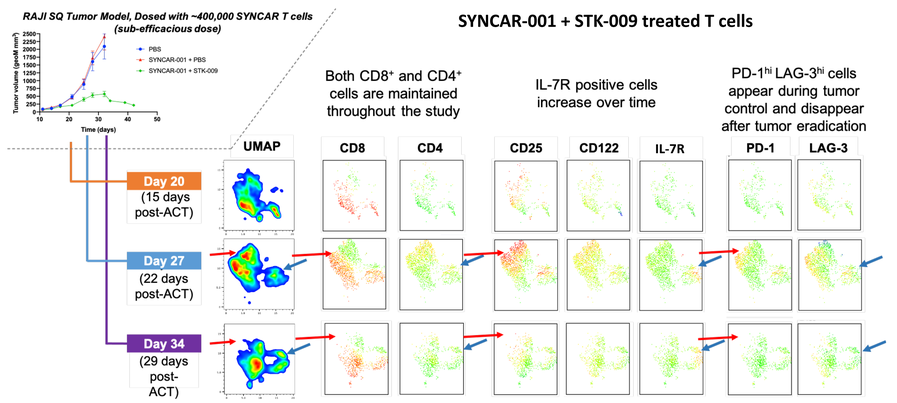


Figure 5. Longitudinal UMAP analysis of SYNCAR T cells during the course of Raji tumor control. SYNCAR-001 + STK-009 treated mice (same dosing regimen as in Figure 4) were bled at the indicated time points. Cells were stained by a panel of indicated markers and and analyzed by flow cytometry. Data was then subjected to UMAP analysis. Red indicates higher relative marker intensity, while green indicates lower relative marker intensity. Red arrow designates transient $CD25^{hi}$ $PD\text{-}1^{hi}$ $LAG3^{hi}$ population. Blue arrow designates $IL\text{-}7R^{hi}$ $PD\text{-}1^{low}$ $LAG\text{-}3^{low}$ population.

STK-009 Significantly Increases Peripheral T Cell Numbers and All T Cell Memory Subtypes Including T_{SCM} Cells

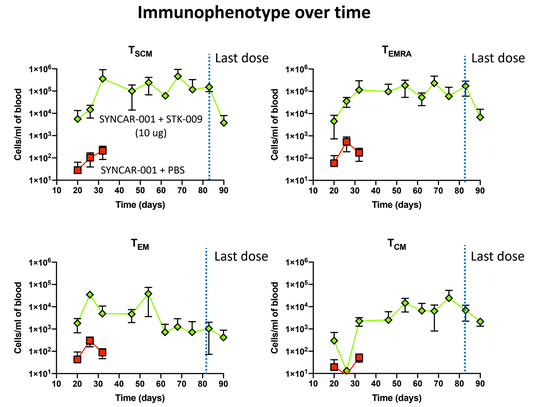


Figure 6. Immunophenotyping of SYNCAR-001 cells. Cells from mice treated with the same SYNCAR-001, PBS, and STK-009 dosing regimen as in Figure 4 were obtained from weekly bleeds and subjected to flow cytometry analysis for CCR7 and CD45RA.

Transcriptome analysis of SYNCAR-001 T cells treated with STK-009 >1 month post-ACT further supports a non-exhausted state and IL-7R upregulation

- STK-009 treatment results in the induction of activation and memory markers
  - Increased expression of CD40L, IL-2Ra, IL-7R, and IL-10RA
- No evidence of an exhausted gene signature or FOXP3 induction upon prolonged STK-009 treatment

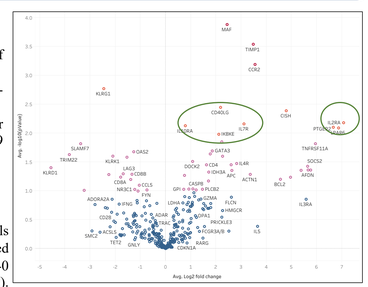


Figure 7. Nanostring analysis. Human CD3 T cells were sorted from mice bearing SC Raji tumors treated with SYNCAR-001 and either STK-009 or PBS 40 days post-ACT (same dosing regimen as Figure 4). Cells were then subjected to Nanostring analysis (human CAR T characterization panel). Significantly differentially expressed transcripts highlighted in red. Key activation and memory markers circled.

CONCLUSIONS

The incorporation of the STK-009/*ortho*IL2Rβ system enables the following advantages over current clinically validated CAR T cell therapies:

- Allows enrichment of CAR-transduced cells during *ex vivo* manufacturing
- Increases and controls persistence and activation of CAR T cells
- Improves anti-tumor efficacy
- Drives a significant increase in CAR T cell counts *in vivo*
  - On-demand and controlled by dosing strategy
- Expands a favorable anti-tumor and persistent CAR T cell immunophenotype

Therefore, the STK-009/SYNCAR platform has the potential to overcome clinically relevant hurdles in cell therapy.