*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 has demonstrated remarkable clinical efficacy against relapsed and refractory hematological malignancies, such as B cell non-Hodgkin lymphoma (NHL) and acute lymphoblastic leukemia (ALL)[1-3]. Despite these advances, prominent barriers including poor T cell effector function, lack of proliferation, and limited CAR T cell persistence prevent CAR T cell therapies from reaching their full curative potential[4].

Interleukin-2 (IL-2) is a potent stimulator of CD4 and CD8 T cell proliferation, survival, and cytotoxic function, thereby making it an attractive molecule to support CAR T cell therapy. However, therapeutic use of IL-2 is limited by systemic toxicity due its promiscuous activation of undesired immune cell populations, including non-tumor reactive T cells and NK cells[5].

To facilitate selective *ex vivo* and *in vivo* expansion of engineered T cells we have developed a human orthogonal (*ortho*) ligand/receptor system consisting of a pegylated, IL-2 mutein (STK-009) and a mutated IL-2 Receptor Beta (*ortho*IL-2Rβ) that selectively bind each another, but does not significantly activate their wild type receptor and cytokine counterparts. This system allows for *in vivo* IL-2 signaling in engineered adoptive cell therapies that express the *ortho*IL-2Rβ while avoiding signaling in non-tumor reactive T cells and NK cells.

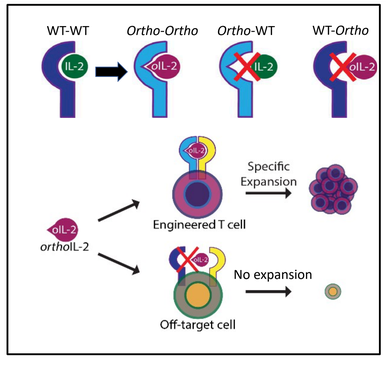
Here, we demonstrate the ability of the STK-009/*ortho*IL-2Rβ ligand/receptor pair to selectively potentiate human *ortho*IL-2Rβ (hoRb) expressing CD19 CAR T cells *in vitro* and *in vivo*. We incorporated hoRβ into a CD19 directed CAR lentiviral construct utilizing a T2A peptide linker, allowing the use of a single lentiviral plasmid to generate CD19 *ortho*CAR T cells. STK-009 subcutaneous administration increased the efficacy of suboptimal doses of CD19 *ortho*CAR T cells against a disseminated Raji mouse model of aggressive lymphoma. STK-009 treatment dramatically increased levels of CD19 *ortho*CAR T cells with a clinically favorable T_{SCM} and T_{EMRA} immunophenotype. STK-009 treatment of large tumors which had relapsed on CD19 *ortho*CAR T cell administration alone, significantly decreased tumor burden. STK-009 treatment was able to rescue CD19 *ortho*CAR T cell efficacy more than 30 days after initial CD19 *ortho*CAR T cell treatment.

When dosage was stopped after complete tumor responses, CD19 *ortho*CAR T cells contracted, as expected. We found that STK-009 re-dosing could reverse this CD19 *ortho*CAR T cell contraction and significantly increased CD19 *ortho*CAR T cell levels. This illustrates the on-demand control of the STK-009/*ortho*CAR T cell platform.

These findings validate a platform that selectively drives potent T cell effector functions of IL-2 without IL-2 mediated toxicities mediated by NK cells or non-tumor specific T cells and improves the efficacy and durability CAR T cell therapy.

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

1



	WT IL-2	oIL-2
WT-IL2Rβ	+	-
Ortho-IL2Rβ (hoRb)	-	+

Figure 1. Orthogonal IL-2/IL-2Rβ schema. Wild type (WT) and *ortho*IL-2Rβ receptors 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.

RESULTS

STK-009 (pegylated human oIL-2) is selective for hoRb expressing cells

2

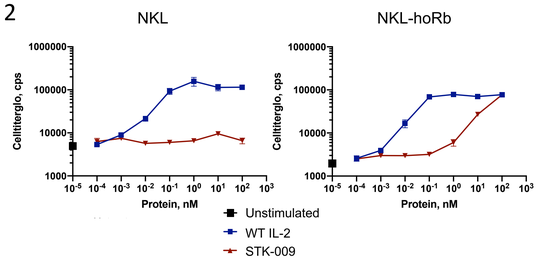


Figure 2. STK-009 specifically induces the proliferation of NKL-hoRb cells and not NKL parental cells. NKL cells are an IL2Rαβγ high cell line that requires IL-2 for proliferation. To generate NKL-hoRB cells, NKL cells were transduced with MSCV-hORB-IRES-YFP virus and YFP$^+$ cells were sorted. Proliferation was assessed by Cellltiter Glo.

Proof of concept CD19 *ortho*CAR construct

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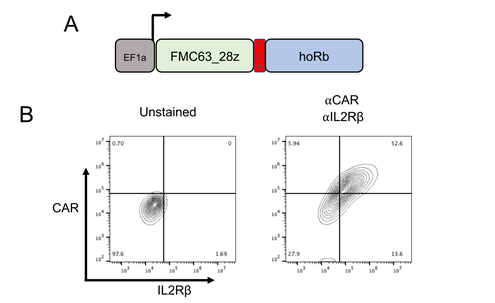


Figure 3. CD19 *ortho*CAR construct and expression check. A) Lentiviral construct containing the anti-CD19 FMC63 scFv, CD28 transmembrane and co-stimulatory domain, followed by CD3zeta. The cleavage peptide T2A and human orthoIL-2Rβ (hoRb) immediately follow the CAR construct and are expressed as a single mRNA. Expression is regulated via an EF1α promoter. **B)** Flow cytometry analysis of CAR and hoRb expression. HEK293T cells (IL2Rβ negative) were transfected with the CD19 *ortho*CAR construct. FMC63 expression was performed via recombinant human biotinylated Fc-CD19/streptavidin-PE. hoRb expression was assessed with antibodies that detect wild type IL2Rβ.

oIL-2 specifically expands hoRb expressing CD19 *ortho*CAR T cells during manufacturing

4

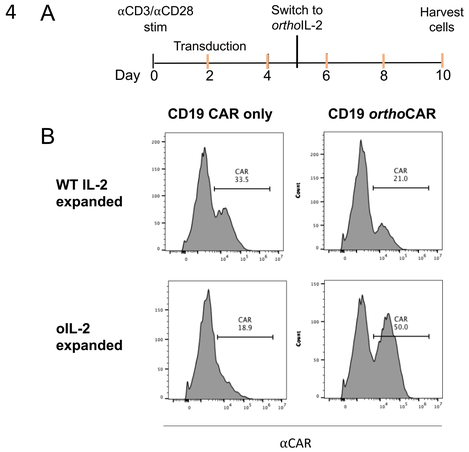
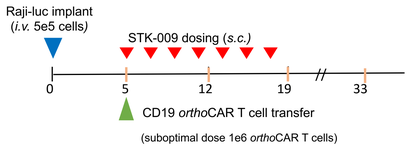


Figure 4. *ortho*IL-2 enrichment of orthoCAR T cells *in vitro*. A) Schema of CAR T cell manufacturing for oIL-2 enrichment of *ortho*CAR T cells. **B)** Flow cytometry analysis detecting CD19 CAR expression.

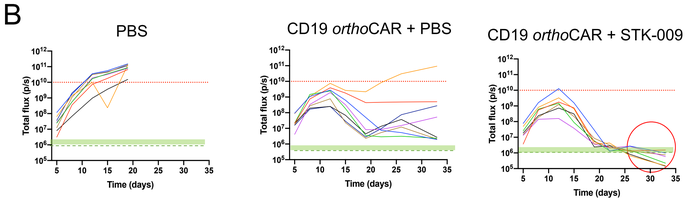
STK-009 administration enhances relapse free anti-tumor efficacy of a suboptimal dose of CD19 *ortho*CAR T cells in a disseminated Raji mouse model of lymphoma

5 A



Group	Cells	Treatment	Mice
1	-	PBS	8
2	CD19 *ortho*CAR T cells (**1e6** *ortho*CAR$^+$)	PBS	8
3	CD19 *ortho*CAR T cells (**1e6** *ortho*CAR$^+$)	STK-009 (q.o.d.)	8

B



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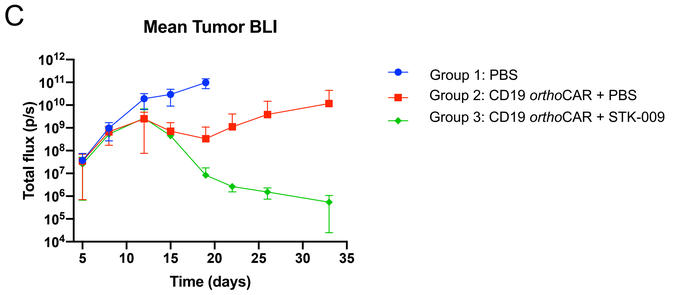
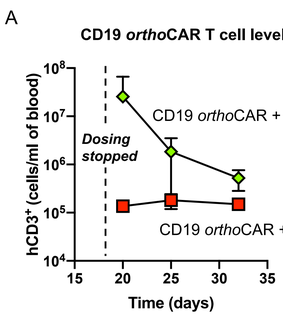


Figure 5. STK-009 + CD19 *ortho*CAR T cells in a stress test mouse model of lymphoma. A) Study design. NOD scid gamma (NSG) were injected with 5E5 Raji-luc cells on Day 0. Mice were imaged with an IVIS imaging system, randomized, and received their respective treatment on Day 5. Mice were dosed with either PBS or STK-009 every other day until Day 17. **B&C)** Mice were imaged twice per week. **(B)** Spider plots for individual mice and **(C)** treatment group means with standard deviations are displayed.

STK-009 treatment significantly increases CD19 *ortho*CAR T cell levels while maintaining a clinically favorable immunophenotype

6 A



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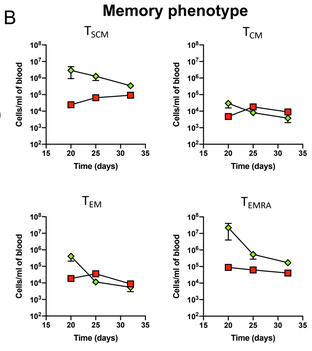
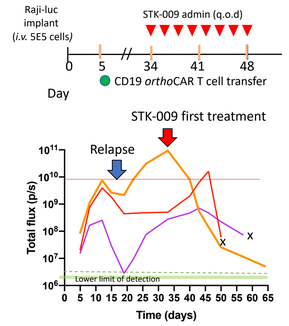


Figure 6. Flow analysis of peripheral blood. A) Human CD3$^+$ cells and **B)** memory phenotype based upon CD45RA and CCR7 expression (T_{SCM}: CD45RA$^+$CCR7$^+$, T_{CM}: CD45RA$^-$ CCR7$^+$, T_{EM}: CD45RA$^-$ CCR7$^-$, T_{EMRA}: CD45RA$^+$ CCR7$^-$. Data represented as total cell numbers/ml of blood.

STK-009 treatment of "STK-009 naïve" CD19 *ortho*CAR T relapsed mice inhibits tumor growth

7 A



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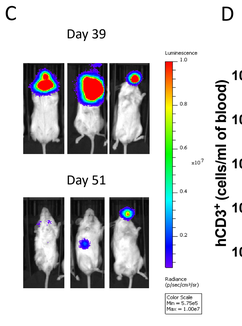

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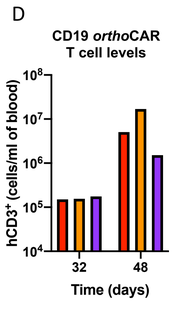
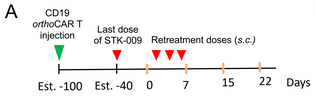


Figure 7. Delayed treatment of STK-009 29 days after CD19 *ortho*CAR T cell administration decreases tumor burden in relapsed mice and expansion of *ortho*CAR T cells. A) Three mice that were treated with CD19 *ortho*CAR T cells alone demonstrated significant relapsed tumor growth. These mice were then treated with STK-009 alone starting on Day 34 of the study. **B)** Tumor burden spider plot of individual mice. X denotes taken off study. **C)** Bioluminescent imaging. **D)** Flow analysis of peripheral blood 3 days prior (Day 32) and 13 days after (Day 48) first dose of STK-009 (Day 35).

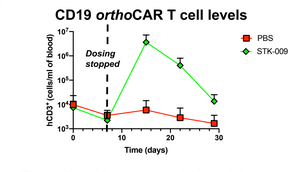
STK-009 retreatment increases CD19 *ortho*CAR T cell levels after a >40-day drug holiday

8 A



Cells	Treatment after drug holiday	Mice
*ortho*CAR T cells (**1E6** *ortho*CAR$^+$)	PBS	4
*ortho*CAR T cells (**1E6** *ortho*CAR$^+$)	STK-009 (q.o.d.)	4

B



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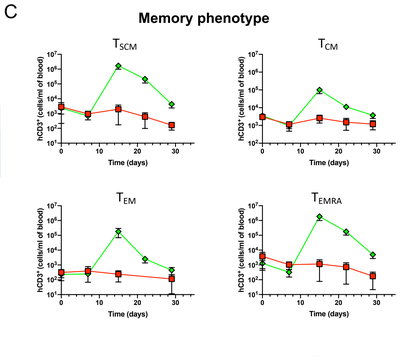

Figure 8. A) Study design. Mice exhibiting no tumor burden from the STK-009 + CD19 *ortho*CAR T cell treated cohorts were put on drug holiday for at least 40 days. Mice were split into two groups receiving either PBS or STK-009. Flow analysis was performed on peripheral blood and analyzed for **B)** human CD3 positive cells and **C)** memory phenotype based upon CD45RA and CCR7 expression.

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 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
  - T_{SCM} for persistence and T_{EMRA} for anti-tumor effector function
- Re-expansion of resting/exhausted CAR T cells
  - Restoration of CAR T cell levels and activity

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

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