# Pre-clinical Pharmacology and Tolerability Characterization of a Novel KIR x ICOS Targeting Bispecific CD8 Treg Modulator

Monica Childs, Minh Pham, Jennifer Gardell, Cathy Tan, Phoenicia Quach, Brent Meengs, Gleda Hermansky, Justin Bowser, Lisa Bogatzki, Jon Therriault, Susan Julien, Dan Boster, Kristine M Swiderek, Catherine J McMahan, and Courtney Crane

MOZART THERAPEUTICS

Orchestrating The Immune System

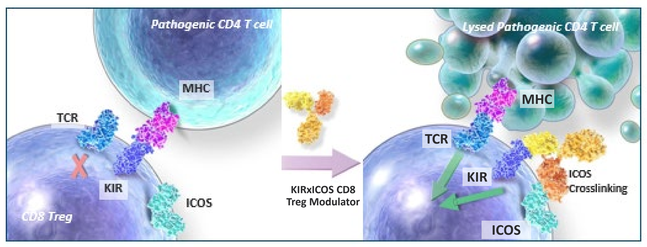
## Abstract

Here we present an Fc-based bispecific CD8 Treg modulator that binds in cis to KIR2DL and ICOS receptors co-expressed by CD8 Treg designed to restore CD8 Treg functions. CD8 Treg dysfunctions include impaired cytotoxicity and insufficient longevity in autoimmune disease-affected tissues. The KIR and ICOS targeting bispecific molecule selectively enhances CD8 Treg killing, promoting their recruitment to and survival within disease-affected tissues.

Through in vitro, ex vivo, and in vivo evaluations in humanized mouse models, we assessed binding, specificity, and function of the KIRxICOS CD8 Treg Modulator, which boosted ICOS signaling in KIR-expressing CD8 Treg without activating immune cells expressing only ICOS. It extended survival and reduced disease score in a humanized acute GvHD model, marked by a reduction of activated CD4 T cells and an increase of CD8 Treg. In antigen stimulated PBMC, the Modulator reduced CD4 T cell derived proinflammatory cytokines and reduced epithelial cell death in Crohn's Disease affected tissues. In vivo, the Modulator demonstrated antibody-like PK parameters. Repeat dose studies in CD34+ NSG-Tg(Hu-IL-15) mice indicated good tolerability without terminal toxicity or induction of proinflammatory serum cytokines. The evaluation of binding, impact on immune cell phenotypes, and pharmacology revealed immune cell binding without activation.

Our preclinical data supports development of the KIR x ICOS CD8 Treg Modulator as a therapeutic for autoimmune disease.

### KIR x ICOS CD8 Treg Modulator is Targeting a Novel Network to Restore Immune Balance in Autoimmune Disease




*In autoimmune disease, pathogenic immune cells escape surveillance*

*Mozart's CD8 Treg Modulators are designed to restore homeostasis*

**Mozart's approach seeks to restore cytolytic activity of dysfunctional CD8 Treg**

## Methods

- Target binding was evaluated by Octet and on human PBMC cell subsets by flow cytometry.
- Activity in ICOS reporter assay was determined using a dose titration.
- Activation in healthy PBMC subsets was assessed by flow cytometry after overnight stimulation with 1 µg/ml anti-CD3 (OKT3) followed by 48hr treatment with 100 nM KIR x ICOS CD8 Treg Modulator or controls.
- Impact to antigen-specific CD4 proliferation in Crohn's disease was assessed with PBMC from Crohn's patients activated by 100 ng/ml flagellin and treated with 100 nM KIR x ICOS CD8 Treg Modulator for 5 days before analysis by flow cytometry.
- Intestinal organoids derived from Crohn's patient biopsies were utilized to assess the effects of the KIR x ICOS CD8 Treg Modulator on 100 ng/ml flagellin and 1 µg/ml $OmpC_{321-340}$ peptide-driven epithelial cell death.
- A dose titration study of the KIR x ICOS CD8 Treg Modulator was done in an acute GvHD model using human PBMC engrafted NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ (NSG) mice. Mice were injected intravenously with 1e7 human PBMCs 4hr post-irradiation (0.5 Gy) and dosed intravenously with the Modulator at 0.02, 0.2 or 2 mg/kg the day after PBMC injection and every seven days until study day 21. Whole blood was collected for immunophenotyping on study days 11, 14, and 42. Spleens were harvested at study endpoint and processed for flow cytometry.
- Efficacy of the Modulator was evaluated in a survival study of acute GvHD. NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Wjl}$/SzJ (NSG) mice were injected intravenously with 1e7 human PBMCs 4 hours post-irradiation (0.75 Gy). Mice were dosed intravenously with 0.2 mg/kg or saline (n=8 per group) the day after PBMC injection and every seven days until study day 35. Blood was collected on study day 14 to evaluate binding to T cell subsets by flow cytometry. Study was terminated at day 42. A Kaplan-Meier survival curve was generated and used to determine survival differences between groups. P-value calculated by Log-rank (Mantel-Cox) test.
- For assessment of toxicity, immunotoxicity, and PD parameters, healthy female NSG-Tg(Hu-IL15) mice engrafted with human CD34+ cord blood cells received repeat doses (weekly, n= 5 doses) of the Modulator (5 or 50 mg/kg) or vehicle intravenously via the tail vein over a period of 4 weeks. Immunotoxicity analyses included flow cytometry and multiplex U-plex Mesoscale Discovery (MSD) based cytokine assays.
- PK profiles were assessed following a single dose of the Modulator at 5 mg/kg in female BALB/cJ mice. Quantitation in serum was performed using a sandwich ELISA.

### KIR x ICOS CD8 Treg Modulator shows target-specific binding and signaling activity

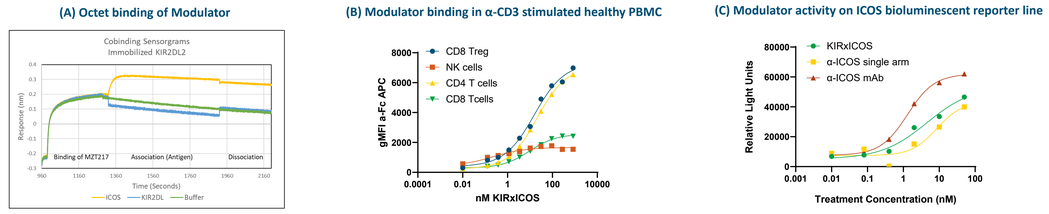



**Figure 1 A)** Binding profile of KIR x ICOS Treg modulator by Octet demonstrating ICOS binding capacity after KIR is engaged. **B)** Binding on healthy human PBMC stimulated with anti-CD3 shows preferential binding to CD8 Treg and CD4 T cells. **C)** The Modulator induces signaling in an ICOS reporter cell line at levels similar to monovalent anti-ICOS and at lower levels than bivalent anti-ICOS.

### KIR x ICOS CD8 Treg Modulator demonstrates functional activity in healthy and diseased human tissue

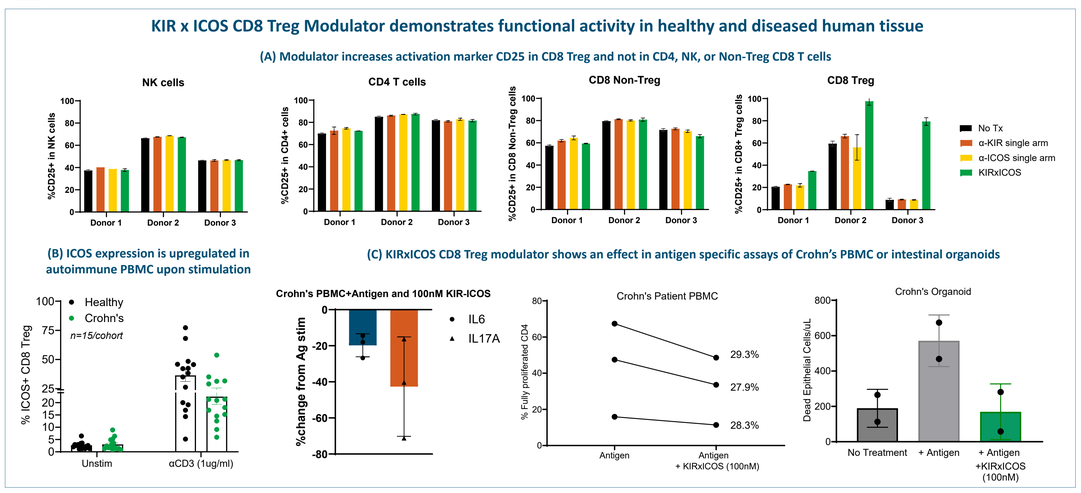



**Figure 2 A)** Healthy donor PBMC treated with 100 nM CD8 Treg Modulator after anti-CD3 stimulation show increased CD25 expression only in CD8 Tregs. **B)** ICOS expression is upregulated in CD8 Treg from autoimmune patients after stimulation with anti-CD3. **C)** PBMC from Crohn's disease patients show decreased antigen-induced CD4 T cell proliferation and cytokine induction with treatment. Intestinal organoids from Crohn's disease patients show decreased antigen-induced epithelial cell death with treatment. Intestinal biopsies were provided by Dr. James Lord at the Benaroya Research Institute in Seattle, WA.

### KIR x ICOS CD8 Treg Modulator shows dose-dependent effects in an acute GVHD model

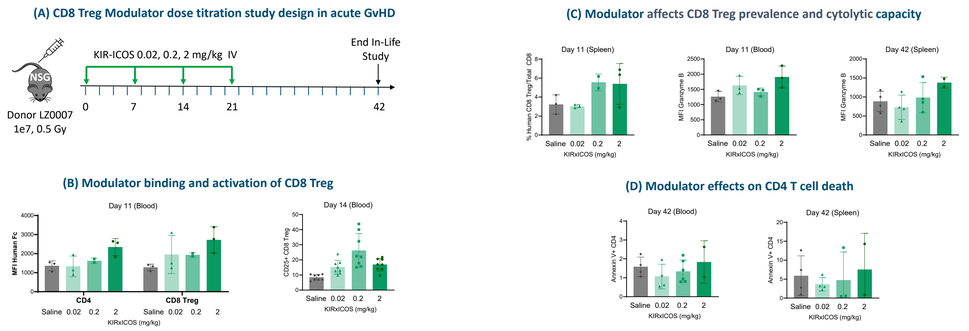



**Figure 3A)** Modulator dose titration study experimental design. **B)** Dose-dependent binding of Modulator on CD4 T cells and CD8 Treg and increased percentage of CD25+ activated CD8 Treg in the blood. **C)** Modulator shows dose-dependent increase in CD8 Treg prevalence and cytolytic capacity as measured by Granzyme B in blood and spleen. **D)** Dose-dependent increase in CD4 death as measured by Annexin V+ in the remaining mice at the terminal time points.

### KIR x ICOS CD8 Treg Modulator improves survival in an acute GVHD study

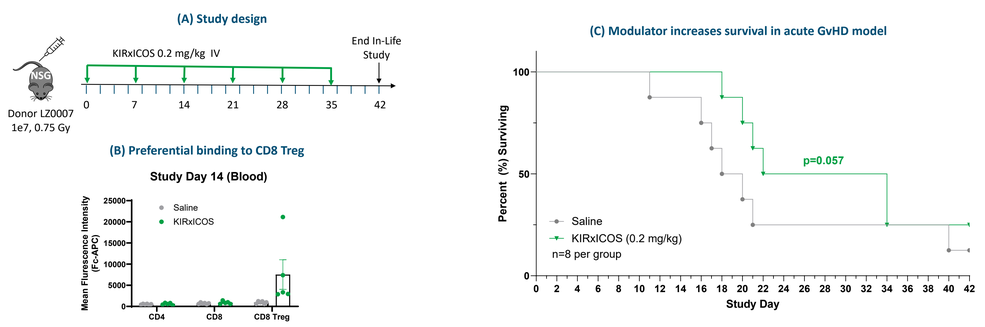



**Figure 4A)** CD8 Treg Modulator survival study experimental design. **B)** Preferential binding of Modulator to CD8 Treg at study day 14 in peripheral blood (pre-dose). **C)** Modulator increased survival relative to the saline control group as shown in the Kaplan-Meier survival curve for the duration of the study.

### Body weight of CD34+ NSG-Tg(Hu-IL-15) mice remains stable following repeat doses of KIR x ICOS CD8 Treg Modulator

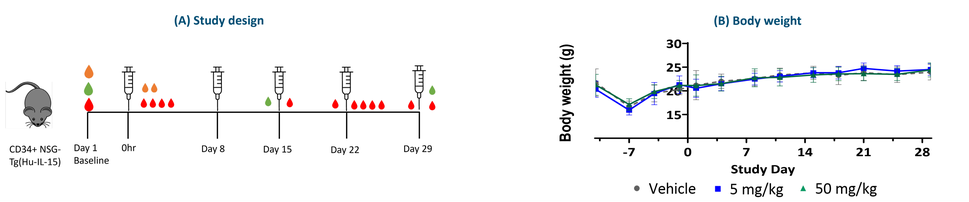



**Figure 5 A)** Blood samples were collected for PK time points (🩸): exposure evaluation pre-dose on Day 1, Day 22 and Day 29 and post dose on Day 1 and 22 at 0.5, 2, 24, 96, 168 hours; time points were also collected post-dose on Day 15 and 29; Flow cytometry time points (🩸): pre-dose on Day 1 and Day 15 and post-dose on Day 29; Serum cytokine time points (🩸): pre-dose on Day 1 and at 8 and 24 hr post-dose. **B)** The body weight of animals was monitored over time.

### After multiple doses of KIR x ICOS CD8 Treg Modulator, the presence of human immune cells in peripheral blood of CD34+ NSG-Tg(Hu-IL-15) mice remains consistent with vehicle group

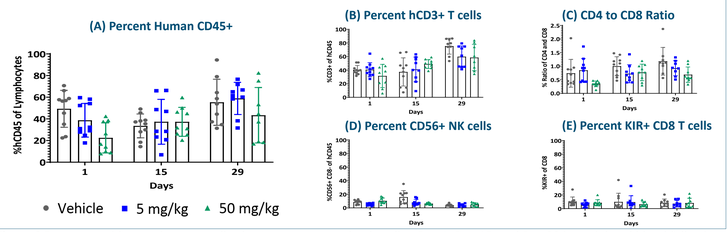



**Figure 6** Frequency of human immune cell subsets were assessed at baseline Day 1, Day 15, and Day 29 in peripheral blood following weekly IV administration of vehicle or KIR x ICOS CD8 Treg modulator at 5 or 50 mg/kg. Total hCD45, CD3, CD4, CD8 T cells, NK (CD56+CD8-) cells and CD8 Treg (KIR+ CD8+ T cells) in blood remained similar between 0, 5 and 50 mg/kg KIR x ICOS CD8 Treg Modulator dosing. Data are presented as the percentage with group mean as bars ± SD; points represent individual animals.

### Binding of KIR x ICOS CD8 Treg Modulator to peripheral blood cells expressing the target

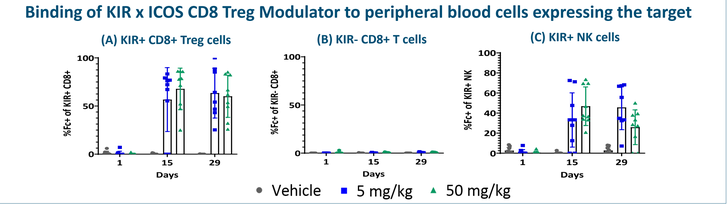



**Figure 7** Binding of KIR x ICOS CD8 Treg Modulator to CD8 Treg, KIR- CD8+ T cells, and KIR+ NK cells via Fc detection using an anti-human IgG1 Fc secondary antibody were found on cells in blood of CD34+ NSG-Tg(Hu-IL-15) mice. Binding was observed to cells expressing target in peripheral blood. As expected, no binding was observed to CD4 T cells in blood (Data not shown here). Data are presented as mean bars ± SD; points represent individual animals.

### No increase of CD69+CD25+ or ICOS

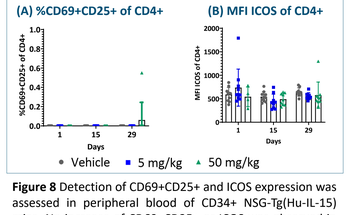
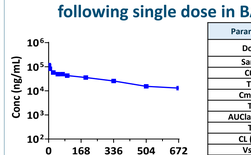



**Figure 8** Detection of CD69+CD25+ and ICOS expression was assessed in peripheral blood of CD34+ NSG-Tg(Hu-IL-15) mice. No increase of CD69+CD25+ or ICOS was observed in CD4+ T cells–or in total CD8, KIR+ CD8+, NK, and KIR+ NK cells after multiple doses of KIR x ICOS CD8 Treg Modulator (not shown). Data are presented as mean bars ± SD; points represent individual animals.

### PK profile of KIR x ICOS Treg Modulator following single dose in BALB/cJ mice




| Parameter (Units) | BALB/cJ |
|---|---|
| Dose mg/kg | 5 |
| Sample type | Serum |
| C0 (µg/ml) | 119 |
| Tmax (hr) | 0.5 |
| Cmax (µg/ml) | 116 |
| Tlast (hr) | 672 |
| AUClast (hr*µg/ml) | 19100 |
| T1/2 (hr) | 328 |
| CL (mL/hr/kg) | 0.201 |
| Vss (mL/kg) | 90.8 |
| VZ (mL/kg) | 95.1 |
| Number evaluated | 12 |

**Figure 9** Blood collection for serum in BALB/cJ mice was staggered as n=3 mice per timepoint, according to IACUC approved protocol. Data are presented as mean of individual animals at individual time points; only mice with AUC%Extrap <20% were used to calculate PK parameters shown in table.

## Results

- Functional activity of the KIR x ICOS CD8 Treg Modulator was specific to CD8 Treg cells despite a similar binding profile to activated CD4 T cells.
- In a Crohn's disease antigen-specific restimulation assay, the Modulator yielded reduction of proinflammatory cytokines and pathogenic CD4 T cells.
- In a human PBMC engrafted acute GvHD model, the Modulator bound to, activated, and increased the cytolytic capacity of CD8 Treg resulting in the elimination of activated CD4 T cells and improved survival.
- In early non-clinical safety studies, the Modulator bound to its targets and was well-tolerated following multiple doses in CD34+ NSG-Tg(Hu-IL-15) mice with no impact on body weight, in-life observations or terminal toxicity assessments.
- The Modulator did not increase CD69+CD25+ and ICOS activation of NK cells, CD4 T cells or CD8 T cells or cause a detectable increase of pro-inflammatory serum cytokines (data not shown) in CD34+ NSG-Tg(Hu-IL15) mice.
- Based on single dose serum concentration data, $T_{1/2}$ of the KIR x ICOS CD8 Treg Modulator was determined to be ~13.7 days. Consistent exposure levels were observed in CD34+ NSG-Tg(Hu-IL15) mice following single or multiple doses (data not shown).

## Conclusions

- In translationally relevant in vivo models of inflammation and in ex vivo Crohn's patient derived intestinal organoids, the Modulator demonstrated target specific selectivity and efficacy supporting its postulated mechanism of action.
- We postulate that the KIR x ICOS CD8 Treg Modulator may represent a novel and selective therapeutic approach for CD8 Treg modulation for the treatment of highly inflammatory autoimmune disease, including IBD.

**Contact:**
Follow up questions can be directed to Kristine Swiderek, Chief Scientific Officer, Mozart Therapeutics kswiderek@mozart-tx.com or by visiting the website at https://www.mozart-tx.com/

**References:**

- Aryee, et al. Enhanced development of functional human NK cells in NOD-scid-IL2rg$^{null}$ mice expressing human IL15. FASEB, 2022. https://doi.org/10.1096/fj.202200045R
- Abeynaike, et al. Human Hematopoietic Stem Cell Engrafted IL-15 Transgenic NSG Mice Support Robust NK Cell Responses and Sustained HIV-1 Infection. Viruses 2023. https://doi.org/10.3390/v15020365
- Li, et al. KIR $^{+}$ CD8 $^{+}$ T cells suppress pathogenic T cells and are active in autoimmune diseases and COVID-19. Science. 2022. DOI: 10.1126/science.abi9591
- Lodes, et al. Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest, 2004. DOI: 10.1172/JCI20295
- Morgan, et al. Flagellin-specific CD4 cytokine production in Crohn's disease and controls is limited to a small subset of antigen-induced CD40L+ T cells. J Immunol, 2021. DOI:10.4049/jimmunol.2000918
- Uchida, et al. Escherichia coli-specific CD4+ T cells have public T cell receptors and low interleukin 10 production in Crohn's Disease. Cell Mol Gastroenterol Hepatol. 2020. DOI:10.1016/j.jcmgh.2020.04.013