MTX-101, a bispecific CD8 Treg Modulator, corrects the dysfunction in Crohn's patient CD8 Treg to restore immune balance.

Meghan Maurer, Jennifer Gardell, Minh Pham, Daniel Boster, Justin Bowser, Monica Childs, Rachael Fasnacht, Gleda Hermansky, Susan Julien, Brent Meengs, Nadine Morgan, Daniel Patton, Phoenicia Quach, Cathy Tan, Jon Therriault, Catherine J McMahan, Courtney Crane, and Kristine M Swiderek

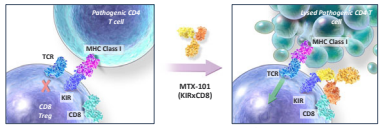
MOZART THERAPEUTICS
Orchestrating The Immune System

BACKGROUND/PURPOSE

In healthy individuals, activation of CD8 Treg leads to selective elimination of self-reactive CD4 T cells to maintain immune balance. CD8 Treg appear dysfunctional in autoimmune (AI) diseases, including Crohn's disease (CD). CD8 Treg from AI patients are insufficient to kill self-reactive CD4 T cells, due to expression of inhibitory KIR2DL1/2/3 that serves as an autoimmune checkpoint.

We have developed MTX-101, a bispecific CD8 Treg modulator targeting CD8 and KIR2DL1/2/3. MTX-101 selectively binds CD8 Treg and enhances their killing of self-reactive CD4 T cells.

CD8 Treg function enhanced by MTX-101 prevents self-reactive CD4 T cell expansion and inflammation, without increasing unwanted immune cell activation or pro-inflammatory cytokines.



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

METHODS

- Prevalence and phenotype of CD8 Treg from healthy and CD donors were examined by flow cytometry.
- Binding of MTX-101 was evaluated by flow cytometry.
- The functional impact of MTX-101 was evaluated using flow cytometry and supernatant analysis in PBMCs isolated from Crohn's disease patients.
- MTX-101 was tested in AI patient intestinal tissue biopsies (celiac and CD) and animal models of acute Graft vs. Host Disease (GvHD).
- Tolerability and pharmacokinetic studies for MTX-101 were performed with NSG-Tg(Hu-IL15) mice engrafted with human CD34+ cord blood cells.

CD8 Treg are present in Crohn's disease patient PBMCs and have decreased cytolytic capacity

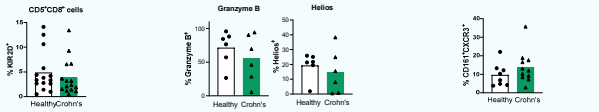


Figure 1: (A) CD8 Treg are present in the peripheral blood of healthy and Crohn's disease donors. **(B)** Peripheral blood CD8 Treg derived from healthy and Crohn's donors were stained for Granzyme B, as a measure of cytolytic capacity, and the transcription factor (TF) Helios. **(C)** Autoreactive CD4 T cells in PBMC from 8 Healthy and 11 Crohn's donors.

MTX-101 enhances CD8 Treg function and reduces proinflammatory cytokines and CD4 T cell expansion in Crohn's PBMC

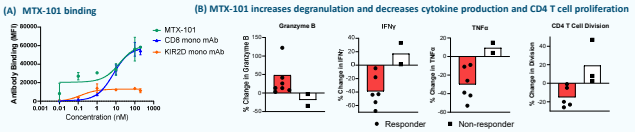


Figure 2: (A) PBMC were incubated with MTX-101 or monospecific constituent molecules. Antibody binding to CD8 Treg was detected using an anti-human IgG1 Fc secondary antibody. **(B)** Crohn's PBMC were incubated for seven days with a mixture of bacterial flagellin and OmpC peptide and IL-7 and IL-15 cytokines +/- MTX-101 at 100nM. The percentage increase in concentration of Granzyme B as detected in the supernatant on study day 5 and decrease in IFNγ and TNFα at day 7 is shown for MTX-101 treated donors relative to untreated for both responders and non-responders in the assay. The percentage decrease in division as determined by measuring fully diluted CFSE is also shown on day 7 for both responders and non-responders relative to untreated.

MTX-101 decreases pathogenic CD4 expansion and proinflammatory cytokine secretion in autoimmune disease tissue-derived organoids

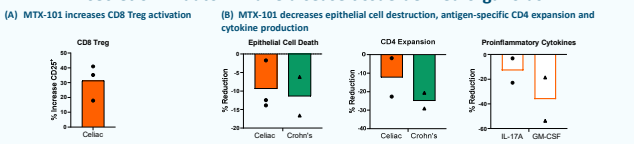


Figure 3: Organoids were cultured with flagellin/OmpC peptide (Crohn's) or gliadin peptides (Celiac) +/- MTX-101. (A) Increase in CD25 expression by CD8 Treg within Celiac organoid following incubation with 100nM MTX-101 compared to untreated control. (B) Reduction in antigen-induced epithelial cell death and CD4 T cell expansion is shown across celiac and Crohn's organoid cultures following MTX-101 treatment. Reduction in IL-17a and GM-CSF detected in celiac organoid supernatant is shown following treatment with MTX-101.

MTX-101 increases survival in human PBMC GvHD model

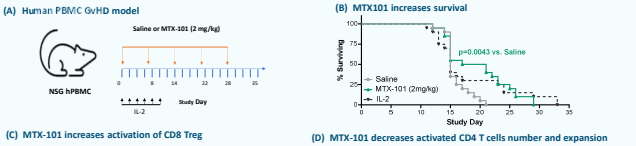


Figure 4: (A) NSG mice were irradiated and injected intravenously with human PBMC from an HLA-DQ2.5 healthy donor on study day 0. MTX-101 (2 mg/kg) or saline were injected every 7 days to study day 28. Low-dose IL-2 (25,000 IU) was injected every other day from study day 0 to study day 10. **(B)** The survival curve for mice across each of the three treatment groups is shown. **(C)** Percentage of CD25+ CD8 Treg cells within the peripheral blood as detected across treatment groups. Granzyme B MFI is shown for MTX-101 and low dose IL-2 treatment groups on study day 20 for CD8 Tregs (no saline mice remained). **(D)** Absolute counts of activated CD25+ CD4 T cells/uL and MFI of Ki67 staining in CD4 T cells are shown for early take down mice across treatment groups on study day 15 within the peripheral blood.

MTX-101 dosing in CD34+ NSG-Tg(Hu-IL15) mice does not result in production of pro-inflammatory cytokines

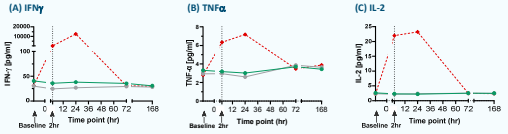


Figure 5: NSG-Tg(Hu-IL15) mice engrafted with CD34+ hematopoietic stem cells (HSC) [CD34+ NSG-Tg(Hu-IL15)] were injected with 5 mg/kg MTX-101 and blood was collected at 2, 24, 72 and 168 hrs. Dosing of mice with anti-CD3 (clone OKT3; 0.5 mg/ml) was used as a positive control for inflammatory cytokine production. Serum levels of **(A)** IFNγ, **(B)** TNFα and **(C)** IL-2 were measured over time.

No MTX-101-related observations in pilot toxicology study following repeated doses up to 50 mg/kg in CD34+ NSG-Tg(Hu-IL15) mice

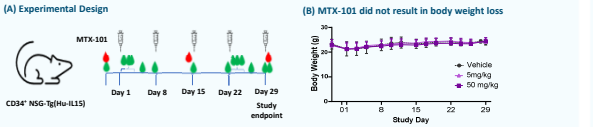


Figure 6: (A) Experimental design for multidose pilot toxicology study in CD34+ NSG-Tg(Hu-IL15) mice engrafted from two independent cord blood donors. At 12 weeks, mice with >25% hCD45, >3% hCD3 and >2% hCD56 were accepted for the study. Animals received IV doses on Days 1, 8, 15, 22, 29 of 0, 5 or 50 mg/kg of MTX-101.

Blood samples were collected for PK (n=4/dose/donor) and immunophenotyping (n=5/dose/donor) at specific time points following dose, and terminal blood and spleen were collected on Day 28.

- PK time points (🩸): pre-dose and at 0.5, 2, 24, 96, 168 hr following dosing on Days 1 and 22; additional PK time point were collected on Day 15, 29 (0.5 hr post-dose)
- Flow cytometry time points (🩸): pre-dose on Day 0 and 15 and post-dose on Day 29

(B) Mouse body weight over time after dosing with MTX-101.

MTX-101 selectively binds CD8 Treg in CD34+ NSG-Tg(Hu-IL15) mice

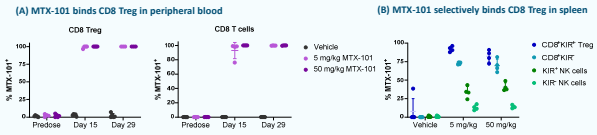


Figure 7: (A) Binding of MTX-101 to CD8 Treg and CD8 T cells in the peripheral blood of CD34+ NSG-Tg(Hu-IL15) mice pre-dose on Day 15 (7 days after Day 8 dosing) and 30 min post-dose on Day 29. **(B)** Binding of MTX101 to CD8 Treg, CD8 and NK cell populations from the spleen of CD34+ NSG-Tg(Hu-IL15) mice at terminal time point (Day 29, 30min post-dose). Experimental design shown in **Figure 6A.**

Evidence of MTX-101-related pharmacology in CD8 Treg observed following dosing in CD34+ NSG-Tg(Hu-IL15) mice

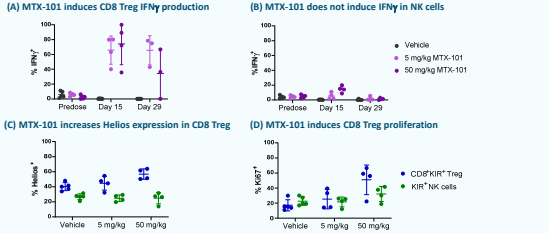


Figure 8: Production of IFNγ by **(A)** CD8 Treg and **(B)** NK cells pre-dose, at day 15 and day 29 as measured by flow cytometry in peripheral blood. **(C)** Proportion of Helios+ cells in the CD8 Treg and populations in splenocytes. **(D)** Proportion of CD8 Treg or NK cells in cell cycle, as measured by expression of Ki67 in splenocytes. Experimental design is shown in **Figure 6A.**

RESULTS

- CD8 Treg from Crohn's disease donors appeared impaired relative to healthy controls, with reduced levels of Granzyme B and Helios.
- MTX-101 selectively bound to and activated CD8 Treg in IBD donor derived PBMC and tissues.
- MTX-101 increased cytolytic capacity and activation of IBD patient-derived CD8 Treg in PBMC and Crohn's donor colon tissues.
- Treatment with MTX-101 reduced production of antigen-specific pro-inflammatory cytokines in IBD donor-derived PBMCs and tissues and did not elevate cytokine production in early safety studies.
- MTX-101 showed selective CD8 Treg binding, without unwanted immune cell activation or body weight loss in early safety studies in a humanized mouse model.
- Treatment with MTX-101 selectively increased the Helios content and proliferation of the CD8 Treg population, but not of NK cells, in splenocytes.

CONCLUSIONS

- MTX-101 enhances CD8 Treg function in vitro, in vivo, and in ex vivo patient tissues.
- MTX-101 may decrease inflammation via selective elimination of pathogenic CD4 T cells in IBD.
- We postulate that CD8 Treg can be targeted by MTX-101 to suppress pathogenic CD4 T cells and may represent a novel and selective therapeutic approach to restore immune balance in patients with Crohn's disease.

Acknowledgements: The authors would like to acknowledge the contributions of Dr. James Lord (Benaroya Research Institute; Seattle, WA) and patients for duodenal biopsies and members of Dr. Calvin Kuo's laboratory (Stanford University; Palo Alto, CA) for assistance with the organoid culturing methods (Figure 3).
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/
Disclosures: All authors are employees of Mozart Therapeutics.
References:
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
Aryee, et al. Enhanced development of functional human NK cells in NOD-scid-IL2rg^{null} mice expressing human IL15. *FASEB*, 2022. DOI: 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. DOI: 10.3390/v15020365