Despite expression of exhaustion markers, CD8 Treg are a cytolytic population that target pathogenic T cells to prevent beta cell destruction by Type 1 Diabetes peripheral blood mononuclear cells

Meghan E. Maurer, Tara N. McCray, Jennifer L. Gardell, Daniel T. Patton, Nadine N. Morgan, Kaelen Encarnacion, Monica M. Childs, Susan H. Julien, Cong Tan, Allison O'Rourke, Catherine J. McMahan, Kristine M. Swiderek, Courtney A. Crane

MOZART THERAPEUTICS

Orchestrating The Immune System

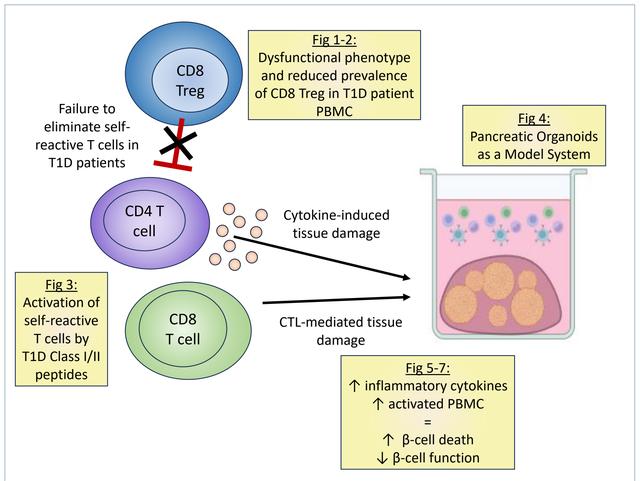
Abstract

CD8 regulatory T cells (CD8 Treg; defined as KIR$^+$ CD8 T cells) play a crucial role in maintaining immune system homeostasis by their cytolytic elimination of pathogenic immune cells. Increased CD8 Treg prevalence in Type 1 Diabetes (T1D) patients correlates with improved outcomes in response to immunotherapies, including teplizumab and alefacept. This study sought to investigate the phenotype of CD8 Treg and explore their role in preventing beta cell (β cell) death in T1D using pancreatic organoids.

CD8 Treg appear to be an exhausted T cell subset (Tex) in healthy controls (HC) and patients with autoimmune diseases, based on expression of canonical markers including PD1, TIM3, LAG3, and KLRG1. However, cells are transcriptionally distinct from Tex and express cytolytic markers consistent with a cytotoxic MOA. CD8 Treg are reduced in peripheral blood from T1D patients and have a dysfunctional phenotype. Stimulation increased activation and cytolytic markers, suggesting: (1) Dysfunctional CD8 Treg can be rescued by activation in T1D patients, and (2) CD8 Treg are a cytolytic population that natively expresses high levels of exhaustion markers, as opposed to a functionally exhausted population.

Stimulation of T1D peripheral blood mononuclear cells (PBMC) with a self-peptide cocktail illustrated that CD8 Treg from T1D patients were unable to control pathogenic T cell responses. Pancreatic organoids provide a model system for studying the interactions between pathogenic T cells and CD8 Treg. In pancreatic organoid cocultures, T1D PBMCs produced proinflammatory cytokines and reduced viable β cells and glucose-stimulated insulin secretion. Our data highlight the therapeutic potential for restoration of CD8 Treg functions in T1D patients to delay or prevent T cell mediated islet β cell destruction.

Poster Overview



Methods

- CD8 Treg and non-Treg populations from the peripheral blood of HC and T1D patients were comparatively assessed using flow cytometry and bulk RNA sequencing
- Functional responses of T1D patient-derived CD8 Treg were evaluated using anti-CD3 or a T1D autoantigen peptide cocktail
- Effects on pathogenic T cells were evaluated using autoantigen peptide restimulation of PBMC
- Effects on disease-affected tissue damage were tested using functional human pancreatic islet-derived organoids in the presence of T1D patient PBMC and T1D autoantigen peptides

CD8 Treg are a Cytolytic Population that are Distinct from Exhausted CD8 T cells

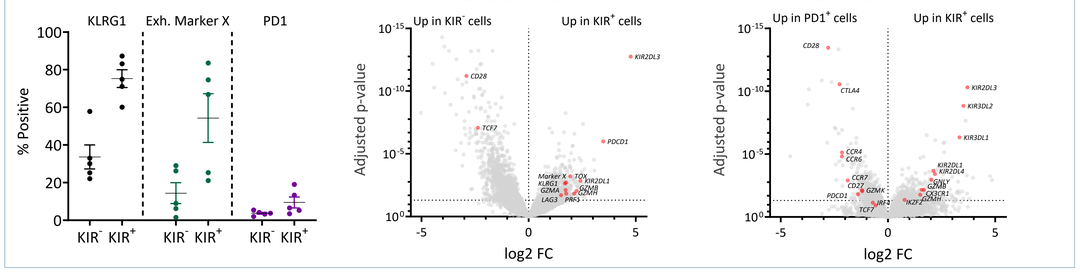


Figure 1: CD8 Treg express exhaustion markers but are highly cytolytic and uniquely distinct from exhausted (PD-1$^+$) T cells (A) KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1 expressing (KIR$^+$) CD8 Treg express surface markers associated with an exhausted phenotype, including KLRG1, Exhaustion marker "X", and PD-1, compared to KIR$^-$ CD8 T cells. **(B)** KIR$^+$ CD8 T cells differentially express exhaustion and cytolytic markers compared to KIR$^-$ CD8 T cells. **(C)** KIR$^+$ and PD-1$^+$ CD8 T cell populations differ by expression of cytolytic molecules, NK cell-associated surface markers, transcription factors, and chemokine receptors, all suggesting distinctly different regulation and function.

In T1D Patients, CD8 Treg are Reduced and Dysfunctional, yet Present and Highly Responsive to anti-CD3 Stimulation

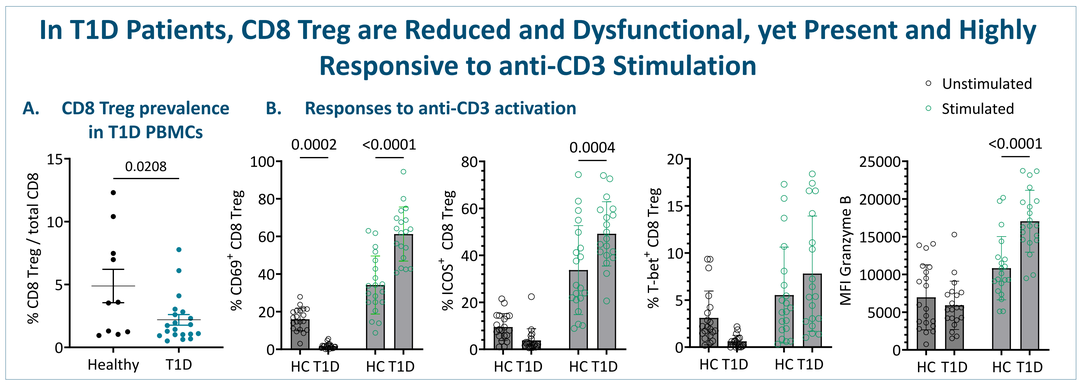


Figure 2: CD8 Treg in T1D patient PBMC have a less activated phenotype at baseline but remain highly responsive to stimulation compared to HC CD8 Treg (A) Proportion of CD8 Treg out of the total CD8 T cell population. **(B)** PBMC from HC (n=20) or T1D donors (n=20) were cultured with and without anti-CD3 for 24 hours and activation status (CD69, T-bet and ICOS) and cytolytic capacity (Granzyme B) were assessed.

Long Term Diagnosed T1D Patients Retain Autoantigen Recall Responses

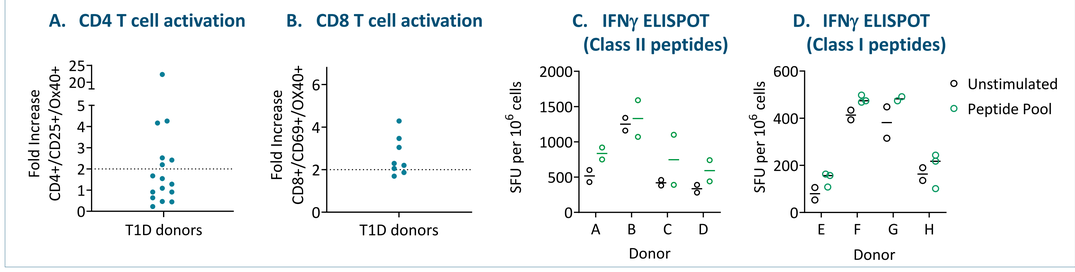


Figure 3: T1D Autoantigen Peptides Induce CD4 and CD8 T cell activation in patients more than 5 years past diagnosis of T1D. T1D patient PBMC were stimulated with a pool of 11 Class II peptides **(A, C)** or a pool of 54 Class I peptides **(B, D)** derived from GAD65, IA2 and PPI . Increase in proportion of CD4+CD25+Ox40+ cells **(A)** or CD8+CD69+OX40+ cells **(B)** relative to unstimulated. PBMC showing >2x fold increase were considered responders. **(C, D)** Number of IFNγ spot forming units (SFU) in a subset of the responding patients from **(A)** and **(B)**.

Pancreatic Organoids Secrete Insulin in Response to Glucose Stimulation

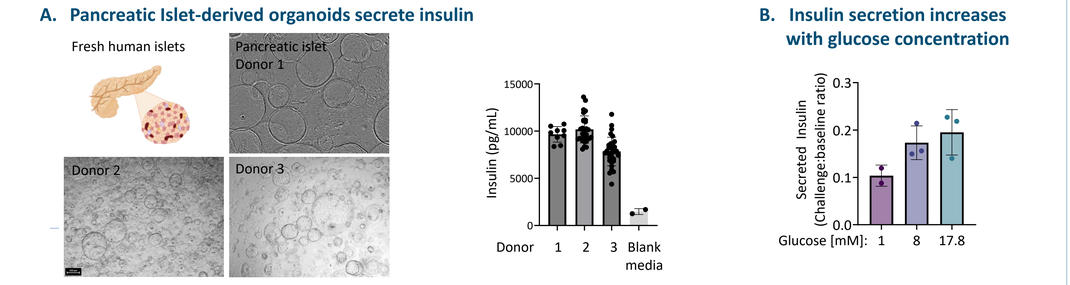


Figure 4: Pancreatic organoids generated from freshly isolated human islets secrete insulin (A) Organoids were generated from three separate islet donors (left; scale bar = 250 µm). Insulin expression is shown as detected in media by MSD. Each dot represents one well. Blank media is the insulin within media recipe. **(B)** Insulin release was measured after organoids were rested in 0mM glucose followed by exposure to glucose for one hour at the concentration shown on X-axis. Each well was normalized to its baseline to account for differences in cell number.

T1D PBMC Produce Inflammatory Cytokines in Coculture with Pancreatic Organoids

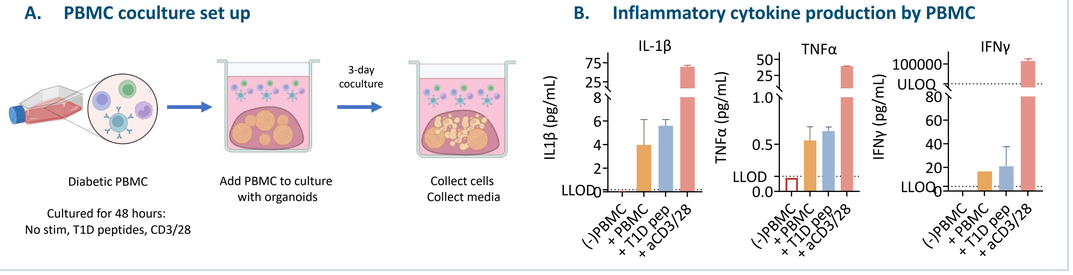


Figure 5: PBMC from T1D patients produce inflammatory cytokines when in coculture with pancreatic organoids (A) T1D PBMC were thawed and cultured for 48hr without stim (+ PBMC), with T1D peptides from **Figure 3** (+T1D pep), or with anti-CD3/28 stim. After 48hr, PBMC were added to established pancreatic organoids and cocultured for 3 days before collecting cells and media for analysis. **(B)** Inflammatory cytokines IL-1β, TNFα and IFNγ increased with addition of T1D PBMC to organoids and increased further with activation of PBMC by T1D peptides or anti-CD3/28 stimulation, a possible driver of beta cell death seen in **Figure 6.**

Cytokines Induce Beta Cell Death in Pancreatic Organoids

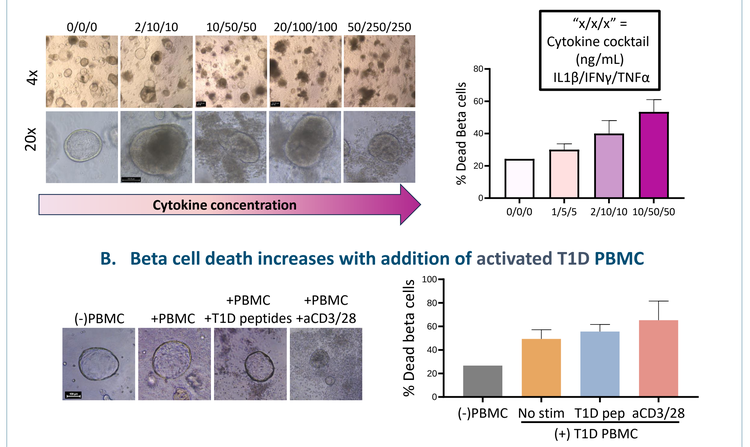


Figure 6: **Diabetogenic cytokines are a possible driver for cell death in pancreatic organoids (A)** In pancreatic organoids, 3 days exposure to cytokines resulted in cell death, as seen under brightfield microscopy (left). Cell death is increased in the proInsulin+ beta cells, as detected by Live/Dead staining by flow cytometry (right). **(B)** Stimulation of T1D PBMC with T1D peptides or anti-CD3/28 results in increased beta cell death in organoid cocultures, possibly due to increased secretion of IFNγ, IL1β, and TNFα, as shown in **Figure 5**. Scale bars = 100 µm.

Glucose-Stimulated Insulin Secretion Decreases in Pancreatic Organoids Exposed to Inflammatory Cytokines

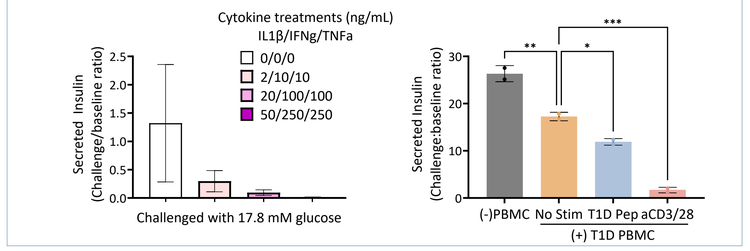


Figure 7: Glucose-stimulated insulin secretion in organoids challenged with T1D cytokines or in coculture with T1D PBMC (A) Pancreatic organoids were cultured in cytokines for three days, followed by a rest in 0mM glucose and a 1hr challenge with 17.8mM glucose prior to measuring insulin secretion. Insulin released after glucose stimulation was reduced in organoids exposed to cytokines. **(B)** Organoids were cocultured with T1D PBMC for three days, followed by a glucose rest and challenge; activation of PBMC reduced the glucose-stimulated insulin release of pancreatic organoids.

Results and Conclusions

- CD8 Treg express typical markers of an exhausted population; however, RNAseq data suggest they are unique and have a cytolytic phenotype
- CD8 Treg from T1D patients are reduced in prevalence and are dysfunctional based on phenotype and inability to control self-reactive CD4 and CD8 T cell activation and cytokine production in T1D PBMC
- CD8 Treg function in T1D PBMC can be restored by activation
- Addition of self-reactive T1D PBMC or inflammatory cytokines induces beta cell damage in pancreatic organoids
- The pancreatic organoid system may provide a human tissue-based model to study T1D immune regulation more broadly, including the role of CD8 Treg
- Our data support the development of therapeutics designed to activate CD8 Treg and thus to restore their function for the treatment of T1D.

Acknowledgements: The authors would like to thank the PBMC and pancreatic tissue organ donors. Illustrated figures created with Biorender.com.

Contact: Questions can be directed to Courtney Crane, SVP, Translational Medicine & Biology, Mozart Therapeutics ccrane@mozart-tx.com or by visiting the website at https://www.mozart-tx.com/

References: (1) 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 Long SA, et al. **(2)** Partial exhaustion of CD8 T cells and clinical response to teplizumab… Sci Immuno. 2016. DOI: 10.1126/sciimmunol.aai7793. **(3)** Driehuis E, et al. Establishment of Pancreatic Organoids from Normal Tissue and Tumors. *STAR Protoc*. 2020. DOI: 10.1016/j.xpro.2020.100192 **(4)** Molano RD, et al. A static glucose-stimulated insulin secretion (sGSIS) assay... *BMJ Open Diabetes Res Care*. 2024. DOI:10.1136/bmjdrc-2023-003897