MOZART THERAPEUTICS

Orchestrating The Immune System

Development of a Whole-Blood Assay based on Peptide-Stimulated Cytokine Release for the Detection of Pathogenic CD4 T cells in Type 1 Diabetes

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Background and Aims

Rapid, simple, and specific detection of autoreactive CD4 T cells improves understanding of type 1 diabetes (T1D) pathogenesis and short-term readout for efficacy of immunotherapy. Previous approaches have either used complex flow cytometry or peptide stimulation that requires extensive manipulation of cells. The development of the Novoleukin™ whole blood antigen-stimulated cytokine release assay in celiac disease was the basis for the development of a similar assay using peptides which activate autoreactive T cells in T1D. In the celiac disease Novoleukin test, the level of IL-2 response correlates with the prevalence of reactive CD4 T cells in patient blood, and could detect one antigen-specific cell in 10^6 CD4 T cells.

Methods and Results

The Novoleukin assay platform utilizes whole blood incubated in a tube containing peptides. This assay was adapted for T1D using peptides derived from GAD65, IA2 and Proinsulin. The peptides were modified to increase solubility and stability. Scrambled peptide was used as a negative control, and PHA as a positive control. Blood from ten T1D patients were transferred to the tubes and incubated for 24hrs at the clinical site, centrifuged, and the resulting plasma stored at -20˚C. Plasma was shipped to a central laboratory and analyzed for cytokines using an MSD S-Plex Multiplex which detected levels of IFN-γ, IL-10, IL-12p70, IL-17A, IL-1β, IL-2, IL-4, IL-6 and TNF-α. The levels of cytokine increased with peptide stimulation, with IL-2 showing the greatest fold-over-background and selectivity, which led to IL-2 selection as the analyte for quantifying autoreactive CD4 T cell responses in the blood. Several other cytokines were of interest from a functional perspective, including IFN-γ, TNF-α, IL-10, and IL-17, enabling additional functional characterization in the future.

The analyses were repeated using the single-plex MSD IL-2 S-Plex assay. This assay utilizing IL-2 was then successfully deployed for exploratory monitoring the response in T1D patients upon dosing with MTX-101.

Self-peptides stimulate activation and cytokine production in T1D donor cells

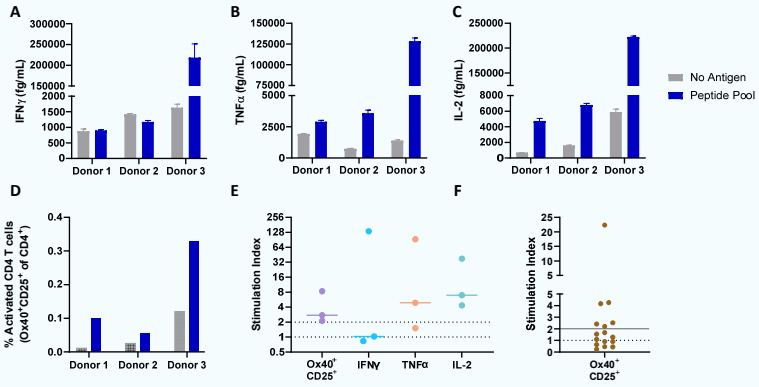


Figure 1. Self-peptides stimulate T1D donor CD4⁺ T Cells
PBMC were obtained from three T1D donors and incubated for 20 hours with peptides derived from GAD65, PPI and IA2. The production of cytokines was assessed in the supernatants by MSD, and the concentration of (A) IFNγ, (B) TNFα and (C) IL-2 assessed. (D) The proportion of activated OX40⁺CD25⁺ cells within the CD4⁺ T cell population was enumerated by flow cytometry. (E) The stimulation index (peptide stimulated wells divided by no antigen) was calculated for the cytokine and activation data in parts A-D. (F) Sixteen further T1D donors were assessed for their activation by the T1D peptides.

T1D peptides stimulate IL-2 release in Novoviah whole blood assay

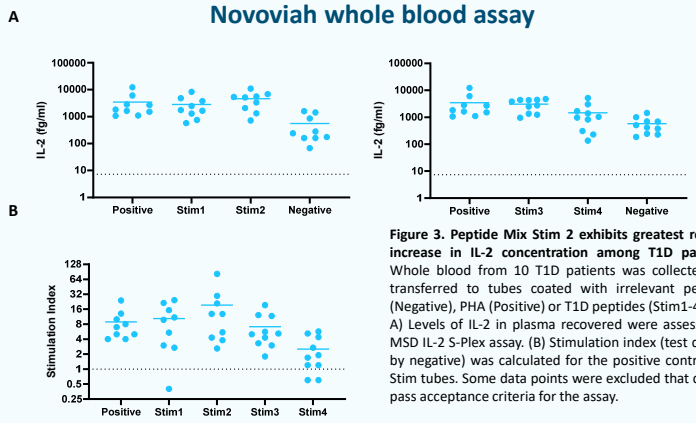
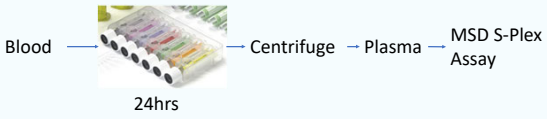


Figure 3. Peptide Mix Stim 2 exhibits greatest relative increase in IL-2 concentration among T1D patients.
Whole blood from 10 T1D patients was collected and transferred to tubes coated with irrelevant peptides (Negative), PHA (Positive) or T1D peptides (Stim1-4).
A) Levels of IL-2 in plasma recovered were assessed by MSD IL-2 S-Plex assay. (B) Stimulation index (test divided by negative) was calculated for the positive control and Stim tubes. Some data points were excluded that did not pass acceptance criteria for the assay.

Novoviah T1D Assay Workflow



Peptide Mix	Peptide Source	Modifications
Stim1	GAD65, PPI, IA2	Solubility
Stim2	GAD65, PPI, IA2	Solubility, Stability
Stim3	PPI	Solubility
Stim4	PPI	Solubility, Stability
Negative	Random Hexamers	None

Figure 2. Workflow for Novoviah T1D assay.
Whole blood was taken in a lithium heparin 4.9ml tube and 4ml transferred via a transfer device to tubes pre-coated with peptides or PHA (Positive Control). After 24 hours incubation at 37˚C, the tubes were then centrifuged at 2000g for 10 minutes and plasma recovered and frozen before running MSD S-Plex assay.

Assay window in Ph1b pre-dose samples

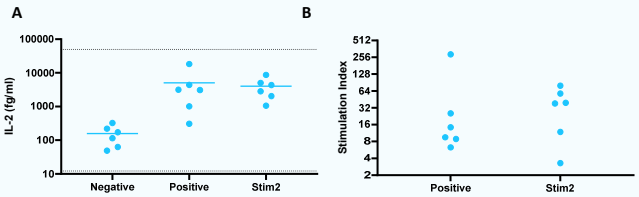


Figure 4. IL-2 based whole blood assay shows activation by peptide in all six patients in Ph1b trial
Whole blood from six T1D patients enrolled in the MTX-101 Phase 1b trial was taken prior to the initial dose of MTX-101. The blood was transferred to tubes coated with irrelevant peptides (Negative), PHA (Positive) or T1D peptides (Stim2). (A) Levels of IL-2 in plasma recovered were assessed by MSD IL-2 S-Plex assay. (B) Stimulation index (test divided by negative) was calculated for the positive and Stim2 tubes.

Conclusions

- Peptides from T1D autoantigens induce IL-2 release in a whole blood assay
- A whole blood ex vivo restimulation assay has potential to serve as a tool to assess autoreactive cells in T1D patients

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