Identification of children with Type 1 Diabetes Suitable for Antigen-specific Immunotherapy

Yassmin Musthaffa¹,², Emma Hamilton-Williams², Mark Harris¹,², Ranjeny Thomas²

¹: Department of Endocrinology and Diabetes, Lady Cilento Children’s Hospital, South Brisbane, QLD.
²: University of Queensland Diamantina Institute, Translational Research Institute, University of Queensland Brisbane, QLD.
Background

What we know about the cause and treatments for Type 1 Diabetes (T1D)

• Autoimmune disease
  • T cell-mediated pancreatic β-cell destruction

• Increasing incidence, Significant biopsychosocial burden

• Current (insulin replacement) therapies inadequate

• Need to stop the autoimmune process → preserve β-cells
  • Better metabolic control, less severe hypoglycaemia, fewer long term complications
  • Better quality of life

* β-cell = Insulin producing cells of the pancreas

DCCT 1993, EDIC 2009
Immune therapy has the potential to cure and/or prevent T1D
Developing Antigen Specific Immunotherapy

The 3 therapeutic targets

1. Identify the auto-antigen

2. Identify individuals in whom self-antigen recognition occurs

3. Change the cross-talk between Dendritic Cells (DC) and T cells (occurs via NF-κB activation)
Developing Antigen Specific Immunotherapy

Dendritic cells (DC) as therapeutic targets in T1D

Regulating the presentation of islet autoantigens by DCs to autoreactive T cells can restore self-tolerance.
Developing Antigen Specific Immunotherapy

The 3 therapeutic targets in T1D

1. Identify the antigenic target
   Proinsulin

2. Identify individuals in whom self-antigen recognition occurs

3. Change the cross-talk between APC and T cells
   (via NF-κB activation)

Calcitriol
   (NF-κB inhibitor)
The T-cell response to Pro-insulin peptides

**Hypothesis**

CD4+ T-cell responses in individuals with T1D will vary according to age, HLA*-type, disease duration, and C-peptide

**Aims**

To (A) identify and (B) characterise individuals with T1D who have CD4+ T-cell responses to established islet auto-antigens

* HLA = Human leucocyte antigen
Project overview and methodology

1. HLA typing
2. Isolate PBMC*
3. Label PBMC with Fluorescent dye CFSE
4. Incubate PBMC with islet autoantigens
   - Proinsulin and Hybrid Insulin Peptides
5. Measure CD4+ T cell proliferation with Flow Cytometry

*PBMC – Peripheral Blood mononuclear cells

Divided cells (dim) Undivided cells (bright)

CD4+ T-cell proliferation in response to (PI33-63)
The different *in vitro* stimulation conditions

1. Negative control: No Antigen

2. Positive control: Human $\alpha$CD3 / Tetanus Toxoid

3. Synthetic Islet Peptides
   - Pro-insulin peptides
     - Name: PI$^{33-63}$
       Sequence: EAEDLQVGQVELGGPGAGSLQPLALEGSLQ
     - Name: PI$^{48-62}$
       Sequence: PGAGSLQPLALEGSL
     - Name: PI$^{40-52}$
       Sequence: GQVELGGGPGAGS

4. Hybrid Insulin peptides (HIPs)
   - Name: hEGGG:C-pep
     Sequence: GQVELGGGEAEDLQV
   - Name: hEGGG:IAPP2
     Sequence: GQVELGGGANVEVLK
   - Name: hEL:A-chain
     Sequence: SLQPLALGIVEQCC

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**Human Proinsulin**

![Human Proinsulin Structure](image)
Calculating the Cell Division Index (CDI)

T cell proliferation in response to antigen stimulation is defined as the CDI

\[
CDI = \frac{\text{number of divided } \text{CD}4^+ \text{ cells per 5,000 CD}4^+ \text{ CFSE}^{\text{undivided}} \text{ from “with antigen” group}}{\text{number of divided (CD}4^+) \text{ from the “without antigen” group}}.
\]
## Results

### Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>All patients</th>
<th>T1D &lt; 3 months (early-onset T1D)</th>
<th>T1D &gt; 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects</strong></td>
<td>10</td>
<td>48</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td><strong>Mean duration of diagnosis</strong></td>
<td></td>
<td></td>
<td>1.44</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Mean age (years; ± SD)</strong></td>
<td>35.14 ± 10</td>
<td>10.1 ± 3.8</td>
<td>9.3 ± 4.1</td>
<td>10.3 ± 3.5</td>
</tr>
<tr>
<td><strong>Mean Age at diagnosis (years)</strong></td>
<td></td>
<td></td>
<td>8.8 ± 3.7</td>
<td>9.1 ± 4.3</td>
</tr>
<tr>
<td><strong>Gender (female:male)</strong></td>
<td>7:3</td>
<td>21:27</td>
<td>7:9</td>
<td>14:18</td>
</tr>
<tr>
<td><strong>Body Mass index (kg/m²± SD)</strong></td>
<td>25.1 ± 5.8</td>
<td>19.72 ± 4.5</td>
<td>17.9 ± 4.1</td>
<td>20.6 ± 4.5</td>
</tr>
<tr>
<td><strong>Mean insulin dose adjusted glycated hemoglobin (% ± SD)</strong></td>
<td></td>
<td></td>
<td>11.6 ± 3.4</td>
<td>10.9 ± 2.6</td>
</tr>
<tr>
<td><strong>Average total daily insulin dose (IU Kg⁻¹ day⁻¹; ±SD)</strong></td>
<td></td>
<td></td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td><strong>Estimated C-peptide¹</strong></td>
<td>0.4 ± 0.2</td>
<td>0.02 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>**</td>
</tr>
</tbody>
</table>

* p<0.05 value compares all T1D and HC  
** p value compares early-onset T1D and T1D > 3 months  

Results: Disease duration

CD4+ T cell responses were detected more frequently in early-onset T1D

CD4+ T CELL DIVISION INDEX ≤ 3 MONTHS

CD4+ T CELL DIVISION INDEX > 3 MONTHS

* \( p = 0.01 \), compares CDI \( \text{PI}_{33-63} \) in early-onset T1D and T1D > 3 months

<table>
<thead>
<tr>
<th>CDI ≥ 3 to any peptide</th>
<th>All Patients (n = 48)</th>
<th>22 (46%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early onset (n= 16)</td>
<td>13 (81%) **</td>
</tr>
<tr>
<td></td>
<td>T1D &gt; 3 months (n= 32)</td>
<td>10 (31%)</td>
</tr>
<tr>
<td>Healthy Controls (n = 11)</td>
<td></td>
<td>4 (36%)</td>
</tr>
</tbody>
</table>

** \( p = 0.03 \), compares CDI for any peptide in early-onset and T1D > 3 months
Results: Peptide specificity

CD4+ T cell responses to PI_{33-63} predominate

CD4+ T-CELL RESPONSES (CDI≥3) TO ISLET PEPTIDES IN EARLY-ONSET T1D

- PI33-63: 24%
- PI48-62: 16%
- PI40-52: 9%
- hEL:A-chain: 16%
- hEGGG: IAPP2: 16%
- hEGGG: C-pep: 15%
- No response: 19%
- Responders: 81%

CD4+ T CELL DIVISION INDEX TO PROINSULIN_{33-63}

Error bars display the mean ± SD. * p = 0.01
CD4+ T cell responses occur equally across age brackets in early onset T1D

Results: The influence of age

Error bars display the mean ± SD.
CD4+ T cell responses to multiple peptides were detected more frequently in early-onset T1D.

**Results:**

- **Multiple peptides:***
  - 1 peptide: 17%
  - 2 peptides: 4%
  - 3 peptides: 8%
  - 4 peptides: 11%
  - 5 peptides: 4%
  - 6 peptides: 2%
  - No response: 54%

**ALL T1D PATIENTS**

**HEALTHY CONTROLS**

<table>
<thead>
<tr>
<th>CDI ≥ 3 to multiple peptides</th>
<th>All Patients (n = 48)</th>
<th>Early onset (n= 16)</th>
<th>T1D &gt; 3 months (n= 32)</th>
<th>Healthy Controls (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 peptide</td>
<td>14 (29%)</td>
<td>8 (50%)</td>
<td>6 (19%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>2 peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 peptides</td>
<td></td>
<td></td>
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<tr>
<td>4 peptides</td>
<td></td>
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</tr>
<tr>
<td>5 peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>14 (29%)</td>
<td>8 (50%)</td>
<td>6 (19%)</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

*p value compares early onset T1D against T1D > 3 months and healthy controls*
Results: The influence of glycaemia

**CD4+ T cell responses correlated negatively with Estimated C-peptide**

<table>
<thead>
<tr>
<th></th>
<th>CDI ≥ 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated C peptide*</td>
<td>r = -0.47 to -0.32 **</td>
</tr>
<tr>
<td>Insulin dose adjusted HbA1c</td>
<td>r = -0.18 to 0.36</td>
</tr>
</tbody>
</table>

*Clinical model incorporating age, gender, BMI-Z score, HbA1c, time since diagnosis and insulin, correlates significantly with 90-minute stimulated C-peptide measurements (adjusted R² = 0.62, P <0.0001). Buchanan et al 2019.

**P < 0.05 using spearman’s test, range provided for different peptides**
Results: Longitudinal CD4+ T cell responses

CD4+ T cell responses diminish with time

*CDI – Cell Division Index
Results: Cytokine responses

CD4+ T cell ‘non’ responders may demonstrate cytokine responses

* IFN-γ = Interferon-gamma, LAP = latency-associated peptide, TGF-β = transforming growth factor beta
^ T1D patient with CD4+ T cell proliferative response at day 0 (diagnosis) but not at day 150 or 330
# Double Antibody positive euglycaemic individual
Summary of preliminary results

• Peptide*-specific CD4+ T-cells can be detected in peripheral blood of most children early-onset T1D, half of whom show responses to multiple islet peptides.
  • CD4+ T cells proliferative responses may diminish with time
  • CD4+ T cells may continue to produce cytokine responses
  • Further evaluation of clinical variables and cytokine profiles is warranted

• Of the peptides tested, CD4+ T-cell responses to Proinsulin\textsubscript{33-63} may be an attractive candidate for a T-cell based biomarker

* Natural Proinsulin peptides or Hybrid Insulin Peptides
Significance of our findings

• Phase Ib Clinical trial of antigen-specific immunotherapy (ASI) in Rheumatoid Arthritis

Thomas Group

• Findings from this study can support the development of ASI in T1D by identifying:
  • the best candidate peptides to incorporate into ASI
  • the patients who are most likely to respond to ASI
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**Thomas Lab**
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**RACP Congress**
RACP Foundation

**T1D patients and families**
New onset T1D team (Queensland Children’s Hospital)

Dr Hendrick Nel, Nathan Stone
Results

CD4+ T cell responses in Healthy controls

CD4+ T CELL DIVISION INDEX

PERCENTAGE OF CD4+ T-CELL RESPONSES (CDI≥2) TO ISLET PEPTIDES IN HEALTHY CONTROLS

CD4+ T cell responses were seen in 3/9 of HC (33%)
Translation into Human T1D

Finding the ‘right’ self antigen to generate a T-cell response

• Proinsulin
  • Major autoantigen in NOD mice

• Humans
  • Insulin gene locus is a T1D susceptibility gene
  • Insulin –specific antibodies are first marker of pre-diabetes
  • Insulitis only seen in islets with insulin positive cells
  • Insulin-specific CD4+ T cells isolated from blood of recent onset T1D, and from islets and pancreatic lymph nodes of donors
  • Same epitopes found from islets of different patients

Bennett 1995; Pugliese 1997
Ziegler 2013
Campbell-Thompson 2016
Mannering 2010, Kent 2005
Babon 2016, Nakayama 2017, Pathiraja/Mannering 2015
Proinsulin-derived epitopes recognized by human islet-infiltrating CD4+ T cells

**Human Proinsulin**

<table>
<thead>
<tr>
<th>Insulin B-Chain</th>
<th>C-Peptide</th>
<th>Insulin A Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVNQHLCGSHLVEALYLVCGERGFYTPKTRREAEDLQVGQVELGGPGAGSLQLALEGSLQKRGIVEQCTSICSLOYQLENYC</td>
<td>DQ8 x10 (5)</td>
<td>Pathiraja, V. et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>DQ8 x2 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DQ8 x2 (1)</td>
<td></td>
</tr>
<tr>
<td>DQ8/DQ8trans x2 (2)</td>
<td>DQ8trans</td>
<td>Michels AW. et al. (2017)</td>
</tr>
</tbody>
</table>

**Hybrid Insulin peptides (HIPs)**

- DQ8
- DQ8
- IAPP2
- NP-Y
- IAPP1
- INS-A (Insulin A chain)
- IAPP2 (Islet amyloid polyprotein 2)
- DR4

Preliminary Human T1D Data

Proinsulin specific CD4+ T cells in islets of people with T1D

• CD4+ T cell clones isolated from the islets of a deceased T1D donor

• These clones recognised epitopes from proinsulin, insulin’s precursor.

• All the pro-insulin specific clones were restricted by HLA-DQ8, or HLA-DQ8 transdimer that only forms with HLA-DQ2/DQ8 APCs

Preliminary Human T1D Data

Proinsulin specific CD4+ T cells in peripheral blood of people with T1D

- CFSE labelled CD4+ T cell proliferation
  - Recent onset: < 100 days T1D diagnosis
  - Long standing: > 100 days T1D diagnosis
  - Healthy control: HLA matched
  - CDI (Cell division index)
    - \( \text{CDI} = \frac{\text{number of cells that have proliferated in response to antigen}}{\text{number of cells that have proliferated in absence of antigen}} \)
    - CDI \( \geq 2 \) traditionally considered a significant response;
    - CDI \( \geq 3 \) was considered to improve the specificity of the results

Michelle So, Stuart Mannering
Subjects and Samples

• Subjects
  • 200 children/adolescents from dedicated New Diagnosis T1D clinic
  • Inclusion criteria:
    • Age 2-16y (male or female) at varying stages following T1D diagnosis
  • Exclusion criteria:
    • Auto-immune disease (except treated thyroid and coeliac disease)

• Participant Samples
  • 5-15 mls blood
    • HLA-typing
    • preparation of peripheral blood mononuclear cells (PBMC) for fresh CFSE labelled CD4+ T cell readouts in response to islet peptides
    • ± Cryopreservation
Future directions

Measuring cytokine responses

- In addition to cell proliferation, CD4+ T cells may produce cytokine responses to proinsulin
  - Particularly relevant to assessing longitudinal responses
  - ? Biomarker for use with frozen samples
## Measuring cytokine responses

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Relevance in Type 1 Diabetes</th>
</tr>
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<tbody>
<tr>
<td>IFN-γ*</td>
<td>Pro inflammatory</td>
</tr>
<tr>
<td></td>
<td>• Promotes CD4+ T cell effector function</td>
</tr>
<tr>
<td></td>
<td>• cytotoxic, cytoytic, cytostatic (inhibits insulin synthesis and</td>
</tr>
<tr>
<td></td>
<td>secretion), or cytocidal actions to pancreatic islets</td>
</tr>
<tr>
<td>TNF-α*</td>
<td></td>
</tr>
<tr>
<td>IL* 17a</td>
<td>Pro inflammatory</td>
</tr>
<tr>
<td></td>
<td>• Enhances IL-1b, IFN-γ, and TNF-α-induced apoptosis in human</td>
</tr>
<tr>
<td></td>
<td>islets</td>
</tr>
<tr>
<td>TGF-β (LAP*) and IL*-10</td>
<td>Anti inflammatory</td>
</tr>
<tr>
<td></td>
<td>• Suppress T cell proliferation and DCs*, Inhibit effector T</td>
</tr>
<tr>
<td></td>
<td>cell responses</td>
</tr>
</tbody>
</table>

* IFN-γ = Interferon-gamma, TNF-α = Tumour necrosis factor alpha, IL = interleukin, LAP = latency-associated peptide, TGF-β = transforming growth factor beta, DC = dendritic cell
Procedures

Incubation Period with antigenic conditions and controls

- Optimal duration of 7 days
  - Shorter periods insufficient proliferation, reduced sensitivity
  - Longer periods increased background proliferation