Proinsulin C-peptide is an autoantigen in people with type 1 diabetes


Immunology and Diabetes Unit, St. Vincent’s Institute of Medical Research, Fitzroy, VIC 3065, Australia; aDepartment of Population Health and Immunology Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia; bDepartment of Medicine, University of Melbourne, St. Vincent’s Hospital, Fitzroy, VIC 3065, Australia; cWestern Centre for Health Research and Education, University of Melbourne, Western Clinical School, Sunshine Hospital, St. Albans, VIC 3021, Australia; dDepartment of Endocrinology and Diabetes, Royal Children’s Hospital, Murdoch Children’s Research Institute, Parkville, VIC 3052, Australia; and eDepartment of Paediatrics, University of Melbourne, VIC 3010, Australia

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Type 1 diabetes (T1D) is an autoimmune disease in which insulin-producing beta cells, found within the islets of Langerhans in the pancreas, are destroyed by islet-infiltrating T cells. Identifying the antigens targets of beta-cell reactive T cells is critical to gain insight into the pathogenesis of T1D and develop antigen-specific immunotherapies. Several lines of evidence indicate that insulin is an important target of T cells in T1D. Because many human islet-infiltrating CD4+ T cells recognize C-peptide-derived epitopes, we hypothesized that full-length C-peptide (P31-43), the peptide excised from proinsulin as it is converted to insulin, is a target of CD4+ T cells in people with T1D. CD4+ T cell responses to full-length C-peptide were detected in the blood of: 14 of 23 (>60%) people with recent-onset T1D, 2 of 15 (>13%) people with long-standing T1D, and 1 of 13 (<8%) HLA-matched people without T1D. C-peptide-specific CD4+ T cell clones, isolated from six people with T1D, recognized epitopes from the entire 31 amino acids of C-peptide. Eighty-six percent (19 of 22) of the C-peptide-specific clones were restricted by HLA-DQ8, HLA-DQ2, HLA-DQ8trans, or HLA-DQ2trans, HLA alleles strongly associated with risk of T1D. We also found that full-length C-peptide was a much more potent agonist of some CD4+ T cell clones than an 18mer peptide encompassing the cognate epitope. Collectively, our findings indicate that proinsulin C-peptide is a key target of autoreactive CD4+ T cells in T1D. Hence, full-length C-peptide is a promising candidate for antigen-specific immunotherapy in T1D.

Significance

Type 1 diabetes (T1D) is an incurable autoimmune disease caused by T cell-mediated destruction of insulin-producing beta cells. The beta-cell antigens recognized by human CD4+ T cells in T1D are poorly defined. Here, we show that C-peptide, derived from proinsulin, is recognized by CD4+ T cells from the blood of >60% of people with recent onset T1D. The majority of C-peptide-derived epitopes were recognized when presented by high-risk T1D HLA alleles. For some T cells, full-length C-peptide was a more potent stimulator than shorter peptides. Hence, our data suggests that full-length C-peptide may be uniquely antigenic in human T1D. C-peptide may be useful in assays to monitor changes in T cell autoimmunity and antigen-specific therapies for T1D.


Conflict of interest statement: A provisional patent has been filed by St. Vincent’s Institute to protect the use of full-length C-peptide in T cell assays and antigen-specific therapies (2017904853).

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1To whom correspondence should be addressed. Email: smanering@svi.edu.au.

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identified proinsulin-derived epitopes that bind to HLA-DQ2 and HLA-DQ8trans (14). However, despite the pivotal role HLA-DQ2 and HLA-DQ8 play in susceptibility to T1D, the epitopes presented by these HLA molecules recognized by CD4+ T cells are largely unknown. For example, to our knowledge, no blood-derived HLA-DQ2 (or HLA-DQ2trans) restricted, and only one HLA-DQ8 (or HLA-DQ8trans) restricted, insulin-specific CD4+ T cell clone has been reported (15).

In contrast to peripheral blood, HLA-DQ2/DQ8 restricted CD4+ T cells have been isolated from the residual pancreatic islets of deceased organ donors who suffered from T1D (16, 17). We found that many islet-infiltrating CD4+ T cell clones recognized epitopes derived from proinsulin C-peptide presented by HLA-DQ8 or HLA-DQ8trans (16). More recently, others have identified similar HLA-DQ8 restricted, islet-derived, T cell receptors (17). Although the numbers of donors are still small at this time, 10 of the 12 proinsulin-specific clones analyzed to date recognize epitopes derived from C-peptide (18).

C-peptide is a 31-aa peptide that is excised from proinsulin during the biosynthesis of insulin (19). In light of the evidence pointing to C-peptide–specific T cells playing a role in the immune pathogenesis of human T1D, we asked if C-peptide–specific CD4+ T cells could be detected in the blood of people with and without T1D. If present, we sought to characterize these cells. Our finding is that C-peptide is an important, but previously overlooked, antigen in T1D. This information will further guide the development of effective antigen-specific therapies for T1D and T cell assays for monitoring disease progression.

Results

CD4+ T Cell Responses to C-Peptide Are Detectable in the Blood of People with T1D. Previously, we identified six C-peptide–derived epitopes were recognized by human islet-infiltrating CD4+ T cells (16). To allow responses to all possible C-peptide–derived epitopes to be detected, with a small volume of blood, we used fully processed C-peptide (PI33–63) to stimulate peripheral blood mononuclear cells (PBMCs) and detected CD4+ T cell responses using the sensitive CFSE-based proliferation assay (20). CD4+ T cell responses [cell division index (CDI) ≥ 3.0] to PI33–63 were detected in 14 of 23 subjects (60.8%) with recent-onset diabetes (within 100 d of diagnosis, stage III; ref. 21), 2 of 15 (13.3%) subjects with long-standing diabetes (greater than 100 d of diagnosis), and 1 of 13 (7.7%) healthy subjects (Fig. 1 and SI Appendix, Fig. S1 and Tables S1–S4). Recent-onset vs. healthy P = 0.005; recent-onset vs. long-standing P = 0.016). The magnitude of the CD4+ T cell responses to PI33–63 was significantly greater in PBMCs from individuals with recent-onset T1D compared with healthy controls (10.8 ± 3.9 vs. 1.9 ± 0.25; P = 0.0024) and compared with long-standing subjects (10.8 ± 3.9 vs. 2.3 P = 0.0196). A CDI cutoff of ≥5.0 gave the greatest disease specificity (92.3%) and sensitivity (60.9%) (SI Appendix, Table S5). Insulin VNTR genotype showed no association with CD4+ T cell responses PI33–63 measured by CFSE-based proliferation assay (SI Appendix, Fig. S1). We found no correlation between CFSE responses and insulin dose-adjusted HbA1c (SI Appendix, Fig. S2).

C-Peptide Harbors Many CD4+ T Cell Epitopes. To further characterize the C-peptide–derived epitopes recognized by peripheral blood CD4+ T cells, clones were isolated using our CFSE-based T cell cloning protocol (22). A total of 32 CD4+ T cell clones that responded to PI33–63 were isolated from the peripheral blood of six subjects with recent-onset T1D. Of the 32, 7 could not be grown for analysis. The remaining 25 clones’ epitope specificities were determined using a panel of five overlapping 18mer peptides spanning C-peptide. The epitope mapping for two clones, H11.5 and K9.5, is shown in Fig. 2. Data for all of the other clones is shown in SI Appendix, Fig. S3. Fifteen of the 25 clones (60%), including H11.5 (Fig. 2A and B), responded to one of the five 18mer peptides encompassing the entire C-peptide (Fig. 24 and SI Appendix, Fig. S3). The minimum epitopes required to stimulate these clones were then determined using a panel of peptides sequentially truncated by one amino acid from either the N or C terminus (Fig. 2B and SI Appendix, Fig. S3). Ten C-peptide–specific clones did not respond to any of the five 18mer peptides, including clone K9.5 (Fig. 2C–F). For these clones, the minimum epitope was then determined by first testing against a panel of deletion variants of the full-length C-peptide (Fig. 2 D and E and SI Appendix, Fig. S4). The minimum epitope was identified by testing the clones against a panel of peptides with a single amino acid substitution expected to impair T cell recognition by disrupting either HLA binding or TCR recognition (Fig. 2F and SI Appendix, Fig. S4). Using this approach, the epitope specificities of 7 of these 10 clones were determined, but the specificity of the remaining 3 clones could not be determined. We concluded that clone H11.5 recognized PL42–43 (Fig. 2B) and clone K9.5 recognized PI42–43 (Fig. 2F). In all, we mapped epitopes recognized by 22 CD4+ T cell clones, from six subjects with recent-onset T1D (Table 1).

Most C-Peptide–Specific Clones Are HLA-DQ2, HLA-DQ8, HLA-DQ2trans, and HLA-DQ8trans Restricted. The HLA restriction of the CD4+ T cell responses to proinsulin was determined by inhibiting responses by blocking HLA-DP, HLA-DQ, and HLA-DR, then testing against HLA-transfected cell lines (SI Appendix, Fig. S5). For the C-peptide–specific clones, 19 of 22 (86.3%) clones were HLA-DQ restricted and the remaining 3 (13.6%) were HLA-DR restricted (SI Appendix, Fig. S5). To define the restricting HLA alleles, panels of T2 or BLS lines transduced with individual HLA-DQ or HLA-DR genes, respectively, were used as antigen-presenting cells. Of the 22 clones analyzed: 9 (40.9%) were HLA-DQ8 restricted, 4 (18.2%) were HLA-DQ2 restricted, 3 (13.6%) were HLA-DR4 restricted, and 3 (13.6%) were HLA-DQ8trans restricted (SI Appendix, Figs. S5 and S6). The three remaining clones exhibited promiscuous recognition; one clone (4.5%) responded equally to both HLA-DQ8-trans and HLA-DQ8-trans expressing antigen-presenting cells (APC) (SI Appendix, Fig. S5) and two clones (9.1%) responded to both HLA-DQ2 and HLA-DQ2trans-expressing APC (SI Appendix, Figs. S5 M and N and S6). All three (13.6%) HLA-DR restricted clones were restricted by HLA-DR4; no HLA-DR3 restricted clones were isolated. Hence, with the exception of HLA-DR3, C-peptide–specific clones were restricted by all of the HLA
alleles strongly associated with risk of T1D; notably 86% (19 of 22) of the clones were restricted by HLA-DQ2, HLA-DQ8 and/or their transdimers (Table 1). A comparison of the epitope specificity of islet-infiltrating and PBMC-derived C-peptide–specific clones is shown in SI Appendix, Table S6.

Private TCRs Encode C-Peptide Recognition. To examine the clonal diversity of the peripherally derived C-peptide–specific CD4+ T cells, TCR genes expressed by the clones were sequenced. The clones used a range of TRAV and TRBV genes (SI Appendix, Table S7). TRAV and TRBV genes were sequenced from 21 of the 22 clones for which an epitope and HLA restriction could be determined. Fifteen distinct TRA/TRB combinations were found (SI Appendix, Table S7). There was no evidence of a “public” TCR. Clones from three of the six (50%) subjects expressed identical TCRs (SI Appendix, Table S8). Clones with identical TCRs had matching epitope specificity and HLA restriction, except T6.6 and T17.1. T6.6 was insufficiently sensitive to respond to HLA-DQ2trans, whereas T17.1 responded to both HLA-DQ2 and HLA-DQ2trans.

Full-Length C-Peptide Is a More Potent Antigen for Some T Cells. To determine the relative potency of PI33–63 as an agonist for CD4+ T cells, we compared the potency of full-length (PI33–63, 31 aa) C-peptide to 18mer peptides incorporating the cognate epitope for eight C-peptide–specific clones. In each case, the minimum epitope was flanked by at least three amino acids at both the N and C termini (SI Appendix, Table S9). Six of eight clones were more sensitive to the full-length C-peptide. Remarkably, for five of eight clones, full-length C-peptide was >100- to 1,000-fold more potent than the 18mer peptide incorporating the cognate epitope (Fig. 3 and SI Appendix, Table S9). In contrast, two of the eight clones tested responded equally to both full-length C-peptide and an overlapping 18mer. The clones that were more sensitive to the full-length C-peptide recognized epitopes toward the C terminus of the C-peptide (SI Appendix, Table S10). The enhanced response to full-length C-peptide is not due to protein splicing because amino acid substitution experiments, using a high concentration (50 μM) of 16mer peptides, show that 7–10 contiguous amino acids are required to stimulate the clones (Figs. 2F and 3I and J and SI Appendix, Fig. S4G). We conclude, that for many C-peptide–specific CD4+ T cells, full-length C-peptide is a much stronger agonist than 18mer peptides incorporating the cognate epitope.

To confirm that the C-peptide–specific clones responded to beta cells, six clones were tested for responses against human islet lysates. Five of six clones responded to islet lysates (SI Appendix, Fig. S7) and, in some cases, weakly to acinar tissue.
Table 1. Epitope mapping and HLA restriction analysis of blood-derived C-peptide–specific CD4+ T cells

| Clone | E | A | D | L | Q | V | G | Q | V | L | G | G | P | G | A | S | L | Q | P | L | A | L | E | G | S | L | Q | HLA restriction* |
| B3.1  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | DQ8 |
| B3.3  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | DQ8 |
| K3.2  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | DQ2 |
| K4.4  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | DQ4 |
| K6.2  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8trans |
| K6.4  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| K9.5  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | DQ2 |
| K9.6  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| D1.1  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| D1.4  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| T6.1  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ2/DQ2trans |
| T6.6  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ2 |
| T17.1 | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ2/DQ2trans |
| H3.3  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8trans |
| H3.7  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| H6.4  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8trans |
| H7.4  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| H8.5  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| H11.5 | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| H12.2 | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8/DQ8trans |
| E2.3  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |
| E2.5  | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | DQ8 |

* Nomenclature referenced by abbreviated HLA type is as follows (indicated in parentheses): HLA-DQ8 (A1*03:01, B1*03:02), HLA-DQ2 (A1*05:01, B1*02:01), HLA-DQ8trans (A1*05:01, B1*03:02), HLA-DQ2trans (A1*03:01, B1*02:01), HLA-DR4 (B1*04:01).
that many islet-infiltrating CD4+ T cell clones are restricted by HLA-DQ2/DQ8 (16–18, 23). Broadly, there is overlap between the HLA-DQ2 and HLA-DQ8 restricted clones isolated from islets and peripheral blood (SI Appendix, Table S6) (16, 17), although fine mapping reveals that very few are identical. HLA-DQ8 restricted HIPs also infiltrate human islets (28). Interestingly, some CD4+ T cells that recognize a hybrid insulin peptide epitope formed by the fusion of the central region of C-peptide with a fragment of IAPP2, cross-react with unmodified C-peptide (28). Although the unmodified C-peptide is less potent than the HIP for these clones, this suggests that responses to HIPs and full-length C-peptide may both be HLA-DQ8 restricted and potentially reenforce each other.

The C-terminal half of C-peptide (PI51–68) has been tested in a phase I trial as a candidate for antigen-specific therapy to curb autoimmune CD4+ T cell responses in T1D (37, 38). This peptide, PI51–68, incorporates epitopes recognized by half (11 of 22) of the full-length C-peptide–specific clones described here. Although the PI51–68 peptide was identified in an HLA-DR4 restricted response (26), our data shows that this peptide contains epitopes presented by HLA-DQ2, HLA-DQ2 trans, and HLA-DQ8 in addition to HLA-DR4 when T cells are stimulated with full-length C-peptide. Compared with PI51–68, full-length C-peptide comprises multiple epitopes, stimulates predominantly HLA-DQ2/8 restricted responses, and is a much more potent antigen.

The primary limitation of this work, like all clinical studies, is that we cannot demonstrate that C-peptide–specific CD4+ T cells cause T1D in humans. A direct demonstration of a pathogenic role for CD4+ T cell responses to C-peptide will require a mouse model of the human T cell responses to C-peptide, which is yet to be developed.

There are two clinical applications of our findings: T cell biomarker assays and antigen-specific therapy protocols.
Developing T cell biomarker assays to monitor changes in beta cell-specific T cells in the blood of people with T1D has been an enduring challenge. Based on the results presented here, we suggest that C-peptide could usefully be included in functional T cell assays, such as ELISPOT or CFSE-based proliferation assays. C-peptide can readily be applied to assays, using larger cohorts, to further delineate the temporal dynamics of CD4+ T cell responses to C-peptide in the blood of people developing T1D. Full-length C-peptide warrants further investigation as an antigen in antigen-specific therapies. As noted above, a shorter section of C-peptide has already been evaluated as an antigen-specific therapy, in a phase Ib clinical trial with HLA-DRB1*04:01 subjects (38). PI33 could readily be tested for prevention of T1D because it is relatively stable, nontoxic, and water soluble, and has already been used in clinical trials for diabetes-associated complications (39).

### Materials and Methods

#### Subjects
Studies received local ethical approval (St. Vincent’s Hospital, HREC-A 135/08, (Royal Melbourne Hospital, 2009/026), and (Monash Health, 121858). All participants provided written informed consent. Participants (aged 3-45 y) without T1D, diagnosed with T1D within 100 d (recent-onset), or greater than 100 d (long-standing) were recruited. T1D was diagnosed according to American Diabetes Association criteria.

#### CFSE-Based Proliferation Assay and CD4+ T Cell Cloning
Blood was obtained by venepuncture and PBMC isolated. CFSE-labeled PBMC were cultured with no antigen, PI33-labeled synthetic peptides and the KJ cell line (22). Cloned CD4+ T cells were incubated with synthetic peptides and the KJ cell line (22). Cloned CD4+ T cells (50,000 per well) were cultured with 20,000 APC with and without peptides. Responses to antigen were measured in triplicate as IFN-gamma (IFN-γ) secretion into the culture media, determined by ELISA (BioLegend).

#### Determining the HLA Restriction of T Cell Clones
The HLA restriction of the C-peptide-specific CD4+ T cell clones was determined by mAb blocking and transfected cell assays (22, 31).

#### Sequencing TCR Genes Expressed by C-Peptide–Specific Clones
Reverse transcription mix, SuperScript VILO Reverse Transcriptase (Invitrogen), was dispensed directly into wells containing five cells. TRA and TRB genes were amplified using pools of forward primers and Taq polymerase (Qiagen) as described by ref. 40.