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**Recognition of mRNA splice variant and secretory granule epitopes by CD4+ T cells in
type 1 diabetes**

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Running Title: Unconventional CD4+ T cell epitopes in diabetes

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Abstract

A recent discovery effort identified novel splice variant and secretory granule antigens within the HLA class I peptidome of human islets and documented their recognition by CD8+ T cells from peripheral blood and human islets. In the present study, we applied a systematic discovery process to identify novel CD4+ T cell epitopes derived from these candidate antigens. We predicted 145 potential epitopes spanning unique splice junctions and within conventional secretory granule antigens and measured their in vitro binding to DRB1*04:01. We generated HLA class II tetramers for the 35 peptides with detectable binding and used these to assess immunogenicity and isolate T cell clones. Tetramers corresponding to peptides with verified immunogenicity were then used to label T cells specific for these putative epitopes in peripheral blood. T cells that recognize distinct epitopes derived from a cyclin I splice variant, neuroendocrine convertase 2, and urocortin-3 were detected at frequencies that were similar to an immunodominant proinsulin epitope. Cells specific for these novel epitopes predominantly exhibited a Th1-like surface phenotype. Among the three epitopes, responses to the cyclin I peptide exhibited a distinct memory profile. Responses to neuroendocrine convertase 2 were detected among pancreatic infiltrating T cells. These results further establish the contribution of unconventional antigens to the loss of tolerance in autoimmune diabetes.

Introduction

Type 1 diabetes is a chronic disease in which pancreatic β -cells are selectively destroyed through autoimmune attack. Although CD8⁺ T cells dominate the pancreatic immune infiltrates (1), the strong genetic association of type 1 diabetes with susceptible HLA class II alleles and the diagnostic importance of islet-specific antibodies both point to a significant role for CD4⁺ T cell responses in this disease (2-4). Numerous studies have sought to define the antigens and epitopes recognized by autoreactive T cells in subjects with type 1 diabetes (5). In general, published studies of CD4⁺ and CD8⁺ T cell responses have focused on a limited number of “classical” islet antigens, such as preproinsulin (PPI), glutamic acid decarboxylase (GAD65), islet antigen (IA)-2, and zinc transporter 8 (ZnT8), all of which are known to be targeted by autoantibodies. Other work has identified CD4⁺ T cell epitopes within additional islet-associated proteins that are not standard antibody targets, such as islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and islet amyloid polypeptide (IAPP) (6,7). T cells that recognize epitopes derived from all of these antigens have been detected in the peripheral blood of subjects with type 1 diabetes using HLA class II tetramers (8) and, in some cases, those specificities have been observed among islet-infiltrating T cells (9,10). However, questions remain about the importance and relative immunodominance of this wide array of conventional epitopes. Furthermore, the epitope specificity for the majority of islet-infiltrating T cells which have been catalogued remains unknown (9), leaving open the possibility that additional disease-relevant antigens are yet to be discovered.

It is increasingly appreciated that, as part of the disease process, pancreatic β -cells undergo inflammatory and metabolic insults, which trigger endoplasmic reticulum (ER) stress in β -cells (11,12). These insults have been shown to promote the formation of neoepitopes, including

enzymatically-modified antigens (7,13,14), oxidized antigens (15,16), hybrid peptide sequences (17), and novel polypeptides formed through translation of alternative open reading frames (18). The formation of such neoepitopes has the potential to increase immune recognition of β -cells, eliciting additional inflammation and creating a feed forward loop of dysfunction and destruction (19). Furthermore, it was recently shown that alternative mRNA splicing represents an additional pathway for generating novel antigenic sequences that are under-represented in the thymus and regarded as non-self. The idea that neoepitope-specific T cells more readily escape negative selection remains an open question, but a recent study demonstrated that a T cell receptor that recognizes a deamidated GAD65 peptide was more readily selected than a T cell receptor that recognizes the corresponding wild type peptide (20). Recent work demonstrates that a distinct alternative mRNA splicing signature can be induced in β -cells by inflammatory stress (21). Combining a rich HLA class I peptidomics dataset obtained from the ECN90 β -cell line with RNA sequencing (RNA-seq) data from primary human islets (either untreated or exposed to inflammatory cytokines) led to the identification of tissue-specific mRNA splice variants and other novel secretory granule antigens that are present within human islets (22,23). CD8⁺ T cells that recognize unique peptide sequences from these proteins were detectable within peripheral blood and pancreatic infiltrates by tetramer staining. Importantly, pancreas-infiltrating T cells reactive to three of these novel epitopes were observed to be enriched in individuals with type 1 diabetes (23). CD8⁺ T cells reactive to the murine orthologues of granule antigens were also observed in the islet infiltrates of prediabetic NOD mice and were diabetogenic upon in vivo transfer into NOD.*scid* mice, with a potency equivalent to that of PPI-reactive CD8⁺ T cells.

In light of these compelling findings, we sought to investigate CD4⁺ T cell responses directed against unique splice variant and secretory granule protein-derived peptides. We screened a library

of peptides to identify novel CD4⁺ T cell epitopes restricted by the prevalent type 1 diabetes-susceptible HLA-DRB1*04:01 allele. We then applied a systematic approach to identify novel immunogenic peptides. We next used the corresponding HLA class II tetramers to evaluate the frequency and phenotype of T cells reactive to these novel epitopes in the peripheral blood of subjects with type 1 diabetes and HLA-matched controls. Collectively, our work supports the relevance of these novel antigens and epitopes in HLA-DRB1*04:01⁺ subjects with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Human Subjects

The study was approved by Benaroya Research Institute's Institutional Review Board (Protocol # IRB07109). All samples were obtained under approved research protocols and with written informed consent. For epitope-defining studies, peripheral blood was collected from 15 individuals with type 1 diabetes (Supplemental Table 1), immediately processed, and used for in vitro T cell assays. Peripheral blood was also collected and cryopreserved from 10 additional individuals with type 1 diabetes (Supplemental Table 2) and 8 age-matched healthy controls (Supplemental Table 3). Cryopreserved samples were thawed in batches to perform direct tetramer staining. All subjects carried at least one HLA-DRB1*04:01 allele. Subject characteristics are summarized in Supplemental Tables 1, 2, and 3. Live pancreas slices from Network for Pancreatic Organ Donors with Diabetes (nPOD) donor 6472 (Supplemental Table 4) were distributed through the nPOD Slice Program under approved research protocols after obtaining written consent for research from organ donor families.

Epitope Prediction and Peptide Synthesis

Putative epitopes from splice variant junction regions and secretory granule proteins were selected using a prediction algorithm as previously described (24,25). Briefly, motif scores were calculated by multiplying coefficients corresponding to each anchor residue for all possible core 9-mers within each unique junction region and peptides spanning each secretory granule protein of interest. All peptides with plausible binding motifs were synthesized (Sigma-Aldrich, St. Louis, MO). A more comprehensive peptide set was synthesized for the secretory granule protein Proprotein Convertase Subtilisin/Kexin Type 2 (PCSK2). As summarized in Supplemental Table 5, a total of 145 peptides were synthesized. All peptides were dissolved in DMSO to a stock concentration of 20 mg/ml and used for binding assays. Those with detectable binding were subsequently used for tetramer production and T cell studies.

Peptide Binding to HLA-DRB1*04:01

Binding was assessed through a competition assay, as previously described (26). Briefly, increasing concentrations of each peptide were incubated in competition with a biotinylated reference influenza hemagglutinin peptide (HA306-318) at 0.02 μ M in wells coated with DR0401 protein. After washing, residual biotin-HA306-318 was detected using europium-conjugated streptavidin (Perkin Elmer, Waltham, MA) and quantified using a Victor2D time resolved fluorometer (Perkin Elmer). Curves were simulated using Prism software (Version 7.0, GraphPad Software Inc., San Diego, CA) and IC50 values calculated as the concentration needed to displace 50% of the reference peptide.

Preparation of HLA Class II Protein and Tetramer Reagents

DR0401 protein was purified from insect cell cultures as previously described (27,28). Monomers were loaded with 0.2 mg/ml peptide at 37°C for 72 h in the presence of 0.2 mg/ml n-Dodecyl- β -maltoside and 1 mM Pefabloc (Sigma-Aldrich, St. Louis, MO). Individual peptide-loaded monomers for all candidate epitopes were conjugated into tetramers using R-PE streptavidin (Invitrogen, Waltham, MA) at a molar ratio of 8:1 and used to stain *in vitro* expanded T cells. For direct *ex vivo* staining, R-PE streptavidin conjugated CCNI-008₁₉₋₃₇ (CCNI-008 p4, HTATPLDFLHIMDSSQLIH), PE-CF594 streptavidin (BD) conjugated PCSK2₂₄₃₋₂₅₇ (PCSK2 p23, QPFMTDIIIEASSISH), PE-Cy5 streptavidin (BD) conjugated UCN3₅₈₋₇₂ (UCN3 p5, SFHYLRSRDASSGEE), PE-Cy7 streptavidin (SouthernBiotech, Birmingham, AL) conjugated PPI₇₆₋₉₀ (SLQPLALEGSLQKRG), and BV421 streptavidin (BioLegend, San Diego, CA) conjugated influenza MP₉₇₋₁₁₆ (MP p54, VKLYRKLKREITFHGAKEIS) labeled tetramers were prepared to provide multi-color, single tube tetramer staining.

In vitro Tetramer Assays and T Cell Clone Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll underlay, counted, and either frozen in 7% DMSO and subsequently re-thawed or immediately used for assays. PBMCs were suspended in T cell medium (RPMI, 10% pooled human serum, 1% Penicillin-Streptomycin, 1% L-glutamine) at 4×10^6 cells/mL and stimulated with peptides (20 μ g/mL total) in 48-well plates for 14 days, adding medium and IL-2 starting on day 7. Cells were stained with individual PE-labeled tetramers for 75 min at 37°C, followed by CD3 BV510 (BioLegend), CD4 BUV395 (BD Biosciences, Franklin Lakes, NJ), CD25 FITC (BioLegend), and a combination of CD14 PerCP-Cy5.5 (ThermoFisher, Waltham, MA), CD19 PerCP-Cy5.5 (BioLegend) and Viaprobe (BD Biosciences) for 15 min at 4°C, run on an LSR II (BD), and analyzed using FlowJo (Tree Star,

Inc., Ashland, OR). Clones were isolated by sorting single tetramer-positive CD4⁺ T cells using a FACSAria (BD) and expanded in 96-well plates in the presence of 1×10⁵ irradiated PBMCs, 2 µg/mL phytohaemagglutinin (PHA; Remel Inc., Lenexa, KS), adding media and IL-2 starting on day 10.

T Cell Clone Maintenance and Characterization

Clones specific for each immunogenic peptide were maintained in RPMI supplemented with 10% human serum and re-stimulated as needed using 2 µg/mL PHA. To assess specificity, T cell clones were re-stained with HLA class II tetramers or assayed to assess peptide-specific proliferation as follows: 10⁴ T cells/well were plated with 1×10⁵ irradiated DR0401+ PBMCs and stimulated in triplicate with 10 µg/mL of peptide. After incubating for 48 h at 37°C and pulsing with medium containing ³[H]-thymidine (1 µCi/well), incorporation was measured 18 h later with a scintillation counter.

Ex vivo Tetramer Staining

Ex vivo tetramer staining and enrichment was accomplished using previously published protocols (29,30). A total of 20 million PBMCs were thawed and treated with Dasatinib for 10 min at 37°C. PBMCs were then stained with 4.5 µl of CCNI-PE, 8 µl PCSK2-PE-CF594, 4.5 µl UCN3-PE-Cy5, 4.5 µl PPI₇₆₋₉₀-PE-Cy7, and 4.5 µl Influenza MP₉₇₋₁₁₆-BV421-labeled tetramers at room temperature for 90 min. Cells were washed and enriched as previously described (29,30), by incubating with anti-PE and anti-Myc magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany; a Myc tag was incorporated in the Influenza MP₉₇₋₁₁₆ tetramer) at 4°C for 20 min, washed again, and a 1/100th fraction was saved for antibody staining (“Pre”). The other fraction

was passed through a MS magnetic column (Miltenyi Biotec). Bound, PE, PE-Cy5, PE-CF594, PE-Cy7 or BV421-labeled cells were flushed and collected. Both enriched (“Post”) and non-enriched (“Pre”) fractions were labeled with Sytox green, anti-CD4-V500 (BD), anti-CD45RA-AF700 (BD), anti-CXCR3-BV785 (BioLegend), anti-CCR4-BV605 (BioLegend), anti-CCR7-BV650 (BD), anti-CD14-FITC (BioLegend), anti-CD19-FITC (BioLegend). Samples were run on a BD LSRII flow cytometer, and data was analyzed using FlowJo software version 10. Single T cells (defined as a Sytox negative and CD4⁺/CD14⁻/CD19⁻) were gated to exclude cells that were positive for more than one tetramer fluorophore. Each tetramer positive T-cell population was then gated to determine the number of positive events and analyzed for surface receptor expression. The frequency (F) of epitope-specific T cells per million CD4⁺ T cells was calculated as follows: $F = (1,000,000 \times \text{tetramer-positive events from enriched tube}) / (100 \times \text{number of CD4}^+ \text{ T cells from the "Pre" fraction})$. T-cell lineages were first assigned based on CD45RA and CCR7 expression: naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻), and terminal effectors (CD45RA⁺CCR7⁻). The memory cell populations were pooled and subdivided into Th1-like (CXCR3⁺, CCR4⁻ and CCR6⁻), Th2-like (CXCR3⁻, CCR4⁺ and CCR6⁻), Th17-like (CXCR3⁻, CCR4⁺ and CCR6⁺), Th1/17-like (CXCR3⁺, CCR4⁻ and CCR6⁺), Th1/2-like (CXCR3⁺, CCR4⁺ and CCR6⁻), or Th1*-like (CXCR3⁺, CCR4⁺ and CCR6⁺) lineages (31-33).

Islet-derived T-Cell Assays

Cell lines from islet-infiltrating T cells were recovered and expanded from nPOD donor 6472 (see Supplemental Table 4). To generate lines, 150 µm live slices of pancreas were digested with 1 mg/ml collagenase-P (Sigma-Aldrich, St. Louis, MO) in HBSS with Mg²⁺/Ca²⁺ (Thermo Fisher,

Waltham, MA) for 10 min in a 37°C water bath with vigorous shaking. Digestion was confirmed by visual inspection. Digested tissue was washed and placed in medium (AIM-V supplemented with 2 mmol/L l-glutamine, 5 mmol/L HEPES, 100 units/mL penicillin, and 100 µg/mL streptomycin, 0.1 mmol/L of each nonessential amino acid, 1 mmol/L sodium pyruvate [all from Lonza, Walkersville, MD] with 5% heat-inactivated human male AB serum [Access Biologicals, Vista, CA]). Individual islets were hand-picked and placed in culture with irradiated PBMCs as feeders, a blocking anti-Fas antibody (1 µg/mL; eBiosciences, now Applied Biosystems, Santa Clara, CA); anti-PD-1 antibody (1 µg/mL; BD Biosciences), and mifepristone (100 nmol/L; Invitrogen). After 12 days of culture, T cell lines were collected, further expanded in culture and then phenotyped by flow cytometry for T cell subsets. B-LCL were generated from autologous splenocytes. Responsiveness was assessed as previously described (17). Briefly, irradiated autologous B-LCL (generated as previously described (9)), were plated (20,000/well) after pulsing with peptides at 50 µg/ml, no peptide, or DMSO volume control and then cultured with T cells (50,000/well) and anti-CD28 (5 µg/ml) for co-stimulation. After 48 h of co-culture, responses were detected by IFN-γ ELISA. The islet T cell line previously reactive with PCSK2 p23 peptide was restimulated with autologous B-LCL pulsed with the peptide and expanded in culture. When resting, the islet T cell line (30,000 viable cells per well) was challenged with autologous B-LCL pulsed with individual peptides and tested for cytokine secretion by IFN-γ immunospot (all reagents from C.T.L) after 24 h of co-culture.

Statistics

All statistical analysis was performed using GraphPad Prism 9.2.0. The tests used (as appropriate) included unpaired t-tests, Wilcoxon signed ranked test, Mann-Whitney tests, and Kruskal Wallis

test with Dunn's multiple comparison test. *P* values below 0.05 were considered significant. Marginal *P* values at or below 0.1 are also provided, when applicable. Biological sex was considered as a variable, but no significant associations were observed.

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Identification of Immunogenic Splice Variant and Secretory Granule Peptides

Through combined human islet transcriptomics and β -cell HLA peptidomics datasets, we previously identified a set of candidate antigens consisting of ten mRNA splice antigens (cyclin I splice variant CCNI-008, glutamate decarboxylase 2 splice variant GAD2-003, Guanine Nucleotide binding protein variants GNAS-002 and GNAS-036, islet amyloid polypeptide variant IAPP-002, Protein Tyrosine Phosphatase Receptor Type N variant PTPRN-021, receptor-type tyrosine-protein phosphatase N2 variant PTPRN2-005, RNA Exonuclease 2 variant REXO2-020, Secretogranin-5 variant SCG5-009, and Solute Carrier Family 30 Member 8 variant SLC30A8-002), peptide splice antigens (an IAPP/IAPP *cis*-spliced antigen and a neuropeptide Y (NPY)/SCG5 *trans*-spliced antigen), and three conventional secretory granule proteins (SCG5, Urocortin-3 (UCN3), and PCSK2) (23). To facilitate efficient screening of the large number of possible peptides from these candidate proteins, we applied a previously described algorithm to identify peptides with predicted HLA-DRB1*04:01 binding motifs (24,25). To predict possible motifs, we considered each unique splice junction within CCNI-008, GAD2-003, GNAS-002,

GNAS-036, IAPP-002, PTPRN-021, PTPRN2-005, REXO2-020, SCG5-009, SLC30A8-002 and the full sequences of SCG5, UCN3, PCSK2, *cis*-spliced IAPP/IAPP, and *trans*-spliced neuropeptide Y (NPY)/SCG5 (also including n-terminal and c-terminal flanking residues to generate a minimum peptide length of 15). We identified a total of 145 candidate peptides (Supplemental Table 3). These were synthesized and tested for their ability to bind HLA-DRB1*04:01. In total, 35 peptides had detectable binding to HLA-DRB1*04:01 (Supplemental Table 3). The reduced fraction of peptides with confirmed binding was expected, because we chose a lower cutoff value than our previous study (34). For these 35 peptides, we next assessed their immunogenicity by stimulating CD4⁺ T cells from PBMCs of HLA-DRB1*04:01+ subjects with type 1 diabetes and staining with each of the corresponding HLA class II tetramers after 2 weeks of *in vitro* expansion. Among the peptides tested, CCNI-008₁₉₋₃₇ (CCNI), PCSK2₂₄₃₋₂₅₇ (PCSK2), and UCN3₅₈₋₇₂ (UCN3) were determined to be immunogenic, based on their ability to elicit a detectable population of tetramer+ CD4⁺ T cells after expanding T cells from subjects with type 1 diabetes (Fig. 1A). Performing T cell expansions in a total of 15 donors with type 1 diabetes (Supplemental Table 1), 5 showed positive staining for CCNI, 4 showed positive staining for PCSK2, and 8 showed positive staining for UCN3. In addition, we were able to isolate T cell clones for these epitopes (by sorting and expanding single tetramer-positive T cells), which proliferated robustly in response to their cognate peptide but not to an irrelevant peptide (Fig. 1B). The sequences and predicted minimal motifs for these peptides are summarized in Table 1. Among these three peptides, the CCNI splice variant peptide is a neoepitope with a sequence that differs substantially from the corresponding conventional CCNI sequence (sharing only 57.9% identity) resulting in a distinct predicted binding register (Table 2). The PCSK2 and UCN3 peptides are conventional epitopes. The remaining 32 peptides were not advanced for further study either

because they failed to elicit measurable responses in more than one subject (29 peptides) or because the tetramer staining pattern was atypical and could not be confirmed by isolating a peptide responsive T cell clone (3 peptides).

Novel Epitope-specific CD4⁺ T Cells are Detectable in Subjects with Diabetes

We next asked whether T cells that recognize these three novel epitopes are detectable in the peripheral blood of individuals with type 1 diabetes (Supplemental Table 1). We directly assessed the frequency and surface phenotype of epitope-specific T cells using a multiplex HLA class II tetramer staining approach that allows *ex vivo* enrichment and detection of multiple tetramer specificities in a single peripheral blood sample and co-staining with cell surface marker antibodies (10). The flow cytometry panel included tetramers corresponding to three novel immunogenic peptides: CCNI, PCSK2, and UCN3 plus a PPI₇₆₋₉₀ (PPI) reference tetramer that was previously reported as an immunodominant self-epitope (8). Each of the three novel tetramers was effective at labeling a distinct population of T cells (Fig. 2A shows a positive staining result for each tetramer), albeit with a lower fluorescence intensity for CCNI. Staining above the limit of detection (previously reported as 1 cell per million for direct HLA class II tetramer staining (35)) was observed in 9/10 subjects for CCNI, 6/10 subjects for PCSK2, 10/10 subjects for UCN3, and 10/10 subjects for PPI (Fig. 2B). Individual subjects had variable frequencies of T cells specific for these epitopes (Fig. 2C). For example, subjects T1D #1 and #6 had comparatively high frequencies of PPI- and PCSK2-specific T cells and lower frequencies of CCNI- and UCN3-specific T cells, whereas T1D #3 had high frequencies of CCNI- and PCSK2-specific T cells and lower frequencies of UCN3- and PPI-specific T cells.

T Cells Specific for Novel Epitopes have Distinct Phenotypes

To draw inferences about T cell lineages, we utilized cell surface marker expression to characterize tetramer+ T cells that recognized these novel specificities. The cell surface markers present in our panel included CD45RA and CCR7, which we utilized to classify T cells as naïve, central memory (T_{CM}), effector memory (T_{EM}), and terminal effectors (T_{EMRA}) (Fig. 3A). To draw inferences about the cell subset distribution for T cells that recognized these novel specificities, we utilized CXCR3, CCR4, CXCR5 and CCR6 expression to define T helper subsets using established lineage definitions: Th1-like (CXCR3+, CCR4- and CCR6-), Th2-like (CXCR3-, CCR4+ and CCR6-), Th17-like (CXCR3-, CCR4+ and CCR6+), Th1/17-like (CXCR3+, CCR4- and CCR6+), Th1/2-like (CXCR3+, CCR4+ and CCR6-), or Th1*-like (CXCR3+, CCR4+ and CCR6+) lineages (31-33) (Fig. 3B). For each novel epitope and PPI, approximately half of the T cells exhibited an apparently naïve phenotype. We observed apparent differences in the memory distribution of CCNI-specific T cells as compared to PCSK2, UCN3, and PPI. In particular, CCNI had a distinct memory profile, with a significantly lower proportion of naïve cells than UCN3 and that trended lower than PCSK2 (Fig. 3C). Subset analysis suggested that CCNI-, PCSK2-, and PPI-specific memory T cells all exhibited a predominantly Th1-like surface phenotype. In contrast UCN3 had a distinct cell subset distribution, with a significantly lower proportion of Th1-like cells than CCNI ($P = 0.04$) and proportion of Th2-like cells that was significantly higher than PCSK2 ($P = 0.03$) and trended higher than CCNI ($P = 0.1$) (Fig. 3C). These results suggest that UCN3-specific T cells may exhibit a different functional phenotype than CCNI-, PCSK2-, and PPI-specific T cells.

T Cells Specific for Novel Epitopes are Present at Higher Frequencies in Subjects with Diabetes

To further assess the relevance of T cells that recognize these novel epitopes, we assessed their frequency in the peripheral blood of age- and HLA-matched controls (demographics in Supplemental Table 2). As expected, some T cells that recognized these epitopes were present in controls. However, the combined frequency of novel epitope reactive T cells in subjects with type 1 diabetes was significantly higher than in controls ($P = 0.0003$) (Fig. 4A). Considering T cell frequencies for individual epitopes, frequencies trended toward being higher in subjects with type 1 diabetes than in controls for PCSK2 and UCN3 (Fig. 4B), but differences only reached statistical significance for CCNI ($P = 0.02$, Šídák's multiple comparisons test). Utilizing CD45RA and CCR7, we classified novel epitope reactive T cells as naïve or memory and compared the ratio (the combined percentage of all memory subsets divided by the percentage that were naïve) in subjects with type 1 diabetes and controls (Fig. 4C). Although subjects with type 1 diabetes showed a very modest trend toward having a higher proportion of epitope-specific memory T cells, there was no significant difference ($P = 0.565$). We next utilized CXCR3, CCR4, and CCD6 to compare the surface phenotype of novel epitope reactive T cells in subjects with type 1 diabetes and controls (Fig. 4D). Unlike subjects with type 1 diabetes, the predominant phenotype in healthy controls was Th2-like (CCR4 single positive). Thus, subjects with established type 1 diabetes had significantly greater numbers of novel epitope reactive CD4⁺ T cells and controls lacked the Th1-like bias seen for some specificities in subjects with type 1 diabetes.

Cells that Recognize Novel Epitopes are Present among Islet Infiltrating T Cells

To further support the relevance of these novel epitopes, we next evaluated whether their cognate T cells are present within human islets by probing the specificity of T cell lines from nPOD donor 6472 (Supplemental Table 4). Multiple T cell lines were expanded as delineated in the Methods

section. We tested the reactivity of these lines toward CCNI-008, PCSK2, and UCN3 by assaying for IFN- γ secretion stimulated by each peptide. Islet-derived CD4⁺ T cell line AG (isolated and expanded from nPOD donor 6472) recognized PCSK2 in the context of autologous splenocyte-derived B-LCL by IFN- γ secretion (Fig. 5A) and by ELISpot (Fig. 5B). The donor was positive for HLA-DRB1*04:04 but not HLA-DRB1*04:01, indicating that PCSK2 can apparently be presented by both of these HLA-DR4 subtypes. These observations establish that CD4⁺ T cells that recognize PCSK2 are present among the islet-infiltrating T cells of this subject, further supporting the relevance of these newly discovered epitopes to disease.

DISCUSSION

During the development of type 1 diabetes, there is a failure of self-tolerance that is most clearly manifested by the appearance of islet autoantibodies, but is also accompanied by loss of T cell tolerance to insulin and other islet-specific antigens (8,36-39). The sequential acquisition of additional islet autoantibodies suggests mounting waves of autoimmune attack that encompass new antigen specificities (36,40). As a consequence of innate and adaptive immune activation and subsequent metabolic dysregulation, pancreatic β -cells experience diverse stressors (41-45). These have been shown to promote the formation of neoepitopes through enzymatic and non-enzymatic post-translational modification of proteins and defective ribosomal initiation (7,13,16,18,45-47). Such neoepitopes are thought to represent a profound challenge to central and peripheral tolerance mechanisms (19,48). Indeed, T cells that recognize citrullinated, deamidated, and hybrid peptide neoepitopes are present at higher frequencies in subjects with type 1 diabetes and are also present among islet-infiltrating T cells and in pancreatic lymph nodes in this setting (7,9,10,17,49).

The induction of altered transcripts and splice variants leads to the generation of neoepitopes (and epitopes from previously understudied secretory granule proteins) that were previously shown to be recognized by CD8⁺ T cells (22,23). In this study, we sought to identify novel epitopes derived from those secretory granule proteins and stress-associated splice variant proteins and to explore CD4⁺ T cell responses directed against these epitopes. Using a systematic approach, we predicted peptides likely to bind to DR0401, assessed their *in vitro* binding and immunogenicity, and verified their validity by isolating peptide responsive T cell clones from subjects with type 1 diabetes. These efforts led us to identify three novel epitopes. One of these epitopes spanned a unique junction from cyclin I splice variant CCNI-008. The other epitopes originated from PCSK2 and UCN3, which are both conventional secretory granule proteins, but which have not previously been appreciated as CD4⁺ T cell targets. Previously identified CD8⁺ T cell epitopes from these antigens were restricted by HLA-A2 (CCNI-008₁₄₋₂₂, PCSK2₃₀₋₃₈ and UCN3₁₋₉) (22) and HLA-A3 (UCN3₄₆₋₅₆) (23), and, for the murine orthologs *Pcsk2* and *Ucn3* (also restricted by K^d) in the islet-infiltrating T cells of prediabetic NOD mice (23) (mRNA splice epitopes were not investigated in mice, as splicing rules display significant inter-species differences). The novel CCNI-008 epitope that was recognized by CD4⁺ T cells (CCNI-008₁₉₋₃₇) overlaps with the CD8⁺ T cell epitope CCNI-008₁₄₋₂₂, raising the possibility of linked recognition. The novel PCSK2 and UCN3 CD4⁺ T cell epitopes (PCSK2₂₄₃₋₂₅₇ UCN3₅₈₋₇₂) did not overlap with the previous CD8⁺ T cell epitopes.

Through direct analysis with HLA class II tetramers, we observed detectable populations of T cells that recognized these peptides in the peripheral blood of subjects with type 1 diabetes that, in aggregate, were present at significantly higher frequencies than in HLA-matched controls. Considering the individual epitopes separately, only CCNI-specific T cells were present at

significantly higher frequencies. Notably, T cells specific for this splice variant-derived CCNI peptide had the highest overall frequencies and exhibited a distinct memory profile (increased proportions of T_{CM} and T_{EM}) compared with the other specificities examined. As a whole, these observations suggest that T cells that recognize this splice variant-derived epitope might be more effectively primed and expanded than T cells that recognize the epitopes derived from conventional secretory granule antigens. Notably, T cells that recognize the PCSK2 epitope were present among the islet-infiltrating T cells of an nPOD donor, supporting the relevance of this specificity. In accord with this, CCNI- and PCSK2-specific T cells predominantly exhibited a Th1-like phenotype whereas a significant proportion of T cells specific for the UCN3 epitope exhibited a Th2-like surface phenotype. Based on the paradigm that Th1 cells have a key role in the pathogenesis of type 1 diabetes, as supported by the foundational work of Katz et al. (50), this observation suggests a pathogenic role for these cells in type 1 diabetes.

T cells that recognize these novel epitopes were also present in HLA-matched control subjects, albeit at lower frequencies and lacking the Th1-like phenotype that was predominant in subjects with type 1 diabetes. In line with previous reports on islet-reactive CD8⁺ T cells (22,23,51), a notable proportion of these novel autoreactive CD4⁺ T cells were naïve in peripheral blood, supporting the possibility that the fraction actively engaged in the autoimmune process may be preferentially sequestered in the pancreas target organ (52,53). These observations indicate incomplete tolerance, consistent with other studies suggesting that having a disease-susceptible HLA is sufficient to cause the selection of a potentially autoreactive T cell repertoire (51,54,55). The observation that self-reactive T cells were not completely limited to a naïve phenotype in healthy donors indicates that limited T cell expansion can occur in the absence of autoimmune disease. This is in accord with the concept of ‘benign’ islet autoimmunity, which postulates that

self-reactive T cells are present in all individuals and that contributing behavior on the part of pancreatic β -cells is required to potentiate disease progression (52,53).

Our study does have limitations. Notably, although we observed diverse frequencies of T cells specific for novel T cell epitopes, our study was not adequately powered to correlate these differences with clinical parameters. Furthermore, our study was limited to peptides with predicted binding to DR0401 and derived from targets that were selected based on HLA class I peptidome data. Although the known β -cell antigens targeted by CD4⁺ and CD8⁺ T cells have a high degree of overlap (5), the approach we followed would omit any self-antigens that are uniquely presented to CD4⁺ T cells. In addition, given that HLA binding predictions are imperfect, there could be epitopes present among the peptides we chose not to synthesize. Since our methods relied on HLA tetramer staining, which preferentially detects high affinity T cell receptors, it is possible that there are additional low affinity specificities that we failed to discover. Also, it is likely that additional epitopes derived from the unique splice variants and secretory granule proteins that we studied are recognized in human subjects with type 1 diabetes in the context of other disease-associated HLA molecules, such as DQ0302 (DQ8). We have developed a method for predicting DQ8-binding peptides, which we plan to utilize in our ongoing studies.

Collectively, our findings introduce UCN3 as a potentially relevant CD4⁺ T cell antigen, and strongly suggest that the CCNI splice variant and PCSK2 are important T cell targets in type 1 diabetes. Among these, CCNI-specific T cells appear to be more extensively expanded in the circulation of subjects with type 1 diabetes. We can speculate that pathogenic CD4⁺ T cells that recognize this tissue-specific splice junction epitope may not be effectively controlled by central and peripheral tolerance mechanisms. Our data support the hypothesis that stress-related changes

that occur in human β -cells can generate neoepitopes, eliciting inflammatory T cell responses, which amplify autoimmunity and promote the progression of type 1 diabetes.

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Author Contributions. EAJ and RM conceptualized and designed the study. RM and DE identified the sequences of candidate splice variant junctions and secretory granule proteins of interest. SL and CS were responsible for subject recruitment, sample collection, and clinical data collection. DAL and PG performed peptide binding assays and T cell studies. AM and SCK performed islet-derived T cell experiments. EAJ and PG wrote the manuscript with assistance from all co-authors. EAJ obtained funding and was responsible for the entire project. EAJ is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Sequences and binding affinities of splice variant and secretory granule epitopes

Peptide	Amino acid sequence^a	Protein Source	IC50^b (μM)	Response Rate^c
CCNI-008 ₁₉₋₃₇	HTATPL DFLHIMDSSQLIH	cyclin I splice variant	7.2	90%
PCSK2 ₂₄₃₋₂₅₇	QPFMT DIEASSISH	Neuroendocrine convertase 2	0.07	60%
UCN3 ₅₈₋₇₂	SFHYLRSDASSGEE	Urocortin-3	0.94	100%

^aThe best predicted minimal epitope is shown in boldface. Secondary motifs are underlined. ^bIC50 represents the peptide concentration that displaces half of the reference peptide. ^cEx vivo responses were examined in a total of 10 subjects with established type 1 diabetes. The response rate (percentage of subjects with a detectable population of tetramer positive T cells) is listed for each novel epitope.

Table 2. Sequence comparison of splice variant and non-variant CCNI

Peptide	Amino acid sequence^a
CCNI-008 ₁₉₋₃₇	HTATPL<u>DFLHIMDSS</u>QLIH
CCNI ₁₄₃₋₁₆₁	HTATPL<u>DFLHIFHAI</u>AVST

^aThe best predicted minimal epitope is underlined. Conserved amino acids are in boldface.

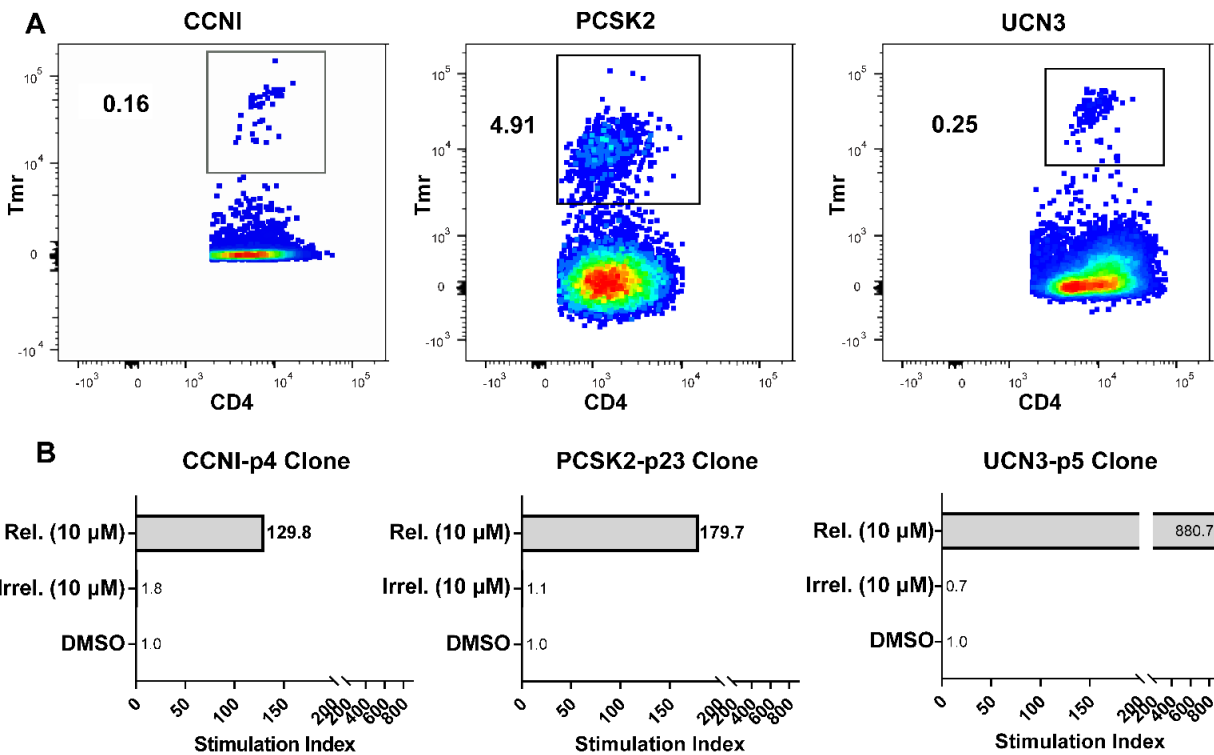


Figure 1 – Recognition of novel epitopes by T cells from subjects with type 1 diabetes. *A*: T cells that recognize epitopes derived from splice variants and novel secretory granule antigens could be expanded from peripheral blood after peptide specific expansion and detected by HLA class II tetramer staining. Representative results from one individual donor per epitope are shown (out of 15 subjects with T1D, Supplemental Table 1) *B*: Epitope-specific T cell clones were isolated by sorting and expanding single tetramer-positive T cells. Each clone proliferated robustly (stimulation index, SI > 100) in response to 10 µg/mL of its cognate peptide and exhibited negligible proliferation (SI < 2) in response to 10 µg/mL of an irrelevant peptide. Data are represented as SI values, calculated by normalizing the proliferation of each clone based on [³H] thymidine incorporation of unstimulated wells. Tmr, Tetramer; Rel., Relevant; Irrel., Irrelevant.

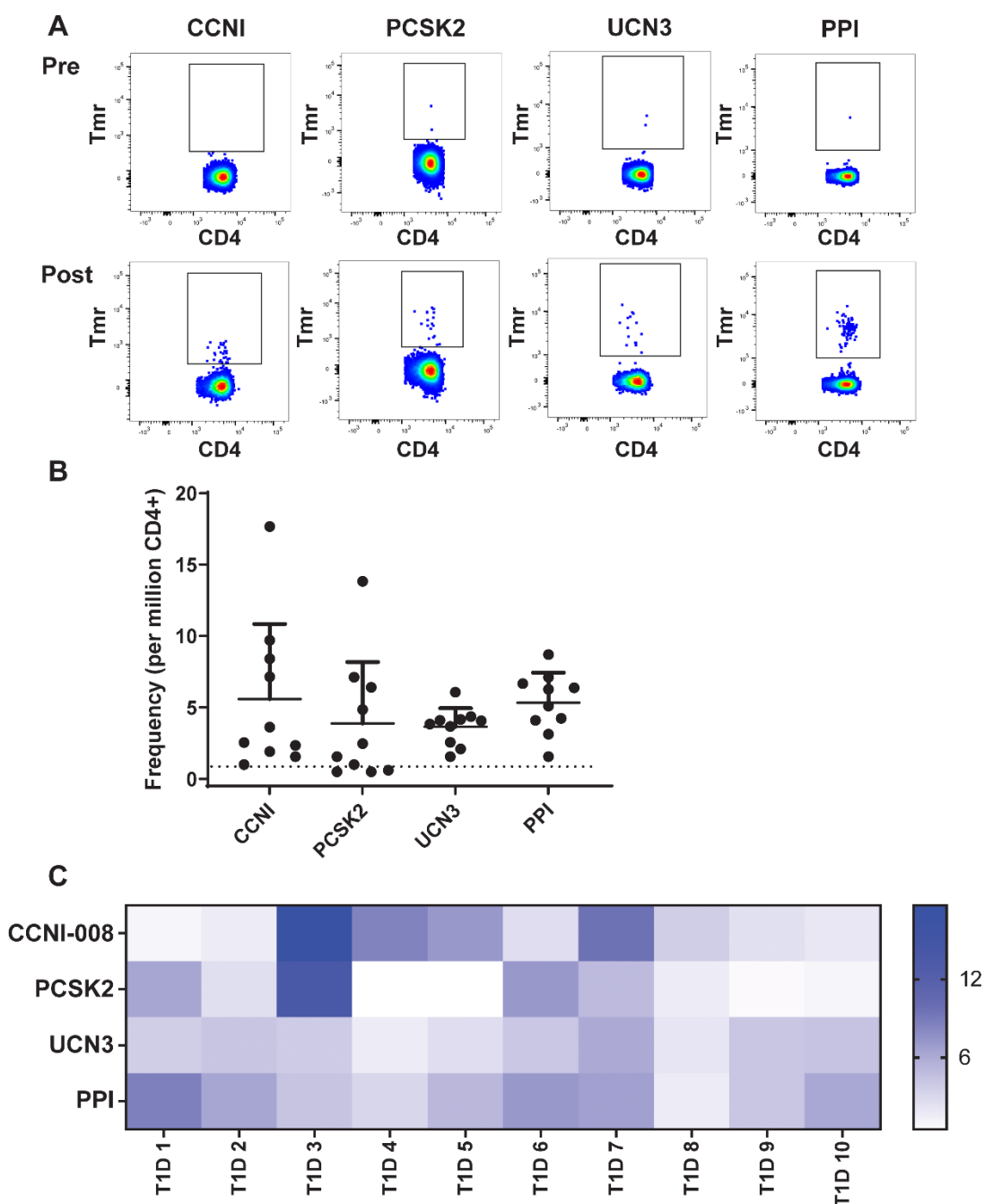


Figure 2 – T cells specific for novel epitopes have variable frequencies in subjects with type 1 diabetes. T cells specific for the novel epitopes identified were directly enumerated in peripheral blood using a magnetic enrichment procedure. *A*: HLA class II tetramers were used to enumerate CD4⁺ T cells for three novel epitopes, plus a reference PPI epitope. Cells were gated based on size, viability and lack of CD14/CD19 expression, CD4 expression, and then displayed on a CD4 versus tetramer (PE, PE-CF594, PE-Cy5, or PE-Cy7-labeled). A typical result, representative of 10 subjects with T1D examined (Supplemental Table 2), is shown. Each upper panel shows a pre-column fraction, used to determine the total number of CD4⁺ T cells in the unmanipulated sample and to set a threshold for positive tetramer staining. Each lower panel shows the corresponding enriched fraction, used to determine the total number of epitope-specific CD4⁺ T cells in the sample. *B*: Summary of ex vivo frequencies for each epitope measured in the peripheral blood of 10 subjects with established type 1 diabetes (Supplemental Table 2). The dotted line indicates the previously reported limit of detection for direct tetramer staining. CCNI and PPI were more frequent (5.6 and 5.3 cells per million, respectively) than PCSK2 and UCN3 (3.9 and 3.6 cells per million, respectively). *C*: A heatmap of the same frequency data reveals unique patterns of reactivity in different subjects with type 1 diabetes. Tmr, Tetramer.

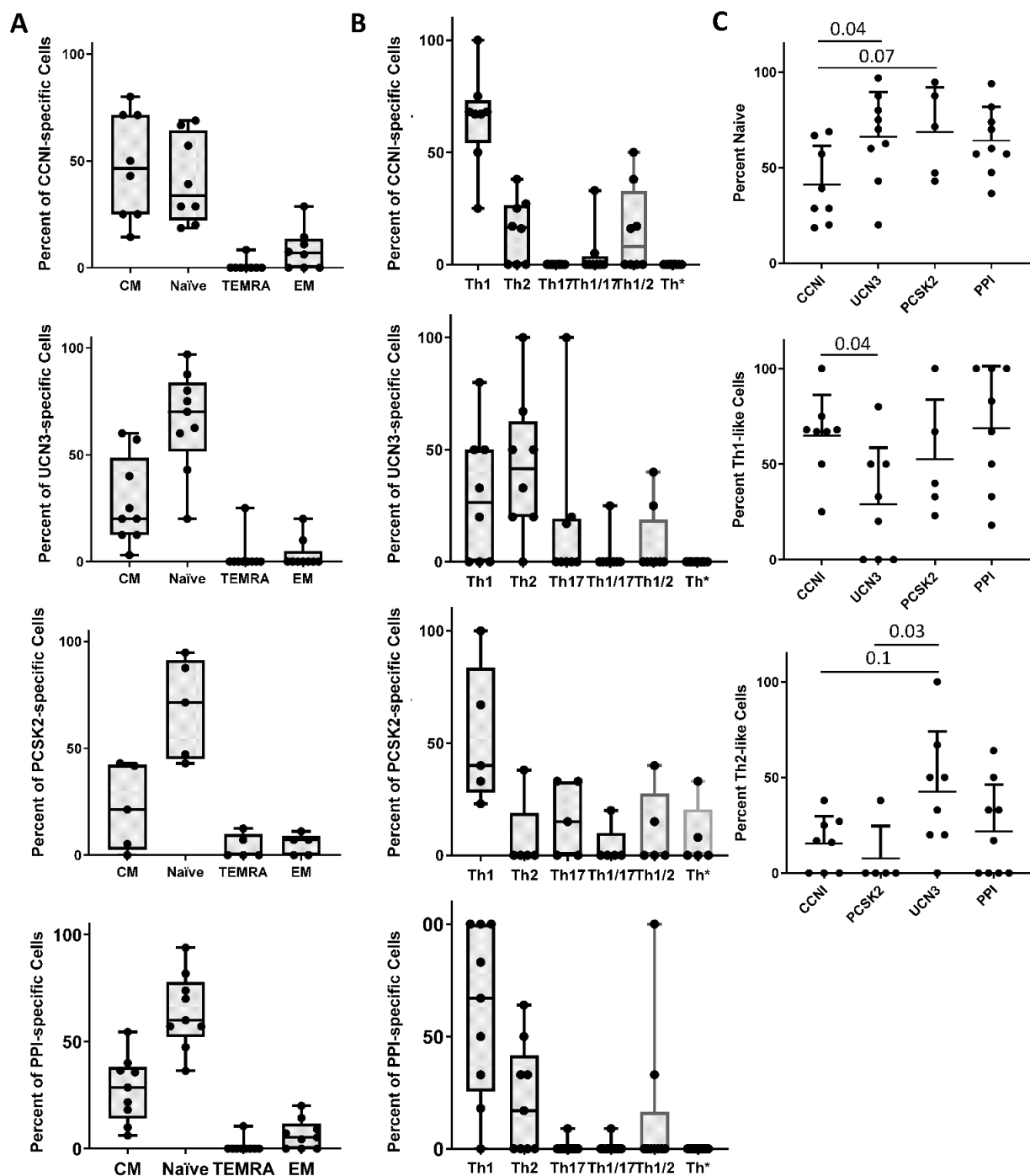


Figure 3 – T cells specific for novel epitopes exhibit different phenotypes. Epitope-specific CD4⁺ T cells in subjects with T1D (Supplemental Table 2) were phenotyped based on their cell surface expression of lineage markers. *A*: CD45RA and CCR7 surface staining was used to classify T cells as naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁺CCR7⁻), effector memory (CD45RA⁻CCR7⁻), or terminal effectors (CD45RA⁺CCR7⁻). This allowed comparison of the memory distributions for CCNI, UCN3, PCSK2, and PPI (as a reference epitope). *B*: Chemokine receptors were used to subdivide memory T cells into Th1-like (CXCR3⁺, CCR4⁻ and CCR6⁻), Th2-like (CXCR3⁻, CCR4⁺ and CCR6⁻), Th17-like (CXCR3⁺, CCR4⁻ and CCR6⁺), Th1/17-like (CXCR3⁺, CCR4⁻ and CCR6⁺), Th1/2-like (CXCR3⁺, CCR4⁺ and CCR6⁻), or Th1*-like (CXCR3⁺, CCR4⁺ and CCR6⁺) states. This allowed comparison of the cell subset distributions for CCNI, UCN3, PCSK2, and PPI (as a reference epitope). *C*: CCNI had a distinct memory profile, with a significantly lower proportion of naïve cells than UCN3 ($P = 0.04$) and trended lower than PCSK2 ($P = 0.07$). UCN3 had a distinct cell subset distribution, with a significantly lower proportion of Th1-like cells than CCNI ($P = 0.04$) and proportion of Th2-like cells that was significantly higher than PCSK2 ($P = 0.03$) and trended higher than CCNI ($P = 0.1$). CM, central memory; TEMRA, terminal effectors; EM, effector memory.

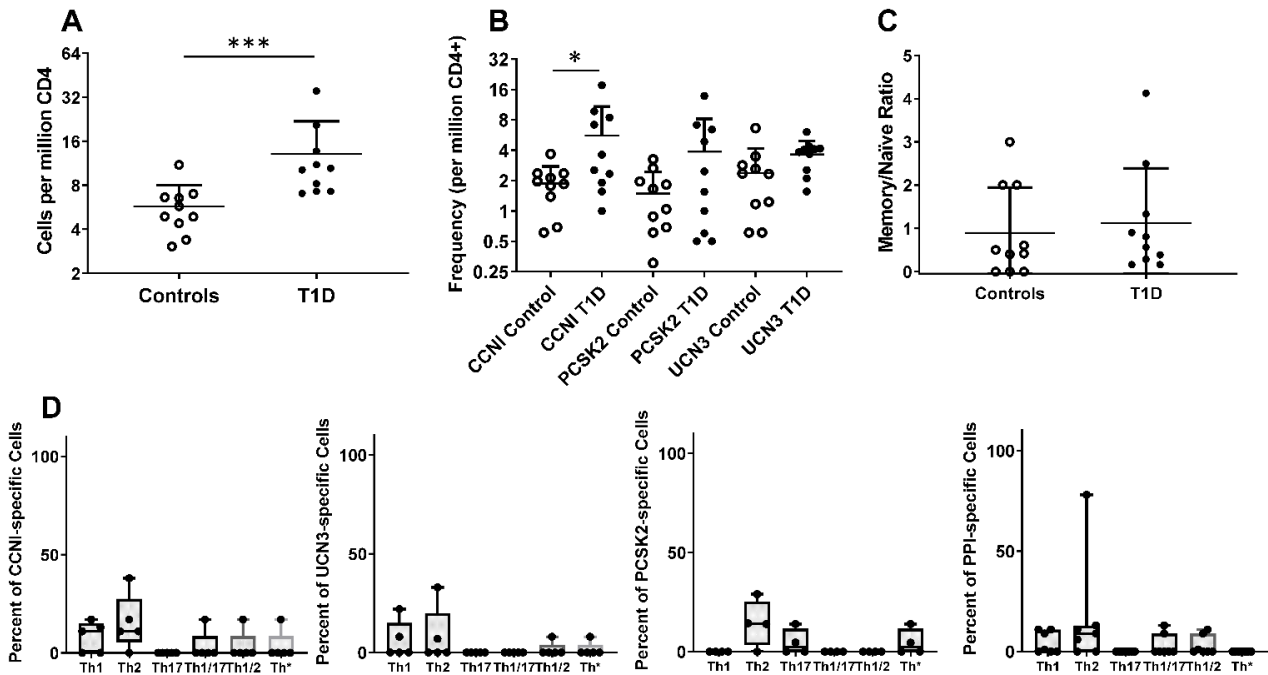


Figure 4 – T cells specific for novel epitopes are more frequent in subjects with type 1 diabetes and exhibit phenotype differences. HLA class II tetramers were used to characterize T cells specific for the three novel epitopes in 10 HLA matched control subjects (Supplemental Table 3). *A*: Combining all three epitopes (CCNI, PCSK2, and UCN3) total frequencies were significantly higher ($P = 0.0003$, Mann Whitney test) in subjects with type 1 diabetes (black circles) than in HLA matched controls (white circles). *B*: Examining individual epitopes, individual frequencies were significantly higher in subjects with type 1 diabetes (black circles, taken from Figure 2B but shown here on a log axis to more effectively depict the low frequencies seen in controls) than in healthy controls (white circles) only for CCNI ($P = 0.021$). *C*: Combining all three epitopes (CCNI, PCSK2, and UCN3) the proportion of T cells that had a memory phenotype (calculated as a ratio of the combined percentage of all memory subsets and percentage that were naïve) was not significantly different ($P = 0.565$, Mann Whitney test) in subjects with type 1 diabetes (black circles) than in HLA-matched controls (white circles). *D*: Chemokine receptors were used to subdivide memory T cells from healthy controls into Th1-like, Th2-like, Th17-like, Th1/17-like, Th1/2-like, or Th1*-like states (just as in Figure 3B for subjects with T1D). Controls lacked the Th1-like bias seen for some specificities in subjects with type 1 diabetes.

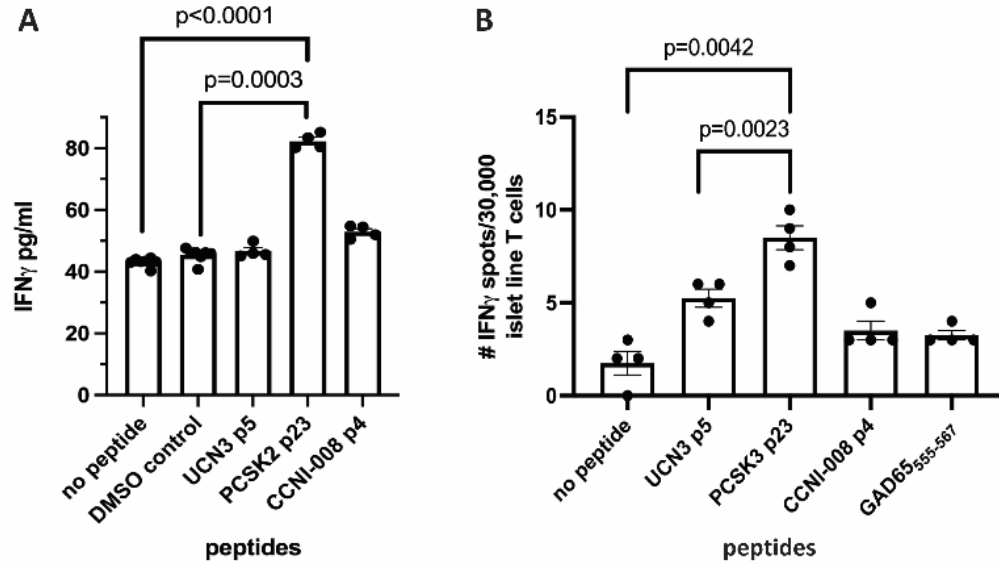
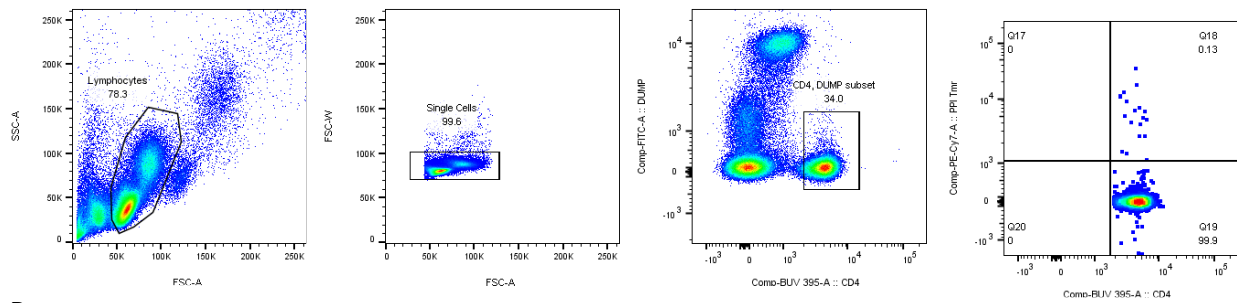
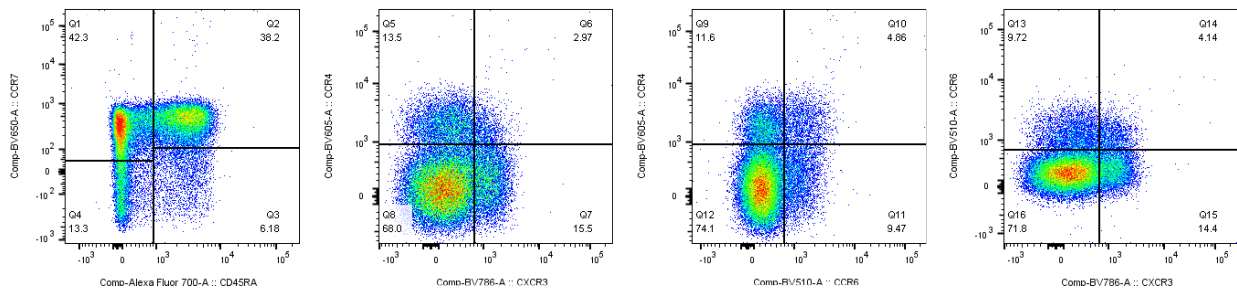


Figure 5 – PCSK2-specific CD4⁺ T cells infiltrate the islets of a type 1 diabetic donor. An islet-derived CD4⁺ T cell line for a donor with T1D recognizes PCSK2 p23 peptide. T cells were isolated and expanded from live, vibratome slices of pancreas tissue from nPOD donor 6472. *A*: Irradiated autologous B-LCL pulsed with peptides at 50 μ g/ml, no peptide, or DMSO volume control and then cultured with T cells and anti-CD28 (5 μ g/ml) for co-stimulation for 48 h, supernatants were collected and responses detected using a standard IFN- γ ELISA. The data shown represent one of 4 similar experiments. *B*: The islet T cell line previously cultured with PCSK3 p23 peptide was restimulated (using peptide pulsed autologous B-LCL as antigen-presenting cells), expanded, and then re-tested by IFN γ ELISpot for reactivity to the peptides. TNF α was not detected by ELISpot. The data shown represent one of two similar experiments.

A**B**

Supplemental Figure 1 – Surface marker gating for T cell analysis. FACS plots from a representative subject with T1D show the gating scheme used for CD4 T cell analysis. **A:** We sequentially gated lymphocytes (FSC-A versus SSC-A), single cells (FSC-A versus FSC-W), and viable CD4+ T cells (CD4 versus Dump). For viable CD4+ T cells, positive thresholds were then set for each tetramer channel, as shown for the representative PE-Cy7 labeled PPI tetramer. **B:** For viable CD4+ T cells, positive thresholds were also set to define naïve and memory subsets (CD45RA versus CCR7) and positive thresholds for CXCR3, CCR4, and CCR6. Using these threshold MFI values, sequential gating was then applied to define Th1-like (CXCR3+, CCR4- and CCR6-), Th2-like (CXCR3-, CCR4+ and CCR6-), Th17-like (CXCR3-, CCR4+ and CCR6+), Th1/17-like (CXCR3+, CCR4- and CCR6+), Th1/2-like (CXCR3+, CCR4+ and CCR6-), or Th1*-like (CXCR3+, CCR4+ and CCR6+) lineages.

Supplemental Table 1. DRB1*4:01+ subjects with type 1 diabetes used for in vitro assays

Subject ID	Age^a	Sex	Time Since Diagnosis^a	Auto-antibody status^b
T1D #1	29	Male	4.3 years	IAA, IA2, ZnT8
T1D #2	20	Female	1.9 years	GAD, IAA, IA2, ZnT8
T1D #3	26	Female	1.4 years	GAD, IAA, IA2
T1D #4	31	Female	5.1 years	GAD, IA2, ZnT8
T1D #5	30	Female	3.2 years	GAD, IAA, IA2, ZnT8
T1D #6	32	Female	7.3 years	GAD, IAA, IA2, ZnT8
T1D #7	29	Male	9.9 years	IAA, IA2
T1D #8	31	Female	3.1 years	IAA, IA2
T1D #9	37	Male	7.1 years	GAD, IAA, IA2, ZnT8
T1D #10	29	Female	12.6 years	GAD, IAA, IA2
T1D #11	32	Male	7.1 years	IAA
T1D #12	39	Female	27.9 years	GAD, IA2
T1D #13	40	Female	4.4 years	GAD
T1D #14	31	Female	3.5 years	GAD, IAA
T1D #15	36	Female	31.5 years	IAA, IA2

^aThe mean age of patients was 31.5 years. The mean time since diagnosis was 8.7 years.

^bGAD denotes glutamic acid decarboxylase 65, IAA denotes (micro) insulin, IA2 denotes tyrosine phosphatase-related islet antigen 2, ZnT8 denotes zinc transporter 8. Autoantibody data is historical and was not in done conjunction with the current study.

Supplemental Table 2. DRB1*4:01+ subjects with type 1 diabetes used for direct staining

Subject ID	Age^a	Sex	Time Since Diagnosis^a	Auto-antibody status^b
T1D #1	23	Female	4.8 years	IAA, IA2, ZnT8
T1D #2	70	Female	14.5 years	IAA
T1D #3	29	Male	5.6 years	GAD, IAA
T1D #4	53	Male	37.8 years	GAD, IAA
T1D #5	56	Female	42.6 years	IAA
T1D #6	25	Male	2.3 years	IAA, IA2, ZnT8
T1D #7	34	Female	31.6 years	IAA
T1D #8	63	Male	48.8 years	No results
T1D #9	42	Male	12.7 years	GAD, IAA
T1D #10	59	Female	14.1 years	GAD

^aThe mean age of patients was 45.4 years. The mean time since diagnosis was 21.4 years.

^bGAD denotes glutamic acid decarboxylase 65, IAA denotes (micro) insulin, IA2 denotes tyrosine phosphatase-related islet antigen 2, ZnT8 denotes zinc transporter 8. Autoantibody data is historical and was not in done conjunction with the current study.

Supplemental Table 3. DRB1*04:01 healthy subjects used for direct staining

Subject ID	Age	Sex
Control #1	62	Male
Control #2	58	Female
Control #3	53	Male
Control #4	42	Female
Control #5	22	Female
Control #6	67	Female
Control #7	34	Female
Control #8	46	Male

The mean age of controls was 48 years.

Healthy subjects under the age of 18 could not be recruited.

Supplemental Table 4. Organ donor with diabetes used for study

Case ID^a	Age	Sex	Time Since Diagnosis	HLA-DR Type
nPOD 6472	10	Female	4 years	DRB1*03:01/04:04

^aCD4+ T cell lines were isolated from vibratome slices of pancreas tissue from this organ donor (Cat# nPOD_6472, RRID:SAMN15879525).

Supplemental Table 5. Splice Variant and Secretory Granule Peptides with Predicted DR0401 Binding Motifs

Peptide	Sequence	Measured IC ₅₀ (μM) ^{a,b}
CCNI-008 p1	SEILRMERIILDKLN	>50
CCNI-008 p2	MERIILDKLNWDLHT	>50
CCNI-008 p3	KLNWDLHTATPLDFL	>50
CCNI-008 p4	HTATPLDFLHIMDSSQLIH	7.2
CCNI-008 p5	ATPLDFLHIMDSSQ	>50
CCNI-008 p6	DFLHIMDSSQLIH	20.1
CCNI-008 p7	LHIMDSSQLIHCRE	15.1
CCNI-008 p8	SSQLIHCRELVAHHL	>50
CCNI-008 p9	RELVAHHLSTLQSSL	11.4
CCNI-008 p10	LSTLQSSLPLNSVYV	>50
GAD2-003 p1	LKYAIKTGIVSSKII	37.3
GAD2-003 p2	KTGIVSSKIIKLFFR	>50
GNAS-002 p1	AQQWRRARHNYNDL	>50
GNAS-002 p2	GRRRAATALLWLSCSI	>50
GNAS-002 p3	ATALLWLSCSIALLRAL	>50
GNAS-002 p4	SCSIALLRALATSNA	>50
GNAS-002 p5	IALLRALATSNAARAQ	0.02
GNAS-002 p6	AQVPESPESEDHE	>50
GNAS-002 p7	ELSLPECLEYEEEFD	>50
GNAS-002 p8	LEYEEEFDYETESE	>50
GNAS-002 p9	EEFDYETESETESE	>50
GNAS-002 p10	EFDYETESETESEIE	>50
GNAS-002 p11	ESEISETDFETEPE	>50
GNAS-002 p12	ETDFETEPETAPPTP	>50
GNAS-002 p13	GPVVPKHSTFGQSLT	>50
GNAS-002 p14	HSTFGQSLTQRLHAL	>50
GNAS-036 p1	DKQLQDEKMGYMCTH	>50
GNAS-036 p2	DEKMGYMCTHRLLL	>50
GNAS-036 p3	THRLLLGAGESGKS	>50
GNAS-036 p4	LLGAGESGKSTIV	>50
GNAS-036 p5	STIVKQMRILHVNGF	>50
GNAS-036 p6	MRILHVNGFNGDEKA	>50
GNAS-036 p7	LKEAIETIVAAMSNL	>50
GNAS-036 p8	IETIVAAMSNLVPPV	0.15
GNAS-036 p9	ENQFRVDYILSVMNV	0.17
GNAS-036 p10	RVDYILSVMNVPDFD	10.9
GNAS-036 p11	DFDFPPEFYEHAKAL	>50
GNAS-036 p12	PEFYEHAKALWEDEG	>50
GNAS-036 p13	KALWEDEGVRACYER	>50

GNAS-036 p14	RACYERSNEYQLID	>50
GNAS-036 p15	SNEYQLIDCAQYFLD	38.6
IAPP-002 p1	SVALNHLKATPIERQ	25.4
IAPP-002 p2	IPVLSRNILLELRGA	>50
IAPP-002 p3	KSKVIRWKSGNATLP	>50
PTPRN-021 p1	AAGVKLLEILAEHVH	>50
PTPRN-021 p2	KLLEILAEHVHMSSGS	0.02
PTPRN2-005 p1	LPPRVLPAAAPSSVPR	>50
PTPRN2-005 p2	GCLLEEGLCGASE	>50
REXO2-020 p1	ALWIVKYYSRNSVHE	>50
REXO2-020 p2	VKYYSRNSVHEDKKF	>50
SCG5-009 p1	YEKMKGGERRRK	>50
SCG5-009 p2	TRAWLDCCGDAWSFLS	>50
SCG5-009 p3	GDAWSFLSGAVNRLK	>50
SCG5-009 p4	WSFLSGAVNRLKPSL	>50
SCG5-009 p5	AVNRLKPSLVGKSQN	>50
SLC30A8-002 p1	MEFLERTYLVNDKAA	>50
SLC30A8-002 p2	RTYLVNDKAAKMYAF	0.08
SLC30A8-002 p3	AKMYAFTLESVELQOK	0.03
SLC30A8-002 p4	GGMYHCHSGSKPTEK	6.0
IAPP trans-S p1	QVFLIVLSVALKLQVF	19.9
SCG5 p1	MVSRMVSTMLSGL	2.4
SCG5 p2	SRMVSTMLSGLLFWL	>50
SCG5 p3	SGLLFWLASGWTPAF	>50
SCG5 p4	LLFWLASGWTPAFAY	>50
SCG5 p5	EADIQRLLHGVMEQL	>50
SCG5 p6	QRLLHGVMEQLGIAR	16.9
SCG5 p7	RVEYPAHQAMNLVGP	>50
SCG5 p8	HEGLQHLGPFGNIPN	>50
SCG5 p9	LQHLGPFGNIPNIVA	>50
SCG5 p10	LGPFGNIPNIVAELT	>50
SCG5 p11	IPNIVAELTGDNIPK	>50
SCG5 p12	PKDFSEDQGYDPDPN	>50
SCG5 p13	TAEFSREFQLHQHLF	0.05
SCG5 p14	SREFQLHQHLFDPEH	>50
SCG5 p15	QHLFDPEHDYPGLGK	>50
SCG5 p16	KLLYEKMKGGERRRK	>50
SCG5 p17	NVVAKKSVPHFSDDED	>50
UCN3 P1	PVHFLLLLLLLLGG	16.8
UCN3 P2	PHKFYKAKPIFSCLN	33.4
UCN3 P3	KPIFSCLNTALSEAE	0.34
UCN3 P4	KRSFHYLRSRDAS	>50
UCN3 P5	SFHYLRSRDASSGEE	0.94
UCN3 P6	KKTFPISGARGGARG	>50

UCN3 P7	RTKFTLSLDVPTNIM	7.3
UCN3 P8	SLDVPTNIMNLLFNI	>50
UCN3 P9	PTNIMNLLFNIKAK	>50
UCN3 P10	LFNIAKAKNLRAQAA	0.05
UCN3 P11	AKNLRAQAAANAHLM	>50
PCSK2 p1	GGCVSQWCAAAGFLF	>50
PCSK2 p2	AAGFLFCVMVFASAE	>50
PCSK2 p3	GFLFCVMVFASAERP	>50
PCSK2 p4	RPVFTNHFLVELHKG	>50
PCSK2 p5	HFLVELHKGGEDKAR	0.32
PCSK2 p6	GFGVRKLPFAEGLYH	>50
PCSK2 p7	LYHFYHNGLAKAKRR	>50
PCSK2 p8	RVKMALQQEGFDRKK	>50
PCSK2 p9	KRGYRDINEIDINMN	>50
PCSK2 p10	RDINEIDINMNDPL	>50
PCSK2 p11	EIDINMNDPLFTKQW	>50
PCSK2 p12	DPLFTKQWYLINTGQ	>50
PCSK2 p13	TKQWYLINTGQADGT	0.13
PCSK2 p14	AEAWEELGYTGKGVTI	>50
PCSK2 p15	GVTIGIMDDGIDYLH	>50
PCSK2 p16	HPDLASNYNAEASYD	>50
PCSK2 p17	ASNYNAEASYDFSSN	>50
PCSK2 p18	SYDFSSNDPYPYPRY	>50
PCSK2 p19	YPRYTDDWFNSHGTR	>50
PCSK2 p20	DDWFNSHGTRCAGEV	>50
PCSK2 p21	AGEVSAAANNNICGV	>50
PCSK2 p22	NNNICGVGVAYNSKV	>50
PCSK2 p23	QPFMTDIEASSISH	0.07
PCSK2 p24	MTDIEASSISHMPQ	>50
PCSK2 p25	ISHMPQLIDIYSASW	>50
PCSK2 p26	LIDIYSASWGPTDNG	>50
PCSK2 p27	SASWGPTDNGKTVDG	>50
PCSK2 p28	ELTLQAMADGVNKGR	>50
PCSK2 p29	SIYVWASGDGGSYDD	>50
PCSK2 p30	DGYASSMWTISINS	>50
PCSK2 p31	ASSMWTISINSAIND	>50
PCSK2 p32	MWTISINSAINDGRT	>50
PCSK2 p33	TALYDESCSSTLAST	0.04
PCSK2 p34	TTDLYGNCTLRHSGT	>50
PCSK2 p35	NCTLRHSGTSAAAPE	0.83
PCSK2 p36	AGVFALALEANLGLT	7.2
PCSK2 p37	GLTWRDMQHLLTVLTS	>50
PCSK2 p38	RDMQHLLTVLTSKRN	>50
PCSK2 p39	VHQWRRNGVGFLEFNH	>50

PCSK2 p40	GLEFNHLFGYGVLDA	>50
PCSK2 p41	NHLFGYGVLDAGAM	>50
PCSK2 p42	GYGVLDAGAMVKM	>50
PCSK2 p43	AKDWKTVPERFHCVG	>50
PCSK2 p44	PERFHCVGGSVQDPE	>50
PCSK2 p45	TGKLVLTLLTDACEG	2.9
PCSK2 p46	KENFVRYLEHVQAVI	>50
PCSK2 p47	VRYLEHVQAVITVN	>50
PCSK2 p48	EHVQAVITVNATTR	>50
PCSK2 p49	QAVITVNATTRGDLN	8.1
PCSK2 p50	RGDLNINMTSPMGTK	20.1
PCSK2 p51	KVGFDKWPFMTTHTW	>50
PCSK2 p52	DKWPFMTTHTWGED	>50
PCSK2 p53	PFMTTHTWGEDAR	>50
PCSK2 p54	RGTWTLELGFGSAP	2.8
PCSK2 p55	ELGFVGSAPQKGVLK	>50
PCSK2 p56	KGVLKEWTLMLHGTQ	>50
PCSK2 p57	KEWTLMLHGTQSAPY	>50

^aIC₅₀ represents the peptide concentration that displaces half of the reference peptide.

^bPeptides selected for in vitro studies (based on IC₅₀ values) are shown in boldface.