ZnT8-Specific CD4\(^+\) T Cells Display Distinct Cytokine Expression Profiles between Type 1 Diabetes Patients and Healthy Adults

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Abstract

Determination of antigen-specific T cell repertoires in human blood has been a challenge. Here, we show a novel integrated approach that permits determination of multiple parameters of antigen-specific T cell repertoires. The approach consists of two assays: the Direct assay and the Cytokine-driven assay. Briefly, human PBMCs are first stimulated with overlapping peptides encoding a given antigen for 48 hours to measure cytokine secretion (Direct assay). Peptide-reactive T cells are further expanded by IL-2 for 5 days; and after overnight starvation, expanded cells are stimulated with the same peptides from the initial culture to analyze cytokine secretion (Cytokine-driven assay). We first applied this integrated approach to determine the type of islet-antigen-specific T cells in healthy adults. Out of ten donors, the Direct assay identified GAD65-specific CD4\(^+\) T cells in three adults and zinc transporter 8 (ZnT8)-specific CD4\(^+\) T cells in five adults. The intracytoplasmic cytokine staining assay showed that these islet-antigen-specific CD4\(^+\) T cells belonged to the CD45RO\(^+\) memory compartment. The Cytokine-driven assay further revealed that islet-antigen-specific CD4\(^+\) T cells in healthy adults were capable of secreting various types of cytokines including type 1 and type 2 cytokines as well as IL-10. We next applied our integrated assay to determine whether the type of ZnT8-specific CD4\(^+\) T cells is different between Type 1 diabetes patients and age/gender/HLA-matched healthy adults. We found that ZnT8-specific CD4\(^+\) T cells were skewed towards Th1 cells in T1D patients, while Th2 and IL-10-producing cells were prevalent in healthy adults. In conclusion, the Direct assay and the Cytokine-driven assay complement each other, and the combination of the two assays provides information of antigen-specific T cell repertoires on the breadth, type, and avidity. This strategy is applicable to determine the differences in the quality of antigen-specific T cells between health and disease.

Citation: Chujo D, Foucat E, Nguyen T-S, Chaussabel D, Banchereau J, et al. (2013) ZnT8-Specific CD4\(^+\) T Cells Display Distinct Cytokine Expression Profiles between Type 1 Diabetes Patients and Healthy Adults. PLoS ONE 8(2): e55595. doi:10.1371/journal.pone.0055595

Editor: Matthias G. von Herrath, La Jolla Institute for Allergy and Immunology, United States of America

Received March 9, 2012; Accepted December 27, 2012; Published February 4, 2013

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Funding: This study was supported by funding from Juvenile Diabetes Research Foundation (grant number: 17-2010-406; http://www.jdrf.org) and Baylor Health Care System. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The increased incidence of immune-mediated diseases and the growing numbers of immunomodulatory agents that are moving from bench to bedside demand development of better tools to study the human immune system. In particular, identifying the type of antigen-specific T cell responses may provide significant insights regarding disease pathogenesis and lead to the development of novel platforms for therapeutic approaches. Yet, the enormous diversity of immune responses renders the assessment of the antigen-specific T cell repertoire a challenge. This is particularly true in humans due to the extensive HLA polymorphism. Currently, several tools are available to identify antigen-specific T cells, including the proliferation assay [1,2], ELISPOT [1,3,4], intracytoplasmic cytokine staining (ICS) [5,6,7], and peptide-HLA tetramers [1,4,8,9,10]. Although all assays permit the detection of antigen-specific T cells in human blood, each assay displays both strengths and shortcomings. \(^{3}\text{H}\)-thymidine incorporation or the CFSE dilution assay can be performed at a low-cost [1,2], but provide little functional information. ELISPOT assays are widely used to quantitate low-frequency antigen-specific T cells [1,3,4], but only one or two soluble factors can be measured in a single assay. ICS assays, in particular when performed with multicolor flow cytometry, permit the detailed assessment of cytokine expression profiles of antigen-specific T cells together with cell phenotype, [5,6,7]; though the sensitivity is limited. Peptide-HLA tetramers sensitively determine the frequency of antigen-specific T cells in blood [1,4,8,9,10], but provide little information regarding their functions.

Overlapping peptides that span a given antigen’s sequence are widely used in multiple platforms to stimulate antigen-specific T cells in vitro irrespective of the HLA alleles. We have previously shown that measurement of multiple cytokines in PBMC cultures stimulated for 48 h with overlapping peptides permits the detection of antigen-specific T cells (here called the Direct assay) [11,12]. In studies with blood samples from melanoma patients, tumor-antigen-specific IFN-\(\gamma\)-secreting CD8\(^+\) T cells were identified through the secretion of IP-10 [11], a chemokine that is
induced in response to IFN-γ. Furthermore, tumor-antigen-specific regulatory T cells were identified through peptide-induced IL-10 secretion [12]. While these studies show that the Direct assay can identify antigen-specific T cells together with some of their functions, the assay will not allow detection of very rare antigen-specific T cells. One approach to circumvent this...
shortcoming is to expand peptide-reactive CD4+ T cells by adding cytokines such as IL-2 to the cultures prior to the assessment of cytokine secretion profiles (here called the Cytokine-driven assay). Using this strategy, we were able to demonstrate the presence of islet-antigen-specific CD4+ and CD8+ T cells in Type 1 diabetes (T1D) patients under immunosuppressive treatments after allogeneic islet transplantation [13]. Notably, the Cytokine-driven assay can be combined with the Direct assay in the same cultures, and the combination of the two assays therefore might permit us to characterize multiple aspects of antigen-specific T cell repertoires.

Type 1 diabetes (T1D) is caused by autoimmune destruction of insulin-producing islet β cells [14,15,16,17,18]. Accordingly, T1D patients display IFN-γ-producing islet-antigen-specific T cells in the blood [19,20,21]. T1D-associated islet antigens include GAD65 [8,22,23], (prepro)insulin (PPI) [24,25,26], insulinoma associated-2 (IA-2) [27], islet-specific glucose-6-phosphate catalytic subunit-related protein (IGRP) [28,29], and zinc transporter-3 (ZnT8) [30]. Previous studies showed that islet-antigen-specific T cells can be also found in healthy adults [20,31,32]. Distinct from T1D patients, however, islet-antigen-specific T cells in healthy adults were shown to be either naive [31,32] or non-inflammatory cells secreting primarily IL-10 [19].

In this present study, we used our integrated assay to characterize islet-antigen-specific T cell repertoires in healthy adults and in T1D patients. Here, we show that our integrated strategy allowed the detection of GAD65- and ZnT8-specific CD4+ T cells in healthy adults as well as in T1D patients. Importantly, the quality of ZnT8-specific CD4+ T cells were different between T1D patients and healthy adults. While ZnT8-specific CD4+ T cells were skewed towards Th1 cells in T1D patients, Th2 and IL-10-producing cells were prevalent in healthy adults. This study provides a proof-of-principle that our integrated strategy permits determination of multiple parameters of antigen-specific T cell repertoires.

Results

The Direct Assay can Detect T cells Recognizing Peptides Derived from Islet-antigens in Healthy Adults

Previous studies showed that healthy individuals also display islet-antigen-specific T cells in the blood [20,31,32]. We examined whether our Direct assay is able to identify antigen-specific T cells in healthy adult blood samples. Fresh blood samples were obtained from 10 healthy adults (HS#1–10; 5 male and 5 female, age: 44.8±2.3). Fifteen-mer overlapping peptide pools were generated for three islet antigens – GAD65, ZnT8, and PPI (Table 1). PBMCs were cultured with peptide pools (4–11 peptides/pool), and cytokines secreted during the 48 h culture were measured. Background levels of IL-2, IL-10, IL-13, and IL-17A in the culture where PBMCs were stimulated with no peptide were less than 10 pg/ml (IL-2:0.1±0.3; IL-10:0.8±1.3; IL-13:0.3±0.5; IL-17A: 0.2±0.4 pg/ml, mean ± SD, n=20). IP-10, a chemokine secreted in response to IFN-γ, was detected at variable levels (159.4±152.3 pg/ml). We defined cytokine secretion in response to stimulation with peptides as “positive”, when 1) the levels of IL-2, IL-10, IL-13, or IL-17A were greater than 10 pg/ml, or 2) the level of IP-10 was more than three times the background. The summary of cytokine levels detected in each culture is shown in Figure 1. The secretion of IL-2, IL-10, IL-13, or IL-17A in response to stimulation with islet-antigen-derived peptides was generally weak, and no cultures scored positive for secretion of IL-10 and IL-17A (range, IL-2 0–103.3 pg/ml, IL-10 0–0.7 pg/ml, IL-13 0–30.1 pg/ml, IL-17A 0–3.46 pg/ml). However, multiple cultures with GAD65 and ZnT8 peptide pools in 5 out of 10 healthy individuals scored positive for IP-10 secretion. PPI-specific T cell responses were not detected in any subjects.

After we identified positive responses at a peptide cluster level, we next cultured PBMCs with single peptides (25 μM) within the positive clusters to identify the peptides that triggered IP-10 secretion. Figure 2A shows an example of results in healthy donor HS#1. GAD65 peptide #73 (p#18) from peptide cluster C(1–2) was found to induce IP-10 secretion (Fig. 2A). This peptide was able to induce the proliferation of CD4+ T cells in the PBMC culture (Fig. 2B). These results suggest the presence of GAD65-specific Th1 cells in the blood of donor HS#4. In donor HS#2, ZnT8 peptides p#2, p#18, p#33, and p#83 were found to induce IP-10 secretion (Fig. 2C and 2E) as well as CD4+ T cell proliferation (Fig. 2D and 2E). Of note, in donor HS#2, while GAD65 peptide #66 was found to induce IP-10 secretion within C(1–7), no peptides were found within GAD65 peptide C(4–9). This was likely due to the fact that multiple peptides in C(4–9) contributed to the secretion of IP-10, but no single peptide was sufficient to score positive for IP-10 secretion. The summary of the identified peptides is shown in Table 2 (The sequence is shown in Table S1). Out of the 10 healthy adults that were tested, 3 adults displayed both GAD65-specific CD4+ T cells and ZnT8-specific CD4+ T cells. Two adults displayed only ZnT8-specific CD4+ T cells. All of the identified peptides induced IP-10 secretion and proliferation of CD4+ T cells, suggesting the presence of specific Th1 cells.

ICS Assay Confirms the Presence of Th1 Cells Reactive to Islet Antigen-peptides

While a recent study showed ZnT8-specific IFN-γ-producing CD4+ T cells were present in healthy adults at a lower frequency than in type 1 diabetes patients [21], we were intrigued by the fact that ZnT8 peptides induced strong IP-10 secretion in 5 out of 10

Table 1. Numbers of peptides and peptide clusters.

| Islet antigen | Number of peptides | Number of peptide clusters | Peptides/cluster |
|---------------|-------------------|---------------------------|-----------------|
| PPI           | 25                | 3                         | 8 (C1–C2)       |
|               |                   |                           | 9 (C3)          |
| GAD65         | 144               | 14                        | 10 (C1–C10)     |
|               |                   |                           | 11 (C11–C14)    |
| ZnT8          | 94                | 16                        | 6 (C1–C15)      |
|               |                   |                           | 4 (C16)         |

PPI, preproinsulin; GAD65, glutamic acid decarboxylase 65; ZnT8, zinc transporter 8; C, peptide cluster. doi:10.1371/journal.pone.0055595.t001
healthy donors (Table 2). To determine the frequency of ZnT8-specific CD4+ T cells in healthy adults, PBMCs were incubated with the identified peptide for 6 hours in the presence of Brefeldin A, and intracytoplasmic expression of IFN-γ and IL-2 was analyzed. In donor HS#4, three ZnT8 peptides, p#18, p#65, and p#68 induced IP-10 secretion (Table 2). The ICS assay showed that as many as 2.6% of CD4+ T cells was found to express IFN-γ in response to p#68 (Fig. 3A). Among IFN-γ-expressing CD4+ T cells, a majority also co-expressed IL-2 (Fig. 3A). ~2.3% and ~0.3% of CD4+ T cells expressed IFN-γ in response to p#65 and p#18, respectively. A quarter of IFN-γ-expressing CD4+ T cells cultured with p#65 and p#18 co-expressed IL-2. In donor HS#7, ~0.7% and ~0.6% of CD4+ T cells expressed IFN-γ in response to ZnT8 p#65 and p#87, respectively (Fig. 3B). While IL-2 was expressed by a fraction of p#65-responding CD4+ T cells, few p#87-responding CD4+ T cells expressed IL-2. In both donors, IFN-γ was expressed solely by CD45RO+ memory T cells (Fig. 3A and 3B).

Thus, ZnT8-specific memory CD4+ T cells expressing IFN-γ and IL-2 can be found in the blood of healthy adults at high
The same epitopes as the Direct assay. In donor HS assay identified ZnT8 p
from the Direct assay. Intracytoplasmic cytokine expression assay confirmed the results of the Direct assay.

1.9 driven assay showed that only p
also found to secrete type 2 cytokines, IL-4, IL-5, and IL-13 (Fig. 4C). ICS assay with the expanded p
Secretion Profiling of Peptide-reactive CD4+ T cells
by induction of IL-2 and IP-10 secretion (Fig. 4A) and by
Cytokine-driven assay allowed for a more detailed cytokine secretion profiling, as the assay demonstrated a fraction of ZnT8 p
that were capable of secreting type 2 cytokines.

We next performed the Cytokine-driven assay on all the GAD65 and ZnT8-specific CD4+ T cells that had been identified in the Direct assay. The summary of cytokine secretion profiles is shown in Table 3. While all of the epitopes were identified by the induction of IP-10 in the Direct assay, indicative of IFN-γ secretion, expanded CD4+ T cells were found to secrete variable types of cytokines in addition to IFN-γ (Fig. 4E and 4F). For example, GAD65 p
were found to secrete IL-10, IL-13, and TNF-α (Fig. 4E, top). The ICS assay showed that GAD65 p
were composed of IFN-γ/IL-13+ Th1 cells, IFN-γ/IL-13+ Th2 cells, and IL-10-producing cells (Fig. 4F).

All the fifteen GAD65 and ZnT8 peptides that induced IFN-γ secretion by specific CD4+ T cells were also able to induce IL-13 secretion (Table 2). Five peptides in 2 adult samples induced IL-10 secretion, while 10 peptides in 4 adult samples induced IL-4 and/or IL-5. Thus, GAD65- and ZnT8-specific CD4+ T cells in healthy adults are capable of secreting multiple types of cytokines, including type 1 and type 2 cytokines as well as IL-10.

Table 2. Summary of the islet-antigen-specific T cell responses in the Direct assay.

| Subject | Antigen | Cluster | Epitope | Secreted Cytokine | CFSE-CD4+ (%) | CFSE-CD4+ (%) |
|---------|---------|---------|---------|------------------|--------------|--------------|
| HS#1    | –       | –       | –       | –                | –            | –            |
| HS#2    | –       | –       | –       | –                | –            | –            |
| ZnT8    | C1      | p2      | –       | –                | –            | –            |
| C3      | p18     | –       | –       | –                | –            | –            |
| C6      | p33     | –       | –       | –                | –            | –            |
| C16     | p93     | –       | –       | –                | 9.74         | 0.11         |
| HS#3    | –       | –       | –       | –                | 1.91         | 0.03         |
| ZnT8    | C1      | p2      | –       | –                | 2.87         | 0.03         |
| C3      | p18     | –       | –       | –                | 1.94         | 0.03         |
| HS#4    | –       | –       | –       | –                | 4.30         | 0.07         |
| ZnT8    | C3      | p18     | –       | –                | 9.44         | 0.07         |
| C11     | p65     | –       | –       | –                | 17.2         | 0.08         |
| C12     | p68     | –       | –       | –                | 47.7         | 0.08         |
| HS#5    | –       | –       | –       | –                | –            | –            |
| HS#6    | –       | –       | –       | –                | –            | –            |
| HS#7    | –       | –       | –       | –                | –            | –            |
| ZnT8    | C11     | p65     | –       | –                | 1.20         | 0.21         |
| C12     | p68     | –       | –       | –                | 1.04         | 0.01         |
| C15     | p87     | –       | –       | –                | 4.14         | 0.21         |
| HS#8    | –       | –       | –       | –                | –            | –            |
| HS#9    | –       | –       | –       | –                | –            | –            |
| HS#10   | –       | –       | –       | –                | –            | –            |
| ZnT8    | C2      | p8      | –       | –                | 2.62         | 0.03         |

HS, healthy subject; C, peptide cluster; p, peptide.

doi:10.1371/journal.pone.0055595.t002

frequencies (>0.5% of total CD4+ T cells). Importantly, this intracytoplasmic cytokine expression assay confirmed the results from the Direct assay.

The Cytokine-driven Assay Permits Detailed Cytokine Secretion Profiling of Peptide-reactive CD4+ T cells

We next used the Cytokine-driven assay to analyze islet-antigen-specific T cells in healthy adults. Briefly, IL-2 was added to the cultures of PBMCs with 25 μM of islet-antigen peptides on day 2. Cells were harvested on day 7, rested overnight in fresh media, and re-stimulated for 24 h with the same peptides. Secreted cytokines were measured with a multiplex cytokine assay. Cytokine secretion in response to stimulation with peptides was defined as “positive”, when the levels of each cytokine were more than ten times those in the background (background values: IL-2 4.3 3.0 pg/ml, IL-5 6.9±6.3 pg/ml, IL-10 10.8±16.5 pg/ml, IL-13 51.2±52.0 pg/ml, IL-17A 3.6±6.6 pg/ml, IL-21 1.9±2.3 pg/ml, TNF-α 45.3±33.2 pg/ml, and IFN-γ: 190.1±280.3 pg/ml; mean ± SD, n = 20).

We first examined whether the Cytokine-driven assay identifies the same epitopes as the Direct assay. In donor HS#4, the Direct assay identified ZnT8 p
restimulation with the peptide (Fig. 4D). IL-13 was also expressed by a minor fraction of IFN-γ-expressing cells. Thus, the results of the Direct assay and the Cytokine-driven assay were consistent, as both assays identified ZnT8 p
CD4+ T cells that were capable of secreting type 2 cytokines.

We next performed the Cytokine-driven assay on all the GAD65 and ZnT8-specific CD4+ T cells that had been identified in the Direct assay. The summary of cytokine secretion profiles is shown in Table 3. While all of the epitopes were identified by the induction of IP-10 in the Direct assay, indicative of IFN-γ secretion, expanded CD4+ T cells were found to secrete variable types of cytokines in addition to IFN-γ (Fig. 4E and 4F). For example, GAD65 p
were found to secrete IL-10, IL-13, and TNF-α (Fig. 4E, top). The ICS assay showed that GAD65 p
were composed of IFN-γ/IL-13+ Th1 cells, IFN-γ/IL-13+ Th2 cells, and IL-10-producing cells (Fig. 4F).
Peptide-reactive T cells Detectable by the Direct Assay Display Higher Avidity than Those Detectable by the Cytokine-driven Assay

Using the Direct and Cytokine-driven assays with 25 μM peptide, we found multiple peptides that induced cytokine secretion only in the Cytokine-driven assay. For example, in donor HS#7, while ZnT8 p#61 and p#65 peptides elicited equivalent levels of IFN-γ secretion in the Cytokine-driven assay, ZnT8 p#61 did not induce IP-10 secretion in the Direct assay (Fig. 5A, left and middle). It is possible that ZnT8 p#61-specific CD4+ T cells are present in blood at a very low frequency and become detectable only after expansion. Another possibility is that ZnT8 p#61-specific CD4+ T cells display an avidity lower than ZnT8 p#65-specific CD4+ T cells and secrete little IFN-γ during the initial stimulation, but produce larger amounts of IFN-γ after expansion with IL-2. In this case, low-avidity CD4+ T cells would require higher concentrations of peptides than high-avidity CD4+ T cells to initiate cell proliferation. To test this hypothesis, we titrated the peptides (from 0.04 to 25 μM) at the initial cultures, and re-stimulated cells at a constant concentration (25 μM). In donor HS#7, while p#61 induced specific IFN-γ secretion only at 25 μM, p#65 induced specific IFN-γ secretion at ~1 μM (Fig. 5A, right). This suggests that p#65-specific CD4+ T cells display higher avidity than p#61-specific CD4+ T cells. Similarly, in donor HS#2, while GAD65 p#93 failed to induce cytokine secretion in the Direct assay, but induced IL-13 secretion in the Cytokine-driven assay. A titration experiment showed that 25 μM GAD65 p#93 was required to expand the specific CD4+ T cells (Fig. 5B, top). In contrast, only a minute amount of ZnT8 p#65 (<0.2 μM) in HS#4 was sufficient to elicit specific IL-13 secretion (Fig. 5B, bottom).

These results show that the Direct assay identifies T cells with relatively high avidities, while the Cytokine-driven assay can identify antigen-specific T cells including with lower avidities.

ZnT8-specific CD4+ T cells Display Distinct Cytokine Expression Patterns between T1D Patients and Healthy Adults

Previous studies reported that islet antigen, GAD65-specific CD4+ T cells with high antigen avidity are prevalent in peripheral blood of T1D patients [8,33]. Here, we compared the ZnT8-specific T cell repertoires between T1D patients and non-diabetic healthy adults, by using our integrated assay. Herein we used a lower peptide concentration (2.5 μM) than in the experiments described above, in order to detect only T cells with high avidity. Age and gender-matched 15 recent-onset T1D patients (days from...
ZnT8-Specific CD4+ T Cells in T1D and Controls

A

IL-2
IL-10
IL-13
IL-17
IP-10

(pg/ml)

60
20
20
20
8000

HS #4 ZnT8 C11

B

ZnT8 p65
Ctrl

CD4

CFSE

17.2
0.08

C

IL-4
IL-5
IL-10
IL-13

(pg/ml)

200
800
200
4000

HS #4 ZnT8 C11

D

IL-17
IL-21
TNF-α
IFN-γ

CD4

CFSE

IL-10

IFN-γ

77.2
0.03
0.07
5.56
0.40
0.13
0.03

E

HS #2 GAD65 p66
HS #2 Ctrl

(pg/ml)

6000
1000
0

2000
500
0

4000
1500
0

IL-4
IL-5
IL-10
IL-13
IL-17
IL-21
TNFα
IFNγ

HS #2 ZnT8 p18
HS #2 Ctrl

HS #3 ZnT8 p18
HS #3 Ctrl

F

HS #2 GAD65 p66

37.6
1.02
0.10
4.31
0.69
2.36
1.23

HS #2 ZnT8 p18

17.6
4.60
0.12
7.88
1.42

HS #3 ZnT8 p18

45.9
1.77
0.08
2.39
0.91
diagnosis: 285.9 ± 247.9) and 15 control subjects were accrued in the study (Table 4 and Table S2). HLA distribution based on the frequency of HLA-DRB1*0301, DRB1*0401, and other DR3/DR4 were not statistically different between two groups (p = 0.60, Pearson’s χ² test).

PBMCs were cultured with 2.5 μM of the identified four ZnT8 peptides [p2, p18, p65, and p68], and cytokines secreted during 48 hours culture were measured (Direct assay). With 2.5 μM peptides, none of the PBMC samples from healthy adults produced IP-10 (Fig. 6A, 6B). The data summary is shown in Table 5), in contrast to the observations with 25 μM peptides (Fig. 1). Seven out of 15 T1D PBMC samples produced IP-10 in response to at least one of the four ZnT8 peptides (Fig. 6A, 6B). Two out of 15 control PBMCs were found to produce IL-10, while T1D PBMCs produced little IL-10 (Fig. 6A, 6B), a consistent result with the observations with 25 μM peptides (p2, p18, p65, and p68), and cytokines secreted during 48 hours culture were measured (Direct assay).

In the first set of study, where the peptides were used at 25 μM concentration, healthy adults were found to display GAD65- and ZnT8-specific memory Th1 cells at high frequencies (>0.5% of total CD4⁺ T cells). This finding was unexpected, as previous reports demonstrated that islet-antigen-specific T cells in healthy adults were either naive, anergic, or exhibited regulatory functions [19,20,32]. Furthermore, a fraction of ZnT8-specific Th1 cells in healthy adults also co-expressed IL-2. Thus, our study suggests that activation of GAD65 and ZnT8-specific CD4⁺ T cells in healthy adults also co-expressed IL-2. Thus, our study suggests that activation of GAD65 and ZnT8-specific CD4⁺ T cells may occur even in healthy individuals. Importantly, neither PPI-specific CD4⁺ T cells nor islet-antigen-specific CD8⁺ T cells were dominant in healthy adults.

### Discussion

In this study, we applied the Direct assay and the Cytokine-driven assay to characterize islet-antigen-specific T cell repertoires in healthy adults and T1D patients.

In the first set of study, where the peptides were used at 25 μM concentration, healthy adults were found to display GAD65- and ZnT8-specific memory Th1 cells at high frequencies (>0.5% of total CD4⁺ T cells). This finding was unexpected, as previous reports demonstrated that islet-antigen-specific T cells in healthy adults were either naive, anergic, or exhibited regulatory functions [19,20,32]. Furthermore, a fraction of ZnT8-specific Th1 cells in healthy adults also co-expressed IL-2. Thus, our study suggests that activation of GAD65 and ZnT8-specific CD4⁺ T cells may occur even in healthy individuals. Importantly, neither PPI-specific CD4⁺ T cells nor islet-antigen-specific CD8⁺ T cells were dominant in healthy adults.

### Table 3. Summary of cytokine secretion profiles in the Cytokine-driven assay.

| Subject | Antigen | Epitope | Secreted Cytokine |
|---------|---------|---------|-------------------|
|         |         |         | IL-4 | IL-5 | IL-10 | IL-13 | IL-17 | IL-21 | TNF-α | IFN-γ |
| HS#2 GAD65 | p66 | – | – | + | – | – | + | + | + |
| ZnT8     | p2     | + | + | + | – | – | – | + | + |
| p18      | + | + | + | – | – | – | – | + | + |
| p33      | + | + | + | – | – | – | – | + | + |
| p93      | + | – | – | + | – | – | + | + | + |
| HS#3 GAD65 | p73 | – | – | – | – | – | – | + | + |
| ZnT8     | p2     | + | + | + | – | – | – | + | + |
| p18      | + | + | + | – | – | – | – | + | + |
| HS#4 GAD65 | p73 | – | – | – | – | – | – | + | + |
| ZnT8     | p18    | + | + | – | + | – | – | – | + |
| p65      | – | – | – | – | – | – | – | – | + |
| p68      | – | – | – | – | – | – | – | – | + |
| HS#7 ZnT8 | p65 | – | – | – | – | – | – | + | + |
| p68      | – | – | – | – | – | – | – | – | + |
| p87      | – | – | – | – | – | – | – | – | + |

HS, healthy subject; p, peptide.

doi:10.1371/journal.pone.0055595.t003
detected in any tested donors. Failure to detect islet-specific CD8+ T cells was not due to the limitation in the assays, as the Cytokine-driven assay was able to detect GAD65-specific CD8+ T cells in multiple T1D patients who underwent islet transplantations [34]. Our observation is largely consistent with previous reports concluding that insulin-specific T cells [26,35] and islet-antigen-specific CD8+ T cells [36] are barely found in healthy individuals.

Recent studies show that insulin-specific CD8+ T cells can kill human pancreatic β cells [37] and insulin or/and IGRP-specific CD8+ T cells are present inside the islets of T1D patients [38]. Therefore, the presence of PPI-specific T cells and islet-specific CD8+ T cells might be more limited to T1D patients when compared to GAD65- or ZnT8-specific CD4+ T cells.

The Cytokine-driven assay showed that GAD65- or ZnT8-specific CD4+ T cells in healthy adults were composed of heterogeneous populations that differentially express IFN-γ, IL-10, and type 2 cytokines, including IL-4, IL-5, and IL-13. While IFN-γ secreted by islet-antigen-specific CD4+ T cells might be prone to induce islet inflammation [17,39], IL-10 secreted by T cells might play an inhibitory role [19,40]. The role of Type 2
cytokine-secreting CD4+ T cells in healthy adults is still unclear. A previous study shows that insulin-specific T cell clones established from pancreatic lymph nodes of T1D patients preferentially secreted IL-13, but not IFN-γ [25], suggesting that these cells might play a pathogenic role. Alternatively, it is possible that Type 2 cytokines play an anti-inflammatory role. For example, a systemic administration of recombinant IL-13 prevents the onset of diabetes in NOD mice [41]. Furthermore, generation of IL-5-producing GAD65-specific CD4+ T cells was shown to protect NOD mice from diabetes development [42].

In this study, we identified two 15-mer GAD65 peptides (p66 and p73) and eight 15-mer ZnT8 peptides (p2, p6, p18, p33, p65, p68, p87, and p93) as epitopes for CD4+ T cells. While we did not test whether CD4+ T cells expanded with the identified peptides also react to naturally processed antigens, the two GAD65 peptides and the most ZnT8 peptides shared sequences with CD4+ T cell epitopes reported previously [21,43,44,45,46]. Nonetheless, our approach also has limitations. While 25 μM ZnT8 peptides induced strong IP-10 production in the PBMC cultures from healthy adults, 2.5 μM peptides did not. This discrepancy might be due to the activation of CD4+ T cells by large amounts of peptides, with relatively low avidity or potentially cross-reactive. Furthermore, previous studies show that truncation and extensions of peptide sequences by a single amino acid might change the quality of expanded T cells in terms of cytokine expression profiles [47]. Therefore, suboptimal peptide ligands might alter the outcome. Thus, to determine the type of antigen-specific T cells with this approach, two points should be considered: testing multiple epitope peptides rather than single peptide from a library and using low concentration of peptides.

Finally, we compared the quality of ZnT8-specific T cell responses between healthy adults and T1D patients by using the identified peptides in this study. Herein we used peptides at a 10-fold less concentration than the first set (2.5 μM), to minimize the activation of low-affinity T cells and possible cross-reactivity. We found that ZnT8-specific Th1 cells were more prevalent in T1D patients compared to healthy adults, which is consistent with a recent study [21]. Furthermore, we found that Th2 and IL-10-producing cells were dominant among ZnT8-specific CD4+ T cells in healthy controls. This is in line with the previous study demonstrating that IA-2 and proinsulin-specific CD4+ T cells in healthy adults secrete IL-10 rather than IFN-γ [19]. Thus, our

**Table 4.** Clinical characteristics of T1D patients and HLA-matched controls.

|                  | T1D  | Ctrl |
|------------------|------|------|
| n.               | 15   | 15   |
| Age (years)      | 37.4±11.8 | 37.2±12.4 |
| Gender (M/F)     | 8/7  | 8/7  |
| Disease duration (days) | 285.9±247.9 | NA |
| HLA-DRB1*0301+ (DQB1*0602-) | 4    | 4    |
| HLA-DRB1*0401+ (DQB1*0602-) | 5    | 2    |
| Other HLA-DR4+   | 2    | 3    |
| HLA-DR3-DR4- or DR3-DQB1*0602+ | 4    | 6    |

T1D, Type 1 diabetes patients; Ctrl, HLA-matched controls; n., number; M, male; F, female; NA, not applicable.

doi:10.1371/journal.pone.0055595.t004

**Table 5.** Number of positive cytokine responses induced by 2.5 μM of ZnT8 peptides.

| Peptide # | IL-10 response | IP-10 response |
|-----------|----------------|----------------|
|           | T1D | Ctrl | T1D | Ctrl |
| p2        | 0   | 0    | 2   | 0    |
| p18       | 0   | 1    | 1   | 0    |
| p65       | 0   | 1    | 5   | 0    |
| p68       | 0   | 0    | 1   | 0    |

doi:10.1371/journal.pone.0055595.t005

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![Figure 6](https://example.com/figure6.png)  
**Figure 6.** Cytokine secretions from ZnT8-specific T cells in T1D patients and healthy adults in the Direct assay. PBMCs from 15 T1D patients and 15 age/gender/HLA-matched controls were stimulated with 2.5 μM of ZnT8 single peptides (p#2, p#18, p#65, and p#68) in duplicates for 48 hours and six cytokines secreted during the stimulation were measured. (A) Representative results of ZnT8-specific IL-10 and IP-10 secretion with T1D patient samples. (B) Summary of cytokine secretion patterns. The cultures that scored positive in any cytokines were selected. Data was transformed into a heat-map format indicating the fold increase from the background.

doi:10.1371/journal.pone.0055595.g006
study further extends this observation, and shows that ZnT8-specific CD4+ T cell subsets are distinctly skewed between T1D patients and healthy adults.

In conclusion, our integrated approach is able to determine the breadth, type, and frequency of antigen-specific T cells simultaneously. Application of this approach might facilitate the characterization of antigen-specific T cell repertoires in many immune-mediated diseases.

Methods

Blood Samples
Peripheral blood samples were obtained from 10 healthy adults (HS #1-#10) in Baylor Research Institute, 15 T1D patients (T1D #1-#15) and 15 age/gender/HLA-matched controls (Ctrl #1-#15) in Benaroya Research Institute, who signed the written informed consent forms. This study was approved by the institutional review board of Baylor Research Institute (project no. IRB71009) and Benaroya Research Institute (project no. IRB71099). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll-Paque PLUS (GE-Healthcare Bio-Sciences, Piscataway, NJ) from sodium-heparinized blood within 24 hours after sampling and kept frozen at −80 C until use.

Overlapping Peptide Libraries
A 15-mer overlapping peptide library was designed to cover the entire amino acid sequence of islet-specific antigens with 11 amino acid (AA) overlaps (BioSynthesis, Lewisville, TX). Three peptide libraries; GAD65 (585 AA, 144 peptides), PPI (110 AA, 25 peptides), and ZnT8 (369 AA, 94 peptides) were used (Table 1). Peptides were dissolved at 10 mM with 50% acetonitrile (Sigma-Aldrich Co., St. Louis, MO) and kept frozen at −80 C.

Cell Cultures
PBMCs were re-suspended at a concentration of 2.5×10^6 cells/ml in complete medium (CM); RPMI 1640 medium (GIBCO, Carlsbad, CA) supplemented with 1% L-glutamine (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 50 μM 2-
β-mercaptoethanol (Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 1% non-essential amino acids (Sigma-Aldrich), and 10% heat-inactivated human AB serum (Gemini Bio-Products, Sacramento, CA). The cell viability was examined with 0.4% trypan-blue solution (Sigma-Aldrich) and was always >95%. Five x 10⁵ cells per well were cultured in a 96-well deep well plate in the presence of single peptides (0.04–25 μM each peptide) or peptide clusters (pooled 4–11 single peptides/cluster at 10 μM each peptide). An equal amount of peptide diluent was used as a negative control. On day 2 of culture, 80 μl of culture supernatant was harvested for the cytokine secretion assay. To expand the antigen-specific T cells, 100 U/ml recombinant human IL-2 (TECIN [Teceleukin]; Roche, Nutley, NJ) was added to the culture at day 2 of culture. Cells were harvested on day 7 of culture, rested overnight in fresh media, and re-stimulated with the same peptides for 6 hours for an intracytoplasmic cytokine expression assay and 24 hours for the cytokine secretion assay.

Intracytoplasmic Cytokine Expression Assay

PBMCs were re-suspended with GM at a concentration of 2.5 x 10⁵ cells/ml, and stimulated with 2.5 or 25 μM of the identified peptides for 6 hours in the presence of CD28/CD49d co-stimulatory antibodies (BD Biosciences, San Jose, CA). Brefeldin A (Golgi Plug™; BD Biosciences) was added for the last 4 hours of culture. After surface staining, cells were fixed, permeabilized, and then stained for intracytoplasmic cytokines. The used antibodies are listed in Table S3. Stained cells were acquired by a FACScantoII™ flow cytometer (BD Biosciences) and the data were analyzed with FLOWJO software (Tree Star, Inc., Ashland, OR). CD28/CD49d co-stimulatory antibodies were not used for ICS in the Cytokine-driven assay.

Cytokine Secretion Assay

Secreted cytokine levels (IL-2, IL-10, IL-13, IL-17A, and IP-10 for the Direct assay; and IL-4, IL-5, IL-10, IL-13, IL-17A, IL-21, TNF-α, and IFN-γ for the Cytokine-driven assay) were measured by a multiplex bead-based cytokine assay (BIO-RAD, Hercules, CA). Data were analyzed using the software GraphPad PRISM. For the visualization of the secreted cytokine levels, the data were transformed into a heat-map format indicating the fold increase from the background.

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