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Analysis of pancreatic beta cell specific CD4+ T cells reveals a predominance of proinsulin specific cells

Gabriele Blahnik[a], Hannes Uchtenhagen[a], I.-Ting Chow[b], Cate Speake[b], Carla Greenbaum[b], William W. Kwok[b], Eddie A. James[a,*]

[a] Translational Research Program, Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA
[b] Diabetes Program, Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA

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ABSTRACT

CD4+ T cell responses are thought to play a role in type 1 diabetes (T1D). However, detection and characterization of T cells that respond to beta cell epitopes in subjects with T1D has been limited by technical obstacles, including the inherently low frequencies in peripheral blood and variable responsiveness of individual subjects to single epitopes. We implemented a multicolor staining approach that allows direct *ex vivo* characterization of multiple CD4+ T cell specificities in a single sample. Here we demonstrate and apply that multicolor approach to directly measure the frequency and phenotype of beta cell specific CD4+ T cells in T1D patients and HLA matched controls. For this work we utilized five DR0401 restricted peptides from proinsulin, GAD65, IA-2, and IGRP, which were previously reported as disease relevant epitopes. Surprisingly, although responses to each of these peptides can be readily detected after *in vitro* expansion, our results indicated that only proinsulin specific T cells were consistently detectable at moderate frequencies in subjects with T1D. Characterization of beta cell specific CD4+ T cells revealed only modest differences between subjects with T1D and healthy controls. Subjects with T1D did have higher proportions of CD45RA negative epitope specific T cells than controls. In patients epitope specific T cells were often CXCR3 positive and a substantial proportion were CCR7 negative, suggesting a Th1-like effector phenotype. Finally, we demonstrated that our multicolor staining approach is compatible with class I multimer analysis, facilitating the characterization of self-reactive CD4+ and CD8+ T cells using a single sample.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive T cells home to the pancreas and contribute to pancreatic beta cell death through secretion of inflammatory cytokines and direct cytotoxicity. The disease is strongly associated with a few susceptible HLA class II haplotypes [1], which are thought to select a potentially autoreactive CD4+ T cell repertoire. Among these, DR0401 – corresponding to a heterodimer formed by the essentially invariant Human leukocyte antigen (HLA) DRA*01:01 alpha chain and HLA-DRB*04:01 beta chain – is extremely well-studied, such that DR0401-restricted epitopes from many beta cell associated antigens (including proinsulin, GAD65, IGRP, and IA-2) have been previously defined [2–5]. Beta cell reactive T cells have been widely researched using a variety of methods, but with conflicting results. Whereas some studies report that auto-reactive T cells occur at higher frequencies and have a more inflammatory phenotype in subjects with T1D than in healthy subjects [6,7], other studies observe modest differences or no differences between patients and controls [3,8].

Given their proposed but indeterminate role in disease etiology, there is a strong rationale for broader attempts to characterize and monitor CD4+ T cell responses in subjects who are at risk for or have been diagnosed with autoimmune disease. Accurate measurement of the frequency and cell surface marker profiles of such cells would be desirable, but technical and biological obstacles have made this task difficult [9]. The first major obstacle is the inherently low frequencies of autoreactive CD4+ T cells [10], which have been reported in ranges as low as 1 cell per million [11]. For this reason, many CD4+ T cell assays utilize an *in vitro* amplification step to increase the limit of detection of these rare cells [12]. Unfortunately, such manipulation limits the reliability of functional readouts through the possible introduction of bias during culture and also has the potential to obfuscate differences

Abbreviations: T1D, Type 1 Diabetes

* Corresponding author at: Benaroya Research Institute at Virginia Mason, 1201 9th Ave, Seattle, WA 98101, USA.
E-mail address: ejames@benaroyaresearch.org (E.A. James).

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between patients and healthy controls through unbalanced expansion of different T cell subsets (e.g. naïve versus memory). Therefore, it would be advantageous to implement an assay that can effectively analyze autoreactive T cells directly *ex vivo*. A second major obstacle is the variable responsiveness that has been reported between individual subjects toward single epitopes [4]. Specifically, a particular epitope specificity might be immunodominant in some subjects but virtually undetectable in others. In light of such variability, assays that offer the possibility of interrogating multiple epitopes are likely to provide useful information. HLA multimer staining is an attractive option for monitoring T cells, as this methodology enables direct characterization, quantification and sorting of defined antigen specific T cells independent of their functional phenotype [13]. Multicolor staining approaches to detect multiple specificities in a single staining tube have been successfully implemented to monitor CD8+ T cells that recognize beta cell antigens [14,15]. With recent developments, the analogous technology can now be applied for analysis of auto-reactive CD4+ T cells.

Several years ago, Day et al. developed a methodology for enriching and analyzing multimer labeled CD4+ T cells using anti-PE magnetic beads [16]. Our group implemented a similar magnetic enrichment methodology and applied this approach to analyze epitope specific CD4+ T cells in a variety of settings, including autoreactive specificities that occur at low frequencies in subjects with type T1D [17–20]. More recently, we developed second generation multimer reagents that include a Myc fusion tag, allowing us to implement an anti-Myc enrichment procedure that facilitates direct *ex vivo* detection of CD4+ T cells using any fluorescent label of interest [21]. Combining these resources, we compared the efficacy of HLA class II multimers assembled using a variety of streptavidin-fluorophore conjugates and devised a multicolor panel that allowed us to efficiently characterize CD4+ T cell responses to the seasonal influenza vaccine in healthy subjects and a limited number of rheumatoid arthritis patients. That work demonstrated the technical reliability of our approach and revealed a consistent frequency hierarchy for influenza hemagglutinin epitopes.

Our own work and other published studies suggest that self-reactive T cells in patients with T1D can be most effectively monitored *in vitro* by using a combination of epitopes from antigens such as proinsulin, GAD65, IGRP, and IA-2 [2–5]. That body of work defined multiple epitopes that are naturally processed and presented and reported to preferentially elicit responses in affected individuals, but no single epitope specificity emerged that was present in every subject. Building on our recent work with influenza specific responses, we reasoned that an analogous approach should be applicable to efficiently characterize beta cell reactive T cells in subjects with T1D. In this present study we apply a multicolor staining strategy to simultaneously characterize beta cell reactive CD4+ T cells in patients with T1D and HLA matched controls. Using this strategy, we measured T cells recognizing five distinct beta cell epitopes (plus one influenza epitope as an internal control) in a single staining tube, directly characterizing their number, memory status, and chemokine receptor expression profile. Our results confirm the feasibility of multicolor labeling for detecting rare autoreactive T cell specificities in a single staining tube and revealed an unexpected frequency hierarchy for the epitopes studied.

2. Materials and methods

2.1. Patients and control subjects

Subjects with T1D and healthy control subjects, all with HLA DR0401 haplotypes were recruited though the Benaroya Research Institute (BRI) diabetes and control registries with informed consent, under protocols approved by the BRI Institutional Review Board. The attributes of these subjects are summarized in Table 1. Subjects with T1D had an average age of 29.8, were sampled an average of 3.5 years after diagnosis, and were typically antibody positive for GAD and at least one other self-antigen. Healthy control subjects had an average age of 49.5. This deficiency in age matching was not ideal; however, there is little evidence to suggest that autoreactive responses diminish with age in healthy subjects.

Table 1
Demographic information for HLA-DR04:01+ participants.

Subject ID	Age	Gender	T1D duration	Positive Ab	HLA-A2+
T1D #1	31	M	3.9 years	Ins/GAD	No
T1D #2	30	M	1.2 years	Ins/GAD	Yes
T1D #3	16	F	3.5 years	Ins/GAD/IA2	No
T1D #4	14	M	1.3 years	Ins/GAD/IA2/ZnT8	Yes
T1D #5	27	F	5.0 years	Ins/GAD/ZnT8	Yes
T1D #6	34	F	4.6 years	Ins/GAD	Yes
T1D #7	34	F	7.3 years	Ins/GAD	No
T1D #8	45	M	4.5 years	GAD	Yes
T1D #9	27	M	1.5 years	GAD/Ins/IA2/ZnT8	Yes
T1D #10	39	F	2.9 years	GAD/Ins/IA2	Yes
T1D #11	43	M	5.7 years	GAD/IA2	No
T1D #12	28	F	2.3 years	GAD/IA2/ZnT8	Yes
T1D #13	19	M	1.7 years	Ins/GAD/IA2/ZnT8	Yes
Control #1	54	F	N.A.	N.A.	N.A.
Control #2	50	M	N.A.	N.A.	N.A.
Control #3	66	F	N.A.	N.A.	N.A.
Control #4	36	F	N.A.	N.A.	N.A.
Control #5	66	F	N.A.	N.A.	N.A.
Control #6	58	F	N.A.	N.A.	N.A.
Control #7	32	F	N.A.	N.A.	N.A.
Control #8	59	F	N.A.	N.A.	N.A.
Control #9	55	F	N.A.	N.A.	N.A.
Control #10	27	M	N.A.	N.A.	N.A.
Control #11	45	F	N.A.	N.A.	N.A.
Control #12	46	F	N.A.	N.A.	N.A.

2.2. HLA class I and HLA class II multimers

Recombinant HLA-DRA*01:01/DRB1*04:01 (DR0401) was produced by the BRI Tetramer core as previously described [21,22]. Briefly, DR0401 or DR0401-myc protein was purified from insect cell culture supernatants by affinity chromatography, biotinylated with biotin ligase, and dialyzed against phosphate buffer (0.0625 M monobasic sodium phosphate, 0.0375 M monobasic sodium phosphate, pH 6.0). To prepare multimers DR0401 or DR0401-myc monomer was incubated with 0.2 mg/mL peptide, 0.2% n-octyl-β-D-glucopyranoside (Sigma) and 1 mM Pefabloc SC (Sigma) at 37 °C for 72 h. After incubation PE, APC, PE-Cy5, PE-CF594, or BV421 streptavidin was conjugated to make multimers. As summarized in Table 2, each beta cell epitope was labeled using a unique pair of fluorochromes. The viral control epitope, influenza matrix protein (MP) 97–116, was labeled with BV421. HLA-A2 multimers were obtained through the NIH Tetramer Core Facility. The epitopes and staining strategy corresponded exactly to those used in other recent studies [15].

2.3. In vitro generation of T cell clones and spiked sample preparation

PBMCs were isolated using a ficoll underlay, plated at 4×10^6 cells/mL in T cell media (standard RPMI supplemented with 10% pooled human serum, 1% penicillin-streptomycin, 1% L-glutamine), and stimulated with groups of beta cell derived peptides in 48 well plates, supplementing with IL-2 after the first week of culture and splitting to new wells as needed. After 14 days cells were stained with the corresponding DR0401 multimers at 37 °C for 75 min and then stained with CD25 FITC (BioLegend, Clone PC61), CD3 APC (BioLegend, Clone UCHT1), and CD4 PerCP (BioLegend, Clone OKT4) at 4 °C for 15 min. Samples were analyzed on a FACSCalibur and FlowJo. Positive wells were restained under the same conditions with the same multimers and antibodies and multimer positive CD4+ T cells were single cell sorted into a 96 well plate using a FACS AriaII. Cells were expanded in 96 well

Table 2
Multicolor multumer panel for HLA-DR04:01 restricted epitopes.

Epitope	Name	Sequence	Color 1	Color 2
1	GAD 113–132	DVMNILLQYVVKSFDRSTKV	PE	PE-Cy5
2	GAD 273–292	LIAFTSEHSHFSLKKGAAAL	PE	PE-CF594
3	PPI 78–92	SLQPLALEGSLQKRG	PE	APC
4	IGRP 241–260	KWCANPDWIHIDTTPFAGLV	PE-CF594	APC
5	IA2 709–732	LAKEWQALCYQAEPNTCATAQGEG	PE-Cy5	APC
6	Flu MP 97–116	VKLYRKLKREITFHGAKEIS	BV421	–

with 1×10^5 irradiated PBMC from an unrelated donor, 2 ug/mL phytohemagglutinin and IL-2 (Roche, 10 units/mL). Plates were checked after 10 days for growth, media was changed and IL-2 added. Expanding cells were stained with multimers to confirm specificity. T cell clones specific for GAD 113, GAD 273, IGRP 241, IA2 709, PPI 78, and MP54 were screened by re-staining with the corresponding multimer (to confirm positive staining) or a mismatched multimer. T cell clones were counted and diluted in the PBMC of a non-DR0401 healthy individual to generate a 20x spiked control sample with each clone at approximately 0.5% of the total cell number. The composition of this 20x spiked control was examined by flow cytometry and further diluted 20-fold with additional PBMC from the same healthy individual. The spiked sample was then frozen and run as a positive control in for Class II multimer staining.

2.4. Ex vivo class II multimer assays

Ex vivo multimer staining was carried out using established protocols [21]. Frozen, cells were thawed in T cell medium (standard RPMI supplemented with 10% pooled human serum, 1% penicillin-streptomycin, 1% L-glutamine) plus Benzonase (1:5000 dilution). Once isolated, 20–30 $\times$ 10^6 PBMCs were resuspended in 200 uL T cell medium and incubated with 50 nmol/L Dasatinib for 10 min at 37 °C to improve the detection of auto-reactive T cells as described by Lissina et al. [23]. Individual preparations of each single multimer were prepared at a final concentration of 0.5 mg/mL as described above. A master mix of multimers was then prepared by combining 5 uL each of GAD113-PE and PE-Cy5, GAD273-PE and PE-CF594, IGRP241-PE-CF594, IA2709-PE-Cy5, PPI78-PE, MP54-BV421 and 8 uL each of IGRP241-APC, IA2709-APC, and PPI78-APC. PBMCs were stained with the multimer master mix for 120 min at room temperature in the dark, washed, then incubated with anti-PE, anti-APC and anti-Myc magnetic beads (Miltenyi Biotec) for 20 min at 4 °C. Cells were washed twice and 2.5% of the cells were saved for analysis. The remaining cells were enriched with a magnetic column, then removed from column, flushed and collected. The enriched and pre-column samples were stained with CD4-V500 (BD Biosciences, Clone RPA-T4), CD45RA-AF700 (BioLegend, Clone HI100), CXCR3-FITC (BioLegend, Clone G025H7), CCR4-BV605 (BioLegend, Clone L291H4), CCR7-APC/Cy7 (BioLegend, Clone G043H7) and a combination of CD14-PerCP-Cy5.5 (BioLegend, Clone M5E2), and CD19-PerCP-Cy5.5 (BioLegend, Clone 6D5) for 15 min at 4 °C. Samples were washed then labeled with ViaProbe (BD Biosciences) and run on a BD LSRII. To classify samples as having a reportable frequency for a given epitope we applied a limit of detection of 1 cell per million, which was previously established by serially diluting a PBMC sample that contained an easily measurable frequency of epitope specific T cells with irrelevant PBMC [17].

2.5. Ex vivo class I multimer assays

Class I multimer staining was carried out using frozen PBMCs that were HLA-A2 positive as recently described [15], except that the previously published IAPP epitope was replaced by a ZnT8 epitope [24]. Vials were thawed at 37 °C then diluted in 50% FBS + 50% TCM. Once washed and counted, 2 million cells were resuspended in 100 uL and incubated with 50 nmol/L Dasatinib for 10 min at 37 °C. After incubation cells were washed and stained with a multimer master mix containing: CMV-EBV-Flu QDot 585 and QDot 800, Negative control QDot 585 and QDot 605, INS B10-18 QDot 605 and QDot 655, PPI 15–24 QDot 705 and QDot 655, GAD 114–122 QDot 800 and QDot 655, IA2 979–805 QDot 705 and QDot 605, IGRP 265–273 QDot 800 and QDot 605, ZNT8 186–194 QDot 705 and QDot 800 (all Qdots from ThermoFisher Scientific). QDot multimers were incubated for 15 min at 37 °C then stained with CD8-APC (BD Biosciences, Clone RPA-T8), CXCR3-BV421 (BioLegend, Clone G025H7), CD45RA-AF700 (BioLegend, Clone HI100), CD4-FITC (BioLegend, Clone GK1.5), CD14-FITC (BioLegend, Clone M5E2), CD16-FITC (BioLegend, Clone 3G8), CD20-FITC (BioLegend, Clone 2H7), and CD40-FITC (BioLegend, Clone 3/23) for 30 min at 4 °C. Cells were washed twice after antibody staining then resuspended in 7-AAD buffer and run on a BD LSRII.

2.6. Statistics

All statistical tests were performed using PRISM version 7 software (GraphPad). Two-way comparisons (e.g. between subjects with T1D and controls) of T cell phenotypes (expressed as the percentage of epitope specific cells that were positive for the marker of interest) were compared using Student's *t*-test, applied to log transformed to render populations quasi-normal. Multiple comparisons of T cell frequencies (expressed as the number of epitope specific cells per million CD4+ T cells) were made by ANOVA (also utilizing log transformed data) and applying Tukey's post-test with multiple testing correction. Contingency analysis and Fisher's exact test was used to evaluate differences in the proportion of detectable responses to beta cell epitopes.

3. Results

3.1. Developing a multicolor HLA class II beta cell multimer panel

Multi-color HLA multimer labeling has been successfully implemented in other studies to detect beta cell specific CD8+ T cells of multiple specificities in a single staining tube [14,15]. More recently, a similar method was applied to characterize influenza specific CD4+ T cells; a magnetic enrichment step was necessary because of the inherently low frequencies of epitope specific CD4+ T cells [21]. To implement an analogous strategy for characterizing beta cell specific CD4+ T cells, we focused on established beta cell antigens (GAD65, IGRP, IA-2, and insulin) for which one or more naturally processed DR0401 restricted epitopes have been previously reported. Among the well-characterized epitopes derived from these antigens, we were able to isolate multimer positive T cell clones for five (GAD 113, GAD 273, IGRP 241, IA2 709, and PPI 78), providing a positive control for each of these epitopes to verify the staining activity of each corresponding DR0401 tetramer. For each of these specificities, multimer staining of the reference T cell clone and an irrelevant T cell clones is shown in Supplementary Fig. 1. Consequently, these specificities were selected for inclusion in our staining panel. In addition, a conserved influenza epitope (MP 97–116) was included as an effective positive control [21]. Using these T cell clones, we also generated a clone spiked control sample (PBMC from a DR4-negative subject spiked with T cell clones

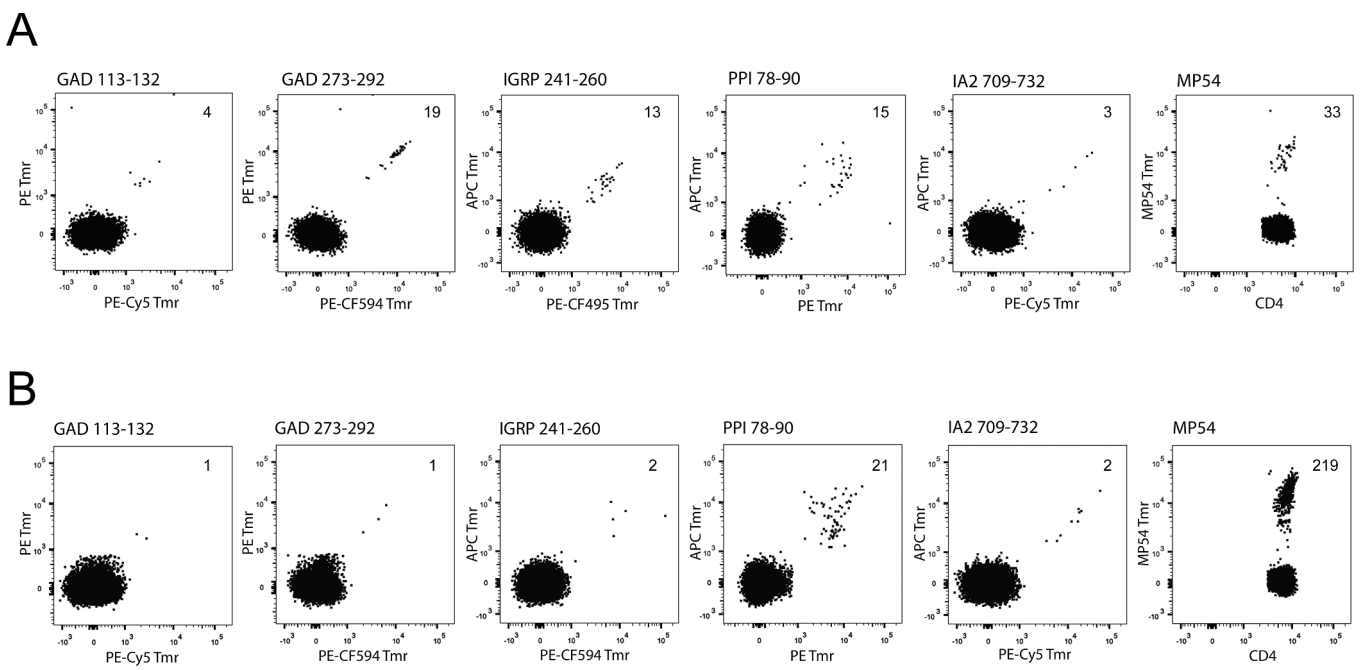

Fig. 1. Multicolor HLA class II multimer staining of clone spiked and peripheral blood samples. (A) *Ex vivo* multimer analysis of peripheral blood mononuclear cells from a DR0401 negative healthy control spiked with T cell clones specific for each respective epitope. The data shown in each panel was Boolean gated for the specified colors and negative gated for all other multimers – except for MP54, which was single stained with BV421. T cell frequencies are shown in the upper right corner of each sub-panel as T cells per million. (B) Multiucolor multimer staining of peripheral blood mononuclear cells from a representative DR0401 positive T1D patient. The data was Boolean gated as in panel A. T cell frequencies are shown in upper right corner of each sub-panel as T cells per million. A previously defined threshold of 1 cell per million was applied to define detectable responses.

specific for all of the epitopes of interest and then serially diluted with additional PBMC) for use as a positive control in our panel development and subsequent experiments.

The beta cell epitopes included in our multiplex staining panel were supported by previously published studies [2–5]. Each of these beta cell epitopes had been reported to elicit a detectable population of tetramer positive T cells following one round of *in vitro* stimulation. However, the frequency of T cells that recognize these beta cell derived epitopes that can be detected in un-manipulated peripheral blood had not been previously characterized. To allow simultaneous assessment of T cell frequencies for all of these specificities, each epitope was labeled by a unique pair of colors, as summarized in Table 2. The resulting class II multimer panel was first evaluated by staining the spiked control sample. As shown in Fig. 1A, the clone spiked sample had positive populations for all of the expected two-color combinations that corresponded to individual beta cell epitopes and for the influenza control. A comparison of the expected spike-in levels and detected frequencies for each T cell clone is provided in Supplementary Table 1. In agreement with the known spike-in levels, observed frequencies varied for the different epitope specificities. Notably, the detection efficiency was somewhat lower for GAD 113 and IA2 709, suggesting that frequencies for these two epitopes could have been underestimated by up to 30 percent. As shown for a representative sample in Fig. 1B, the class II multimer panel was also able to detect populations of T cells for each epitope within PBMC samples from T1D samples. Consistent with previously published approaches, we utilized Boolean gating as part of our analysis to exclude cells that had a positive for three or more multimer channels [14,21]. A data overlay showing events that were included or excluded form analysis based on Boolean gating is shown in Supplementary Fig. 2.

To verify that the T cell frequencies detected by the simultaneous multicolor class II multimer approach are consistent with those that would be detected through the more traditional approach of examining a single epitope per staining tube, we obtained larger blood draws from a limited number (n = 2) of T1D patients and stained PBMC using both the "single tube" multicolor staining approach and by staining each epitope with PE-labeled tetramers in separate tubes. As summarized in Supplementary Fig. 3, the epitope frequencies detected using the multicolor multimer panel agreed closely with the single PE tetramer staining results (R square = 0.993, $P < 0.0001$), supporting the reliability of this approach.

3.2. Analysis of beta cell specific T cells in patients

Having shown that the multicolor class II multimer panel is technically reliable, we next examined T cell frequencies in 13 subjects with T1D, all with DR0401 haplotypes (Table 1). Influenza MP specific T cells were detected at high frequencies in every subject, with an observed mean frequency of 202 per million (Fig. 2A). As expected, the mean frequencies for beta cell epitopes were considerably lower, varying between 0.9 per million CD4 cells (for GAD 113) and 13.3 (for PPI 78). Observed mean T cell frequencies for most of the beta cell epitopes (GAD 113, IGRP 241, and GAD 273, and IA2 709) were all well below 10 cells per million. This was somewhat surprising, given that *in vitro* responses to each of these epitopes could be detected in prior studies [2–5]. However, similar (or even lower) *ex vivo* frequencies have been reported for other disease relevant epitopes, such as Insulin B 9–23 [11]. In contrast, the observed frequencies for PPI 78 were significantly higher (all adjusted p values were < 0.0001, Tukey's post-test with multiple testing correction) than the frequencies of all other beta cell epitopes, exceeding 10 cells per million in most subjects. Therefore, PPI 78 was the predominant specificity among these subjects with established T1D. By cataloguing epitope specific T cell frequencies in each individual subject through the heat map (Fig. 2B) individual patterns of T cell reactivity were evident in different subjects. Each subject had a detectable frequency of PPI specific T cells and in many cases this was the predominant specificity; subjects 5 and 9 were notable exceptions in that their highest frequencies were GAD 273 and IA2 709, respectively. All subjects had detectable T cell frequencies for PPI 78 and at least one additional epitope (Supplementary Table 2, top

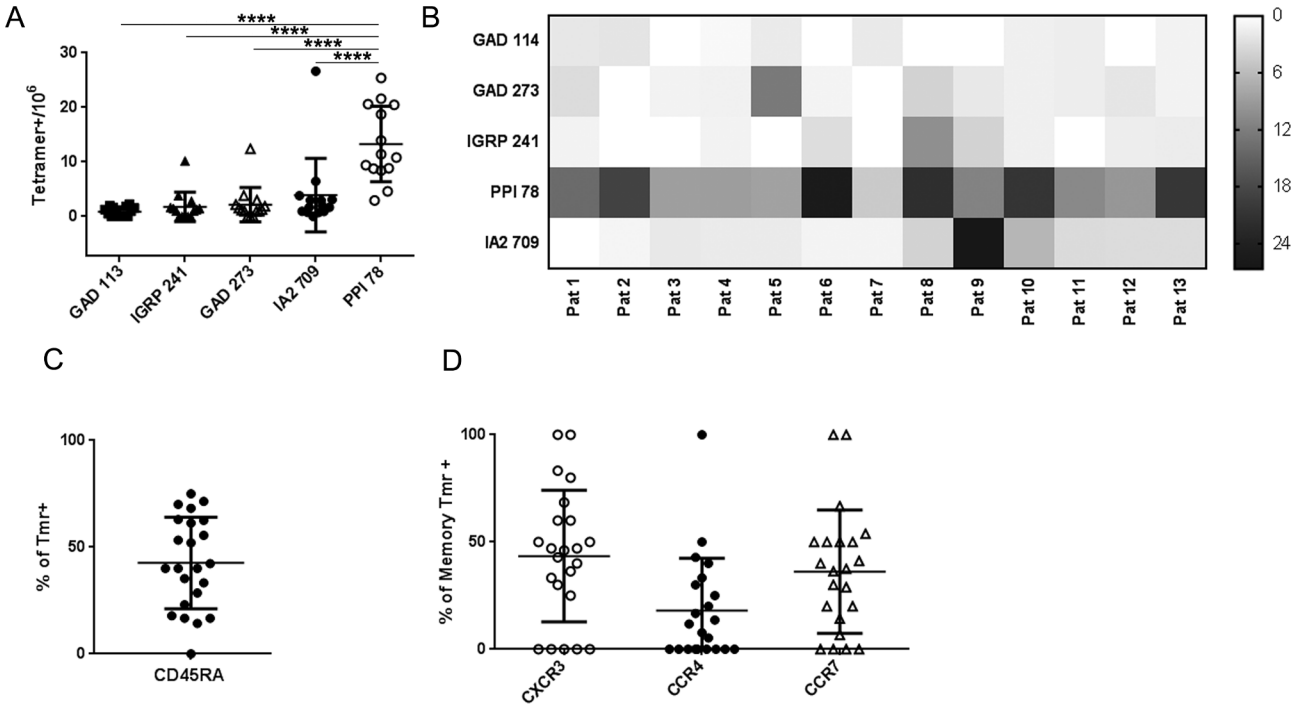

Fig. 2. Frequency and phenotype of beta cell specific T cells in subjects with T1D. (A) *Ex vivo* frequencies of epitope specific T cells from individuals with T1D (n = 13). CD4 T cell frequencies are shown for all five epitopes as cells per million total CD4+ cells. The PPI epitope was significantly more frequent than all other epitopes ($p < 0.0001$). (B) Heat map depicting the *ex vivo* frequencies of epitope specific T cells from individuals with T1D (shown in panel A as grouped data). As indicated by the legend key, T cell frequencies for individual epitopes ranged from 0 to 24 cells per million. In each column, the darkest shaded box indicates the predominant specificity. (C) The percentage of CD45RA expression for pooled epitope specific T cells from the same individuals. Epitopes with fewer than five events were excluded from this analysis. (D) Surface phenotype of epitope specific T cells. The percentage of epitope specific T cells that express CXCR3, CCR4 and CCR7 (gated on the CD45RA negative population) is shown for each population of epitope specific cells. Epitopes with fewer than five events were excluded from this analysis.

portion), but nearly every subject lacked a detectable frequency for at least one epitope.

In addition to the multimer channels used to examine frequency, our staining panel also included cell surface markers to reveal some aspects of the phenotype of multimer positive cells. These included CD45RA (to draw inferences about antigen experience) CCR7 (to draw inferences about effector status), CXCR3 (associated with a Th1-like status), and CCR4 (associated with a Th2-like status). Representative cell surface staining results for MP54 or PPI 78 multimer positive cells are shown in Supplementary Fig. 4. With the exception of PPI 78, the frequency of T cells was relatively low for beta cell epitopes (Fig. 2A). Consequently, it was necessary to pool all beta cell specificities together to attempt a meaningful surface marker analysis. Doing this, we observed highly variable percentages of epitope specific T cells that were CD45RA positive among the different subjects with T1D (Fig. 2C), suggesting varying degrees of exposure to cognate antigen *in vivo*. Among the memory-enriched CD45RA negative population (Fig. 2D), beta cell specific T cells tended to be CXCR3 positive rather than CCR4 positive, which could be taken to suggest a Th1-like bias. Surprisingly, variable percentages of CD45RA negative epitope specific T cells were CCR7 negative, suggesting that in some subjects with established T1D an appreciable fraction of T cells persist in an effector-like state.

3.3. Comparison of beta cell specific T cells in patients and controls

We performed the same analysis for 12 healthy subjects with DR0401 haplotypes (Table 1). Consistent with a recent report that examined islet-reactive CD8+ T cell frequencies [8], we observed no significant differences between healthy subjects and T1D subjects in the frequencies of islet specific CD4+ T cell (Fig. 3A). All healthy subjects had detectable T cell frequencies for PPI 78 and at least one other epitope (Supplementary Table 2, bottom portion), and although the proportion of detectable responses was consistently lower than in subjects with T1D, those differences did not reach statistical significance (Fisher's exact test). With respect to surface marker expression, subjects with T1D had a significantly lower percentage of CD45RA+ T cells than HLA matched controls ($p = 0.0173$) (Fig. 3B), suggesting that autoreactive T cells have undergone more antigen exposure in subjects with T1D. Among memory T cells we observed no statistically significant differences in surface expression of CXCR3, CCR4, or CCR7. For subjects with T1D there were trends toward lower percentages of CCR7 positive cells (which would be consistent with a more effector-like state) and higher percentages of CXCR3 positive T cells (which would be consistent with a more th1-like bias) than for healthy subjects (Fig. 3C). However, given the relatively modest numbers of epitope specific cells, these findings should be interpreted with caution.

3.4. Combined HLA class I and class II multimer analysis of beta cell specific T cells

Since it would be desirable to characterize autoreactive CD4+ and CD8+ T cells in the same samples, we also performed a multicolor class I multimer analysis for the nine subjects with T1D in our study who were HLA-A2 positive, using a previously published HLA class I Qdot multimer staining panel, except that the previously published IAPP epitope was replaced by a ZnT8 epitope (summarized in Supplementary Table 3) [15,24,25]. As in our prior study, class I multimer staining was considered positive for a given multimer if its staining was more than twice the staining level of the negative control (non-interfacing) multimer observed in that subject (typically a threshold of ~0.05%). The class I multimer staining results for these HLA-A2 positive subjects are

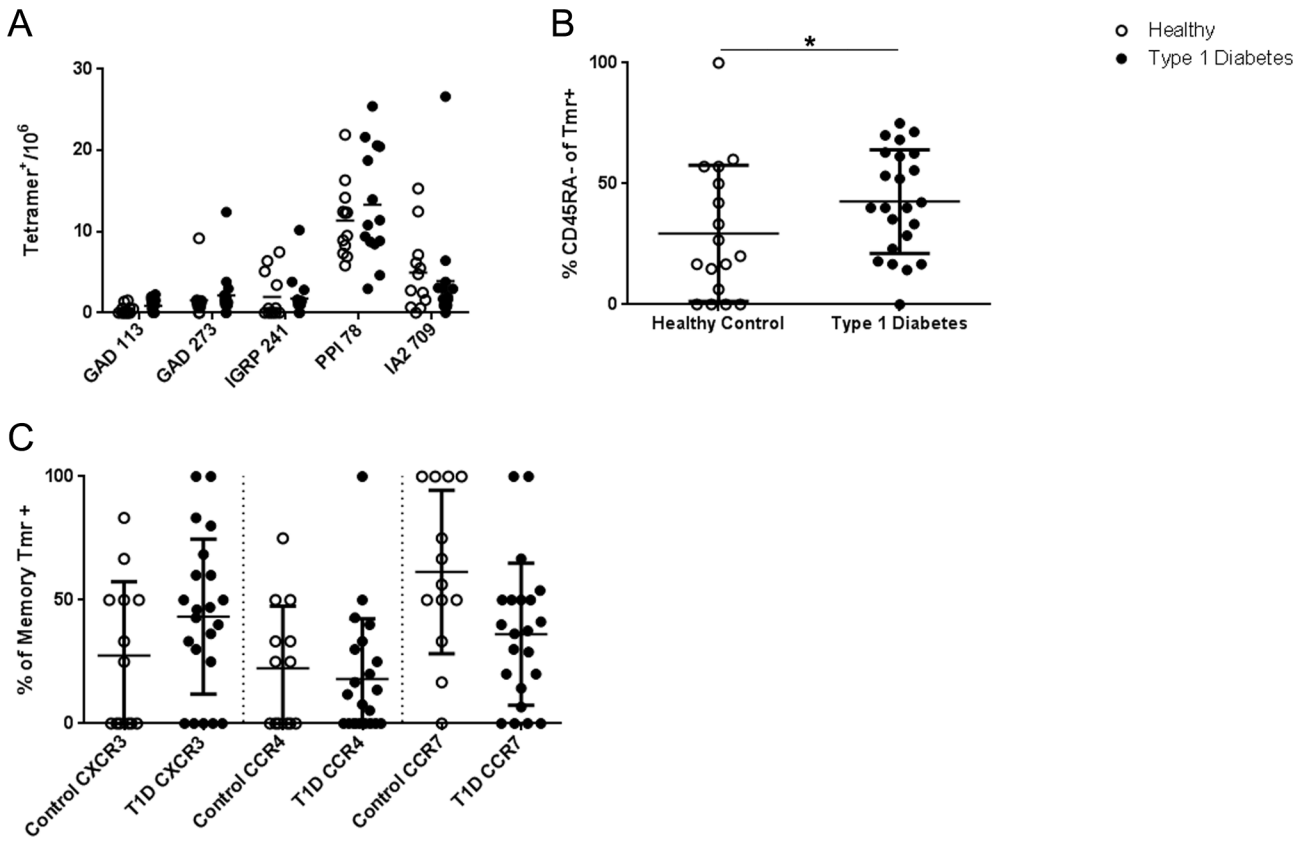

Fig. 3. Comparison of beta cell specific T cells in subjects with T1D and controls. (A) *Ex vivo* frequencies of epitope specific T cells tended to be higher in individuals with T1D (n = 13) than healthy controls (n = 12) but were not significantly different. Frequencies are shown as cells per million total CD4+ cells (B) The percentage of beta cell specific T cells with a memory-associated CD45RA- phenotype were significantly higher in subjects with T1D than in healthy controls (p = 0.017). (C) Surface phenotype of epitope specific memory T cells in subjects with T1D and healthy controls. The percentage of T cells that express CXCR3, CCR4 and CCR7 (gated on the CD45RA negative population) is shown for each population of epitope specific cells. Epitopes with fewer than five events were excluded from this analysis. Expression of CXCR3 tended to be higher and expression of CCR7 tended to be lower in subjects with T1D but these differences were not significant.

summarized in Supplementary Table 4 (the corresponding frequencies of class II multimer positive cells are summarized in Supplementary Table 2). In our previous study, nearly every subject had detectable multimer staining for the HLA class I viral epitopes and, among the beta cell epitopes, Ins B and GAD had the highest proportions of detectable responses, followed by IGRP and PPI (IAPP and IA-2 were rarely detected). Likewise, in the current study, all subjects were positive for the HLA class I viral epitopes and 8/9 were positive for Ins B and GAD whereas IGRP and PPI were only positive in 5/9 and 4/9 subjects respectively. Surprisingly, IA-2 class I multimer staining was detectable in 6/9 subjects for this group of patients. ZnT8, which was not included in our previous study was positive in two patients.

PPI, GAD, IA2 and IGRP multimers were present in both the class II and class I panels, allowing us to look for broad similarities between autoreactive CD4+ and CD8+ T cells (Table 3). The observed degree of overlap was modest. The level of agreement observed for IA2 was 66.7% (6/9); among the 9 samples from HLA-A2 positive subjects, 5 had detectable multimer staining for both class I and class II and 1 had coinciding negative class I and class II staining. The level of agreement for IGRP was 55.6% (5/9); 4 samples had detectable multimer staining for both class I and class II and one sample had coinciding negative class I and class II staining. The level of agreement for PPI was 44.4% (4/9); 4 samples had detectable multimer staining for both class I and class II and none of the samples had coinciding negative class I and class II staining. The similarity seen for GAD was more difficult to assess, given that we analyzed one HLA class I epitope and two class II epitopes. Considering just the GAD 113 epitope, which overlaps with the HLA-A2 epitope the level of agreement was 33.3% (3/9); 3 samples had detectable multimer staining for both class I and class II and none of the samples had coinciding negative class I and class II staining. Considering just the non-overlapping GAD 273 epitope, the level of agreement was 88.8% (8/9); 7 samples had detectable multimer staining for both class I and class II and one of the samples had coinciding negative class I and class II staining. We did note that subjects who had detectable multimer staining for multiple CD8+ T cell epitopes also tended to have a higher number of detectable CD4+ T cell epitopes. Consequently, we performed a correlation analysis by linear regression to determine whether this relationship was statistically significant (Supplementary Fig. 5). This analysis indicated that the number

Table 3
Combined analysis with HLA class I and class II multimers.

Subject*	HLA class I				HLA class II			
	PPI	GAD	IA2	IGRP	PPI	GAD	IA2	IGRP
T1D #2	−	−	−	−	+	−	−	−
T1D #4	−	+	−	+	+	−	−	−
T1D #5	−	+	−	+	+	+	−	−
T1D #6	−	+	+	−	+	+	−	+
T1D #8	−	+	+	+	+	+	+	+
T1D #9	+	+	+	+	+	−	+	+
T1D #10	+	+	+	+	+	−	+	−
T1D #12	+	+	+	−	+	+	+	−
T1D #13	+	+	+	−	+	−	+	−

* Subjects who are HLA-A2 negative are omitted from this table.

of detectable beta cell epitope specific CD8+ T cell populations and the number of beta cell epitope specific CD4+ T cell populations were significantly correlated ($p = 0.026$).

4. Discussion

Autoreactive CD4+ T cells have been implicated in the etiology of T1D, but there is an important remaining need to clarify their role by studying epitope specific cells in human samples. HLA multimer staining is an important technique for assessing the relative magnitude and probing phenotypic properties of antigen specific T cells. However, application of this technique to samples from clinical trials and observational studies, particularly those in the pediatric setting, has been limited by limited blood volumes, the inherently low frequencies of autoreactive T cells, and the wide breadth of epitope specificities that have been described. Prior studies have relied primarily on assays that include an *in vitro* expansion step to facilitate detection of inherently rare epitope specific cells within the diverse repertoire of T cells within peripheral blood. In aggregate these published studies have supported the immunogenicity, HLA restriction, and natural processing and presentation of numerous epitopes, including those that were selected for our study. Utilizing our recently published approach for multicolor HLA class II multimer staining we performed parallel *ex vivo* staining and analysis of five beta cell epitopes (plus an influenza control) in a single staining tube. This staining protocol facilitated T cell frequency assessment for each epitope and also allowed a limited characterization of the surface phenotype of epitope specific cells.

Consistent with other recently published studies, T cells that recognize the beta cell epitopes in our multimer panel were generally present at comparatively modest frequencies in peripheral blood. However, PPI 78 stood out as having comparatively higher frequencies in subjects with T1D. This finding was somewhat surprising, given that T cell responses toward all of these epitopes were easily and comparably detectable in assays that include an *in vitro* expansion step. Therefore, assays which utilize an *in vitro* expansion step apparently have the potential hazard of obfuscating the relative importance of different epitope specificities. The essentially universal presence of PPI 78 could be considered as a basis for pursuing this specificity in antigen specific tolerance approaches.

Comparing subjects with T1D to HLA matched controls, the observed frequencies were not significantly different. This was not completely unexpected, given a recent study which indicated that frequencies of beta cell specific CD8+ T cells may be similar in patients and controls [8]. Notably, all subjects with T1D had detectable T cell frequencies for PPI and at least one other epitope. Therefore, it would be feasible to monitor longitudinal changes in these T cell frequencies in the context of clinical trials or during the natural history of the disease. When all beta cell epitopes were combined, subjects with T1D had a significantly higher proportion of beta cell epitope specific T cells that exhibited a memory-associated CD45RA negative phenotype than controls. Although there were no statistically significant differences in surface expression of CXCR3, CCR4, or CCR7 between controls and subjects with T1D, we observed trends toward higher proportions of CXCR3 positive (possibly suggesting Th1-like responses) and lower proportions of CCR7 positive cells (which is consistent with an effector-like status). Since diverse levels of CXCR3 and CCR7 expression were observed for the subjects in this study, monitoring longitudinal changes in these surface markers could be informative in some settings.

Due in part to technical limitations, relatively little is known about the degree of similarity between autoreactive CD4+ and CD8+ T cell responses. Our study sought to address that question (albeit superficially) through a strategy that combined HLA class I and HLA class II multimer staining, providing frequency and phenotype data across six CD8+ and six CD4+ T cell specificities from a single sample. Although our study was not able to provide definitive conclusions, our findings suggest some relationship between the autoreactive CD4+ and CD8+ T cell specificities present in individuals with T1D. Specifically, our findings indicated that the number of detectable beta cell epitope specific CD8+ and CD4+ T cell populations observed in subjects with T1D were directly correlated. With the techniques presented here, further studies along these lines are clearly feasible.

Our study does have limitations which should be noted. With respect to our methodology, while the use of HLA multimers has the inherent advantage of unambiguously detecting T cells with the desired HLA/peptide specificity, this method is not capable of effectively labeling T cells with low affinity receptors and consequently may underestimate antigen-reactive T cell populations [26]. With respect to our study population, although our study utilized HLA matched samples from subjects with T1D and controls, we were not able to age match these groups (average ages were 29.8 and 49.5 respectively). At more advanced ages (especially beyond age 70) increases in homeostatic T cell proliferation and decreases in the diversity of the T cell pool have been documented [27]; these factors could have an influence on observed T cell frequencies in different age groups. Importantly, our study was only able to interrogate peripheral blood. Especially in light of recently published findings, important differences may exist between observations in the periphery and within disease proximal tissues [8].

In summary, we present the implementation and application of a multicolor HLA class II multimer staining protocol to analyze multiple auto-reactive CD4+ T cell specificities within a single staining tube. This approach can be performed on previously frozen samples, a necessary attribute for its practical application to meaningful sample sets. Our results indicate that among the specificities tested, proinsulin specific T cells were the predominant specificity in subjects with T1D, recommending this epitope as having the most potential for longitudinal monitoring and for tolerance induction strategies. We demonstrated that our approach is compatible with HLA class I multimer analysis, allowing complementary analysis of autoreactive CD8+ T cells. In agreement with other recently published studies, differences between patients and controls were modest. However, this does not negate the possibility that longitudinal monitoring of changes in T cell frequencies could be informative. Along those lines, tracking the expression of key surface markers such as CXCR3 or CCR7 could provide additional insights. Although additional refinement may be necessary to ensure inclusion of the most disease relevant epitopes, we conclude that applying this strategy could yield important insights about T cells in the setting of autoimmunity, providing an efficient and effective means of characterizing precious samples from clinical trials and other specialized research settings.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.11.004.

References

[1] J.A. Noble, A.M. Valdes, Genetics of the HLA region in the prediction of type 1 diabetes, Curr. Diab. Rep. 11 (2011) 533–542.

[2] J. Yang, N.A. Danke, D. Berger, S. Reichstetter, H. Reijonen, C. Greenbaum, et al., Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD4+ T cells in human subjects, J. Immunol. (Baltimore, Md: 1950) (176) (2006) 2781–2789.

[3] J. Yang, N. Danke, M. Roti, L. Huston, C. Greenbaum, C. Pihoker, et al., CD4+ T cells from type 1 diabetic and healthy subjects exhibit different thresholds of activation to a naturally processed proinsulin epitope, J. Autoimmun. 31 (2008) 30–41.

[4] J. Yang, E.A. James, S. Sanda, C. Greenbaum, W.W. Kwok, CD4+ T cells recognize diverse epitopes within GAD65: implications for repertoire development and diabetes monitoring, Immunology 138 (2013) 269–279.

[5] M. Peakman, E.J. Stevens, T. Lohmann, P. Narendran, J. Dromey, A. Alexander, et al., Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4, J. Clin. Invest. 104 (1999) 1449–1457.

[6] V. Oling, J. Marttila, J. Ilonen, W.W. Kwok, G. Nepom, M. Knip, et al., GAD65- and proinsulin-specific CD4+ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects, J. Autoimmun. 25 (2005) 235–243.

[7] S. Arif, F. Moore, K. Marks, T. Bouckenooghe, C.M. Dayan, R. Planas, et al., Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated beta-cell death, Diabetes 60 (2011) 2112–2119.

[8] S. Culina, A.I. Lalanne, G. Afonso, K. Cerosaletti, S. Pinto, G. Sebastiani, et al., Islet-reactive CD8(+) T cell frequencies in the pancreas, but not in blood, distinguish type 1 diabetic patients from healthy donors, Sci. Immunol. (2018) 3.

[9] B.O. Roep, M. Peakman, Surrogate end points in the design of immunotherapy trials: emerging lessons from type 1 diabetes, Nat. Rev. Immunol. 10 (2010) 145–152.

[10] R. Mallone, G.T. Nepom, Targeting T lymphocytes for immune monitoring and intervention in autoimmune diabetes, Am. J. Ther. 12 (2005) 534–550.

[11] J.A. Spanier, N.L. Sahli, J.C. Wilson, T. Martinov, T. Dileepan, A.L. Burrack, et al., Increased effector memory insulin-specific CD4+T-cells correlate with insulin autoantibodies in recent-onset type 1 diabetic patients, Diabetes (2017).

[12] S.I. Mannering, F.S. Wong, I. Durinovic-Bello, B. Brooks-Worrell, T.I. Tree, C.M. Cilio, et al., Current approaches to measuring human islet-antigen specific T cell function in type 1 diabetes, Clin. Exp. Immunol. 162 (2010) 197–209.

[13] J.D. Altman, P.A. Moss, P.J. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, et al., Phenotypic analysis of antigen-specific T lymphocytes, Science (New York, NY) 274 (1996) 94–96.

[14] J.H. Velthuis, W.W. Unger, J.R. Abreu, G. Duinkerken, K. Franken, M. Peakman, et al., Simultaneous detection of circulating autoreactive CD8+ T-cells specific for different islet cell-associated epitopes using combinatorial MHC multimers, Diabetes 59 (2010) 1721–1730.

[15] E.A. James, J.R.F. Abreu, J.W. McGinty, J.M. Odegard, Y.E. Fillie, C.N. Hocter, et al., Combinatorial detection of autoreactive CD8(+) T cells with HLA-A2 multimers: a multi-centre study by the Immunology of Diabetes Society T Cell Workshop, Diabetologia (2017).

[16] C.L. Day, N.P. Seth, M. Lucas, H. Appel, L. Gauthier, G.M. Lauer, et al., Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers, J. Clin. Invest. 112 (2003) 831–842.

[17] W.W. Kwok, V. Tan, L. Gillette, C.T. Littell, M.A. Soltis, R.B. LaFond, et al., Frequency of epitope-specific naive CD4(+) T cells correlates with immunodominance in the human memory repertoire, J. Immunol. (Baltimore, Md: 1950) (188) (2012) 2537–2544.

[18] W.W. Kwok, M. Roti, J.H. Delong, V. Tan, E. Wambre, E.A. James, et al., Direct ex vivo analysis of allergen-specific CD4+ T cells, J. Allergy Clin. Immunol. 125 (1407–9) (2010) e1.

[19] J.W. McGinty, I.T. Chow, C. Greenbaum, J. Odegard, W.W. Kwok, E.A. James, Recognition of posttranslationally modified GAD65 epitopes in subjects with type 1 diabetes, Diabetes 63 (2014) 3033–3040.

[20] I.T. Chow, J. Yang, T.J. Gates, E.A. James, D.T. Mai, C. Greenbaum, et al., Assessment of CD4+ T cell responses to glutamic acid decarboxylase 65 using DQ8 tetramers reveals a pathogenic role of GAD65 121–140 and GAD65 250–266 in T1D development, PLoS ONE 9 (2014) e112882.

[21] H. Uchtenhagen, C. Rims, G. Blahnik, I.T. Chow, W.W. Kwok, J.H. Buckner, et al., Efficient ex vivo analysis of CD4+ T-cell responses using combinatorial HLA class II tetramer staining, Nat. Commun. 7 (2016) 12614.

[22] E.J. Novak, A.W. Liu, G.T. Nepom, W.W. Kwok, MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen, J. Clin. Invest. 104 (1999) R63–R67.

[23] A. Lissina, K. Ladell, A. Skowera, M. Clement, E. Edwards, R. Seggewiss, et al., Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers, J. Immunol. Methods 340 (2009) 11–24.

[24] M. Scotto, G. Afonso, E. Larger, C. Raverdy, F.A. Lemonnier, J.C. Carel, et al., Zinc transporter (ZnT)8(186–194) is an immunodominant CD8(+) T cell epitope in HLA-A2(+) type 1 diabetic patients, Diabetologia 55 (2012) 2026–2031.

[25] J.H. Velthuis, W.W. Unger, J.R.F. Abreu, G. Duinkerken, K. Franken, M. Peakman, et al., Simultaneous detection of circulating autoreactive CD8(+) T-cells specific for different islet cell-associated epitopes using combinatorial MHC multimers, Diabetes 59 (2010) 1721–1730.

[26] C. Rius, M. Attaf, K. Tungatt, V. Bianchi, M. Legut, A. Bovay, et al., Peptide-MHC class I tetramers can fail to detect relevant functional T cell clonotypes and underestimate antigen-reactive T cell populations, J. Immunol. (Baltimore, Md: 1950) (200) (2018) 2263–2279.

[27] K. Naylor, G. Li, A.N. Vallejo, W.W. Lee, K. Koetz, E. Bryl, et al., The influence of age on T cell generation and TCR diversity, J. Immunol. (Baltimore, Md: 1950) (174) (2005) 7446–7452.