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# A pilot study of preproinsulin peptides reactivity in Chinese patients with type 1 diabetes

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## Abstract

**Background:** The aim of our study is to investigate whether preproinsulin (PPI) could trigger a proinflammatory $CD4^+$ T cell response in Chinese patients with type 1 diabetes (T1D).

**Methods:** Peripheral blood mononuclear cells were stimulated by a pool of 13 PPI peptides. Additional five PPI peptides previously proved to be antigenic in other cohorts of patients with T1D were also used. PPI reactive T cell responses were measured by interferon (IFN)-γ ELISPOT assay.

**Results:** Fifty-one Chinese patients with T1D were enrolled in this study and 72.34% of them were positive for at least one islet autoantibody. The stimulation index (SI) value of IFN-γ response to PPI peptide pool or peptides with dominant epitopes was below 3 in patients when SI≥3 was used as the positive cut-off value. Two peptides (B9-23 and C19-A3) restricted to DQ8 or DR4 molecule failed to induce positive IFN-γ response in patients with high-risk HLA-DQ8 or HLA-DR4/DR9 alleles. RNA-seq analysis of PPI specific $CD4^+$ T cell lines further showed that most of the IFN-γ associated genes remained unchanged.

**Conclusions:** This is the first report of $CD4^+$ T cell epitope mapping of PPI in Chinese T1D. The lack of positive IFN-γ response to PPI peptides indicates that PPI might not be the principal antigenic candidate for autoreactive $CD4^+$ T cells in Chinese T1D. Therefore, the efficacy of PPI-based immunotherapies in attenuating proinflammatory $CD4^+$ T cell response requires further investigation.

**KEYWORDS**

IFN-γ, overlapping peptides, preproinsulin, T cell response, type 1 diabetes

## 1 | INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by pathogenic T cell–mediated destruction of insulin-producing islet β cells.

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The disease is dramatically increased in incidence, doubling in the last two decades, especially in young children.[1] Genetic and environmental factors both contribute to the increasing prevalence in T1D. Although nonimmune genes contribute to the pathogenesis of T1D, immune genes, in particular, human leukocyte antigen (HLA) class II region, are considered to be the most susceptible candidate by mounted association studies.[2] By far, patients with T1D carrrying DR4 (DRB1*04)-DQ8 (DQB1*03:02) haplotype are the most investigated population manifested with activation of antigen-specific $CD4^+$ T cells.[3,4]

Based on the autoantibody detection, insulin, glutamate decarboxylase 65 (GAD 65), insulinoma-associated tyrosine phosphatase-like protein-2 (IA-2), and zinc transporter 8 (ZnT8) are the major islet autoantigens, and insulin is the only pancreatic β cell–specific protein. Genetic studies in patients with T1D reveal that the disease susceptibility is strongly conferred by a polymorphism of a variable number of tandem repeat upstream of insulin gene (INS-VNTR; odds ratio, approximately 2.5),[5] which is the second risk gene only after HLA-DQB1 locus (odds ratio, approximately 6.8).[6] Besides, higher levels of peripheral proinsulin-specific T cells have been detected in patients with susceptible INS-VNTR gene polymorphisms than those with protective ones.[7] Strong antigenic property of insulin in activating $CD4^+$ T cells from pancreatic lymph nodes for a proinflammatory phenotype has been well described in Caucasian T1D population.[8] Peripheral blood mononuclear cells (PBMCs) isolated from patients were polarized to interferon (IFN)-γ producing T helper type 1 (Th1) cells under stimulation of preproinsulin (PPI) peptides.[9,10] Moreover, (pre)proinsulin-specific $CD4^+$ T cell response is closely linked to the HLADR4-DQ8 haplotype.[11,12]

In the Chinese population, patients with T1D present with unique characteristics in genetic susceptibility as well as clinical manifestation. Our recent study shows that the incidence of Chinese T1D is much lower than that in Caucasians.[13] Although the variation in T1D incidence across multiple ethnic groups may be partly associated with environmental determinants,[14] it could also be attributed to the differences in HLA-II haplotype/genotype frequences.[15,16] The DRB1*09:01 is the dominant risk allele in Chinese, which is barely present in Caucasians.[17] Moreover, patients in China were characterized by older age at onset and a higher prevalence of diabetic ketoacidosis (DKA) at onset compared with those in regions with a high incidence of T1D, such as northern Europe.[18] However, only two studies were available regarding the immunogenetic effects of high-risk HLA-II alleles in Chinese T1D. One study demonstrated that HLA-A33-DR3 haplotype was associated with a reduction in the helper-to-cytotoxic T cell ratio in Han Chinese patients.[19] Another study found the discordant association of autoantibodies with high-risk HLA gene.[17] Whether differences in clinical phenotype between Caucasian and Chinese patients are attributed to immunogenetic effects of HLA-II genes is still unknown.

Oral insulin has been evaluated in two large clinical trials over the last two decades but shows no effect on delaying the progression to T1D in subjects with islet autoantibodies.[20,21] Interestingly, a recent Pre-POINT study in primary prevention found that oral insulin appeared to induce (pro)insulin-responsive regulatory T cells in genetically at-risk healthy children,[22] indicating that oral insulin attenuating PPI-specific autoreactive Th1 responses might be effective in Caucasians with disease-predisposing HLA-II alleles. Due to the genetic variability between Chinese and Caucasians, we hypothesize that Chinese patients might exhibit distinct epitope and phenotype of $CD4^+$ T cell response to PPI peptides from those in Caucasians, which potentially affects the translation of PPI-based immunotherapy into Chinese population. Therefore, to determine the immunogenicity of PPI, enzyme-linked immunospot (ELISPOT) assay was performed using serial overlapping PPI peptides in patients with T1D. To our best knowledge, this is the first study investigating autoreactive $CD4^+$ T cell response to peptides from the full length of PPI in Chinese patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Subjects

Patients included in this study had to be insulin dependent, diagnosed with T1D by an endocrinologist, and met at least one of the following criteria: (a) apparent symptoms of a diabetes-related metabolic disorder; (b) previous diabetic ketosis or ketoacidosis; (c) tested positive for diabetes autoantibodies; and (d) fasting and stimulated C peptide levels < 200 pmol/L. A total of 51 patients with Chinese Han origin were recruited at the Third Affiliated Hospital of Sun Yat-sen University. Clinical characteristics of patients were shown in Table 1. For the generation of a receiver-operator characteristic (ROC) curve,[9] six healthy control subjects with no family history of T1D (median age, 22.4 y; range, 5-25 y) were investigated. Fresh PBMCs were isolated on density gradients (Lymphoprep; Nycomed Pharma, Oslo, Norway) according to the manufacturer's protocol. These studies were carried out with the approval of the Local Research Ethics Committee. The study protocol was approved by the Institutional Review Board. Written informed consent was obtained from all patients and their guardians in accordance with the Declaration of Helsinki.

### 2.2 | Autoantibodies measurements

Serum levels of autoantibodies against GAD 65, IA-2, and ZnT8 were detected by radioimmunoassay confirmed by the Islet autoantibody Standardization Program as previously described.[23] The cut-off for positivity for GADA, IA2A, and ZnT8A was defined as a value above 0.05, 0.02, and 0.011, respectively.

### 2.3 | HLA analysis

The genomic DNA was extracted by QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Langenhagen, Germany) from 200 μL of frozen blood. HLA class II alleles for DRB1, DQA1, and DQB1 were genotyped by polymerase chain reaction sequence–specific oligonucleotide probe (One Lambda Inc, Canoga Park, California) according to the manufacturer's instructions. Data were interpreted by the HLA 3.0 Fusion Research software (One Lambda, USA).

**TABLE 1** Clinical characteristics, islet autoantibody status, and HLA genotype of study participants

| | All (n = 51)[a] | Duration ≤ 1 y (n = 19) | Duration > 1 y (n = 32) | *P* Value |
|---|---|---|---|---|
| Age at onset, y | 11.25 (13.83) | 10.75 (10.34) | 13.25 (15.16) | .592 |
| Ketoacidiosis at onset, n (%) | 31 (60.78) | 12 (63.18) | 19 (59.37) | |
| Duration, y | 1.5 (2.67) | 0.50 0.75) | 2.87 (2.29) | NA |
| HbA1c, % | 7.4 (1.53) | 7.70 (1.65) | 7.40 (1.50) | .313 |
| Fasting C peptide, nmol/L | 0.20 (0.11) | 0.21 (0.05) | 0.20 (0.13) | .922 |
| 2-h postprandial C peptide, nmol/L | 0.17 (0.09) | 0.18 (0.04) | 0.17 (0.11) | .807 |
| Insulin dosage, IU/kg/d | 0.66 (0.26) | 0.63 (0.28) | 0.67 (0.25) | .688 |
| Autoantibody | | | | |
| Positivity of GADA, n (%) | 25 (53.19) | 8 (53.33) | 17 (53.13) | .98 |
| Positivity of IA-2A, n (%) | 20 (42.55) | 7 (46.67) | 13 (40.63) | .696 |
| Positivity of ZnT8A, n (%) | 15 (31.91) | 6 (40.00) | 9 (28.13) | .416 |
| Frequency of DRB1 alleles, % | | | | |
| DRB1*09:01 | 19.61% | 13.16% | 23.44% | |
| DRB1*03:01 | 37.25% | 42.11% | 34.38% | |
| DRB1*04:01/04:04/04:05 | 19.61% | 18.42% | 20.31% | |

*Note*. Data are expressed as mean (SD), median (interquartile range), or as n (%) unless otherwise indicated. NA, not applicable.

[a]Missing data: ketoacidosis at onset, 4 (7.69%); HbA1c, 9 (17.65%); fasting C peptide, 35 (68.63%); 2-hour postprandial C peptide, 41 (80.39%); insulin dosage, 16 (31.37%). Autoantibody at onset: GADA, 4 (7.8%); IA-2A, 4 (7.8%); ZnT8A, 4 (7.8%).

## 2.4 | Peptides synthesis and antigens

A series of adjacent peptides based on the human PPI sequence consisting of 110 amino acids were synthesized by Shanghai Sangon Biotech Company (Shanghai, China). PPI peptides were 14 to 16 amino acids long and overlapped by eight amino acids spanning the complete sequence of PPI (Table 2). All of the 13 peptides were pooled together as a peptide mixture (PM). Given that the different regions of PPI peptides are likely to have different magnitudes in triggering IFN-γ–producing response, a set of five peptides with dominant epitopes previously reported to show positive $CD4^+$ T cells response were used separately: B9-23, C19-A3, B1-16, B10-24, and B30-C13 (Table S1). The purity of peptides was >95% using reverse phase HPLC and mass spectrometry. Pentaxim (Sanofi-Aventis, Paris, France), containing diphtheria toxoid, haemophilus influenzae type B capsular polysaccharide, pertussis toxoid, tetanus toxoid, amd poliomyelitis, was a childhood vaccine widely used across China. It was adopted as a recall antigen, and OKT3 antibody was used as positive control antigen. The selected MHC class II peptide pool PLUS (Axxora, LCC, USA) containing peptides from human cytomegalovirus, Epstein-Barr virus, influenza virus and tetanus toxin was also used for the specificity of T cell response.

## 2.5 | Cytokine ELISPOT analysis

Detection of IFN-γ production by PBMCs was carried out by an ELISPOT assay as previously described.[9] Briefly, fresh PBMCs were dispensed into 48-well plates at a density of $2 \times 10^6$ in 0.5 mL in RPMI-1640 medium supplemented with antibiotic/antimycotic (TC medium, Life Technology Ltd) and 10% human AB serum (Gemini, USA). Cells were seeded with peptide mixture of different concentrations as 1, 10, and 100 μg/mL or with single peptide to a final concentration of 20μM. Cells were then incubated at 37°C, 5% $CO_2$ atmosphere in a humidified incubator, tilted by 5°. Control wells contained TC medium with an equivalent concentration of peptide diluent alone (DMSO), Pentaxim, MHC class II peptide pool PLUS, or anti-CD3 antibody (OKT3, 1 μg/mL, ebioscience). On day 1,

**TABLE 2** Amino acid sequences of truncated preproinsulin peptides

| Peptides | Sequence | Peptides | Sequence |
|---|---|---|---|
| L1-16 | MALWMRLLPLLALLAL | C1-16 | EAEDLQVGQVELGGGP |
| L9-24 | PLLALLALWGPDPAAA | C9-24 | QVELGGGPGAGSLQPL |
| L17-B8 | WGPDPAAAFVNQHLCG | C17-K88 | GAGSLQPLALEGSLQK |
| B1-16 | FVNQHLCGSHLVEALY | C25-A7 | ALEGSLQKRGIVEQCC |
| B9-24 | SHLVEALYLVCGERGF | R89-A15 | RGIVEQCCTSICSLYQ |
| B17-R56 | LVCGERGFFYTPKTRR | A8-A21 | TSICSLYQLENYCN |
| B25-C8 | FYTPKTRREAEDLQVG | | |

0.5-mL prewarmed TC medium/10% AB serum was added, and on day 2, nonadherent cells were resuspended using prewarmed TC medium/2% AB serum, washed, brought to a concentration of $10^6$/300 μL, and 100 μL dispensed in triplicate into wells of 96-well ELISA plates (Nunc MaxiSorp; Thermo Fisher) precoated with monoclonal anti–IFN-γ (U-Cytech, Utrecht, The Netherlands). After captured at 37°C, 5% $CO_2$ for 24 hours, cells were lysed in ice-cold water, plates washed in PBS/Tween 20, and spots developed according to the manufacturer's instructions. Plates were dried, and spots of 80 to 120 μm were counted in a CTL ImmunoSpot reader (Shaker Heights, USA). Data are expressed as the mean number of spots per triplicates and compared with the mean spot number in the presence of diluent alone to derive a stimulation index (SI). A response is considered positive when the SI value is ≥3.

### 2.6 | Generation of PPI-specific $CD4^+$ T cell line

$CD4^+$ T cells were isolated from PBMCs using $CD4^+$ T cell isolation kit (Miltenyi Biotec, Germany). To generate PPI peptide-specific $CD4^+$ T cell line, $CD4^+$ T cells (0.14-0.45 × $10^6$ per well) were cultured in 48-well plate with 0.1 × $10^5$ autologous adherent non-$CD4^+$ T cells as antigen-presenting cells. Cells were stimulated with 20 μg/mL of each truncated peptide (Tables 2 and S1) or peptide mixture and cultured in human T cell medium (RPMI1640 with 10% human AB serum) in the presence of recombinant human IL-2 (10 U/mL; PeproTech, USA) . At day 7, cells were fed with human T cell medium containing 10 U/mL of human IL-2 and were refed with medium and IL-2 every week. After four to six stimulation cycles, lymphocytes were harvested for RNA-seq assays. Autologous PBMCs as negative control were stored frozen until use.

### 2.7 | BGISEQ-500 RNA-Seq assays

PPI-specific $CD4^+$ T cell lines were harvested after long-term culture in vitro. Cells were washed with 1× PBS and then resuspended by Tripure (Roche, USA). mRNA extraction and sequencing were conducted using BGISEQ-500 sequencing platform (BGI, Wuhan, China).[24]

### 2.8 | Statistical analysis

Sample characteristics were summarised as the mean ± SD or as the median and interquartile range (IQR) (Table 1). Categorical variables were presented as the number and percentage of patients affected. Participants' characteristics were described using descriptive statistics. To examine the significance of differences in demographic and clinical characteristics between patients with disease duration ≤1 year and those with disease duration >1 year, a two-sample *t* test for continuous variables and a chi-squared test for categorical variables were used. Statistical significance was defined as two-tailed $P < .05$. Data were analysed with GraphPad Prism 5 software or in statistical software environment such as SPSS version 22.

## 3 | RESULTS

### 3.1 | Clinical characteristics of patients with T1D

Descriptions of patients in this study were listed in Table 1. The average age at onset was 11.25 (range, 0.75-47.1) years, and 60.78% (31 of 51) were diagnosed before 15 years of age. The mean duration of diabetes was 1.5 (range, 0.03-7.5) years, and 37.25% of patients were diagnosed of T1D for no more than 1 year. As for T1D susceptible DRB1 alleles, DRB1*03:01 were significantly more frequent than DRB1*09:01/04. Regarding anti-islet autoantibody positivity, the overall positive ratio was 72.34% (34 of 47). Among them, 19.15% (9 of 47) was positive to all antibodies (GADA, IA-2A, and ZnT8A). As for single autoantibody positivity, GADA ranked the highest at 53.19% (5 of 47). Besides, DKA was manifested in 60.78% of the 51 patients at diagnosis. Regarding the endogenous islet β cell function, both fasting and stimulated C peptide levels were as low as approximately 0.2 nmol/L. In respect to glycaemic control, the median of glycosylated haemoglobin ($HbA_{1c}$) levels was 7.4% (IQR, 5.9%-14.4%). The mean value of daily insulin dose was 0.65 IU/kg (IQR, 0.17-1.53). No significant difference was present in $HbA_{1c}$ and daily insulin dose between patients with new-onset T1D (disease duration ≤ 1 y) and those with established T1D (disease duration > 1 y).

### 3.2 | Overlapping PPI peptides-reactive T cell response in the ELISPOT assay

PBMCs obtained from patients with T1D were assayed for peptide recognition by IFN-γ ELISPOT assays. Basal response reflecting background IFN-γ production was tested by using peptide diluent (DMSO: medium = 1:5000), which was low (median number of spots, 4.09; range, 0-38/300 000 cells). All patients showed significant IFN-γ responses to OKT3 stimulation with the median SI value as 278.69 ranging from 14 to 1050, and 96% of patients showed positive responses to Pentaxim with a median SI value of 70 (Figure 1A). Six out of seven patients (85.72%) showed a positive IFN-γ response to the MHC class II peptide pool (Table S2). However, overlapping PPI peptides mixture (PM) with a range of concentrations (ie, 1, 10, and 100 μg/ml) did not induce positive response as indicated by each SI value below 3 (Figure 1A). Additional five peptides (Table S1), which stably induced Th1 response in the Caucasian population, did not induce specific IFN-γ–producing $CD4^+$ T cells activity (Figure 1A) in our cohort. Moreover, no positive response could be detected in six healthy controls (Figure 1A). Representative cytokine ELISPOT responses from a patient with homozygous DRB1* 09:01 were showed in Figure 1B (a-f).

### 3.3 | IFN-γ response to PPI peptides with dominant epitope in patients with high-risk HLA alleles

Previous study showed that the responsiveness of dominant epitopes as B9-23 was highly restricted to susceptible HLA-DQ8 molecule, and

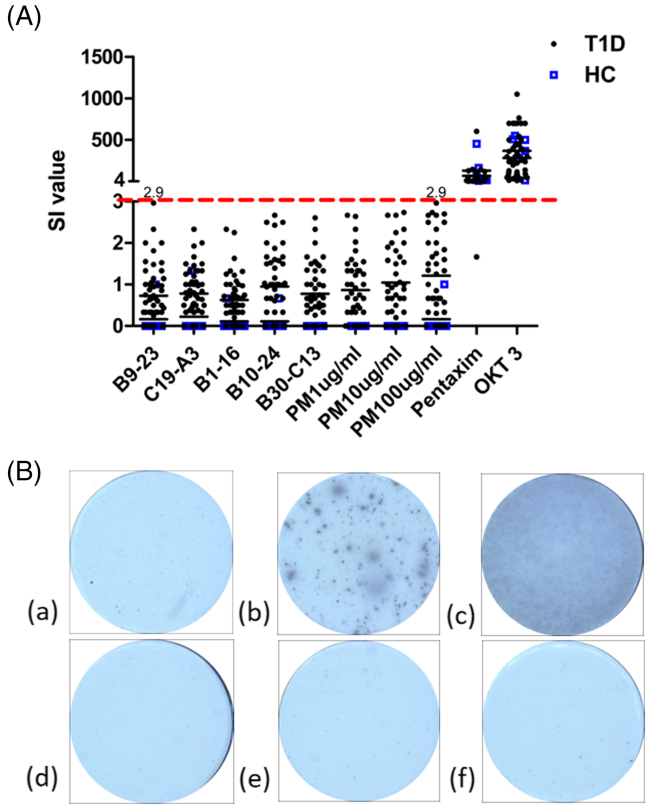


**FIGURE 1** Overlapping PPI peptides-reactive T cell response in the ELISPOT assay. PBMCs from patients with T1D and health controls (HC) were freshly isolated and incubated with peptide or diluent alone for 48 hours after which IFN-γ measured using the ELISPOT. (A) IFN-γ ELISPOT analysis for PPI mixture (PM), Pentaxim, and anti-CD3 antibody (OKT 3) stimulation in patients with T1D and healthy controls. An SI value was calculated as the ratio of the mean response in the presence of peptide to the mean response in the presence of diluent alone. Each dot shows an SI value of triplicate wells on each subject. Horizontal dashed line represents cut-off of positivity (SI ≥ 3 when compared with diluent alone). (B) Representative IFN-γ ELISPOT results from a patient with recent-onset T1D carrying homozygous DRB1*09:01. Treatments are (a) diluent alone, (b) Pentaxim (SI = 71), (c) anti-CD3 antibody (SI = 278.6), (d) PPI peptide mixture in a fixed concentration of 10 μg/mL, (e) B9-23 (20 μM), and (f) C19-A3 (20 μM)

its mean SI value of positive IFN-γ response was 159 in Caucasian patients with DQ8 alleles.[11] The mean SI value of positive IFN-γ response to another peptide C19-A3, which was naturally processed and presented by DR4 molecule, was 13 in patients with DR4 alleles.[9] We added these data from samples of Caucasian patients tested in previous studies into Figure 2 for comparison with our result to show the overall IFN-γ secretion to these two PPI peptides according to the distribution profile of high-risk DR and DQ genotypes in our cohort. Neither DQ8 nor DR4 carriers in our study showed a positive IFN-γ response to B9-23 (Figure 2A). Similarly, C19-A3 failed to induce a positive IFN-γ response from patients with DR4 alleles (Figure 2B). Together, PPI peptides with epitopes antigenic to Caucasians could not induce specific IFN-γ response in Chinese patients with T1D predisposing HLA-DQ8 or HLA-DR4/DR9 alleles.

### 3.4 | IFN-γ–associated gene expression in PPI peptide-specific CD4$^+$ T cell lines

Considering proportions of antigen-specific CD4$^+$ T cells from freshly isolated PBMCs were low, specific T cell lines were generally generated by continuous antigen stimulation to increase the frequency of the corresponding peptide-specific T cells. We, therefore, generated PPI-specific CD4$^+$ T cell lines followed by RNA-seq assays to investigate the regulation of IFN-γ secretion further. Fifty-six CD4$^+$ T cell lines specific to 13 PPI peptides sufficient for RNA-seq analysis were generated from 11 patients (Table S3). Up-regulated mRNA expressions of IFN-γ (*IFNG*) were detected upon eight peptide-specific T cell lines, while down-regulated expressions of IFN-γ (*IFNG*) were seen in two cell lines (dots with number "1" in Figure 3). The expression of IFN receptors (*IFNGR2* and *IFNLR1*) was slightly down-regulated (dots with numbers "2" and "3" in Figure 3). The majority of the mRNA expression associated with IFN signalling molecules remained unchanged. The low level of IFN-γ production was not attributed to T cell exhaustion because the mRNA levels of genes associated with T cell exhaustion were significantly down-regulated or unchanged in PPI mixture-specific CD4$^+$ T cell lines when compared with autologous PBMCs (Table S4). As for T cell effector function, genes related to IL17 signalling pathway including IL17C, IL17RD, IL23A, and IL6 were obviously down-regulated, while the majority of Th17 signature genes remained unchanged (Table S5). In contrast, the anti-inflammatory Th2 signature genes including IL4 and IL9 were significantly up-regulated (Table S5). These results indicated that PPI failed to induce a positive pro-inflammatory Th1 response in Chinese T1D.

## 4 | DISCUSSION

This is the first report describing IFN-γ response of CD4$^+$ T cells to overlapping human PPI peptides in Chinese patients with T1D. Peptide truncation here can cover almost all the candidate epitopes,[25,26] including those reported to be antigenic in Caucasians (eg, B1-16 and A1-15).[25,27] We apply ELISPOT assay for detection of IFN-γ secreting cells, which demonstrates good performance characteristics regarding sensitivity and reproducibility in reports of the Third Immunology of Diabetes Society T-Cell Workshop.[28,29] However, no positive IFN-γ response can be detected in patients with T1D, indicating that in terms of natural peptide repertoire, PPI is not likely to be the dominant proinflammatory autoantigen in the pathogenesis of T1D in Chinese.

In the current study, the majority of patients has susceptible types of DRB1 genes (DRB1*04/03:01/09:01), and their frequencies are similar to those previously reported in Chinese.[17,30] Meanwhile, the profile of positive anti-islet autoantibodies is in accordance with other studies in Chinese.[17,31] Therefore, subjects examined in our study are considered representative of Chinese patients with T1D and show differences from Caucasian T1D in the aspect of genetic background and islet autoimmunity.[16]

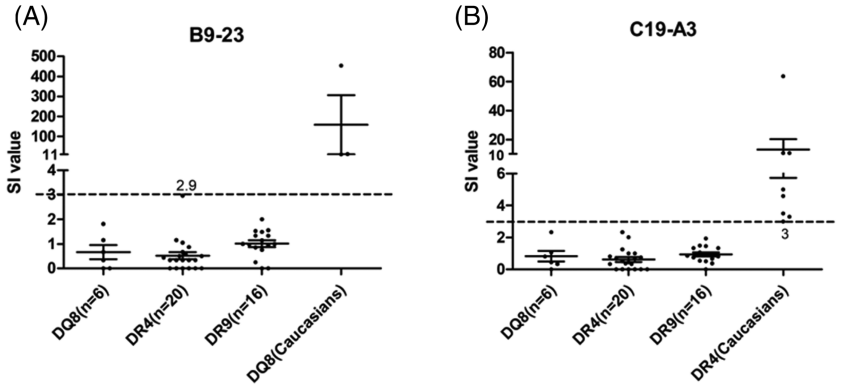


**FIGURE 2** IFN-γ response to PPI peptides with dominant epitope in patients with high risk HLA alleles. (A) IFN-γ responses to B9-23 in Chinese patients (the first three columns) carrying DQ8/DR4/DR9 alleles and Caucasian patients with DQ8 alleles (the last column). (B) IFN-γ responses to C19-A3 in Chinese patients (the first three columns) carrying DQ8/DR4/DR9 alleles and Caucasian patients with DR4 alleles (the last column). IFN-γ responses in Caucasian patients (the last column of each panel) were referenced from previous reports and not run in our experiment. Individual spot indicates the SI value of triplicates with peptide treatment. Horizontal dashed line represents cut-off of positivity (SI ≥ 3 when compared with diluent alone)

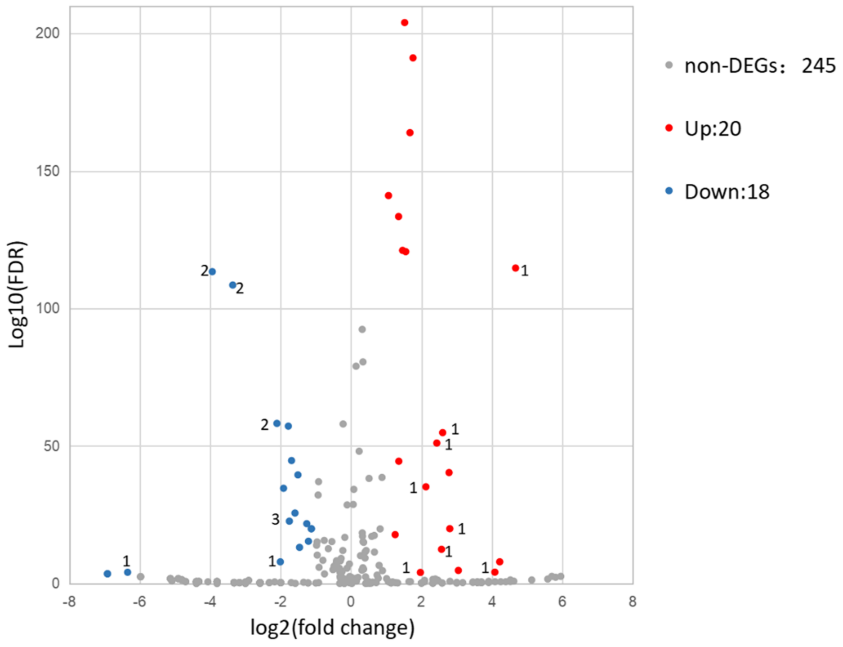


**FIGURE 3** IFN-γ–associated gene expression in PPI peptide-specific $CD4^+$ T cell lines. Volcano plot for RNA-seq analysis of IFN-γ–associated genes in PPI-specific $CD4^+$ T cell lines. Selected genes relevant to interferon (IFN) were filtered by the key word "IFN" within a gene symbol. X axis represents log2 transformed fold change. Y axis represents −log10 transformed significance. Red and blue dots were detected as significant differential expression between PBMCs and peptide-specific $CD4^+$ T cell lines with false discovery rate (FDR) for each *P* value ≤ .001. Red points represent up-regulated differentially expressed gene (DEGs) with $\log_2$ (fold change) ≥ 1. Blue points represent down-regulated DEGs with $\log_2$ (fold change) ≤ −1. Grey points represent unchanged genes (non-DEGs). Number "1" represents up-regulated mRNA expressions of IFN-γ (IFNG) in eight PPI peptide-specific T cell lines (L9-L24, L17-B8, B1-B16, B9-B24, B25-C8, C19-A3, and PPI mix) and down-regulated mRNA expressions of IFN-γ (IFNG) in two PPI peptide-specific T cell lines (R89-A15, A8-A21). Numbers "2" and "3" represent down-regulated mRNA expressions of IFN receptors (IFNGR2 and IFNLR1, respectively) in PPI peptide-specific T cell line

Rising studies have focused on the association between T cell epitope of PPI peptides and HLA restriction. In Caucasians, B9-23–specific IFN-γ response could be detected in all of the three patients with DQ8 alleles.[11] Nevertheless, in another recent study, only 2 of 14 (14.29%) new-onset patients with DQ8 alleles were responsive to this peptide.[32] Another peptide C19-A3 has been proved to be a prominent target of Th1 response in 8 of 17 (47%) patients with DR4 alleles.[9] No homogeneous characteristics in HLA genotype could be found in those patients without positive IFN-γ secretion to B9-23 or C19-A3 stimulation. Although there was no definite association between PPI-specific autoreactive Th1 response and susceptible HLA-II alleles, previous study showed that each register of a HLA-DQ8 cis/trans candidate epitope exhibited a distinct binding affinity to all four HLA DQ dimers.[33] The original study by van Lummel et al suggested that DQ8trans molecule might be the dominantly good binder to native B30-C13.[33] However, in our current study, six patients with DQ8 allele also presented DQ2 allele; these changes in the formation of DQ dimers[34] might account for the different levels of IFN-γ-producing $CD4^+$ T cell responses to PPI peptides between our patient cohort and Caucasians with DQ8 alleles.

Up to now, limited research was available for PPI-specific T cell response in Asian patients carrying the unique risk allele DRB1*09:01.[2,30] A Japanese study showed insulin B9-23–stimulated IFN-γ spots were observed in 31.8% (7 of 22) of patients with T1D.[35] Another similar study performing T cell epitope mapping of insulin in Japanese juvenile patients with T1D discovered that patients were responsive to B10-24 more frequently than B9-23.[36] However, ELISPOT subtraction analysis was applied for these studies instead of stimulation index to define the positive responses. It should be noted that the stimulation index did not achieve sufficient value to be defined as a positive response in these two studies.

SI value presents the fold change of IFN-γ production dependent upon background response in each individual correspondingly. A cut-off of SI $\geq$ 3 has been used in some studies to discriminate PPI-induced IFN-γ production of Caucasian patients from controls with the greatest sensitivity (72%) and specificity (92%).[9,37] In fact, an SI value $\geq$ 1.5 is chosen as the cut-off in our cohort (data not shown) using the ROC curve approach reported previously.[9] By using SI $\geq$ 1.5, sensitivity in our study (49%) is much lower than that in Caucasians (72%),[9] despite similar specificity (100% and 92%, respectively). As this is the pilot study to investigate PPI-specific Th1 response in Chinese population, we use a well-established ELISPOT cut-off, SI $\geq$ 3, as the positive standard reference.

Most studies prefer to enrol patients with short-term T1D for detection of autoreactive T cell responses due to the hypothesis that residual β cell function in the early stage of disease may act as a source of autoantigen for the activation and maintenance of islet autoantigen-specific T cell response.[38] However, lack of IFN-γ–producing $CD4^+$ T cell response to PPI peptides is not likely to be due to long disease duration since 19 subjects in this cohort are recent-onset patients with T1D duration $\leq$ 1 year. Moreover, studies have shown that T cells with persistent memory to islet autoantigens are a permanent population in peripheral blood several years after disease development.[39,40]

Our current study is encouraging but has several limitations. Firstly, it will be better to interpret the difference in PPI-specific IFN-γ response between Chinese and Caucasian populations by using samples from Caucasian patients who were reported to respond to B9-23 or C19-A3 as positive controls. Secondly, serial peptides overlapped by eight amino acids in this study might miss some potential additional epitopes of PPI. As accumulating evidence supports that post-transcriptional modifications (PTM) of PPI may contribute to T1D by the generation of antigens escaping tolerance induction,[33,41,42] other forms of PPI, including PTM and hybrid insulin peptides, might also be antigenic for $CD4^+$ T cell in Chinese patients. Finally, in addition to PPI, other putative targets (eg, GAD, IA-2) for autoreactive Th1 cells remain to be identified.

In conclusion, the absence of PPI-specific Th1 response in patients with T1D here indicates that PPI is not the crucial disease-causing autoantigen in our cohort. The underlying immunological mechanism triggering autoreactive $CD4^+$ T cell response requires further investigation into other putative islet antigens in Chinese T1D. Based on the immunological heterogeneity of PPI-specific T cell response in both different populations and different individuals from the same study cohort, it is noteworthy to optimize patient selection in PPI-based clinical trial powered for T1D prevention.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHOR CONTRIBUTIONS

Y.X. conceived experiments, researched data, and wrote and reviewed the manuscript. H.X. conceived and designed the study, contributed to data analysis and interpretation, manuscript preparation. Y.G. contributed to discussion and reviewed and edited the manuscript. J.Y., F.X., and B.Y. contributed to the study design. J.L. contributed to the recruitment and blood sample collection of T1D patients. W.R., Q.H., and Z.J. researched data. J.W. supervised this study and contributed to study design, discussion, and writing the manuscript. J.W. 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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## PRIOR PRESENTATION

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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