JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
ORIGINAL ARTICLE

Diagnostic value of combined islet antigen-reactive T cells and autoantibodies assays for type 1 diabetes mellitus

Wei Tang[1,2†], Huiying Liang[1,3†], Ying Cheng[1], Jiao Yuan[1], Gan Huang[1], Zhiguang Zhou[1], Lin Yang[1*]

[1]National Clinical Research Center for Metabolic Diseases, Key Laboratory of Diabetes Immunology, Ministry of Education, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, Hunan, China, [2]Department of Metabolism and Endocrinology, The First People's Hospital of Huaihua, Huaihua, Hunan, China, and [3]Affiliated Dongguan People's Hospital, Southern Medical University (Dongguan People's Hospital), Dongguan, Guangdong, China

Keywords
Autoantibodies, Enzyme-linked immunospot assay, Type 1 diabetes mellitus

***Correspondence**
Lin Yang
Tel.: +86-731-8529-2154
Fax: +86-731-8536-7220
E-mail address:
yanglin_nfm@csu.edu.cn

J Diabetes Investig 2021; 12: 963–969

doi: 10.1111/jdi.13440

ABSTRACT

Aims/Introduction: Type 1 diabetes mellitus is a T cell-mediated autoimmune disease. However, the determination of the autoimmune status of type 1 diabetes mellitus relies on islet autoantibodies (Abs), as T-cell assay is not routinely carried out. This study aimed to investigate the diagnostic value of combined assay of islet antigen-specific T cells and Abs in type 1 diabetes mellitus patients.

Materials and Methods: A total of 54 patients with type 1 diabetes mellitus and 56 healthy controls were enrolled. Abs against glutamic acid decarboxylase (GAD), islet antigen-2 and zinc transporter 8 were detected by radioligand assay. Interferon-γ-secreting T cells responding to glutamic acid decarboxylase 65 and C-peptide (CP) were measured by enzyme-linked immunospot.

Results: The positive rate for T-cell responses was significantly higher in patients with type 1 diabetes mellitus than that in controls ($P < 0.001$). The combined positive rate of Abs and T-cell assay was significantly higher than that of Abs assay alone (85.2% vs 64.8%, $P = 0.015$). A significant difference in fasting CP level was found between the T^+ and T^- groups (0.07 ± 0.05 vs 0.11 ± 0.09 nmol/L, $P = 0.033$). Furthermore, levels of fasting CP and postprandial CP were both lower in the Ab^-T^+ group than the Ab^-T^- group (fasting CP 0.06 ± 0.05 vs 0.16 ± 0.12 nmol/L, $P = 0.041$; postprandial CP 0.12 ± 0.13 vs 0.27 ± 0.12 nmol/L, $P = 0.024$).

Conclusions: Enzyme-linked immunospot assays in combination with Abs detection could improve the diagnostic sensitivity of autoimmune diabetes.

INTRODUCTION

Type 1 diabetes mellitus has been widely perceived as an autoimmune disease characterized by T cell-mediated destruction of islet β-cells, based on genetic susceptibility and triggered by environmental factors. Type 1 diabetes mellitus, also known as autoimmune diabetes, can currently be screened by a variety of islet autoantibodies (Abs), such as glutamic acid decarboxylase autoantibodies (GADA), islet antigen-2 autoantibodies (IA-2A) and zinc transporter 8 autoantibodies (ZnT8A)[1]. However, some patients with type 1 diabetes mellitus accompanied by acute ketosis or ketoacidosis and poor islet function are negative for multiple islet Abs detection, temporarily classified as nonimmune-mediated "idiopathic type 1 diabetes mellitus". Previous studies showed that GAD-reactive T-cell assay could improve, to some extent, the diagnostic efficiency of autoimmune diabetes[2–5], but the sensitivity was still limited. Full-length islet antigens, such as GAD65 and C-peptide (CP), both of which are key targets of type 1 diabetes mellitus-specific autoreactive T cells[6,7], can cover more epitope information without human leukocyte antigen restriction, significantly expanding the testing population. Furthermore, the combinatorial detection of two islet antigens-specific T cells might further increase the sensitivity. In the present study, we detected interferon (IFN)-γ-secreting T-cell responses to antigen GAD65 and CP by enzyme-linked immunospot (ELISPOT) assay, and

†Wei Tang and Huiying Liang contributed equally to this work.
Received 28 May 2020; revised 16 September 2020; accepted 12 October 2020

investigated the diagnostic value of combined assay of islet antigen-reactive T cells and Abs for immunophenotyping in type 1 diabetes mellitus.

METHODS

Participants

Patients with type 1 diabetes mellitus were recruited in the Department of Metabolism and Endocrinology at the Second Xiangya Hospital of Central South University, Changsha, Hunan, China. The following were the inclusion criteria for type 1 diabetes mellitus: (i) diagnosis of diabetes according to the World Health Organization 1999 criteria[8]; (ii) spontaneous ketosis or ketoacidosis within half a year after diagnosis; and (iii) insulin dependence since diagnosis. Exclusion criteria: (i) acute infections within 2 weeks before blood draw; (ii) gestational diabetes and other specific types of diabetes; and (iii) other autoimmune-related diseases. A total of 54 patients with type 1 diabetes mellitus were enrolled in the present study, including 24 male and 30 female patients. At baseline, the levels of fasting CP (FCP) and postprandial CP (PCP) in most cases of type 1 diabetes mellitus were <0.1 and <0.2 nmol/L, respectively. All patients were followed up for at least 24 months to eliminate the possibility of ketosis-prone type 2 diabetes, latent autoimmune diabetes in adults and latent autoimmune diabetes in the young, as well as the disturbance of the partial remission phase when judging the insulin-dependent state. The mean age of patients was 28.6 years (standard deviation ±12.9 years), and the median duration of diabetes was 3 months (25th percentile to 75th percentile, 1–8 months).

The inclusion criteria for healthy controls were as follows: (i) fasting blood glucose <6.1 mmol/L and 2 h blood glucose <7.8 mmol/L during oral glucose tolerance test; and (ii) no family history of diabetes or other autoimmune diseases. There were 56 individuals with a mean age of 29.2 years (standard deviation ±12.3 years) selected as healthy controls.

The Ethics Committee of the Second Xiangya Hospital of Central South University approved the study (Approval No. 2017028), and the guidelines of the Helsinki Declaration were followed. Written informed consent was obtained from all individual participants (or their parent in the case of minor participants).

Clinical data, such as sex, age, duration of diabetes, insulin dose and body mass index (BMI), as well as laboratory data including glycosylated hemoglobin A1c (HbA1c), fasting blood glucose (FBG), FCP and 2-h PCP, triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were collected with standardized methods.

Autoantibodies assays

GADA, IA-2A and ZnT8A were detected in duplicate using radioligand assay, as previously described[9,10], which showed high sensitivity and specificity in the Islet Autoantibody Standardization Program 2012 (78.0% and 96.7% for GADA, 74.0% and 96.7% for IA-2A, and 70.0% and 98.9% for ZnT8A, respectively).

ELISPOT assay

IFN-γ-secreting T-cell responses to GAD65 and CP were determined by ELISPOT, as described previously[11]. In brief, human peripheral blood mononuclear cells were separated within 4 h of blood collection by density gradient centrifugation with a lymphocyte separation medium (Sigma, St. Louis, MO, USA). After overnight coating at 4°C with anti-IFN-γ capture Ab (U-Cytech, Utrecht, the Netherlands) according to the manufacturer's instructions, polyvinylidene difluoride membrane plates (Millipore MSIPS4510, County Cork, Ireland) were blocked with RPMI (Gibco, Carlsbad, CA, USA) containing 10% human AB serum. Antigens were added to triplicate wells as follows in 20 μL volume per well: GAD65 (Diamyd, Stockholm, Sweden; 25 μg/mL final concentration) and its diluents (RPMI medium) as the negative control, and CP (GL Biochem, Shanghai, China; 1 mmol/L). Positive control tetanus toxoid (Sanofi, Marcy-l'Étoile, France; 1 μg/mL) were added in duplicate wells. Peripheral blood mononuclear cells were resuspended with an AIM-V medium (Invitrogen, Carlsbad, CA, USA) containing recombinant human interleukin-2 (R&D, Minneapolis, MN, USA; 2.5 U/mL), seeded at 3×10^5 cells/well and cultured at 37°C for 40–48 h. IFN-γ secretion was visualized with biotinylated anti-IFN-γ detection Ab (U-CyTech), ExtrAvidin-Alkaline Phosphatase (Sigma) and color developer NBT-BCIP tablets (Roche, Mannheim, Germany). Spots were automatically counted by an ELISPOT plate reader (CTL, Cleveland, OH, USA). A stimulation index (SI) was calculated as the ratio of mean value of spots in experimental wells divided by the mean value of spots in negative control wells. The lowest value (0.5) was substituted if the mean number of spots in negative control wells was zero[12]. A response is considered positive when stimulation index >3.2 for GAD and stimulation index >6.3 for CP, respectively[11] base on the best cut-off selection by receiver operating characteristic plot analysis. The endotoxin contents were low in GAD65 and CP (both negative in limulus lysate assay). The Intra-assay and interassay coefficients of variation were evaluated by measuring spot numbers for positive control tetanus toxoid using repeated measures within the same assay and repeated measures between assays[13], using the same donor in 2 weeks. The intra-assay coefficient of variation was 12.2%, and the interassay coefficient of variation was 17.7% in the present study.

HLA genotyping

Genomic deoxyribonucleic acid was extracted from ethylenediaminetetraacetic acid anticoagulant blood using the phenol–chloroform method. HLA-DRB1, DQA1 and DQB1 genotypes were determined by polymerase chain reaction direct sequencing. According to our previous data in Chinese[14], DRB1*0301-DQA1*05-DQB1*0201 (DR3), DRB1*0405-DQA1*03-DQB1*0302 or DRB1*0405-DQA1*03-DQB1*0401 (DR4) and DRB1*0901-DQA1*03-DQB1*0303 (DR9) were defined as susceptible haplotypes. DRB1*0803-DQA1*0103-DQB1*0601 (DR8), DRB1*1101-DQA1*05-DQB1*0301 (DR11),

DRB1*1202-DQA1*0601g-DQB1*0301 (DR12) and DRB1*1401-DQA1*0101g-DQB1*0502 (DR14) were considered as protective haplotypes.

Statistical analysis

Data analysis was carried out using IBM SPSS Statistics 20 software (IBM Corporation, Armonk, NY, USA). Continuous data are expressed as the mean ± standard deviation, median (25th percentile to 75th percentile) or as indicated. Categorical variables are presented as the number or percentage. Comparison between groups was carried out with an independent Student's *t*-test if their normality were not rejected, or the Mann–Whitney *U*-test was used otherwise. The χ^2-test was carried out to compare categorical data. Spearman rank correlation analysis was carried out to explore the relationship between Abs and T-cell assays. A two-sided *P*-value <0.05 was considered statistically significant.

RESULTS

Sensitivity and specificity of ELSIPOT assay

The IFN-γ secretion induced by GAD and CP were presented as dot plots in Figure S1. Among the 54 patients with type 1 diabetes mellitus, 35.2% (19/54) and 38.8% (21/54) were tested positive for GAD or CP-reactive T-cell responses, respectively, both significantly higher than that in healthy controls (vs 3.6% [2/56] for GAD and 1.8% [1/56] for CP, both $P < 0.001$). In all, 30 of 54 patients with type 1 diabetes mellitus showed positive T-cell responses for GAD and/or CP (Table 1), which was higher than that in healthy controls (55.6% [30/54] vs 5.4% [3/56], $\chi^2 = 33.0$, $P < 0.001$). The sensitivity and specificity of T-cell assay was 55.6% (30/54) and 94.6% (53/56), respectively.

Significance of combined ELISPOT and Abs detection

Of 54 patients with type 1 diabetes mellitus, 35 had at least one kind of islet Ab (35 cases with GADA positivity, 11 with IA-2A positivity and 1 with ZnT8A positivity); that is, 64.8% (35/54) sensitivity, and one of 56 healthy controls were GADA-positive with 98.2% specificity. Of 19 patients with idiopathic type 1 diabetes mellitus, 11 were showed positive T-cell responses by ELISPOT (2 cases reactive to GAD alone, 5 reactive to CP alone, and 4 responsive to both GAD and CP); 19 of 35 patients with autoimmune type 1 diabetes mellitus had positive T-cell responses for islet antigens (seven reactive to GAD alone, six responsive to CP alone and six reactive to both; Figure 1). For more information about the overlap to T cells and islet Abs in autoimmune type 1 diabetes mellitus, please see Figure S2. There were no statistically significant differences of sensitivity or specificity between T-cell assay and Abs (sensitivity 55.6% vs 64.8%, $\chi^2 = 0.92$, $P = 0.33$; Table 1; specificity 94.6% vs 98.2%, $\chi^2 = 1.04$, $P = 0.31$). In addition, 11 patients showed positive T-cell responses among 19 patients with idiopathic type 1 diabetes mellitus (negative for all three Abs). However, 16 patients were positive for at least one of three Abs among the 24 patients with negative T-cell responses, whereas eight participants with type 1 diabetes mellitus showed negative both for Abs and T-cell assay. The Spearman rank correlation coefficient was −0.03 ($P = 0.83$), suggesting no significant correlation between Abs and T-cell assays. Combining Abs and T-cell assay increased the diagnostic sensitivity of autoimmune diabetes significantly than Abs detection alone (85.2% [46/54] vs 64.8%, $\chi^2 = 5.9$, $P = 0.015$) with 94.6% (53/56) specificity. A total of 15 patients with autoimmune type 1 diabetes mellitus and eight patients with idiopathic type 1 diabetes mellitus were genotyped, and the correlation between HLA-DR-DQ haplotypes and the positivity of ELISPOT assay is shown in Table S1. Interestingly, positive T-cell responses were observed in three of the four idiopathic type 1 diabetes mellitus patients and in one autoimmune type 1 diabetes mellitus patients with protective haplotypes.

Clinical manifestation in subgroups of type 1 diabetes mellitus

According to the results of ELISPOT, 54 patients with type 1 diabetes mellitus were divided into two groups; that is, T^+ and T^- groups. There were no significant differences in sex, age of onset, duration of diabetes, insulin dose, BMI, HbA1c, FBG, TG, LDL-C and PCP levels between these groups (Table 2; Figure 2a). However, FCP levels in the T^+ group were lower as compared with the T^- group (0.07 ± 0.05 vs 0.11 ± 0.09 nmol/L, $P = 0.033$).

To further explore the manifestations of idiopathic type 1 diabetes mellitus, we compared the clinical features of the Ab^-T^+ group with the Ab^-T^- group. No significant differences

Table 1 | T cell and antibodies assays in type 1 diabetes mellitus

	Abs		Total, *n* (%)	Combined positivity (%)
	+ (*n*)	− (*n*)		
T-cell assay				
+ (*n*)	19	11	30 (55.6)	85.2*
− (*n*)	16	8	24 (44.4)	
Total, *n* (%)	35 (64.8)	19 (35.2)	54	

Data were expressed as number and percentage. *$P < 0.05$, versus antibodies (autoantibodies) detection alone (χ^2-test).

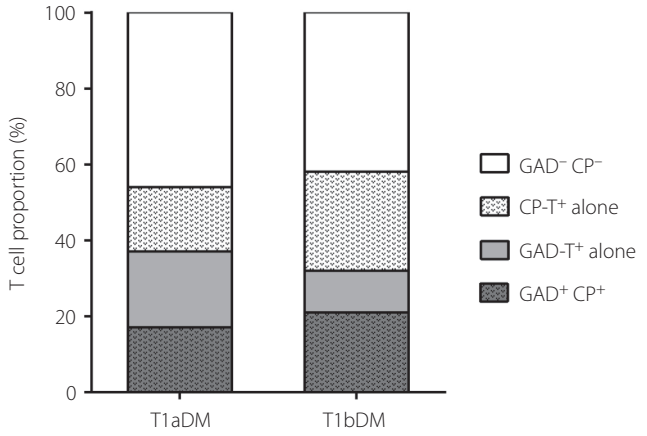

Figure 1 | Constituent ratio of T-cell positivity in autoimmune type 1 diabetes mellitus (T1aDM) and idiopathic type 1 diabetes mellitus (T1bDM) groups. CP, C-peptide; GAD, glutamic acid decarboxylase.

Table 2 | Clinical features of T^+ and T^- groups in type 1 diabetes mellitus

	T^+ group (n = 30)	T^- group (n = 24)
Men/women (n)	16/14	12/12
Age of onset (years)	27.3 ± 11.9	30.4 ± 14.4
Duration of diabetes (months)	4 (2–12)	2 (1–6)
Insulin dose (U/kg/day)	0.6 ± 0.1	0.6 ± 0.2
BMI (kg/m^2)	19.7 ± 3.0	19.8 ± 3.2
HbA1c (%)	10.9 ± 2.9	10.0 ± 3.2
FBG (mmol/L)	9.43 ± 1.16	9.44 ± 1.45
TG (mmol/L)	0.89 ± 0.43	0.93 ± 0.61
LDL-C (mmol/L)	2.00 ± 0.59	2.01 ± 0.47
FCP (nmol/L)	0.07 ± 0.05*	0.11 ± 0.09
PCP (nmol/L)	0.13 ± 0.10	0.20 ± 0.16

Data are presented as the number, mean ± standard deviation or median (25th percentile to 75th percentile). BMI, body mass index; FBG, fasting blood glucose; FCP, fasting C-peptide; HbA1c, glycosylated hemoglobin A1c; LDL-C, low-density lipoprotein cholesterol; PCP, 2-h postprandial C-peptide; TG, triglyceride. *P < 0.05, vs T^- group (Student's t-test).

were found in sex, age of onset, duration of diabetes, insulin dose, BMI, HbA1c, FBG, TG and LDL-C between the Ab^-T^+ group and Ab^-T^- group, except that FCP and PCP levels were both lower in the Ab^-T^+ group than that of the Ab^-T^- group (FCP 0.06 ± 0.05 vs 0.16 ± 0.12 nmol/L, P = 0.041; PCP 0.12 ± 0.13 vs 0.27 ± 0.12 nmol/L, P = 0.024; Figure 2b).

To further investigate the characteristics of the Ab^-T^+ group, we also compared it with the autoimmune type 1 diabetes mellitus Ab^+ group. No significant difference was observed in clinical features; for example, sex, age of onset, duration of diabetes, insulin dose, BMI, HbA1c, FBG, TG, LDL-C and islet β-cell function between the Ab^-T^+ group and Ab^+ group (all P> 0.05).

DISCUSSION

As we know, type 1 diabetes mellitus was widely perceived as a T-cell-mediated autoimmune disease. However, the diagnosis of autoimmune diabetes is currently mainly based on islet Abs, which are absent in idiopathic type 1 diabetes mellitus. In the present study, combining T-cell assay and Abs detection can improve the diagnostic sensitivity of autoimmune diabetes.

Actually, islet Abs are just fluid autoimmune markers, not required for β-cell destruction in the pathogenesis of type 1 diabetes mellitus[15,16], whereas the islet antigen autoreactive T cells are the prime culprits[17–20]. In addition, due to different races and positive thresholds, and so on, the positive rates of islet Abs in diabetes are distinct, suggesting that patients who are negative for islet Abs could not exclude the possibility of autoimmune diabetes absolutely, even if multiple Abs are determined[21–23]. Idiopathic type 1 diabetes mellitus, accounting for 40–50% of type 1 diabetes mellitus in China, is more prevalent than that among white people. Hence, it is a high priority to improve the diagnostic efficiency of autoimmune diabetes by other methods, such as islet antigen autoreactive T-cell assay, which aims at the primary effectors of β-cell lesions. Nevertheless, it is difficult to measure islet antigen autoreactive T cells in circulation as a result of their very low frequency and functional avidity[24]. Fortunately, the promising technique, ELISPOT, allows detection of antigen autoreactive T cells qualitatively and quantitatively at single-cell resolution[25,26]. It is endowed with high accuracy (86% sensitivity, 91% specificity) when evaluating $CD8^+$ T-cell responses in type 1 diabetes mellitus, whose sensitivity reached 100% when combined with Ab determinations[27]. However, $CD8^+$ T-cell enzyme-linked immunospot (ISL8Spot) restricts the study to individuals that express a common allele, such as HLA-A2[27–29], accounting for <20% in China. Unlike ISL8Spot, T-cell ELISPOT in the present study applied full-length antigens rather than peptides, endowed with HLA-unrestricted investigation of T-cell responses, making it valuable in clinical practice. Kotani *et al.*[30] and our team have both detected positive T-cell responses to GAD65 in fulminant type 1 diabetes mellitus patients with negative Abs by ELISPOT, suggesting that cellular immune abnormalities might be involved in the pathogenesis of fulminant type 1 diabetes mellitus[5,31] (whereas, we did not include patients with fulminant type 1 diabetes mellitus in the present study). By analyzing the clinical characteristics, these patients with positive T cells were similar to autoimmune type 1 diabetes mellitus patients. Therefore, the current definition of idiopathic type 1 diabetes mellitus is only an exclusive diagnosis. However, whether these patients have T-cell abnormality is not conclusive. This study aimed to explore the diagnostic value of combined assay of ELISPOT and Abs detection in type 1 diabetes mellitus accordingly.

Herein, further T-cell assay could greatly improve the diagnostic efficiency of autoimmune diabetes (from 64.8% to 85.2%) based on traditional Abs detection. T-cell assays and Abs detection had similar sensitivity, as well as specificity, but

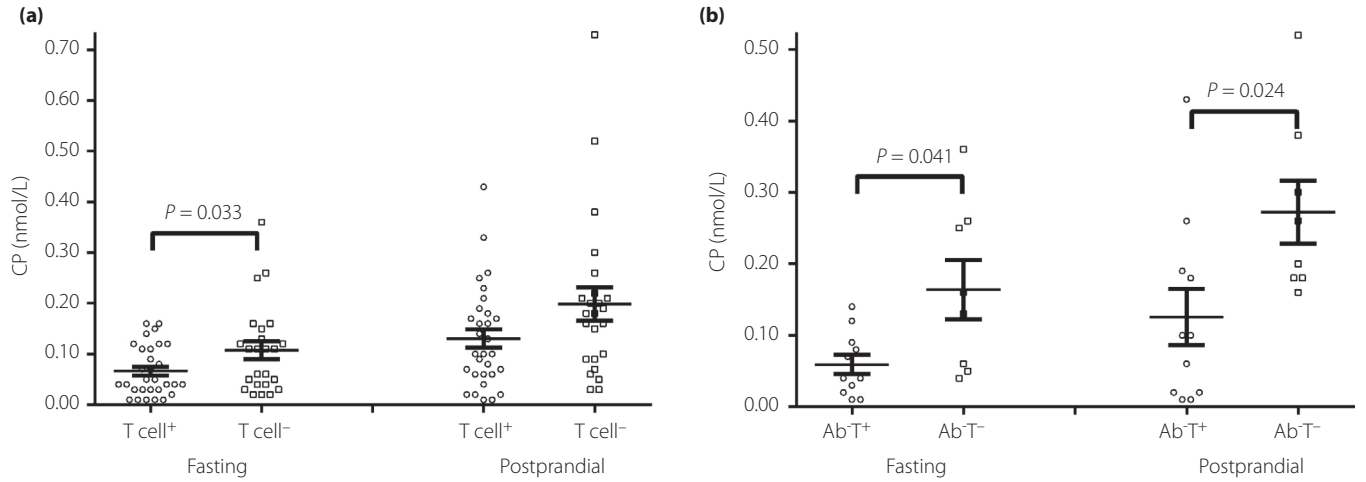

Figure 2 | Islet function in subgroups of type 1 diabetes mellitus. (a) Islet β-cell function in T^+ (n = 30) and T^- (n = 24) groups independent of autoantibodies (Abs) status. (b) Islet β-cell function in Ab^-T^+ (n = 11) and Ab^-T^- (n = 8) groups. Data are presented as dot plots with indications of mean and standard error of the mean. CP, C-peptide.

more importantly, with few correlations, indicating the high priority of further measuring islet autoreactive T cells in Ab-negative patients. Previous studies showed no correlation of T-cell reactivity with Abs[32], which was further confirmed in the present study (Spearman rank correlation coefficient was −0.03, P = 0.83), suggesting that T-cell assays could provide a diagnostic value beyond Abs detection.

In this research, we found that 11 of 19 (57.9%) Ab^- type 1 diabetes mellitus patients had positive T-cell responses to islet antigens, which could not be identified when measured even by three islet Abs (GADA, IA-2A and ZnT8A). Here, independent of Ab status, the T^+ group with type 1 diabetes mellitus showed significantly lower levels of PCP than the T^- group. Additionally, the Ab^-T^+ group, with similar islet β-cell function compared with Ab^+ group, autoimmune type 1 diabetes mellitus, had significantly lower levels of FCP and PCP than Ab^-T^- group. In short, T-cell reactivity in type 1 diabetes mellitus may be associated with poorer islet β-cell function. Similarly, Pfleger *et al.*[32] reported that IFN-γ-secreting T-cell responses negatively correlated with islet β-cell function in the initial diagnosis by ELISPOT, implying that T-cell reactivity could predict the failure of islet function and patients positive for T-cell assays should require more rigorous follow up.

In the present study, the Ab^-T^+ patients, with similar phenotype with autoimmune type 1 diabetes mellitus, in the strict sense, should be diagnosed as autoimmune diabetes rather than idiopathic type 1 diabetes mellitus, and might obtain additional benefits from immune therapy; for example, islet function preservation. Unfortunately, the lack of follow up precluded us from identifying the effect of T-cell reactivity on the progressive β-cell dysfunction in type 1 diabetes mellitus patients. In addition, other limitations to the present study include the relatively small sample size. Therefore, further prospective studies with larger sample size are required for confirmation in the future.

In conclusion, combining ELISPOT assays and islet Abs detection is helpful to improve the diagnostic sensitivity of autoimmune diabetes.

ACKNOWLEDGMENTS

We are indebted to the individuals who participated in this study and all our colleagues, especially Dr Zhiguo Xie, Dr Shuoming Luo, Dr Ying Xia and Dr Keyu Guo who greatly assisted us in HLA genotyping. This work was supported by the National Key R&D Program of China (2018YFC2001005) and the European Foundation for the Study of Diabetes (EFSD/CDS/Lilly Collaborative Grant Programme-2009).

DISCLOSURE

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

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

Table S1 | Correlation between HLA-DR-DQ haplotypes and the positivity of enzyme-linked immunospot assay.