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Significance of peripheral mononuclear cells producing interferon-γ in response to insulin B:9–23-related peptides in subtypes of type 1 diabetes

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ABSTRACT

Type 1 diabetes is largely caused by β-cell destruction through anti-islet autoimmunity. Reportedly, interferon (IFN)-γ-secreting peripheral blood mononuclear cells (PBMCs) specific to four insulin B-chain amino acid 9–23-related peptides (B:9–23rPep) were increased in type 1 diabetes participants. This study aimed to investigate the PBMC frequencies in subtypes of type 1 diabetes using enzyme-linked immunospot assay. In this cross-sectional study, peripheral blood samples were obtained from 148 participants including 72 with acute-onset type 1 diabetes (AT1D), 51 with slowly progressive insulin-dependent diabetes mellitus (SPIDDM), and 25 with type 2 diabetes. The frequency of B:9–23rPep-specific IFN-γ-producing PBMCs was significantly higher in AT1D participants than in SPIDDM and type 2 diabetes participants. Meanwhile, a significant inverse correlation was observed between the PMBC frequencies and insulin secretion capacity in SPIDDM participants. These findings suggest that the increased peripheral B:9–23rPep-specific IFN-γ immunoreactivity reflects decreased functional β-cell mass and greater disease activity of type 1 diabetes.

1. Introduction

The most common cause of type 1 diabetes is the destruction of β-cells via anti-islet autoimmunity that eventually progresses to an insulin-dependent state [1]. Insulin, glutamic acid decarboxylase (GAD), insulinoma-associated tyrosine phosphatase-like protein-2, and zinc transporter 8 are considered as major autoantigens in participants with type 1 diabetes [2] and many studies have reported the detection of autoreactive T-cells against these antigens [3]. Insulin is a representative pancreatic β-cell-specific protein and the most important islet-associated autoantigen. Insulin B-chain amino acid 9–23 (B:9–23) peptide is the main epitope targeted by T-cells in both mouse and human type 1 diabetes.

Non-obese diabetic (NOD) mouse is an excellent animal model of human type 1 diabetes. In these mice, insulin-specific $CD4^+$ T-cells have been strongly implicated in β-cell destruction. In pre-diabetic NOD mice, approximately 50% of the T-cell clones established from islet-infiltrating lymphocytes were insulin-specific, and majority of these clones produced interferon (IFN)-γ and recognized the insulin B:9–23 epitope [4,5]. Moreover, substitution of a single residue in the B:9–23 region abrogated development of diabetes in transgenic NOD mice [6,7]. Thus, B:9–23 epitope is considered pivotal to the development of diabetes in NOD mice.

In humans, increased B:9–23-specific IFN-γ-producing T-cells were first reported in peripheral lymphocytes obtained from patients with recent-onset type 1 diabetes and from pre-diabetes subjects at high risk

Abbreviations: ANCOVA, analysis of covariance; AT1D, acute-onset type 1 diabetes; B:9–23, insulin B-chain amino acid 9–23; B:9–23rPep, B:9–23-related peptides; BCG, Bacillus Calmette–Guérin; BMI, body mass index; CPI, C-peptide index; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot assay; FT1D, fulminant type 1 diabetes; GAD, glutamic acid decarboxylase; GADA, anti-GAD antibody; HbA1c, hemoglobin A1c; HLA, human leukocyte antigen; IAA, insulin autoantibody; IFN, interferon; IL, interleukin; LADA, latent autoimmune diabetes in adults; NGT, normal glucose tolerance; NOD, non-obese diabetic; N.S., not significant; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; RIA, radioimmunoassay; SD, standard deviation; SFC, spot-forming cell; SPIDDM, slowly progressive insulin-dependent (type 1) diabetes mellitus; T2D, type 2 diabetes; Tx, therapy

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for the disease [8]. The insulin B:9–23 amino acid sequence is identical in mice and humans, suggesting that this epitope plays an immunodominant and pathogenic role in human type 1 diabetes as well. B:9–23-specific IFN-γ-producing peripheral blood mononuclear cells (PBMCs) were also detected in Japanese type 1 diabetes pediatric participants by enzyme-linked immunospot (ELISpot) assay [9]. Intriguingly, IFN-γ-producing PBMCs were also detected with other insulin B-chain amino acids adjacent to B:9–23, such as B:10–24, B:11–25 and B:12–26 [9].

In terms of onset pattern of hyperglycemia, type 1 diabetes can be classified into three types – acute-onset type 1 diabetes (AT1D), slowly progressive insulin-dependent (type 1) diabetes mellitus (SPIDDM), and fulminant type 1 diabetes (FT1D) [10,11]. While viral infection and the subsequent immune reaction cause β-cell destruction in FT1D [12], most AT1D and SPIDDM result from anti-islet autoimmune destruction of the β-cells [1,13]. To our knowledge, there is no report on the four B:9–23-related peptides (hereinafter referred to as 'B:9–23rPep')-specific IFN-γ-producing PBMCs in participants with the two onset patterns of autoimmune type 1 diabetes, namely, AT1D and SPIDDM. Therefore, we distinguished the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in the two subtypes of type 1 diabetes participants using ELISpot assay and clarified the clinical significance of the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in type 1 diabetes. Participants with fulminant type 1 diabetes, a subtype of type 1 diabetes, characterized by an extremely abrupt onset, were excluded from this study due to remarkably few participants.

2. Material and methods

2.1. Participants

This is a cross-sectional study, and the period of investigation was from June 1, 2013 to December 31, 2018. Peripheral blood samples were obtained with informed consent from 148 insulin-treated Japanese diabetes participants, who regularly visited Saitama Medical University Hospital or Tokyo Saiseikai Central Hospital. Furthermore, their data on clinical characteristics (sex, age, body mass index (BMI), disease duration, ad lib plasma glucose level, hemoglobin A1c (HbA1c) level, ad lib serum C-peptide level and human leukocyte antigen (HLA)-DRB1 allele) were obtained. The diabetic condition was classified into the following three categories: AT1D ($n = 72$), SPIDDM or latent autoimmune diabetes in adults (LADA) ($n = 51$), and type 2 diabetes ($n = 25$). Since exogenous insulin itself could induce cellular immunity against the administered insulin molecule, insulin-treated type 2 diabetes participants were considered as appropriate control subjects.

2.2. Diagnostic criteria for diabetes mellitus

AT1D, SPIDDM and type 2 diabetes were diagnosed according to the diagnostic criteria of the Japan Diabetes Society [14–16]. Briefly, patients who develop ketosis or diabetic ketoacidosis within 3 months after the onset of hyperglycemic symptoms, who require insulin treatment continuously after the diagnosis of diabetes, and who have anti-islet autoantibodies are diagnosed with AT1D. In addition, those whose endogenous insulin secretion is exhausted (fasting serum C-peptide immunoreactivity < 0.6 ng/mL) without verifiable anti-islet autoantibodies are also diagnosed with AT1D [14]. The following two criteria are required for a definitive diagnosis of SPIDDM: 1) the presence of anti-GAD antibodies (GADA) and/or islet cell antibodies (ICAs) at some time during the patient's clinical course, and 2) the absence of ketosis or ketoacidosis at the onset (or diagnosis) of diabetes mellitus without the need for insulin treatment to correct hyperglycemia upon diagnosis [15]. All type 2 diabetes participants tested negative for GADA.

2.3. Measurement of serum GADA level

Serum GADA levels were initially measured using GADA-radioimmunoassay (RIA) kit (RSR Ltd., Cardiff, UK). However, from December 2015 onwards, GADA measurements in Japan were shifted from RIA to enzyme-linked immunosorbent assay (ELISA). Thus, serum GADA levels for some participants were determined using the GADA-ELISA kit (RSR Ltd.). The cut-off values for GADA-RIA and GADA-ELISA tests were 1.5 U/mL and 5.0 U/mL, respectively.

2.4. C-peptide index

C-peptide index which is the ratio of serum C-peptide to plasma glucose concentrations, is a readily measured index for β-cell function and is calculated as: 100 × serum C-peptide level (ng/mL)/plasma glucose level (mg/dL) [17]. Here, the C-peptide indices comprised of both fasting and postprandial C-peptide components. As with fasting C-peptide index, postprandial C-peptide index is also reported to be a practical index that reflects β-cell functional capacity [17].

2.5. HLA typing

HLA typing of DRB1 alleles was performed using a locus-specific polymerase chain reaction (PCR) amplification procedure as described previously [18].

2.6. Peptide synthesis and antigens

Four consecutive overlapping insulin peptides from human insulin B-chain – B:9–23 (insulin B-chain peptide with amino acid 9–23; SHLVEALYLVCGERG), B:10–24 (HLVEALYLVCGERGF), B:11–25 (LVEALYLVCGERGFF), and B:12–26 (VEALYLVCGERGFFY), were chemically custom synthesized by Japan Bio Services Co., Ltd. (Saitama, Japan). The purity of these insulin peptides was > 95%. Purified protein derivative (PPD) of *Mycobacterium tuberculosis* was obtained from Japan BCG laboratory (Tokyo, Japan). In Japan, universal Bacillus Calmette–Guérin (BCG) vaccination in infants and re-vaccination in school children have been conducted since the 1950s [19]. Consequently, the tuberculosis test usually shows a positive test result in most Japanese population. Therefore, PPD antigen was used as a positive control in ELISpot assay as described below.

2.7. ELISpot assay

The ELISpot assay was performed using a part of ELISpot assay tools packaged in the T-SPOT.TB kit (Oxford Immunotec Limited, UK), according to the manufacturer's instructions. Briefly, 10 mL of heparinized peripheral blood samples were collected from each participant and PBMCs were isolated from whole blood samples by centrifugation over a Ficoll density gradient (GE Healthcare Life Sciences, Pittsburgh, PA). The cells were re-suspended in AIM-V serum-free medium supplemented with L-glutamine/gentamycin/streptomycin (Gibco, Grand Island, NY) and adjusted to a concentration of 2.5×10^6 cells/mL. In total, 2.5×10^5 cells/well were seeded in 96-well plates pre-coated with anti-IFN-γ capture monoclonal antibody. The four insulin peptides – B:9–23, B:10–24, B:11–25, and B:12–26 – were added individually to the cells in duplicate, anti-human CD28 antibody (Clone: L293) (eBioscience, San Diego, CA) was added to the wells at a final concentration of 10 μg/mL, and then the plates were incubated for 24 h at 37 °C with 5% CO_2. The final concentration of each stimulant was 10 μg/mL. PBMCs in medium alone or stimulated with PPD at 10 μg/mL were used as negative or positive controls, respectively. Then, the alkaline phosphatase-conjugated secondary antibody was added for 1 h. After a washing step, the chromogenic substrate was added, and the individual spot-forming cells (SFC) were counted by use of an automated image analysis system, ELISpot reader (CTL ImmunoSpot S6

TATC Analyzer, CTL Analyzers LLC, OH). Each SFC represented an antigen-specific PBMC producing IFN-γ. The number of SFCs obtained as a result of antigen stimulation was determined as [(mean number of SFCs in the presence of stimulant) − (mean number of SFCs obtained without stimulation)]. The results of ELISpot assay were described by the number of SFCs per 2.5×10^5 cells. The cut-off value of SFC numbers for a positive result was set to 2.5 SFCs, which was determined as a mean number of SFCs +2 standard deviations (SD), using pooled data from all the numbers of IFN-γ SFCs detected by stimulation using each of the four insulin peptides in 25 insulin-treated type 2 diabetes participants. The mean absolute value of the difference ± SD for all paired SFC numbers was 0.06 ± 1.07 SFCs in response to insulin peptides. According to the manufacturer's instructions, the background number of SFCs in negative control well for PBMC was supposed to be < 10 SFCs due to limiting accuracies of measurement. Therefore, cases where the SFC numbers in negative control wells were > 10 SFCs, were excluded from the analysis. The frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in each participant was defined as the maximum number of IFN-γ SFCs obtained by ELISpot upon treatment with each of the four insulin B:9–23-related peptides and used for all analyses, as described previously [9].

2.8. Ethics

The study protocol was in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Saitama Medical University Hospital (Approval No. 15-123-5) and the Institutional Review Board of Tokyo Saiseikai Central Hospital (Approval No. 164).

2.9. Statistical analysis

All statistical analyses were performed using the IBM SPSS statistical software, version 23 (SPSS Inc., Chicago, IL). Data are presented as mean ± SD. Differences in SFC numbers between the three groups were analyzed for statistical significance by Kruskal–Wallis test followed by Dunnett's multiple comparison test. Differences in SFC numbers and C-peptide index between two groups were analyzed for statistical significance by Mann–Whitney *U* test. Difference in SFC numbers between SPIDDM participants with significantly different C-peptide index was analyzed for statistical significance by Student's *t*-test. Differences between categorical data were evaluated using Pearson's chi-square test or Fisher's exact test. Spearman's correlation coefficient (r_s) was used when comparing two nonparametric variables. A trend analysis (Jonckheere-Terpstra test) was performed for statistical significance of trends between AT1D, SPIDDM and type 2 diabetes participants. Analysis of covariance (ANCOVA) was used to determine the relationship between age, diabetes, duration, and the number of IFN-γ SFC. A value of $P < .05$ was considered significant.

3. Results

3.1. Clinical background of participants

There were significant differences in the age, diabetes duration, HbA1c, and C-peptide index between AT1D, SPIDDM, and type 2 diabetes participants (Table 1), suggesting the existence of heterogeneity in their clinical backgrounds. As for HLA, the frequency of DRB1*04:05 was significantly lower in type 2 diabetes participants than in AT1D and SPIDDM participants. Moreover, the frequencies of DRB1*15:01 or DRB1*15:02 were significantly lower in AT1D participants and significantly higher in type 2 diabetes participants.

3.2. Higher frequency of B:9–23rPep-specific IFN-γ-producing PBMCs was observed in AT1D participants

To investigate the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in AT1D, SPIDDM and type 2 diabetes participants, IFN-γ ELISpot assay was performed. There was a significant difference in IFN-γ SFC numbers between the three types of diabetes (Fig. 1; $P < .01$ by Kruskal–Wallis test). IFN-γ SFC numbers were significantly higher in AT1D participants than in SPIDDM (2.59 ± 3.04 vs. 1.55 ± 2.02 SFC; $P < .05$ by Dunnett's multiple comparison test) and type 2 diabetes participants (2.59 ± 3.04 vs. 1.00 ± 1.13 SFC; $P < .05$ by Dunnett's multiple comparison test). There was no significant difference in PPD-specific IFN-γ SFC numbers between AT1D, SPIDDM and type 2 diabetes participants (28.8 ± 32.8, 30.6 ± 33.7, 39.7 ± 68.4 SFC, respectively) (Supplementary Fig. 1).

As shown in Table 1, 14 of 72 AT1D participants were GADA negative. Therefore, we next investigated the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in GADA-positive AT1D ($n = 58$), GADA-negative AT1D ($n = 14$), SPIDDM, and type 2 diabetes participants in the same way. As shown in Supplementary Fig. 2, the number of IFN-γ SFCs was significantly higher in GADA-positive AT1D participants than in type 2 diabetes participants (2.53 ± 3.10 vs. 1.00 ± 1.13 SFC; $P < .05$ by Dunnett's multiple comparison test). However, there was no significant difference in the number of IFN-γ SFC between GADA-negative AT1D participants and type 2 diabetes participants (2.82 ± 3.10 vs. 1.00 ± 1.13), between GADA-positive AT1D and SPIDDM participants (2.53 ± 3.10 vs. 1.55 ± 2.02 SFC), or between GADA-negative AT1D and SPIDDM participants (2.82 ± 3.10 vs. 1.55 ± 2.02 SFC). This could be due to insufficient statistical power based on the small number of participants in each group.

A cut-off value of 2.5 SFCs was determined based on data from insulin-treated type 2 diabetes participants. Based on this cut-off, IFN-γ ELISpot was positive in 44.4% (32 out of 72) of AT1D participants, 21.6% (11 out of 51) of SPIDDM participants, and 8.0% (2 out of 25) of type 2 diabetes participants. The positivity rate in AT1D participants was significantly higher than those in SPIDDM and type 2 diabetes participants ($P < .05$ and $P < .01$, respectively, by chi-square test). Meanwhile, no significant difference was found in the positivity rate between SPIDDM and type 2 diabetes participants. These findings suggest that the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs is higher in AT1D participants, and these cells are associated with the pathogenesis of AT1D.

3.3. SPIDDM participants with increased frequency of B:9–23rPep-specific IFN-γ-producing PBMCs show decreased insulin secretion capacity

To clarify the clinical significance of the peripheral frequency of B:9–23rPep-specific IFN-γ-producing PBMCs, we evaluated the relationship between clinical parameters and IFN-γ SFC numbers in AT1D, SPIDDM and type 2 diabetes participants. While there were no significant correlations between IFN-γ SFC numbers and BMI, diabetes duration, plasma glucose level, or HbA1c level in any of the three types of diabetes, a significant positive correlation was observed between IFN-γ SFC numbers and age exclusively in SPIDDM participants ($r_s = 0.318$, $P < .05$), but not in AT1D and type 2 diabetes participants (Supplementary Table 1). Meanwhile, there were no significant differences in the age, sex, plasma glucose level, HbA1c level, BMI, and diabetes duration of participants showing positive or negative results in the IFN-γ ELISpot assay, in any of the three types of diabetes.

As for the insulin secretion capacity, there was a significant inverse correlation between IFN-γ SFC numbers and C-peptide index in SPIDDM participants ($r_s = -0.278$, $P = .049$ by Spearman's correlation coefficient test), but not in AT1D and type 2 diabetes participants (Supplementary Table 1, Fig. 2). Moreover, there was a significant difference in C-peptide index between SPIDDM participants showing positive or negative results in the IFN-γ ELISpot assay, but not in AT1D

Table 1
Clinical backgrounds of participants.

		AT1D (n = 72)	SPIDDM (n = 51)	T2D (n = 25)	P
Clinical characteristics					
Age (years)		46.2 ± 14.7	57.0 ± 14.8	63.3 ± 13.1	< 0.01[a]
Sex (Male/Female)		34/38	23/28	18/7	N.S.[b]
Disease duration (years)		8.8 ± 10.7	10.9 ± 10.0	17.0 ± 10.2	< 0.01[a]
Body mass index (kg/m^2)		22.3 ± 3.4	22.8 ± 5.1	24.3 ± 4.8	N.S.[a]
Laboratory findings					
Plasma glucose level	(mmol/L)	11.5 ± 7.5	9.6 ± 4.4	8.6 ± 2.5	N.S.[a]
	(mg/dL)	207.4 ± 135.4	173.8 ± 78.9	154.6 ± 45.1	
HbA1c	(mmol/mol)	80.6 ± 27.4	71.4 ± 20.9	60.7 ± 11.3	< 0.01[a]
	(%)	9.5 ± 2.5	8.7 ± 1.9	7.7 ± 1.0	
Serum C-peptide	(nmol/L)	0.16 ± 0.19	0.36 ± 0.42	0.61 ± 0.38	< 0.01[a]
	(ng/mL)	0.48 ± 0.58	1.07 ± 1.27	1.85 ± 1.15	
C-peptide index		0.29 ± 0.43	0.70 ± 0.97	1.25 ± 0.90	< 0.01[a]
GADA positivity (positive/negative)		58/14	48/3	0/25	< 0.01[b]
Presence of HLA DRB1*04:05 (Yes/No)		33/39	25/26	3/22	< 0.01[b]
Presence of HLA DRB1*09:01 (Yes/No)		40/32	23/28	9/16	N.S.[b]
Presence of HLA DRB1*08:02 (Yes/No)		9/63	4/47	0/25	N.S.[b]
Presence of HLA DRB1*15:01 or DRB1*15:02 (Yes/No)		1/71	4/47	6/19	< 0.01[b]

Data are shown as mean ± standard deviation (SD). AT1D, acute-onset type 1 diabetes; GADA, anti-glutamic acid decarboxylase antibody; HLA, human leukocyte antigen; SPIDDM, slowly progressive insulin-dependent (type 1) diabetes mellitus; T2D, type 2 diabetes.
[a] Kruskal–Wallis test.
[b] Chi-square test.

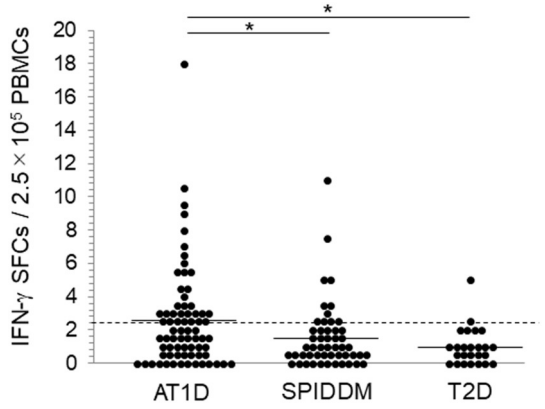

Fig. 1. ELISpot assay showing SFC numbers of B:9–23rPep-specific IFN-γ-producing PBMCs in the different categories of diabetes.
Each dot indicates the maximum number of IFN-γ SFCs obtained from each participant upon stimulation with each of the four B:9–23-related peptides, as determined by ELISpot assay. Horizontal solid line represents the mean IFN-γ SFC number. Horizontal dotted line represents a cut-off value of IFN-γ SFC numbers. Participants with AT1D (n = 72); participants with SPIDDM (n = 51); participants with type 2 diabetes (n = 25). *P < .05 between the two categories by Dunnett's multiple comparison test. AT1D, acute-onset type 1 diabetes; IFN, interferon; PBMC, peripheral blood mononuclear cell; SFC, spot forming cell; SPIDDM, slowly progressive insulin-dependent (type 1) diabetes mellitus; T2D, type 2 diabetes.

and type 2 diabetes participants (Fig. 3). Further, C-peptide indices were lower in AT1D participants and higher in type 2 diabetes participants than in SPIDDM participants, irrespective of their ELISpot assay results for IFN-γ (Fig. 3).

Next, all SPIDDM participants were divided into two categories based on low (≤ 0.479; n = 26) or high (> 0.479; n = 25) C-peptide indices, and their IFN-γ SFC numbers were evaluated. The median value C-peptide index was 0.479 in SPIDDM participants. Upon this categorization, the IFN-γ SFC numbers were significantly higher in SPIDDM participants with lower C-peptide index than in those with higher C-peptide index (2.14 ± 2.56 vs. 0.94 ± 0.96 SFC, respectively; P < .05 by Student's t-test) (Supplementary Fig. 3), indicating that SPIDDM participants with an increased frequency of B:9–23rPep-specific IFN-γ-producing PBMCs show decreased insulin secretion capacity. These findings suggest that higher frequency of B:9–23rPep-specific IFN-γ-producing PBMCs reflects a greater degree of β-cell destruction and an advanced stage of clinical condition in SPIDDM, though no significant difference in the PBMC frequency between SPIDDM and type 2 diabetes participants as shown in Fig. 1.

3.4. Detection of B:9–23rPep-specific IFN-γ-producing PBMCs is not associated with HLAs linked with type 1 diabetes

In Japan, HLA-DRB1*04:05, DRB1*08:02 and DRB1*09:01 are associated with type 1 diabetes development [20]. To clarify the relationship between the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs and HLA class II alleles associated with type 1 diabetes, we categorized IFN-γ SFC numbers according to the presence or absence of the three HLA alleles. There was no significant difference in the IFN-γ SFC numbers either in the presence or absence of the DRB1*04:05 and DRB1*08:02 HLA alleles in AT1D and SPIDDM participants (Supplementary Fig. 4A and B). However, IFN-γ SFC numbers were significantly higher in SPIDDM participants without DRB1*09:01 than those with it (Supplementary Fig. 4B). Moreover, we observed the same results if the participants with HLA-DRB1*15:01 and DRB1*15:02 (AT1D; n = 1, SPIDDM; n = 4), which are protective for type 1 diabetes [20], were excluded from the analysis. These findings suggest that B:9–23rPep-specific IFN-γ-producing PBMCs are detected independently of the HLA alleles associated with type 1 diabetes.

4. Discussion

This study demonstrated that the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs is higher in AT1D participants than in SPIDDM and insulin-treated type 2 diabetes participants. To our knowledge, there is no previous study that has investigated B:9–23-specific or B:9–23rPep-specific peripheral cellular immunity in type 1 diabetes participants classified according to the onset pattern of the disease. In addition, this is the first report investigating the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in SPIDDM participants and comparing the PBMC frequency between AT1D and SPIDDM participants. Although Strom et al. previously attempted to examine the frequency of B:11–23-specific IFN-γ-producing or interleukin (IL)-13-producing PBMCs using ELISpot assay in AT1D, LADA, type 2 diabetes and healthy control participants, they could not uncover any difference

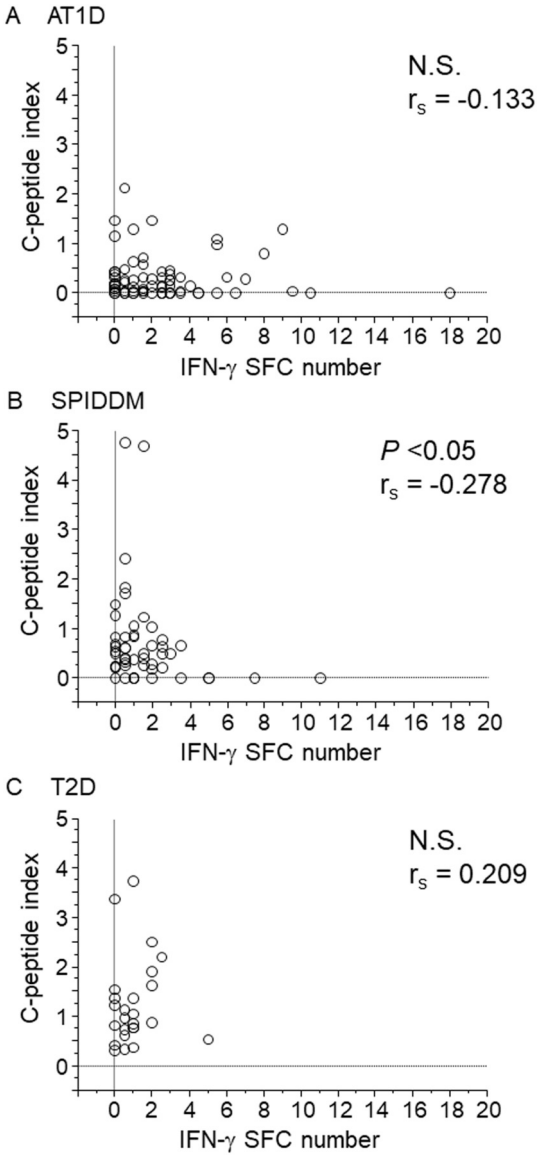

Fig. 2. Correlation between C-peptide index and SFC numbers of B:9–23rPep-specific IFN-γ-producing PBMCs in different categories of diabetes.
Dot-plots show a correlation between C-peptide index and the maximum number of IFN-γ SFCs obtained from each participant after stimulation with each of the B:9–23-related peptides using ELISpot assay. Participants with AT1D (n = 72); participants with SPIDDM (n = 51); participants with type 2 diabetes (n = 25). A significant inverse correlation between the two parameters was observed in SPIDDM participants (B) (Spearman's correlation coefficient, denoted as $r_s = -0.278$, $P < .05$), but not in AT1D participants (A) and type 2 diabetes participants (C). AT1D, acute-onset type 1 diabetes; IFN, interferon; N.S., not significant; PBMC, peripheral blood mononuclear cell; r_s, Spearman's rank correlation coefficient; SFC, spot forming cell; SPIDDM; slowly progressive insulin-dependent (type 1) diabetes mellitus; T2D, type 2 diabetes.

in the cell frequencies among them because of the low SFC numbers that could not be distinguished from the background levels [21]. Moreover, we found an inverse correlation between the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs and endogenous insulin secretion capacity (C-peptide index) in SPIDDM participants. These findings suggest that the increased frequency of B:9–23rPep-specific IFN-γ-producing PBMCs reflects a decreased functional β-cell mass and greater disease activity of type 1 diabetes. Meanwhile, there was no significant correlation between the PBMC frequency and C-peptide index in AT1D participants. The C-peptide index was significantly

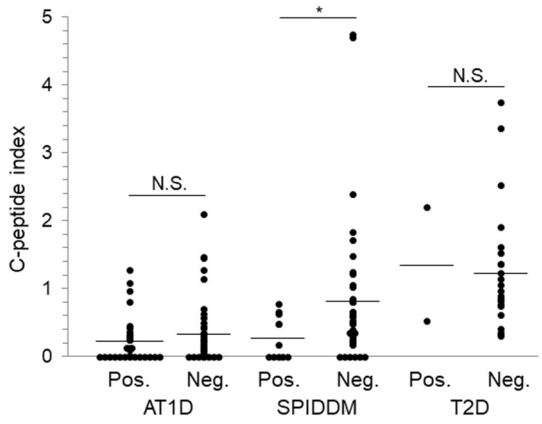

Fig. 3. C-peptide index classified according to positive and negative results of IFN-γ ELISpot assay in different categories of diabetes.
Each dot indicates C-peptide index in each participant. Horizontal solid line represents mean IFN-γ SFC number. The cut-off value of SFC number for the positive result was set to 2.5 SFC/2.5 × 10^5 PBMCs. AT1D participants with positive result (n = 32) and negative result (n = 40); SPIDDM participants with positive result (n = 11) and negative result (n = 40); type 2 diabetes participants with positive result (n = 2) and negative result (n = 23). $^*P < .05$ by Mann–Whitney U test. AT1D, acute-onset type 1 diabetes; IFN, interferon; PBMC, peripheral blood mononuclear cell; Pos., positive; Neg., negative; SFC, spot forming cell; SPIDDM, slowly progressive insulin-dependent (type 1) diabetes mellitus, T2D, type 2 diabetes.

distributed in a lower range in AT1D participants than in SPIDDM participants ($P < .01$ by Mann–Whitney U test) (Fig. 2; Y-axis). Consequently, it is challenging to clearly demonstrate an inverse correlation between C-peptide index and the PBMC frequency in AT1D participants.

As shown in Supplementary Fig. 2, the number of IFN-γ SFC was significantly higher in GADA-positive AT1D participants, but not in GADA-negative AT1D participants, compared to type 2 diabetes participants. These findings suggest that disease activity might be greater in GADA-positive AT1D than GADA-negative AT1D participants, although there was no significant difference in the number of IFN-γ SFC between the two AT1D groups, probably owing to the small sample size. Future studies using a larger number of participants could clarify the difference in disease activity among GADA-positive AT1D and GADA-negative AT1D participants.

The maximum number of IFN-γ SFCs in a set of ELISpot data with each of the four insulin B:9–23-related peptides was defined as a frequency of B:9–23rPep-specific IFN-γ-producing PBMCs in each participant. Accordingly, we separately analyzed all ELISpot assay data according to the four insulin peptides. Subsequently, a significant difference in the SFC numbers were obtained between AT1D and type 2 diabetes participants upon B:12–26 stimulation (1.20 ± 1.71 vs. 0.40 ± 0.63 SFCs, respectively; $P < .05$ by Dunnett's multiple comparison test) (Supplementary Fig. 5D). When the participants were classified according to those without DRB1*09:01 and/or without DRB1*08:02, similar results were observed. However, no significant differences in the SFC numbers were observed between AT1D and type 2 diabetes participants with DRB1*09:01 and/or with DRB1*08:02, and with or without DRB1*04:05. These findings suggest that the B:12–26-specific immune responses occur independently of HLA alleles susceptible for type 1 diabetes. With regards to B:9–23, B:10–24 and B:11–25, there were no significant differences in the IFN-γ SFC numbers among participants with the three type of diabetes (Supplementary Fig. 5A, B and C). The low peripheral frequencies of insulin peptide-specific IFN-γ-producing mononuclear cells may individually fail to show significant differences when categorized according to the four insulin peptides; this is a major limitation of this study using primary cultured PBMCs, although these immune responses may develop in the body. Studies using

in vitro expanded or cloned insulin peptide-specific T-cells may be required for clarification of the above results.

More IFN-γ-producing PBMCs were detected previously in recent-onset pediatric type 1 diabetes participants than established ones [9]. Here, there was no significant inverse correlation between disease duration and IFN-γ SFC numbers in type 1 diabetes. The discrepancies between the current and previous studies could be due to the difference in the degree of insulin peptide-specific immune responses between pediatric and adult type 1 diabetes participants. Indeed, the positivity rate of insulin autoantibody (IAA) measurements has been reported to be higher in pediatric type 1 diabetes participants than adult ones [22]. Moreover, the titer of IAA is inversely correlated to age of pediatric participants [23], suggesting the existence of a stronger immune reaction to insulin molecules in pediatric type 1 diabetes participants than adult ones, leading to the increase in IFN-γ-producing PBMCs in recent-onset pediatric, but not in adult type 1 diabetes participants with shorter disease duration, in this study. Alternatively, other islet-associated antigens or neoantigens might act as crucial antigens in the development of type 1 diabetes, or the balance between the expression/secretion of proinflammatory cytokines (IFN-γ) and immunoregulatory cytokines (IL-10 or IL-4) by B:9–23rPep-stimulated PBMCs might be more strongly associated with the immunological disease activity of type 1 diabetes. An investigation focusing on such a balance may help to clarify the role of B:9–23-related peptides in the early phase of clinical manifestation of autoimmune diabetes.

There were significant differences in participants' age, diabetes duration, and HbA1c levels among AT1D, SPIDDM and type 2 diabetes participants (Table 1), indicating the existence of a heterogeneity of participants' clinical background among the three types of diabetes. This clinical heterogeneity could confound the appropriate analysis of association between IFN-γ SFC numbers and types of diabetes. Trend analysis using Jonckheere-Terpstra test showed significantly lower mean age, lower mean diabetes duration and higher mean HbA1c levels in the following order – AT1D, SPIDDM, type 2 diabetes ($P < .01$). However, there were no significant inverse correlations between IFN-γ SFC numbers and age, and between IFN-γ SFC numbers and diabetes duration in overall participants. Similarly, there were no significant positive correlations between IFN-γ SFC numbers and HbA1c levels in overall participants. In addition, we used ANCOVA to evaluate whether age and diabetes duration can influence the number of IFN-γ SFC and found that these were not confounding factors influencing the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs (F-value, 1.48; $P = .23$, and F-value, 0.28; $P = .60$, respectively). Taken together, these findings suggest that age, diabetes duration, and HbA1c levels are not associated with the number of IFN-γ SFC.

Here, there was no difference in the frequency of B:9–23rPep-specific IFN-γ-secreting PBMCs between SPIDDM and insulin-treated type 2 diabetes participants (Fig. 1). We surmise that exogenous insulin itself may induce cellular immunity against insulin molecules, probably contributing to no difference in the frequency of B:9–23rPep-specific IFN-γ-secreting PBMCs between the two groups. Meanwhile, we collected preliminary data from eight participants with normal glucose tolerance (NGT) (male/female, 6/2; age, 48.4 ± 11.9 years) and nine type 2 diabetes participants without insulin therapy (male/female, 7/2; age, 60.8 ± 13.3 years; diabetes duration, 11.8 ± 9.8 years) and analyzed their IFN-γ SFC numbers. We did not find any significant difference in IFN-γ SFC numbers among the following three groups – NGT participants, type 2 diabetes participants with and without insulin therapy (0.69 ± 0.46, 1.00 ± 1.13, 1.11 ± 0.78 SFC, respectively) (Supplementary Fig. 6). These findings suggest the presence of a mixture of B:9–23rPep-specific and non-specific immune responses in the relatively low range of IFN-γ SFC numbers. It is possible that our ELISpot system does not have sufficient sensitivity to distinguish between the two immune responses, and hence cannot resolve differences in the frequencies of B:9–23rPep-specific IFN-γ-secreting PBMCs between SPIDDM and insulin-treated type 2 diabetes participants. However, given that the increased frequency of B:9–23rPep-specific IFN-γ-secreting PBMCs may reflect decreased functional β-cell mass in SPIDDM (Figs. 2, 3 and Supplementary Fig. 3), but not in insulin-treated type 2 diabetes participants, we believe that the cell frequency in SPIDDM participants may be of clinical and immunological significance at a range even below the cut-off value for IFN-γ SFC numbers.

There was a significant positive correlation between participants' age and IFN-γ SFC numbers in SPIDDM participants (Supplementary Table 1). Considering that participants' age was also inversely correlated with C-peptide index ($r_s = -0.336$, $P < .05$ by Spearman's correlation coefficient test), increased age may be associated with greater disease activity of SPIDDM, which may be supported by a previously demonstrated finding that increased age is one of the risk factors for LADA [24].

Here, we could not clarify the association between B:9–23-related peptides and HLA alleles susceptible for Japanese type 1 diabetes. Furthermore, while we could demonstrate that the frequency of B:9–23rPep-specific IFN-γ-secreting PBMCs was significantly higher in SPIDDM participants without DRB1*09:01 than those with it (Supplementary Fig. 4B), we could not determine HLA alleles other than DRB1*09:01 that might interact with B:9–23rPep. Previous studies demonstrated that B:9–23-specific immune responses can be observed in an HLA DQ8 (DQB1*03:02)-restricted manner in human type 1 diabetes [8]. HLA DQ8 confers high risk for type 1 diabetes exclusively in Caucasian populations [25]. However, in the current study, it remains to be elucidated whether B:9–23rPep-specific immune responses are DQ8-restricted or not. B:9–23-specific IFN-γ-producing lymphocytes were detected even in type 1 diabetes participants without DQ8 [8], suggesting that B:9–23rPep-specific immune responses are not necessarily observed in DQ8-restricted manner. To clarify the detailed mechanisms of B:9–23rPep-specific immune responses observed in this study, studies focusing on the binding of B:9–23rPep to HLA molecules will be needed in the future.

A previous study revealed that insulin B:9–23-specific IFN-γ-producing T-cells were increased in peripheral lymphocytes obtained from participants with recent-onset type 1 diabetes, but not from normal participants [8]. To verify these findings in our study, we compared the insulin B:9–23-specific number of IFN-γ SFC between recent-onset AT1D participants with diabetes duration of ≤1 year ($n = 25$) and NGT participants ($n = 8$). Using the Mann–Whitney U test, we found no significant difference between the recent-onset AT1D and NGT participants (0.56 ± 0.87 vs. 0.44 ± 0.42 SFCs, respectively). We also found no significant difference in the number of IFN-γ SFC between the recent-onset AT1D and type 2 diabetes ($n = 25$) participants (0.56 ± 0.87 vs. 0.30 ± 0.60 SFCs, respectively). The discrepancy in findings between our study and the previous study [8] might be caused by a difference in HLA (Japanese participants in our study vs. Caucasian participants in the previous study [8]) and a difference in age (adult participants in our study vs. pediatric participants in the previous study [8]). Another possible explanation for this difference is that other islet-associated antigens might act as crucial antigens early in the disease process of AT1D specifically in the Japanese population; this idea warrants further investigation.

Several other limitations exist in the current study in addition to the ones discussed above. First, the small sample size obtained from only two institutions, raises concerns about generalization of the data, and thus warrants further investigation. Second, due to the nature of the cross-sectional study design, the causal correlation between IFN-γ SFC numbers and β-cell functions remains unknown. To clarify this, a longitudinal prospective follow-up study will be needed in the future. Third, we did not examine the immune responses induced by insulin peptides besides the four B:9–23-related peptides. The frequency of other insulin peptide-specific IFN-γ-producing PBMCs is very rare [9]. Thus, although we believe that using these four insulin B:9–23-related peptides is sufficient for detecting insulin peptides-related immune responses, further investigations are necessary. Moreover, as discussed

above, secretion of immunoregulatory cytokines like IL-10 or IL-4 by PBMCs should also be investigated to clarify the immunological significance of B:9–23-related peptides in the development of type 1 diabetes. Finally, we did not distinguish the B:9–23rPep-specific immune responses using ELISpot assay according to sub-sets of T-cells – $CD4^+$/$CD8^+$ T-cells or immunoregulatory T-cells. Knowing which type of immune cells produce inflammatory or anti-inflammatory cytokines may help us to elucidate the detailed mechanisms for the development of type 1 diabetes.

5. Conclusions

In conclusion, the frequency of B:9–23rPep-specific IFN-γ-producing PBMCs was higher in AT1D participants than in SPIDDM and insulin-treated type 2 diabetes participants, irrespective of the presence of HLA susceptible for type 1 diabetes. Moreover, an inverse correlation between the PBMC frequency and endogenous insulin secretion capacity was observed in SPIDDM participants, though the very low reactivity. These findings suggest that the increased peripheral B:9–23rPep-specific IFN-γ immune reactivity reflects a decreased functional β-cell mass in type 1 diabetes and greater disease activity of type 1 diabetes, thereby playing a key role in the development of type 1 diabetes.

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Declaration of competing interest

A.Sh. has received lecture fees from Novo Nordisk Pharma Inc., Sanofi K.K., and Eli Lilly Japan K.K. M.N. has received lecture fees from Novo Nordisk Pharma, Inc., and MSD K.K. Y.H. has received research grants from Cosmic Corporation Co., Ltd., MSD K.K., Mitsubishi Tanabe Pharma Corporation, Novo Nordisk Pharma Inc., and Ono Pharmaceutical Co., Ltd. The other authors declare that there is no duality of interest associated with this manuscript.

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Informed consent

Written informed consent was obtained from all the participants.

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Author contributions

Y.O. analyzed data and wrote the manuscript. K.S. performed ELISpot assays. A.Sa., A.H., T.K., Y.H., I.I., M.N., and A.Sh. reviewed the manuscript and contributed to the discussion. Y.O. is the guarantor of this work and 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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