Adult-onset type 1 diabetes patients display decreased IGRP-specific Tr1 cells in blood

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ABSTRACT

The breakdown of immune tolerance against islet antigens causes type 1 diabetes (T1D). The antigens associated with adult-onset T1D (AT1D) remain largely undefined. It is possible that AT1D patients display a unique type of CD4^{+} T cells specific for a certain islet antigen. Here we analyzed the cytokine production profiles of CD4^{+} helper T (Th) cells that are specific for three islet antigens; GAD65, preproinsulin, and IGRP in patients with AT1D, juvenile-onset T1D (JT1D), and age-, gender- and human leukocyte antigen (HLA)-matched control adults. While IGRP-specific Th cells in AT1D patients were dominantly Th1 cells, IGRP-specific Th cells in control adults and JT1D patients were dominantly Th2 and T regulatory type 1 (Tr1) cells. Notably, the frequency of IGRP-specific Tr1 cells was significantly lower in AT1D patients than in control adults and JT1D patients. In conclusion, our study suggests that IGRP-specific Th cells play a unique pathogenic role in AT1D.

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1. Introduction

Autoreactive T cells play a major role in the destruction of insulin-producing islet β cells in type 1 diabetes (T1D) [1–5]. Autoreactive T cells developed in T1D patients recognize various islet-associated antigens including glutamic acid decarboxylase 65 (GAD65) [6–8], preproinsulin (PPI) [9–11], insulinoma associated-2 (IA-2) [12], islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP) [13,14] and zinc transporter-8 (ZnT8) [15,16]. Islet-specific T cells found in T1D patients contain inflammatogenic IFN-γ-producing CD4^{+} helper T (Th) type 1 cells [16–18]. While the onset of T1D usually occurs during the first decade of life (juvenile T1D, JT1D), some adults develop diabetes accompanied by the generation of autoantibodies reactive to islet antigens (adult-onset T1D (AT1D)). The reason why AT1D patients develop diabetes much later in life than JT1D patients is unknown.

It is possible that the immune mechanism associated with the development of anti-islet autoimmunity differs between JT1D and AT1D patients. Accordingly, the dominant islet antigens associated with the development of JT1D and AT1D might also be different. In line with this hypothesis, in a Belgian T1D patient cohort, more than 90% of the patients less than 10 years old were positive for serum anti-insulin antibodies, while only approximately 40% were positive among the patients between 10 and 39 years old [19]. Furthermore, while approximately 90% of cases of latent autoimmune diabetes in adults were positive for only one islet autoantibody, JT1D patients often display multiple islet autoantibodies [20–22].

In this study, we hypothesized that the dominant islet antigen associated with AT1D was different from that associated with JT1D, and accordingly, AT1D patients would display CD4^{+} helper T (Th) cell subsets against certain islet antigen(s) distinct from those of JT1D patients. To address this hypothesis, we assessed the cytokine production profiles of CD4^{+} T cells specific for the islet antigens GAD65, PPI and IGRP in AT1D, JT1D and control adults. We herein report that the dominant type of IGRP-specific CD4^{+} T cells was largely distinct between AT1D and control adults JT1D.

2. Materials and methods

2.1. Blood samples

Peripheral blood samples were obtained from patients with JT1D, AT1D and age- and gender-matched healthy adults after informed consent was obtained. The protocol was approved by the institutional review board (IRB7109-92). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll-Paque PLUS (GE-Healthcare Bio-Sciences, Piscataway, NJ) from sodium-heparinized blood within 24 h after sampling. Then, PBMCs were cryopreserved in FBS with 10% of DMSO at the concentration of 10–20 million cells/ml and stored in liquid nitrogen tanks. The duration of the disease at sampling (235.8 ± 210.4 days vs 243.0 ± 168.3 days, $p = 0.89$ by Fisher's exact test) and the haplotype distribution ($p = 0.15$) were similar between JT1D and AT1D groups.

2.2. Peptides

Well-characterized immunodominant class II peptides (13–20-mers, BioSynthesis) were used in this study: six GAD65 [6,23–29], four PPI [9–11,17,30,31] and four IGRP [13] peptides (Table 1). The peptides were dissolved with 50% acetonitrile at 2.0 mM (Sigma-Aldrich), and the generated peptides were pooled according to the antigen type (GAD65 peptides were split into two pools), which resulted in the generation of four peptide clusters (C1 and C2: GAD65 peptide clusters, C3: PPI peptide cluster, C4: IGRP peptide cluster; Table 1). Peptides were kept frozen at -80 °C.

2.3. Assessment of antigen-specific Th repertoire

To determine the type of islet antigen-specific Th cells in the blood samples, we applied an integrated approach that we described recently [32]. This approach consists of two assays: the Direct assay and the Cytokine-driven assay. The sensitivity in the detection of specific Th cells is higher in the Cytokine-driven assay due to the inclusion of an expansion phase of peptide-reactive Th cells [32].

2.4. The Direct assay

The detailed protocols were described previously [32]. Briefly, frozen PBMCs were thawed in a water bath at 37 °C, washed three times with $1 \times$ PBS, and re-suspended at a concentration of 2.5×10^6 cells/ml in complete RPMI medium (CM). After incubating cells at 37 °C for 15 min, 5×10^5 cells per well were cultured in a 96-well deep well plate in the presence of peptide clusters or single peptides (final concentration of 2.5 μM per peptide). Equal amount of peptide diluent

(50% acetonitrile) was used as negative controls. On day 2 of the culture, culture supernatant was harvested to measure cytokine levels (IL-2, IL-10, IL-13, IL-17A, IL-21 and IP-10) by a multiplex bead-based Cytokine assay (BIO-RAD). We defined cytokine secretion in response to stimulation with peptides as "positive" when the levels of each cytokine were more than twofold that of the background (PBMCs cultured with the diluent alone). 5 pg/ml in IL-2, IL-10, IL-13, IL-17A or IL-21 and 10.0 pg/ml in IP-10 were used as background when the background levels were less than those values [32].

2.5. The Cytokine-driven assay

The detailed protocols were described previously [32]. Briefly, PBMCs stimulated for two days with peptides or peptide diluent (in the Direct assay) were further cultured in the presence of recombinant human IL-2 (100 U/ml) to expand the antigen-specific T cells. The cells were harvested on day 8, starved for 24 h in serum-free media, resuspended with CM to a concentration of 1.0×10^6 cells/ml and then were re-stimulated for 6 h with the same peptides (2.5 μM per peptide) or peptide diluent in the presence of Brefeldin A during the last 4 h. After surface staining was performed, the cells were stained for intracytoplasmic cytokines. CD3 FITC (SK7), CD8 PerCP (SK1) and IL-10 PE (JES3-19F1) were obtained from BD Pharmingen. CD4 PE-Cy7 (S3.5) and LIVE/DEAD fixable aqua dead cell stain kit were obtained from Invitrogen. IL-13 APC (JES10-5A2) and IFN-γ Pacific Blue (4S.B3) were obtained from Biolegend. Stained cells were analyzed with a FACSCantoII™ flow cytometer (BD Biosciences) and the data was analyzed using the FLOWJO software.

2.6. Statistical analysis

Values were dichotomized based on their value equal to 1 or not. A Fisher's exact test was used to test the association between this derived variable and patient status. The raw and Bonferroni adjusted p-values are reported. Data were log (base 10) transformed and analyzed using Multivariate Analysis of Variance. Wilks' Lambda was used to test the overall difference between the two indicated groups. If the overall test was significant, univariate Analysis of Variance was used to test for differences between the groups. The frequency of positive responders and distribution of HLA genotypes between the cohorts was compared using Fisher's exact test. Bar graphs in the figures show the mean $\pm$ SD.

3. Results

3.1. Different IGRP-specific Th response between AT1D patients and controls

We first accrued 15 recent-onset AT1D patients (blood sampling days following diagnosis: 285.9 ± 247.9 days, age 37 ± 12 years old, mean $\pm$ SD) and 15 age-, gender- and HLA-class II-matched control adults (Supplementary Tables 1 and 2), and analyzed the Th responses against peptide clusters (C1–C4) or single peptides (p1–p14) from three islet antigens; GAD65, PPI and IGRP (Table 1).

Fig. 1A shows an example of the cytokine data from the Direct assay. In the culture of PBMCs from control adult #3 (Ctrl #3), IL-10 but not IP-10, was produced in response to stimulation with the PPI and IGRP peptide clusters (hereafter called an IL-10 response). In contrast, in the culture of PBMCs from AT1D patient #2 (AT1D #2), IP-10 but not IL-10, was produced in response to stimulation with the IGRP peptide cluster (hereafter called an IP-10 response, which reflects the presence of a specific Type 1 response [32]). Other cytokines (IL-2, IL-13, IL-17A and IL-21) were not produced at detectable levels in these cultures (not shown).

The amount of cytokines in the PBMC cultures from 15 AT1D patients and 15 control adults are summarized in Fig. 1B. The positive PBMC cultures were selected where cytokines were produced more than twofold over the background in response to any antigen peptides. Upon culture with GAD65 peptides (C1 and C2), an IP-10 response was

Table 1
Layout of islet-specific peptides and peptide clusters.

Antigen	Cluster no.	Peptide no.	Position	AA sequence
GAD65	C1	p1	115–127	MNILLQYVVKSFD
		p2	247–266	NMYAMMIARFKMFPEVKEKG
		p3	274–286	IAFTSEHSHFSLK
	C2	p4	491–510	YEMVFDGKPQHTNVCFWYIP
		p5	505–519	CFWYIPPSLRTLEDN
		p6	555–567	NFFRMVISNPAAT
PPI	C3	p7	B9–B23	SHLVEALYLVCGERG
		p8	B20–C4	GERGFFYTPKTRREAED
		p9	C19–A3	GSLQPLALEGSLQKRGIV
		p10	A1–A15	GIVEQCCTSICSLYQ
IGRP	C4	p11	13–25	QHLQKDYRAYYTF
		p12	23–35	YTFLNFMSNVGDP
		p13	226–238	RVLNIDLLWSVPI
		p14	247–259	DWIHIDTTPFAGL

No., number; AA, amino acid; GAD65, glutamic decarboxylase 65; PPI, preproinsulin; IGRP, islet-specific glucose 6 phosphatase catalytic subunit-related protein.