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Identification of Novel IGRP Epitopes Targeted in Type 1 Diabetes Patients

Irene Jarchum^a, Lynn Nichol^b, Massimo Trucco^b, Pere Santamaria^c, and Teresa P. DiLorenzo^{a,d,*}

^aDepartment of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461

^bDivision of Immunogenetics, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213

^cDepartment of Microbiology and Infectious Diseases and Julia McFarlane Diabetes Research Centre, University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada

^dDepartment of Medicine (Division of Endocrinology), Albert Einstein College of Medicine, Bronx, NY 10461

Abstract

CD8⁺ T cells play an important role in the development of type 1 diabetes (T1D) in NOD mice and humans. IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein) has emerged in recent years as a major antigen in NOD mice. Therefore, we aimed to determine if IGRP is an antigen in T1D patients and to identify the HLA-A2-restricted IGRP epitopes targeted. Using IFN- γ ELISPOT assay, we tested PBMC from recent-onset pediatric T1D patients and healthy controls for reactivity to four IGRP peptides directly *ex vivo*. Importantly, 65% of patients and 0% of controls were positive for at least one IGRP peptide. Two of these have not been reported previously. These data provide evidence that IGRP is a CD8⁺ T cell antigen in humans, contributing to the understanding of the underlying disease process as well as to future directions for diagnosis and monitoring disease progression in T1D patients.

Keywords

T cells; Antigens; Human; Autoimmunity; Diabetes; IGRP

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by immune-mediated destruction of pancreatic beta cells, resulting in permanent beta cell loss and insulin deficiency. In the NOD mouse model of T1D, CD8⁺ T cells play an essential role in the development of the disease [1]. This is evidenced in several models of NOD mice lacking CD8⁺ T cells, such as NOD. $\beta 2m^{null}$ mice [2], CD8 α -deficient mice [3] and mice treated with anti-CD8 antibodies [4], which are protected from disease. CD8⁺ T cells are also thought to play a crucial role in disease progression in humans, where they are a predominant cell type found in infiltrated islets of T1D patients and in islet grafts of patients with recurrent autoimmune disease [5–8].

*Corresponding author. Dr. Teresa P. DiLorenzo, Albert Einstein College of Medicine, Microbiology and Immunology, 1300 Morris Park Ave., Bronx, NY 10461; telephone: 718-430-2014; FAX: 718-430-8711. E-mail address: dilorenz@aeom.yu.edu.

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In addition to environmental factors of elusive nature, many genetic factors contribute to T1D susceptibility [9]. Of these, class II MHC alleles are the major single contributor in mice and humans. However, class I MHC alleles are also involved in susceptibility to T1D [10–14]. The class I allele HLA-A2 is one of the most common alleles and it represents an additional risk factor in T1D patients with high risk class II alleles [13]. Importantly, HLA-A*0201 transgenic NOD mice have accelerated disease and generate HLA-A*0201-restricted CD8⁺ T cell responses [15;16].

Because of the crucial role that CD8⁺ T cells and their class I MHC-restricted targets play in disease progression to T1D, the development of specific monitoring and therapeutic strategies requires the knowledge of the antigenic peptides recognized by pathogenic CD8⁺ T cells. However, this work is often limited by the difficulty in accessing tissues where a high frequency of autoreactive T cells is found (*e.g.*, pancreas), by the size of blood samples obtained from patients that are often young children, and by the fact that at the time of clinical symptoms, up to 80% of islets have been destroyed [17]. In spite of this, efforts to study the peptide targets of CD8⁺ T cells have resulted in the identification of epitopes derived from several antigens, including proinsulin [18;19], human islet amyloid polypeptide precursor protein (ppIAPP) [20;21], and more recently, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) [21–23].

The work described here, which was nearing completion at the time of publication of the latter work mentioned, was aimed to identify epitopes of IGRP in T1D patients. This autoantigen is a non-essential ER-resident protein [24] that was identified by our group in recent years as a target of the autoimmune attack in NOD mice when we found that IGRP 206–214 is the antigen recognized by the highly diabetogenic clone 8.3 [25–28]. Importantly, the frequency of IGRP-reactive T cells in the blood of NOD mice can predict development of the disease [29;30] and IGRP-reactive CD8⁺ T cells are the most prevalent specificity early during graft rejection [31]. Interestingly, single-cell sorting and TCR sequencing was used to determine that the repertoire of IGRP-reactive T cells in the blood of both diabetic and non-diabetic mice is representative of that found in pancreatic lymph nodes and islets [32]. Although so far this has only been demonstrated for IGRP 206–214-reactive T cells in NOD mice [32], the results suggest that this may reflect the underlying biology of autoreactive T cells in organ-specific autoimmune disease and that T cells specific for epitopes as identified using peripheral blood may be found in the pancreas of diabetic patients.

Upon finding that IGRP is a major antigen in NOD mice, we decided to begin addressing the possibility that IGRP plays a role in the autoimmune disorder in T1D patients. To this end, we utilized HLA-A*0201 transgenic mice to identify epitopes of IGRP that are targeted during spontaneous progression to diabetes by isolating islet-infiltrating T cells and identifying their peptide targets [15]. Using this model, we found 3 epitopes that elicit an HLA-A*0201-restricted T cell response. In an effort to determine whether IGRP plays a role in the development of T1D in humans, we screened PBMC obtained from recent-onset HLA-A2⁺ T1D patients and healthy controls with a panel of putative IGRP epitopes that included one of the peptides targeted in HLA-A*0201-transgenic mice [15] as well as human IGRP peptides predicted to bind to HLA-A*0201 by computer algorithms. As we face the need for the development of diagnostic, monitoring, and therapeutic approaches in T1D, the increasing knowledge of the antigenic peptides targeted by T cells is a key contribution.

Materials and Methods

Subjects

Blood was collected from 20 patients with recent onset T1D (average age, 9.5 years old $\pm$ 4.0) and 10 healthy subjects without a history of first-degree relatives with T1D (average age, 30.8

± 5.4 years old). Patients were recruited from The Children's Hospital at Montefiore (Bronx, NY). All patients were lean and presented at diagnosis with acute onset of symptoms, and have required permanent insulin treatment since diagnosis. Unfractionated PBMC were obtained by Ficoll-Paque (Amersham Biosciences, Pittsburgh, PA) separation as described by the manufacturer, within 3 hours from venisection. PBMC were frozen in 10% DMSO, 20% FCS/RPMI. Islet cell antibodies were measured by indirect immunofluorescence, and insulin and glutamic acid decarboxylase antibodies were measured by radioimmunoassay (American Medical Laboratories, Chantilly, VA). Low-resolution typing of HLA-A, -B, -C, -DQ, and -DR was performed by PCR followed by sequence-specific oligonucleotide probing (SSOP) using LABType SSO Typing Tests (One Lambda, Canoga Park, CA). High-resolution typing of HLA-A2 alleles was performed by PCR with sequence-specific primers (SSP) using a PEL-FREEZ SSP UniTray kit (Dynal, Brown Deer, WI). All typing was performed at the Children's Hospital of Pittsburgh (Pittsburgh, PA). Informed consent was obtained from all individuals and their parents or guardians, and the study was approved by the relevant ethics committees.

Peptides

Peptides were synthesized by standard solid-phase methods using Fmoc chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA), and their identities were confirmed by mass spectrometry. Concentrated stocks (10 mM) were prepared in DMSO, and 10 μ M working stocks were obtained by dilution in PBS.

IFN- γ ELISPOT

ELISPOT plates (MAHA S45 10; Millipore, Billerica, MA) were precoated with anti-human IFN- γ mAb (RnD Systems, Minneapolis, MN) and blocked with 1% BSA (Fraction V; Sigma-Aldrich, St. Louis, MO). Frozen unfractionated PBMC were thawed, washed and added at an average of $2.3 \times 10^5 \pm 1.5 \times 10^5$ cells/well as they were available. Anti-CD28 mAb (CD28.2; BD Pharmingen, San Jose, CA) was added to the cells at a concentration of 1 μ g/ml to improve the costimulatory environment that may be suboptimal in the periphery [33]. Cells were pulsed with 1 μ M peptide and plates were incubated at 37° C for 40 hours. IFN- γ secretion was detected with a second, biotinylated anti-human IFN- γ mAb (RnD Systems) and spots were developed using streptavidin-alkaline phosphatase (Zymed Laboratories, Carlsbad, CA) and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate (Sigma-Aldrich). All peptides were tested in duplicate or triplicate as cells were available. HLA-A2-binding peptides from HIV (used for only 2 patients, both with negative responses to the peptide) or HCV were used as likely irrelevant negative controls. Two of the patients were not tested for reactivity against the HCV Core 132–140 or HIV Gag 77–85. However, they were included in the study because they were negative for at least 2 of the IGRP peptides tested. Spots with a minimum size of 0.01 mm² were counted using an automated ELISPOT reader system (Autoimmun Diagnostika, Strassberg, Germany). To obtain the stimulation index (SI), the mean number of spots in test wells was divided by the mean number of spots in PBS wells. When the mean number of spots in PBS wells was zero, which was the case for a single patient, it was equaled to one. The average number of spots in PBS wells was similar for patients (8.6 ± 9.8 ; range, 0–33) and controls (16.8 ± 6.9 ; range, 8.5–31.5).

Peptide-binding assay

Peptide binding to the HLA-A2 molecule was determined as previously described [34]. Briefly, T2 cells were incubated overnight at 26° C and washed in serum-free medium. Cells (1×10^5) in 70 μ l serum-free RPMI were added to round bottom 96-well plates with 10 μ l peptide (at 10X to achieve the final concentrations shown in Fig. 2) and 20 μ l human beta-2-microglobulin in PBS (final concentration 15 μ g/ml; Sigma-Aldrich). Cells were incubated at 37° C for 20 h, transferred to V-bottom 96-well plates and incubated at 50° C for 3 min. The cells were washed

twice in cold PBS, stained with FITC-conjugated anti-HLA-A2 mAb BB7.2 (BD Pharmingen), and analyzed by flow cytometry. The up-regulation of HLA-A2 is reported as the fluorescence index, defined as the mean fluorescence intensity (MFI) of the experimental sample divided by the MFI in the absence of peptide. Results shown are representative of 3 independent experiments.

Statistical analysis

Wilcoxon rank-sum tests were used to compare mean SI between patients and controls.

Results

Patients

To determine whether IGRP-reactive CD8⁺ T cells can be found in the blood of T1D patients, we examined the IFN- γ response of PBMC to a panel of candidate HLA-A2-restricted IGRP epitopes directly *ex vivo*. We collected blood from recent onset (median duration of disease, 80 days; range 5–398) T1D patients and healthy controls and used high-resolution HLA typing for HLA-A2 and low-resolution typing to determine other HLA alleles. While HLA-A*0201 was the most common HLA-A2 subtype in both patients and controls, other subtypes were also represented in our population (Table 1). This was not surprising, given that our Bronx population of patients and controls is 57% Hispanic, 23% African American and 17% Caucasian, and in light of the fact that the HLA-A*0201 allele is present in 96% of Caucasian but in only 59% of African American and 73% of Hispanic individuals carrying an HLA-A2 allele [35]. Interestingly, the other subtypes present in our population belong to the HLA-A2 supertype and are all characterized by the same peptide binding preference as HLA-A*0201 [36]. We therefore reasoned that other A2 alleles may present the same peptides, and that inclusion of non-HLA-A*0201 individuals in our study would broaden the applicability of our findings to a larger ethnic population.

As shown in Table 1, 80% (16/20) of patients and 50% (5/10) of controls carried the high risk class II MHC alleles DR3 or DR4 [37], and 20% (4/20) of patients and none of the controls were DR3/DR4 heterozygotes.

Rationale for construction of the IGRP peptide panel

One of the peptides that we identified in HLA-A*0201 transgenic NOD mice, IGRP 265–273, is completely conserved between mouse and human IGRP and is an excellent candidate for evaluating IGRP antigenicity in humans. However, this mouse model did not allow us to identify IGRP epitopes that are unique to human IGRP, and in order to identify these candidate epitopes, we used 4 computer algorithms. Since the HLA-A*0201 molecule is the only molecule belonging to the HLA-A2 supertype available from multiple algorithms, this member of the supertype was used for peptide binding predictions. Because the blood volume available from pediatric patients is small, only a limited number of peptides can be screened directly *ex vivo*. Therefore, SYFPEITHI [38], BIMAS [39], RankPep [40], and SVMHC [41] were used to identify 3 epitopes (IGRP 211–219, IGRP 215–223, and IGRP 222–230) that ranked first, second, third or fourth in at least 2 of the algorithms. When available, we determined IGRP peptide binding predictions for other A2 alleles in the supertype and found that all 3 peptides ranked first, second, third or fourth for binding to at least one other allele of the A2 supertype. We also included in our panel ppIAPP 5–13, which was previously reported to elicit IFN- γ production from unfractionated PBMC from T1D patients [20]. In addition, because individuals at the clinic where we recruited patients are vaccinated against the flu upon T1D diagnosis, we suspected that some of them may have been vaccinated before we obtained the sample, and decided to include the influenza-derived peptide MP 58–66 as a control (Table 2).

IFN- γ response to IGRP peptides in T1D patients

We then performed IFN- γ ELISPOT directly *ex vivo* to establish if any of the IGRP peptides are antigenic in T1D patients. To determine the best cut-off for positivity, a receiver operating characteristic (ROC) plot, which takes into account the sensitivity (percentage of patients positive for at least 1 IGRP peptide) and specificity (percentage of healthy controls negative for all IGRP peptides) of the assay was used (not shown). A stimulation index (SI) ≥ 2.5 was chosen as the most appropriate cut-off. We chose the SI to express the results, as we and others [42] consider this method to be a fitting representation of the degree of signal over background when working in systems such as the one reported here.

Responses to all 4 IGRP epitopes were observed (Fig. 1A), with 65% of patients versus 0% of healthy controls testing positive for at least one IGRP peptide and 35% of patients versus 0% of controls positive for 2 or more IGRP peptides (data not shown). Three patients tested positive for IGRP 265–273, while none of the controls tested positive. The largest number of positive responses was seen to IGRP 222–230, to which 45% of patients and 0% of controls tested positive (9/20 and 0/10, respectively), and IGRP 215–223, with 35% of patients and 0% of controls testing positive (7/20 and 0/10, respectively). Three of the 4 patients heterozygous for DR3/DR4 showed reactivity to IGRP. Interestingly, all 4 HLA-A2⁺ patients carrying non-A*0201 alleles were positive for at least one IGRP peptide, suggesting these are targeted in T1D in the context of other alleles in the HLA-A2 supertype. One healthy control and 5 patients tested positive for ppIAPP 5–13. Responses to Flu MP 58–66 were seen in both patients and controls and the difference was not statistically significant ($p = 0.093$). All patients and controls showed equally strong responses to PHA (Fig. 1B and data not shown).

Binding affinity of IGRP peptides to HLA-A2

We then investigated the ability of our panel of IGRP peptides to bind to HLA-A2 using a T2 cell-based MHC stabilization assay. T2 cells are deficient for TAP1/2, so HLA-A2 is unstable on the surface of the cells at 37° C. Peptide binding affinity can be determined as a measure of the degree of MHC stabilization they achieve on the surface of the cells. Using this system, the binding of the 4 IGRP peptides to HLA-A2 was assessed (Fig. 2). IGRP 215–223 demonstrated strong binding affinity as compared to Flu MP 58–66, followed by IGRP 265–273 and IGRP 222–230. IGRP 211–219, although it ranked as a predicted excellent binder using computer algorithms, showed little to no binding in this assay. This is, however, not the first time that a peptide showing poor binding in an assay of this type is found to elicit CD8⁺ T cell reactivity; a similar case was noted for a major H-2K^d-restricted epitope in NOD mice (*i.e.*, Ins B15–23) [43].

Discussion

We report here the presence of IGRP-reactive T cells capable of producing an IFN- γ response directly *ex vivo* in the blood of pediatric T1D patients. Our findings confirm recent reports, only one of which was performed with pediatric patients, of reactivity to IGRP in recent onset T1D patients [22;23;44]. Of particular interest is IGRP 265–273, an epitope we previously identified as being targeted by CD8⁺ T cells in HLA-A*0201 transgenic mice [15]. Importantly, our data show that reactivity to this epitope is present in the pediatric T1D population, complementing recently reported results demonstrating reactivity in the adult T1D population [23]. The highest reactivity was to a novel epitope, IGRP 222–230, to which reactivity was seen in 45% of patients and 0% of the controls. We also report here for the first time an IFN- γ response to IGRP 211–219 and to the previously described IGRP 215–223 [22;44]. Of note is that a considerable percentage of patients respond to the individual peptides, and 65% of patient versus 0% of healthy control PBMC produce IFN- γ in response to at least 1 of the IGRP epitopes studied here, suggesting that IGRP reactivity alone can identify a large percentage of

T1D patients. In addition, while other studies often exclude non-HLA-A*0201 individuals [20;22;23;44] or sometimes include all HLA-A2 individuals without high-resolution typing of the HLA-A2 allele [18] (which would include HLA-A2 alleles not present in the supertype [36]), here we identify and include only individuals with non-HLA-A*0201 alleles that are members of the HLA-A2 supertype. Importantly, inclusion of non-HLA-A*0201 individuals in the study allows us to conclude that the IGRP epitopes described here may be used to identify T1D patients not only carrying the HLA-A*0201 allele but also other alleles of the HLA-A2 supertype.

Unfortunately, because of blood volume limitations resulting from working with samples from pediatric patients, we were unable to provide direct evidence that CD8⁺ T cells are responsible for the production of IFN- γ in our assay. However, several reasons strongly suggest the responses observed here were generated by HLA-A2-restricted responses to the peptides. First, we have successfully refolded recombinant HLA-A2 with each of the IGRP peptides described here (data not shown). Second, robust binding of IGRP 215–223 to HLA-A2 was also noted using our cell based assay (Fig. 2), and IGRP 265–273 has been previously characterized by us as a CD8⁺ T cell target using HLA-A2 transgenic NOD mice [15]. Third, all epitopes described here are 9mers, and while peptides binding class II MHC usually vary between 13 and 17 amino acids in length [47], those binding class I MHC are generally 8 to 10 amino acids long [48].

Recently, the pattern of expression of IGRP splice variants in human tissues was investigated, and while expression of full-length IGRP was found in pancreas, the only transcripts detected in human thymus were lacking exon 4 [45]. This would result in a frameshift mutation, preventing expression not only of exon 4, but also of the last and largest exon (exon 5), and a failure to establish tolerance to IGRP epitopes found in this region of the protein. Interestingly, all epitopes reported here lie within exon 5, and epitopes 211–219, 215–223, and 222–230 may represent a “hot spot” for T cell reactivity. The previously described epitope 152–160 [22] is encoded by exon 4. In addition, IGRP lies near a putative T1D susceptibility locus, *IDDM7* [46], and the possibility that its expression and that of its splice variants is regulated and contributes to diabetes susceptibility has not been excluded.

It is widely recognized today that as immune-targeted interventions become available, new approaches are needed for the development of tools to monitor disease progression. Although useful for the prediction and diagnosis of T1D, autoantibodies against islet antigens have not proven useful in monitoring disease progression during immune interventions such as treatment with cyclosporine [49] or anti-CD3 antibodies [50]. It is, therefore, of primary importance to identify markers of the disease that may be employed for this purpose. Our results presented here confirm recent reports suggesting that IGRP is a CD8⁺ T cell antigen targeted in humans [22;23;44], contributing to the understanding of the immunopathology of T1D. In addition, the epitopes described here in the context of the *ex vivo* IFN- γ ELISPOT may be a useful tool in monitoring the immune system in patients undergoing therapeutic interventions.

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Fig. 1. Recent-onset T1D patient PBMC but not healthy control PBMC produce IFN- γ in response to IGRP peptides

Patient (filled symbols) and control (open symbols) responses to the peptide panel used in the study (A). The dashed line marks the cut-off for a positive response as determined by a receiver operating characteristic (ROC) plot. Asterisks denote positive responses from non-HLA-A*0201 patients. Differences between patient and control responses were statistically significant only for IGRP 215–223 and IGRP 222–230. Duplicate ELISPOT wells from a healthy control and a patient are shown in B and numbers represent spot number counts for each well.

Fig. 2. IGRP peptides show variable binding affinity to HLA-A2

A T2-cell-based assay was used to determine the relative binding affinity of IGRP peptides. Flu MP 58–66 was used as a positive control.

Table 1
Characteristics of patients (PT) and healthy control (HC) individuals included in the study

Patient/ Healthy control	Gender	Age at diagnosis (years)	Duration of disease (days)	Antibodies ^a	HLA-DR	HLA- DQB1	HLA-A	HLA-B	HLA-Cw
PT1	M	10	318	N/A ^b	04, 07	02, 03	0201, 24	49, 51	07, 14
PT2	F	7	54	IAA+	07, 13	02, 03	0201, 24	44, 49	05, 06
PT3	F	14	105	ICA-, IAA-, GADA+	03, 04	02, 03	0201, 26	15, 57	03, 06
PT4	M	7	157	ICA+, GADA+	09, 09	02	0202, 23	07, 13	07, 08
PT5	F	6	137	GADA+	04, 15	03, 06	0201, 03	15, 18	04, 16
PT6	F	13	124	ICA+, GADA+	03, 13	02, 06	0201, 30	51, 57	06, 16
PT7	F	7	32	ICA-, IAA-, GADA+	01, 04	03, 05	0201, 24	44, 44	02, 05
PT8	F	18	14	ICA-, IAA-, GADA+	03, 04	02, 03	0201	15, 47	03, 07
PT9	M	7	107	ICA-, GADA+	11, 12	03	0201	15, 51	02, 15
PT10	F	5	398	ICA-, IAA-, GADA+	01, 03	02, 05	0201, 03	18, 35	04, 05
PT11	F	7	292	ICA+, IAA-, GADA+	04	03, 02	0201, 26	15, 73	07, 15
PT12	F	14	10	ICA-, IAA-, GADA+	03, 13	02, 06	0201, 24	15, 18	05, 07
PT13	M	1	44	ICA+, IAA+, GADA+	03, 04	02, 03	0201, 01	08, 14	07, 08
PT14	F	13	7	ICA-, IAA-, GADA+	04, 04	03	0202, 11	52, 58	07, 07
PT15	F	9	32	ICA-, GADA+	04, 13	03, 06	0201, 11	35, 53	04, 12
PT16	M	12	5	IAA-, GADA+	04, 12	03, 03	0201	07, 15	03, 07
PT17	M	12	13	GADA+	03, 04	02, 03	0204, 30	08, 39	07, 07
PT18	F	10	136	ICA-, IAA-, GADA+	08, 09	02, 04	0201, 68	15, 18	03, 07
PT19	M	13	45	N/A	01, 04	03, 05	0201, 03	44, 49	05, 07
PT20	F	7	234	ICA-, IAA-, GADA-	01, 04	02, 05	0204, 29	14, 41	06, 08
HC1	M	29		N/A	03, 07	02, 04	0202, 03	07, 13	07, 08
HC2	M	32		N/A	07	02, 03	0201, 01	44, 57	04, 06
HC3	F	35		N/A	04, 12	03, 03	0206, 11	15, 51	01, 04
HC4	F	41		N/A	07, 13	02, 06	0201, 01	15, 44	07, 16
HC5	F	20		N/A	01, 11	03, 05	0201, 24	18, 51	12, 15
HC6	M	33		N/A	03, 13	02, 06	0205, 23	07, 58	07, 07
HC7	F	31		N/A	07	02	0205, 32	13, 51	06, 16
HC8	M	27		N/A	10, 11	03, 05	0201, 33	58, 78	06, 16
HC9	M	29		N/A	03, 07	02, 03	0201, 23	08, 44	04, 07
HC10	M	31		N/A	03, 09	02, 03	0206, 33	51, 58	03, 14

^aICA, islet cell antibodies; IAA, insulin autoantibodies; GADA, glutamic acid decarboxylase antibodies.

^bN/A, not available. Not all antibodies were available for all patients.

Table 2

Candidate IGRP peptides and additional epitopes screened with patient and control samples

Peptide	Sequence
IGRP 265–273	VLFGGLGFAI
IGRP 211–219	NLFLFLFAV
IGRP 215–223	FLFAVGFYL
IGRP 222–230	YLLLRVLNI
Flu MP 58–66	GILGFVFTL
ppIAPP 5–13	KLQVFLIVL