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(54) Title: A METHOD FOR TREATING TYPE 1 DIABETES

(57) Abstract: The present disclosure relates to a method for the treatment or prevention of Type 1 diabetes including a condition associated with Type 1 diabetes and to therapeutic and prophylactic agents useful for same. The present disclosure further teaches diagnostic assays in the detection and monitoring of Type 1 diabetes and its associated conditions.

A METHOD FOR TREATING TYPE 1 DIABETES

FILING DATA

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2013902683, filed on 19 July 2013, entitled "Method of treating Type I diabetes", the entire contents of which, are incorporated herein by reference.

BACKGROUND

FIELD

[0002] The present disclosure relates to a method for the treatment or prevention of Type 1 diabetes including a condition associated with Type 1 diabetes and to therapeutic and prophylactic agents useful for same. The present disclosure further teaches diagnostic assays in the detection and monitoring of Type 1 diabetes and its associated conditions.

DESCRIPTION OF PRIOR ART

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Type 1 diabetes is an autoimmune disease associated with activation of lymphocytes against pancreatic β-cells. Whilst Type 1 diabetes has been the focus of decades of research, it is still ill defined in human subjects (Faideau *et al.* (2005) *Diabetes 54(2)*:S87-S96). The autoimmune disease is highly multigenic (Todd (2010) *Immunity*

32(4):457-467; Rogner *et al.* (2001) *Genomics 72(2)*:163-171). A feature of human Type 1 diabetes is the long preclinical phase that precedes hyperglycemia (Ziegler and Nepom (2010) *Immunity 32(4)*:468-478). There is also a high level of recurrence of autoimmunity in long-standing patients who have been treated with exogenous insulin (Sibley *et al.* (1985) *Laboratory Investigation 53(2)*:132-144).

[0006] β-Cell-specific $CD4^+$ and $CD8^+$ T-cells maintain immunological memory for years after onset of Type 1 diabetes. Key autoantigens associated with Type 1 diabetes are insulin and its precursor forms, proinsulin and preproinsulin (Faideau *et al.* (2005) *supra*; Homo-Delarche and Boitard (1996) *Immunology Today 17(10)*:456-460).

[0007] Type 1 diabetes is a debilitating, chronic disease. Antigen-specific therapies are a promising approach in the treatment and prevention of Type 1 diabetes. This approach requires a knowledge of epitopes recognized by pathogenic T-cells and B-cells. In turn, this would also lead to better diagnostic assays to detect and monitor Type 1 diabetes. However, progress towards the identification of disease-relevant epitopes using T-cells and B-cells isolated from peripheral blood has been slow.

SUMMARY

[0008] A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

[0009] Islet-infiltrating T-cells have been used to detect T-cell epitopes on proinsulin which, when modified, are useful in the treatment or prevention of Type 1 diabetes or a condition associated with Type 1 diabetes in mammalian subjects such as humans. Peptides encompassing the identified t-cell epitopes are modified by an amino acid substitution. The modified peptides result in a more sensitive, efficacious and/or active response from T-cells from diabetic subjects. These epitopes are also useful in diagnostic assays for autoreactive T-cells, B-cells and antibodies. The amino acid sequence in the proinsulin-derived peptides is modified by a glutamine (Q) to glutamic acid (E) substitution. This substitution can be made during the chemical synthesis of the peptides, by recombinant means, or by recombinant means followed by posttranslational modification. The peptides may also comprise a chemical analog of, or a non-naturally occurring amino acid substitute of, one or more amino acid residues in the sequence.

[0010] Hence, the instant disclosure teaches a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject comprising the administration to the mammalian subject a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. It is proposed that the peptide comprises an epitope which, when modified by the glutamine to glutamic acid substitution, is far more sensitive to recognition by antigen-presenting cells and lymphocytes. This is indicated by the level of response by activated T-cells in the presence of the peptide compared to a peptide comprising a non-modified sequence.

[0011] Further enabled herein is an isolated T-cell or antigen-presenting cell derived from a mammalian subject presenting a peptide comprising an amino acid sequence of at

least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution.

[0012] The isolated T-cell or antigen-presenting cell is useful for treating or preventing Type 1 diabetes or an associated condition wherein the T-cells or antigen-presenting cells are autologous to the subject being treated. Reference to a "T-cell" or "T-lymphocyte" includes a $CD4^+$ T-cell and a $CD8^+$ T-cell. This aspect includes regulatory T-cells (Treg cells) useful to enhance a response to the modified peptide. Similarly, peptide-HLA Class II tetramers may be prepared and used for inducing tolerance and/or to assess $CD4^+$ T-cell autoreactivity. A condition associated with Type 1 diabetes includes a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, hearing impairment, an infection and a complication in pregnancy. In an embodiment, the antigen-presenting cell is serologically HLA-DQ8. In an embodiment, the antigen-presenting cell comprises the haplotype HLA-DQ A*03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ A*03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A*05:01, DQ B*03:02 and/or DQ A*03:01; DQ B*02:01), HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

[0013] Further taught is a therapeutic or prophylactic composition comprising a peptide comprising an amino acid sequence of at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution and one or more pharmaceutically acceptable carriers, diluents and/or excipients. The subject peptide may also be complexed with an HLA Class II tetramer.

[0014] The present specification is also instructional on the use of a peptide comprising at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a

glutamine to glutamic substitution in the manufacture of a medicament for the treatment of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject.

[0015] In an embodiment, the mammalian subject is a human male or female of any age.

[0016] Diagnostic assays are also enabled by the present disclosure. Taught herein is a method of detecting autoreactive T-cells including $CD4^+$ T-cells which recognize an epitope on proinsulin with a modification comprising a glutamine to glutamic acid substitution with greater responsiveness compared to the same peptide but without the glutamine to glutamic acid substitution. $CD8^+$ T-cells can also be detected. HLA Class II tetramers may also be used to interrogate $CD4^+$ T-cells. The tetramers are complexed with a subject peptide such as SEQ ID NO:94.

[0017] Enabled herein is a method for detecting the presence of autoreactive T-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising contacting a sample from the subject comprising immune cells with a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive T-cell, if present, to be stimulated into producing an immune effector molecule, and then detecting for the presence or level of the immune effector molecule, wherein the presence or level of the immune effector molecule is indicative of Type 1 diabetes or its state or risk of progression. The level of response in a peptide comprising a glutamine to glutamic acid substitution is greater than the same peptide without this substitution.

[0018] In an embodiment, the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121.

[0019] In an embodiment, the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

[0020] In an embodiment, the peptide may be further modified to include a non-naturally occurring amino acid in place of or in addition to an existing amino acid residue. For example, an arginine may be converted to a citrulline such as set forth in a sequence selected from the list consi8sting of SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95. In an embodiment, the peptide is SEQ ID NO:95. Other amino acid substitutions may also be made including the substitution of a natural amino acid with a non-naturally occurring amino acid residue.

[0021] The instant disclosure further contemplates detecting autoreactive antibodies to insulin, proinsulin or preproinsulin as well as generating antibodies to the modified peptides of the instant disclosure.

[0022] Further enabled herein is a method for screening for autoreactive antibodies associated with Type 1 diabetes or a condition associated therewith in a subject, comprising contacting body fluid from the subject with a peptide comprising an amino acid sequence encompassing an epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive antibody, if present, to bind to the peptide and then screening for the presence of the peptide-antibody complex. The presence or level of the antibody is indicative of Type 1 diabetes or its state or risk of progression. The glutamine to glutamic acid substitution results in higher sensitivity in antibody binding.

[0023] A peptide of the present disclosure comprising an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121 may also be conjugated to or embedded in a targeting moiety and/or a carrier molecule. In an embodiment, the targeting moiety and the carrier molecule are the same

entity. A targeting moiety is useful in facilitating the deployment of the peptide to a particular cell type or tissue type. Examples of cell types include antigen-presenting cells, and $CD4^+$ T-cells or other lymphocytes such as $CD8^+$ T-cells. Examples of targeting moieties include antibodies or antigen-binding fragments or derivatives thereof specific for an antigen on the target cell or tissue, receptor ligands, a glycosaminoglycan, heparan or heparin or a fragment thereof, a cytokine or other molecule capable of specific binding to a desired cell type. HLA Class II tetramers may also be used. Carrier molecules include adjuvants, molecules which increase the half life in the blood stream and the like. Other carrier molecules include HLA-DQ8 complexes such as a complex with HLA-DQ A*03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A*05:01, DQ B*03:02 and DQ A*03:01; DQ B*02:01), HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

[0024] Enabled herein is an agent comprising a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution in an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121, the peptide conjugated at its N- or C-terminal end to a targeting moiety or carrier molecule.

[0025] In an alternative embodiment, a peptide is provided comprising an amino acid sequence of at least seven consecutive amino acid residues encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises an arginine to citrulline substitution in an amino acid sequence selected from SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95, the peptide embedded in a targeting moiety or carrier molecule.

[0026] Enabled herein is an agent comprising a peptide comprising an amino acid sequence encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin having a glutamine to glutamic acid substitution and comprising an amino acid sequence

set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121, the peptide conjugated at its N- or C-terminal end to a targeting moiety or carrier molecule.

[0027] In accordance with these embodiments, one particular peptide is as defined by SEQ ID NO:94 or comprises the amino acid sequence of SEQ ID NO:94.

[0028] In an embodiment, the subject specification contemplates a method for detecting the presence of autoreactive T-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition associated with Type 1 diabetes in a subject, the method comprising contacting a sample from the subject comprising immune cells with a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive T-cell, if present, to be stimulated into producing an immune effector molecule, and then detecting the presence of the immune effector molecule, wherein the presence or level of the immune effector molecule is indicative of Type 1 diabetes or its state or risk of progression or a condition associated with Type 1 diabetes.

[0029] In an embodiment, the subject specification contemplates a method for detecting the presence of autoreactive B-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition associated with Type 1 diabetes in a subject, the method comprising contacting a sample from the subject comprising immune cells with a peptide comprising an amino acid sequence encompassing an epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive B-cell, if present, to be stimulated into producing an antibody, and then detecting the presence of the antibody, wherein the presence or level of the antibody is indicative of Type 1 diabetes or its state or risk of progression or a condition associated with Type 1 diabetes.

[0030] In an embodiment, the subject is a human.

[0031] In an embodiment, the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121.

[0032] In an embodiment, the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

[0033] In an embodiment, the condition associated with Type 1 diabetes is selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

[0034] In an embodiment, the peptide has a minimum length of 13 amino acid residues and encompasses an epitope which is recognized by and binds to an antigen-presenting cell which is serologically HLA-DQ8 such as an antigen-presenting cell which comprises the haplotype HLA-DQA*03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A*05:01, DQ B*03:02 and DQ A*03:01; DQ B*02:01, HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx). Reference to a minimum length of 13 includes from 13 to 40 including from 13 to 20. There is a proviso that the amino acid sequence comprises at least one glutamine to glutamic acid substitution (i.e. a deamidation).

[0035] Enabled herein is an isolated HLA Class II tetramer incorporating a peptide comprising at least 7 consecutive amino acids comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin, wherein the peptide comprises a glutamine to glutamic acid substitution.

[0036] In an embodiment the peptide comprises at least 13 amino acid residues.

[0037] In an embodiment the peptide comprises a sequence selected from SEQ ID NO:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.

[0038] In an embodiment the peptide comprises SEQ ID NO:94.

Table 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
1	Amino acid sequence of human proinsulin
2	Amino acid sequence of modified proinsulin-derived peptide (FVN**E**HLC)
3	Amino acid sequence of modified proinsulin-derived peptide (VN**E**HLCG)
4	Amino acid sequence of modified proinsulin-derived peptide (N**E**HLCGS)
5	Amino acid sequence of modified proinsulin-derived peptide (**E**HLCGSH)
6	Amino acid sequence of modified proinsulin-derived peptide (YLVCGE**X**)
7	Amino acid sequence of modified proinsulin-derived peptide (LVCGE**X**G)
8	Amino acid sequence of modified proinsulin-derived peptide (VCGE**X**GF)
9	Amino acid sequence of modified proinsulin-derived peptide (CGE**X**GFF)
10	Amino acid sequence of modified proinsulin-derived peptide (GE**X**GFFY)
11	Amino acid sequence of modified proinsulin-derived peptide (E**X**GFFYT)
12	Amino acid sequence of modified proinsulin-derived peptide (**X**GFFYTP)
13	Amino acid sequence of modified proinsulin-derived peptide (FYTPKT**X**)
14	Amino acid sequence of modified proinsulin-derived peptide (YTPKT**X**R)
15	Amino acid sequence of modified proinsulin-derived peptide (YTPKTR**X**)
16	Amino acid sequence of modified proinsulin-derived peptide (YTPKT**XX**)
17	Amino acid sequence of modified proinsulin-derived peptide (TPKT**X**RE)
18	Amino acid sequence of modified proinsulin-derived peptide (TPKTR**X**E)
19	Amino acid sequence of modified proinsulin-derived peptide (TPKT**XX**E)
20	Amino acid sequence of modified proinsulin-derived peptide (PKT**X**REA)
21	Amino acid sequence of modified proinsulin-derived peptide (PKTR**X**EA)
22	Amino acid sequence of modified proinsulin-derived peptide (PKT**XX**EA)
23	Amino acid sequence of modified proinsulin-derived peptide (KT**X**REAE)
24	Amino acid sequence of modified proinsulin-derived peptide (KTR**X**EAE)
25	Amino acid sequence of modified proinsulin-derived peptide (KT**XX**EAE)

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
26	Amino acid sequence of modified proinsulin-derived peptide (TXREAED)
27	Amino acid sequence of modified proinsulin-derived peptide (TRXEAED)
28	Amino acid sequence of modified proinsulin-derived peptide (TXXEAED)
29	Amino acid sequence of modified proinsulin-derived peptide (XREAEDL)
30	Amino acid sequence of modified proinsulin-derived peptide (RXEAEDL)
31	Amino acid sequence of modified proinsulin-derived peptide (XXEAEDL)
32	Amino acid sequence of modified proinsulin-derived peptide (REAEDLE)
33	Amino acid sequence of modified proinsulin-derived peptide (XEAEDLQ)
34	Amino acid sequence of modified proinsulin-derived peptide (XEAEDLE)
35	Amino acid sequence of modified proinsulin-derived peptide (EAEDLEV)
36	Amino acid sequence of modified proinsulin-derived peptide (AEDLEVG)
37	Amino acid sequence of modified proinsulin-derived peptide (EDLEVGQ)
38	Amino acid sequence of modified proinsulin-derived peptide (EDLQVGE)
39	Amino acid sequence of modified proinsulin-derived peptide (EDLEVGE)
40	Amino acid sequence of modified proinsulin-derived peptide (DLEVGQV)
41	Amino acid sequence of modified proinsulin-derived peptide (DLQVGEV)
42	Amino acid sequence of modified proinsulin-derived peptide (DLEVGEV)
43	Amino acid sequence of modified proinsulin-derived peptide (LEVGQVE)
44	Amino acid sequence of modified proinsulin-derived peptide (LQVGEVE)
45	Amino acid sequence of modified proinsulin-derived peptide (LEVGEVE)
46	Amino acid sequence of modified proinsulin-derived peptide (EVGQVEL)
47	Amino acid sequence of modified proinsulin-derived peptide (QVGEVEL)
48	Amino acid sequence of modified proinsulin-derived peptide (EVGEVEL)
49	Amino acid sequence of modified proinsulin-derived peptide (VGEVELG)
50	Amino acid sequence of modified proinsulin-derived peptide (GEVELGG)
51	Amino acid sequence of modified proinsulin-derived peptide (EVELGGG)
52	Amino acid sequence of modified proinsulin-derived peptide (PGAGSLE)
53	Amino acid sequence of modified proinsulin-derived peptide (GAGSLEP)

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
54	Amino acid sequence of modified proinsulin-derived peptide (AGSLEPL)
55	Amino acid sequence of modified proinsulin-derived peptide (GSLEPLA)
56	Amino acid sequence of modified proinsulin-derived peptide (SLEPLAL)
57	Amino acid sequence of modified proinsulin-derived peptide (LEPLALE)
58	Amino acid sequence of modified proinsulin-derived peptide (EPLALEG)
59	Amino acid sequence of modified proinsulin-derived peptide (ALEGSLE)
60	Amino acid sequence of modified proinsulin-derived peptide (LEGSLEK)
61	Amino acid sequence of modified proinsulin-derived peptide (EGSLEKR)
62	Amino acid sequence of modified proinsulin-derived peptide (EGSLQKX)
63	Amino acid sequence of modified proinsulin-derived peptide (EGSLEKX)
64	Amino acid sequence of modified proinsulin-derived peptide (GSLEKRG)
65	Amino acid sequence of modified proinsulin-derived peptide (GSLQKXG)
66	Amino acid sequence of modified proinsulin-derived peptide (GSLEKXG)
67	Amino acid sequence of modified proinsulin-derived peptide (SLEKRGI)
68	Amino acid sequence of modified proinsulin-derived peptide (SLQKXGI)
69	Amino acid sequence of modified proinsulin-derived peptide (SLEKXGI)
70	Amino acid sequence of modified proinsulin-derived peptide (LEKRGIV)
71	Amino acid sequence of modified proinsulin-derived peptide (LQKXGIV)
72	Amino acid sequence of modified proinsulin-derived peptide (LEKXGIV)
73	Amino acid sequence of modified proinsulin-derived peptide (EKRGIVE)
74	Amino acid sequence of modified proinsulin-derived peptide (QKXGIVE)
75	Amino acid sequence of modified proinsulin-derived peptide (EKXGIVE)
76	Amino acid sequence of modified proinsulin-derived peptide (KRGIVEE)
77	Amino acid sequence of modified proinsulin-derived peptide (KXGIVEQ)
78	Amino acid sequence of modified proinsulin-derived peptide (KXGIVEE)
79	Amino acid sequence of modified proinsulin-derived peptide (RGIVEEC)
80	Amino acid sequence of modified proinsulin-derived peptide (XGIVEQC)
81	Amino acid sequence of modified proinsulin-derived peptide (XGIVEXC)

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
82	Amino acid sequence of modified proinsulin-derived peptide (GIVEECC)
83	Amino acid sequence of modified proinsulin-derived peptide (IVEECCT)
84	Amino acid sequence of modified proinsulin-derived peptide (VEECCTS)
85	Amino acid sequence of modified proinsulin-derived peptide (EECCTSI)
86	Amino acid sequence of modified proinsulin-derived peptide (ECCTSIC)
87	Amino acid sequence of modified proinsulin-derived peptide (SICSLYE)
88	Amino acid sequence of modified proinsulin-derived peptide (ICSLYEL)
89	Amino acid sequence of modified proinsulin-derived peptide (CSLYELE)
90	Amino acid sequence of modified proinsulin-derived peptide (SLYELEN)
91	Amino acid sequence of modified proinsulin-derived peptide (LYELENY)
92	Amino acid sequence of modified proinsulin-derived peptide (YELENYC)
93	Amino acid sequence of modified proinsulin-derived peptide (ELENYCN)
94	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSLEPL)
95	Amino acid sequence of modified proinsulin-derived peptide (QPLALEGSLQKXGI)
96	Amino acid sequence of modified proinsulin-derived peptide (GQVELGGGPGAGSLEPL)
97	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSLQPL)
98	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSLEP)
99	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSLE)

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
100	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSL)
101	Amino acid sequence of modified proinsulin-derived peptide (ELGGGPGAGSLEPLAL)
102	Amino acid sequence of modified proinsulin-derived peptide (ELGGGPGAGSLEPL)
103	Amino acid sequence of modified proinsulin-derived peptide (ELGGGPGAGSLEPL)
104	Amino acid sequence of modified proinsulin-derived peptide (VELGGGPGAGSLEPL)
105	Amino acid sequence of modified proinsulin-derived peptide (GQVELGGGPGAGSLEPL)
106	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAGSLEPA)
107	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAGSLEAL)
108	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAGSLAPL)
109	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAGSAEPL)
110	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAGKLEPL)
111	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGAKSLEPL)
112	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPGKGSLEPL)
113	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGPKAGSLEPL)

SEQUENCE ID NO:	DESCRIPTION *X = citrulline
114	Amino acid sequence of modified proinsulin-derived peptide (QVELGGGAGAGSLEPL)
115	Amino acid sequence of modified proinsulin-derived peptide (QVELGGKPGAGSLEPL)
116	Amino acid sequence of modified proinsulin-derived peptide (QVELGKGPGAGSLEPL)
117	Amino acid sequence of modified proinsulin-derived peptide (QVELKGGPGAGSLEPL)
118	Amino acid sequence of modified proinsulin-derived peptide (QVEAGGGPGAGSLEPL)
119	Amino acid sequence of modified proinsulin-derived peptide (QVALGGGPGAGSLEPL)
120	Amino acid sequence of modified proinsulin-derived peptide (QKELGGGPGAGSLEPL)
121	Amino acid sequence of modified proinsulin-derived peptide (AVELGGGPGAGSLEPL)

[0039] Single and three letter abbreviations are used in the subject specification and are defined in Table 2.

Table 2

Amino acid single and three letter designations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Pyrrolysine	Pyl	O
Selenocysteine	Sec	U
Citrulline	Cit	X (or R*)

BRIEF DESCRIPTION OF THE FIGURES

[0040] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0041] **Figure 1** is a photographic representation of T-cells emerging from human islets.

[0042] **Figure 2** is a representation of an overview of the cloning of human islet infiltrating T-cells.

[0043] **Figure 3** is a diagrammatic representation of an analysis of the antigen specific of T-cell clones.

[0044] **Figure 4** is a graphical representation of clonal expansion of islet-infiltrating T-cells.

[0045] **Figure 5** is a graphical representation of the screening for responses to posttranslational modified peptides.

[0046] **Figure 6** is a graphical representation of the response of clone A2.11 to an epitope formed by posttranslational modification.

[0047] **Figure 7** is a graphical representation of the titration of Q25E peptides.

[0048] **Figure 8** is a graphical representation showing that Pool E stimulates clone A4.7.

[0049] **Figure 9** is a graphical representation showing that R65**X** is the active peptide.

[0050] **Figure 10** is a graphical representation showing that a monoclonal antibody specific for HLA-DQ inhibits an INFγ-based response to a peptide derived and modified from proinsulin (SEQ ID NO:94).

[0051] **Figure 11** is a graphical representation showing that a T-cell clone's response to a peptide requires an antigen-presenting cell (APC) which expresses HLA DQ8 (HLA-DQ A*03:01, DQ B*03:02).

[0052] **Figure 12** is a graphical representation of a fine epitope mapping. Panel A: these data show that the minimum number of amino acid residues required to stimulate a T-cell clone is 13. Panel B: to confirm the results in Panel A, peptides covering the sequence of one particular epitope but with each amino acid in turn substituted with an alanine (A) or lysine (K) were tested. The results showed which residues could be modified without impairing the T-cell response.

[0053] **Figure 13** is a graphical representation of $CD4^+$ T-cell responses measured using a CFSE-based proliferation assay in (A) typical diabetes; and (B) healthy controls. The results show the ratio of the number of $CD4^+$ T-cells that have proliferated in response to a non-modified peptide (with glutamine [Q]) compared to a glutamic acid (E) substitution.

[0054] **Figure 14** is a graphical representation showing the effects of 15mer peptide covering an epitope in an unmodified (glutamine [Q]) or modified (glutamic acid [E]) on IFNγ response from a T-cell clone.

DETAILED DESCRIPTION

[0055] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any element or integer or method step or group of elements or integers or method steps.

[0056] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a single peptide, as well as two or more peptides; reference to "an epitope" includes a single epitope, as well as two or more epitopes; reference to "the disclosure" includes a single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the claimed invention.

[0057] The present specification teaches the generation of peptides comprising an amino acid sequence having at least seven consecutive amino acid residues comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin. The amino acid sequence of the peptides is modified with a glutamine to glutamic acid substitution. The peptide may contain other modifications to the amino acid residues such as a chemical modification to an amino acid residue or a substitution of an amino acid residue for a non-naturally occurring amino acid. The peptide may alternatively or in addition comprise an arginine to citrulline substitution. The glutamine to glutamic acid substitution in the amino acid sequence of the epitope results in a higher degree of recognition by antigen-presenting cells and lymphocytes and antibodies. This can be detected by a greater response by sensitized T-cells to the peptides compared to the same peptides without the modification. The peptide epitopes derived from insulin, proinsulin or preproinsulin are used to induce tolerance of autoreactive T-cells and/or other immune cells in the treatment or prophylaxis of Type 1 diabetes or an associated condition in a mammalian subject. The modified peptides are also useful in diagnostic assays to detect autoreactive antibodies and the

peptides and/or the antibodies are also useful to measure the level of autoreactive T-cells such as $CD4^+$ T-cells or other T-cells such as $CD8^+$ T-cells. Such autoreactive antibodies or T-cells lead to the development or progression of Type 1 diabetes or a condition associated therewith. Hence, their direct detection or indirect detection assists in determining the presence of Type 1 diabetes, such as prior to hyperglycemia and/or the state of progression of diabetes. Antibodies may also be generated specifically to the modified peptides. Such antibodies are useful in diagnostic assays.

[0058] Conditions associated with Type 1 diabetes include cardiovascular conditions such as angina, heart attack, stroke, atherosclerosis and hypertension; neuropathy; retinopathy; nephropathy; hearing impairment; chronic gum infection; and complications in pregnancy such as miscarriage, preeclampsia and diabetic ketoacidosis.

[0059] Enabled herein is a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution.

The peptides comprise epitopes which are recognized by $CD4^+$ T-cells, $CD8^+$ T-cells and/or antigen-presenting cells as well as B-cells in mammalian subjects sensitized to insulin, proinsulin or preproinsulin. In an embodiment, the T-cells are $CD4^+$ T-cells. By "recognized" is meant that the peptide induces a T-cell mediated response statistically significant relative to a T-cell control using the same peptide to which the mammal is not sensitized or an unrelated peptide in a sensitized or non-sensitized mammal or is bound by autoreactive antibodies directed to insulin, proinsulin or preproinsulin. The amino acid sequence is "derived from" insulin, proinsulin or preproinsulin meaning the peptide is a fragment of one of these molecules or is chemically synthesized based on the amino acid sequence of the desired region or is produced by recombinant means alone or by recombinant means followed by posttranslational chemical modification. In an

embodiment, the peptide with the glutamine to glutamic acid substitution is more strongly recognized by a serologically HLA-DQ8 antigen-presenting cell such as an antigen-presenting cell which comprises the haplotype HLA-DQ A* 03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A* 05:01, DQ B* 03:02 and DQ A* 03:01; DQ B* 02:01) or HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

[0060] Hence, in an embodiment, the peptide binds to HLA-DQ A*03:01; DQ B*03:02. In another embodiment, the peptide binds to another HLA molecule selected from one or more of HLA DQ2, HLA DQ 2/8 transdimer, DR3 and/or DR4. In an embodiment, the peptide binds to an antigen-presenting cell which comprises the haplotype DQ A*05:01, DQ B*03:02 and/or DQ A*03:01, DQ B*02:01.

[0061] The peptides of the instant disclosure comprise from 7 to 40 consecutive amino acid residues meaning 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues. In relation to a peptide for use in an antibody assay, the only requirement is a glutamine to glutamic acid substitution. In this case, the "peptide" may contain from 2 to 40 amino acid residues provided it contains this substitution. In an embodiment, the seven consecutive amino acids represented in SEQ ID NOs:2 to 93 are comprised within a larger 10 to 20 amino acid sequence. Examples of larger amino acid sequences include SEQ ID NOs:94 to 121. By "10 to 20" means 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid residues. It is taught herein that the peptides are useful in tolerizing or down-regulating the priming and/or activity of T-cells of a mammal with Type 1 diabetes. The peptides are also useful in diagnostic assays.

[0062] In an embodiment, the peptide has a minimum of 13 amino acid residues, hence from 13 to 40 including 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues.

[0063] Accordingly, enabled herein is a method for the treatment or prophylaxis of

Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of at least 13 consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. By "at least 13" means from "13 to 40" and in particular "13 to 20". There is a proviso that the amino acid sequence comprises at least one glutamine to glutamic acid substitution (i.e. a deamidation).

[0064] Hence, enabled herein is a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of from 13 to 40 consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. By "at least 13" means from "13 to 40" and in particular "13 to 20". There is a proviso that the amino acid sequence comprises at least one glutamine to glutamic acid substitution (i.e. a deamidation).

[0065] Further enabled herein is a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of from 13 to 20 consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. By "at least 13" means from "13 to 40" and in particular "13 to 20". There is a proviso that the amino acid sequence comprises at least one glutamine to glutamic acid substitution (i.e. a deamidation).

[0066] In an embodiment, the peptide binds to an antigen-presenting cell which comprises the haplotype HLA-DQ A*03:01, DQ B*03:02. In an embodiment the peptide is part of an HLA Class II tetramer.

[0067] Reference to a "mammalian subject" includes a human or other primate, laboratory test animal such as a mouse, rat, rabbit or guinea pig, a farm animal such as a

horse, donkey, pig, cow or sheep, a companion animal such as a dog or cat and a captive wild animal. In an embodiment, the mammalian subject is a human. The human may be an infant, child, young adult, adult or elderly person. The human may be male or female.

[0068] Hence, enabled herein is a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. As indicated above, the term "at least seven consecutive amino acids" includes the aspect where a peptide of 10 to 20 amino acid residues including a peptide of at least 13 amino acid residues comprises the seven amino acid sequences. An example includes SEQ ID NOs:94 or a peptide selected from SEQ ID NO:96 to 121.

[0069] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, the method comprising administering to the subject a peptide comprising an amino acid sequence of at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution wherein the peptide comprises an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121. In an embodiment, the peptide comprises at least 13 amino acid residues. In an embodiment, the peptide binds to an antigen-presenting cell which comprises the haplotype HLA-DQ A* 03:01, DQ B*03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A* 05:01, DQ B* 03:02 and/or DQ A* 03:01; DQ B* 02:01), HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx). In an embodiment, the peptide is or comprises SEQ ID NO:94.

[0070] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a

mammalian subject, the method comprising administering to the subject a peptide having a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94. In an embodiment, the peptide consists of SEQ ID NO:94. In another embodiment, the peptide comprises the sequence selected from SEQ ID NOs:96 to 121.

[0071] The peptide may contain further chemical modifications such as the chemical addition of amino acid residues in a synthetic reaction designed to generate peptides of a defined amino acid sequence, chemically modifying an amino acid residue. Another form of chemical modification is substituting an existing amino acid with a non-naturally occurring amino acid residue. The peptides defined by reference to SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121 and SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 and SEQ ID NOs:96 to 121 includes those having the glutamine to glutamic acid/or arginine to citrulline substitution but may also comprise a chemically modified amino acid or contain a non-naturally occurring amino acid substitution or another natural amino acid substitution.

[0072] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a peptide comprising at least seven consecutive amino acids encompassing a T-cell epitope derived from insulin, proinsulin or wherein the amino acid sequence comprises a glutamine to glutamic acid substitution wherein the peptide is chemically synthesized and comprises an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 or 96 to 121.

[0073] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a chemically synthesized peptide encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin comprises or consists of an amino acid sequence set forth in SEQ ID NO:94.

[0074] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a chemically synthesized peptide encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin comprises an amino acid sequence set forth in SEQ ID NOs:96 to 121.

[0075] In another embodiment, the present specification teaches a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a peptide comprising at least seven consecutive amino acids having a T-cell epitope derived from insulin, proinsulin or wherein the amino acid sequence comprises a arginine to citrulline substitution wherein the peptide is chemically synthesized and comprises an amino acid sequence selected from SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95.

[0076] The present specification is further instructional on a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a chemically synthesized peptide encompassing a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the peptide comprises or consists of an amino acid sequence set forth in SEQ ID NO:95.

[0077] Reference to "tolerance" includes a reduction in a T-cell response and/or a B-cell response which is specific for insulin, proinsulin or preproinsulin. Tolerance may occur by any of a number of mechanisms including reducing autoreactive T-cells or B-cells, inducing ignorance of insulin, proinsulin or preproinsulin, mechanisms that imprint an intrinsic status on tolerant lymphocytes in the form of anergy and immunedeviation and on extrinsic mechanisms involving regulating cells. Molecular interactions in the presentation of autoantigen in the periphery are central to the tolerance process and in strategies aiming at restoring or inducing immune tolerance in autoimmunity. It is proposed herein that the peptides facilitate removal, elimination or reprogramming of

deleterious autoreactive T-cells and antigen-presenting cells and activation of regulatory cells to control autoimmune effectors. Tolerance may also be induced using complexes between peptides such as SEQ ID NO:95 and HLA Class II tetramers.

[0078] In an embodiment, the peptides or nucleic acid molecules encoding same are useful in the treatment or prophylaxis of Type 1 diabetes or an associated condition in a mammalian subject as well as a condition associated with Type 1 diabetes. The treatment of Type 1 diabetes includes the amelioration of symptoms of diabetes. In general, the peptides are recognized by $CD4^+$ T-cells, $CD8^+$ T-cells and/or or antigen-presenting cells and/or B-cells from a mammalian subject having or at risk of developing Type 1 diabetes. In an embodiment, the T-cells are $CD4^+$ T-cells. In an embodiment, the peptide is at least 13 amino acids in length such as from 13 to 40, and it binds to HLA-DQ A* 03:01, DQ B* 03:02. In another embodiment, T-cells respond to the peptide presented by another HLA molecule, such as but not limited to HLA DQ2 and HLA DQ 2/8 transdimers (DQ A* 05:01, DQ B* 03:02 and/or DQ A* 03:01; DQ B* 02:01), HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx). By "at least 13 amino acids in length" means 13 to 40 such as 13 to 20.

[0079] The present specification is instructional on a method of tolerizing or down-regulating the priming or activity of T-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0080] The present specification is instructional on a method of tolerizing or down-regulating the priming or activity of T-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:96 to 121 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0081] The present specification is instructional on a method of tolerizing or down-

regulating the priming or inactivity of T-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide comprising the amino acid sequence set forth in SEQ ID NO:95.

[0082] The present specification teaches a method of tolerizing or down-regulating the priming or activity of T-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 wherein the peptide comprises an arginine to citrulline substitution and/or an arginine to citrulline substitution.

[0083] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of T-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide comprising the amino acid sequence set forth in SEQ ID NO:95.

[0084] The present specification is instructional on a method of tolerizing or down-regulating the priming or activity of B-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0085] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide comprising the amino acid sequence set forth in SEQ ID NO:94.

[0086] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide comprising the amino acid sequence set forth in SEQ ID NO:96 to 121.

[0087] The present specification is instructional on a method of tolerizing or down-regulating the priming or activity of B-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs: 6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 96 wherein the peptide comprises an arginine to citrulline substitution.

[0088] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in mammalian subjects with or at risk of developing Type 1 diabetes by the administration of a peptide comprising the amino acid sequence set forth in SEQ ID NO:95. In addition, tolerization may employ the use of an HLA Class II tetramer comprising a peptide of the present invention such as, but not limited to, SEQ ID NO:94.

[0089] In relation to these embodiments, the peptides may further comprise other modifications such as to an amino acid or by the substitution of a non-naturally occurring amino acid residue.

[0090] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of T-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0091] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of T-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide comprising the amino acid sequence set forth in SEQ ID NO:94.

[0092] The present specification is instructional on a method of tolerizing or down-

regulating the priming or inactivity of T-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:96 to 121 wherein the peptide comprises an arginine to citrulline substitution.

[0093] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of T-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 wherein the peptide comprises an arginine to citrulline substitution.

[0094] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of T-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide comprising the amino acid sequence set forth in SEQ ID NO:95.

[0095] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0096] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:96 to 121 wherein the peptide comprises a glutamine to glutamic acid substitution.

[0097] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in human subjects with or at risk of

developing Type 1 diabetes by the administration of a chemically synthesized peptide comprising the amino acid sequence set forth in SEQ ID NO:94.

[0098] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide selected to comprise an amino acid sequence set forth in SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 wherein the peptide comprises an arginine to citrulline substitution.

[0100] The present specification is instructional on a method of tolerizing or down-regulating the priming or inactivity of B-cells in human subjects with or at risk of developing Type 1 diabetes by the administration of a chemically synthesized peptide comprising the amino acid sequence set forth in SEQ ID NO:95.

[0101] As indicated above, the peptide may further comprise one or more chemical modifications to one or more amino acid residues including an amino acid addition and/or deletion as well as a substitution of a naturally occurring or non-naturally occurring amino acid for one or more of the amino acid residues.

[0102] Once isolated and characterized, derivatives, e.g., chemically derived derivatives, of a given peptide can be readily prepared. For example, amides of the peptide or peptide variants of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor to an amide. One preferred method for amide formation at the C-terminal carboxyl group is to cleave the peptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

[0103] Salts of carboxyl groups of a peptide or peptide variant may be prepared in the usual manner by contacting the peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate

or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

[0104] N-acyl derivatives of an amino group of the peptide or peptide variants may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired.

[0105] Formyl-methionine, pyroglutamine and trimethyl-alanine may be substituted at the N-terminal residue of the peptide or peptide variant. Other amino-terminal modifications include aminooxypentane modifications (see Simmons *et al.* (1997) *Science 276*:276).

[0106] In addition, the amino acid sequence of a peptide can be modified so as to result in a peptide variant (see above). The modification includes the substitution of at least one amino acid residue in the peptide for another amino acid residue, including substitutions which utilize the D rather than L form, as well as other well known amino acid analogs. These analogs include phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, gamma-carboxyglutamate; hippuric acid, octahydroindole-2-carboxylic acid, statine, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, penicillamine, ornithine, citrulline, α-methyl-alanine, *P*-benzoyl-phenylalanine, phenylglycine, propargylglycine, sarcosine, and tert-butylglycine.

[0107] Regardless of the presence of any other modifications, the amino acid sequence of the peptide will comprise one or more of a glutamine to glutamic acid substitution and/or an arginine to citrulline substitution.

[0108] One or more of the residues of the peptide can be altered, so long as the peptide variant is biologically active in the sense it retains its epitopic function. Conservative

amino acid substitutions include, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

[0109] Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic; trp, tyr, phe.

[0110] Acid addition salts of the peptide or variant peptide, or of amino residues of the peptide or variant peptide, may be prepared by contacting the peptide or amine with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the peptides may also be prepared by any of the usual methods known in the art.

[0111] A list of unnatural amino acids, contemplated herein and which may be incorporated into the peptide is shown in Table 3.

Table 3

Non-conventional amino acids

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg

Non-conventional amino acid	Code	Non-conventional amino acid	Code
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug

Non-conventional amino acid	Code	Non-conventional amino acid	Code
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

[0112] Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n = 1 to n = 6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N $_\alpha$-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogs by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

[0113] The peptides may be produced by the stepwise addition of amino acid residues by chemical synthetic means or by recombinant means or by recombinant means followed by posttranslational modification. In relation to the chemical synthesis of peptides, any

number of methods may be employed including using 9-fluoronylmethoxy-carbonyl (Fmoc) synthesis. Purity can be readily determined by HPLC and peptide masses determined by, for example, mass spectrometry.

[0114] For example, the peptides may be synthesised by standard solution phase methodology, as described in Hruby *et al.* (1998) *Chemical synthesis of peptides. University of Arizona*, USA. Editor(s): Hecht, Sidney, M. Bioorganic Chemistry: Peptides and Proteins :27-64, Oxford University Press, New York, N. Y.

[0115] Linear peptides may also be synthesised by solid phase methodology using Boc chemistry, as described by Schnolzer *et al.* (1992) *Int J Pept Protein Res 40*:180-193. Following deprotection and cleavage from the solid support the reduced peptides are purified using preparative chromatography.

[0116] As indicated above, peptides may also be synthesised by solid phase methodology using Fmoc chemistry, as described below:

1) Peptide is synthesized by Fmoc solid-phase peptide synthesis using an automatic synthesizer.

2) Peptide is synthesized from its C-terminus by stepwise addition of amino acids.

3) The first Fmoc-amino acid is attached to an insoluble support resin *via* an acid labile linker.

4) After deprotection of Fmoc by treatment with piperidine, the second Fmoc-amino acid is coupled utilizing a pre-activated species or *in situ* activation.

5) After the desired peptide is synthesized, the resin bound peptide is deprotected and detached from the resin *via* TFA cleavage.

6) Following deprotection and cleavage from the solid support the reduced peptides are purified using preparative chromatography.

[0117] Following deprotection and cleavage from the solid support the reduced peptides are purified using preparative chromatography.

[0118] In terms of production of peptides by recombinant means, sources of nucleotide sequences from which a nucleic acid molecule encoding a peptide or variant thereof include total or polyA$^+$ RNA from a mammalian (e.g. human) source from which cDNAs can be derived by methods known in the art. Other sources of DNA molecules include genomic libraries derived from a mammalian (e.g. human) source.

[0119] Moreover, DNA molecules may be prepared *in vitro*, e.g., by synthesizing an oligonucleotide or by subcloning a portion of a DNA segment that encodes a particular peptide.

[0120] As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a DNA, peptide or polypeptide molecule from its natural cellular o biological environment, and from association with other components of the cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. For example, an "isolated, preselected nucleic acid" is RNA or DNA that encode at least a portion of a peptide selected from SEQ ID NOs:1 through 121, or a RNA or DNA complementary thereto, that is complementary or hybridizes, respectively, to RNA or DNA encoding the peptide, or polypeptide comprising the peptide, and remains stably bound under stringent conditions, as defined by methods well known in the art, e.g. in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. Once expressed and a peptide produced, it then undergoes posttranslational modification. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell.

[0121] As used herein, the term "recombinant nucleic acid" or "preselected nucleic acid," e.g., "recombinant DNA sequence or segment" or "preselected DNA sequence or

segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate tissue source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been transformed with exogenous DNA. An example of preselected DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from the source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

[0122] Nucleic acid molecules encoding amino acid sequence variants of a peptide of the invention are prepared by a variety of methods known in the art. These methods include, but are not limited to, preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the preselected peptide. Posttranslational modification may also be used to alter the amino acid sequence.

[0123] Oligonucleotide-mediated mutagenesis is a method for preparing amino acid substitution variants of a peptide. This technique is described by Adelman *et al.* (1983) *DNA 2*:183. Briefly, DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the preselected DNA.

[0124] To prepare expression cassettes for transformation herein, the recombinant or preselected DNA sequence or segment may be circular or linear, double-stranded or single-stranded. Generally, the preselected DNA sequence or segment is in the form of chimeric

DNA, such as plasmid DNA, that can also contain coding regions flanked by control sequences which promote the expression of the preselected DNA present in the resultanT-cell line.

[0125] The peptides including their salts, are generally administered so as to achieve a reduction in activity of autoreactive T-cells to thereby ameliorate at least one symptom associated with Type 1 diabetes. To achieve this effect, the peptide or a variant thereof is administered at dosages of at least about 0.001 to about 100 mg/kg including about 0.01 to about 10 mg/kg including about 0.1 to about 5 mg/kg, of body weight, although other dosages may provide beneficial results. The amount administered will vary depending on various factors including, but not limited to, the peptide selected, the stage of the disease, the weight, the physical condition, and the age of the mammal, whether prevention or treatment is to be achieved, and whether the peptide is chemically modified. Such factors can be readily determined by the clinician employing animal models or other test systems which are well known to the art. In an embodiment, the mammal is a human. Formulations suitable for peptides and for adaptive immunity are well known in the art. The subject peptides or peptide-all complexes or peptide-HLA-DQ A*03:01, DQ B*03:02 complexes can be formulated using standard techniques. Other complexes contemplated herein include peptide complexes with HLA DQ 2 and DQ 2/8 transdimers (DQ A* 05:01, DQ B* 03:02 and/or DQ A* 03:01; DQ B* 02:01), HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

[0126] Administration of sense nucleic acid molecules is also contemplated herein, accomplished through the introduction of cells transformed with an expression cassette comprising the nucleic acid molecule (see, for example, WO 93/02556) or the administration of the nucleic acid molecule (see, for example, Feigner *et al.* U.S. Pat. No. 5,580,859, Pardoll *et al.* (1995) *Immunity 3*:165; Stevenson *et al.* (1995) *Immunol. Rev. 145*:211; Molling (1997) *J. Mol. Med. 75*:242; Donnelly *et al.* (1995) *Ann. N.Y. Acad. Sci. 772*:40; Yang *et al.* (1996) *Mol. Med, Today 2*:476; Abdallah *et al.* (1995) *Biol, Cell 85*:1). Pharmaceutical formulations, dosages and routes of administration for nucleic acids are generally disclosed, for example, in Feigner *et al. supra.*

[0127] Administration of the therapeutic or prophylactic agents in accordance with the instant disclosure may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the peptides may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated.

[0128] To prepare the composition, peptides are synthesized or otherwise obtained, purified and then lyophilized and stabilized. The peptide can then be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given peptide included in a unit dose of a tolerogen can vary widely. For example, about 0.01 to about 10 mg, including about 0.5 to about 5 mg, of at least one peptide comprising an epitope sequence from insulin, proinsulin or preproinsulin, can be administered. A unit dose of the tolerogen may be administered either *via* a mucous membrane, e.g., by respiratory, e.g., nasal (e.g., instill or inhale aerosol) or genitourinary tract administration, or orally, although other routes, such as subcutaneous and intraperitoneal are envisioned to be useful to induce tolerance.

[0129] Thus, one or more suitable unit dosage forms comprising the therapeutic or prophylactic peptides, which, as discussed below, may optionally be formulated for sustained release (for example using microencapsulation, see WO 94/07529, and U.S. Pat. No. 4,962,091), can be administered by a variety of routes including oral, or parenteral, including by rectal, transdermal, subcutaneous, intravenous, intramuscular, intraperitoneal, intrathoracic, intrapulmonary and intranasal (respiratory) routes. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic or prophylactic peptide with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

[0130] When the peptides are prepared for oral administration, they are combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. Conveniently, orally administered peptides are formulated for sustained release, e.g., the agents are microencapsulated. The total active ingredients in such formulations comprise from 0.1 to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipient, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for oral administration may be present as a powder or as granules; as a solution, a suspension or an emulsion; or in achievable base such as a synthetic resin for ingestion of the active ingredients from a chewing gum. The active ingredient may also be presented as a bolus, electuary or paste.

[0131] Pharmaceutical formulations containing the therapeutic or prophylactic peptide can be prepared by procedures known in the art using well known and readily available ingredients. For example, the agent can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose, HPMC and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

[0132] For example, tablets or caplets containing the peptides can include buffering agents such as calcium carbonate, magnesium oxide and magnesium carbonate. Caplets and tablets can also include inactive ingredients such as cellulose, pregelatinized starch, silicon dioxide, hydroxy propyl methyl cellulose, magnesium stearate, microcrystalline

cellulose, starch, talc, titanium dioxide, benzoic acid, citric acid, corn starch, mineral oil, polypropylene glycol, sodium phosphate, and zinc stearate, and the like. Hard or soft gelatin capsules comprising a therapeutic or prophylactic peptide can contain inactive ingredients such as gelatin, microcrystalline cellulose, sodium lauryl sulfate, starch, talc, and titanium dioxide, and the like, as well as liquid vehicles such as polyethylene glycols (PEGs) and vegetable oil. Moreover, enteric coated caplets or tablets of an agent of the invention are designed to resist disintegration in the stomach and dissolve in the more neutral to alkaline environment of the duodenum.

[0133] The therapeutic or prophylactic peptides can also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

[0134] The pharmaceutical formulations of the peptide can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

[0135] The therapeutic or prophylactic peptide may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

[0136] These formulations can contain pharmaceutically acceptable vehicles and adjuvants which are well known in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl

alcohol, glycol ethers such as the products sold under the name "Dowanol", polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, preferably ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol", isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

[0137] The compositions described herein can also contain thickening agents such as cellulose and/or cellulose derivatives. They can also contain gums such as xanthan, guar or carbo gum or gum arabic, or alternatively polyethylene glycols, bentones and montmorillonites, and the like.

[0138] It is possible to add, if necessary, an adjuvant chosen from antioxidants, surfactants, other preservatives, film-forming, keratolytic or comedolytic agents, perfumes and colorings. Also, other active ingredients may be added, whether for the conditions described or some other condition.

[0139] For example, among antioxidants, t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and .alpha.-tocopherol and its derivatives may be mentioned. The galenical forms chiefly conditioned for topical application take the form of creams, milks, gels, dispersion or microemulsions, lotions thickened to a greater or lesser extent, impregnated pads, ointments or sticks, or alternatively the form of aerosol formulations in spray or foam form or alternatively in the form of a cake of soap.

[0140] Additionally, the agents are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal or respiratory tract, possibly over a period of time. The coatings, envelopes, and protective matrices may be made, for example, from polymeric substances, such as polylactide-glycolates, liposomes, microemulsions, microparticles, nanoparticles, or waxes. These coatings, envelopes, and protective matrices are useful to coat indwelling devices, e.g., stents, catheters, peritoneal dialysis tubing, and the like.

[0141] The therapeutic peptides disclosed herein can be delivered *via* patches for transdermal administration. See U.S. Pat. No. 5,560,922 for examples of patches suitable for transdermal delivery of a therapeutic agent. Patches for transdermal delivery can comprise a backing layer and a polymer matrix which has dispersed or dissolved therein a therapeutic agent, along with one or more skin permeation enhancers. The backing layer can be made of any suitable material which is impermeable to the peptide. The backing layer serves as a protective cover for the matrix layer and provides also a support function. The backing can be formed so that it is essentially the same size layer as the polymer matrix or it can be of larger dimension so that it can extend beyond the side of the polymer matrix or overlay the side or sides of the polymer matrix and then can extend outwardly in a manner that the surface of the extension of the backing layer can be the base for an adhesive means. Alternatively, the polymer matrix can contain, or be formulated of, an adhesive polymer, such as polyacrylate or acrylate/vinyl acetate copolymer. For long-term applications it might be desirable to use microporous and/or breathable backing laminates, so hydration or maceration of the skin can be minimized.

[0142] Therapeutic or prophylactic peptides released from a transdermal delivery system must be capable of penetrating each layer of skin. In order to increase the rate of permeation of a therapeutic agent, a transdermal drug delivery system must be able in particular to increase the permeability of the outermost layer of skin, the stratum corneum, which provides the most resistance to the penetration of molecules. The fabrication of patches for transdermal delivery of therapeutic agents is well known to the art.

[0143] For topical administration, the therapeutic or prophylactic peptides may be formulated as is known in the art for direct application to a target area. Conventional forms for this purpose include wound dressings, coated bandages or other polymer coverings, ointments, creams, lotions, pastes, jellies, sprays, and aerosols. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents. The active

ingredients can also be delivered *via* iontophoresis, e.g., as disclosed in U.S. Pat. Nos. 4,140,122; 4,383,529; or 4,051,842. The percent by weight of a therapeutic agent of the invention present in a topical formulation will depend on various factors, but generally will be from 0.01% to 95% of the total weight of the formulation, and typically 0.1-25% by weight.

[0144] Drops, such as eye drops or nose drops, may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered *via* a simple eye dropper-capped bottle, or *via* a plastic bottle adapted to deliver liquid contents dropwise, *via* a specially shaped closure.

[0145] The therapeutic or prophylactic peptide may further be formulated for topical administration in the mouth or throat. For example, the active ingredients may be formulated as a lozenge further comprising a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the composition in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the peptide composition in a suitable liquid carrier.

[0146] The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water.

[0147] A peptide of the present disclosure comprising an amino acid sequence selected from SEQ ID NOs:2 through 121 inclusive may be conjugated to or embedded in a targeting moiety and/or a carrier molecule. In an embodiment, the targeting moiety and the carrier molecule are the same entity. A targeting moiety is useful in facilitating the deployment of the peptide to a particular cell type or tissue type. Examples of cell types include antigen-presenting cells and $CD4^+$ T-cells or other lymphocytes such as $CD8^+$ T-

cells. Examples of targeting moieties include antibodies or antigen-binding fragments or derivatives thereof specific for an antigen on the target cell or tissue, receptor ligands, a glycosaminoglycan, heparan or heparin or a fragment thereof, a cytokine or other molecule capable of specific binding to a desired cell type. Carrier molecules include adjuvants, molecules which increase the half life in the blood stream and the like.

[0148] Enabled herein is an agent comprising a peptide comprising an amino acid sequence having a T-cell epitope derived from insulin, proinsulin or preproinsulin having a glutamine to glutamic acid substitution and comprising an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 inclusive, the peptide conjugated at its N- or C-terminal end to a targeting moiety or carrier molecule.

[0149] In another embodiment, a peptide is provided comprising an amino acid sequence having a T-cell epitope derived from insulin, proinsulin or preproinsulin having a glutamine to glutamic acid substitution and comprising an amino acid sequence set forth in SEQ ID NOs:96 to 121, the peptide embedded in a targeting moiety or carrier molecule.

[0150] In an alternative embodiment, a peptide is provided comprising an amino acid sequence derived comprising a T-cell epitope from insulin, proinsulin or preproinsulin having a glutamine to glutamic acid substitution and comprising an amino acid sequence set forth in SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94, the peptide embedded in a targeting moiety or carrier molecule.

[0151] Enabled herein is an agent comprising a peptide comprising an amino acid sequence having a T-cell epitope derived from insulin, proinsulin or preproinsulin having an arginine to citrulline substitution and comprising an amino acid sequence set forth in SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 inclusive, the peptide conjugated at its N- or C-terminal end to a targeting moiety or carrier molecule.

[0152] In an alternative embodiment, a peptide is provided comprising an amino acid sequence having an epitope derived from insulin, proinsulin or preproinsulin having an

arginine to citrulline acid substitution and comprising an amino acid sequence set forth in SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95, the peptide embedded in a targeting moiety or carrier molecule.

[0153] In an embodiment, the peptide comprises or consists of SEQ ID NO:94. In another embodiment, the peptide comprises or consists of SEQ ID NO:95.

[0154] In an embodiment, the targeting moiety is a moiety which specifically targets an antigen-presenting cell or a T-cell such as a $CD4^+$ T-cell or $CD8^+$ T-cell. Examples include an antibody, receptor ligand, cytokine or a glycosaminoglycan.

[0155] In an embodiment, the carrier molecule is human serum albumin. In an embodiment, the carrier molecule is a cyclic peptide. In the case of the latter, the peptide may be embedded in the cyclic peptide to facilitate increasing its blood half-life and/or to enhance stability.

[0156] In another embodiment, the peptide is conjugated to an antigen-presenting cell or T-cell such as a $CD4^+$ T-cell, cell or other cell of the immune system.

[0157] The conjugated peptide or peptide-cell complex is useful in a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a peptide having an amino acid sequence comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution and comprising an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 wherein the peptide is conjugated to or embedded in a targeting moiety, carrier molecule or immune cell.

[0158] The conjugated peptide or peptide-cell complex is useful in a method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a human subject, the method comprising administering to the subject a peptide having an amino acid sequence comprising a T-cell epitope derived from insulin, proinsulin or

preproinsulin wherein the amino acid sequence comprises an arginine to citrulline substitution and comprising an amino acid sequence selected from SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 wherein the peptide is conjugated to or embedded in a targeting moiety, carrier molecule or immune cell.

[0159] It is proposed herein that such a conjugated entity is useful in inducing tolerance as hereinbefore defined. Pharmaceutical compositions described in relation to the peptide equally apply to a conjugated or embedded peptide. As indicated above, the peptides may comprise other chemical modifications to the amino acid residue.

[0160] The peptides described herein including those having an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 are also useful in diagnostic assays to detect or monitor the progression of Type 1 diabetes. In an embodiment, Type 1 diabetes is detected prior to onset of hyperglycemia. The peptides are also useful in monitoring T-cell responses in a patient's blood, monitoring reactivity to pancreatic islet cells and to detect autoreactive antibodies.

[0161] The peptides described herein including those having an amino acid sequence selected from SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 are also useful in diagnostic assays to detect or monitor the progression of Type 1 diabetes. In an embodiment, Type 1 diabetes is detected prior to onset of hyperglycemia. The peptides are also useful in monitoring T-cell responses in a patient's blood, monitoring reactivity to pancreatic islet cells and to detect autoreactive antibodies.

[0162] The peptides described herein including those having an amino acid sequence selected from SEQ ID NOs:96 to 121 are also useful in diagnostic assays to detect or monitor the progression of Type 1 diabetes. In an embodiment, Type 1 diabetes is detected prior to onset of hyperglycemia. The peptides are also useful in monitoring T-cell responses in a patient's blood, monitoring reactivity to pancreatic islet cells and to detect autoreactive antibodies.

[0163] The assay may be conducted in any number of ways including incubating the peptides with a sample of peripheral blood mononuclear cells or a cellular fraction thereof and screening for T-cell activation. This can be determined, for example, by T-cell proliferation or the presence of immune effector molecules.

[0164] Enabled herein is a method for measuring a cell-mediated immune response in a subject, the method comprising collecting a sample from the subject wherein the sample comprises cells of the immune system which are capable of producing immune effector molecules following stimulation by an autoantigen selected from a modified peptide comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin, incubating the sample with the antigen and then measuring the presence of or elevation in the level of an immune effector molecule wherein the presence or level of the immune effector molecule is indicative of the capacity of the subject to mount an autoreactive a cell-mediated immune response.

[0165] In an embodiment, the subject specification contemplates a method for detecting the presence of autoreactive T-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition associated with Type 1 diabetes in a subject, the method comprising contacting a sample from the subject comprising immune cells with a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin or a chemical analog of, or a non-naturally occurring amino acid substitute of, one or more amino acid residues in the sequence wherein the amino acid sequence comprises a glutamine to glutamic acid substitution and/or an arginine to citrulline substitution for a time and under conditions sufficient for an autoreactive T-cell, if present, to be stimulated into producing an immune effector molecule, and then detecting the presence of the immune effector molecule, wherein the presence or level of the immune effector molecule is indicative of Type 1 diabetes or its state or risk of progression.

[0166] Further enabled herein is a method for screening for autoreactive antibodies associated with Type 1 diabetes or a condition associated therewith in a subject, comprising contacting body fluid from the subject with a peptide comprising an amino acid sequence having a T-cell epitope derived from insulin, proinsulin or preproinsulin or a chemical analog of, or a non-naturally occurring amino acid substitute of, one or more amino acid residues in the sequence wherein the amino acid sequence comprises an arginine to citrulline substitution for a time and under conditions sufficient for an autoreactive antibody, if present, to bind to the peptide and then screening for the presence of the peptide-antibody complex. The presence or level of the antibody is indicative of Type 1 diabetes or its state or risk of progression. Examples of body fluid include serum, whole blood, tissue fluid, sputum, urine, and the like.

[0167] In an embodiment, the subject is a human.

[0168] In an embodiment, the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NO: SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94.

[0169] In an embodiment, the peptide comprises an amino acid sequence set forth in SEQ ID NO:94. In an aspect, the peptide consists of SEQ ID NO:94.

[0170] In an embodiment, the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95.

[0171] In an embodiment, the peptide comprises an amino acid sequence set forth in SEQ ID NO:95.

[0172] In an embodiment, the peptide comprises an amino acid sequence set forth in SEQ ID NOs:96 to 121.

[0173] In an embodiment, the condition associated with Type 1 diabetes is selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

[0174] In an embodiment, the immune effector molecule is detected by an antibody labeled with a reporter molecule, capable of providing a detectable signal.

[0175] Reference to "immune cells" includes cells such as lymphocytes including natural killer (NK) cells, T-cells, ($CD4^+$ and/or $CD8^+$ cells), B-cells, macrophages and monocytes, dendritic cells or any other cell which is capable of producing an effector molecule in response to direct or indirect antigen stimulation. Conveniently, the immune cells are lymphocytes and includes $CD4^+$ T-cells.

[0176] The immune effector molecules for T-cell assays may be any of a range of molecules which are produced in response to cell activation or stimulation by the peptide antigen. Immune effector molecules include interferon (e.g. IFN-γ), a range of cytokines such as interleukins (IL), e.g. IL-2, IL-4, IL-10 or IL-12, tumor necrosis factor alpha (TNF-α), a colony stimulating factor (CSF) such as granulocyte (G)-CSF or granulocyte macrophage (GM)-CSF amongst many others such as complement or components in the complement pathway.

[0177] The sample collected from the subject is generally deposited into a collection tube. A collection tube includes a blood draw tube or other similar vessel. Conveniently, when the sample is whole blood, the blood collection tube is heparinized. Alternatively, heparin is added to the tube after the blood is collected. Notwithstanding that whole blood is the most convenient sample, the present disclosure extends to other samples containing immune cells such as lymph fluid, cerebral fluid, tissue fluid and respiratory fluid including nasal and pulmonary fluid.

[0178] The use of blood collection tubes is useful since they are compatible with standard automated laboratory systems and these are amenable to analysis in large-scale

and random access sampling. Blood collection tubes also minimize handling costs and reduce laboratory exposure to whole blood and plasma and, hence, reduce the risk of laboratory personnel from contracting a pathogenic agent such as HIV or hepatitis B virus (HBV).

[0179] The incubation step with the peptide antigen may be from 5 to 50 hours, including 5 to 40 hours and 8 to 24 hours or a time period in between.

[0180] Another aspect enabled herein contemplates a method for measuring a cell-mediated immune response in a subject including a human subject, the method comprising collecting a sample of whole blood from the subject, incubating the whole blood sample with a peptide comprising an amino acid sequence selected from SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 and then measuring the presence or elevation in level of an immune effector molecule such as IFN-γ wherein the presence or level of the immune effector molecule is indicative of the capacity of the subject to mount a cell-mediated immune response to insulin, proinsulin or preproinsulin leading to Type 1 diabetes.

[0181] Another aspect enabled herein contemplates a method for measuring a cell-mediated immune response in a subject including a human subject, the method comprising collecting a sample of whole blood from the subject, incubating the whole blood sample with a peptide comprising an amino acid sequence selected from SEQ ID NOs:96 to 121 and then measuring the presence or elevation in level of an immune effector molecule such as IFN-γ wherein the presence or level of the immune effector molecule is indicative of the capacity of the subject to mount a cell-mediated immune response to insulin, proinsulin or preproinsulin leading to Type 1 diabetes.

[0182] Another aspect enabled herein contemplates a method for measuring a cell-mediated immune response in a subject including a human subject, the method comprising collecting a sample of whole blood from the subject, incubating the whole blood sample with a peptide comprising an amino acid sequence selected from SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71, 72, 74, 75, 77, 78, 80, 81 and 95 and then measuring the

presence or elevation in level of an immune effector molecule such as IFN-γ wherein the presence or level of the immune effector molecule is indicative of the capacity of the subject to mount a cell-mediated immune response to insulin, proinsulin or preproinsulin leading to Type 1 diabetes.

[0183] In an embodiment, the peptide employed is or comprises SEQ ID NO:94.

[0184] The ability to measure an autoreactive cell-mediated immune response is important for assessing a subject's likelihood of mounting an autoimmune response to insulin, proinsulin or preproinsulin and developing Type 1 diabetes and its associated conditions.

[0185] Detection of the immune effector molecules may be measured at the protein or nucleic acid levels. Consequently, reference to "presence or level of the immune effector molecule" includes direct and indirect data. For example, high levels of IFN-γ mRNA is indirect data showing increased levels of IFN-γ.

[0186] Ligands such as antibodies to the immune effectors are useful in detecting and/or quantitating these molecules. Similarly, autoreactive antibodies may be detected such as by anti-IgG antibodies, labeled with a reporter molecule. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays, ELISA and ELISpot. Reference to "antibodies" includes parts of antibodies, mammalianized (e.g. humanized) antibodies, recombinant or synthetic antibodies and hybrid and single chain antibodies.

[0187] Both polyclonal and monoclonal antibodies to the effector molecule or autoreactive antibodies are obtainable by immunization with the immune effectors or antigenic fragments thereof and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the immune effector, or antigenic part thereof, collecting serum from the animal and isolating

specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favored because of the potential heterogeneity of the product.

[0188] The use of monoclonal antibodies in an immunoassay is useful due to the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

[0189] Another aspect of the present invention contemplates, therefore, a method for detecting an immune effector in response to autoreactive T-cells to a peptide derived from insulin, proinsulin or preproinsulin and comprising an amino acid sequence as set forth in SEQ ID NOs:2 through 121 or a chemically modified form of the amino acid sequence in a sample comprising immune cells from a subject, the method comprising incubating the sample with the peptide and capturing any immune effector molecules produced by stimulated T-cells with an antibody specific for the immune effector or antigenic fragment and then detecting the capture immune effector.

[0190] Another aspect of the present invention contemplates, therefore, a method for detecting an autoreactive antibody in response to autoreactive B-cells to a peptide derived from insulin, proinsulin or preproinsulin and comprising an amino acid sequence as set forth in SEQ ID NOs:2 through 121 or a chemically modified form of the amino acid sequence in a sample comprising immune cells from a subject, the method comprising incubating the sample with the peptide and capturing any autoreactive antibodies produced by stimulated B-cells with an antibody specific for the autoreactive antibody or antigenic fragment and then detecting the capture immune effector.

[0191] A sample includes whole blood. This method includes micro-arrays and macro-arrays on planar or spherical solid supports. A micro- or macro-array is useful.

[0192] A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653.

[0193] In one type of assay, either a monoclonal or polyclonal capture antibody is employed which is coated aseptically onto the surface of well. These antibodies have specificity for the immune effector molecule. The plate is blocked, usually with a serum protein that is non-reactive with any of the antibodies in the assay. After this, immune cells (e.g. from a blood sample) are plated out at varying densities, along with a peptide comprising an amino acid sequence selected from SEQ ID NOs:2 through 121, or a modified form of the peptide comprising amino acid substitution, addition or deletion or comprising a non-naturally occurring amino acid residue, and then placed in a humidified 37°C CO_2 incubator for a specified period of time.

[0194] The immune effector molecule secreted by activated cells is captured locally by the coated antibody on the high surface area microplate. Alternatively, synthetic peptides comprising an arginine to citrulline substitution are coated to the surface of a reaction vessel. After washing the wells to remove cells, debris, and media components, an antibody labeled with a reporter molecule and specific for the immune effector or autoreactive antibody is added to the wells. This antibody is reactive with a distinct epitope of the immune effector or autoreactive antibody and is used to detect the captured immune effector or autoreactive antibody. Following a wash to remove any unbound labeled antibody, the detected immune effector or autoreactive antibody is then visualized using a detection system. The spots can be counted manually (e.g. with a dissecting microscope) or using an automated reader to capture the microwell images and to analyze spot number size. Such an assay can also be used to quantitate the number of T-cells producing immune effectors or to quantitate or quantitate the presence of autoreactive antibodies. The production of immune effectors means the presence of autoreactive T-cells and/or the preparation of autoreactive antibodies means the potential for, or state of, Type 1 diabetes.

[0195] In these assays, the immobilized antibody having specificity for the instant immune effector or the synthetic peptide is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride, polyvinylidene fluoride or polypropylene. The solid supports may be in the form of tubes, beads, spheres, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-120 minutes or where more convenient, overnight) and under suitable conditions (e.g. for about 20°C to about 40°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the antigen. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the antibody-immune effector complex or peptide-autoreactive antibody complex.

[0196] By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of immune effector-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules. In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and horse radish peroxidase. It is also possible to employ fluorogenic substrates, which yield a

fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labeled antibody is added to the first antibody-immune effector complex or peptide-autoreactive antibody complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-immune effector-antibody or peptide-autoreactive antibody-antibody. The substrate reacts with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of immune effector or autoreactive antibody which was present in the sample. Again, the present invention extends to a substantially simultaneous assay.

[0197] Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labeled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. The fluorescent labeled antibody is allowed to bind to the first antibody-immune effector complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the antigen of interest. Immunofluorescene and EIA techniques are both well established in the art. Other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

[0198] There are a range of other detection systems which may be employed including colloidal gold and all such detection systems are encompassed herein.

[0199] The present disclosure also contemplates genetic assays such as involving PCR analysis to detect RNA expression products of a genetic sequence encoding an immune effector or autoreactive antibody.

[0200] In one embodiment, PCR is conducted using pairs of primers, one or both of which are generally labeled with the same or a different reporter molecule capable of

giving a distinguishable signal. The use of fluorophores is useful in the practice of this aspect of the invention.

[0201] Any suitable method of analyzing fluorescence emission is encompassed herein. In this regard, the instant disclosure contemplates techniques including but not restricted to 2-photon and 3-photon time resolved fluorescence spectroscopy as, for example, disclosed by Lakowicz *et al.* (1997) *Biophys. J. 72:*567, fluorescence lifetime imaging as, for example, disclosed by Eriksson *et al.* (1993) *Biophys. J. 2:*64 and fluorescence resonance energy transfer as, for example, disclosed by Youvan *et al.* (1997) *Biotechnology et elia 3:*1-18.

[0202] Luminescence and phosphorescence may result respectively from a suitable luminescent or phosphorescent label as is known in the art. Any optical means of identifying such label may be used in this regard.

[0203] Infrared radiation may result from a suitable infrared dye. Exemplary infrared dyes that may be employed in the invention include but are not limited to those disclosed in Lewis *et al.* (1999) *Dyes Pigm. 42(2):*197; Tawa *et al. Mater. Res. Soc. Symp. Proc. 488* [Electrical, Optical and Magnetic Properties of Organic Solid-State Materials IV], 885-890; Daneshvar *et al.* (1999) *J. Immunol. Methods 226(1-2):*119-128; Rapaport *et al.* (1999) *Appl. Phys. Lett. 74(3):*329-331 and Durig *et al.* (1993) *J. Raman Spectrosc. 24(5):*281-285. Any suitable infrared spectroscopic method may be employed to interrogate the infrared dye. For instance, fourier transform infrared spectroscopy as, for example, described by Rahman *et al.* (1998) *J. Org. Chem. 63:*6196 may be used in this regard.

[0204] Suitably, electromagnetic scattering may result from diffraction, reflection, polarization or refraction of the incident electromagnetic radiation including light and X-rays. Such scattering can be used to quantitate the level of mRNA or level of protein.

[0205] Flow cytometry is also useful in analyzing fluorophore emission.

[0206] As is known in the art, flow cytometry is a high throughput technique which involves rapidly analyzing the physical and chemical characteristics of particles (e.g. labeled mRNA, DNA or proteins) as they pass through the path of one or more laser beams while suspended in a fluid stream. As each particle intercepts the laser beam, the scattered light and fluorescent light emitted by each cell or particle is detected and recorded using any suitable tracking algorithm.

[0207] A modern flow cytometer is able to perform these tasks up to 100,000 cells/particles s^{-1}. Through the use of an optical array of filters and dichroic mirrors, different wavelengths of fluorescent light can be separated and simultaneously detected. In addition, a number of lasers with different excitation wavelengths may be used. Hence, a variety of fluorophores can be used to target and examine, for example, different immune effectors within a sample or immune effectors from multiple subjects.

[0208] Suitable flow cytometers which may be used in the methods of the present invention include those which measure five to nine optical parameters using a single excitation laser, commonly an argon ion air-cooled laser operating at 15 mW on its 488 nm spectral line. More advanced flow cytometers are capable of using multiple excitation lasers such as a HeNe laser (633 nm) or a HeCd laser (325 nm) in addition to the argon ion laser (488 or 514 nm).

[0209] For example, Biggs *et al.* (1999) *Cytometry 36:*36-45 have constructed an 11-parameter flow cytometer using three excitation lasers and have demonstrated the use of nine distinguishable fluorophores in addition to forward and side scatter measurements for purposes of immunophenotyping (i.e. classifying) particles. The maximum number of parameters commercially available currently is 17: forward scatter, side scatter and three excitation lasers each with five fluorescence detectors. Whether all of the parameters can be adequately used depends heavily on the extinction coefficients, quantum yields and amount of spectral overlap between all fluorophores (Malemed *et al.* (1990) *"Flow cytometry and sorting"*, 2nd Ed., New York, Wiley-Liss). However, it will be understood

that the present invention is not restricted to any particular flow cytometer or any particular set of parameters. In this regard, the invention also contemplates use in place of a conventional flow cytometer, a microfabricated flow cytometer as, for example, disclosed by Fu *et al.* (1999) *Nature Biotechnology 17*:1109-1111.

[0210] The instant disclosure further contemplates autoreactive antibodies which have a binding affinity to the modified peptides derived from insulin, proinsulin or preproinsulin wherein the modified peptides comprise an arginine to citrulline substitution. Antibodies may also be generated to such modified peptides for use *inter alia* for diagnostic purposes.

[0211] Hence, in an embodiment, enabled herein is an assay to screen for autoreactive antibodies to insulin, proinsulin or preproinsulin, the assay comprising contacting a biological sample such as serum from a subject to be tested with a peptide derived insulin, proinsulin or preproinsulin wherein the peptide comprises an arginine to citrulline substitution, so as to form a complex between an autoreactive antibody if present in the sample and the peptide and then detecting the presence of the antibody-peptide complex.

[0212] In an embodiment, the sample is selected from serum, whole blood, sputum, urine or tissue fluid.

[0213] In an embodiment, the presence of an autoreactive antibody or the level or presence of the antibody is an indicator of Type 1 diabetes or a stage of developed Type 1 diabetes such as early stage Type 1 diabetes.

[0214] The present specification further teaches animal models such as mouse, rat, rabbit, guinea pig, hamster or non-human primate models for Type 1 diabetes. These animal models may comprise humanized insulin or humanized human components in a diabetic model. These animal models are useful for testing the peptides in treatment protocols and in diagnostic assays. Existing animal models may be used or readily adapted. Examples of existing animal models are disclosed in Example 11.

[0215] The present specification further teaches immunotherapies and immunodiagnostics based on HLA Class II tetramers. In particular, peptide-labeled HLA Class II tetramers are used to elucidate the role of antigen-specific Tregs in diabetes autoimmunity and to monitor insulin-specific $CD4^+$ T-cells. Peptide-HLA Class II tetramers display the antigen peptide in a form which is a surrogate for the recognition events which occur between T-cells and antigen-presenting cells. They are useful to interrogate $CD4^+$ T-cells and in desensitization protocols (Nepom (2012) *J. Immunol. 188(6)*:2477-2482).

[0216] Enabled herein is an isolated HLA Class II tetramer incorporating a peptide comprising at least 7 consecutive amino acids comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin, wherein the peptide comprises a glutamine to glutamic acid substitution.

[0217] In an embodiment the peptide comprises at least 13 amino acid residues.

[0218] In an embodiment the peptide comprises a sequence selected from SEQ ID NO:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.

[0219] In an embodiment the peptide comprises SEQ ID NO:94.

EXAMPLES

[0220] Embodiments contemplated herein are now described by the following non-limiting Examples.

EXAMPLE 1

Selection of islet-infiltrating T-cells

[0221] T-cells are isolated from within pancreatic islets due to the fact that this constitutes evidence that a T-cell contributes to beta-cell autoimmunity and destruction. Analysis of islet-infiltrating T-cells leads to the identification of disease-relevant epitopes. These epitopes are prime candidates for developing T-cell assays and antigen-specific therapies for Type 1 diabetes. Islet T-cells are shown in Figure 1. The cloning of human islet T-cells is shown in Figure 2.

EXAMPLE 2

Insolating islet-infiltrating T-cells

[0222] A summary of T-cell clones from Type 1 diabetic organ donors is provided in Table 4. The methodology is shown in Figure 3.

Table 4

Donor	Disease duration	HLA Class I	HLA Class II	$CD4^+$ clones	$CD8^+$ clones
A	3 years	A1, 2, B8, 51	DRB1*03:01, 04:04 DQB1*02:01; 03:02	62	27
B	>20 years	A2, 30, B*18:01, 44:02	DRB1*01:01, 03:01 DQB1*0201,05:01	153	129
C	19 years	A*02:01 -, B*18:01, 39:01/46	DRB1*03:01, 04:01 DQB1*02:01, 03:02	29	58
D	8 years	A1, A23, B8, 50	DRB1*03:01:01G,-	60	30
E	8years	A*02:01,-, B*15:01, 40:01	DRB1*01:01, 04:01 DQB1*03:02, 05:01	85	17
F	15 years	A*02:01, 23:01 B*15:01, 50:01	DRB1*0301, 0401 DQ1*0201,0302	103	

EXMAPLE 3

Searching for modifications that fit the p9 pocket

[0223] Pools of two peptides (Pools A-E) are made and screened against Donor A's clones (Figure 4).

[0224] A summary of epitope mapping-epitope formed by posttranslational modification is provided in Table 5 and Figures 5 to 9. A glutamine to glutamic acid substitution is referred to herein as a deamidation.

Table 5

B-chain	C-peptide	A-chain
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN		
Unmodified control 1	ELGGGPGAGSLQPLALEG	
Unmodified control 2	GAGSLQPLALEGSLQKRG	
Q25E short peptide	VELGGGPGAGSLEPL	
Q25E long peptide	TRREAEDLQVGQVELGGGPGAGSLEPLALEGSLQK	

[0225] Table 6 is a summary of epitope mapping-epitope formed by posttranslational modification. See also Figure 6.

Table 6

Donor A

B-chain	C-peptide	A-chain	No. of clones
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN			
	VELGGGPGAGSLEPL		1

[0226] Table 7 provides a summary of epitope mapping-Epitope formed by posttranslational modification. See also Figures 8 and 9.

Table 7

Donor A

B-chain	C-peptide	A-chain	No. of clones
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN			
		QPLALEGSLQKR*GI	1

R*= citrulline

[0227] The data show that citrulline (Cit or R*) is recognized by autoreactive antibodies generated in Type 1 diabetes.

EXAMPLE 4

HLA blocking

[0228] To determine which HLA molecules present the peptide to T-cells mAb specific for HLA-DR, -DP or DQ are added to the peptides stimulation assay. The results are shown in Figure 10. The data show that only the mAB specific for HLA-DQ inhibits the response to the peptide [SEQ ID NO:94] (Figure 10).

EXAMPLE 5

HLA restriction

[0229] Data show that the T-cell clone's response to peptide [SEQ ID NO:94] requires an antigen-presenting cell that expresses HLA DQ8 (HLA-DQ A*03:01, DQ B*03:02) [Figure 11]. In another embodiment, the peptide binds to HLA DQ2 and/or HLA DQ 2/8 transdimer such as DQ A*05:01, DQ B*03:02 and/or DQ A*03:01, DQ B*02:01, HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

EXAMPLE 6

Fine epitope mapping

[0230] The minimum sequence of amino acids required to stimulate the T-cell clone was determined by tested and panel of peptide of differing length (Panel A). To confirm this result, a panel of peptides covering the sequence of the epitope set forth in SEQ ID NO:94, but with each amino acid in turn substituted by alanine or lysine, whichever is most different from the native amino acid was tested. This confirmed the epitope mapping and showed which residues could be modified without impairing the T-cell response.

EXAMPLE 7

Response to deamidated peptide in the peripheral blood from people with and without Type 1 diabetes

[0231] $CD4^+$ T-cell responses were measured using a carboxy fluorescein succinimidyl ester (CFSE)-based proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood samples from individuals with Type 1 diabetes, or those without Type 1 diabetes ("healthy controls"). The results show the ratio of the number of $CD4^+$ cells that have proliferated in response to the peptides relative to control samples cultured without peptide. The ratio is known as a "Cell Division Index" or CDI (Mannering *et al.* (2003) *J. Immunol Methods 283*:173-183). A CDI> 2.0 is considered to be a positive response (Figure 13).

EXAMPLE 8

T-cell assay

[0232] Figure 14 provides the results of an INF-γ-based assay detected from sensitive T-cells in response to the modified peptide of SEQ ID NO:94 (with the glutamine to glutamic acid substitution) compared to a non-modified peptide. The modified peptide was significantly more active in stimulating a response.

EXAMPLE 9

Epitope characterization

[0233] $CD4^+$ T-cells are isolated and characterized specific for the deamidated epitopes derived from human proinsulin. The epitopes are subjected to assays to determine specificity, HLA restriction and binding to T-cell receptors determination. The SEQ ID NO:94 peptide is specific for HLA-DQ A*03:01, DQ B*03:02.

EXAMPLE 10

Role of regulatory T-cells

[0234] The role of regulatory T (Treg) cell responses against the deamidated epitopes and their unmodified counterparts is investigated using standard procedures. Therapies combining peptide delivery and Treg-cell modification or regulation is contemplated. Furthermore, Treg-cells and effector T-cell responses, against modified and unmodified peptides, in people with Type 1 diabetes and healthy subject.

EXAMPLE 11

Animal model

[0235] A transgenic NOD mouse model of human $CD4^+$ T-cell responses against deamidated proinsulin epitopes is developed. During development, standard mouse models can be employed such as described by Niens *et al.* (2011) *Diabetes 60*:1229-1236; Daniel *et al. (2011)* J. Exp. Med. 10.1084/jem.20110574; Unger *et al.* (2012) *7(11)*:e49213.doi:10.1371/Jounal.Pone.0049213; and Scotto *et al.* (2012) *Diabetologia 55*:2026-2031.

EXAMPLE 12

Effects of physiological stress agents

[0236] Deamidated proinsulin peptides are analyzed in human beta cells when the islets are exposed to different physiological stressors (e.g. cytokines, elevated glucose concentrations). This leads to combination therapies and protocols to augmentation therapies.

[0237] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

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CLAIMS:

1. A method for the treatment or prophylaxis of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject, said method comprising administering to said subject a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution.

2. The method of Claim 1 wherein the mammalian subject is a human.

3. The method of Claim 1 or 2 wherein the peptide comprises from 13 to 40 amino acid residues with the proviso that it contains at least one glutamine to glutamic acid substitution.

4. The method of Claim 1 or 2 wherein the peptide comprises from 13 to 20 amino acid residues with the proviso that it contains at least one glutamine to glutamic acid substitution.

5. The method of Claim 1 or 2 wherein the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121.

6. The method of Claim 1 or 2 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

7. The method of Claim 1 wherein the peptide alternatively comprises or contains in addition an arginine to citrulline substitution.

8. The method of Claim 7 wherein the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:6 to 31, 33, 34, 62, 63, 65, 66, 68, 69, 71,

72, 74, 75, 77, 78, 80, 81 and 95.

9. The method of Claim 8 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:95.

10. The method of any one of Claims 1 to 9 wherein the peptide binds to an antigen-presenting cell which comprises the haplotype HLA-DQ A*03:01; DQ B*03:02.

11. The method of any one of Claims 1 to 9 wherein the peptide binds to an HLA molecule selected from one or more of HLA DQ 2, HLA DQ 2/8 transdimer, HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

12. The method of Claim 11 wherein the haplotype is DQ A*05:01, DQ B*03:02 and/or DQ A*03:01, DQ B*01:01.

13. The method of Claim 1 wherein the condition associated with Type 1 diabetes is selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

14. The method of Claim 1 wherein the peptide is conjugated to or embedded in a targeting moiety, carrier molecule or an immune cell.

15. An isolated T-cell or antigen-presenting cell derived from a mammalian subject presenting a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine or glutamic acid substitution.

16. The isolated T-cell or antigen-presenting cell of Claim 15 wherein the mammalian subject is a human.

17. The isolated T-cell or antigen-presenting cell of Claim 16 wherein the peptide is from 13 to 40 amino acid residues in length.

18. The isolated T-cell or antigen-presenting cell of Claim 17 wherein the peptides is from 13 to 20 amino acids in length.

19. The isolated T-cell or antigen-presenting cell of Claim 18 wherein the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.

20. The isolated T-cell or antigen-presenting cell of Claim 19 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

21. The isolated T-cell or antigen-presenting cell of any one of Claims 15 to 20 for treating or preventing Type 1 diabetes or a condition associated with Type 1 diabetes wherein the T-cells or antigen-presenting cells are autologous to the subject being treated.

21. The isolated T-cell or antigen-presenting cell of Claim 21 wherein the condition associated with Type 1 diabetes is selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

23. A therapeutic or prophylactic composition comprising a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

24. The therapeutic or prophylactic composition of Claims 1 to 23 wherein the peptide is from 13 to 40 amino acids in length.

25. The therapeutic or prophylactic composition of Claim 23 wherein the peptide is from 13 to 20 amino acids in length.

26. The therapeutic or prophylactic composition of Claim 23 wherein peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.

27. The therapeutic or prophylactic composition of Claim 26 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

28. The therapeutic or prophylactic composition of Claim 23 wherein the peptide binds to an antigen-presenting cell which comprises the haplotype HLA-DQ A*03:01; DQ B*03:02.

29. The therapeutic or prophylactic composition of Claim 28 wherein the peptide binds to an HLA molecule selected from one or more of HLA DQ 2, HLA DQ 2/8 transdimer, HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

30. The therapeutic or prophylactic composition of Claim 23 is in the form of an HLA Class II tetramer.

31. The therapeutic or prophylactic composition of Claim 23 wherein the peptide is conjugated to or embedded in a targeting moiety, carrier molecule or an immune cell.

32. Use of a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising an epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution in the manufacture of a medicament for the treatment of Type 1 diabetes or a condition associated with Type 1 diabetes in a mammalian subject.

33. Use of Claim 22 wherein the mammalian subject is a human.

34. Use of Claim 32 or 33 wherein the peptide is from 13 to 40 amino acids in length.

35. Use of Claim 34 wherein the peptide is from 13 to 20 amino acids in length.

36. Use of Claim 35 wherein the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79, 82 to 94 and 96 to 121.

37. Use of Claim 35 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

38. Use of Claim 32 wherein the peptide binds to an antigen-presenting cell which comprises the haplotype HLA-DQ A*03:01, DQ B*03:02.

39. Use of Claim 33 wherein the peptide binds to an HLA molecule selected from one or more of HLA DQ 2, HLA DQ 2/8 transdimer, HLA-DR3 (DRB1*03xx) and/or HLA-DR4 (DRB1*04xx).

40. The method of Claim 39 wherein the haplotype is DQ A*05:01, DQ B*03:02 and/or DQ A*03:01, DQ B*01:01.

41. Use of Claim 32 wherein a condition associated with Type 1 diabetes is selected from selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

42. Use of Claim 32 wherein the peptide is conjugated to or embedded in a targeting moiety, carrier molecule or an immune cell.

43. A method for detecting the presence of autoreactive T-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition

associated with Type 1 diabetes in a subject, said method comprising contacting a sample from said subject comprising immune cells with a peptide comprising an amino acid sequence of at least seven consecutive amino acid residues comprising a t-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive T-cell, if present, to be stimulated into producing an immune effector molecule, and then detecting the presence of said immune effector molecule, wherein the presence or level of the immune effector molecule is indicative of Type 1 diabetes or its state or risk of progression or a condition associated with Type 1 diabetes.

44. An assay to screen for autoreactive antibodies to insulin, proinsulin or preproinsulin, the assay comprising contacting a biological sample such as serum from a subject to be tested with a peptide comprising a T-cell epitope derived insulin, proinsulin or preproinsulin wherein the peptide comprises a glutamine to glutamic acid substitution, so as to form a complex between an autoreactive antibody if present in the sample and the peptide and then detecting the presence of the antibody-peptide complex.

45. A method for screening for autoreactive antibodies associated with Type 1 diabetes or a condition associated therewith in a subject, comprising contacting body fluid from the subject with a peptide comprising an amino acid sequence comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises an glutamine to glutamic acid substitution for a time and under conditions sufficient for an autoreactive antibody, if present, to bind to the peptide and then screening for the presence of the peptide-antibody complex.

46. A method for detecting the presence of autoreactive B-cells indicative of the presence of, or a predisposition for the development of, Type 1 diabetes, or a condition associated with Type 1 diabetes in a subject, the method comprising contacting a sample from the subject comprising immune cells with a peptide comprising an amino acid sequence comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution for a

time and under conditions sufficient for an autoreactive B-cell, if present, to be stimulated into producing an antibody, and then detecting the presence of the antibody, wherein the presence or level of the antibody is indicative of Type 1 diabetes or its state or risk of progression or a condition associated with Type 1 diabetes.

47. The method of any one of Claims 43 to 46 wherein the subject is a human.

48. The method of Claim 47 wherein the presence of autoreactive antibodies is an indication of Type 1 diabetes or its stage of development.

49. The method of Claim 47 wherein the peptides is from 13 to 40 amino acids in length.

50. the method of Claim 47 wherein the peptide is from 13 to 20 amino acids in length.

51. The method of Claim 50 wherein the peptide comprises an amino acid sequence selected from the list consisting of SEQ ID NOs:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.

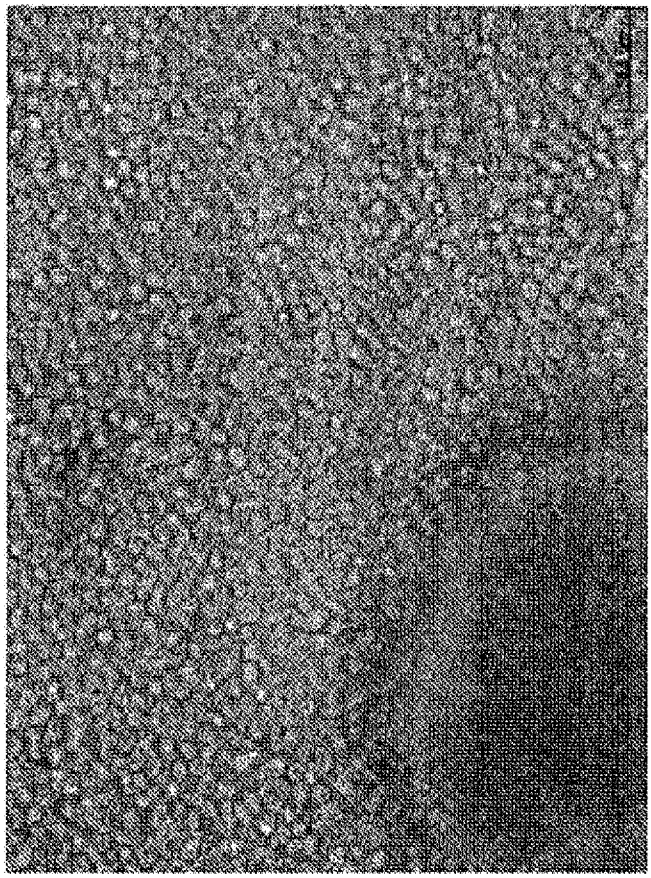
52. The method of Claim 50 wherein the peptide comprises an amino acid sequence set forth in SEQ ID NO:94.

53. The method of any one of Claims 43 to 46 wherein the condition associated with Type 1 diabetes is selected from the list consisting of a cardiovascular condition, a neuropathy, a retinopathy, a nephropathy, infection and a complication in pregnancy.

54. An isolated HLA Class II tetramer incorporating a peptide comprising at least 7 consecutive amino acids comprising a T-cell epitope derived from insulin, proinsulin or preproinsulin, wherein the peptide comprises a glutamine to glutamic acid substitution.

55. The isolated HLA Class II tetramer of Claim 54 wherein the peptide comprises at least 13 amino acid residues.

56. The insulin HLA Class II tetramer of Claim 55 wherein the peptide comprises a sequence selected from SEQ ID NO:2 to 5, 32, 35 to 61, 64, 67, 70, 73, 76, 79 and 82 to 94 or 96 to 121.



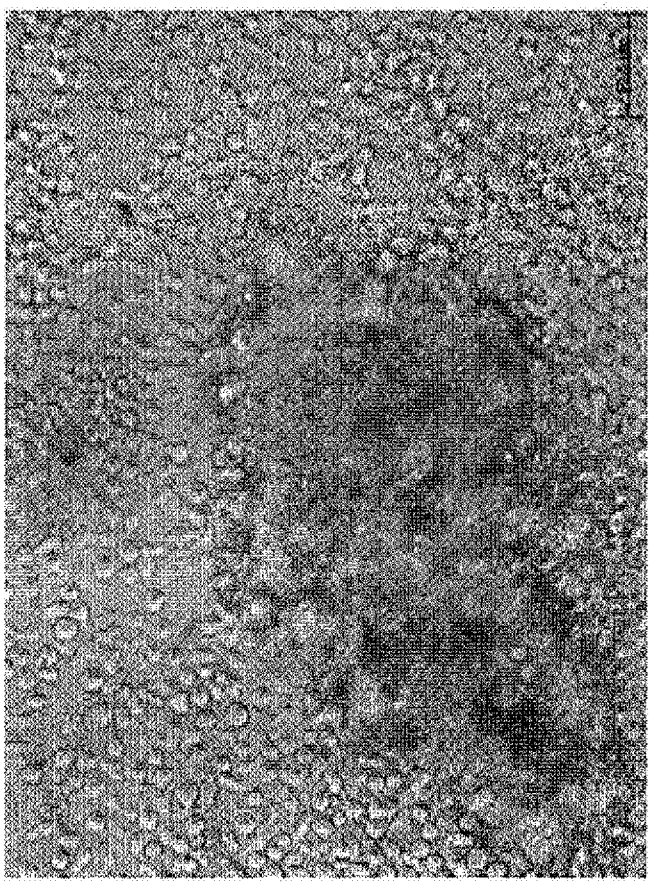


Figure 1

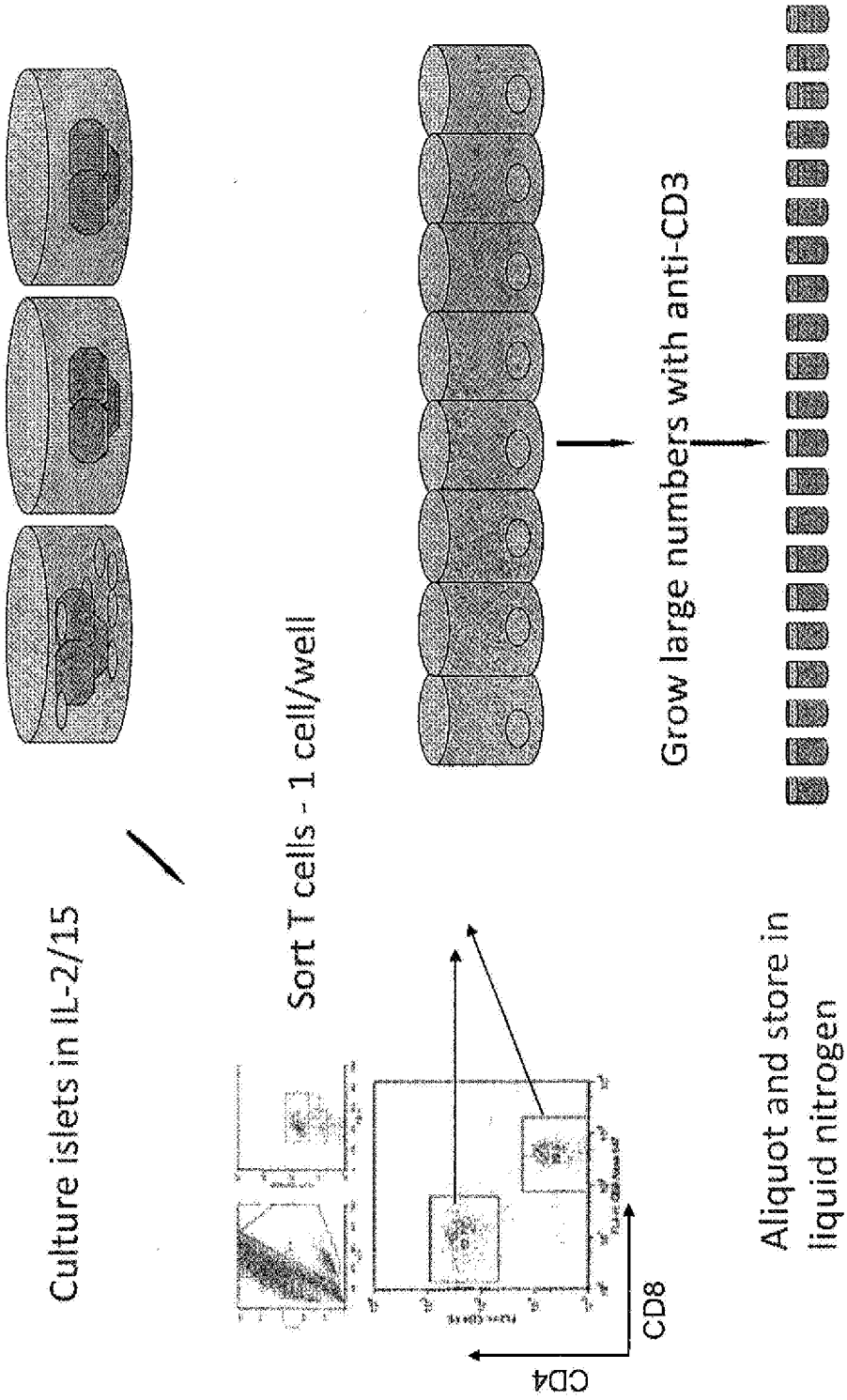

Culture islets in IL-2/15
Sort T cells - 1 cell/well
CD4
CD8
Grow large numbers with anti-CD3
Aliquot and store in liquid nitrogen

Figure 2

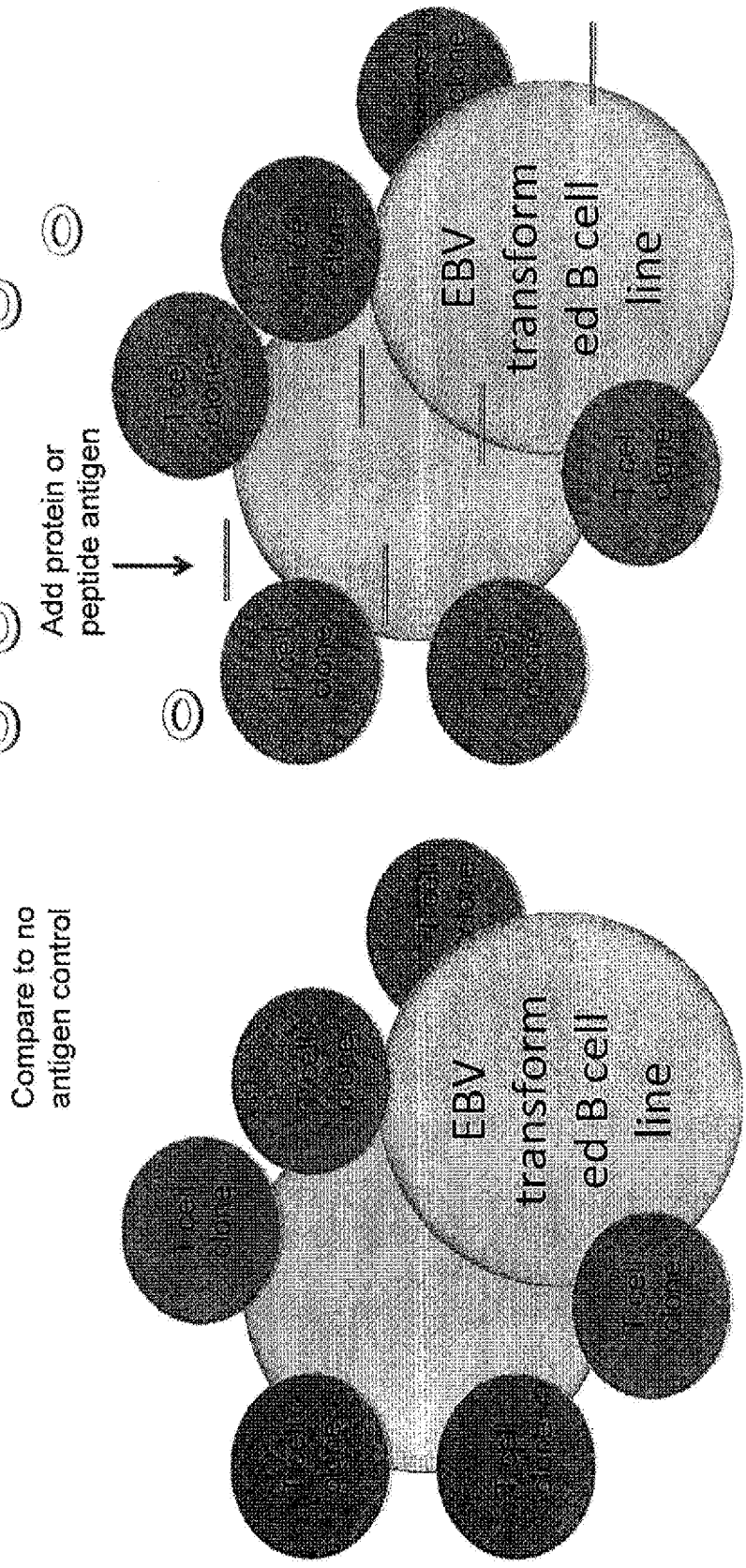

Compare to no antigen control
EBV transform ed B cell line
T cell clone
Add protein or peptide antigen
Secreted IFNγ
EBV transform ed B cell line
T cell clone
T cell clone
T cell clone

Figure 3

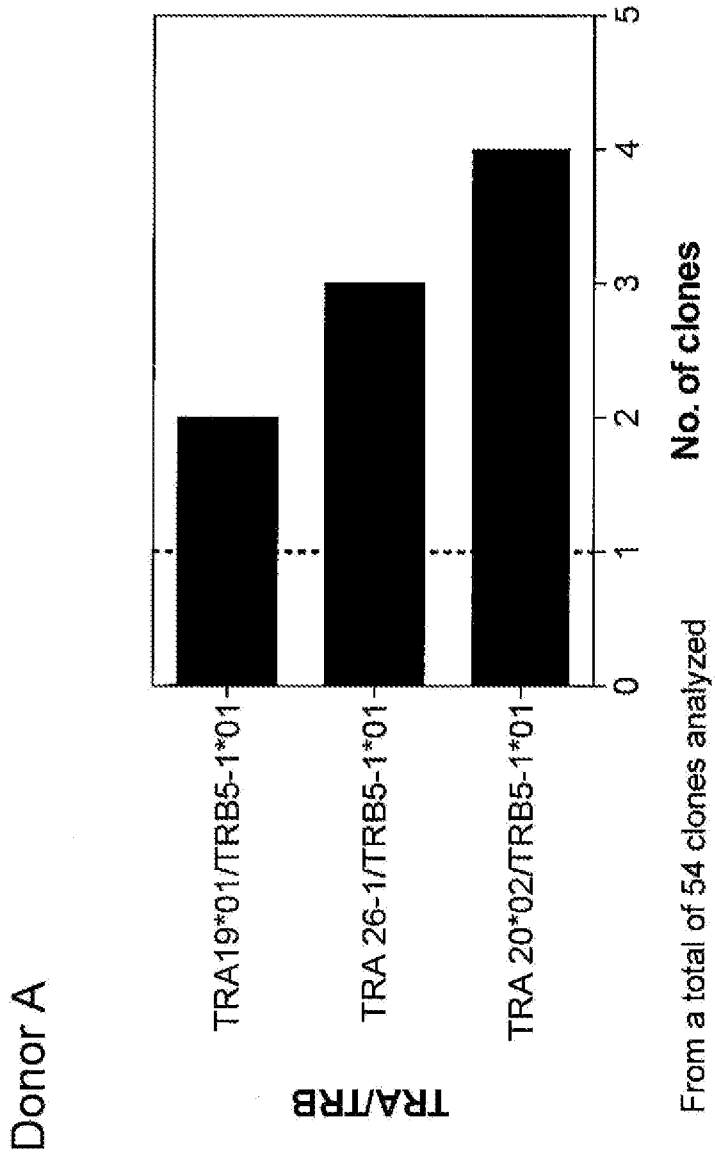

Donor A
TRA/TRB
TRA19*01/TRB5-1*01
TRA 26-1/TRB5-1*01
TRA 20*02/TRB5-1*01
0
1
2
3
4
5
No. of clones
From a total of 54 clones analyzed

Figure 4

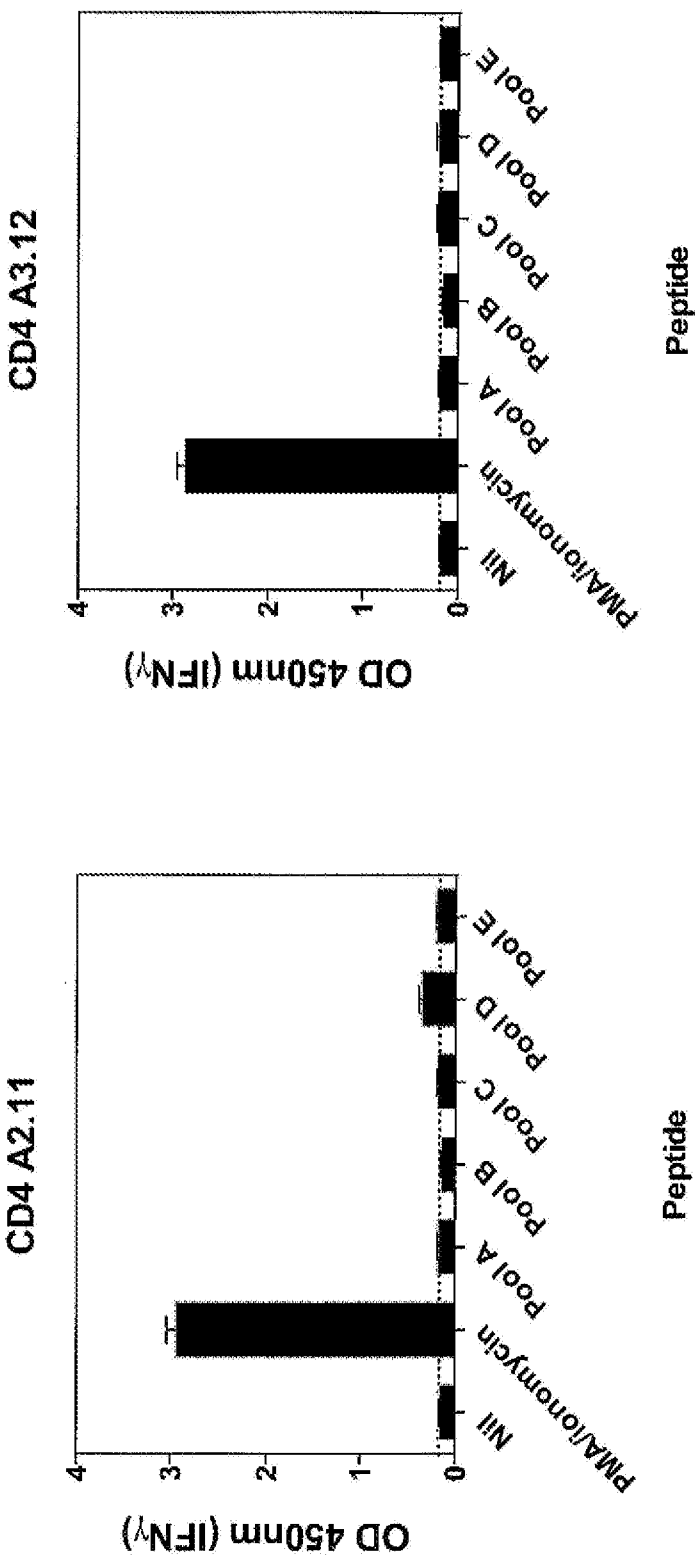

CD4 A3.12
OD 450nm (IFNγ)
0
1
2
3
4
Nil
PMA/ionomycin
pool A
pool B
pool C
pool D
pool E
Peptide
CD4 A2.11
OD 450nm (IFNγ)
0
1
2
3
4
Nil
PMA/ionomycin
pool A
pool B
pool C
pool D
pool E
Peptide

Figure 5

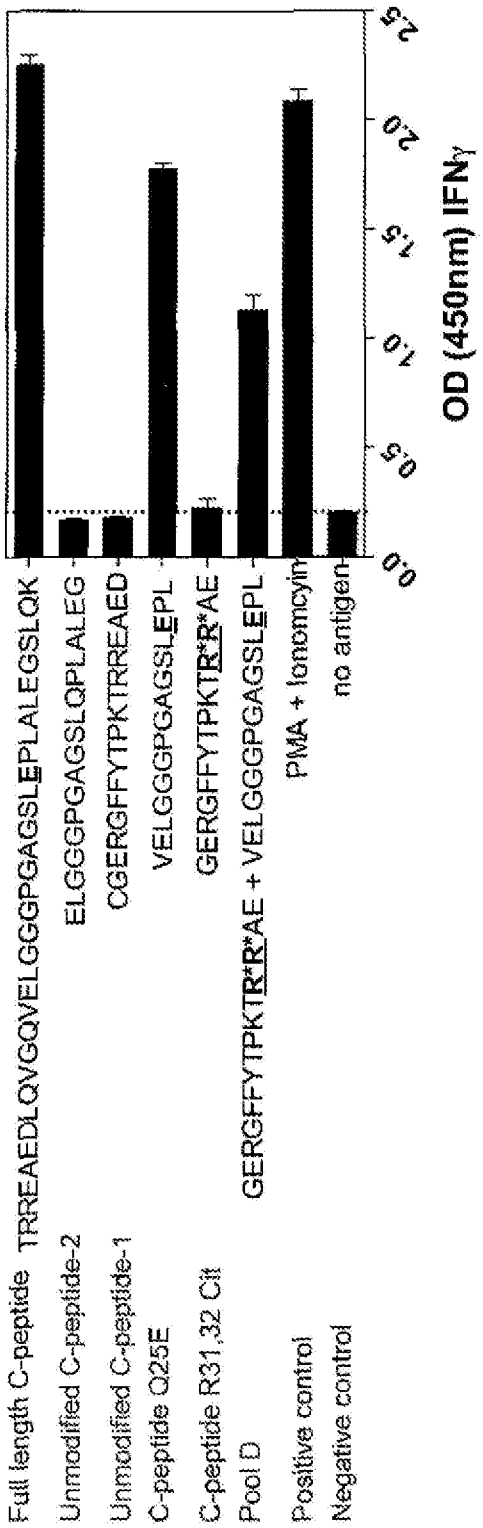

CD4 A 2.11
Full length C-peptide TRREAEDLQVGQVELGGGPGAGSLEPLALEGSLQK
Unmodified C-peptide-2 ELGGGPGAGSLQPLALEG
Unmodified C-peptide-1 CGERGFFYTPKTRREAED
C-peptide Q25E VELGGGPGAGSLEPL
C-peptide R31,32 Cit GERGFFYTPKTRR*AE
Pool D GERGFFYTPKTRR*AE + VELGGGPGAGSLEPL
Positive control PMA + Ionomcyin
Negative control no antigen
0.0
0.5
1.0
1.5
2.0
2.5
OD (450nm) IFNγ

Figure 6

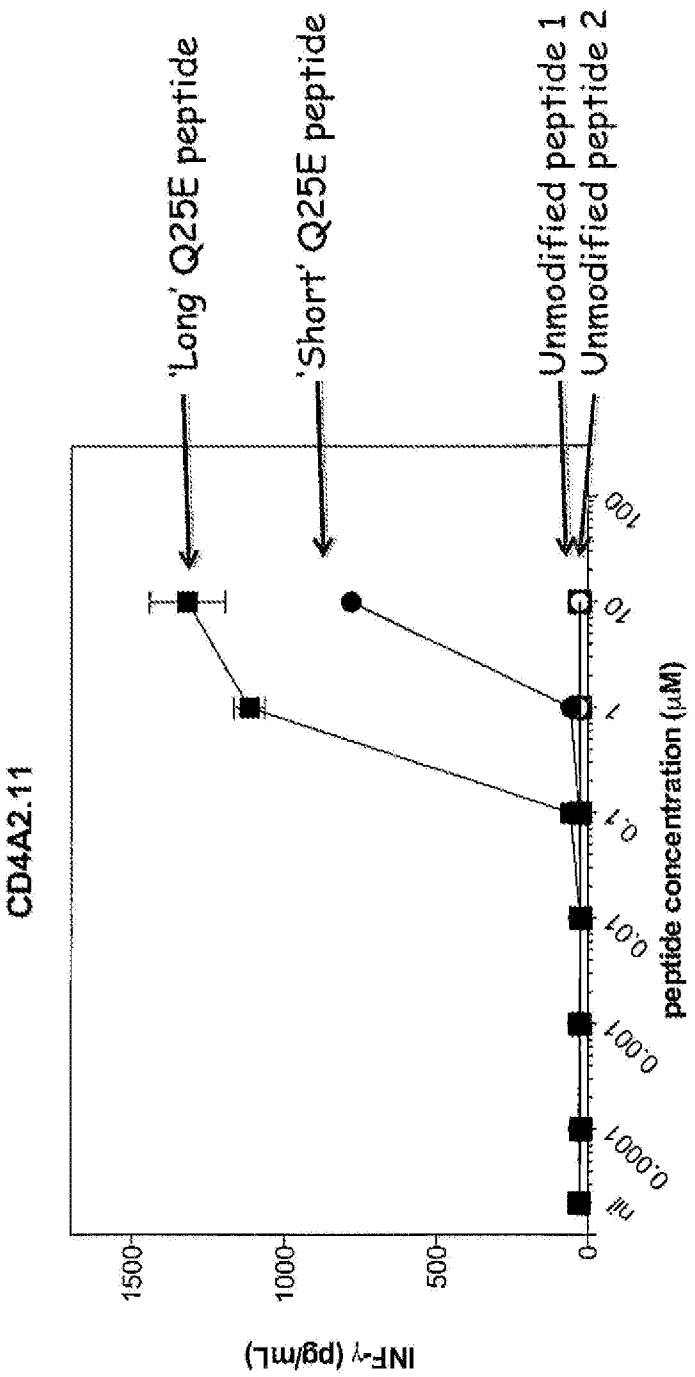

'Long' Q25E peptide
'Short' Q25E peptide
Unmodified peptide 1
Unmodified peptide 2
CD4A2.11
INF-γ (pg/mL)
1500
1000
500
0
peptide concentration (μM)
100
10
1
0.1
0.01
0.001
0.0001
nil

Figure 7

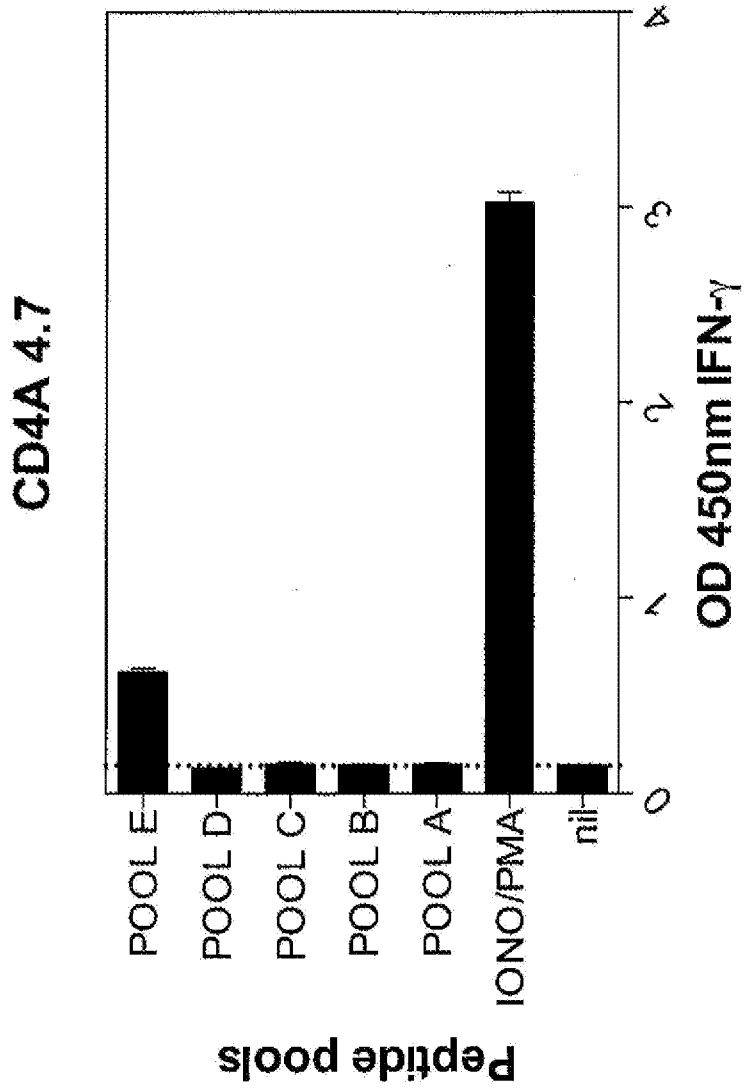

CD4A 4.7
OD 450nm IFN-γ
0
1
2
3
4
Peptide pools
POOL E
POOL D
POOL C
POOL B
POOL A
IONO/PMA
nil

Figure 8

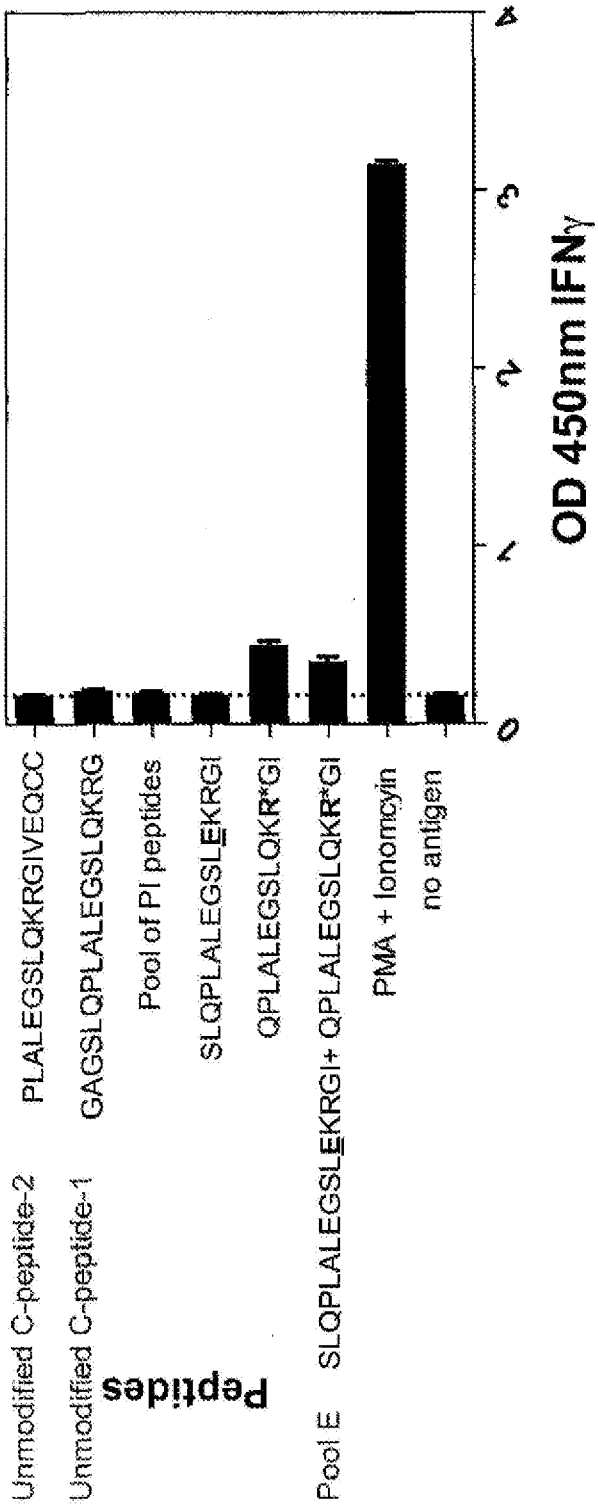

OD 450nm IFNγ
0
1
2
3
4
Peptides
Unmodified C-peptide-2
Unmodified C-peptide-1
Pool E
PLALEGSLQKRGIVEQCC
GAGSLQPLALEGSLQKRG
Pool of PI peptides
SLQPLALEGSLEKRGI
QPLALEGSLQKR*GI
SLQPLALEGSLEKRGI+ QPLALEGSLQKR*GI
PMA + Ionomoyin
no antigen

Figure 9

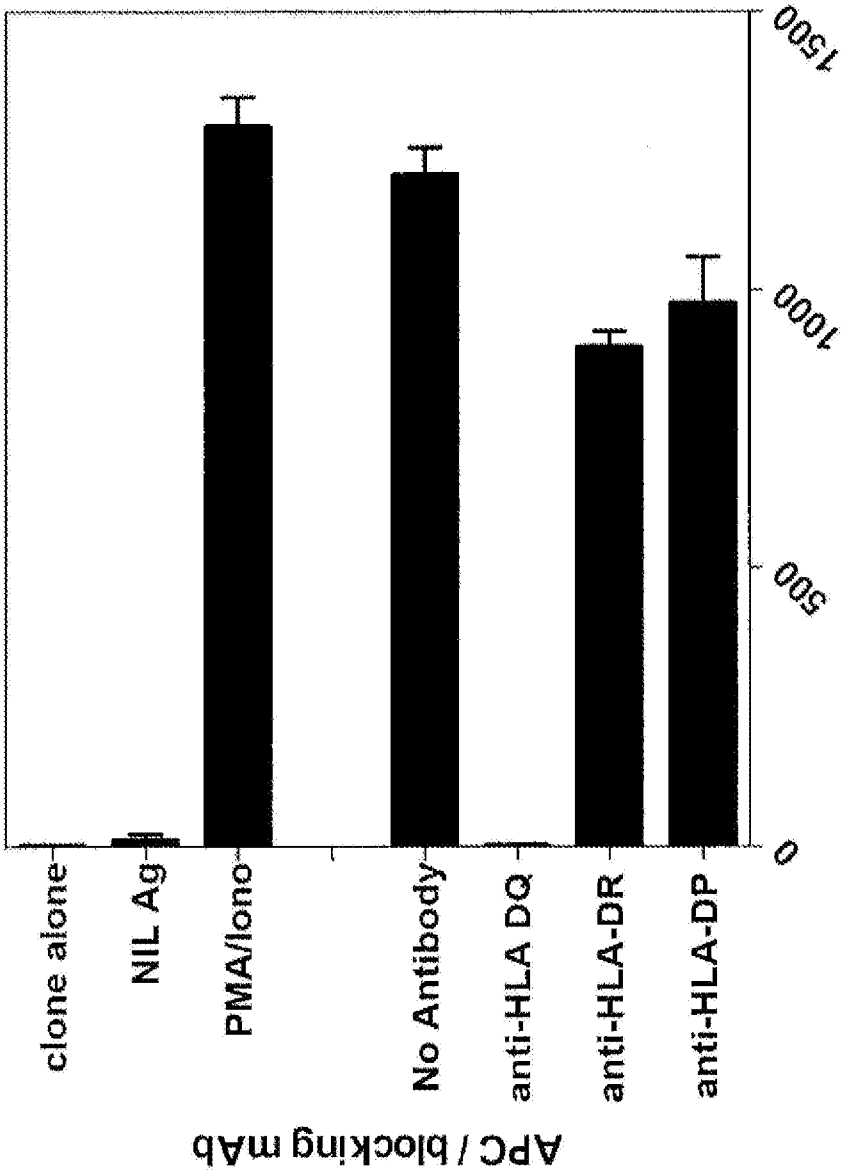

1500
1000
500
0
INF-γ pg/mL
clone alone
NIL Ag
PMA/Iono
No Antibody
anti-HLA DQ
anti-HLA-DR
anti-HLA-DP
APC / blocking mAb

Figure 10

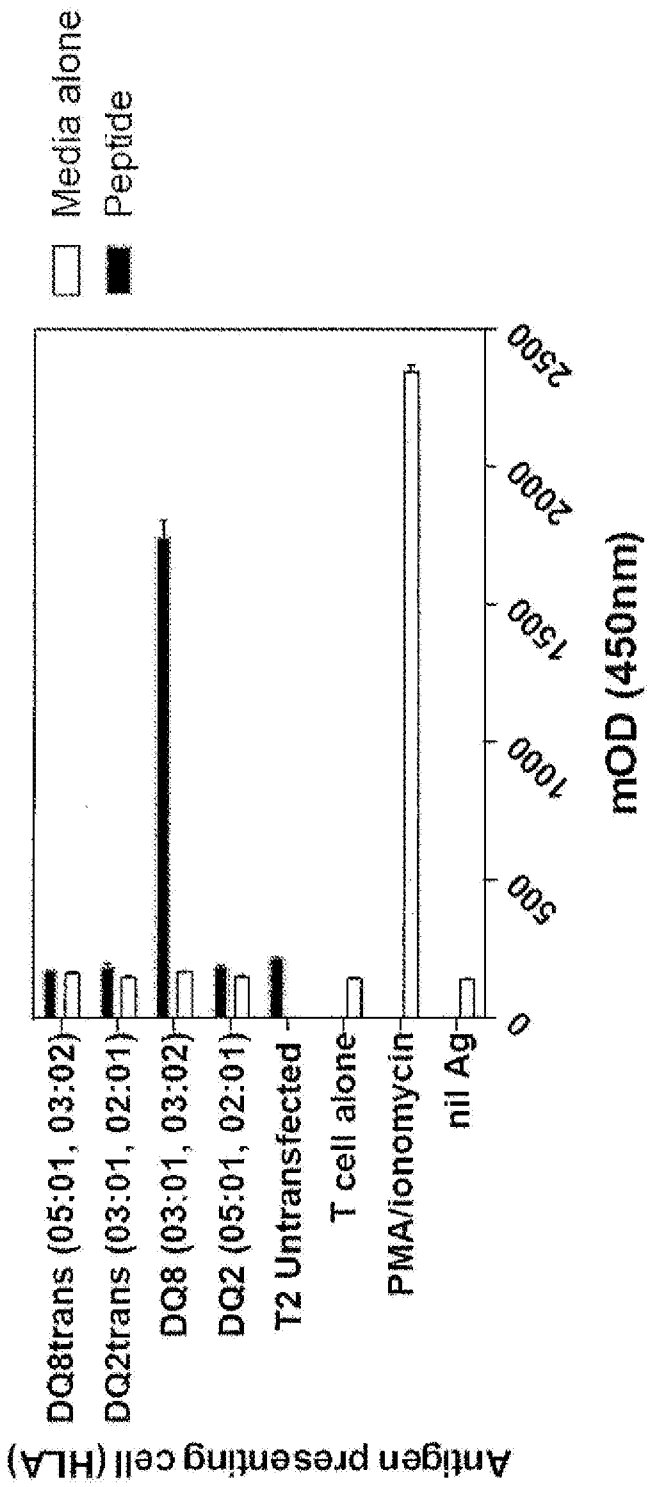
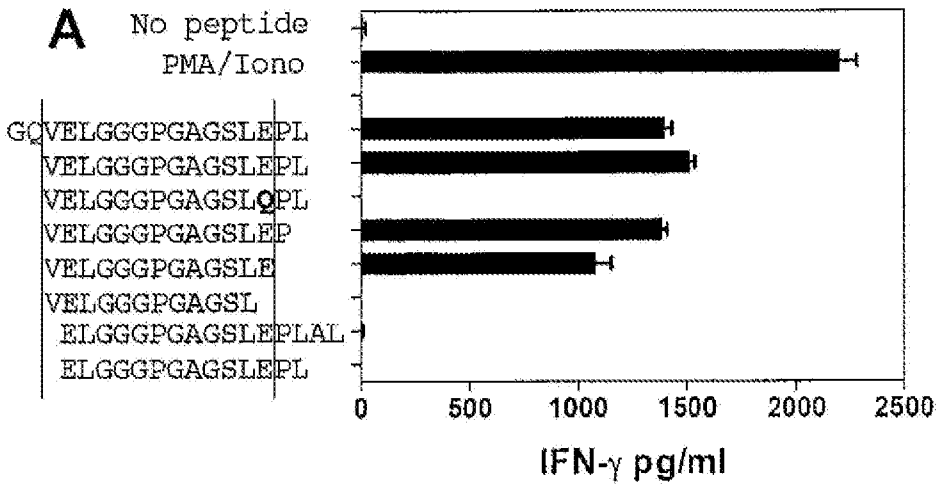

Media alone
Peptide
Antigen presenting cell (HLA)
DQ8trans (05:01, 03:02)
DQ2trans (03:01, 02:01)
DQ8 (03:01, 03:02)
DQ2 (05:01, 02:01)
T2 Untransfected
T cell alone
PMA/ionomycin
nil Ag
0
500
1000
1500
2000
2500
mOD (450nm)

Figure 11


A
No peptide
PMA/Iono
GQVELGGGPGAGSLEPL
VELGGGPGAGSLEPL
VELGGGPGAGSLQPL
VELGGGPGAGSLEP
VELGGGPGAGSLE
VELGGGPGAGSL
ELGGGPGAGSLEPLAL
ELGGGPGAGSLEPL
0
500
1000
1500
2000
2500
IFN-γ pg/ml

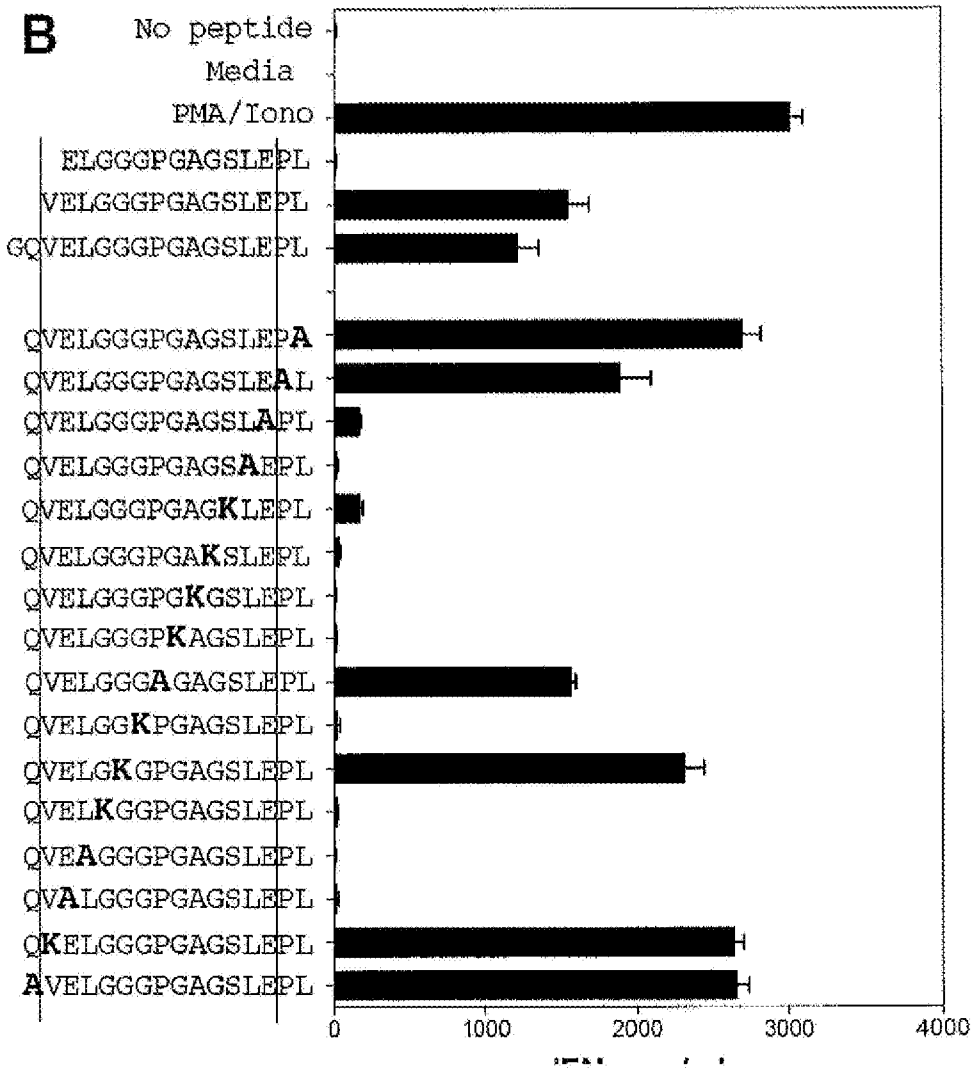

B
No peptide
Media
PMA/Iono
ELGGGPGAGSLEPL
VELGGGPGAGSLEPL
GQVELGGGPGAGSLEPL
QVELGGGPGAGSLEPA
QVELGGGPGAGSLEAL
QVELGGGPGAGSLAPL
QVELGGGPGAGSAEPL
QVELGGGPGAGKLEPL
QVELGGGPGAKSLEPL
QVELGGGPGKGSLEPL
QVELGGGPKAGSLEPL
QVELGGGAGAGSLEPL
QVELGGKPGAGSLEPL
QVELGKGPGAGSLEPL
QVELKGGPGAGSLEPL
QVEAGGGPGAGSLEPL
QVALGGGPGAGSLEPL
QKELGGGPGAGSLEPL
AVELGGGPGAGSLEPL
0
1000
2000
3000
4000

Figure 12

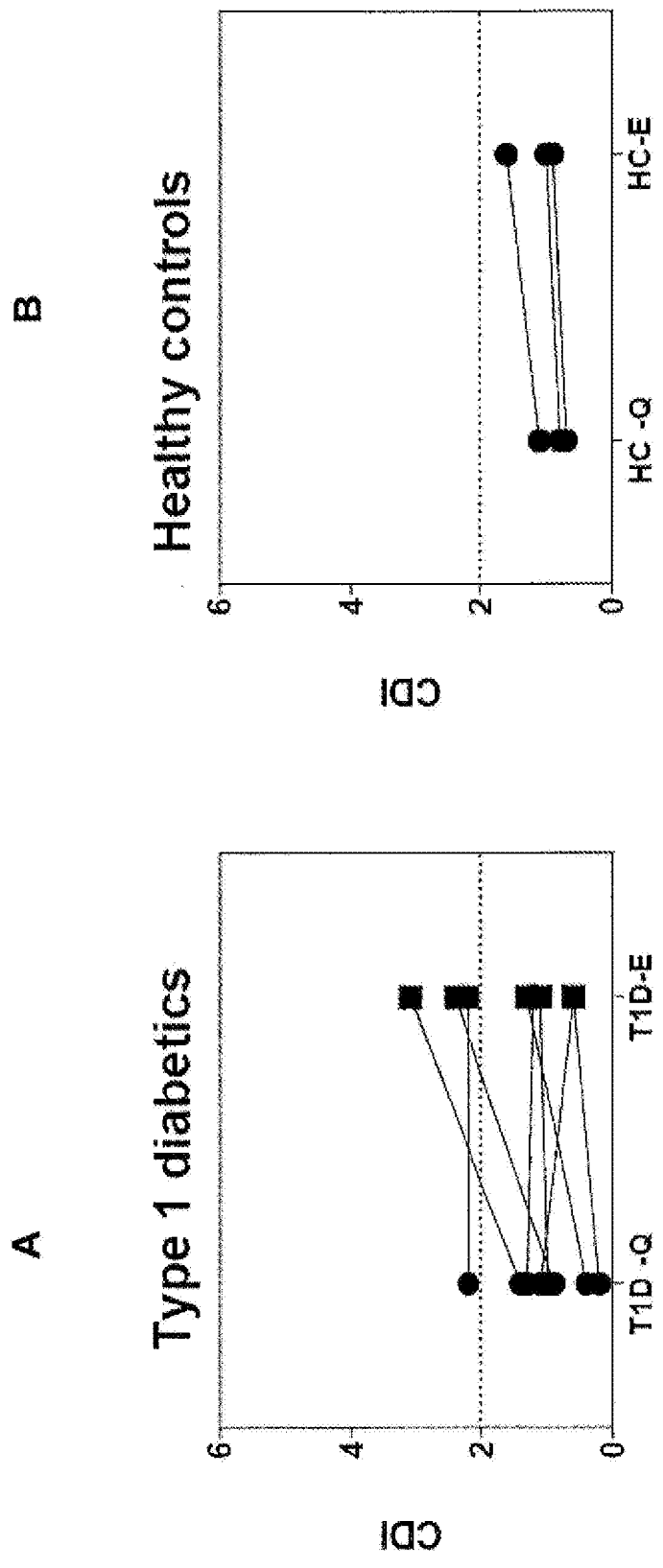

A
Type 1 diabetics
CDI
6
4
2
0
T1D -Q
T1D-E
B
Healthy controls
CDI
6
4
2
0
HC -Q
HC-E

Figure 13

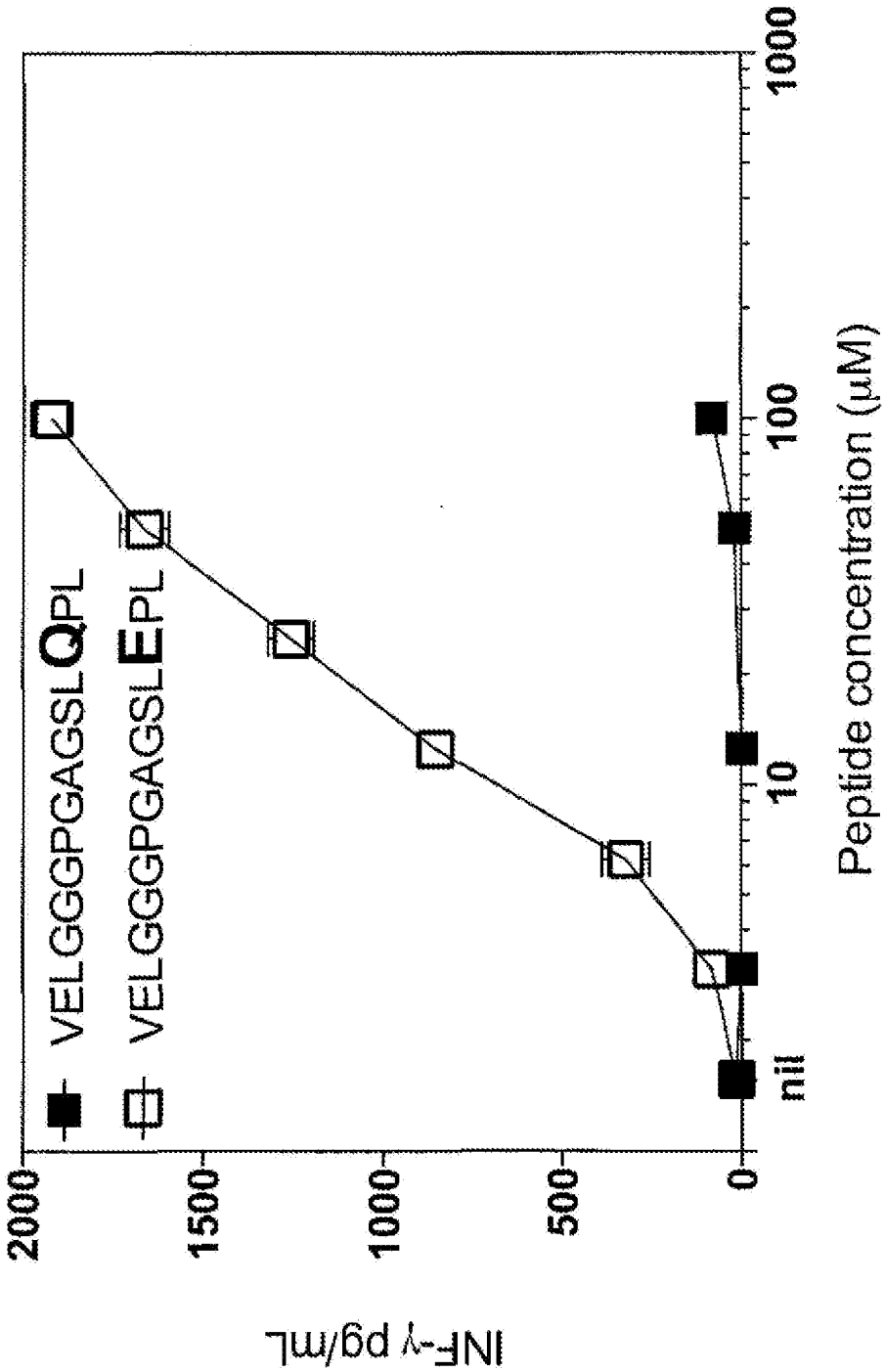

INF-γ pg/mL
2000
1500
1000
500
0
nil
10
100
1000
Peptide concentration (μM)
VELGGGPGAGSLQPL
VELGGGPGAGSLEPL

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050136

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/28 (2006.01) A61K 39/00 (2006.01) A61P 3/10 (2006.01) C12N 5/0783 (2010.01) G01N 33/53 (2006.01) G01N 33/564 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, and MEDLINE: Keywords: insulin, epitope, regulatory, Treg, CD25, tolerance, glutamine, glutamic acid, and related terms.

Espacenet and PubMed: Applicant and Inventor name search.

GenomeQuest: SEQ ID NOs: 52-58, 94, 96, 98, 99, 101-107, and 109-121.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 2014	15 September 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Michael Amon AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832083

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2014/050136**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DURINOVIC-BELLO, I., *et al.*, "DRB1*0401-restricted human T cell clone specific for the major proinsulin 73-90 epitope expresses a down-regulatory T helper 2 phenotype," Proceedings of the National Academy of Sciences of the United States of America, 2006, Vol. 103, No. 31, pages 11683-11688. See, in particular: Abstract; page 11685, 'Influence of Flanking Amino Acids on Activation of the Clones', 2nd paragraph; page 11687, 'Mouse T Cell Hybridoma' section ; and Figures 2a and 3.	15, 17-19, 21-26, 28, 29, and 31
A	WO 2001/030378 A1 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH) 03 May 2001 See, in particular: Abstract; page 5, lines 6-20; and Examples.	1-56
A	EVERY, A. L., *et al.*, "Intranasal Vaccination with Proinsulin DNA Induces Regulatory $CD4^+$ T Cells That Prevent Experimental Autoimmune Diabetes," The Journal of Immunology, 2006, Vol. 176, pages 4608-4615. See, in particular: Abstract and page 4608, paragraph 1.	1-56
L	WO 2013/096386 A1 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION) 27 June 2013 Lack of Unity	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050136

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050136

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-56 as they relate to the invention defined by Group 5.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features, they define different inventions.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the claims is a peptide comprising an epitope derived from insulin, proinsulin or preproinsulin wherein the amino acid sequence comprises a glutamine to glutamic acid substitution. However, this feature is not novel in light of:

D4: WO 2013/096386 A1 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION) 27 June 2013

D4 discloses single chain insulin analogs for the treatment of diabetes such as SEQ ID NO: 85 (also identified as DP32 in D4) which comprises a sequence of at least seven consecutive amino acid residues identified within the present application as SEQ ID NO:5 (Abstract; and page 43, lines 6-20). D4, Example 19 on pages 183-185 further discloses that SEQ ID NO:85 has a glutamic acid (E) substitution in place of the glutamine residue(Q) present in either of SEQ ID NOs: 83 or 84.

This means that the common feature can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature, it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore, the claims do not satisfy the requirement of unity of invention *a posteriori*.

This International Searching Authority has therefore found that there are multiple different inventions. As the identified general concept is not novel, each glutamine to glutamic acid substitution across insulin is considered to be a special technical feature and therefore each glutamine to glutamic acid substitution across insulin represents a separate invention.

Group 1: SEQ ID NOs: 2-5 (all in full)

Group 2: SEQ ID NOs: 32, 35-37, 40, 43, and 46 (all in full)

Group 3: SEQ ID NOs: 38, 41, 44, 47, 49-51 (all in full)

Group 4: SEQ ID NOs: 39, 42, 45, and 48 (all in full)

Group 5: SEQ ID NOs: 52-58, 94, 96, 98, 99, 101-107 and 109-121 (all in full)

Group 6: SEQ ID NOs: 59-61, 64, 67, 70, and 73 (all in full)

Group 7: SEQ ID NOs: 76, 79, and 82-86 (all in full)

Group 8: SEQ ID NOs: 87-93 (all in full)

It is considered that search and examination for each additional invention will require more than negligible additional search and examination effort over that of the main invention. As there are 8 points within the insulin peptide wherein a peptide of at least 7 amino acids in length and having a glutamine to glutamic acid substitution may be derived, a total of 8 such searches are required to search all of the inventions.

For the fee already paid, the invention defined by Group 5 was searched.

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU2014/050136

Supplemental Box

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2014/050136**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2001/030378 A1	03 May 2001	AU 767688 B2	20 Nov 2003
		AU 1115301 A	08 May 2001
		CA 2387652 A1	03 May 2001
		EP 1225912 A1	31 Jul 2002
		JP 2003512435 A	02 Apr 2003
WO 2013/096386 A1	27 June 2013	None	

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.