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(54) **Title**: METHODS FOR THE TREATMENT AND DIAGNOSIS OF TYPE 1 DIABETES

(57) **Abstract**: The present invention relates to methods for the treatment and diagnosis of Type 1 Diabetes. In particular the present invention relates to a human preproinsulin peptide selected from the group consisting of: - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15") - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30") - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35") - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40") - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97") - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110").

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METHODS FOR THE TREATMENT AND DIAGNOSIS OF TYPE 1 DIABETES

FIELD OF THE INVENTION:

The present invention relates to methods for the treatment and diagnosis of Type 1 Diabetes.

BACKGROUND OF THE INVENTION:

Type 1 Diabetes (T1DM) is a major health problem due to onset before the age of 18 in 50% of patients, an increasing incidence in all countries that have been scrutinized, the absence of treatment other than lifelong insulin replacement therapy, the high vascular burden relating with residual hyperglycaemia in insulin-treated patients and a major social impact. Despite increasing knowledge of T1D pathogenesis, mostly based on studies in the NOD mouse [1], efforts to apply immunotherapy [2] have been unsuccessful when translated into clinical trials. In addition, major differences have been reported between the diabetes phenotype seen in animal models and the human disease [3]. In the human, immunosuppression has shown limited efficacy in preserving β-cells from destruction in recent-onset T1D patients, but significant side effects, precluding their use in the long-term range [4]. Therapeutic approaches aiming at restoring immune tolerance to β-cells should thus be prioritized as allowing the specific blockade of self-reactive lymphocytes without impairing immune responses to unrelated antigens, especially infectious or tumoral antigens. This may prove of outmost importance considering that insulin replacement therapy has gained in efficacy and safety over years, explaining a progressively decreasing mortality gap with the general population [5]. Antigen and peptide-specific immunotherapy are likely to allow optimal risk/benefit ratios. However, defining antigen or peptide-specific therapies to prevent T1DM are still needed in particular due to a lack of new T1DM preclinical models [6].

SUMMARY OF THE INVENTION:

The present invention relates to methods for the treatment and diagnosis of Type 1 Diabetes. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to human preproinsulin peptide for use in a method for treating Type 1 Diabetes in a subject in need thereof.

As used herein, the term “insulin-dependent diabetes mellitus," "IDDM," "type I diabetes mellitus," and "T1DM," refer to diseases characterized by the autoimmune destruction of the β cells in the pancreatic islets of Langerhans.

In some embodiments, the subject was early diagnosed with T1DM. When used herein, the expression "early diagnosis of T1DM" or "early diagnosed" T1DM refers to the patient in whom T1DM has either been recently or newly diagnosed, e.g. wherein the patient has been diagnosed with T1DM within about 3 months of initial treatment, and/or wherein the patient's T1DM is in early stages or is not advanced, e.g. wherein the patient is determined to have functioning beta cells, for instance as determined by a blood test such as C-peptide in which a detectable level of C-peptide (e.g. > 0.03 nMol/L either fasting or stimulated by a caloric load such as a mixed-meal tolerance test (MMTT) indicates the presence of functioning beta cells and a MMTT-stimulated C-peptide level >0.2 nMol/L indicates a clinically meaningful level of beta cell function.

As used herein, the term “treatment” or “treating a subject” is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease. Treatment can slow, cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. For example, treatment of a subject, e.g., a human subject, with a composition described herein, can slow, improve, or stop the ongoing autoimmunity, e.g., a reaction against pancreatic β-cells, in a subject before, during, or after the clinical onset of type 1 diabetes. Therefore the method of the invention can prevent diabetes mellitus, or prevent or delay loss of residual β-cell mass, providing a longer remission period reducing short term complications and/or delaying the onset of diabetes-related complications at a later stage of the life. The onset of T1DM may be delayed by the method as described herein such that insulin is not needed by the subject for a longer length of time. Alternatively or in addition, the present method may extend the “honeymoon phase” in an already diabetic subject. The honeymoon

phase is where insulin is secreted by the pancreas, causing high blood sugar levels to subside, and resulting in normal or near normal glucose levels due to responses to insulin injections and treatment. The method of the present invention is also used to arrest the autoimmune destruction of tissue, e.g., pancreatic β-cells. The method of the present invention is suitable to arrest the autoimmune destruction, even at a late stage at the time of clinical onset of type 1 diabetes mellitus or after clinical onset. For example, at the time of clinical onset of type 1 diabetes mellitus, significant number of insulin producing β-cells are destroyed. If the autoimmune process can be arrested even in this late stage or as far as residual secretion can be restored, these cells can be preserved. The β-cells have some limited capacity to replicate and precursors may form new β-cells. The phrase "delaying the progression" as used herein in the context of delaying the progression of diabetes mellitus means that the loss of functional residual β-cell mass, after the clinical onset of Type 1 diabetes is delayed. The delayed progression of T1DM can be measured, for example, by measuring C-peptide production.

As used herein the term "preproinsulin" has its general meaning in the art. The insulin mRNA is translated as a 110 amino acid single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin which consists of the A and B chain. Insulin and free C peptide are packaged in the Golgi into secretory granules which accumulate in the cytoplasm. An exemplary amino acid sequence for the human preproinsulin polypeptide is SEQ ID NO:1.

SEQ ID NO:1 (Homo sapiens)
MALWMRLLPL LALLALWGPD PAAAFVNQHL CGSHLVEALY LVCGERGFFY TPKTRREAED
LQVGQVELGG GPGAGSLQPL ALEGSLQKRG IVEQCCTSIC SLYQLENYCN

According to the invention the human preproinsulin peptide is a HLA-DQ8-restricted hPPI epitope selected from the group consisting of:

- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")

- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")
- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")
- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")
- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")
- a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110")

In some embodiments, the human preproinsulin peptide of the present invention comprises 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, or 35 amino acids.

In some embodiments, the human preproinsulin peptide of the present invention is selected from the group consisting of

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")
- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")
- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")
- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")
- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110").

According to the invention a first amino acid sequence having at least 90% of identity with a second amino acid sequence means that the first sequence has 90; 91; 92; 93; 94; 95; 96; 97; 98; 99; or 100% of identity with the second amino acid sequence. Amino acid sequence identity is typically determined using a suitable sequence alignment algorithm and default parameters, such as BLAST P (Karlin and Altschul, 1990).

In some embodiments, the human preproinsulin peptide of the present invention is selected from the group consisting of

- a peptide consisting of the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")
- a peptide consisting of the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")
- a peptide consisting of the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")
- a peptide consisting of the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")
- a peptide consisting of the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")
- a peptide consisting of the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110").

In some embodiments, the human preproinsulin peptide of the present invention consists of the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 30 in SEQ ID NO:1.

In some embodiments, the human preproinsulin peptide of the present invention is fused to a heterologous polypeptide to form a fusion protein. As used herein, a "fusion protein"

comprises all or part (typically biologically active) of a human preproinsulin peptide of the present invention operably linked to a heterologous polypeptide (i.e., a polypeptide other than the human preproinsulin). Within the fusion protein, the term "operably linked" is intended to indicate that the human preproinsulin peptide of the present invention and the heterologous polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C-terminus of the human preproinsulin peptide of the present invention.

In some embodiments, the preproinsulin peptide of the present invention is fused either directly or via a linker to the heterologous polypeptide. As used herein, the term "directly" means that the (first or last) amino acid at the terminal end (N or C-terminal end) of the human preproinsulin polypeptide of the present invention is fused to the (first or last) amino acid at the terminal end (N or C-terminal end) of heterologous polypeptide. As used herein, the term "linker" refers to a sequence of at least one amino acid that links the human preproinsulin peptide of the present invention with the heterologous polypeptide. Linker are well known to one of ordinary skill in the art and typically comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acids.

In some embodiments, the heterologous polypeptide comprises at least one redox motif C—(X)2-[CST] or [CST]-(X)2-C. In some embodiments, the C—(X)2-[CST] or [CST]-(X)2-C motif is positioned N-terminally of the human preproinsulin peptide of the present invention. In some embodiments, the fusion protein of the invention contains the sequence motif C—X(2)-[CS] or [CS]—X(2)-C. In some embodiments the fusion protein of the invention contain the sequence motif C—X(2)-S, S—X(2)-C or C—X(2)-C. C—(X)2-[CST] or [CST]-(X)2-C motif.

As used herein, the symbol X is used for a position where any amino acid is accepted. Alternatives are indicated by listing the acceptable amino acids for a given position, between square brackets (‘[ ]’). For example: [CST] stands for an amino acid selected from Cys, Ser or Thr. The different elements in a motif are separated from each other by a hyphen “-“. Repetition of an identical element within a motif can be indicated by placing behind that element a numerical value or a numerical range between parentheses. For example: X(2) corresponds to X—X, X(2, 4) corresponds to X—X or X—X—X or X—X—X—X , A(3) corresponds to A-A-A.

In some embodiments, C represents either cysteine or another amino acids with a thiol group such as mercaptovaline, homocysteine or other natural or non-natural amino acids with a thiol function. In order to have reducing activity, the cysteines present in the redox motif should not occur as part of a cystine disulfide bridge. Nevertheless, the redox motif may comprise modified cysteines such as methylated cysteine, which is converted into cysteine with free thiol groups in vivo.

In some embodiments, each of the amino acids X in the C—(X)2-[CST] or [CST]-(X)2-C motif can be any natural amino acid, including S, C, or T or can be a non-natural amino acid, whereby the two amino acids X are either the same or different. In some embodiments X is an amino acid with a small side chain such as Gly, Ala, Ser or Thr. In some embodiments, X is not an amino acid with a bulky side chain such as Tyr. In some embodiments at least one X in the [CST]-X(2)-[CST] motif is His or Pro.

In some embodiments, the redox motif is placed either immediately adjacent to the human preproinsulin peptide sequence within the fusion protein, or is separated from the human preproinsulin peptide by a linker as defined herein. In some embodiments, the linker comprises an amino acid sequence of 7 amino acids or less. In some embodiments, the linker comprises 1, 2, 3, or 4 amino acids. In some embodiments, a linker may comprise 6, 8 or 10 amino acids. Typical aminoacids used in linkers are serine and threonine. Example of peptides with linkers in accordance with the present invention are CXXC-G-human preproinsulin peptide, CXXC-GG- human preproinsulin peptide, CXXC—SSS-e human preproinsulin peptide, CXXC—SGSG- human preproinsulin peptide and the like.

In some embodiments, the redox motif occurs several times (1, 2, 3, 4 or even more times) in the fusion protein, for example as repeats of the redox motif which can be spaced from each other by one or more amino acids (e.g. CXXC X CXXC X CXXC), as repeats which are adjacent to each other (e.g. CXXC CXXC CXXC) or as repeats which overlap with each other (e.g. CXXCXXCXXC or CXCCXCCXCC). In some embodiments, one or more motifs are provided at both the N and the C terminus of the human preproinsulin peptide of the present invention. Other variations envisaged for the fusion proteins of the present invention include fusion proteins containing repeats of a human preproinsulin peptide of the present invention wherein each peptide is preceded and/or followed by the redox motif (e.g. repeats of “motif-

peptide" or repeats of "motif-peptide-motif"). Herein the redox motifs can all have the same sequence but this is not obligatory.

In some embodiments, the fusion protein of the invention further comprises an amino acid sequence facilitating uptake of the peptide into (late) endosomes for processing and presentation. The late endosome targeting is mediated by signals present in the cytoplasmic tail of proteins and correspond to well-identified peptide motifs such as the dileucine-based [DE]XXXL[LI] or DXXLL motif (e.g. DXXXLL), the tyrosine-based YXXØ motif or the so-called acidic cluster motif. The symbol Ø represents amino acid residues with a bulky hydrophobic side chains such as Phe, Tyr and Trp. The late endosome targeting sequences allow for processing and efficient presentation of the human preproinsulin peptide of the present invention by antigen presenting cells (APC). Such endosomal targeting sequences are contained, for example, within the gp75 protein (Vijayasaradhi et al. (1995) J Cell Biol 130, 807-820), the human CD3 gamma protein, the HLA-DM β (Copier et al. (1996) J. Immunol. 157, 1017-1027), the cytoplasmic tail of the DEC205 receptor (Mahnke et al. (2000) J Cell Biol 151, 673-683). Other examples of peptides which function as sorting signals to the endosome are disclosed in the review of Bonifacio and Traub (2003) Annu. Rev. Biochem. 72, 395-447. In some embodiments, the sequence can be that of a subdominant or minor T cell epitope from a protein, which facilitates uptake in late endosome without overcoming the T cell response towards the alloantigen-derived T cell epitope.

In some embodiments, the fusion protein of the present is a fusion protein as described in WO2008035217. Accordingly, in some embodiments, the fusion protein of the present invention comprises:

- an IgG binding moiety consisting of two or more IgG binding domains of streptococcal protein G placed in a tandem arrangement and optionally separated by a peptide linker, and
- a cargo moiety comprising the human preproinsulin of the present invention.

In some embodiments, the IgG binding moiety of a fusion protein of the invention comprises three IgG binding domains of streptococcal protein G. In some embodiments, it comprises four of five IgG binding domains of streptococcal protein G. The IgG binding domains of streptococcal protein G may be identical or different. Any combination of these two or more domains among the Bl, B2, and B3 domains, in any order, can be used. Typical combinations include Bl-Bl, Bl-Bl-Bl, Bl- B2, B1-B2-B1, B2-B1, B2-B1-B2, B2-B2 and B2-B2-B2. In some

enbodiments, the human preproinsulin peptide of the present invention is also fused to an ubiquitin domain to its N-terminal end, in order to enhance its degradation in the proteasome-dependent MHC class I antigen processing pathway. If one uses naturally occurring ubiquitin, having a glycine at position 76, and lysines at positions 29 and 48, the presence of an N-terminal ubiquitin molecule may, upon protein translocation into the cytosol, enhance degradation of the polypeptide of interest by cellular proteasome complexes. This enhancing effect on degradation will not occur if one uses an ubiquitin variant wherein both the lysines at positions 29 and 48 are substituted by other amino-acid residues, e.g. arginine or alanine; such an ubiquitin variant may however play a part in the stabilization of the fusion protein. Conversely, non cleavable ubiquitin-antigen junctions, obtained by replacing the C-terminal glycine of the ubiquitin sequence by another amino-acid residue (such as valine or cysteine), can be used if the objective is to induce more potent CTL responses, using the pathway of "ubiquitin fusion degradation".

The peptides and fusion proteins of the invention may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination. Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said polypeptides, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions. Alternatively, the polypeptides and fusions proteins of the invention can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired (poly) peptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

In some embodiments, the human preproinsulin peptide of the present invention is fused or conjugated to an antibody for forming an "immunoconjugate".

As used herein, the term "antibody" is thus used to refer to any antibody-like molecule that has an antigen binding region, and this term includes antibody fragments that comprise an antigen binding domain such as Fab', Fab, F(ab')2, single domain antibodies (DABs or VHH), TandAbs dimer, Fv, scFv (single chain Fv), dsFv, ds-scFv, Fd, linear antibodies, minibodies,

diabodies, bispecific antibody fragments, bibody, tribody (scFv-Fab fusions, bispecific or trispecific, respectively); sc-diabody; kappa(lamda) bodies (scFv-CL fusions); DVD-Ig (dual variable domain antibody, bispecific format); SIP (small immunoprotein, a kind of minibody); SMIP ("small modular immunopharmaceutical" scFv-Fc dimer; DART (ds-stabilized diabody "Dual Affinity ReTargeting"); small antibody mimetics comprising one or more CDRs and the like. In some embodiments, the antibody is a chimeric antibody, a humanized antibody or a human antibody. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')2 fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation. Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab') 2 Fab, Fv and Fd fragments. Antibodies can be indeed fragmented using conventional techniques. For example, F(ab')2 fragments can be generated by treating the antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')2, scFv, Fv, dsFv, Fd, dAbs, TandAbs, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques or can be chemically synthesized. Techniques for producing antibody fragments are well known and described in the art. For example, each of Beckman et al., 2006; Holliger & Hudson, 2005; Le Gall et al., 2004; Reff & Heard, 2001 ; Reiter et al., 1996; and Young et al., 1995 further describe and enable the production of effective antibody fragments. The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to

IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgGl, IgG2, IgG3 and IgG4. The present invention also includes so-called single chain antibodies. The term “single domain antibody” (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called “nanobody®”. According to the invention, sdAb can particularly be llama sdAb.

Typically, the antibody is directed against a surface antigen of an antigen presenting cell so that the human preproinsulin peptide of the present invention is targeted to said cell to elicit an immune response (e.g. tolerance). As used herein the term "Antigen Presenting Cells" (APC) are cells that are capable of activating T- cells, and include, but are not limited to, certain macrophages, B cells and dendritic cells. In some embodiments, the antibody is directed against a surface antigen of a dendritic cell. "Dendritic cells" (DCs) refer to any member of a diverse population of morphologically similar cell types found in lymphoid or non-lymphoid tissues. These cells are characterized by their distinctive morphology, high levels of surface MHC-class II expression (Steinman, et al., Ann. Rev. Immunol. 9:271 (1991); incorporated herein by reference for its description of such cells). These cells can be isolated from a number of tissue sources, and conveniently, from peripheral blood, as described herein. Accordingly, the antibody is selected from an antibody that specifically binds to dendritic cell immunoreceptor (DCIR), MHC class I, MHC class II, CD1, CD2, CD3, CD4, CD8, CDl lb, CD14, CD15, CD16, CD19, CD20, CD29, CD31, CD40, CD43, CD44, CD45, CD54, CD56, CD57, CD58, CD83, CD86, CMRF-44, CMRF-56, DCIR, DC-ASPGR, CLEC-6, CD40, BDCA-2, MARCO, DEC-205, mannose receptor, Langerin, DECTIN-1, B7-1, B7-2, IFN-γ receptor and IL-2 receptor, ICAM-1, Fey receptor, LOX-1, and ASPGR. In some embodiments, the antibody is specific for a cell surface marker of a professional antigen presenting cell. Preferably, the antibody is specific for a cell surface marker of a dendritic cell, for example, CD83, CMRF-44 or CMRF-56. The antibody may be specific for a cell surface marker of another professional antigen presenting cell, such as a B cell or a macrophage. CD40 is expressed on both dendritic cells, B cells, and other antigen presenting cells so that a larger number of antigen presenting cells would be recruited.

Techniques for conjugating molecule to antibodies, are well-known in the art (See, e.g., Arnon et al., “Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy,” in Monoclonal Antibodies And Cancer Therapy (Reisfeld et al. eds., Alan R. Liss, Inc., 1985);

Hellstrom et al., "Antibodies For Drug Delivery," in Controlled Drug Delivery (Robinson et al. eds., Marcel Deiker, Inc., 2nd ed. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications (Pinchera et al. eds., 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody In Cancer Therapy," in Monoclonal Antibodies For Cancer Detection And Therapy (Baldwin et al. eds., Academic Press, 1985); and Thorpe et al., 1982, Immunol. Rev. 62:119-58. See also, e.g., PCT publication WO 89/12624.) Typically, the nucleic acid molecule is covalently attached to lysines or cysteines on the antibody, through N-hydroxysuccinimide ester or maleimide functionality respectively. Methods of conjugation using engineered cysteines or incorporation of unnatural amino acids have been reported to improve the homogeneity of the conjugate (Axup, J.Y., Bajjuri, K.M., Ritland, M., Hutchins, B.M., Kim, C.H., Kazane, S.A., Halder, R., Forsyth, J.S., Santidrian, A.F., Stafin, K., et al. (2012). Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. Proc. Natl. Acad. Sci. USA 109, 16101–16106.; Junutula, J.R., Flagella, K.M., Graham, R.A., Parsons, K.L., Ha, E., Raab, H., Bhakta, S., Nguyen, T., Dugger, D.L., Li, G., et al. (2010). Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. Clin. Cancer Res.16, 4769–4778.). Junutula et al. (2008) developed cysteine-based site-specific conjugation called ''THIOMABs'' (TDCs) that are claimed to display an improved therapeutic index as compared to conventional conjugation methods. Conjugation to unnatural amino acids that have been incorporated into the antibody is also being explored for ADCs; however, the generality of this approach is yet to be established (Axup et al., 2012). In particular the one skilled in the art can also envisage Fc-containing polypeptide engineered with an acyl donor glutamine-containing tag (e.g., Gin-containing peptide tags or Q- tags) or an endogenous glutamine that are made reactive by polypeptide engineering (e.g., via amino acid deletion, insertion, substitution, or mutation on the polypeptide). Then a transglutaminase, can covalently crosslink with an amine donor agent (e.g., a small molecule comprising or attached to a reactive amine) to form a stable and homogenous population of an engineered Fc-containing polypeptide conjugate with the amine donor agent being site- specifically conjugated to the Fc-containing polypeptide through the acyl donor glutamine- containing tag or the accessible/exposed/reactive endogenous glutamine (WO 2012059882).

The peptides, fusion proteins and immunoconjugates as described herein may be administered as part of one or more pharmaceutical compositions. The term "pharmaceutical

composition" refers to a composition described herein, or pharmaceutically acceptable salts thereof, with other agents such as carriers and/or excipients. The pharmaceutical compositions as provided herewith typically include a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical-Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional carrier medium is incompatible with the peptides of the present invention, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatine; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil, sesame oil; olive oil; corn oil and soybean oil; glycols; such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogenfree water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

In particular, the peptides, fusion proteins and immunoconjugates as described herein are particularly suitable for preparing vaccine composition. For the purpose of the present invention, the term "vaccine composition" is intended to mean a composition which can be administered to humans or to animals in order to induce an immune system response; this immune system response can result in the activation of certain cells, in particular antigen-presenting cells, T lymphocytes and B lymphocytes. As used herein, the vaccine composition of the present invention is thus particularly suitable for the treatment of T1DM.

Accordingly, in some embodiments, the vaccine composition of the present invention comprises an adjuvant. The term "adjuvant" can be a compound that lacks significant activity administered alone but can potentiate the activity of another therapeutic agent. In some embodiments, an adjuvant is selected from the group consisting of buffers, anti-microbial preserving agents, surfactants, antioxidants, tonic regulators, antiseptics, thickeners and viscosity improvers.

In some embodiments, the adjuvant is Incomplete Freund's adjuvant (IFA) or other oil based adjuvant is present between 30-70%, preferably between 40-60%, more preferably between 45-55% proportion weight by weight (w/w). In some embodiments, the active ingredient (i.e. peptides, fusions proteins or immunoconjugates) and IFA or other oil based adjuvant are present in about a 50/50 weight by weight ratio. In some embodiments, the pharmaceutical composition is free of contaminants, e.g., pyrogens. In some embodiments, the active ingredient (i.e. peptides, fusions proteins or immunoconjugates) is lyophilized. The oil-based carrier is a composition that includes at least 10% by weight of a natural or synthetic oil suitable for administration to a human in conjunction with a therapeutic agent is one preferred embodiment. In some embodiments, the carrier includes at least 20, 30, 50, 70, 80, 90, 95, 98, or 99% oil by weight. In some embodiments, the oil-based carrier can include less than 70, 60, 50, 40, or 20% oil by weight. In preferred embodiments, the oil will be in the range of 10 to 95%, preferably 20 to 90% or 30 to 70% oil by weight. The oil should be chosen such that it provides for sustained release of a substance dispersed within it when administered to a subject. Suitable oils include mineral oil (e.g., Drakeol 6 VR light mineral oil), vegetable oil, squalene, or liquid paraffin. In some embodiments, the oil-based carrier can contain more than one type of oil. In some embodiments, the oil-based carrier can include an immune stimulator, e.g., an immunostimulating glucan, but it is much preferred that the oil-based carrier does not include an immune stimulator, e.g., an immunostimulating glucan, a bacterial component, e.g., a mycobacterial component. In a preferred embodiment, the oil-based carrier does not include an alum component. Oil based carriers optionally include an emulsifier or surfactant component. The emulsifier or surfactant (and the amount of emulsifier or surfactant) is chosen such that it facilitates the mixture or dispersion of a substance, e.g., an antigen preparation, with the oil. An oil-based carrier can include 0.1 to 50%, preferably 1 to 30%, more preferably 5 to 20% by weight of a surfactant or emulsifier. Examples of emulsifiers or surfactants include Arlacel A, mannide oleate (e.g., Montanide 80-mannide monooleate), anhydrous mannitol/oleic acid ester, polyoxyethylene or polyoxypropylene. An oil-based carrier or adjuvant typically consists of

two components: (1) an oil, and (2) an emulsifier or surfactant, mixed with water. Suitable oils and emulsifiers are known in the art. For example, the oil can be mineral oil, vegetable oil, squalene or liquid paraffin. The emulsifier or surfactant can be, e.g., Arlacel A, mannide oleate, anhydrous mannitol/oleic acid ester, polyoxyethylene or polyoxypropylene. Exemplary oil-based adjuvants include conventional IFA, Montanide ISA adjuvants, or Hunter's TiterMax adjuvant. In preferred embodiments, the adjuvant includes 20 to 95%, preferably 30 to 90%, more preferably 40 to 70% by weight of an oil phase, and 0.1 to 50%, preferably 1 to 30%, more preferably 5 to 20% by weight of a surfactant or emulsifier. Various types of oil-based adjuvants are described, e.g., in U.S. Pat. No. 5,814,321, U.S. Pat. No. 6,299,884, U.S. Pat. No. 6,235,282, and U.S. Pat. No. 5,976,538.

Other typical adjuvants include those as described after. Montanide ISA Adjuvants (Seppic, Paris, France) are a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two. They are prepared for use as an emulsion with aqueous Ag solution. The surfactant for Montanide ISA 50 (ISA=Incomplete Seppic Adjuvant) is mannide oleate, a major component of the surfactant in Freund's adjuvants. The surfactants of the Montanide group undergo strict quality control to guard against contamination by any substances that could cause excessive inflammation, as has been found for some lots of Arlacel A used in Freund's adjuvant. The various Montanide ISA group of adjuvants are used as water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. The different adjuvants accommodate different aqueous phase/oil phase ratios, because of the variety of surfactant and oil combinations. Hunter's TiterMax (CytRx Corp., Norcross, Ga.) is an oil/surfactant-based adjuvant prepared as a water-in-oil emulsion in a manner similar to that used for conventional Freund's adjuvants. However, it uses a metabolizable oil (squalene) and a nonionic surfactant that has good protein antigen-binding capacity as well as adjuvant activity. The adjuvant activity may relate, in part, to the surfactant's ability to activate complement and bind complement components, as this helps target the Ag to follicular dendritic cells in the spleen and lymph nodes. The surfactant used in the commercially available adjuvant is one of a number of synthetic nonionic block copolymers of polyoxyethylene and polyoxypropylene developed by Hunter (Hunter et al., 1991 Vaccine 9:250-256). The utilization of copolymer-coated microparticles to stabilize the emulsion permits formation of stable emulsions with less than 20% oil, an important factor in minimizing total adjuvant injected.

In some embodiments, the vaccine composition of the present invention comprise at least one Toll-Like Receptor (TLR) agonist which is selected from the group consisting of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and TLR8 agonists.

The pharmaceutical compositions of the present invention can be delivered to a patient using a wide variety of routes or modes of administration. Suitable routes of administration include, but are not limited to, inhalation, transdermal, oral, rectal, transmucosal, intestinal and parenteral administration, including intramuscular, subcutaneous and intravenous injections.

The pharmaceutical compositions (e.g. vaccine composition) may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Injection (intravenous or subcutaneous) is a preferred method of administration for the compositions of the current invention. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions 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 may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. In some embodiments, pharmaceutical composition herein may be administered parenterally, by injection subcutaneously, or intramuscularly. In some embodiments, a preferred mode of administration is intramuscularly. For example, the Type 1 diabetes autoantigen as described herein can be given as an intramuscular injection, preferably a deep intramuscular injection, in a small volume, e.g., 1 ml. The autoantigen can be administered once, or more than once. It can be given, for example, before, or after the onset of Type 1 diabetes mellitus. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents, which increase the solubility of the compounds to allow for the preparation of highly, concentrated solutions. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's

solution, or physiological saline buffer. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

Typically, the active ingredient of the present invention (i.e. peptides, fusion proteins and immunoconjugates as described herein) is administered to the subject at a therapeutically effective amount. By a "therapeutically effective amount" is meant a sufficient amount of the active ingredient of the present invention to treat T1DM at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. In particular, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, in particular from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In some embodiments, the peptides, fusions proteins and immunoconjugates as herein described are used in combination with, for example, any known therapeutic agent or method for treating T1DM. Non-limiting examples of such known therapeutics for treating T1DM include insulin, insulin analogs, islet transplantation, stem cell therapy including PROCHYMAL®, non-insulin therapies such as IL-lbeta inhibitors including (Anakinra, Kineret®), Diamyd, alefacept (Ameviv®), anti-CD3 antibodies such as Otelixizumab, DiaPep277 (Hsp60 derived peptide), Alpha 1- Antitrypsin, Prednisone, azathioprine,

Ciclosporin, El-INT (an injectable islet neogenesis therapy comprising an epidermal growth factor analog and a gastrin analog), statins including Zocor®, Simlup®, Simcard®, Simvacor®, Sitagliptin (dipeptidyl peptidase (DPP-4) inhibitor), Anti-CD3 mAb (e.g., Teplizumab), Anti IL- lBeta (Canakinumab), Anti-CD20 mAb (e.g, rituximab). In some embodiments, the peptides, fusions proteins and immunoconjugates as herein described are used in combination with a GABA agonist. Illustrative GABA receptor agonists include, but are not limited to, certain barbiturates (e.g., thiopental, thiamylal, pentobarbital, secobarbital, hexobarbital, butobarbital, amobarbital, barbital, mephobarbital, phenobarbital, primidone, and the like), certain benzodiazepines (e.g., midazolam, triazolam, lometazepam, flutazolam, nitrazepam, fluritrazepam, nimetazepam, diazepam, medazepam, oxazolam, prazeam, tofisopam, rilmazafonoe, lorazepam, temazepam, oxazepam, fluidazepam, chlordizaepoxide, cloxazolam, flutoprazepam, alprazolam, estazolam, bromazepam, flurazepam, clorazepate potassium, haloxazolam, ethyl loflazepate, qazepam, clonazepam, mexazolam, and the like), certain thienodiazepiens (e.g., etizolam, brotizolam, clotizaepam, and the like), certain dialkylphenols (e.g., propofol, fospropofol, and the like), certain non-benzodiazepines (e.g., Zolpidem, zopiclone, exzopiclone, etc.), and the like. In some embodiments, the peptides, fusion proteins or immunoconjugates as described herein are used in combination with a CTLA1 molecule. As used herein, a “CTLA4 molecule” is a molecule comprising a cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) extracellular domain. In some embodiments, the extracellular domain of CTLA4 comprises a portion of the CTLA4 protein that recognizes and binds to at least one B7 (CD80/86) antigen such as a B7 antigen expressed on B cells and on antigen presenting cells (APCs). The B-cells and APCs may be activated. The extracellular domain may also include fragments or derivatives of CTLA4 that bind a B7 antigen. The CTLA4 extracellular domain can also recognize and bind CD80 and/or CD86. The extracellular domain may also include fragments or derivatives of CTLA4 that bind a binds CD80 and/or CD86. The CTLA4 molecule may be a fusion protein, where a fusion protein is defined as one or more amino acid sequences joined together using methods well known in the art. The joined amino acid sequences thereby form one fusion protein. In some embodiments, the CTLA4 molecule contains at least a portion of an immunoglobulin, such as the Fc portion of an immunoglobulin. In some embodiments, the CTLA4 molecule is an isolated and purified CTLA4 molecule. In some embodiments, the CTLA4 molecule is a protein containing at least a portion of an immunoglobulin, such as the Fc portion of an immunoglobulin. In some embodiments, the CTLA4 molecule is an isolated and purified CTLA4 molecule. In some preferred embodiments, the CTLA4 molecule is abatacept. Abatacept is a soluble fusion protein that consists of the

extracellular domain of human CTLA-4 linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1). Abatacept is produced by recombinant DNA technology in a mammalian cell expression system. The apparent molecular weight of abatacept is 92 kilodaltons. Abatacept was developed by Bristol-Myers Squibb and is disclosed, for example, in U.S. Pat. No. 5,851,795, U.S. Pat. No. 7,455,835, and U.S. Pat. Pub. 20011/311529.

A further object of the present invention also relates to a nucleic acid molecule encoding for a peptide or a fusion protein as herein described for use in a method for treating Type 1 Diabetes in a subject in need thereof.

Typically, said nucleic acid molecule is a DNA or RNA molecule, which may be included in any suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.

A wide variety of methods exist to deliver nucleic acid molecules to subjects, as defined herein. For example, the acid nucleic molecule of the present invention can be formulated with cationic polymers including cationic liposomes. Other liposomes also represent effective means to formulate and deliver self-acid nucleic molecule. Alternatively, the DNA can be incorporated into a viral vector, viral particle, or bacterium for pharmacologic delivery. Viral vectors can be infection competent, attenuated (with mutations that reduce capacity to induce disease), or replication-deficient. Methods utilizing DNA to prevent the deposition, accumulation, or activity of pathogenic self proteins may be enhanced by use of viral vectors or other delivery systems that increase humoral responses against the encoded autoantigen. In some embodiments, the DNA can be conjugated to solid supports including gold particles, polysaccharide-based supports, or other particles or beads that can be injected, inhaled, or delivered by particle bombardment (ballistic delivery). Methods for delivering nucleic acid preparations are known in the art. See, for example, U.S. Patent Nos. 5,399,346, 5,580,859, and 5,589,466. A number of viral based systems have been developed for transfer into mammalian cells. For example, retroviral systems have been described (U.S. Patent No. 5,219,740; Miller et al, Biotechniques 7:980-990 (1989); Miller, Human Gene Therapy 1 :5-14, (1990); Scarpa et al, Virology 180:849-852 (1991); Burns et al, Proc. Natl Acad. Sci. USA 90:8033-8037 (1993); and, Boris-Lawrie and Temin, Cur. Opin. Genet. Develop. 3: 102-109 (1993). A number of adenovirus vectors have also been described, see e.g., (Haj-Ahmad et al., J. Virol. 57:267-274

(1986); Bett et al., J. Virol. 67:591 1-5921 (1993); Mittereder et al, Human Gene Therapy 5:717-729 (1994); Seth et al., J. Virol. 68:933-940 (1994); Barr et al, Gene Therapy 1 :51-58 (1994); Berkner, BioTechniques 6:616-629 (1988); and, Rich et al, Human Gene Therapy 4:461-476 (1993). Adeno-associated virus (AAV) vector systems have also been developed for nucleic acid delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g. , U.S. Patent Nos. 5,173,414 and 5,139,941 ; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski et al, Molec. Cell Biol. 8:3988-3996 (1988); Vincent et al , Vaccines 90 (Cold Spring Harbor Laboratory Press) (1990); Carter, Current Opinion in Biotechnology 3:533-539 (1992); Muzyczka, Current Topics in Microbiol. And Immunol. 158:97-129 (1992); Kotin, Human Gene Therapy 5:793-801 (1994); Shelling et al., Gene Therapy 1 : 165-169 (1994); and, Zhou et al. , J. Exp. Med. 179: 1867-1875 (1994).

In some embodiments, the nucleic acid molecule of the present invention is delivered without a viral vector. For example, the nucleic acid molecule can be packaged in liposomes prior to delivery to the subject. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, e.g. , Hug et al , Biochim. Biophys. Acta. 1097: 1-17 (1991); Straubinger et al. , in Methods of Enzymology, 101 : 512-527 (1983). Alternatively, the nucleic acid molecule is delivered via electroporation (i.e. muscular delivery by electroporation).

In some embodiments, the acid nucleic molecule is delivered by intramuscular ("IM") injection. In some embodiments, the acid nucleic molecule of the present invention is delivered intranasally, orally, subcutaneously, intradermally, intravenously, mucosally, impressed through the skin, or attached to particles delivered to or through the dermis. Alternatively, nucleic acid molecules can be delivered into skin cells by topical application with or without liposomes or charged lipids. Yet another alternative is to deliver the nucleic acid as an inhaled agent. Typically, the acid nucleic molecule is formulated in solutions containing higher quantities of Ca++, e.g., between 1 mM and 2M. The acid nucleic molecule may be formulated with other cations such as zinc, aluminum, and others. Alternatively, or in addition, the acid nucleic molecule may be formulated either with a cationic polymer, cationic liposome-forming compounds, or in non- cationic liposomes. Examples of cationic liposomes for DNA delivery include liposomes generated using 1,2-bis(oleoyloxy)-3-(trimethylammionio) propane (DOTAP) and other such molecules.

In some embodiments, the human preproinsulin peptide of the present invention is loaded in MHC class II multimers. Typically MHC class II multimers are well known in the art (Fierabracci A. The potential of multimer technologies in type 1 diabetes prediction strategies. Diabetes Metab Res Rev. 2011 Mar;27(3):216-29.) and include but are not limited to dimers, tetramers, pentamers, streptamers, dextramers and octmaers. According to the invention, the HLA class II multimers according to the invention are preferably HLA-DQ8 multimers loaded with the human preproinsulin peptide of the present invention. Examples of MHC class II multimers include (i) the ectopic expression of a membrane-bound MHC class II heterodimer on the surface of eukaryotic cells by use of a lipid tether (GPI anchor), see Wettstein et al., 1991, J. of Exp. Medicine, 174: 219-228; (ii) the expression of MHC class II ectodomains in insect cells, see Wallny et al., 1995, Eur. J. Immunology, 25: 1262-1266; (iii) the introduction of heterologous dimerization motifs, such as the leucine zipper, C-terminally into the MHC class II molecules, in combination with insect cell production, see Quarsten et al., 2001, J. Immunol., 167: 4861-4868, and Crawford et al., 2006, Immunological Reviews, 210: 156-170; (iv) the production of antibody/MHC class II chimeras in combination with insect cell production, see Casares et al., 1997, Protein Engineering, 10: 1295-1301; (v) the use of bacterial expression systems that allow formation of functional pMHC trimers through refolding approaches from inclusion bodies, see Arimilli et al., 1995, J. Biol. Chem., 270:971-977; or (vi) the use of a truncated bacterial produced single chain MHC class II format which is comprised of the α1 and β1 domains only, that allows formation of functional MHC molecules through refolding approaches from inclusion bodies, see Burrows et al., 1999, Protein Engineering, 12: 771-778. In addition, Landais et al., 2009, J. Immunol., 183:7949-7957, describes an insect cell expression system which uses internal artificial disulphide bridges in conjunction with exogenous leucine zippers for producing stabilized murine I-Ad OVA MHC class II tetramers. Other examples include those described in US20130171668 that consist of a recombinant MHC class II molecule, which comprises (i) all or part of the extracellular portion of an MHC class II α chain; (ii) all or part of the extracellular portion of an MHC class II β chain; wherein (i) and (ii) provide a functional peptide binding domain and wherein (i) and (ii) are linked by a disulphide bond between cysteine residues located in the α2 domain of said α chain and the β2 domain of said β chain, wherein said cysteine residues are not present in native MHC class II α2 and β2 domains. The native MHC class II molecules comprise an α- and a β-chain, both of which have transmembrane regions and belong to the immunoglobulin (Ig) superfamily. The extracellular portion of each chain is composed of two domains, each consisting of

approximately 90 amino acid residues, of which the two membrane distal domains, the α1 and β1 domains, form an inter-latticed α/β structure essential for the peptide binding property of the T cell epitopes. The two membrane proximal domains, the α2 and the β2 domains, both form discrete Ig domains. In both the α and the β chain, a stretch of approximately 20 amino acid residues spans the cell membrane and on the cytoplasmic side of the membrane a fairly short peptide segment is located. The molecules of US20130171668 comprise all or part of the extracellular portion of an MHC class II α chain and all or part of the extracellular portion of an MHC class II β chain. The extracellular portion of an MHC class II α chain comprises a signal sequence, a membrane distal α1 domain and a membrane proximal α2 domain (which forms a discrete Ig domain). There is also a spacer region between the transmembrane domain and the α2 domain. Likewise, the extracellular portion of an MHC class II β chain comprises a signal sequence, a membrane distal β1 domain, a membrane proximal β2 domain (which forms a discrete Ig domain) and a spacer region. Other examples include dextramer reagents commercially available from Immudex (Copenhagen, Denmark - http://www.immudex.com/about-products/dextramer-descrip.aspx). Typically multimers thus comprise two or three or four or five or more recombinant MHC class II molecules associated with one another. Such association can be carried out using methods known and described in the art but is generally mediated via another linkage such as a linker molecule. Suitable linker molecules are well known and described in the art and particularly appropriate linker molecules will have multiple binding sites to which the recombinant molecules can be attached. For example, multiple attachment molecules such as avidin and streptavidin (or any other molecule which binds biotin in a multivalent manner) may be used which each have multiple binding sites for biotin. Thus, incorporation of biotin into the recombinant MHC molecules by methods known in the art (e.g. using AviTag or other BirA substrates to enable enzymatic biotinylation) will allow the formation of multimers, e.g. tetramers of MHC molecules. Labels can also conveniently be incorporated into multimeric forms, e.g. using fluorescent streptavidin or by the fusing of a label to a phage coat protein, for example a different type of coat protein than the one chosen for MHC class II molecule display. Incorporation of such labels allows ready detection of the tetramers by various known techniques. For example use of a fluorescent label allows flow cytometry techniques such as FACS analysis to be used, which is particularly advantageous. Typically labels include fluorochromes such as, for example, fluorescein isothiocyanate (FITC), phycoerythrin, phycocyanin or allophycocyanin.

The peptides and multimers as described herein are also suitable for preparing polyclonal T cells and T cell lines or clones recognizing a human preproinsulin peptide of the invention. In some embodiments, the human preproinsulin peptides of the present invention are suitable for preparing Treg cells that can be used in adoptive T cell transfer. As used herein, "regulatory T cells" or "Tregs" has its general meaning in the art and refers to the subset of T cells which actively suppress or tolerize activation of the immune system (e.g. reactive immune cells), maintain immune system homeostasis and/or prevent pathological self- reactivity. Regulatory T cells of the present invention include cells that express e.g. CD8 and CD122, or CD4, CD25, Foxp3, CD45RBlow, CD62Lhigh and/or TCDap (e.g. naturally occuring CD4+CD25+FoxP3+ regulatory T cells). However, the term T regulatory cells also encompass other T cells that have suppressive function. The regulatory T cells of the present invention encompass both "naturally-occurring" Tregs as well as Tregs generated in vitro. Tregs of the present invention are typically capable of suppressing a variety of cells, such as other T cells (e.g. effector T cells), B cells (e.g. effector B cells) and/or antigen presenting cells (e.g. effector APCs such as monocytes and dendritic cells). Typically Tregs exert their function via TCR/MHC class II interaction following cell to cell contact, however, Tregs may also suppress effector cells by secretion of immunosuppressive cytokines (e.g. TGF-beta and IL-10). In some embodiments, the T cells are prepared according to the method described in WO 2010119307. In some embodiments, the a method for producing T cells displaying specific immunological properties from a subject comprising the steps consisting of culturing said blood or PBMC sample in an appropriate culture medium which comprises an amount of Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) and/or IL-4 and/or FMS-like tyrosine kinase 3 (Flt-3) ligand and/or IL-1beta and an amount of a least human preproinsulin peptide of the present invention and isolating at least one T cell displaying a specificity for the human preproinsulin peptide. Said specific immunological properties include, but are not limited to, recognition by the isolated T cells of the peptide. By way of example, another possible specific immunological property can be the production of IL-10. T cells producing IL-10 can be used as regulatory T cells for the treatment T1DM by adoptive T cell transfer. The person skilled in the art is also familiar with methods suitable to isolate said Ag-specific T cells in a viable state based on different immunological properties. For example, selection of IL-10-producing T cells may be obtained by Miltenyi cytokine capture assays. As yet another example, said T cells can be isolated by means of MHC Class I or Class II multimers [Mallone et al., *Diabetes* 53:971, 2004; Mallone et al., *Blood* 106:2798, 2005; Skowera et al., *J.Clin.Invest.* 118:3390, 2008; Ladell et al., *Immunity* 38:425, 2013].

The peptides as described herein are also suitable for detecting autoreactive T cells specific for a human preproinsulin polypeptide of the invention. Therefore the human preproinsulin polypeptides of the present invention are thus particularly suitable for diagnosing T1DM in a subject. In some embodiments, the diagnostic method of the present invention is performed as described in WO 2010119307. In some embodiments, the method comprises the steps consisting of culturing a blood or PBMC sample obtained from the subject in an appropriate culture medium which comprises an amount of Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) and/or IL-4 and/or FMS-like tyrosine kinase 3 (Flt-3) ligand and/or IL-1beta and an amount of a least human preproinsulin peptide of the present invention and detecing at least one T cell displaying a specificity for the human preproinsulin peptide. Methods for the detection of stimulated T cells are known to the skilled person (e.g. Enzyme-linked immunospot (ELISpot), proliferation assay, Supernatant cytokine assay…). Alternatively, the diagnostic method of the present invention contemplated use of a human preproinsulin peptide of the present invention that is loaded on multimers as described above, so that the isolated CD8+ T cells from the subject are bringing into contact with said tetramers. There is no requirement for in vitro T cell activation or expansion. Following binding, and washing of the T cells to remove unbound or non-specifically bound multimer, the number of CD8+ cells binding specifically to the HLA-peptide tetramer may be quantified by standard flow cytometry methods, such as, for example, using a FACSCalibur Flow cytometer (Becton Dickinson). The multimers can also be attached to paramagnetic particles or magnetic beads to facilitate removal of non-specifically bound reporter and cell sorting. Such particles are readily available from commercial sources (eg. Beckman Coulter, Inc., San Diego, Calif , USA). Multimer staining does not kill the labeled cells; therefore cell integrity is maintained for further analysis.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. *Ex vivo* proliferative responses to hPPI in YES-RIP-hB7.1 mice. Full-length hPPI and a series of hPPI peptides covering the whole molecule were tested, $hPPI_{1-15}$,

$hPPI_{16-30}$, $hPPI_{20-35}$, $hPPI_{25-40}$ and $hPPI_{33-47}$. Splenocytes from diabetic YES-RIP-hB7.1 (●), non-diabetic YES-RIP-hB7.1 (●) and YES mice (○) were re-stimulated *in vitro* for 3 days and proliferation was evaluated by measuring BrdU incorporation. ns, non-significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; Mann-Whitney test.

EXAMPLE 1:

Material & Methods

Mice:

YES mice were obtained by crossing the hINS mouse that lacks the expression of the two mINS genes, expresses the human insulin gene (hINS) as a transgene under the control of 353 bp of the human insulin gene promoter on a DBA/CBA ($H\text{-}2^k$, IA^k) mix genetic background (kindly given by J Jami and R Joshi, Institut Cochin, Paris) [15] and a double transgenic HLA-A*02:01/HLA-DQ8 mouse with C57BL/6J as main genetic background. The double transgenic HLA-A*02:01/HLA-DQ8 mouse was obtained (F Lemonnier) by crossing a *H-2D^b*, mouse ß2-microglobuline (*mß2m*) double KO mice lacking the expression of mouse H-2 class I molecules, expressing under the control of HLA-A*02:01 gene promoter a unique species of MHC class I molecules (a chimeric HLA-A*02:01 HHD monochain containing the HLA-A*02:01 α1 and α2 domains and the $H2\text{-}D^b$ α3 domain linked to the human β2m by a 15-residue linker peptide) [16] with a mouse lacking the expression of the mouse *IA^b* and *IE^b* genes [17], but expressing the HLA-DQ8 DQA1*03:01 α and DQB1*03:02 β chains under the control of their natural MHC class II promoter [18]. YES-RIP-hB7.1 mice were obtained by lentiviral transgenesis at Institut Pasteur, Paris. The RIP-hB7.1 transgene was inserted in a recombinant lentiviral vector (LV-RIP-hB7.1; 4,640 bp) derived from Human Immunodefiency Virus as previously reported [19]. Retroviral pseudotypes were injected in fertilized eggs obtained from superovulated YES female mice mated with YES-male mice. Fertile and transduced eggs reimplanted into pseudopregnant C57BL/6-CBA F1 mice. All mice were maintained under specific pathogen-free conditions at the Hospital Cochin-St Vincent de Paul animal facility, and experimental studies were performed in accordance with the Institutional Animal Care and Use Guidelines and accredited by the ethic committee under the number CEEA34.CB.024.11.

Genotyping:

Real time quantitative PCR based on 3-point dilutions using QuantiTect SybrGreen PCR Kit (Qiagen) on LC480 (Roche) were performed to select mice carrying a single allele of *mINS1* and/or *mINS2* genes for further crosses. In addition, cell-surface stainings were realized to screen for the loss H2-D^k class I MHC and IA^k-IE^k class II MHC molecules in the successive crosses using anti-mouse-TCR-APC-labeled and anti-mouse-H-$2D^k$-FITC-labelled antibodies (BD-Pharmingen) or with anti-mouse-CD19-APC-labelled and anti-mouse-IA^k/IE^k-FITC-labelled antibodies (BD Pharmingen). Data were collected with a LSR-FORTESSA flow cytometer (Becton Dickinson) and analyzed using FlowJo 8.7.3 software (FlowJo, Tree Star Inc).

Nimblegen sequence capture of LV-RIP-B7.1 transgene:

Sequence capture of genomic DNA targets was realised for two founders of YES-RIP-hB7.1 mice and a descendant of each founder with NimbleGen SeqCap EZ Developer Libraries (ROCHE). Selecting target for probes design was 97,6% of the primary target of LV-RIP-hB7.1 construction (bp 9-1,957; bp 1,985-2,741; bp 2,754-4,577 with valid overlapping probes leaving 113 bp uncovered). Roche® Probes were optimised after performing a preliminary Comparative Genomic Hybridization (CGH) experiment. 1µg of each genomic DNA was used for sample library preparation (Illumina TruSeq DNA Sample Preparation Kit, ROCHE) and was amplified using Ligated-Mediated PCR (LM-PCR) in preparation for hybridization to the SeqCap EZ Developer Library. Captured multiplex DNA samples were recovered, amplified and captured-sequences reads on a Roche 454 GS-FLX System at ICM, Paris. All reads were analysed by IGV software with C57BL/6NJ genome as reference. Captured sequences were filtered by complete alignment with the primary sequence LV-RIP-hB7.1 sequence in BLASTN. We reference the beginning and the ending of the primary sequence LV-RIP-hB7.1 with a word size of 50 for captured the transgene insertion. We aligned the unfiltered sequence with the 2 reference sequences named Begin-LV-RIP-hB7.1 and End-LV-RIP-hB7.1, respectively (Table 1). Conserved sequences were then aligned against scaffold of C57BL/6NJ lineage to obtain alignment of Begin-LV-RIP-hB7.1 and End-LV-RIP-hB7.1 in IGV software. Profiles showing decreasing accumulations of reads on both ends of transgene insertion were sought and a 10kb for each region was extracted and amplified by Long-Range PCR (LR-PCR). A nested PCR for hB7.1 transgene was performed on the Long-Range PCR product to highlight the presence of the transgene as for genotyping except that we performed hB7.1 screening using SYBRGreen PCR Master Mix (Applied Byosystems) on LC480 real time PCR device (Roche) for a high throughput.

Expression of human and mouse pre-pro-insulin:

After RNA Later-stabilisation, RNA extractions were performed on liver, pancreas, thymus, spleen, kidney and inguinal lymph node with RNeasy Mini Prep and DnaseI treatment (Qiagen). One-step real time RT-PCR were performed with RT-PCR one-step QuantiTect SYBRGeen RT-PCR Kit (Qiagen) and passed on Light Cycler LC480 (Roche).

Flow cytometry:

Spleens, thymus, inguinal lymph nodes, lymphocytes and bone marrow cells were re-suspended at 10^6 cells/ml in RPMI 1640 / FCS 10 and stained with the following antibodies: anti-mouse-CD3ε-PE, anti-mouse-CD3ε-APC, anti-mouse-CD8α-PerCP, anti-mouse-CD4-efluor450, anti-mouse-CD44-FITC, anti-mouse-CD19-APC, anti-mouse-CD11c-APC, anti-mouse-CD11b-efluor450, anti-mouse-CD11b-APC, anti-human-β2microglobulin-FITC (clone TÜ99, BD-Biosciences), anti-human-HLA-DQ-Biot (Hybridoma SVPL3), SAV-FITC, anti-$H2\text{-}D^k$, anti-IA^k, anti-mouse-NKp46-PE, anti-mouse-CD25-PE, anti-mouse-FoxP3-FITC, anti-mouse-TCRβ-APC, anti-mouse-NK1.1-APC, anti-mouse-LY6G-PE-labelled, anti-mouse-TER-119-FITC (home made), anti-mouse-CD45.2-PE, anti-mouse-CD90.2-FITC, anti-mouse-CD62L-Biot and SAV-PE-Cy7 from e-biosciences if not specified. Data were collected with a LSR-FORTESSA flow cytometer (Becton Dickinson) and analyzed using FlowJo 8.7.3 software (FlowJo, Tree Star Inc).

Immunisation procedures:

Immunizations were performed by injecting YES mice with either of Keyhole Limpet Hemocyanin (KLH), 50μg in complete Freund's adjuvant (CFA), or 100μg denaturised (3 min at 94°C) hINS, or 100μg HLA-A*02:01-restricted Influenza matrix protein 2 peptide GILGFVFTL ($MatA2_{58\text{-}66}$) in CFA, or 100μg of $hPPI_{6\text{-}14}$, $hPPI_{15\text{-}24}$ or $hPPI_{33\text{-}42}$ peptides with 140μg HLA-DQ8-restricted-helper Nef peptide $Cyst_{66\text{-}97}$ in incomplete Freund's adjuvant (IFA); or 100μg hPPI or 50μg HLA-DQ8-restricted $hPPI_{1\text{-}31}$, $hPPI_{1\text{-}15}$, $hPPI_{16\text{-}30}$, $hPPI_{18\text{-}30}$, $hPPI_{20\text{-}35}$, $hPPI_{25\text{-}40}$, $hPPI_{33\text{-}47}$, $hPPI_{55\text{-}70}$, $hPPI_{80\text{-}97}$ or $hPPI_{92\text{-}110}$ peptides in CFA subcutaneously at the base of the tail, followed by 3 injections of the same peptide in IFA every other week. DNA immunizations were performed by pre-treating YES mice with 10μM cardiotoxin (Carloxan Lab) intramuscularly, and with inject i.m 5 days later 100μg of pCMV-B10-hPPI DNA vector or pCMV-B10-CTL DNA vector as control followed by a boost two weeks after.

Mice were anesthetized during each injection. Control immunizations were realized injecting PBS1X under the same conditions.

Cytotoxicity assay:

Ex vivo cytotoxicity was evaluated on HHD-transfected P815 cells pre-pulsed with 10µg peptide for 2h at 37°C, and in some cases pre-incubated by 50µg/ml of anti-HLA-A*02:01 antibody (BB7.2 clone), using the LDH Cytotoxicity Detection Kit ^{PLUS} (Roche) or the LIVE/DEAD Cell-Mediated Cytotoxicity Kit (Invitrogen).

Enzyme-linked immunospot (ELISpot) assay:

IFNγ-ELIspot assays were performed as previously reported [20]. Spots were counted using Bioreader 5000 Pro-SF (Bio-Sys GmbH). Data are mean of triplicate wells and expressed as spot-forming cells (SFC) per 10^6 cells evaluating the background IFNγ responses from triplicates in the absence of peptide. Positive controls were triplicates of cells stimulated by 1µg/ml ConA (Sigma-Aldrich). In some experiments, the response was inhibited using 50µg/ml anti-HLA-A*02:01 antibody (BB7.2) for 20 min.

Cell proliferation assay:

10^5 spleen cells/well were incubated with 0.5µg antigen/well or 0.1µg peptide/well for 72h at 37°C in triplicate. Proliferation was evaluated with BrdU Cell Proliferation Assay Kit (Cell Signaling) and expressed as proliferation index (PI). Background and positive controls were evaluated in triplicate wells containing 10^5 cells/well incubated without antigen or in the presence of 10µg/ml final concentration of anti-CD3ε antibody. In some experiments, the response was inhibited adding 50µg/ml of anti-HLA-DQ8 antibody (Hybridoma SVPL3) prior.

Metabolic evaluations:

Intraperitoneal glucose tolerance (IGTT) (2g/kg body weight) tests were performed after 12h-16h fasting. Intraperitoneal insulin tolerance tests (IITT) (0.75U/kg body weight) were performed after 4h fasting. Blood glucose was measured using a glucometer (BG Star) at 0, 30, 60 and 120 min. Glycaemic values are in mg/dl. Assessment of total ß-cell mass was performed on scan stained-microscope slides with ImageJ software using a guinea pig anti-human insulin antibody (DAKO) as the ratio between ß-cell surface (μm^2)/pancreas surface (μm^2) multiplied by pancreas weight (mg).

Immunostainings:

Immunostainings were performed on formalin-fixed paraffin pancreas sections that were deparaffinised in Xylene and dehydrated by Ethanol. After washing, antigen retrieval was realized by hot incubation, followed by permeabilization (20 min in PBS1X/0,4% TritonX100) and saturation (20 min PBS1X/1% Horse Serum) before immunostaining with biotynilized rat anti-human CD3ε (ABDSerotec) and polyclonal rabbit anti-glucagon (DAKO) antibodies overnight. Slides were washed with PBS1X/1%BSA/0.1%TritonX100 and stained with an anti-rabbit-FITC antibody (Abcam) and SAV-Cy3 (Abcam) at RT. Sections were mounted in Vectashield Mounting Medium for fluorescence with DAPI (Vector Laboratory). Observations were made with a spinning disk confocal apparatus at the Institute Cochin imaging platform and pictures analyzed with the ImageJ software.

Tetramer assays:

Tetramers (TMrs) associating the chimeric HLA-A*02:01-HHD to hPPI peptides were obtained from the NIH Tetramer Core Facility. Stainings were performed as previously reported [21]. Incubation of cells with TMRs was performed for 30 min at RT. The following antibodies were used: anti-CD3ε-AlexaFluo700, anti-CD8α-APC, anti–CD19-PerCP-Cy5 and anti–CD14-PercP-Cy5 antibodies (BD/Pharmingen and eBioscience). Cells were analysed with a BD LSR Fortessa flow cytometer and the FlowJo software (Tree Star Inc).

Single-cell RT- PCR

Staining was performed on 10^8 spleen cells by adding 5-10 µg/ml of PE-labeled TMr at RT for 30 min. CD8α-APC-labeled, anti–CD19-PerCP-Cy5.5-labeled and anti–CD14-PercP-Cy5.5-labeled antibodies were then added for 15 min at 4°C. Lymphocytes were gated according to forward/side scatter profiles. $CD8^+/TMr^+$ T-cells were sorted with a FACSAria II at 1 cell/well into 96-well PCR plates containing 5µl PBS1X treated with diethyl pyrocarbonate (DEPC) (Sigma) and immediately frozen at -80°C. Single-cell RT-PCR was performed as previously described [21]. Perforin (*Prf1*), granzyme A (*Gzma*), granzyme B (*Gzmb*), Fas ligand (*tnfsf6*), IFN-γ (*Ifng*), TGF-β (*Tgfb*), TGF-β R1 (*Tgfb-r1*), TGF-β R2 (*Tgfbr2*), TGF-β R3 (*Tgfbr3*), TNF-α (*Lta*), IL-2 (*Il2*), IL-7R (*Il7r*), IL-10Rα (*Il10ra*), IL-15 (*Il15*), IL15-R (*Il15r*), IL-21 (*Il21*), CCR7 (*Ccr7*), and CD3ε (*Cd3e*) mRNAs were analysed.

Islet isolation

Islets of Langerhans were isolated as described previously [22]. Once handpicked, islets were dissociated with Cell Dissociation Solution Non-Enzymatic (SIGMA) 30 min at RT. Cells were filtered and stained with anti-mouse-HLA-DQ-FITC (BD Biosciences), anti-human-ß2m-PE (BD Biosciences), guinea pig-anti-bovine-insulin (DAKO), anti-guinea pig-Biot and SAV-PE-Cy7, anti-mouse CD4-AlexaFluo700, anti-mouse-CD8-APC anti-mouse-CD19-PercpCy5.5 and anti-mouse-TCRß-efluo450 antibodies (e-biosciences). Acquisition was done with a BD FACSAria flow cytometer and analyses using FlowJo software (Tree Star Inc).

Infiltrate staining

Handpicked islets were pooled in 24-well-plate well for 24h in RPMI supplemented with 10% FCS, 1% penicillin-streptomycin and 5.10^{-5}M ß-mercapto ethanol allowing a spontaneous extrusion of infiltrating lymphocytes. Infiltrating lymphocytes were recovered and stained with anti-mouse-CD3ε-AlexaFluo700, anti-mouse-CD19-PerCP-Cy5, anti-mouse-CD11b-efluo450 and anti-mouse-CD11c-APC antibodies, anti-mouse-CD103-FITC, anti-HLA-DQ-Biot (SVPL3 Hybridoma), SAV-BrillantViolet650 (Biolegend), anti-mouse-CD4-PE, anti-mouse-CD8α-PE-Cy7 antibodies from e-biosciences. Acquisition was done with a BD FACSAria flow cytometer and analyses using FlowJo software (Tree Star).

Results

DEVELOPMENT OF HLA-A*02:01/HLA-DQ8/hins YES MICE AND TRANSGENE EXPRESSION

To obtain YES mice, F1 crosses of HLA-A*02:01/HLA-DQ8 and hINS transgenic mice were backcrossed with hINS transgenic mice and female breeders were progressively selected for full loss of *mINS1* and *mINS2* alleles, expression of at least one allele of the *hINS* transgene and one allele of *HLA-A2*02:01* and *HLA-DQ8* transgenes along with full loss of *H2-D^k*, *IA^k-IE^k and mβ2m* alleles. Additional crosses allowed us to obtain homozygous YES mice with the following genotype: *mINS1$^{-/-}$ mINS2$^{-/-}$ hINS$^{+/+}$ mβ2m$^{-/-}$ H2D$^{-/-}$ IA$^{-/-}$ IE$^{-/-}$ A2.1$^{+/+}$ DQ8$^{+/+}$*. Twenty brother-sisters crosses led to inbred YES mouse. We confirmed by RT-PCR that YES mice lack the expression of *mINS1* and *2* genes in the pancreas and in the thymus. We observed a strong expression of the *hINS* transgene in the pancreas and a weak expression in the thymus and liver. We confirmed the loss of murine MHC class I (H2-D^k and H2-K^k) and class II (IA^k)

and the presence of the transgenic HLA-A*02:01 molecule on T-cells and of the transgenic HLA-DQ8 molecule on B-cells using flow cytometry. We also observed the expression of HLA-A*02:01 on $CD45^-$ $Insulin^+$ islet cells from YES mice to a level that was comparable to murine H2-D^b in conventional C57BL/6 mice.

The expression of HLA-A*02:01/HHD was further confirmed by flow cytometry on immune cell subsets in spleen, blood, and iLN. In all subsets analyzed, the expression of HLA-A*02:01 was comparable in YES mice and in parental HLA-A*02:01/HLA-DQ8 double transgenic mice (data not shown). Several differences were seen when comparing the expression of HLA-A*02:01/HHD on immune cells in YES mice (n=6) to that of H-$2D^b$ in the corresponding subsets in C57BL/6 mice (n=3). In the basal state, a lower percentage of spleen B and DCs cells and of blood $CD4^+$ T cells expressed HLA-A*02:01 in YES mice as compared to the corresponding cells subsets that expressed H2-D^b in C57BL/6 mice. In addition, an increased percentage of iLN macrophages expressed HLA-A*02:01 in YES mice. The expression of HLA-DQ8 was comparable in YES and in parental HLA-A*02:01/HLA-DQ8 double transgenic mice (data not shown). As for HLA-A*02:01, several differences were seen when comparing the expression of HLA-DQ8 on immune cells in YES mice to that of H-$2IA^b$ in C57BL/6 mice. In the basal state, a lower percentage of spleen and blood DCs and of spleen macrophages expressed HLA-DQ8 as compared to the corresponding subsets that expressed H-$2IA^b$ in C57BL/6 mice.

DISTRIBUTION OF IMMUNE CELL SUBSETS IN YES MICE

Absolute numbers of spleen, iLN and thymic cells were comparable in YES mice and in parental HLA-A*02:01/HLA-DQ8 double transgenic mice (data not shown). The distribution of most immune cell subsets was comparable in YES mice and in C57BL/6 mice, with few differences. The percentage of T-cells was lower in YES as compared to C57BL/6 mice in spleen (13.0% ± 2.4 *vs* 29.0% ± 2.1) and iLN (47.0% ± 8.5 *vs* 53.45% ± 5.6), in contrast to B-lymphocytes (65% ± 4.5 *vs* 60% ± 3.2 in spleen and 42% ± 8.2 *vs* 38% ± 4.1 in iLN). In peripheral blood, T-cells were underrepresented in YES as compared to C57BL/6 mice (12.0% ± 9.8 *vs* 48.0% ± 7.6, *p=0.0238 with Mann Whitney Test). In contrast, dendritic cells (12.0% ± 1.83 *vs* 0.89% ± 1.1) and macrophages (30.0% ± 8.1 *vs* 8.83%± 3.4) were overrepresented. An inverted ratio of $CD3^+CD4^+$/$CD3^+CD8^+$ T-cells was seen in YES as compared to C57BL/6 mice in spleen (0.7 and 1.35, respectively), iLN (0.5 and 1.25, respectively) and peripheral blood (0.69 and 1.3, respectively). Analysis of the thymus showed no difference in YES mice as compared to C57BL/6 mice. In the thymus, the dominant $CD3^{+low}$ T-cell subset, which is

mainly constituted of $CD3^{+low}CD4^{+}CD8^{+}$ double positive cells was comparable in YES and C57BL/6 mice (92% and 88%, respectively). When absolute numbers were considered, no significant difference was seen between YES mice and C57BL/6 mice. Respective numbers of spleen B and T-cells were $34,4x10^6 \pm 8,1$ and $6,9x10^6 \pm 1,6$, respectively, in YES mice, as compared to $45x10^6 \pm 6,9$ and $21,7x10^6 \pm 3,2$, respectively, in C57BL/6 mice. In the thymus, the total number of T-cells was $16,2x10^6 \pm 2,5$ in YES mice, as compared to $29,4x10^6 \pm 4$ in C57BL/6 mice. We further analysed $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T-cells which were not significantly different in YES and in C57BL/6 mice.

In YES spleen, a significant fraction of cells (9.0% ± 3.9) were $CD3^{-}$ $CD4^{-}$ $CD8^{-}$ $CD19^{-}$ $CD11b^{-}$ $CD11c^{-}$ $Nk1.1^{-}$ (Negative cells (NC)). Unexpectedly, a corresponding population was not observed in the parental HLA-A*02:01/HLA-DQ8 double transgenic mice, but present in the parental hINS transgenic mouse (8.0% ± 2.9). NC cells were hematopoietic cells, as they expressed CD45 that is present on all leucocytes. We characterized DN cells using CD44 and CD25 markers that track early developing T-cells [22]. In YES spleens, a majority of cells were analogous to DN3 ($CD44^{-}CD25^{+}$) pre-T cells (59.3%) while a minority were analogue to DN1 ($CD44^{+}CD25^{-}$, 17.2%), DN2 ($CD44^{+}CD25^{+}$, 5.2%) pro-T cells and DN4 ($CD44^{+}CD25^{-}$, 16.3%) pre-T cells as compared to 3.4%, 32.2%, 3.1% and 61.2%, respectively in hINS transgenic mice. In YES spleens, DN cells were $CD62L^{-}$, suggesting that they correspond to immature T-cells blocked at an immature stage and devoid of migratory capacity to the peripheral lymph node.

YES RESPONSES TO CONVENTIONAL ANTIGENS AND hPPI

Since YES mice showed differences in the composition of their peripheral immune system, we evaluated immune responses to conventional antigens. We tested HLA-A*0201-restricted $CD8^{+}$ T cell responses to Influenza $MatA2_{58-66}$. After co-immunization with Nef peptide $Cyst_{66-97}$ in IFA, followed with three recall injections in IFA, spleen cells from immunized mice were shown cytotoxic against $MatA2_{58-66}$-pulsed target cells. Cytotoxicity was inhibited by pre-incubating target cells with an anti-HLA-A*02:01 antibody (BB7.2). Spleen HLA-A*02:01-restricted $CD8^{+}$ T-cells recovered from $MatA2_{58-66}$-immunized YES mice were further analysed using an IFNγ-ELISpot assay. Incubating $MatA2_{58-66}$-pulsed cells with BB7.2 antibody inhibited the strong IFNγ response seen in $MatA2_{58-66}$- immunized YES mice. We previously reported that two hPPI leader sequence peptides and a third peptide located within the insulin B-chain, namely $hPPI_{6-14}$, $hPPI_{15-24}$ and $hPPI_{33-42}$, are frequently recognized by $CD8^{+}$ T-cells in human T1D [23]. We thus tested whether $CD8^{+}$ T-cells were elicited against $hPPI_{6-14}$,

$hPPI_{15-24}$ and $hPPI_{33-42}$ in YES mice. After immunization against individual hPPI peptides, a significant response was detected in YES mice against $hPPI_{6-14}$ ($p \leq 0.03$) and $hPPI_{33-42}$ ($p \leq 0.04$) using an IFNγ-ELIspot assay. To evaluate $CD4^+$ T-cell responses to hPPI, YES mice were immunized against a series of hPPI peptides covering a large part of the hPPI sequence: $hPPI_{1-15}$, $hPPI_{16-30}$, $hPPI_{18-30}$, $hPPI_{20-35}$, $hPPI_{25-40}$, $hPPI_{33-47}$, $hPPI_{55-70}$, $hPPI_{80-97}$and $hPPI_{92-110}$. Proliferative responses were obtained against B-chain peptides $hPPI_{25-40}$ and $hPPI_{33-47}$ and against the leader sequence-B-chain junction $hPPI_{20-35}$. Altogether, these experiments indicate that YES mice respond against conventional antigens as expected. In addition, they respond to hPPI peptides upon immunization. They show proliferative responses that cluster around a region overlapping the leader sequence and B-chain sequence.

YES MICE SHOW CONSERVED GLUCOSE HOMEOSTASIS

As hINS binding to the mouse insulin receptor is in the normal range [24], the secretion of hINS was expected to allow normoglycemia in YES mice. YES mice maintained a normal weight in the long-term range when compared to hINS transgenic and HLA-A*02:01/HLA-DQ8 double transgenic parental strains. The overall morphology of the islets of Langerhans was comparable in YES and HLA-A*02:01/HLA-DQ8 parental mice, as were insulin, glucagon and PDX1 stainings. The β-cell mass was comparable in YES mice and parental hINS and HLA-A*02:01/HLA-DQ8 mice at 15 weeks of age. When YES mice were submitted to a 2mg/g i.p. GTT at 6 months of age, no significant difference was observed as compared to HLA-A*02:01/HLA-DQ8 parental mice. We tested the sensitivity to human insulin in both strains. There was a significant difference in the glycaemic responses to 0.75 U/Kg body weight i.p. insulin between YES male mice and parental HLA-A*02:01/HLA-DQ8 male mice, while no difference was observed in the response of female mice. Most differences seen between YES and control mice were minor, and are likely to represent the expected variability between different strains. In the long-term range, we have not seen the development of diabetes or insulitis in YES mice up to 56 weeks of age.

YES-RIP-hB7.1 MICE DEVELOP INSULITIS AND DIABETES

YES mice remained normoglycemic up to more than one year of age and showed no evidence of insulitis within the same age range, indicating that they were basically immune tolerant to the hINS transgene. In order to enforce the development of T1D in YES mice, we introduced the human *B7.1* gene under the control of the rat insulin gene promoter (RIP) using the LV-RIP-hB7.1 vector. We initially selected 27 mice that were positive for the hB7.1

transgene. To stabilize lineages, we backcrossed YES-RIP-hB7.1 mice onto the YES background and selected three founders (F-10, F-23 and F-36) that expressed the *hB7.1* transgene. We lost the F-10 and F-23 lineages in which we had a high diabetes incidence but inefficient reproduction. Insulitis and spontaneous diabetes developed in F-36 offsprings. After more than 20 brother-sister matings, we analysed pancreas paraffin sections by hematoxylin-eosin staining from diabetic and non-diabetic YES-RIP-hB7.1 mice. The number of islets that was amenable to observation was lower in diabetic than in non-diabetic mice (16.14 ± 10.1 *versus* 21.61 ± 10.5, respectively, $p \leq 0.03$). Insulitis was observed in 10.4 ± 9.8 (i.e. 64.6%) islets in diabetic mice, as compared to 2.4 ± 4.5 (i.e. 11.1%) in non-diabetic mice. Insulitis was confirmed by immuno-fluorescence using a rabbit anti-glucagon antibody to locate remnant islets and an anti-CD3ε antibody to detect T cell infiltration. Both peri-insulitis and intra-islet infiltration were seen in pancreases showing insulitis. Glucagon-positive cells were dispersed in the islet area rather than located in the islet periphery, as opposed to figures observed in normal mouse islets, due to the dislocation of the islet architecture. In 3 non-diabetic YES-RIP-hB7.1 mice from which infiltrated islets were recovered, infiltrating cells were composed of 37.8 to 84.7% $CD3^+$ T-cells, of which 65.7-84.2% were $CD8^+$ T-cells and 9.9-24.0% were $CD4^+$ T-cells. Other cells included 4.1-7.3% $CD4^-CD8^{hi}CD11c^+CD11b^-$, 3.4-11.0% $CD4^-CD8^{lo}CD11c^+CD11b^+$ DCs and 0.8-2.9% $CD19^+$ cells.

To further characterize the F-36 lineage, we realized a NimbleGen Sequence Capture of the *hB7.1* transgene that covered 97.6% of the primary target region on the F-36 founder and F-36 offspring. Relevant sequences were identified as indicated in Table 1, matching with regions located on chr. 19, 16, 14 and 11 for the F-36 founder, and chr.19 for the F-36 offspring. We screened 128 mice from the F-36 progeny for the selected insertion sites. In the F-36 progeny, 46 out of 128 YES-RIP-hB7.1 mice developed diabetes. The highest diabetes prevalence was seen in mice carrying the 11qB5 transgene insertion, in association with either the 19qA or 14qE5 insertions, 7/9 and 17/35, respectively. Noticeably, only 16/53 mice carrying the 11qB5, 14qE5 and 19qA insertions developed diabetes, but corresponding mice were obtained early along the F-36 lineage and were thus unlikely to carry homozygous LV-RIP-hB7. The age at onset of hyperglycaemia was variable from one mouse to another, spanning from 6 weeks to 51weeks independently of the lineage expansion and diabetes was definitive in all mice observed. In the three groups carrying two transgene insertions, the overall prevalence of diabetes was similar in female (53.8%) and in male (50.0%) YES-RIP-hB7.1 mice.

hPPI-SPECIFIC T-LYMPHOCYTES ARE DETECTED IN YES-RIP-hB7.1 MICE

We previously characterized hPPI peptides that bind to HLA-A*02:01[20,23]. Using an IFNγ-ELIspot assay, we observed that most selected hPPI peptides were recognized by T-cells obtained from diabetic YES-RIP-hB7.1 mice. A significant response was detected against hPPI$_{6-14}$, hPPI$_{15-24}$, and hPPI$_{34-42}$. When considering responses that were over the mean ± 3SD of responses seen in YES mice as controls, 56.2% diabetic YES-RIP-hB7.1 mice responded to hPPI$_{6-14}$, hPPI$_{15-24}$ and hPPI$_{34-42}$. In addition, individual responses were observed to hPPI$_{2-11}$ (43.7%), hPPI$_{30-39}$ (6.2%), hPPI$_{33-42}$ (18.7%), hPPI$_{42-51}$ (37.5%) and hPPI$_{101-109}$ (12.5%) although they did not reach statistical significance in the whole mouse population analysed (Table 2). Considering individual mice, 93.75% YES-RIP-hB7.1 diabetic mice showed a positive response, defined as over mean ± 3SD of responses seen in YES mice, against at least one hPPI peptide (Table 2). As indicated previously, insulitis was detected in non-diabetic YES-RIP-hB7.1 mice, although to a lower extent than in diabetic YES-RIP-hB7.1 mice. Individual non-diabetic YES-RIP-hB7.1 mice showed a positive IFNγ-ELIspot response, defined as previously, to either hPPI$_{2-11}$ (1/6), hPPI$_{6-14}$ (2/6), hPPI$_{15-24}$ (0/6), hPPI$_{30-39}$ (2/6), hPPI$_{33-42}$ (1/6), hPPI$_{34-42}$ (1/6), hPPI$_{42-51}$ (1/6) and hPPI$_{101-109}$ (1/6), thus 50% non-diabetic YES-RIP-hB7.1 mice showed a response to at least one hPPI peptide (Table 2). Using HLA-A*02:01 TMrs, we detected significant expansions of $CD8^+$ T-cells that were specific for hPPI$_{6-14}$, hPPI$_{15-24}$ and hPPI$_{33-42}$. 47.1%, 29.4% and 47.1% YES-RIP-hB7.1 mice showed a significant $CD8^+$ T cell expansion against hPPI$_{6-14}$, hPPI$_{15-24}$ and hPPI$_{33-42}$ when compared to YES mice. Considering individual mice, an expansion of $CD8^+$ T-cells was seen in 70.6% YES-RIP-hB7.1 mice against hPPI$_{6-14}$, hPPI$_{15-24}$ or hPPI$_{33-42}$, which does not preclude that expansions driven by other hPPI peptides may have occurred. Single hPPI-specific $CD8^+$ T-cells showed gene expression profiles that were characteristic of memory T-cells. hPPI-specific $CD8^+$ T-cells (59.38%) from diabetic YES-RIP-hB7.1 mice expressed *GzmA*, among which 100% co-expressed *Ccr7*, while only 4.33% hPPI-specific $CD8^+$ T-cells from YES-RIP-hB7.1 non-diabetic mice or YES control mice expressed *GzmA*, of which 76.67% co-expressed *Ccr7*, suggesting a naïve T-cells profile of most hPPI-specific $CD8^+$ T-cells in non-diabetic mice. This result was strengthened by the increased percentage of cells expressing *Foxo1* observed in hPPI-specific $CD8^+$ T-cells from non-diabetic mice as compared to the hPPI-specific $CD8^+$ T-cells from diabetic mice and by the significant level of *Foxo1* expression detected in 10-cell and single-cell batches from non-diabetic YES-RIP-hB7.1 mice when normalized to the *CD3ε* expression.

In addition to HLA-A*02:01-restricted responses, we studied HLA-DQ8-restricted T cell responses to hPPI in YES-RIP-hB7.1 mice. Using a proliferation assay, a significant

response to full-length hPPI was detected in diabetic and non-diabetic YES-RIP-hB7.1 mice, as compared to YES mice. Dominant hPPI regions were located in the leader sequence, at the leader sequence-B chain junction and within the B chain (**Figure 1**) as indicated by significant T-cell proliferations detected in the presence of $hPPI_{1-15}$, $hPPI_{16-30}$, $hPPI_{20-35}$, $hPPI_{25-40}$ and $hPPI_{33-47}$ peptides in YES-RIP-hB7.1 diabetic mice as compared to YES controls and similar proliferative responses, although not significant, observed in non-diabetic YES-RIP-hB7.1 mice. As for $CD8^+$ T-cell responses, individual responses were observed against peptides covering the whole hPPI sequence. When considering responses that were over the mean ± 3SD percentile of control responses seen in YES mice individual responses were observed to $hPPI_{1-15}$ (20,83%), $hPPI_{16-30}$ (16,67%), $hPPI_{20-35}$ (29,17%), $hPPI_{25-40}$ (29,17%), $hPPI_{33-47}$ (16,67%) and $hPPI_{101-109}$ (12.5%) in diabetic YES-RIP-hB7.1 mice (Table 3).

DISCUSSION

The clinical management of patients with T1D is hampered by the lack of fully accurate biomarkers to diagnose autoimmunity in all recent onset diabetic patients or predict diabetes in all prediabetic individuals and the lack of therapies that would directly address the autoimmune process responsible for the destruction of β-cells. Major improvements in glucose monitoring and in insulin preparations that are currently used as a replacement therapy in insulin-dependent patients have narrowed the mortality gap with the general population and have shifted the safety line balancing risks and benefits of any immunotherapy towards the safest approaches. Immunosuppression has shown limited efficacy in preserving β-cells in recent-onset T1D patients, but carry the risk of side effects that preclude their use in the long-term range. Meanwhile, antigen-specific immunotherapy has failed in preserving β-cells from further destruction in recent-onset T1D patients or in preventing T1D in autoantibody-positive subjects at risk for its development. The autoantigen dose, the delivery route, the stage of the disease process at which immunotherapy is applied and the use of peptides rather than full-length autoantigens remain difficult issues in the human. In addition, preclinical models that would allow direct translation of experimentally-defined peptides in human are presently lacking and the NOD mouse shows a unique phenotype that is unlikely to summarize T1D heterogeneity.

We developed a new model in which we introgressed genes encoding for HLA-A*02:01, HLA-DQ8 and the human insulin genes into mice that were deficient for murine class I, class II and insulin genes. Mice carrying the same hINS transgene have been reported as presenting with normal insulin levels and glucose homeostasis [25]. YES mice indeed remain

normoglycemic up to one year of follow up, maintain normoglycemia to an intraperitoneal glucose challenge in the long-term range, have a normal β-cell mass and show a glucose response to insulin that is in the range of control mice up to 6 months of age. In contrast with the human, mice express two insulin genes located on chromosome 19 and chromosome 2, respectively. Diabetes has been observed at several crosses in males expressing a single mINS1 allele, as reported on the NOD mouse background [26]. Indeed, we observed acute diabetes in male mice at some crosses in which mice expressed a single mINS1 allele. However, diabetes was not observed at further crosses and in mice with complete lack of murine insulin genes that express two copies of the hINS transgene. YES mice are able to mount normal immune responses to conventional antigens despite showing an inverted $CD4^+/CD8^+$ T-cell ratio, presumably due to a lower efficiency of class II HLA-DQ8 than class I HLA-A*02:01 to select in the thymus and maintain in periphery the HLA-restricted T-cells. The YES immune phenotype is basically identical to that of the parental HLA-A*02:01/HLA-DQ8 mice strain. Despite expression of the class II HLA-DQ8 diabetes high susceptibility gene, YES mice remain tolerant to hPPI and to islets, including the human insulin transgene, and remain insulitis-free in the long-term range. Given available data indicating that enforced expression of hB7.1 on β-cells favors the development of autoimmunity to β-cells [27], induction of hB7.1 expression on β-cells in YES mice has been obtained by injecting a HIV-derived recombinant lentiviral vector in which a RIP-hB7.1 transgene has been inserted. We obtained several founders from which four insertions were detected in 19qA, 14qE5, 11qB5 and 16qB4. All insertions did not show the same efficiency in promoting spontaneous diabetes, with two combinations - 11qB5/19qA and 14qE5/11qB5 - providing the highest diabetes incidence. Diabetes was seen within a large age-range, from 6 weeks of age in some mice up to 51 weeks of age in others. The incidence of diabetes was comparable in males and females. Diabetic YES-RIP-hB7.1 mice showed extensive insulitis that was composed of both $CD8^+$ and $CD4^+$ infiltrating T-cells, and of $CD11c^+$ cells among which over 38% $CD4^-CD8^{hi}CD11c^+CD11b^-$ lymphoid DCs (data not shown). Insulitis was also detected in non-diabetic YES-RIP-hB7.1 mice, although to a lower extent than in diabetic mice, with a minority of mice showing extensive insulitis, presumably at an autoimmune stage that was close to diabetes. The T-cell infiltrate was predominantly composed of $CD8^+$ T-cells in non-diabetic YES-RIP-hB7.1 mice. A $CD8^+$ T-cell response was observed against a wide array of hPPI epitopes using an IFNγ-ELIspot assay, with a predominant response to two leader sequence peptides, $hPPI_{6-14}$ and $hPPI_{15-24}$, and to the B chain peptide $hPPI_{34-42}$. But overall, YES mice showed responses to a diverse peptide repertoire and individual mice showed responses to up to 3 peptides. Responses

were observed against epitopes spanning the whole hPPI sequence. $CD8^+$ T-cell responses were also observed in a minority of non-diabetic YES-RIP-hB7.1 mice. Interestingly, in a HLA-A*02:01 NOD transgenic mouse, responses have been reported against peptides that were largely overlapping with the HLA-A*02:01-restricted peptides that we have identified, in particular $hPPI_{3-11}$ and $hPPI_{5-14}$ [28]. Using HLA-A*02:01-restricted TMrs, we detected expansions of $CD8^+$ T-cells against the three dominants HLA-A*02:01-restricted hPPI peptides. The study of gene expression in $hPPI_{6-14}$, $hPPI_{15-24}$ and $hPPI_{33-42}$ specific $CD8^+$ T-cells allow discriminating diabetic, in which 40 to 80% hPPI-specific single $CD8^+$ T-cells expressed *gzma*, from non-diabetic YES-RIP-hB7.1 mice, in which *gzma*$^+$ cells were absent. Fifty five to 95% $CD8^+$ T-cells were shown to express *ccr7*, suggesting that $CD8^+$ T-cells were mostly central memory cells in diabetic YES-RIP-hB7.1 mice, while $CD8^+$ T-cells mostly showed a naïve phenotype in non-diabetic YES-RIP-hB7.1 mice, as confirmed by increased expression of *Foxo1*. HLA-DQ8-restricted $CD4^+$ T-cell responses were also detected against full-length hPPI. A majority of responses were seen against a region overlapping the B-chain and B-chain-C-peptide junction. Significant T-cell proliferations were detected to $hPPI_{20-35}$, $hPPI_{25-40}$ and $hPPI_{33-47}$ peptides in YES-RIP-hB7.1 diabetic mice, although not in non-diabetic YES-RIP-hB7.1 mice as compared to YES controls. But, as in the case of $CD8^+$ T-cell responses, $CD4^+$ T-cell responses were observed in individual diabetic YES-RIP-hB7.1 mice against peptides covering most of the hPPI sequence.

The development of diabetes in YES-RIP-hB7.1 mice was expected given previous reports in which transgenic expression of hB7.1 in β-cells triggered spontaneous diabetes. While less than 2% conventional RIP-hB7.1 transgenic mice developed diabetes by 8 months of age [29], spontaneous diabetes was commonly observed in double transgenic mice co-expressing the RIP-hB7.1 transgene in addition to other transgenes, in particular transgenes encoding for additional β-cell antigens [30]. Seemingly, co-expression of RIP-hB7.1 in addition to HLA-DQ8 transgene has been shown to allow the development of diabetes on a C57BL/6 genetic background [31-33]. In non-diabetic YES-RIP-hB7.1 mice in which an infiltrate was recovered, islet infiltrating $CD8^+$ T-cells were largely predominant, suggesting that $CD8^+$ T-cells were a major driving force in the diabetes process. In contrast, in diabetic YES-RIP-hB7.1 mice, a significant $CD4^+$ T-cell infiltrate was seen in addition to $CD8^+$ T-cells. In addition, an expansion of hPPI-specific $CD8^+$ T-cells was not only seen in fully diabetic mice, but also in some non-diabetic mice. In previously reported HLA-A*02:01/HLA-DQ8 double transgenic mice, diabetes was largely $CD4^+$ T-cell-independent as in transgenic mice that lacked the expression of class II molecules and $CD4^+$ T-cells [34].

The hPPI epitopes that were identified in non-diabetic and diabetic YES-RIP-hB7.1 mice are likely to be involved in the human disease. In YES-RIP-hB7.1 mice, $CD8^+$ T-cells were detected against a wide array of epitopes covering the hPPI sequence. The dominant responses were observed against the same epitopes as those that we previously identified in the human. In the case of the two leader sequence epitopes, i.e. $hPPI_{6-14}$ and $hPPI_{15-24}$, and the B-chain peptide $hPPI_{34-42}$, we and others have previously provided evidence that they are naturally processed as $hPPI_{6-14}$, $hPPI_{15-24}$, and $hPPI_{34-42}$-specific $CD8^+$ T-cells are cytotoxic either to hPPI-transfected HLA-A*02:01-P815 target cells [23] or to human HLA-A*02:01 islets [35]. However, as in the human, the repertoire of hPPI epitopes that were recognized by $CD8^+$ T-cells was very diverse. As we previously reported in the human, there was no correlation between the affinity of peptides for soluble HLA-A*02:01 and the prevalence of $CD8^+$ T-cell responses in diabetic mice [19,20,23].

In contrast with class I-restricted epitopes, HLA-DQ8-restricted hPPI epitopes have been ill defined in the human. The dominant responses that were characterized by immunization of YES mice against hPPI peptides were comparable to the most frequent responses detected in diabetic YES-RIP-hB7.1 mice and corresponded to $hPPI_{20-35}$, $hPPI_{25-40}$ and $hPPI_{33-47}$. It has previously been reported that I-A^{g7}-restricted and HLA-DQ8-restricted T-cell responses were observed in the NOD mouse [10,36] and in the human [10] against the B-chain peptide B_{9-23}, that is identical to $hPPI_{33-47}$. Epitopes located in the C-peptide region have also been characterized as recognized by T cell clones obtained from patients with T1D [37]. By contrast, the proliferative responses to $hPPI_{20-35}$ and $hPPI_{25-40}$ that overlap the hPPI leader sequence peptide and B-chain have not been previously reported. Furthermore, individual mice showed responses to $hPPI_{1-15}$, $hPPI_{16-30}$, $hPPI_{55-70}$ and $hPPI_{92-110}$ spanning all over the hPPI sequence. In addition to the previous detection of proliferative responses to $hPPI_{33-47}$ (B_{9-23}) in the human, $CD4^+$ T-cell responses have been detected against $hPPI_{1-24}$ and $hPPI_{44-63}$ but not against $hPPI_{33-47}$ in conventional HLA-DQ8 transgenic mice [38]. Such responses were not observed with YES and YES-RIP-hB7.1 mice. Such marked differences might be related to the fact that by contrast with YES and YES-RIP-hB7.1 mice, conventional HLA-DQ8 transgenic mice expressed murine insulin genes. The $hPPI_{33-47}$ sequence only differs from mINS1 by a S/P substitution at position 33 and is identical to the mINS2 sequence. $hPPI_{20-35}$ and $hPPI_{25-40}$ differ by 2 positions at $hPPI_{27}$ and $hPPI_{33}$ from mINS1 and by one position at $hPPI_{27}$ from mINS2, respectively. In any case, differences between YES or YES-RIP-hB7.1 mice and parental HLA-DQ8 transgenic mice, which have not been reported to respond to $hPPI_{33-47}$, possibly result from differences at the selection level in the thymus. $hPPI_{25-40}$ and $hPPI_{33-47}$ both carry a glutamic acid at position

37 and 45, respectively, which are frequent anchor residues with the HLA-DQ8 P9 binding pocket [39,40].

In conclusion, the YES-RIP-hB7.1 mouse is a new T1D model that will allow evaluating hPPI-based immunotherapy that may directly apply to the human. The YES mouse does not develop spontaneous diabetes, but we have preliminary evidence that T1D is possibly induced in YES mice following immunization against recombinant hPPI in combination with the injection of TLR agonists. Interestingly, in contrast with the NOD mouse, the YES mouse does not develop spontaneous diabetes, probably due to the absence of selection of T1D susceptibility genes other than the HLA-DQ8 gene. Deciphering mechanisms of induction of T1D in the YES model may thus prove informative as referred to the search for environmental factors that may combine to allow breaking of immune tolerance to the islets of Langerhans in the human.

Table 1 LV-RIP-B7.1 insertions

	F-23 offspring		F-36 Founder		F-36 offspring	
	Begin	End	Begin	End	Begin	End
Nb seq in init FASTA/Q file	46385		50831		63167	
% reads mapped (Samtools flagstat)	18,60%	34,25%	41,11%	40,37%	28,69%	24,58%
reads mapped (Samtools flagstat)	257	372	876	1042	683	632
reads	629		1918		1315	
Scaffold sequence alignment	sca-43319		sca-273750 sca-12477 sca-231303 sca-123866		sca-273750	
Region	chr15:11692802-11696801		19qA, insertion A 14qE5, insertion B 11qB5; insertion C 16qB4, insertion D		chr19:15483421-15487432	
Genes in region	None		No Tm9sf2 Smg6 Naa50		None	

Table 2 IFNgamma-ELISpot median values of stimulation scores in diabetic YES-RIP-hB7.1 and YES mice

Peptide	Responses (spot number)				Frequencies of recognition (±3SD)			
	diabetic YES-RIP-B7.1 mice (n=16)	YES mice (n=6)	non-diabetic YES-RIP-B7.1 mice (n=6)	*P* value	diabetic YES-RIP-B7.1 mice	YES mice	non- diabetic YES-RIP-B7.1 mice	*P* value
hPPI $_{2-11}$	133.9 (0-445)	83.50 (0-129)	58.00 (0-440)		7/16	0/6	1/6	
hPPI $_{6-14}$	177.1 (0-440)	49.17 (0-129)	123.2 (0-287)	p≤0.008	9/16	0/6	2/6	≤ 0.005
hPPI $_{15-24}$	135.9 (0-454)	29.50 (0-100)	58.00 (0-195)	p≤ 0.02	9/16	0/6	0/6	≤ 0.005
hPPI $_{30-39}$	83.56 (0-245)	41.83 (0-192)	83.17 (0-379)		1/16	1/6	2/6	
hPPI $_{33-42}$	147.1 (0-487)	102.5 (12-425)	164.8 (42-637)		3/16	1/6	1/6	
hPPI $_{34-42}$	182.3 (0-692)	48.50 (5-104)	94.17(0-420)	p≤ 0.008	9/16	0/6	1/6	≤ 0.005
hPPI $_{42-51}$	160.9 (0-1302)	56.83 (4-104)	62.83 (0-287)		6/16	0/6	1/6	
hPPI $_{101-109}$	171.8 (0-1137)	72.67 (0-175)	163.5 (20-604)		2/16	0/6	1/6	
Control peptide	79.38 (0-298)	78.17 (0-195)	103.2 (0-343)		4/16	1/6	1/6	

Table 3. Anti-hPPI proliferative responses in diabetics YES-RIP-hB7.1 and YES mice.

Peptide	Responses (proliferation index)				Frequencies of recognition (±3SD)				
	diabetics YES-RIP-B7.1 mice (n=24)	YES mice (n=26)	no-diabetics YES-RIP-B7.1 mice (n=25)	*P* value	diabetics YES-RIP-B7.1 mice	YES mice	no diabetics YES-RIP-B7.1 mice	*P* value
hPPI $_{1-15}$	1.63 (0.5-4.2)	1.01 (0.6-1.9)	1.43 (0.3-4.5)	p≤0.005	5/24	0/26	3/25	≤ 0.05
hPPI $_{16-30}$	1.44 (0.5-3.5)	0.97 (0.2-1.9)	1.24 (0.1-2.4)	p≤0.005	4/24	0/26	2/25	≤ 0.05
hPPI $_{18-30}$	1.01 (0.7-1.5)	0.92 (0.6-1.4)	0.9 (0.3-2)		0/24	0/26	0/25	
hPPI $_{20-35}$	1.76 (0.8-3.1)	1.14 (0.7-2.3)	1.46 (0.5-5)	p≤ 0.0001	7/24	1/26	2/25	≤ 0.05
hPPI $_{25-40}$	1.78 (0.5-3.9)	1.04 (0.5-1.9)	1.74 (0.2-6)	p≤ 0.0005	7/24	0/26	5/25	≤ 0.005
hPPI $_{33-47}$	1.45 (0.4-2.6)	0.99 (0.3-1.9)	1.35 (0.2-2.9)	p≤ 0.0025	4/24	0/26	3/25	≤ 0.05
hPPI $_{55-70}$	1.18 (0.5-3.5)	1.02 (0.6-1.9)	0.83 (0.3-1.6)		1/24	0/26	0/25	
hPPI $_{80-97}$	1.45 (0.6-2.6)	1.11 (0.5-2.7)	1.21 (0.6-3.6)	p≤0.01	4/24	1/26	1/25	
hPPI $_{92-110}$	1.72 (0.5-4.9)	1.11 (0.5-2)	1.63 (0.2-6.1)	p≤0.04	6/24	0/26	3/25	≤ 0.01
hPPI #	1.41 (0.4-3)	0.94 (0.4-1.7)	1.41 (0.1-2.7)	p≤ 0.005	4/24	1/26	5/25	≤ 0.05
Nef $_{Cys66-97}$	1.48 (0.4-2.8)	1.0 (0.3-2)	0.95 (0.1-2.1)		2/24	0/26	0/25	

EXAMPLE 2:

Identifying peptides derived from autoantigens and that are presented to T lymphocytes, especially CD4+ T-cells, is a key issue in type 1 diabetes. It will open new avenues in setting up assays that will be used as tests in diabetes diagnosis and in developing strategies to induce immune tolerance to prevent type 1 diabetes in prediabetic individuals or dampen the autoimmune process in diabetic patients. Characterization of peptides derived from human insulin or its precursors (human preproinsulin, hPPI) is especially important considering the direct (Thebaut-Baumont et al. J Clin Invest 2003) and indirect evidence that it is the key autoantigen in the disease process. The YES mouse that we now report will be instrumental in both avenues. It has allowed us to define peptides that are recognized by $CD8^+$ T cells upon their presentation by the class I allele HLA*0201, that is the most common in Caucasians and shows a genetic association with type 1 diabetes (see Table 3 as above mentioned). Among the many HLA*0201-restricted PPI peptides that we identified in YES mice that develop autoimmune diabetes upon expression of the T-cell coactivator hB7.1 under the control of the rat insulin promotor (RIP), in particular two peptides located in the leader sequence and a peptide located in the insulin B chain, all were confirmed as relevant to the human disease whatever the detection techniques that we used in the human: either γ-interferon elispot assays or peptide-HLA*0201 tetramers that both detected an increased number of peptide-specific $CD8^+$ T cells in the human (Luce et al. Diabetes 60:3289-3299, 2011). Beyond further exemplifying the key role of hPPI as a key autoantigen in the disease process, these data validate the YES mouse and its YES-RIP-hB7.1 counterpart as relevant models to characterize PPI peptides that are recognized by T cells and are relevant to the human disease. It also allows characterizing the immune phenotype of autoreactive PPI-specific T-cells. Indeed, the analysis of these cells has enabled us to highlight that PPI-specific $CD8^+$ T-cells express central memory profile in diabetic patients, suggesting that PPI-specific $CD8^+$ T-cells were present in inflammatory site at the beginning of autoimmune reaction. It is thus likely that human PPI peptides that we have defined in YES and YES-RIP-hB7.1 mice as restricted by the diabetes high susceptibility class II allele HLA-DQ8, which is encoded by the gene endowed with the highest susceptibility score in type 1 diabetes, will also directly apply the human disease. Based on data obtained studying $CD8^+$ T-cells, corresponding tools for studying PPI-specific $CD4^+$ T-cell are indeed expected to bring similar advances and open the way to define new bioassays and new perspective in immunotherapy of human type 1 diabetes.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A HLA-DQ8-restricted human preproinsulin peptide selected from the group consisting of:

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")

   - a peptide consisting of 8 consecutive amino acids selected in the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110")

2. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 which comprises 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, or 35 amino acids.

3. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 which is selected from the group consisting of

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")

- a peptide consisting of a sequence having at least 90% of identity with the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110").

4. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 which is selected from the group consisting of

- a peptide consisting of the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 15 in SEQ ID NO:1 ("hPPI1-15")

- a peptide consisting of the sequence ranging from the amino acid residue at position 16 to the amino acid residue at position 30 in SEQ ID NO:1("hPPI16-30")

- a peptide consisting of the sequence ranging from the amino acid residue at position 20 to the amino acid residue at position 35 in SEQ ID NO:1("hPPI20-35")

- a peptide consisting of the sequence ranging from the amino acid residue at position 25 to the amino acid residue at position 40 in SEQ ID NO:1("hPPI25-40")

- a peptide consisting of the sequence ranging from the amino acid residue at position 80 to the amino acid residue at position 97 in SEQ ID NO:1("hPPI80-97")

- a peptide consisting of the sequence ranging from the amino acid residue at position 92 to the amino acid residue at position 110 in SEQ ID NO:1("hPPI92-110").

5. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 which consists of the sequence ranging from the amino acid residue at position 1 to the amino acid residue at position 30 in SEQ ID NO:1.

6. The HLA-DQ8-restricted human preproinsulin peptide according to any one of claims 1-5 which is fused to a heterologous polypeptide to form a fusion protein.

7. The HLA-DQ8-restricted human preproinsulin peptide of claim 6 wherein the heterologous polypeptide comprises at least one redox motif C—(X)2-[CST] or [CST]-(X)2-C.

8. The HLA-DQ8-restricted human preproinsulin peptide according to any one of claims 1-5 which is fused or conjugated to an antibody for forming an immunoconjugate.

9. A nucleic acid encoding for the HLA-DQ8-restricted human preproinsulin peptide peptide of claim 1

10. A method for treating Type 1 Diabetes in a subject in need thereof comprising the subject with a therapeutically effective amount of the HLA-DQ8-restricted human preproinsulin peptide peptide of claim 1 or the nucleic acid of claim 9.

11. A vaccine composition comprising the HLA-DQ8-restricted human preproinsulin peptide peptide of claim 1 or the nucleic acid of claim 9.

12. The vaccine composition which comprises an adjuvant which is Incomplete Freund's adjuvant (IFA) or other oil based adjuvant.

13. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 which is loaded in MHC class II multimers.

14. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 or 13 for preparing polyclonal T cells and T cell lines or clones recognizing the HLA-DQ8-restricted human preproinsulin peptide of claim 1.

15. The HLA-DQ8-restricted human preproinsulin peptide of claim 1 or 13 for detecting autoreactive T cells specific for the human preproinsulin polypeptide of claim 1.

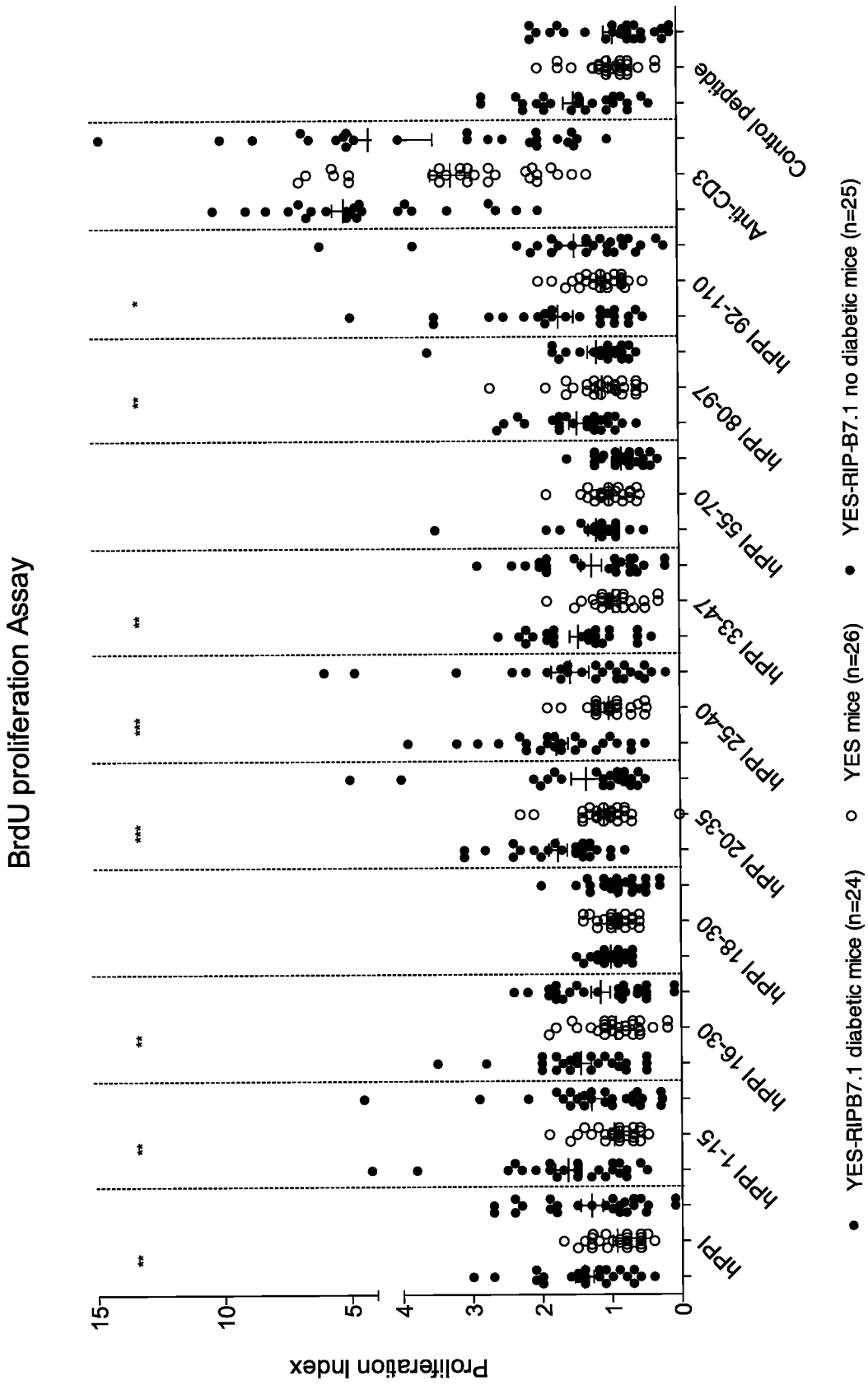
BrdU proliferation Assay
Proliferation Index
0
1
2
3
4
5
10
15
hPPI
hPPI 1-15
hPPI 16-30
hPPI 18-30
hPPI 20-35
hPPI 25-40
hPPI 33-47
hPPI 55-70
hPPI 80-97
hPPI 92-110
Anti-CD3
Control peptide
YES-RIPB7.1 diabetic mice (n=24)
YES mice (n=26)
YES-RIP-B7.1 no diabetic mice (n=25)

Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/053446

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/00 C07K14/74
ADD.

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)
A61K C07K

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)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 27 May 2010 (2010-05-27), "Human insulin B chain peptide, SEQ ID 8.", XP002742580, retrieved from EBI accession no. GSP:AXX42529 Database accession no. AXX42529 sequence ----- -/--	1-4,9

[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
18 March 2016	11/04/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 18 July 2013 (2013-07-18), "Human insulin A-chain (residues 90-110), SEQ ID 6.", XP002742581, retrieved from EBI accession no. GSP:BAP08311 Database accession no. BAP08311 sequence -----	1-4,9
X	ROBERTO MALLONE ET AL: "T Cell Recognition of Autoantigens in Human Type 1 Diabetes: Clinical Perspectives", CLINICAL AND DEVELOPMENTAL IMMUNOLOGY, vol. 356, no. 9229, 1 January 2011 (2011-01-01), pages 545-16, XP055187114, ISSN: 1740-2522, DOI: 10.1016/S0168-8227(00)00225-4 the whole document table 2 -----	1-15
X	RAJU RAGHAVANPILLAI ET AL: "T cell recognition of human pre-proinsulin peptides depends on the polymorphism at HLA DQ locus: A study using HLA DQ8 and DQ6 transgenic mice", HUMAN IMMUNOLOGY, NEW YORK, NY, US, vol. 58, no. 1, 1 November 1997 (1997-11-01), pages 21-29, XP002677789, ISSN: 0198-8859 the whole document -----	1-15
X	ASTILL T P ET AL: "Promisuous binding of proinsulin peptides t type 1 diabetes-permissive and -protective HLA class II molecules", DIABETOLOGIA, SPRINGER, BERLIN, DE, vol. 46, no. 4, 1 April 2003 (2003-04-01), pages 496-503, XP008094668, ISSN: 0012-186X the whole document -----	1-15
X	WO 2015/006833 A1 (ST VINCENTS INST MED RES [AU]) 22 January 2015 (2015-01-22) paragraphs [0007], [0009], [0034] - [0035], [0068] - [0076], [0160] - [0161] abstract table 1 example 3 figures 11,12,14 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/053446

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015006833 A1	22-01-2015	NONE	