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(54) Title: DIAGNOSTIC AGENTS AND USES THEREFOR

(57) Abstract: The present invention discloses methods and agents for diagnosing the presence or risk of developing type 1 diabetes in animals, especially vertebrate animals. Also disclosed are novel surrogate markers for this disease including members of the nuclear factor kB (NF-kB) pathway. The present invention also extends to methods for treating or preventing type 1 diabetes, which employ the diagnostic method of the invention.

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“DIAGNOSTIC AGENTS AND USES THEREFOR”

FIELD OF THE INVENTION

[0001] The present invention relates generally to methods and agents for
5 diagnosing the presence or risk of development of type 1 diabetes in animals, especially
vertebrate animals. More particularly, the present invention relates to the use of
members of the nuclear factor κ B (NF- κ B) pathway as surrogate markers for this
disease. The present invention also extends to methods for treating or preventing type 1
diabetes, which employ the diagnostic methods and agents of the invention.

BACKGROUND OF THE INVENTION

[0002] In the case of autoimmune disease, it is the antigen presenting cells
(APC), such as dendritic cells (DC) which initiate T-cell mediated immune responses.
Dendritic cells originate from hematopoietic progenitors in the bone marrow and traffic
into the blood to peripheral tissues. Their numbers may be increased in circulation by
15 granulocyte colony-stimulating factor (G-CSF), exercise, and surgical stress, with
kinetics similar to those of granulocytes (Ho *et al.*, 2001, *Blood*, 98; 140). DC are
important immunostimulatory and immunomodulatory antigen presenting cells
regulating health, but also play important roles in the initiation and perpetuation of
autoimmune diseases (Thompson *et al.*, 2002, *Immunol. Cell. Biol.* 80; 164-169;
20 Summers *et al.*, 2003, *Ann N Y Acad Sci*, 1005; 226-229). Mature DC express high
major histocompatibility complex (MHC) and costimulatory molecules, secrete
interleukin (IL)-12, and stimulate various T-helper 1 responses (Ma *et al.*, 2000,
Diabetes, 52; 1976-1985). By contrast, immature DC expressing low costimulatory
molecules induce antigen specific hyporesponsiveness by triggering T-cell apoptosis
25 (Lu *et al.*, 1995, *Transplantation*, 60; 1539-1545). DC also participate in tolerance,
through thymic negative selection of autoreactive T-cells, and presentation of self-
antigens (Ag) in the periphery, in a tolerogenic fashion. In infection and stress they
mature rapidly in response to pathogen associated molecular patterns (Gallucci and
Matzinge, 2001, *Curr Opin Immunol* 13(1); 114-119), and acquire both active NF- κ B,
30 and the ability to stimulate Ag specific pathogenic immunity.

[0003] The NF- κ B family of molecules is essential for cell differentiation,
viability, and activation. The family is also a key transducer of inflammatory signals

and is important in the maturation and activation of DC (Rescigno *et al.*, 1998, *J Exp Med*, 188; 2175-2180). NF- κ B is a transcription factor family which exists as homo or heterodimers of p50/p105, p52/p100, p65 (RelA), RelB and c-Rel. RelA, RelB and c-Rel contain transcriptional activation domains at their C-terminus, allowing p50 and p52 to form dimers with the three molecules which consequently lose their C-terminal domains when processed from p105 or p100 respectively (Xiao *et al.*, 2001, *Mol Cell*, 7(2); 401-409; Betts and Nabel, 1996, *Mol Cell Biol*, 16(11); 6363-6371). In resting cells, NF- κ B dimers are present in an inactive form in the cytoplasm bound to inhibitor proteins of the I- κ B family. The I- κ B family consists of I- κ B β and NF- κ B essential modulator (NEMO) in the classical activation pathway and the p100 inhibitory precursor of p52, which participates with RelB in the alternative activation pathway, complexing with I- κ B α (Baldwin, 1996, *Ann Rev Immunol*, 14; 649-683). In the classical pathway I κ B is ubiquitinated and degraded in the proteasome after stimulation by I κ B kinases IKK- β and IKK- γ . In the alternative pathway, NF- κ B inducing kinase (NIK) and IKK- α stimulate the phosphorylation and processing of p100 (Senftleben *et al.*, 2001, *Science*, 293(5534); 1495-1499) to p52 in the proteasome, releasing p52/RelB dimers for nuclear translocation (Solan *et al.*, 2001, *J Biol Chem*, 277(2); 1405-1418).

[0004] Various stimuli, including cytokines, lipopolysaccharides (LPS), and mitogens, lead to the dissociation of the NF- κ B/I κ B complex by degrading I κ B and allowing the nuclear translocation of free NF- κ B (Rescigno *et al.*, 1998, *J Exp Med*, 188; 2175-2180). For example, tumour necrosis factor alpha (TNF- α), LPS and colony stimulating factor 154 (CD154) signal through the adaptor molecule TNF receptor-associated factor 6 (TRAF6) and activate NF- κ B allowing translocation of released NF- κ B dimers to the nucleus and binding of NF- κ B to a specific DNA motif that regulates transcription of target genes (Baldwin, 1996, *Ann Rev Immunol*, 14; 649-683; O'Sullivan *et al.*, 2002, *J Immunol*, 168(11): 5491-5498). TRAF6 functions as a ubiquitin ligase, recruiting various kinases into a multimeric complex after cell activation, leading to the phosphorylation of I κ B, p100 and p38 MAPK, through MKK6. Activated NF- κ B promotes the expression of many genes, of which the majority participate in the regulation of host immune systems designated as 'central mediators of the immune response' (Pahl, 1999, *Oncogene*, 18(49); 6853-6866).

[0005] SHP-1 is an NF- κ B pathway inhibitor (Neznanov *et al.*, 2004, *DNA Cell Biol*, 23(3); 175-182), potentially acting upstream of NF- κ B at the level of the adaptor molecule TRAF6. In contrast, another similar tyrosine phosphatase, Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) is a positive
5 regulator of NF- κ B (Neznanov *et al.*, 2004, *DNA Cell Biol*, 23(3); 175-182; Khaled *et al.*, 1998, *Clin Immunol Immunopath*, 86(2); 170-179; Massa and Wu, 1998, *J Interferon Cytokine Res*, 18(7); 499-507). SHP-1 and SHP-2 are very similar classical non-receptor protein tyrosine phosphatases, abundant in haematopoietic cells with about 59% sequence homology, which mainly differ in the 100 amino acids residues in the C-
10 terminus, and play negative and positive regulatory roles in cell signalling respectively (Poole and Jones, 2005, *Cell Signal*, 17(11); 1323-1332).

[0006] While other NF- κ B molecules contribute to the immune response, RelB has been most directly associated with DC differentiation and functional maturation, and its expression is upregulated early during differentiation from a variety
15 of progenitors. RelB is detected in the nucleus of mature interdigitating DC of the lymphoid organs, in inflamed tissues, and in *in vitro* systems. Genes specifically activated or repressed by RelB are largely unknown. However, RelB has been shown to transcriptionally activate the major histocompatibility complex class I (MHC class I) gene and RelB deficient DC specifically lack expression of CD40 (Martin *et al.*, 2003, *Immunity*, 18(1); 155-167), (Platzer *et al.*, *Blood*, 104(12); 3655-3663; O'Sullivan *et al.*,
20 2000, *Proc Natl Acad Sci USA*, 97(21); 11421-11426).

[0007] Furthermore, RelB-containing NF- κ B complexes transactivate MHC Class I promoters in breast cancer cells suggesting RelB regulates MHC class I protein expression (Dejardin *et al.*, 1998, *Oncogene*, 16(25): 3299-3307). CD40 plays an
25 important role in maintaining tolerance to self antigen and CD40 and CD154^{-/-} mice are predisposed to autoimmune disease development (Kumanogoh *et al.*, 2001, *J Immunol*, 166(1): 353-360).

[0008] By contrast, mice deficient in RelB, NF- κ B inducing kinase (NIK) or TRAF6 develop a complex inflammatory phenotype, including multi-organ
30 autoimmune inflammatory disease, sialitis, pancreatitis, atopic dermatitis-like lesions, and susceptibility to *Listeria* infection (Weih *et al.*, 1997, *J Exp Med*, 185(7); 1359-1370; Barton *et al.*, 2000, *Eur J Immunol*, 30(8); 2323-2332). These mice also have reduced numbers of thymic dendritic cells, thymic medullary epithelial cells, and

display reduced negative selection and increased numbers of autoreactive T-cells in the periphery (Burkly *et al.*, 1995, *Nature*, 373(6514); 531-536; Weih *et al.*, 1995, *Cell*, 80(2); 331-340). Recent studies demonstrate that RelB inhibition impairs monocyte-derived DC development, with no effect on other myeloid differentiation pathways *in vitro* (Platzer *et al.*, 2004, *Blood*, 104(12); 3655-3663).

[0009] Deficient differentiation of monocyte-derived DC, with low yields and low B7 expression, have been demonstrated in recently diagnosed autoimmune disease patients and first-degree relatives (Takahashi *et al.*, 1998, *J Immunol*, 161(5); 2629-2635; Jansen *et al.*, 1995, *Lancet*, 345(8948); 491-492). Furthermore, a decrease in human leukocyte antigen (HLA)-DR, CD11c and TNF- α expression in DC from newly diagnosed autoimmune disease children has also been identified (Skarsvik *et al.*, 2004, *Scand J Immunol*, 60(6); 647-652).

[0010] There are a number of different therapies currently in use for treating autoimmune disease and preventing progression of the disease. These include the use of immunosuppressive drugs such as cyclosporine A (CsA), which blocks T-cell activation through the inhibition of calcineurin (Skyler *et al.*, 1992, *J Diabetes*, 6; 77-88); restoration of islet cell function by directly targeting autoreactive T-cells or by inducing regulatory cells or mechanisms that re-establish the tolerant state using a short course of treatment (Masteller and Bluestone, 2002, *Curr Opin Immunol*, 14; 652-659) and insulin replacement strategies (Ergun-Longmire *et al.*, 2004, *Ann N Y Acad Sci*, 1029; 260-277).

[0011] Several self-molecules have been identified as target antigens in autoimmune diseases. Since lack or loss of tolerance to these molecules is one of the key events promoting autoimmunity, researchers are exploring the possibility that the administration of antigens or peptides may stimulate tolerogenic mechanisms and delay or prevent the full phenotypic expression of autoimmune diseases, including type 1 diabetes (Pugliese, 2003, *J Clin Invest*, 111; (1280-1282).

[0012] Type 1 diabetes mellitus (T1DM) is an organ specific autoimmune disease which occurs when the immune systems T-cells mistakenly attack the insulin secreting β -cells of the islets of Langerhans within the pancreas (Ma *et al.*, 2003, *Diabetes*, 52; 1976-1985). The nature of immune dysregulation leading to β -cell destruction remains poorly understood, but is clearly influenced by multiple genetic and immunological factors (Knip, 1997, *Ann Med*, 29; 447-451).

[0013] The present invention is based in part on the discovery of novel surrogate markers of T1DM and the likely role of altered signaling thresholds in the pathogenesis for this disease. This discovery has been reduced to practice in the form of surrogate markers for patients with T1DM and methods for treating or preventing these diseases, as described hereafter.

SUMMARY OF THE INVENTION

[0014] The present invention discloses methods for detecting T1DM. Surrogate markers for this disease in the form of genes and their products have been identified and are described. These genes and gene products can be used in gene expression assays, protein expression assays, whole cell assays, and in the design and manufacture of therapies. They can also be used to determine the presence or risk of T1DM in animals with or without clinical signs of disease. It is proposed that such assays, when used frequently as an indicator of risk to T1DM or response to this disease or its progression, will lead to better management decisions and treatment regimes.

[0015] The present invention represents a significant advance over current technologies for the management of subjects having or at risk of developing T1DM. In certain advantageous embodiments, it relies upon measuring the level of certain markers in cells, including white blood cells, of the host rather than detecting autoantibodies. As such, these methods are suitable for widespread screening of symptomatic and asymptomatic subjects. In certain embodiments where circulating leukocytes are the subject of analysis, the detection of T1DM, or its risk of development, is feasible at very early stages of its progression, before autoantibodies can be detected in serum. Advantageous embodiments involve monitoring the expression of certain genes in peripheral leukocytes, which may be reflected in changing patterns of RNA levels or protein production that correlate with the presence or risk of development of T1DM.

[0016] Accordingly, in one aspect, the present invention provides methods for diagnosing the presence or risk of development of T1DM, in a subject. These methods generally comprise detecting in the subject aberrant signaling through the NF- κ B pathway in response to a pro-inflammatory signal. Representative members of the NF- κ B pathway include, but are not limited to, BTK, LYN, BCR Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK, RelA/p65, P105/p50, c-Rel, RelB, p52, NIK, Leu13, CD81,

CD19, CD21 and its ligands in the complement and coagulation cascade, ubiquitin mediated proteolysis, TRAF6, ubiquitin ligase, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, Slp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP-2, SAP, SLAM, PKR, 2B4, SHP-1, SHIP, PIR-B, CD22, CD72, 5 Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl, KIR3DL1, KIR3DL2, KIR2DL and Csk. Illustrative pro-inflammatory signals include tumor necrosis factor (*e.g.*, TNF α), C5a, interleukin-1 (*e.g.*, IL-1 β), CD154 and lipopolysaccharide (LPS).

[0017] Aberrant signaling of the NF- κ B pathway is suitably detected by detecting aberrant expression of a gene involved in or belonging to that pathway.

10 Typically, such aberrant expression is detected by: (1) measuring in a biological sample obtained from the subject the level or functional activity of an expression product of at least one gene belonging to the NF- κ B pathway and (2) comparing the measured level or functional activity of each expression product to the level or functional activity of a corresponding expression product in a reference sample obtained from one or more 15 normal subjects or from one or more subjects lacking disease, wherein a difference in the level or functional activity of the expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the presence or risk of development of T1DM in the subject. In some embodiments, the methods further comprise diagnosing the 20 presence, stage or degree or risk of development of T1DM in the subject when the measured level or functional activity of the or each expression product is different than the measured level or functional activity of the or each corresponding expression product. In these embodiments, the difference typically represents an at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, or even an at least about 100%, 25 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% increase, or an at least about 10%, 20%, 30% 40%, 50%, 60%, 70%, 80%, 90%, 92%, 94%, 96%, 97%, 98% or 99%, or even an at least about 99.5%, 99.9%, 99.95%, 99.99%, 99.995% or 99.999% decrease in the level or functional activity of an individual expression product as compared to the level or functional activity of an individual corresponding 30 expression product. In illustrative examples of this type, the presence or risk of development of T1DM is determined by detecting a decrease in the level or functional activity of an expression product of at least one gene involved in or belonging to the NF- κ B pathway, which is suitably selected from genes that encode BTK, LYN, BCR

Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK, RelA/p65, P105/p50, c-Rel, RelB, p52, NIK, Leu13, CD81, CD19, CD21 and its ligands in the complement and coagulation cascade, TRAF6, ubiquitin ligase, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, Slp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP2, SAP, SLAM, PKR and 2B4. In specific embodiments, the presence or risk of development of T1DM is determined by detecting a decrease in the level or functional activity of an expression product of at least one gene selected from genes that encode RelA/p65, P105/p50, c-Rel, RelB or p52.

[0018] In other illustrative examples, the presence or risk of development of T1DM is determined by detecting an increase in the level or functional activity of an expression product of at least one gene belonging to the NF- κ B pathway, which is suitably selected from genes that encode SHP1, SHIP, PIR-B, CD22, CD72, Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl, KIR3DL1, KIR3DL2, KIR2DL and Csk. In specific embodiments, the presence or risk of development of T1DM is determined by detecting an increase in the level or functional activity of an expression product of a gene that is common to both the canonical and non-canonical NF- κ B pathways. In illustrative examples of this type, the gene is the SHP-1 gene.

[0019] In some embodiments, the method further comprises diagnosing the absence of T1DM or a low risk of developing that disease when the measured level or functional activity of the or each expression product is the same as or similar to the measured level or functional activity of the or each corresponding expression product. In these embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%.

[0020] As used herein, polynucleotide expression products of genes involved in or belonging to the NF- κ B pathway are referred to herein as "T1DM marker polynucleotides." Polypeptide expression products of gene members of the NF- κ B pathway are referred to herein as "T1DM marker polypeptides."

[0021] In some embodiments, the methods comprise measuring the level or functional activity of individual expression products of at least about 2, 3, 4, 5, 6, 7, 8,

9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or even 30, 40 or 50 genes belonging to the NF- κ B pathway. For example, the methods may comprise measuring the level or functional activity of a T1DM marker polynucleotide either alone or in combination with as much as 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 other T1DM marker polynucleotide(s). In another example, the methods may comprise measuring the level or functional activity of a T1DM marker polypeptide either alone or in combination with as much as 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 other T1DM marker polypeptides(s).

[0022] Advantageously, the biological sample comprises blood, especially peripheral blood, which suitably includes leukocytes. In certain embodiments, the expression product is selected from a RNA molecule or a polypeptide. In some embodiments, the expression product is the same as the corresponding expression product. In other embodiments, the expression product is a variant (*e.g.*, an allelic variant) of the corresponding expression product. In illustrative examples, the biological sample comprises cells, which have been exposed to a pro-inflammatory signal. In these examples, the reference sample also comprises cells, which have been exposed to the pro-inflammatory signal.

[0023] In certain embodiments, the expression product or corresponding expression product is a target RNA (*e.g.*, mRNA) or a DNA copy of the target RNA whose level is measured using at least one nucleic acid probe that hybridizes under at least low, medium, or high stringency conditions to the target RNA or to the DNA copy, wherein the nucleic acid probe hybridizes to at least 15 contiguous nucleotides of a T1DM marker polynucleotide. In these embodiments, the measured level or abundance of the target RNA or its DNA copy is normalized to the level or abundance of a reference RNA or a DNA copy of the reference RNA that is present in the same sample. Suitably, the nucleic acid probe is immobilized on a solid or semi-solid support. In illustrative examples of this type, the nucleic acid probe forms part of a spatial array of nucleic acid probes. In some embodiments, the level of nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by hybridization (*e.g.*, using a nucleic acid array). In other embodiments, the level of nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by nucleic acid amplification (*e.g.*, using a polymerase chain reaction (PCR)). In still other embodiments, the level of

nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by nuclease protection assay.

[0024] In other embodiments, the expression product or corresponding expression product is a T1DM marker polypeptide whose level is measured using at least one antigen-binding molecule that is immuno-interactive with the T1DM marker polypeptide. In these embodiments, the measured level of the T1DM marker polypeptide is normalized to the level of a reference polypeptide that is present in the same sample. Suitably, the antigen-binding molecule is immobilized on a solid or semi-solid support. In illustrative examples of this type, the antigen-binding molecule forms part of a spatial array of antigen-binding molecule. In some embodiments, the level of antigen-binding molecule that is bound to the T1DM marker polypeptide is measured by immunoassay (*e.g.*, using an ELISA).

[0025] In still other embodiments, the expression product or corresponding expression product is a T1DM marker polypeptide whose level is measured using at least one substrate for that polypeptide with which it reacts to produce a reaction product. In these embodiments, the measured functional activity of the T1DM marker polypeptide is normalized to the functional activity of a reference polypeptide that is present in the same sample.

[0026] In other embodiments, the expression product or corresponding expression product is a T1DM marker polypeptide whose level is measured using at least one oligonucleotide that binds to a nucleic acid binding site of the marker polypeptide (*e.g.*, RelB, p65, p50 etc). In illustrative examples, the oligonucleotide comprises a detectable label and the level of oligonucleotide that is bound to the T1DM marker polypeptide is measured by quantifying the amount of detectable label that associates with the polypeptide (*e.g.*, by autoradiography, fluorimetry, luminometry, or phosphoimage analysis).

[0027] In other embodiments, aberrant signaling of the NF- κ B pathway is detected by detecting aberrant phosphorylation of a polypeptide involved in or belonging to the NF- κ B signaling pathway. In specific embodiments, aberrant phosphorylation is detected by: (1) determining in a biological sample obtained from the subject the phosphorylation state of at least one phosphorylatable polypeptide selected from IRAK, TAK1, TAB1, TAB2, PKR, Akt, Cot, IKK α , IKK β and IKK γ ; and (2) comparing each determined phosphorylation state to the phosphorylation state of a

corresponding phosphorylatable polypeptide in a reference sample obtained from one or more normal subjects or from one or more subjects lacking disease, wherein a difference in the phosphorylation state of the phosphorylatable polypeptide in the biological sample as compared to the phosphorylation state of the corresponding
5 phosphorylatable polypeptide in the reference sample is indicative of the presence or risk of development of T1DM in the subject.

[0028] In another aspect, the present invention provides methods for treating, preventing or inhibiting the development of T1DM in a subject. These methods generally comprise detecting in the subject aberrant signaling through the NF- κ B
10 pathway in response to an inflammatory signal, and administering to the subject an effective amount of an agent that treats or ameliorates the symptoms or reverses or inhibits the development of T1DM in the subject. Representative examples of such treatments or agents include, but are not limited to, anti-CD3 therapy (*e.g.*, monoclonal antibody hOKT3gamma1 (Ala-Ala)) and antigen-specific tolerogenic therapies.

15 [0029] In still another aspect, the present invention provides probes for interrogating nucleic acid for the presence of a T1DM marker polynucleotide as broadly described above. These probes generally comprise a nucleotide sequence that hybridizes under at least low stringency conditions to a T1DM marker polynucleotide as broadly described above.

20 [0030] In yet another aspect, the present invention provides oligonucleotides for interrogating protein samples for the presence of a T1DM marker polypeptide that comprises a nucleic acid binding site. These oligonucleotides generally comprise a nucleotide sequence that binds to the nucleic acid binding site of the T1DM marker polypeptide (*e.g.*, RelB, p65, p50 etc).

25 [0031] In related aspects, the invention provides a solid or semi-solid support comprising at least one nucleic acid probe as broadly described above or at least one polypeptide-binding oligonucleotide as broadly described above immobilized to the support. In some embodiments, the solid or semi-solid support comprises a spatial array of nucleic acids immobilized thereon.

30 [0032] Still a further aspect of the present invention provides an antigen-binding molecule that is immuno-interactive with a T1DM marker polypeptide as broadly described above.

[0033] In a related aspect, the invention provides a solid or semi-solid support comprising at least one antigen-binding molecule as broadly described above immobilized thereon. In some embodiments, the solid or semi-solid support comprises a spatial array of antigen-binding molecules immobilized thereon.

5 [0034] Still another aspect of the invention provides the use of one or more T1DM marker polynucleotides as broadly described above, or the use of one or more probes as broadly described above, or the use of one or more T1DM marker polypeptides as broadly described above, or the use of one or more antigen-binding molecules as broadly described above, or the use of one or more polypeptide-binding
10 oligonucleotides as broadly described above, in the manufacture of a kit for diagnosing the presence of T1DM in a subject.

[0035] The aspects of the invention are directed to the use of the diagnostic methods as broadly described above, or one or more T1DM marker polynucleotides as broadly described above, or the use of one or more probes as broadly described above,
15 or the use of one or more T1DM marker polypeptides as broadly described above, or the use of one or more antigen-binding molecules as broadly described above, or the use of one or more polypeptide-binding oligonucleotides as broadly described above, for diagnosing the presence or risk of development of T1DM in animals, especially vertebrates animals including mammals.

20 BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Figure 1a is a photographic representation of an immunoblot and a Ponceau S protein stained gel. The immunoblot shows dysregulated LPS-induced nuclear translocation and RelB DNA binding but not transcription of NF- κ B subunits in T1DM (autoimmune disease) dendritic cells (DC). Cytoplasmic or nuclear extracts were
25 prepared from 72 h cultured DC from autoimmune disease or healthy control individuals with or without 100 ng/mL LPS for the last 24 h of culture. 20 μ g protein was loaded to each well of the gel and electrophoresed then nitrocellulose membranes were immunoblotted with individual NF- κ B subunit antibodies, stripping and blocking between each one. Representative of five experiments using DC from different donors.
30 The Ponceau S protein stained gel shows that DC from T1DM patient populations behaved similarly to DC from healthy controls.

[0037] Figure 1b is a graphical representation showing that RelB DNA binding was significantly lower in T1DM DC than healthy control DC after 24h treatment with LPS. 10 µg of nuclear extract from DC prepared from healthy controls or patients with autoimmune disease, RA or T2DM which were added to wells of a NF-κB consensus oligonucleotide coated ELISA plate. The absorbance of the ELISA was read at 650 nm. Data was analyzed using unpaired t tests with statistical confidence measures of (* p < 0.05, ** p < 0.01, *** p < 0.001).

[0038] Figure 2 is a graphical representation showing that in all groups, RelB DNA binding after LPS was significantly higher than that of T1DM patients. 10 µg of nuclear extract from DC prepared from healthy controls or patients with autoimmune disease, RA or T2DM which were added to wells of a NF-κB consensus oligonucleotide coated ELISA plate. The absorbance of the ELISA was read at 650 nm. Data was analyzed using unpaired t tests with statistical confidence measures of (* p < 0.05, ** p < 0.01, *** p < 0.001).

[0039] Figure 3 is a graphical representation showing that there were no significant differences between T1DM DC and healthy controls in the relative mRNA levels of RelA, RelB, c-Rel or p50 after 24 h of LPS. DC were prepared from healthy controls and patients with autoimmune disease. NF-κB gene expression levels were measured as fold over untreated samples by real-time PCR. The bar represents the mean value of the data and there were no significant differences between controls and autoimmune disease patients.

[0040] Figure 4 is a graphical representation showing a normal p38 MAPK response in DC from patients with T1DM. Phosphorylation of p38 MAPK was detected with a specific mAb before or after LPS treatment for 5-30 min. Phosphorylation of p38 MAPK (T180/Y182) in 100ng/mL of LPS was measured at the indicated time points and seems to be similar in both autoimmune disease DC and control DC.

[0041] Figure 5 is a graphical representation showing that when monocyte-derived DC from T1DM or control subjects were differentiated for 72 h, they expressed CD1a, low levels of surface CD40, HLA-DR, MHC class I, CD86, and CD80, but neither CD14 nor CD83 consistent with an immature DC phenotype. Samples were taken from healthy controls or patients with autoimmune disease, RA or T2DM and were further stained for CD40, MHC class I, HLA-DR, CD80, CD86 and CD83 expression. Each sample was also analysed by flow cytometry (a). ELISA

measurements of culture supernatants to test for the production of IL-1 β , TNF α , IL-10 and IL-12 (b). Data were analyzed using paired t tests comparing groups with and without LPS treatment, and unpaired t tests comparing different groups.

[0042] Figure 6 is a graphical representation showing that LPS-induced CD40 expression is increased in me^v/me^v splenic DC and that SHP-1 is dysregulated in T1DM PBMC. Purified CD11c⁺ splenic DC from me^v/me^v or wt mice were cultured with or without 100 ng/mL of LPS overnight. Samples were stained for CD40 and CD86 expression (a). PBMC from 13 healthy control subjects and 18 T1DM patients were cultured for 48h, then permeabilised and stained for SHP-1. Data are expressed as Δ MFI, determined by subtracting the mean fluorescence intensity (MFI) of the isotype control from that of SHP-1-stained cells in gated monocytes. ** p < 0.01 (b). PBMC from 7 healthy control subjects and 4 T1DM patients were cultured for 30 min with or without 100 ng/mL of LPS, then permeabilised and stained for phospho-I- κ B α . Data are expressed as Δ MFI, determined by subtracting the MFI of the isotype control from that of phospho-I- κ B α -stained cells in gated monocytes. ** p < 0.01 (c) PBMC from 31 healthy control or T1DM patients were cultured for 48h, then permeabilised and stained for SHP-1. Δ MFI of SHP-1 was determined for gated lymphocytes and monocytes in each sample, and then plotted. R² = 0.4573, p < 0.0001 (d).

[0043] Figure 7 is a graphical representation showing reduced LPS-induced NF- κ B activity in T1DM DC. Nuclear extracts were prepared from DC cultured for 72 h from four adult T1DM patients or healthy control (HC) subjects without or with addition of 1 μ g/mL LPS for the last 24 h of culture. 10 μ g of the nuclear extract were bound to wells of a NF- κ B consensus oligonucleotide-coated chemiluminescent ELISA plate, and revealed with anti-RelB, anti-p65 or anti-p50 antibody. Luminescence was measured using a luminometer and expressed in photon units with relation to time in seconds.

[0044] Figure 8 is a graphical representation showing that CD1a expression is increased in T1DM DC relative to healthy control DC. DCs were cultured for 72 h from four adult T1DM patients or healthy control (HC) subjects with or without addition of 1 μ g/mL LPS for the last 24 h of culture. Either nuclear extracts were prepared or cells were stained with CD1a and analyzed by FACS.

[0045] Figure 9 is a graphical representation showing increased TGF- β production by LPS treated T1DM DC. DCs were cultured for 72 h from four adult T1DM patients or healthy control (HC) subjects with or without addition of 1 μ g/mL LPS for the last 24 h of culture. For the NF- κ B activity, 10 μ g of the nuclear extract were bound to wells of a NF- κ B consensus oligonucleotide-coated chemiluminescent ELISA plate, and revealed with anti-RelB, anti-p65 or anti-p50 antibody. Luminescence was measured using a luminometer and expressed in photon units with relation to time in seconds.

[0046] Figure 10 is a graphical representation showing reduced LPS-induced NF- κ B activity in PBMC of at-risk T1DM family members. Peripheral blood was collected from members of families in which one child has T1DM. Two families were studied. PBMC were extracted from blood and treated for 24 h without or with 1 μ g/mL of LPS. Nuclear extracts were prepared. 10 μ g of the nuclear extract were bound to wells of a NF- κ B consensus oligonucleotide-coated chemiluminescent ELISA plate, and revealed with anti-RelB antibody. Luminescence was measured using a luminometer and expressed in photon units with relation to time in seconds.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

[0047] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[0048] The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0049] The term “aberrant expression” is used herein to describe the over-expression or under-expression of genes belonging to the NF- κ B pathway, relative to the level of expression of the such genes in cells obtained from a healthy subject or from a subject lacking T1DM, and/or to a higher or lower level or functional activity of

a NF- κ B pathway gene product (*e.g.*, transcript or polypeptide) in a tissue sample or body fluid obtained from a healthy subject or from a subject lacking T1DM. In particular, a specific NF- κ B pathway gene is aberrantly expressed if the level of expression of such a gene product is at least about 10% ($\frac{1}{10}$), 20% ($\frac{1}{5}$), 30% ($\frac{3}{10}$), 40% ($\frac{2}{5}$), 50% ($\frac{1}{2}$), 60% ($\frac{3}{5}$), 70% ($\frac{7}{10}$), 80% ($\frac{4}{5}$) or 90% ($\frac{9}{10}$), or even at least about 100% (1-fold), 200% (2-fold), 300% (3-fold), 400% (4-fold), 500% (5-fold), 600% (6-fold), 700% (7-fold), 800% (8-fold), 900% (9-fold) or 1000% (10-fold) higher than the level of expression of a corresponding NF- κ B pathway gene product in a tissue sample or body fluid obtained from a healthy subject or from a subject without autoimmune disease, especially T1DM. Alternatively, a specific NF- κ B pathway gene is aberrantly expressed if the level of expression of such a gene product is about $\frac{9}{10}$, $\frac{4}{5}$, $\frac{7}{10}$, $\frac{3}{5}$, $\frac{1}{2}$, $\frac{2}{5}$, $\frac{3}{10}$, $\frac{1}{5}$, $\frac{1}{10}$, or even about $\frac{1}{100}$, $\frac{1}{200}$, $\frac{1}{300}$, $\frac{1}{400}$, $\frac{1}{500}$, $\frac{1}{600}$, $\frac{1}{700}$, $\frac{1}{800}$, $\frac{1}{900}$, $\frac{1}{1000}$ or less of the level of expression of a corresponding NF- κ B pathway gene product in a tissue sample or body fluid obtained from a healthy subject or from a subject without autoimmune disease, especially T1DM.

[0050] By “about” is meant a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[0051] The term “amplicon” refers to a target sequence for amplification, and/or the amplification products of a target sequence for amplification. In certain other embodiments an “amplicon” may include the sequence of probes or primers used in amplification.

[0052] By “antigen” is meant all, or part of, a protein, peptide, or other molecule or macromolecule capable of eliciting an immune response in a vertebrate animal, preferably a mammal. Such antigens are also reactive with antibodies from animals immunised with said protein, peptide, or other molecule or macromolecule.

[0053] By “antigen-binding molecule” is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

[0054] Reference herein to “a level or functional activity” in the context of a transcript or polypeptide produced by a specified cell is to be taken in its broadest sense and includes a level or functional activity of a transcript or polypeptide that is produced in a single cell or in a plurality or population of cells. In the latter case, therefore, it will
5 be understood that the phrase will encompass a mean level or functional activity of transcript or polypeptide produced by a plurality or population of cells.

[0055] The term “allogeneic” as used herein refers to cells, tissues, organisms etc that are of different genetic constitution.

[0056] By “autologous” is meant something (e.g., cells, tissues etc) derived
10 from the same organism.

[0057] The term “biological sample” as used herein refers to a sample that may be extracted, untreated, treated, diluted or concentrated from an animal. The biological sample may include a biological fluid such as whole blood, serum, plasma, saliva, urine, sweat, ascitic fluid, peritoneal fluid, synovial fluid, amniotic fluid,
15 cerebrospinal fluid, tissue biopsy, and the like. In certain embodiments, the biological sample is blood, especially peripheral blood.

[0058] “*Cis* binding site”, also referred to herein as “*cis* site,” refers to a defined nucleic acid sequence (or sequence motif) that is capable of associating with an endogenous or exogenously supplied nucleic acid binding factor or protein, whereby the
20 specific complex formed is typically used by the cell to regulate a cellular process involving gene expression. Examples of such cellular processes include transcription, RNA processing such as RNA capping and splicing, and translation.

[0059] Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the
25 inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. Thus, use of the term “comprising” and the like indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of”. Thus,
30 the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present. By “consisting essentially of” is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the

listed elements. Thus, the phrase “consisting essentially of” indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

5 **[0060]** By “effective amount”, in the context of modulating an immune response or treating or preventing a disease or condition, is meant the administration of that amount of composition to an individual in need thereof, either in a single dose or as part of a series, that is effective for that modulation, treatment or prevention. The effective amount will vary depending upon the health and physical condition of the
10 individual to be treated, the taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

[0061] The terms “expression” or “gene expression” refer to either
15 production of RNA message or translation of RNA message into proteins or polypeptides. Detection of either types of gene expression in use of any of the methods described herein are part of the invention.

[0062] As used herein, the term “functional activity” generally refers to the ability of a molecule (*e.g.*, a transcript or polypeptide) to perform its designated
20 function including a biological, enzymatic, or therapeutic function. In certain embodiments, the functional activity of a molecule corresponds to its specific activity as determined by any suitable assay known in the art.

[0063] The term “gene” is used in its broadest context to include both a genomic DNA region corresponding to the gene as well as a cDNA sequence
25 corresponding to exons or a recombinant molecule engineered to encode a functional form of a product.

[0064] Reference herein to “immuno-interactive” includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the molecules is, or mimics, a component of the immune system.

30 **[0065]** By “isolated” is meant material that is substantially or essentially free from components that normally accompany it in its native state.

[0066] By “modulating” is meant increasing or decreasing, either directly or indirectly, the immune response of an individual.

[0067] By “obtained from” is meant that a sample such as, for example, a cell extract or nucleic acid or polypeptide extract is isolated from, or derived from, a particular source. For instance, the extract may be isolated directly from biological fluid or tissue of a subject.

[0068] By “pharmaceutically-acceptable carrier” is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in topical or systemic administration.

[0069] The term “polynucleotide” or “nucleic acid” as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotides in length.

[0070] “Polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers.

[0071] By “primer” is meant an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerizing agent. The primer is preferably single-stranded for maximum efficiency in amplification but can alternatively be double-stranded. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerization agent. The length of the primer depends on many factors, including application, temperature to be employed, template reaction conditions, other reagents, and source of primers. For example, depending on the complexity of the target sequence, the primer may be at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, 500, to one base shorter in length than the template sequence at the 3' end of the primer to allow extension of a nucleic acid chain, though the 5' end of the primer may extend in length beyond the 3' end of the template sequence. In certain embodiments, primers can be large polynucleotides, such as from about 35 nucleotides to several kilobases or more. Primers can be selected to be “substantially complementary” to the sequence on

the template to which it is designed to hybridize and serve as a site for the initiation of synthesis. By “substantially complementary”, it is meant that the primer is sufficiently complementary to hybridize with a target polynucleotide. Desirably, the primer contains no mismatches with the template to which it is designed to hybridize but this is not
5 essential. For example, non-complementary nucleotide residues can be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary nucleotide residues or a stretch of non-complementary nucleotide residues can be interspersed into a primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to
10 hybridize therewith and thereby form a template for synthesis of the extension product of the primer.

[0072] “Probe” refers to a molecule that binds to a specific sequence or sub-sequence or other moiety of another molecule. Unless otherwise indicated, the term “probe” typically refers to a polynucleotide probe that binds to another polynucleotide,
15 often called the “target polynucleotide”, through complementary base pairing. Probes can bind target polynucleotides lacking complete sequence complementarity with the probe, depending on the stringency of the hybridization conditions. Probes can be labeled directly or indirectly and include primers within their scope.

[0073] By “regulatory lymphocyte” is meant a lymphocyte that is involved in
20 controlling responses and actions of other cells, especially of other immune cells such as B lymphocytes and T helper lymphocytes.

[0074] As used herein, the term “related” when used with respect to subjects indicates that the subjects are known to share a common line of descent: that is, the subjects have a known ancestor in common. Illustrative examples of related subjects
25 include siblings (brothers and sisters), parents, grandparents, children, grandchildren, aunts, uncles, cousins, second cousins and third cousins. Subjects less closely related than third cousins are typically not sufficiently related to be useful as “related” subjects for the methods of this invention, even if they share a known ancestor, unless some related individuals that lie between the distantly related subjects are also included. Thus,
30 for a group of related individuals, each subject shares a known ancestor within three generations or less with at least one other subject in the group, and desirably with all other subjects in the group or has at least that degree of consanguinity due to multiple known common ancestors. In specific embodiments, subjects share a common ancestor

within two generations or less, or otherwise have equivalent level of consanguinity. Conversely, as used herein the term “unrelated,” when used in respect of subjects, refers to subjects who do not share a known ancestor within 3 generations or less, or otherwise have known relatedness at that degree.

5 **[0075]** The terms “subject” or “individual” or “patient”, used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, primates, avians, livestock animals (*e.g.*, sheep, cows, horses, donkeys, pigs), laboratory
10 test animals (*e.g.*, rabbits, mice, rats, guinea pigs, hamsters), companion animals (*e.g.*, cats, dogs) and captive wild animals (*e.g.*, foxes, deer, dingoes). A preferred subject is a mammal, especially a primate and more especially a human. However, it will be understood that the aforementioned terms do not imply that symptoms are present.

[0076] As used herein the term “surrogate marker” refers to a biological or
15 clinical parameter that is measured in place of the biologically definitive or clinically most meaningful parameter. In comparison to definitive markers, surrogate markers are generally either more convenient, less expensive, provide earlier information or provide pharmacological or physiological information not directly obtainable with definitive markers. Examples of surrogate biological parameters include testing cell NF- κ B levels
20 or the presence of autoimmune antibodies for GAD65, ICA512, and mIAA in subjects having or at risk of developing T1DM, whereas examples of definitive biological parameters include insulin independence, blood glucose and C peptide production. The measurement of a surrogate marker may be an endpoint in a clinical study or clinical trial, hence “surrogate endpoint”.

25 **[0077]** The term “template” as used herein refers to a nucleic acid that is used in the creation of a complementary nucleic acid strand to the “template” strand. The template may be either RNA and/or DNA, and the complementary strand may also be RNA and/or DNA. In certain embodiments, the complementary strand may comprise all or part of the complementary sequence to the “template,” and/or may include mutations
30 so that it is not an exact, complementary strand to the “template”. Strands that are not exactly complementary to the template strand may hybridize specifically to the template strand in detection assays described here, as well as other assays known in the art, and

such complementary strands that can be used in detection assays are part of the invention.

[0078] The term “treat” is meant to include both therapeutic and prophylactic treatment.

5 [0079] The terms “wild-type” and “normal” are used interchangeably to refer to the phenotype that is characteristic of most of the members of the species occurring naturally and contrast for example with the phenotype of a mutant.

2. *Surrogate markers of type 1 diabetes*

[0080] The present invention concerns the early detection, diagnosis,
10 monitoring, or prognosis of T1DM. Surrogate markers for this disease, in the form of RNA molecules of specified sequences, or polypeptides expressed from these RNA molecules in cells, including blood cells (*e.g.*, peripheral blood cells), of subjects with or susceptible to T1DM, are disclosed. These markers are indicators of T1DM and, when differentially expressed as compared to their expression in normal subjects or in
15 subjects lacking T1DM, are diagnostic for the presence or risk of development of this disease in tested subjects. Such markers provide considerable advantages over the prior art in this field. In certain advantageous embodiments where peripheral blood is used for the analysis, it is possible to T1DM before serum autoantibodies are detected.

[0081] It will be apparent that the T1DM polynucleotides disclosed herein
20 will find utility in a variety of applications in T1DM detection, diagnosis, prognosis and treatment. Examples of such applications within the scope of the present disclosure comprise amplification of T1DM marker polynucleotides using specific primers, detection of T1DM marker polynucleotides by hybridization with oligonucleotide probes and development of immunological reagents corresponding to marker encoded
25 products.

[0082] The identified T1DM marker polynucleotides may in turn be used to design specific oligonucleotide probes and primers. Such probes and primers may be of any length that would specifically hybridize to the identified marker gene sequences and may be at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,
30 27, 28, 29, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, 500 nucleotides in length and in the case of probes, up to the full length of the sequences of the marker genes identified

herein. Probes may also include additional sequence at their 5' and/or 3' ends so that they extend beyond the target sequence with which they hybridize.

[0083] When used in combination with nucleic acid amplification procedures, these probes and primers enable the rapid analysis of biological samples (*e.g.*, peripheral blood samples) for detecting marker genes or for detecting or quantifying marker gene transcripts. Such procedures include any method or technique known in the art or described herein for duplicating or increasing the number of copies or amount of a target nucleic acid or its complement.

[0084] One of ordinary skill in the art could select segments from the identified marker genes for use in the different detection, diagnostic, or prognostic methods, vector constructs, antigen-binding molecule production, kit, and/or any of the embodiments described herein as part of the present invention. Marker genes that are desirable for use in the present invention are involved in or belong to the NF- κ B signaling pathway and include, for example, genes encoding BTK, LYN, BCR Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK, RelA/p65, P105/p50, c-Rel, RelB, p52, NIK, Leu13, CD81, CD19, CD21 and its ligands in the complement and coagulation cascade, TRAF6, ubiquitin ligase, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, SIp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP2, SAP, SLAM, PKR, 2B4, SHP1, SHIP, PIR-B, CD22, CD72, Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl, KIR3DL1, KIR3DL2, KIR2DL or Csk

3. *Methods of detecting aberrant T1DM marker gene expression*

[0085] The present invention is predicated in part on the discovery that patients with clinical evidence of T1DM have aberrant signaling through the NF- κ B pathway, which manifests for example in aberrant expression of genes (referred to herein as T1DM marker genes) involved in that pathway, illustrative examples of which are listed above. Accordingly, in certain embodiments, the invention features a method for diagnosing the presence, absence, degree, activity, stage or risk of developing T1DM or a related condition in a subject (*e.g.*, a mammal such as a human), by detecting aberrant expression of a T1DM marker gene in a biological sample obtained from the subject. Illustrative examples of related conditions include celiac disease,

Addison's disease, autoimmune polyendocrinopathies, obesity, increased cholesterol, kidney-related disorders, decreased liver glucokinase activity and the like.

[0086] In order to make such diagnoses, it is generally desirable to qualitatively or quantitatively determine the levels of T1DM marker polynucleotide transcripts or the level or functional activity of T1DM marker polypeptides. In some embodiments, the presence, degree, stage or risk of development of T1DM is diagnosed when a T1DM marker gene is expressed at a detectably lower level in the biological sample as compared to the level at which that gene is expressed in a reference sample obtained from normal subjects or from subjects lacking T1DM. In illustrative examples of this type, the gene is selected from genes encoding BTK, LYN, BCR Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK, RelA/p65, P105/p50, c-Rel, RelB, p52, NIK, Leu13, CD81, CD19, CD21 and its ligands in the complement and coagulation cascade, TRAF6, and other ubiquitin ligases, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, SIp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP-2, SAP, SLAM, PKR and 2B4. In other embodiments, the presence, degree, stage or risk of development of T1DM is diagnosed when a T1DM marker gene is expressed at a detectably higher level in the biological sample as compared to the level at which that gene is expressed in a reference sample obtained from normal subjects or from subjects lacking T1DM. In illustrative examples of this type, the gene is selected from genes encoding SHP-1, SHIP, PIR-B, CD22, CD72, Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl, KIR3DL1, KIR3DL2, KIR2DL and Csk. Generally, such diagnoses are made when the level or functional activity of a T1DM marker gene product in the biological sample varies from the level or functional activity of a corresponding T1DM marker gene product in the reference sample by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 92%, 94%, 96%, 97%, 98% or 99%, or even by at least about 99.5%, 99.9%, 99.95%, 99.99%, 99.995% or 99.999%, or even by at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%.

[0087] The corresponding gene product is generally selected from the same gene product that is present in the biological sample, a gene product expressed from a variant gene (*e.g.*, an homologous or orthologous gene) including an allelic variant, or a splice variant or protein product thereof. In some embodiments, the method comprises measuring the level or functional activity of individual expression products of at least

about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 T1DM marker genes.

[0088] In some embodiments, the biological sample contains blood, especially peripheral blood, or a fraction or extract thereof. Typically, the biological sample comprises blood cells such as mature, immature and developing leukocytes, including lymphocytes, polymorphonuclear leukocytes, neutrophils, monocytes, reticulocytes, basophils, coelomocytes, haemocytes, eosinophils, megakaryocytes, macrophages, dendritic cells natural killer cells, or fraction of such cells (*e.g.*, a nucleic acid or protein fraction). In specific embodiments, the biological sample comprises leukocytes including peripheral blood mononuclear cells (PBMC).

[0089] The diagnostic tests of the invention may be used to diagnose the presence, degree, stage or risk of development of T1DM in any subject. In specific embodiments, these tests are used with subjects related to a patient with clinical signs (one or more definitive markers) of T1DM. These tests are desirably used at a stage and frequency to enable early detection of T1DM, its progression, or to diagnose the risk of developing that disease.

3.1 Nucleic acid-based diagnostics

[0090] Nucleic acids used in polynucleotide-based assays can be isolated from cells contained in the biological sample, according to standard methodologies (Sambrook, *et al.*, 1989, *supra*; and Ausubel *et al.*, 1994, *supra*). The nucleic acid is typically fractionated (*e.g.*, poly A+ RNA) or whole cell RNA. Where RNA is used as the subject of detection, it may be desired to convert the RNA to a complementary DNA. In some embodiments, the nucleic acid is amplified by a template-dependent nucleic acid amplification technique. A number of template dependent processes are available to amplify the T1DM marker sequences present in a given template sample. An exemplary nucleic acid amplification technique is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, Ausubel *et al.* (*supra*), and in Innis *et al.* ("PCR Protocols", Academic Press, Inc., San Diego Calif., 1990). Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, Taq polymerase. If a cognate T1DM marker sequence is present in a sample, the primers will bind to the marker and the polymerase

will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

5 A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989, *supra*. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction
10 methodologies are well known in the art.

[0091] In certain advantageous embodiments, the template-dependent amplification involves the quantification of transcripts in real-time. For example, RNA or DNA may be quantified using the Real-Time PCR technique (Higuchi, 1992, *et al.*, Biotechnology 10:413-417). By determining the concentration of the amplified products
15 of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundance of the specific mRNA from which the target sequence was derived can be determined
20 for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundance is only true in the linear range of the PCR reaction. The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA.

25 [0092] Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR,
30 bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Pat. No. 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

[0093] Q β Replicase, described in PCT Application No. PCT/US87/00880, may also be used. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be
5 detected.

[0094] An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5' α -thio-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*,
10 (1992, Proc. Natl. Acad. Sci. U.S.A 89:392-396).

[0095] Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted
15 for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is
20 present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

[0096] Still another amplification method described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, may be used. In the former
25 application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released
30 intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

[0097] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based

amplification (NASBA) and 3SR (Kwoh *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A., 86:1173; Gingeras *et al.*, PCT Application WO 88/10315). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for
5 isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific
10 primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete,
15 indicate target specific sequences.

[0098] Davey *et al.*, EPO No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is
20 elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology
25 to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many
30 RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

[0099] Miller *et al.* in PCT Application WO 89/06700 disclose a nucleic acid sequence amplification scheme based on the hybridisation of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M. A., In: "PCR Protocols: A Guide to Methods and Applications", Academic Press, N.Y., 1990; Ohara *et al.*, 1989, Proc. Natl Acad. Sci. U.S.A., 86:5673-567).

[0100] Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used for amplifying target nucleic acid sequences. Wu *et al.*, (1989, Genomics 4:560).

[0101] Depending on the format, the T1DM marker nucleic acid of interest is identified in the sample directly using a template-dependent amplification as described, for example, above, or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994, J Macromol. Sci. Pure, Appl. Chem., A31(1):1355-1376).

[0102] In some embodiments, amplification products or "amplicons" are visualized in order to confirm amplification of the T1DM marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation. In some embodiments, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified T1DM marker sequence. The probe is suitably conjugated to a chromophore but may be radiolabeled. Alternatively, the probe is conjugated to a binding partner, such as an antigen-binding molecule, or biotin, and the other member of the binding pair carries a detectable moiety or reporter molecule.

The techniques involved are well known to those of skill in the art and can be found in many standard texts on molecular protocols (*e.g.*, see Sambrook *et al.*, 1989, *supra* and Ausubel *et al.* 1994, *supra*). For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

5 **[0103]** In certain embodiments, target nucleic acids are quantified using blotting techniques, which are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting of RNA species. Briefly, a probe is used to
10 target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by “blotting” on to the filter. Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and
15 rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

[0104] Following detection/quantification, one may compare the results seen in a given subject with a control reaction or a statistically significant reference group of
20 normal subjects or of subjects lacking T1DM. In this way, it is possible to correlate the amount of a T1DM marker nucleic acid detected with the progression or severity of the disease.

[0105] Also contemplated are genotyping methods and allelic discrimination methods and technologies such as those described by Kristensen *et al.* (Biotechniques
25 30(2):318-322), including the use of single nucleotide polymorphism analysis, high performance liquid chromatography, TaqMan™, liquid chromatography, and mass spectrometry.

[0106] Also contemplated are biochip-based technologies such as those described by Hacia *et al.* (1996, Nature Genetics 14:441-447) and Shoemaker *et al.*
30 (1996, Nature Genetics 14:450-456). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ biochip technology to segregate target molecules as high density arrays and screen these molecules on the

basis of hybridization. See also Pease *et al.* (1994, Proc. Natl. Acad. Sci. U.S.A. 91:5022-5026); Fodor *et al.* (1991, Science 251:767-773). Briefly, nucleic acid probes to T1DM marker polynucleotides are made and attached to biochips to be used in screening and diagnostic methods, as outlined herein. The nucleic acid probes attached to the biochip are designed to be substantially complementary to specific expressed T1DM marker nucleic acids, *i.e.*, the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. This complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the nucleic acid probes of the present invention. However, if the number of mismatches is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. In certain embodiments, more than one probe per sequence is used, with either overlapping probes or probes to different sections of the target being used. That is, two, three, four or more probes, with three being desirable, are used to build in a redundancy for a particular target. The probes can be overlapping (*i.e.* have some sequence in common), or separate.

[0107] As will be appreciated by those of ordinary skill in the art, nucleic acids can be attached to or immobilized on a solid support in a wide variety of ways. By “immobilized” and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By “non-covalent binding” and grammatical equivalents herein is meant one or more of either, electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By “covalent binding” and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

[0108] In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

5 [0109] The biochip comprises a suitable solid or semi-solid substrate or solid support. By "substrate" or "solid support" is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by practitioners in the art, the number of possible substrates are very large,
10 and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon™, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general,
15 the substrates allow optical detection and do not appreciably fluoresce.

[0110] Generally the substrate is planar, although as will be appreciated by those of skill in the art, other configurations of substrates may be used as well. For example, the probes may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Similarly, the substrate may be flexible,
20 such as a flexible foam, including closed cell foams made of particular plastics.

[0111] In certain embodiments, oligonucleotides probes are synthesized on the substrate, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques can be used. In an illustrative example, the nucleic acids are synthesized in situ, using well known photolithographic
25 techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Pat. Nos. 5,700,637 and 5,445,934; and references cited within; these methods of attachment form the basis of the Affymetrix GeneChip™ technology.

[0112] In an illustrative biochip analysis, oligonucleotide probes on the biochip are exposed to or contacted with a nucleic acid sample suspected of containing
30 one or more T1DM polynucleotides under conditions favoring specific hybridization. Sample extracts of DNA or RNA, either single or double-stranded, may be prepared from fluid suspensions of biological materials, or by grinding biological materials, or following a cell lysis step which includes, but is not limited to, lysis effected by

treatment with SDS (or other detergents), osmotic shock, guanidinium isothiocyanate and lysozyme. Suitable DNA, which may be used in the method of the invention, includes cDNA. Such DNA may be prepared by any one of a number of commonly used protocols as for example described in Ausubel, *et al.*, 1994, *supra*, and Sambrook, *et al.*, 1989, *supra*.

[0113] Suitable RNA, which may be used in the method of the invention, includes messenger RNA, complementary RNA transcribed from DNA (cRNA) or genomic or subgenomic RNA. Such RNA may be prepared using standard protocols as for example described in the relevant sections of Ausubel, *et al.* 1994, *supra* and Sambrook, *et al.*, 1989, *supra*).

[0114] cDNA may be fragmented, for example, by sonication or by treatment with restriction endonucleases. Suitably, cDNA is fragmented such that resultant DNA fragments are of a length greater than the length of the immobilized oligonucleotide probe(s) but small enough to allow rapid access thereto under suitable hybridization conditions. Alternatively, fragments of cDNA may be selected and amplified using a suitable nucleotide amplification technique, as described for example above, involving appropriate random or specific primers.

[0115] Usually the target T1DM marker polynucleotides are detectably labeled so that their hybridization to individual probes can be determined. The target polynucleotides are typically detectably labeled with a reporter molecule illustrative examples of which include chromogens, catalysts, enzymes, fluorochromes, chemiluminescent molecules, bioluminescent molecules, lanthanide ions (*e.g.*, Eu^{34}), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like. Illustrative labels of this type include large colloids, for example, metal colloids such as those from gold, selenium, silver, tin and titanium oxide. In some embodiments in which an enzyme is used as a direct visual label, biotinylated bases are incorporated into a target polynucleotide. Hybridization is detected by incubation with streptavidin-reporter molecules.

[0116] Suitable fluorochromes include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red. Other exemplary fluorochromes include those discussed by

Dower *et al.* (International Publication WO 93/06121). Reference also may be made to the fluorochromes described in U.S. Patents 5,573,909 (Singer *et al.*), 5,326,692 (Brinkley *et al.*). Alternatively, reference may be made to the fluorochromes described in U.S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663,
5 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218. Commercially available fluorescent labels include, for example, fluorescein phosphoramidites such as Fluoreprime™ (Pharmacia), Fluoredate™ (Millipore) and FAM (Applied Biosystems International)

[0117] Radioactive reporter molecules include, for example, ³²P, which can
10 be detected by an X-ray or phosphorimager techniques.

[0118] The hybrid-forming step can be performed under suitable conditions for hybridizing oligonucleotide probes to test nucleic acid including DNA or RNA. In this regard, reference may be made, for example, to NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH (Homes and Higgins, Eds.) (IRL
15 press, Washington D.C., 1985). In general, whether hybridization takes place is influenced by the length of the oligonucleotide probe and the polynucleotide sequence under test, the pH, the temperature, the concentration of mono- and divalent cations, the proportion of G and C nucleotides in the hybrid-forming region, the viscosity of the medium and the possible presence of denaturants. Such variables also influence the time
20 required for hybridization. The preferred conditions will therefore depend upon the particular application. Such empirical conditions, however, can be routinely determined without undue experimentation.

[0119] In certain advantageous embodiments, high discrimination hybridization conditions are used. For example, reference may be made to Wallace *et al.*
25 (1979, Nucl. Acids Res. 6:3543) who describe conditions that differentiate the hybridization of 11 to 17 base long oligonucleotide probes that match perfectly and are completely homologous to a target sequence as compared to similar oligonucleotide probes that contain a single internal base pair mismatch. Reference also may be made to Wood *et al.* (1985, Proc. Natl. Acad. Sci. USA 82:1585) who describe conditions for
30 hybridization of 11 to 20 base long oligonucleotides using 3M tetramethyl ammonium chloride wherein the melting point of the hybrid depends only on the length of the oligonucleotide probe, regardless of its GC content. In addition, Drmanac *et al.* (*supra*) describe hybridization conditions that allow stringent hybridization of 6-10 nucleotide

long oligomers, and similar conditions may be obtained most readily by using nucleotide analogues such as 'locked nucleic acids (Christensen *et al.*, 2001 Biochem J 354:481-4).

[0120] Generally, a hybridization reaction can be performed in the presence of a hybridization buffer that optionally includes a hybridization optimizing agent, such as an isostabilizing agent, a denaturing agent and/or a renaturation accelerant. Examples of isostabilizing agents include, but are not restricted to, betaines and lower tetraalkyl ammonium salts. Denaturing agents are compositions that lower the melting temperature of double stranded nucleic acid molecules by interfering with hydrogen bonding between bases in a double stranded nucleic acid or the hydration of nucleic acid molecules. Denaturing agents include, but are not restricted to, formamide, formaldehyde, dimethylsulphoxide, tetraethyl acetate, urea, guanidium isothiocyanate, glycerol and chaotropic salts. Hybridization accelerants include heterogeneous nuclear ribonucleoprotein (hnRP) A1 and cationic detergents such as cetyltrimethylammonium bromide (CTAB) and dodecyl trimethylammonium bromide (DTAB), polylysine, spermine, spermidine, single stranded binding protein (SSB), phage T4 gene 32 protein and a mixture of ammonium acetate and ethanol. Hybridization buffers may include target polynucleotides at a concentration between about 0.005 nM and about 50 nM, preferably between about 0.5 nM and 5 nM, more preferably between about 1 nM and 2 nM.

[0121] In some embodiments, a hybridization mixture containing the target T1DM marker polynucleotides is placed in contact with the array of probes and incubated at a temperature and for a time appropriate to permit hybridization between the target sequences in the target polynucleotides and any complementary probes. Contact can take place in any suitable container, for example, a dish or a cell designed to hold the solid support on which the probes are bound. Generally, incubation will be at temperatures normally used for hybridization of nucleic acids, for example, between about 20° C and about 75° C, example, about 25° C, about 30° C, about 35° C, about 40° C, about 45° C, about 50° C, about 55° C, about 60° C, or about 65° C. For probes longer than 14 nucleotides, 20° C to 50° C is desirable. For shorter probes, lower temperatures are preferred. A sample of target polynucleotides is incubated with the probes for a time sufficient to allow the desired level of hybridization between the target

sequences in the target polynucleotides and any complementary probes. For example, the hybridization may be carried out at about 45° C +/-10° C in formamide for 1-2 days.

[0122] After the hybrid-forming step, the probes are washed to remove any unbound nucleic acid with a hybridization buffer, which can typically comprise a hybridization optimizing agent in the same range of concentrations as for the hybridization step. This washing step leaves only bound target polynucleotides. The probes are then examined to identify which probes have hybridized to a target polynucleotide.

[0123] The hybridization reactions are then detected to determine which of the probes has hybridized to a corresponding target sequence. Depending on the nature of the reporter molecule associated with a target polynucleotide, a signal may be instrumentally detected by irradiating a fluorescent label with light and detecting fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be detected using a spectrophotometer; or detection of a dye particle or a colored colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label or chemiluminescent molecule employing a radiation counter or autoradiography. Accordingly, a detection means may be adapted to detect or scan light associated with the label which light may include fluorescent, luminescent, focussed beam or laser light. In such a case, a charge couple device (CCD) or a photocell can be used to scan for emission of light from a probe:target polynucleotide hybrid from each location in the micro-array and record the data directly in a digital computer. In some cases, electronic detection of the signal may not be necessary. For example, with enzymatically generated colour spots associated with nucleic acid array format, visual examination of the array will allow interpretation of the pattern on the array. In the case of a nucleic acid array, the detection means is suitably interfaced with pattern recognition software to convert the pattern of signals from the array into a plain language genetic profile. In certain embodiments, oligonucleotide probes specific for different T1DM marker polynucleotide products are in the form of a nucleic acid array and detection of a signal generated from a reporter molecule on the array is performed using a 'chip reader'. A detection system that can be used by a 'chip reader' is described for example by Pirrung et al (U.S. Patent No. 5,143,854). The chip reader will typically also incorporate some signal processing to determine whether the signal at a particular array position or feature is a true positive or maybe a spurious signal. Exemplary chip

readers are described for example by Fodor et al (U.S. Patent No., 5,925,525).
Alternatively, when the array is made using a mixture of individually addressable kinds of labeled microbeads, the reaction may be detected using flow cytometry.

3.2 Protein-based diagnostics

5 **[0124]** Consistent with the present invention, the presence of an aberrant concentration of a T1DM marker protein is indicative of the presence, degree, activity, stage or risk of development of T1DM or related condition. T1DM marker protein levels in biological samples can be assayed using any suitable method known in the art. For example, when a T1DM marker protein is an enzyme, the protein can be quantified
10 based upon its catalytic activity or based upon the number of molecules of the protein contained in a sample. Antibody-based techniques may be employed, such as, for example, immunohistological and immunohistochemical methods for measuring the level of a protein of interest in a tissue sample. For example, specific recognition is provided by a primary antibody (polyclonal or monoclonal) and a secondary detection
15 system is used to detect presence (or binding) of the primary antibody. Detectable labels can be conjugated to the secondary antibody, such as a fluorescent label, a radiolabel, or an enzyme (*e.g.*, alkaline phosphatase, horseradish peroxidase) which produces a quantifiable, *e.g.*, colored, product. In another suitable method, the primary antibody itself can be detectably labeled. As a result, immunohistological labeling of a tissue
20 section is provided. In some embodiments, a protein extract is produced from a biological sample (*e.g.*, tissue, cells) for analysis. Such an extract (*e.g.*, a detergent extract) can be subjected to western-blot or dot/slot assay of the level of the protein of interest, using routine immunoblotting methods (Jalkanen *et al.*, 1985, J. Cell. Biol. 101:976-985; Jalkanen *et al.*, 1987, J. Cell. Biol. 105:3087-3096).

25 **[0125]** Other useful antibody-based methods include immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a protein-specific monoclonal antibody, can be used both as an immunoabsorbent and as an enzyme-labelled probe to detect and quantify a T1DM marker protein of interest. The amount of such protein present in a sample can be
30 calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm (see Lacobilli *et al.*, 1988, Breast Cancer Research and Treatment 11:19-30). In other embodiments, two different monoclonal antibodies to the

protein of interest can be employed, one as the immunoabsorbent and the other as an enzyme-labeled probe.

[0126] Additionally, recent developments in the field of protein capture arrays permit the simultaneous detection and/or quantification of a large number of proteins. For example, low-density protein arrays on filter membranes, such as the universal protein array system (Ge, 2000 Nucleic Acids Res. 28(2):e3) allow imaging of arrayed antigens using standard ELISA techniques and a scanning charge-coupled device (CCD) detector. Immuno-sensor arrays have also been developed that enable the simultaneous detection of clinical analytes. It is now possible using protein arrays, to profile protein expression in bodily fluids, such as in sera of healthy or diseased subjects, as well as in subjects pre- and post-drug treatment.

[0127] Protein capture arrays typically comprise a plurality of protein-capture agents each of which defines a spatially distinct feature of the array. The protein-capture agent can be any molecule or complex of molecules which has the ability to bind a protein and immobilize it to the site of the protein-capture agent on the array. The protein-capture agent may be a protein whose natural function in a cell is to specifically bind another protein, such as an antibody or a receptor. Alternatively, the protein-capture agent may instead be a partially or wholly synthetic or recombinant protein which specifically binds a protein. Alternatively, the protein-capture agent may be a protein which has been selected in vitro from a mutagenized, randomized, or completely random and synthetic library by its binding affinity to a specific protein or peptide target. The selection method used may optionally have been a display method such as ribosome display or phage display, as known in the art. Alternatively, the protein-capture agent obtained via in vitro selection may be a DNA or RNA aptamer which specifically binds a protein target (see, *e.g.*, Potyrailo *et al.*, 1998 Anal. Chem. 70:3419-3425; Cohen *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:14272-14277; Fukuda, *et al.*, 1997 Nucleic Acids Symp. Ser. 37:237-238; available from SomaLogic). For example, aptamers are selected from libraries of oligonucleotides by the Selex™ process and their interaction with protein can be enhanced by covalent attachment, through incorporation of brominated deoxyuridine and UV-activated crosslinking (photoaptamers). Aptamers have the advantages of ease of production by automated oligonucleotide synthesis and the stability and robustness of DNA; universal fluorescent protein stains can be used to detect binding. Alternatively, the in vitro selected protein-capture agent may be a

polypeptide (*e.g.*, an antigen) (see, *e.g.*, Roberts and Szostak, 1997 Proc. Natl. Acad. Sci. USA, 94:12297-12302).

[0128] An alternative to an array of capture molecules is one made through 'molecular imprinting' technology, in which peptides (*e.g.*, from the C-terminal regions of proteins) are used as templates to generate structurally complementary, sequence-specific cavities in a polymerizable matrix; the cavities can then specifically capture (denatured) proteins which have the appropriate primary amino acid sequence (*e.g.*, available from ProteinPrint™ and Aspira Biosystems).

[0129] Exemplary protein capture arrays include arrays comprising spatially addressed antigen-binding molecules, commonly referred to as antibody arrays, which can facilitate extensive parallel analysis of numerous proteins defining a proteome or subproteome. Antibody arrays have been shown to have the required properties of specificity and acceptable background, and some are available commercially (*e.g.*, BD Biosciences, Clontech, BioRad and Sigma). Various methods for the preparation of antibody arrays have been reported (see, *e.g.*, Lopez *et al.*, 2003 J. Chromatogr. B 787:19-27; Cahill, 2000 Trends in Biotechnology 7:47-51; U.S. Pat. App. Pub. 2002/0055186; U.S. Pat. App. Pub. 2003/0003599; PCT publication WO 03/062444; PCT publication WO 03/077851; PCT publication WO 02/59601; PCT publication WO 02/39120; PCT publication WO 01/79849; PCT publication WO 99/39210). The antigen-binding molecules of such arrays may recognize at least a subset of proteins expressed by a cell or population of cells, illustrative examples of which include growth factor receptors, hormone receptors, neurotransmitter receptors, catecholamine receptors, amino acid derivative receptors, cytokine receptors, extracellular matrix receptors, antibodies, lectins, cytokines, serpins, proteases, kinases, phosphatases, ras-like GTPases, hydrolases, steroid hormone receptors, transcription factors, heat-shock transcription factors, DNA-binding proteins, zinc-finger proteins, leucine-zipper proteins, homeodomain proteins, intracellular signal transduction modulators and effectors, apoptosis-related factors, DNA synthesis factors, DNA repair factors, DNA recombination factors, cell-surface antigens, hepatitis C virus (HCV) proteases and HIV proteases.

[0130] Antigen-binding molecules for antibody arrays are made either by conventional immunization (*e.g.*, polyclonal sera and hybridomas), or as recombinant fragments, usually expressed in *E. coli*, after selection from phage display or ribosome

display libraries (*e.g.*, available from Cambridge Antibody Technology, BioInvent, Affitech and Biosite). Alternatively, 'combibodies' comprising non-covalent associations of VH and VL domains, can be produced in a matrix format created from combinations of diabody-producing bacterial clones (*e.g.*, available from Domantis).

- 5 Exemplary antigen-binding molecules for use as protein-capture agents include monoclonal antibodies, polyclonal antibodies, Fv, Fab, Fab' and F(ab')₂ immunoglobulin fragments, synthetic stabilised Fv fragments, *e.g.*, single chain Fv fragments (scFv), disulphide stabilised Fv fragments (dsFv), single variable region domains (dAbs) minibodies, combibodies and multivalent antibodies such as diabodies
10 and multi-scFv, single domains from camelids or engineered human equivalents.

[0131] Individual spatially distinct protein-capture agents are typically attached to a support surface, which is generally planar or contoured. Common physical supports include glass slides, silicon, microwells, nitrocellulose or PVDF membranes, and magnetic and other microbeads.

- 15 [0132] While microdrops of protein delivered onto planar surfaces are widely used, related alternative architectures include CD centrifugation devices based on developments in microfluidics (*e.g.*, available from Gyros) and specialised chip designs, such as engineered microchannels in a plate (*e.g.*, The Living Chip™, available from Biotrove) and tiny 3D posts on a silicon surface (*e.g.*, available from Zyomyx).

- 20 [0133] Particles in suspension can also be used as the basis of arrays, providing they are coded for identification; systems include colour coding for microbeads (*e.g.*, available from Luminex, Bio-Rad and Nanomics Biosystems) and semiconductor nanocrystals (*e.g.*, QDots™, available from Quantum Dots), and barcoding for beads (UltraPlex™, available from Smartbeads) and multimetal
25 microrods (Nanobarcodes™ particles, available from Surromed). Beads can also be assembled into planar arrays on semiconductor chips (*e.g.*, available from LEAPS technology and BioArray Solutions). Where particles are used, individual protein-capture agents are typically attached to an individual particle to provide the spatial definition or separation of the array. The particles may then be assayed separately, but
30 in parallel, in a compartmentalised way, for example in the wells of a microtiter plate or in separate test tubes.

[0134] In operation, a protein sample, which is optionally fragmented to form peptide fragments (see, *e.g.*, U.S. Pat. App. Pub. 2002/0055186), is delivered to a

protein-capture array under conditions suitable for protein or peptide binding, and the array is washed to remove unbound or non-specifically bound components of the sample from the array. Next, the presence or amount of protein or peptide bound to each feature of the array is detected using a suitable detection system. The amount of protein bound to a feature of the array may be determined relative to the amount of a second protein bound to a second feature of the array. In certain embodiments, the amount of the second protein in the sample is already known or known to be invariant.

[0135] For analyzing differential expression of proteins between two cells or cell populations, a protein sample of a first cell or population of cells is delivered to the array under conditions suitable for protein binding. In an analogous manner, a protein sample of a second cell or population of cells to a second array, is delivered to a second array which is identical to the first array. Both arrays are then washed to remove unbound or non-specifically bound components of the sample from the arrays. In a final step, the amounts of protein remaining bound to the features of the first array are compared to the amounts of protein remaining bound to the corresponding features of the second array. To determine the differential protein expression pattern of the two cells or populations of cells, the amount of protein bound to individual features of the first array is subtracted from the amount of protein bound to the corresponding features of the second array.

[0136] In an illustrative example, fluorescence labeling can be used for detecting protein bound to the array. The same instrumentation as used for reading DNA microarrays is applicable to protein-capture arrays. For differential display, capture arrays (*e.g.* antibody arrays) can be probed with fluorescently labelled proteins from two different cell states, in which cell lysates are labeled with different fluorophores (*e.g.*, Cy-3 and Cy-5) and mixed, such that the color acts as a readout for changes in target abundance. Fluorescent readout sensitivity can be amplified 10-100 fold by tyramide signal amplification (TSA) (*e.g.*, available from Perkin Elmer Lifesciences). Planar waveguide technology (*e.g.*, available from Zeptosens) enables ultrasensitive fluorescence detection, with the additional advantage of no washing procedures. High sensitivity can also be achieved with suspension beads and particles, using phycoerythrin as label (*e.g.*, available from Luminex) or the properties of semiconductor nanocrystals (*e.g.*, available from Quantum Dot). Fluorescence resonance energy transfer has been adapted to detect binding of unlabelled ligands,

which may be useful on arrays (*e.g.*, available from Affibody). Several alternative readouts have been developed, including adaptations of surface plasmon resonance (*e.g.*, available from HTS Biosystems and Intrinsic Bioprobes), rolling circle DNA amplification (*e.g.*, available from Molecular Staging), mass spectrometry (*e.g.*, available from Sense Proteomic, CIPHERgen, Intrinsic and Bioprobes), resonance light scattering (*e.g.*, available from Genicon Sciences) and atomic force microscopy (*e.g.*, available from BioForce Laboratories). A microfluidics system for automated sample incubation with arrays on glass slides and washing has been co-developed by NextGen and Perkin Elmer Life Sciences.

[0137] In some embodiments, nucleic acids (typically oligonucleotides) can be used as a protein capture agents, in which the nucleic acids bind to or otherwise complex with a nucleic acid-binding domain of an NF- κ B pathway transcription factor (*e.g.*, RelB, cRel, p65, p50 etc). The complexing of a transcription factor with a nucleic acid (*e.g.*, RNA or DNA) generally requires a specific nucleic acid sequence that corresponds to a *cis* site to which the transcription factor binds. Such *cis* sites are known to those of skill in the art and nucleic acids corresponding to these sites can be prepared using standard procedures. The nucleic acids can be used in any suitable nucleic acid-binding protein assay, non limiting examples of which include the assays described by Haukanes B I and Kvam C (Biotechnology, 1993 Jan 11 60-63), Alberts B *et al.* (Molecular Biology of the Cell, 1994, 3rd Edn., Garland Publications Inc), Kirigiti P and Machida C A (2000 Methods Mol Biol, 126, 431-51), Molecular Probes handbook and references therein (Molecular Probes, Inc., 4849 Pitchford Ave., Eugene, USA) and in Example 3 *infra*.

[0138] In other embodiments, aberrant signaling of the NF- κ B pathway is detected by detecting aberrant phosphorylation of a phosphorylatable polypeptide (*e.g.*, IRAK, TAK1, TAB1, TAB2, PKR, Akt, Cot, IKK α , IKK β and IKK γ) involved in or belonging to the NF- κ B signaling pathway. The phosphorylation state of the phosphorylatable polypeptide is indicative of the activity of the NF- κ B signaling pathway. Thus, if the phosphorylation state of the phosphorylatable polypeptide in the biological sample is higher or lower than the phosphorylation state of the same polypeptide in a reference sample, then this indicates that the phosphorylation state of phosphorylatable polypeptide in the biological sample is abnormal, indicative of aberrant signaling through the NF- κ B pathway. In specific embodiments, aberrant

signaling through the NF- κ B pathway is detected when the phosphorylation state of a NF- κ B associated phosphorylatable polypeptide such as but not limited to IRAK, TAK1, TAB1, TAB2, PKR, Akt, Cot, IKK α , IKK β and IKK γ , is lower than the phosphorylation state of the corresponding polypeptide in the reference sample.

5 Methods of detecting the phosphorylation state of a protein are well-known in the art. However, it is desirable that antigen-binding molecules that specifically recognize phosphorylated or unphosphorylated forms of the phosphorylatable polypeptide be used. Non-limiting examples of such antigen-binding molecules are available from Cell Signaling Technology, Inc.(Danvers, Massachusetts).

10 [0139] In certain embodiments, the techniques used for detection of T1DM marker gene expression products will include internal or external standards to permit quantitative or semi-quantitative determination of those products, to thereby enable a valid comparison of the level or functional activity of these expression products in a biological sample with the corresponding expression products in a reference sample or
15 samples. Such standards can be determined by the skilled practitioner using standard protocols. In specific examples, absolute values for the level or functional activity of individual expression products are determined.

4. Kits

[0140] All the essential materials and reagents required for detecting and
20 quantifying T1DM maker gene expression products may be assembled together in a kit. In some embodiments, the kit comprises: a) primers designed to produce double stranded DNA complementary to at least a portion of a T1DM marker gene; wherein at least one of the primers contains a sequence which hybridizes to RNA, cDNA or an EST corresponding to the marker gene to create an extension product and at least one
25 other primer that hybridizes to the extension product; b) an enzyme with reverse transcriptase activity, and c) an enzyme with thermostable DNA polymerase activity; wherein the primers are used to detect the expression levels of the marker gene in a test subject. In other embodiments, the kit comprises at least one oligonucleotide which hybridizes to RNA, cDNA or an EST corresponding to a T1DM marker gene, wherein
30 the oligonucleotide is used to detect the expression levels of the marker gene in a test sample. In still other embodiments, the kit comprises at least one oligonucleotide that binds to or otherwise complexes with a nucleic acid-binding domain of a T1DM marker

polypeptide (*e.g.*, an NF- κ B pathway transcription factor), wherein the oligonucleotide is used to detect the level of the transcription factor in a test sample.

[0141] The kits may optionally include appropriate reagents for detection of labels, positive and negative controls, washing solutions, blotting membranes, microtitre
5 plates dilution buffers and the like. For example, a nucleic acid-based detection kit may include (i) a T1DM marker polynucleotide (which may be used as a positive control), (ii) a primer or probe that specifically hybridizes to a T1DM marker polynucleotide. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (Reverse Transcriptase, Taq, Sequenase™ DNA ligase etc. depending on
10 the nucleic acid amplification technique employed), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits also generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each primer or probe. Alternatively, a protein-based detection kit may include (i) a T1DM marker polypeptide (which may be used as a positive control) and
15 (ii) an antigen-binding molecule that is immuno-interactive with a T1DM marker polynucleotide, or (iii) an oligonucleotide that binds to or otherwise complexes with a nucleic acid-binding domain of the T1DM marker polypeptide (*e.g.*, an NF- κ B pathway transcription factor). The kit can also feature various devices and reagents for performing one of the assays described herein; and/or printed instructions for using the
20 kit to quantify the expression of a T1DM marker polynucleotide or the level of a T1DM polypeptide.

5. Treatment and prevention of type 1 diabetes

[0142] The present invention also extends to the prevention and treatment of T1DM, its management or prevention of further progression of the disease, or
25 assessment of the efficacy of therapies in subjects following positive diagnosis for the presence, stage or risk of development of T1DM in the subjects. Illustrative treatment and prevention options include immune suppressors and anti-inflammatory agents (corticosteroids, cytostatic / cytotoxic and immunomodulatory drugs, or self antigens delivered to suppress specific immunity), drugs which promote insulin sensitivity, blood
30 exchange procedures (plasmapheresis, immunoadsorption) and lifestyle change. Treatment and prevention of autoimmune disease may also include specific lifestyle change and adjustment. Lifestyle change may include nutritional supplements, exercise

regimes, dietary changes, weight loss, factors to reduces stress as well as reduced calorie intake.

[0143] In some embodiments, the management of T1DM is based at least in part using an immune suppressor.

5 5.1 Antigens

[0144] In illustrative examples of this type, the immune suppressor is an anti-CD3 antigen-binding molecule, which has been shown to be a potent immunomodulator and to have unique immunomodulatory properties. For example, the immunomodulatory humanized anti-CD3 monoclonal antibody (hOKT3gamma1[Ala-
10 Ala]) can maintain insulin production for up to 18 months when administered to patients with recent onset T1DM.

[0145] In other illustrative examples, the immune suppressor is an antigen, which is suitably in soluble form and which corresponds to at least a portion of an antigen that correlates with the presence of T1DM. Non-limiting examples of such
15 antigens include self antigens such as but not limited to proinsulin, insulin, IA2 and GAD65. In some embodiments, the antigen is in the form of one or more peptides. Usually, such peptides are at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30 amino acid residues in length and suitably no more than about 500, 200, 100, 80, 60, 50, 40 amino acid residues in length. In some embodiments in which more that two or more
20 peptides are used, the peptides can be in the form of a plurality of contiguous overlapping peptides whose sequences span at least a portion of a target antigen. Suitably, the peptide sequences are derived from at least about 30, 40, 50, 60, 70, 80, 90, 91, 92, 93, 94. 95, 96, 97, 98, 99% of the sequence corresponding to the target antigen. In some embodiments, each peptide of the plurality of contiguous overlapping
25 peptide fragments can be 30-90 amino acids in length, *e.g.*, 30, 35, 40, 45, 50, 55, 60, 65, 70, 73, 75, 80, 81, 85, 86 and 90 amino acids in length. In various embodiments, the amino acid sequences of contiguous overlapping peptide fragments in the plurality overlap by about 10 to about 15 amino acids, *e.g.*, 10, 11, 12, 13, 14 and 15 amino acids. Exemplary methods for producing such peptide antigens are described, for
30 example, by Astori *et al.* (2000 J. Immunol. 165, 3497-3505; and references cited therein) and in U.S. Patent Application Publication No. 2004/0241178.

[0146] In some embodiments, the antigen is used in an amount that induces desensitization or suppression of an immune response to the corresponding target antigen. Routine methods for determining desensitizing or immune-suppressing amounts of antigen are known to persons of skill in the art. For example, a suitable dose of antigens needed to stimulate the suppression of an immune response to a target antigen in a patient can be determined by: (1) introducing a series of compositions containing varying concentrations of soluble antigen into the skin of the patient; (2) introducing a positive-control and a negative-control into the skin of the patient; (3) checking for development of a wheal or flare at the introduction site; and (4) comparing the size of the papule (<5 mm) and flare produced by the varying concentrations of the antigen to the positive-control and negative-control, thereby determining the dose of antigen needed to stimulate the suppression of the immune response to the target antigen.

[0147] In some embodiments, T cells are cultured with antigen and at least one proliferating agent that stimulates the proliferation of T lymphocytes (*e.g.*, anti-CD3 antigen-binding molecules and anti-CD28 antigen-binding molecules) and with one or more immunosuppressive agents (*e.g.*, dexamethasone and vitamin D3). In these embodiments, the antigen, proliferating agent and/or immunosuppressive agent(s) are useful in stimulating the proliferation of antigen-specific suppressor T lymphocytes.

5.2 Antigen-presenting cells

[0148] In other embodiments, the immune suppressor is an antigen-presenting cell that presents the antigen for modulation, especially suppression, of lymphocytes. Thus, in these embodiments, the immune suppressor is an antigen-presenting cell, which has been contacted with an antigen, or with a polynucleotide from which the antigen is expressible, for a time and under conditions sufficient for the antigen or a processed form thereof to be presented by the antigen-presenting cell. The resulting 'antigen-specific' antigen-presenting cell is suitably autologous or syngeneic. Typically, the antigen presentation is restricted by major histocompatibility (MHC) molecules. In some embodiments, the antigen-presentation is CD1-restricted. In specific embodiments, the antigen-presenting cell is a tolerogenic antigen-presenting cell, especially a tolerogenic dendritic cell.

[0149] Antigen-presenting cells or their precursors can be isolated by methods known to those of skill in the art. The source of such cells will differ

depending upon the antigen-presenting cell required for modulating a specified immune response. In this context, the antigen-presenting cell can be selected from dendritic cells, macrophages, monocytes and other cells of myeloid lineage. In specific embodiments, the antigen-presenting cell is a tolerogenic antigen-presenting cell, which is produced, for example, according to methods disclosed in International Publication WO2004015056, which comprise contacting a precursor of an antigen-presenting cell with an inhibitor of CD40 or NF- κ B and with an antigen that corresponds to a target antigen, or with a polynucleotide from which the antigen is expressible, for a time and under conditions sufficient to differentiate an antigen-presenting cell from the precursor and to inhibit or otherwise reduce the level or functional activity of CD40 in the cell, wherein the antigen or a processed form thereof is presented by the antigen-presenting cell so produced.

[0150] In other embodiments, tolerogenic antigen-presenting cells are produced *in situ* by delivery vehicles comprising inhibitors of CD40 and optionally an antigen or a nucleic acid construct from which the antigen is expressible. Suitable vehicles include, but are not restricted to, liposomes, dendrimers, antigen-binding molecules and nanoparticles, as for example disclosed by Copland *et al.* (2003, Vaccine 21, 883); Slobbe *et al.* (2003, Immunol Cell Biol 81, 185), Quintana *et al.* (2002, Pharm Res 19, 1310) and Thomas *et al.* (2004, Biomacromolecules 5, 2269).

[0151] In still other embodiments, naturally tolerogenic antigen-presenting cell subsets or their precursors are targeted using a targeting vehicle that comprises antigen. Illustrative vehicles include antigen-binding molecules that are immuno-interactive with an antigen-presenting cell-specific receptor (*e.g.*, DEC205 and DCL-1, macrophage mannose R, DC-SIGN or other DC or myeloid specific (lectin) receptors, as for example disclosed by Hawiger *et al.* (2001, J Exp Med 194, 769), Kato *et al.* (2003, J Biol Chem 278, 34035), Benito *et al.* (2004, J Am Chem Soc 126, 10355) and Schjetne, *et al.* (2002, Int Immunol 14, 1423).

5.3 Suppressor T lymphocytes

[0152] In still other embodiments, the immune suppressor is a suppressor T lymphocyte that suppresses an immune response to the target antigen. Such “antigen-specific” suppressor T lymphocytes can be prepared using the antigen-presenting cell broadly described above. For example, antigen-specific suppressor T lymphocytes can be obtained by contacting a population of T lymphocytes, or their precursors (*e.g.*,

peripheral blood mononuclear cells; PBMC), with an antigen-specific antigen-presenting cell, especially an antigen-specific antigen-presenting cell, as broadly described above for a time and under conditions sufficient to produce suppressor T lymphocytes that suppress an immune response to the target antigen. Alternatively, the suppressor T lymphocytes can be obtained simply by isolating, or isolating and stimulating the proliferation of a population of suppressor T lymphocytes in culture, wherein the isolated population comprises one or more suppressor T lymphocytes that suppress the immune response to the target antigen. The stimulation can be performed *in vivo* in a subject or *ex vivo* in culture. Suitably, the suppressor T lymphocyte is capable of suppressing a proliferative and/or cytokine response of other immune cells, including but not restricted to effector T lymphocytes and B lymphocytes. In some embodiments, the suppressor T lymphocyte expresses markers of constitutive regulatory or suppressor T lymphocytes, including CD4, CD25, CD62L, GITR, CTLA4 and the transcription factor FoxP3.

[0153] Suppressor T lymphocytes can be prepared, for example, by contacting T lymphocytes with an antigen-specific antigen-presenting cells as described in Section 5.2 for an adequate period of time and optionally one or more cytokines (*e.g.*, IL-2 or IL-15) to stimulate the proliferation of suppressor T lymphocytes with specificity to the antigen or antigens presented by those antigen-presenting cells. This period will usually be at least about 1 day, and up to about 5 days. Where the isolated T cell preparation consists of CD4+CD25+ regulatory T lymphocytes, the preferred antigen-specific antigen presenting cell with which to contact those lymphocytes is a mature dendritic cell (Oldenhove 2003, J. Exp. Med 198, 259-66).

[0154] In other embodiments, a population of immature antigen-presenting cell (*e.g.* immature dendritic cells) is cultured in the presence of an antigen and an heterogeneous population of T lymphocytes, typically in the presence of a pro-inflammatory cytokine signalling pathway antagonist as described in Section 2.1, for a period of time and under conditions sufficient for: the antigen, or processed form thereof, to be presented by the antigen-presenting cells and the antigen-presenting cells to stimulate the proliferation of a subpopulation of regulatory T lymphocytes with specificity to the antigen.

[0155] In still other embodiments, a population of antigen-specific suppressor T lymphocytes is obtained by contacting a purified population of constitutive suppressor

lymphocytes (*e.g.*, CD4⁺CD25⁺ suppressor T lymphocytes) with at least one proliferating agent that stimulates the proliferation of suppressor T lymphocytes (*e.g.*, with an anti-CD3 antigen-binding molecule and optionally anti-CD28 antigen-binding molecule) or antigen-loaded tetramers, optionally in the presence of one or more

5 cytokines (*e.g.*, IL-2 and IL-15), to stimulate non-specifically the proliferation of a population of suppressor T lymphocytes which comprises a subpopulation of antigen-specific suppressor T lymphocytes. In these embodiments, the population of suppressor T lymphocytes before expansion comprises one or more suppressor T lymphocytes that suppress the immune response to a target antigen. Illustrative methods of this type are

10 described in Bolt *et al.* (1993, European Journal of Immunology 23, 403), Belghith *et al.* (2003, Nat Med 9, 1202), Herold *et al.* (2002, N Engl J Med 346, 1692), Bluestone *et al.* (2004, Proc Natl Acad Sci U S A 101 Suppl 2, 14622), Tang *et al.* (2004, J Exp Med 199, 1455) and Tarbell *et al.* (2004, J Exp Med 199, 1467). In illustrative

15 examples, the proliferation agent(s) (*e.g.* anti-CD3) is administered to a patient *in vivo*, to stimulate the proliferation of the antigen-specific suppressor T lymphocytes, together with a pro-inflammatory cytokine signaling pathway antagonist. Alternatively, proliferation of the antigen-specific suppressor T lymphocytes can be achieved *ex vivo* by contacting the heterogeneous population of T lymphocytes in culture medium with at least one suppressor T lymphocyte-proliferating agent, and optionally in the presence of

20 a pro-inflammatory cytokine signaling pathway antagonist.

[0156] In some embodiments, the antigen-specific suppressor T lymphocytes are CD4⁺CD25⁺. The invention also contemplates within its scope other regulatory T lymphocytes that inhibit the response of other (effector) lymphocytes in an antigen-specific manner including, for example, Tr1 lymphocytes, Th3 lymphocytes, Th2

25 lymphocytes, CD8⁺CD28⁻ regulatory T lymphocytes, natural killer (NK) T lymphocytes and $\gamma\delta$ T lymphocytes.

[0157] Tr1 lymphocytes can emerge from CD4⁺ or CD4⁺CD25⁻ T lymphocyte precursors after several rounds of stimulation of human blood T cells by allogeneic monocytes in the presence of IL-10. This subpopulation secretes high levels

30 of IL-10 and moderate levels of TGF- β but little IL-4 or IFN- γ (Groux *et al.*, 1997, *Nature* 389, 737-742).

[0158] In some embodiments, a population of antigen-specific suppressor T lymphocytes is obtained by contacting an heterogeneous population of T lymphocytes

(*e.g.*, peripheral blood), or CD4⁺CD25⁻ naive T lymphocyte precursors with an antigen, an antigen presenting cell (*eg.* a peripheral blood monocyte or dendritic cell) and at least one differentiation-modulating agent (*e.g.*, TGF- β and IL-10) that stimulates the differentiation of the precursors into Tr1 suppressor T lymphocytes, for a time and under condition sufficient to obtain antigen-specific suppressor T lymphocytes. Illustrative methods of this type are described in Groux *et al.* (1996, J Exp Med 184, 195) and Levings *et al.* (2004, Blood Oct 12).

[0159] In still other embodiments, a population of antigen-specific suppressor Tr1 type regulatory lymphocytes is obtained by contacting an heterogeneous population of T lymphocytes with at least one proliferating agent that stimulates the proliferation of suppressor T lymphocytes (*e.g.*, anti-CD3 antigen-binding molecules and anti-CD28 antigen-binding molecules) and with one or more immunosuppressive agents (*e.g.*, dexamethasone and vitamin D3) for a time and under conditions sufficient to obtain antigen-specific suppressor T lymphocytes.

[0160] The Th3 regulatory subpopulation refers to a specific subset induced following antigen delivery *via* the oral (or other mucosal, such as intra-nasal) route. They produce predominantly TGF β , and only low levels of IL-10, IL-4 or IFN γ , and provide specific help for IgA production (Weiner *et al.*, 2001, Microbes Infect 3, 947-954). They are able to suppress both Th1 and Th2-type effector T cells.

[0161] Th2 lymphocytes produce high levels of IL-4, IL-5 and IL-10 but low IFN γ and TGF β . Th2 lymphocytes are generated in response to a relative abundance of IL-4 and lack of IL-12 in the environment at the time of presentation of their cognate peptide ligands (O'Garra and Arai, 2000, Trends Cell Biol 10, 542-550). T lymphocyte signaling by CD86 may also be important for generation of Th2 cells (Lenschow *et al.*, 1996, Immunity 5, 285-293; Xu *et al.*, 1997, J Immunol 159, 4217-4226).

[0162] A distinct CD8⁺CD28⁻ regulatory or "suppressor" subset of T lymphocytes can be induced by repetitive antigenic stimulation *in vitro*. They are MHC class I-restricted, and suppress CD4⁺ T cell responses.

[0163] NK T lymphocytes, which express the NK cell marker, CD161, and whose TCR are V α 24J α Q in human and V α 14J α 281 in mouse, are activated specifically by the non-polymorphic CD1d molecule through presentation of a glycolipid antigen (Kawano *et al.*, 1997, Science 278, 1626-1629) or the natural ligand

lysosomal isoglobotrihexosylceramide (Zhou *et al.*, 2004, *Science* 306, 1786-9). They have been shown to be immunoregulatory in a number of experimental systems. They are reduced in number in several autoimmune models before disease onset, and can reduce incidence of disease upon passive transfer to non-obese diabetic (NOD) mice.

- 5 Repetitive administration of the glycolipid, α -galactosyl ceramide (α -gal cer), presented by CD1d, also results in accumulation of NKT lymphocytes and amelioration of diabetes in these mice (Naumov *et al.*, 2001, *Proc Natl Acad Sci U S A* 98, 13838-13843).

- [0164] $\gamma\delta$ T lymphocytes have been implicated in the downregulation of
10 immune responses in various inflammatory diseases and in the suppression of inflammation associated with induction of mucosal tolerance. The tolerance induced by mucosal antigen was transferable to untreated recipient mice by small numbers of $\gamma\delta$ T cells (McMenamin *et al.*, 1995, *J Immunol* 154, 4390-4394; McMenamin *et al.*, 1994, *Science* 265, 1869-1871). Moreover, mucosal tolerance induction was blocked by the
15 administration of the GL3 antibody that blocks $\gamma\delta$ T cell function (Ke *et al.*, 1997, *J Immunol* 158, 3610-3618).

- [0165] The suppression provided by the regulatory T lymphocytes as produced by the methods of the invention involves a much lowered proliferative responsiveness to antigen, *e.g.*, less than about 50% response, usually less than about
20 40% response, more usually less than about 5-10% response or less, as compared to regulatory T lymphocytes exposed to constitutive IL-1 or IL-18 signaling pathway stimulation.

5.4 Pharmaceutical compositions

- [0166] The above immune suppressors are suitably administered in the form
25 of pharmaceutical compositions that optionally comprise a pharmaceutically effective carrier. The immune suppressors are typically contained in an effective amount to achieve their intended purpose. The dose of active compounds administered to a patient should be sufficient to achieve a beneficial response in the patient over time such as a reduction in at least one symptom associated with T1DM (*e.g.*, a reduction in at least
30 one of blood glucose, insulin requirement, obesity or an increase in insulin sensitivity or C-peptide production). The quantity of the pharmaceutically active compounds(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of immune

modulator for administration will depend on the judgement of the practitioner. In determining the effective amount of the active compound(s) to be administered in the treatment or prophylaxis of T1DM, the practitioner may evaluate inflammation, pro-inflammatory cytokine levels, lymphocyte proliferation, cytolytic T lymphocyte activity and regulatory T lymphocyte function. In any event, those of skill in the art may readily determine suitable dosages of the antagonist and antigen.

[0167] In some embodiments, and dependent on the intended mode of administration, antigen-containing pharmaceutical compositions will generally contain about 0.1% to 90%, about 0.5% to 50%, or about 1% to about 25%, by weight of antigen, the remainder being suitable pharmaceutical carriers and/or diluents etc and pro-inflammatory cytokine signaling pathway antagonist. Usually, a nasal or oral dose of insulin in may be from about 10-100 IU/day per day for a patient of approximately 75 kg in weight..

[0168] Depending on the specific condition being treated, the active compounds may be formulated and administered systemically, topically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. For injection, the therapeutic agents of the invention may be formulated in aqueous solutions, suitably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0169] Alternatively, the compositions of the invention can be formulated for local or topical administration. In this instance, the subject compositions may be formulated in any suitable manner, including, but not limited to, creams, gels, oils, ointments, solutions and suppositories. Such topical compositions may include a penetration enhancer such as benzalkonium chloride, digitonin, dihydrocytochalasin B, capric acid, increasing pH from 7.0 to 8.0. Penetration enhancers which are directed to enhancing penetration of the active compounds through the epidermis are preferred in

this regard. Alternatively, the topical compositions may include liposomes in which the active compounds of the invention are encapsulated.

[0170] The compositions of the present invention may be formulated for administration in the form of liquids, containing acceptable diluents (such as saline and sterile water), or may be in the form of lotions, creams or gels containing acceptable diluents or carriers to impart the desired texture, consistency, viscosity and appearance. Acceptable diluents and carriers are familiar to those skilled in the art and include, but are not restricted to, ethoxylated and nonethoxylated surfactants, fatty alcohols, fatty acids, hydrocarbon oils (such as palm oil, coconut oil, and mineral oil), cocoa butter waxes, silicon oils, pH balancers, cellulose derivatives, emulsifying agents such as non-ionic organic and inorganic bases, preserving agents, wax esters, steroid alcohols, triglyceride esters, phospholipids such as lecithin and cephalin, polyhydric alcohol esters, fatty alcohol esters, hydrophilic lanolin derivatives, and hydrophilic beeswax derivatives.

[0171] Alternatively, the active compounds of the present invention can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration, which is also preferred for the practice of the present invention. Such carriers enable the compounds of the invention to be formulated in dosage forms such as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. These carriers may be selected from sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

[0172] 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 that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilisers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0173] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as., for example, maize starch, wheat starch, rice starch, potato starch, gelatine, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more therapeutic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, eg. by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0174] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterise different combinations of active compound doses.

[0175] Pharmaceuticals which can be used orally include push-fit capsules made of gelatine, as well as soft, sealed capsules made of gelatine and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

[0176] Dosage forms of the active compounds of the invention may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of an active compound of the invention may be achieved by coating

the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, controlled release may be achieved by using other polymer matrices, liposomes and/or microspheres.

5 [0177] The immune suppressors may be administered over a period of hours, days, weeks, or months, depending on several factors, including the severity of the neuropathic condition being treated, whether a recurrence of the condition is considered likely, *etc.* The administration may be constant, *e.g.*, constant infusion over a period of hours, days, weeks, months, *etc.* Alternatively, the administration may be intermittent, 10 *e.g.*, active compounds may be administered once a day over a period of days, once an hour over a period of hours, or any other such schedule as deemed suitable.

 [0178] The compositions of the present invention may also be administered to the respiratory tract as a nasal or pulmonary inhalation aerosol or solution for a nebuliser, or as a microfine powder for insufflation, alone or in combination with an 15 inert carrier such as lactose, or with other pharmaceutically acceptable excipients. In such a case, the particles of the formulation may advantageously have diameters of less than 50 micrometers, suitably less than 10 micrometers.

 [0179] When antigen-specific antigen-presenting cells or antigen-specific regulatory T cells are employed, the cells can be introduced into a patient by any means 20 (*e.g.*, injection), which produces the desired modified immune response to an antigen or group of antigens. The cells may be derived from the patient (*i.e.*, autologous cells) or from an individual or individuals who are MHC-matched or -mismatched (*i.e.*, allogeneic) with the patient. In specific embodiments, autologous cells are injected back into the patient from whom the source cells were obtained. The injection site may be 25 subcutaneous, intraperitoneal, intramuscular, intradermal, or intravenous. The cells may be administered to a patient already suffering from the unwanted immune response or who is predisposed to the unwanted immune response in sufficient number to prevent or at least partially arrest the development, or to reduce or eliminate the onset of, that response. The number of cells injected into the patient in need of the treatment or 30 prophylaxis may vary depending on *inter alia*, the antigen or antigens and size of the individual. This number may range for example between about 10^3 and 10^{11} , and more preferably between about 10^5 and 10^8 cells (*e.g.*, dendritic cells or T lymphocytes). Single or multiple administrations of the cells can be carried out with cell numbers and

pattern being selected by the treating physician. The cells should be administered in a pharmaceutically acceptable carrier, which is non-toxic to the cells and the individual. Such carrier may be the growth medium in which the cells were grown, or any suitable buffering medium such as phosphate buffered saline. The cells may be administered
5 alone or as an adjunct therapy in conjunction with other therapeutics known in the art for the treatment or prevention of unwanted immune responses for example but not limited to glucocorticoids, methotrexate, D-penicillamine, hydroxychloroquine, gold salts, sulfasalazine, TNF α or interleukin-1 inhibitors, and/or other forms of specific immunotherapy.

10 **[0180]** In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

EXAMPLE 1

LPS-INDUCED NF- κ B ACTIVITY AND CELL SURFACE MARKER EXPRESSION IN T1DM DC

5 **[0181]** As NF- κ B is required for the functional differentiation of DC, the inventors screened NF- κ B expression by immunoblotting cytoplasmic and nuclear extracts of DC generated in the presence of GM-CSF and IL-4 for 48 h from peripheral blood monocytes of T1DM patients and healthy controls. The inventors have previously demonstrated low levels of nuclear NF- κ B in monocyte-derived DC cultured for 48 h,
10 as compared to 7 days (O'Sullivan *et al.*, 2002, *J Immunol* 168:5491-5498). However, at 48 h, DC efficiently translocate NF- κ B to the nucleus after activation with LPS, TNF- α or CD154 signaling. To further reduce background NF- κ B of 48 h DC, cell culture was carried out in serum free medium. In nuclear extracts, RelB, RelA, c-Rel, p52 and p50 increased in response to LPS in DC from healthy control but not from T1DM
15 patients (Figure 1a). Additionally, binding of nuclear RelB to DNA was reduced after 24 h of LPS exposure (Figure 1b). To further examine the ability of NF- κ B to bind DNA and therefore to exert transcriptional activity, DNA binding of RelB was analyzed by ELISA using nuclear extracts from T1DM patients. While RelB DNA binding of resting DC was similar to that of healthy controls, control DC but not T1DM DC
20 significantly increased RelB DNA binding in response to LPS. Since a reduced DC responsiveness to LPS might be a characteristic of cells derived from a hyperglycemic environment, or might occur in patients with autoimmune diseases in general, the inventors compared RelB DNA binding in DC from patients with T2DM or rheumatoid arthritis (RA). DC from these patient populations behaved similarly to DC from healthy
25 controls, and in all groups RelB DNA binding after LPS was significantly higher than that of T1DM patients (Figure 2).

[0182] RelB is regulated both transcriptionally, including auto-regulation by RelA, and post-translationally (Bren *et al.*, 2001, *Oncogene* 20:7722-7733). Transcription of NF- κ B family members in response to LPS was therefore examined by
30 quantitative real-time PCR. All mRNA levels were initially normalized to GAPDH, and induced transcription after 2 or 24 hours of LPS stimulation was expressed as the fold increase over untreated. There were no significant differences between T1DM DC and

healthy controls in the relative mRNA levels of RelA, RelB, c-Rel or p50 after 2 (not shown) or 24 h of LPS (Figure 3). Taken together, the data demonstrate a specific reduction in nuclear NF- κ B activation in T1DM DC in response to LPS, likely due to post-translational mechanisms. Since NF- κ B and p38 MAPK both contribute to DC
5 activation and cytokine production (O'Sullivan *et al.*, 2002, *supra*; Arrighi *et al.*, 2001, *J Immunol* 166:3837-3845), LPS-induced phospho-p38 MAPK was also examined. p38 was induced equivalently 15-30 min after stimulation of healthy control and T1DM DC with LPS (Figure 4).

[0183] When differentiated for 72 h, monocyte-derived DC from T1DM or
10 control subjects expressed CD1a, low levels of surface CD40, HLA-DR, MHC class I, CD86, and CD80, but neither CD14 nor CD83 consistent with an immature DC phenotype (Figure 5a). When stimulated with LPS for the last 24 h of culture, DC from healthy controls, T2DM and RA patients increased expression of HLA-DR, CD86, CD80, CD83, CD40 and MHC class I. In contrast, augmentation of CD40 and MHC
15 class I expression, but not HLA-DR, CD80, CD86 and CD83, was significantly reduced in T1DM DC in response to LPS (Figure 5b). There was no significant difference in the production of IL-12p70, IL-1 β , TNF- α and IL-10 by resting or LPS-stimulated DC when comparing T1DM DC with DC from healthy controls, T2DM or RA patients (Figure 2b). The data demonstrate a specific reduction of LPS-induced CD40 and MHC
20 class I expression in T1DM DC. Moreover, a similar reduction in CD40 expression in response to either the classical pathway signal TNF α and the predominant alternative pathway signal soluble CD154 was observed in T1DM but not control DC. While LPS predominantly activates the classical pathway in B cells, previous studies have shown that in DC, LPS activates both pathways (Zarnegar *et al.*, 2004, *Proc Natl Acad Sci USA*
25 101:8108-8113; Mordmuller *et al.*, 2003, *EMBO Rep* 4:82-87). These data, together with the global reduction in NF- κ B nuclear translocation in response to LPS treatment of DC, suggest a post-translational deficiency in NF- κ B responsiveness to signaling through diverse cell surface receptors. Intact p38 MAPK signaling is likely to account for the capacity of the DC to induce several costimulatory molecules and cytokines in
30 response to LPS.

EXAMPLE 2**SHP-1 EXPRESSION IS INCREASED IN T1DM HEMOPOIETIC CELLS**

[0184] Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1) has been shown to be an NF- κ B pathway inhibitor (Neznanov *et al.*, 2004, *DNA Cell Biol* 23:175-182), potentially acting upstream of NF- κ B at the level of the adaptor molecule TRAF6. In contrast, another similar tyrosine phosphatase, SHP-2 expression is a positive regulator of NF- κ B (Neznanov *et al.*, 2004, *supra*; Khaled *et al.*, 1998, *Cell Immunol* 185:49-58; Massa *et al.*, 1998, *J Interferon Cytokine Res* 18:499-507). SHP-1 and SHP-2 are very similar classical non-receptor protein tyrosine phosphatases, abundant in hemopoietic cells with about 59% sequence homology, which mainly differ in the 100 amino acids residues in the C-terminus, and play negative and positive regulatory roles in cell signaling respectively (Poole, 2005, *Cell Signal* 17:1323-1332). To assess the relationship of SHP-1 to DC maturation, CD11c⁺ splenic DC from me^v/me^v (SHP-1 mutant motheaten viable mice) and wild type (wt) mice were stimulated with LPS. me^v/me^v DC displayed a pattern of activation resembling T1DM DC in reverse, in that CD40 but not CD86 expression was increased compared with the response of wt DC to LPS (Figure 6a). These data are consistent with the previously-observed increase in NF- κ B activation in me^v/me^v mice. The tyrosine phosphatase SHP-1 is expressed in most cell types. Therefore, SHP1 expression in T1DM and healthy control DC (not shown) or PBMC was examined. The level of SHP-1 in T1DM PBMC was higher than in control PBMC (Figure 6b). However, there was no difference in LPS-induced expression of another src homology 2 domain-containing protein tyrosine phosphatase, SHP-2, which is known to be a positive regulator of NF- κ B. (Figure 6b). Of 15 diabetics examined for LPS-stimulated SHP-1, the abnormal regulation was present in 11 (73%). One patient with normal SHP-1 regulation developed T1DM due to non-autoimmune amyloid infiltration of the pancreas. A further patient with islet cell antibodies and a family history, but normal glucose tolerance also displayed the SHP-1 regulatory abnormality. Conversely, 1 of 8 healthy controls displayed abnormal SHP1 regulation. Consistent with the regulatory role of SHP-1 on NF- κ B activity, SHP-1 expression was negatively correlated with I- κ B phosphorylation after LPS treatment of monocytes from healthy control and T1DM subjects (Figure 6c). The data indicate that NF- κ B, as determined by reduced LPS-induced nuclear translocation and DNA binding of RelA, p50, c-Rel, Rel and p52, and

reduced LPS-induced I- κ B phosphorylation, is abnormally regulated in hemopoietic cells of the majority of the T1DM patients, as compared to healthy controls. In contrast, p38 MAPK regulation is intact in T1DM monocytes. Elevated SHP-1 is associated with the NF- κ B abnormality.

5

DISCUSSION OF EXAMPLES 1 AND 2

THE IMPACT OF NF- κ B DEFICIENCY ON IMMUNOREGULATION

[0185] DC play important antigen presenting roles in thymic selection, to determine the repertoire of potentially self-reactive and immunoregulatory T-cells, as well as in peripheral presentation of self and foreign antigens (Lipscomb and Masten, 2002, *Physiol Rev*, 82(1); 97-130). Therefore, pathways controlling DC function are critical elements impacting on the T-cell repertoire, and the peripheral immune responses which must both control infections and prevent the development of autoimmune disease. NF- κ B and MAPK are the major pathways for DC differentiation and maturation (Ouaaz *et al.*, 2002, *Immunity*, 16(2); 257-270; Platzner *et al.*, 2004, *Blood*, 104(12); 3655-3663; Lyakh, 2000; Xie *et al.*, 2003, *J Immunol*, 171(9); 4792-4800).

[0186] In particular, RelB is required for the development of monocytes and monocyte-derived DC in humans, and of CD4⁺ DC in mouse. RelB activity correlates with functional DC maturation, and DC developing *in vitro* in the absence of RelB stimulate the development of antigen-specific regulatory T cells in the periphery *in vivo* (Ardeschna *et al.*, 2000, *Vox Sang*, 79(1); 46-52; Lyakh *et al.*, 2000, *J. Immunol*, 165(7); 3647-3655; Martin *et al.*, 2003, *Immunity*, 18(1); 155-167).

[0187] Furthermore, RelB plays a critical role in the development of medullary thymic epithelial cells, and these cells are deficient in RelB^{-/-} mice, leading to reduced negative selection of autoreactive T-cells and severe systemic autoimmune inflammation (Weih *et al.*, 1997, *J Exp Med*, 185(7); 1359-1370; Barton *et al.*, 2000, *Eur J Immunol*, 30(8); 2323-2332). Both RelA and RelB contribute to CD40 expression by myeloid cells (Tone *et al.*, 2002, *J Biol Chem*, 277(11); 8890-8897; Martin *et al.*, 2003, *Immunity*, 18(1); 155-167).

[0188] Additionally, RelB-containing NF- κ B complexes transactivate MHC Class I promoters in breast cancer cells suggesting RelB regulates MHC class I protein expression (Dejardin *et al.*, 1998, *Oncogene*, 16(25); 3299-3307). CD40 plays an

important role in maintaining tolerance to self antigen and CD40 and CD154^{-/-} mice are predisposed to autoimmune disease development (Kumanogoh *et al.*, 2001, *J Immunol*, 166(1): 353-360).

[0189] In the current studies, the DC differentiation protocol was designed to optimise the detection of NF- κ B from low levels in very immature DC derived over two days from monocytes in the absence of serum (O'Sullivan *et al.*, 2002, *J Immunol*, 168(11); 5491-5498). While, LPS, TNF or CD154-induced NF- κ B activity was dysregulated, NF- κ B expression by immature DC and LPS-induced transcription was intact, indicating that the regulatory abnormality involves the phosphorylation, ubiquitination or proteasomal degradation of I- κ B/p100, while sparing p38 MAPK. The demonstrated dysregulation of autoimmune disease DC and lymphocytes in response to activation signals provides a strong basis for the abnormal activation of T-cells in response to self or foreign antigen and in the maintenance tolerance to self-antigen in this disease.

IMMUNOREGULATORY ABNORMALITIES IN PATIENTS WITH TYPE 1 DIABETES

[0190] In autoimmune disease, there is an imbalance between autoreactive and regulatory T-cells, such that islet antigen-autoreactive T-cells are polarised to Th1 type proinflammatory cytokine production (Kriegel *et al.*, 2004, *J Exp Med*, 199(9); 1285-1291; Lindley *et al.*, 2005, *Diabetes*, 54(1); 92-99; Arif *et al.*, 2004, *J Clin Invest*, 113(3); 451-463). In keeping with the local inflammatory response, DC infiltrate pancreatic tissue of autoimmune disease patients (Summers *et al.*, 2003, *Ann N Y Acad Sci*, 1005; 226-229). Deficient T-cell proliferation and impaired primary immune responses have been shown in autoimmune disease (Takahashi *et al.*, 1998, *Int Immunol*, 10(12); 1969-1980; Eibl *et al.*, 2002, *Int Immunol*, 10(12); 1969-1980). Since SHP-1 is a negative regulator of TCR signalling, the inventors propose that the increased levels of SHP-1 increase the threshold for T-cell activation (Stefanova *et al.*, 2003, *Immunol Rev*, 191; 97-106). Moreover, decreased activation of NF- κ B in DC and T-cells may impair T-cell co-stimulation and proliferation and the development and activation of CD25⁺CD4⁺ regulatory T cells and NKT cells. Furthermore, SHP-1 also negatively modulates glucose homeostasis, through inhibition of insulin receptor signalling in liver and muscle (Dubois *et al.*, 2006, *Nature Medicine* 12(5): 549-556). Thus, the inventors propose that the increased levels of SHP-1 also increase the

resistance of target tissues to the reduced levels of insulin secreted by pancreatic islets damaged by autoimmune inflammation in T1DM.

[0191] In the non-obese diabetic spontaneous mouse model of autoimmune disease, predisposition to disease occurs at least in part through reduced thymic negative selection. The inventors propose that the altered T cell and DC signal threshold shown here alters thymic selection in those individuals with demonstrated dysregulation. The current data also challenge the notion that genetic or signaling defects responsible for autoimmune disease in NOD mice are necessarily representative of those in humans with autoimmune disease, as NF- κ B signaling in NOD mice has generally been found to be increased in response to signals such as LPS (and unpublished data) but this may vary depending on the signals. Nevertheless, in both mice and humans with autoimmune disease, alterations in the signaling threshold for selection, cell activation and cell death modulated by NF- κ B may affect the balance of thymic selection and peripheral T cell activation and tolerance.

MATERIALS AND METHODS RELATING TO EXAMPLES 1 AND 2

CELL PREPARATION AND CELL CULTURE

[0192] Heparinized peripheral blood was collected from patients with T1DM (autoimmune disease) and from patients without any condition which were referred to as healthy control (HC). Individuals ranged in age from 8 to 80 years. The study was approved by the human ethics committee of the Princess Alexandra Hospital. Selected diabetic patients had either long-standing autoimmune disease, or were newly diagnosed. Autoimmune disease patients were examined and were well apart from the presence of diabetes. There was no sign of any related disorders and their blood glucose was controlled while taking the samples. PBMC were prepared by density gradient centrifugation using Ficoll (Sigma).

[0193] For DC, CD14 positive cells were prepared from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec, GmbH, Germany). The measured purity was more than 97%. Monocytes were cultured in 24 well flat bottom tissue culture plates in X-VIVO 20 (Biowhittaker) with 800 U/mL of rhGM-CSF and 600 U/mL of rh IL-4 (Schering-Plough, Sydney, Australia) for 72 hours at 37° C and 5% CO₂. Approximately 100 ng/mL of LPS was added to the wells for the last 24 hours of the culture period. CD11c⁺ DC were purified from me^v/me^v mice and wt mice spleens

using CD11c microbeads (Miltenyi Biotec), then cultured overnight with or without 100 ng/mL of LPS in RPMI + 10% FCS.

CELL EXTRACTS, IMMUNOBLOTTING AND RELB DNA BINDING ELISA

5 **[0194]** Cytoplasmic and nuclear extracts were prepared from 72 hour cultured DC or freshly-isolated PBMC according to the iso-osmotic/nonidet P-40 method described (Pettit *et al.*, 1997, *J Immunol*, 159(8); 3681-3691). The whole cell lysate was prepared using the RIPA buffer which contained, (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholic acid, 0.1% SDS, NP-40 and Triton® X-100), Protease inhibitor cocktail from Sigma-Aldrich, complete mini tablets, a protease inhibitor
10 cocktail from Roche Applied Science, Indianapolis and 100 mM Phenylmethylsulphonyl fluoride (PMSF). Extracts were preserved in storage at -70° C and protein concentrations were measured using a Protein Assay kit (Bio-rad, Hercules, CA).

15 **[0195]** For immunoblotting, 20 µg of protein were separated on 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Amersham Biosciences, Sunnyvale, CA). The membrane was stained with 0.1% Ponceau S stain in 5% acetic acid to compare protein loading. After several washes, the membrane was blocked with skimmed milk and then incubated with rabbit NF-κB antibodies RelA (sc-372), RelB (sc-226), c-Rel (sc-70), p50 (sc-7178) from Santa Cruz Biotechnology
20 (Santa Cruz, CA) and p52 from Upstate (Lake Placid, NY) and the src homology 2 domain-containing protein tyrosine phosphatases, SHP-1 and SHP-2 from Upstate (Lake Placid, NY). Binding of antibodies was revealed with HRP-conjugated anti-rabbit antibody using the enhanced chemiluminescence (ECL) reagents (Amersham Biosciences UK Limited, Buckinghamshire, England).

25 **[0196]** ELISA-based format BD™ Transfactor Kits (BD biosciences Clontech, Palo Alto, CA) were used to measure the NF-κB DNA binding capacity. Transfactor Kits were coated with oligonucleotides containing the consensus of the DNA binding sequences for the NF-κB transcription factors. Approximately 10 µg of nuclear extract was added to each ELISA plate. Bound transcription factor from the
30 nuclear extract was allowed to bind with the RelB antibody followed by the horseradish peroxidase-conjugated secondary antibodies and detected by colour development of

tetramethylbenzidine substrate, read at 650 nm using a Multiscan plate reader (Labsystems, Chicago, IL).

FLOW CYTOMETRY

[0197] Monocyte-derived DC cultured for 72 h were stained with the following mAb: CD1a FITC, CD86 FITC, isotype control IgG1 PE (Cymbus Biotechnology, Chandlers Ford, United Kingdom), CD11c FITC, HLA-A,B,C FITC, CD83 PE, isotype control IgG1 FITC (BD Pharmingen, San Diego, CA), CD40 FITC, HLA-DR FITC, CD80 FITC, and mouse IgG2a FITC (Biolegend, San Diego, CA). Cell surface markers were analysed using a FACScalibur flow cytometer and Cell Quest software (BD Pharmingen). Purified murine CD11c⁺ DC were stained with CD40 FITC (Santa Cruz Biotechnology), CD86 FITC (BD Pharmingen) and rat IgG2a isotype as a control. Delta geometric mean fluorescence intensity (δ MFI) was measured by subtracting MFI of isotype-matched controls from the specific marker MFI of each of the samples.

INTRACELLULAR STAINING FOR P38 MAPK AND SHP-1

[0198] DC and/or PBMC were fixed with 2% PFA and permeabilized with 90% cold methanol for 30 min. The buffer used for washing and incubation was BD Perm/WashTM buffer (BD Pharmingen, San Diego, CA). For phospho-p38 staining, cells were stained with anti-phospho-p38 MAPK (T180/Y182): Alexa Fluor[®] 647 (BD Pharmingen), and for SHP-1, fixed and permeabilised cells were first incubated with SHP-1 antibody (Upstate, Lake Placid, NY), and then with anti-rabbit biotin (biotinylated goat anti-rabbit Ig, Dako Cytomation, CA), and finally with streptavidin FITC (Biolegend).

CYTOKINE MEASUREMENT

[0199] Cytokines in DC supernatants were measured using ELISA kits for IL-10, IL-1 β , IL-12p70 from BD Pharmingen (OPEIA kits, BD Pharmingen, San Diego, CA) and TNF- α from Biolegend (MaxTM Set Standard, Biolegend, San Diego CA).

REAL-TIME PCR

[0200] Total RNA was extracted from 10⁶ DC using TRIzol reagent (Invitrogen Australia Pty Limited, Vic, Australia). First strand cDNA was synthesised using random primers (Promega, NSW, Australia), dNTPs, Rnase Out, and

superScriptTM III Reverse Transcriptase (Invitrogen Australia Pty Limited, Vic, Australia) and used as a template for real-time PCR. GAPDH and all NF- κ B Primers were as described (O'Sullivan and Thomas, 2002, *J Immunol*, 168(11); 5491-5498) The 25 μ l of PCR reaction contains PCR 1x SYBR Green Master Mix (Applied Biosystems, Foster city, CA) and 100 pmol of each primer. PCR cycling conditions were initially 50° C for 2 minutes and 95° C for 10 minutes followed by 95° C for 15 seconds and 60° C for 1 min for 40 cycles conducted on an ABI PRISM 7700 thermal cycler (PE Applied Biosystems, Foster city, CA). Standard curves were generated for GAPDH and NF- κ B using LPS-stimulated DC cDNA with a serial dilution (1:5, 1:50, 1:500). mRNA level is measured using the threshold cycle and the corresponding standard curves and the relative amount of mRNA is calculated as for each NF- κ B normalized to GAPDH. The relative expression of LPS-induced NF- κ B genes in 2h or 24h time periods were measured as fold increase over untreated.

EXAMPLE 3

REDUCED LPS-INDUCED NF- κ B ACTIVITY IN DC FROM T1DM PATIENTS AND IN PBMC OF AT-RISK T1DM FAMILY MEMBERS

[0201] To quantitate the activation of the different NF- κ B family members in response to LPS with greater sensitivity, DC were generated from peripheral blood (PB) monocytes from either healthy control or T1DM subjects. The capacity of NF- κ B protein in nuclear extracts to bind a consensus NF- κ B oligonucleotide after 24 h culture with or without LPS, was assessed by a highly sensitive luminometric assay. The binding capacity of nuclear RelB, p65 and p50 all increased after LPS treatment of the healthy control DC (Figure 7, black lines). In contrast, RelB did not increase after LPS treatment of T1DM DC (blue, purple, red and green lines). Furthermore, using this assay the binding capacity of p65 and p50 was shown to decrease after LPS treatment, indicating that LPS activates NF- κ B in healthy DC but represses p65 and p50 in T1DM DC (Figure 7). The basal NF- κ B activity, without LPS, varied between individuals. In the same experiment, T1DM DCs were shown to have higher levels of expression of CD1a than healthy control DCs. LPS had no effect on CD1a expression (Figure 8). T1DM DCs in a proportion of patients secreted increased levels of TGF- β in response to LPS in contrast to healthy control DCs in which LPS was associated with a reduction in TGF- β (Figure 9).

[0202] To assess NF- κ B activation by LPS in those at risk of T1DM, the same assay for RelB was carried out using PBMC extracted from small volumes of blood of children and adults from 3 families in which one child has T1DM but the other family members are asymptomatic. While at least one parent from each family showed an increased RelB response to LPS, the diabetic proband and siblings showed no such increase (Figure 10). In family 2, PBMC from both mother and father were assessed, but only the mother showed a RelB increase in response to LPS (Figure 10). These data indicate that within families at increased genetic risk of T1DM, NF- κ B responsiveness to LPS can be used to identify individuals with responses which resemble those of T1DM patients.

MATERIALS AND METHODS RELATING TO EXAMPLE 3

PBMC EXTRACTION:

[0203] Heparinized peripheral blood was collected from T1DM or healthy controls (HC). The study was approved by the human ethics committees of the Princess Alexandra Hospital and Mater Hospital. Blood was collected from insulin-treated T1DM patients (age range 7-72 years, mean age 33 years, 45% female) or from families in which one child has T1DM. PB mononuclear cells (PBMC) were prepared from 2 mL heparinised blood as described (5). For dendritic cells (DC), monocytes were purified from PBMC extracted from 40 mL adult heparinised blood, using CD14 microbeads (Miltenyi Biotec, GmbH, Germany), then cultured in 24 well plates in X-VIVO 20 (BioWhittaker Walkersville, MD) with 800 U/mL rhGM-CSF and 600 U/mL rhIL-4 (Schering-Plough, Sydney, Australia) for 72 hours. 1 ug/mL LPS were added to some wells for the last 24 hours of the culture period.

NF- κ B DNA BINDING:

[0204] Cytoplasmic and nuclear extracts were prepared from 48 hour cultured DC stimulated for 24 h with or without LPS, according to the iso-osmotic/nonidet P-40 method described (5, 13, 14). Extracts were preserved at -70 °C and protein was estimated using a micro BSA Protein Assay kit (Bio-rad, Hercules, CA). Transfactor NF- κ B p50/p65 chemiluminescent Kit (Clontech Laboratories, Mountain View, CA, USA) measured NF- κ B DNA binding using rabbit NF- κ B antibodies RelA (sc-372), RelB (sc-226), c-Rel (sc-70), p50 (sc-7178) from Santa Cruz Biotechnology (Santa Cruz, CA) and p52 (Upstate, Lake Placid, NY) as described (5). Absorbance data

measured using a luminometer (Berthold, Bundoora, Vic, Australia) are expressed as photon units in relation to absorbance acquisition time in seconds.

[0205] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[0206] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

5 [0207] Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of
10 the present invention. All such modifications and changes are intended to be included within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for diagnosing the presence or risk of development of type 1 diabetes, in a subject, the method comprising detecting in the subject aberrant signaling through the NF- κ B pathway in response to a pro-inflammatory signal.
- 5 2. A method according to claim 1, wherein the aberrant signaling is detected by detecting aberrant expression of at least one gene belonging to the NF- κ B pathway.
3. A method according to claim 2, wherein the at least one gene is selected from genes encoding BTK, LYN, BCR Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK,
- 10 RelA/p65, P105/p50, c-Rel, RelB, p52, NIK, Leu13, CD81, CD19, CD21 and its ligands in the complement and coagulation cascade, TRAF6, ubiquitin ligase, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, Slp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP-2, SAP, SLAM, PKR, 2B4, SHP-1, SHIP, PIR-B, CD22, CD72, Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl,
- 15 KIR3DL1, KIR3DL2, KIR2DL and Csk,.
4. A method according to claim 1, wherein the pro-inflammatory signals is selected from tumor necrosis factor, C5a, interleukin-1, CD154 and lipopolysaccharide (LPS).
5. A method according to claim 2, wherein aberrant expression is detected by:
- 20 (1) measuring in a biological sample obtained from the subject the level or functional activity of an expression product of at least gene belonging to the NF- κ B pathway and
- (2) comparing the measured level or functional activity of each expression product to the level or functional activity of a corresponding expression product in a reference sample obtained from one or more normal subjects or from one or more subjects lacking
- 25 disease, wherein a difference in the level or functional activity of the expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the presence or risk of development of type 1 diabetes in the subject.
6. A method according to claim 5, further comprising diagnosing the presence, stage or degree or risk of development of type 1 diabetes in the subject when the
- 30 measured level or functional activity of the or each expression product is different than the measured level or functional activity of the or each corresponding expression product.

7. A method according to claim 6, wherein the difference represents an at least about 10% increase or decrease in the level or functional activity of an individual expression product as compared to the level or functional activity of an individual corresponding expression product.

5 8. A method according to claim 7, wherein the presence or risk of development of type 1 diabetes is determined by detecting a decrease in the level or functional activity of an expression product of at least one gene selected from genes that encode BTK, LYN, BCR Ig α , BCR Ig β , Syk, Blnk, PLC γ 2, PKC β , DAG, CARMA1, BCL10, MALT1, PI3K, PIP3, AKT, COT, IKK α , IKK β , IKK γ , NIK, RelA/p65, P105/p50, c-
10 Rel, RelB, p52, NIK, Leu13, CD81, CD19, CD21 and its ligands in the complement and coagulation cascade, TRAF6 ubiquitin ligase, Tab2, TAK1, NEMO, NOD2, RIP2, Lck, fyn, Zap70, LAT, GRB2, SOS, CD3 zeta, Slp-76, GADS, ITK, PLC γ 1, PKC θ , ICOS, CD28, SHP-2, SAP, SLAM, PKR and 2B4.

 9. A method according to claim 7, wherein the presence or risk of development
15 of type 1 diabetes is determined by detecting a decrease in the level or functional activity of an expression product of at least one gene selected from genes that encode RelA/p65, P105/p50, c-Rel, RelB or p52.

 10. A method according to claim 7, wherein the presence or risk of development of type 1 diabetes is determined by detecting an increase in the level or functional
20 activity of an expression product of at least one gene selected from genes that encode SHP-1, SHIP, PIR-B, CD22, CD72, Fc γ RIIB, I κ B, P100, CTLA4, CD1a, TGF- β , PD-1, Cbl, KIR3DL1, KIR3DL2, KIR2DL and Csk.

 11. A method according to claim 7, wherein the presence or risk of development of type 1 diabetes is determined by detecting an increase in the level or functional
25 activity of an expression product of the SHP-1 gene.

 12. A method according to claim 5, further comprising measuring the level or functional activity of individual expression products of at least about 2 genes belonging to the NF- κ B pathway.

 13. A method according to claim 5, wherein the biological sample comprises
30 leukocytes.

 14. A method according to claim 5, wherein the expression product or corresponding expression product is a target RNA or a DNA copy of the target RNA whose level is measured using at least one nucleic acid probe that hybridizes under at least low stringency conditions to the target RNA or to the DNA copy, wherein the

nucleic acid probe hybridizes to at least 15 contiguous nucleotides of a T1DM marker polynucleotide as herein defined.

15. A method according to claim 5, wherein the expression product or corresponding expression product is a T1DM marker polypeptide as herein defined
5 whose level is measured using at least one antigen-binding molecule that is immuno-interactive with the target polypeptide.

16. A method according to claim 5, wherein the expression product or corresponding expression product is a T1DM marker polypeptide as herein defined
10 whose level is measured using at least one substrate for that polypeptide with which it reacts to produce a reaction product.

17. A method according to claim 5, wherein the expression product or corresponding expression product is a T1DM marker polypeptide as herein defined
whose level is measured using at least one oligonucleotide that binds to a nucleic acid binding site of the maker polypeptide.

18. A method according to claim 1, wherein the aberrant signaling is detected
15 by detecting aberrant phosphorylation of a polypeptide involved in or belonging to the NF- κ B signaling pathway.

19. A method according to claim 18, wherein the aberrant phosphorylation is detected by: (1) determining in a biological sample obtained from the subject the
20 phosphorylation state of at least one phosphorylatable polypeptide selected from IRAK, TAK1, TAB1, TAB2, PKR, Akt, Cot, IKK α , IKK β and IKK γ ; and (2) comparing each determined phosphorylation state to the phosphorylation state of a corresponding phosphorylatable polypeptide in a reference sample obtained from one or more normal subjects or from one or more subjects lacking disease, wherein a difference in the
25 phosphorylation state of the phosphorylatable polypeptide in the biological sample as compared to the phosphorylation state of the corresponding phosphorylatable polypeptide in the reference sample is indicative of the presence or risk of development of T1DM in the subject.

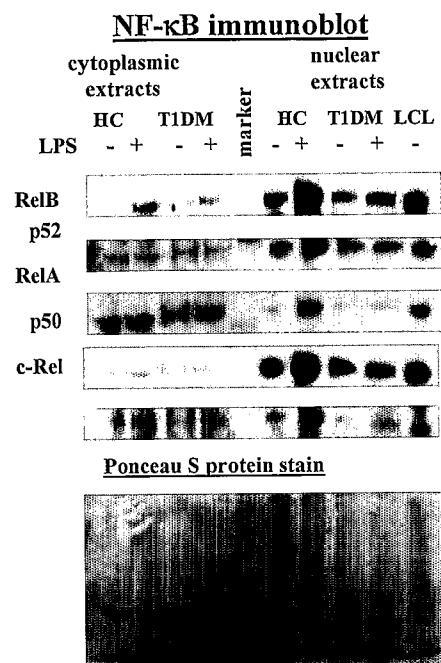
20. A method for treating, preventing or inhibiting the development of type 1
30 diabetes in a subject, the method comprising detecting in the subject aberrant signaling through the NF- κ B pathway in response to an inflammatory signal, and administering to the subject an effective amount of an agent that treats or ameliorates the symptoms or reverses or inhibits the development of type 1 diabetes in the subject.

21. A method according to claim 20, wherein the treatments or agents are selected from anti-CD3 therapy, and antigen-specific tolerogenic therapies.

22. A method according to claim 20, wherein the antigen-specific tolerogenic therapy is selected from a tolerogenic antigen-presenting cell and a suppressor T
5 lymphocyte.

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a



b

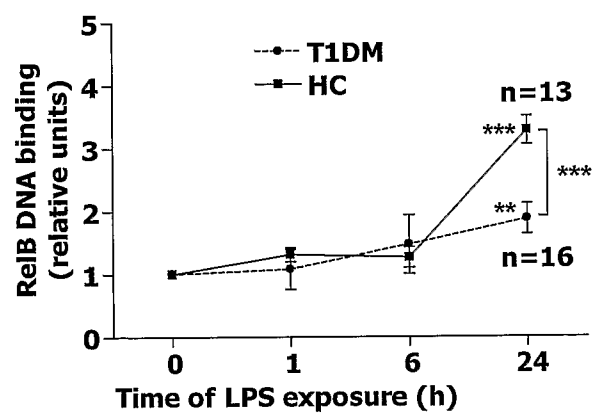


FIGURE 1

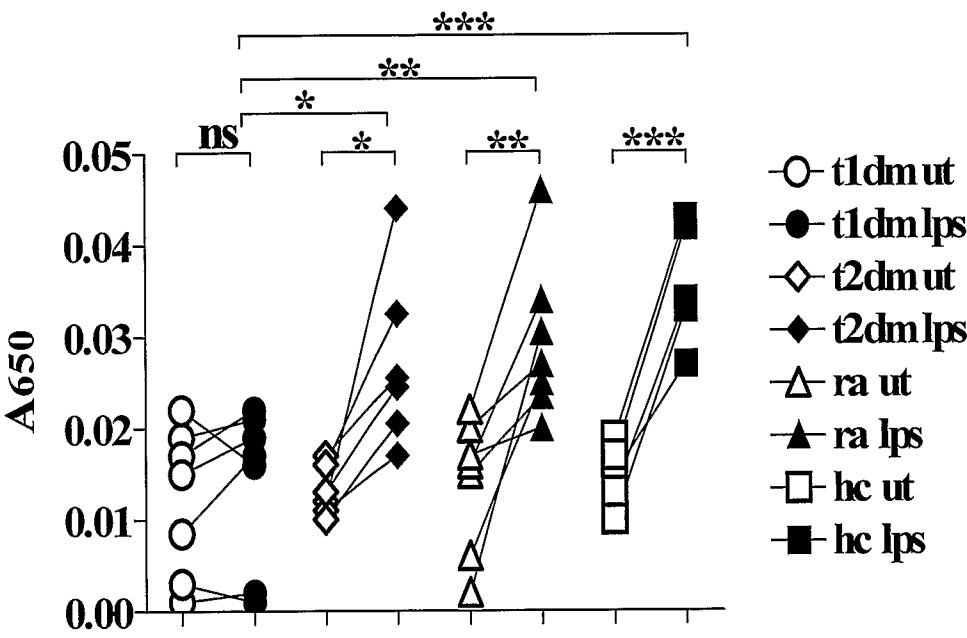


FIGURE 2

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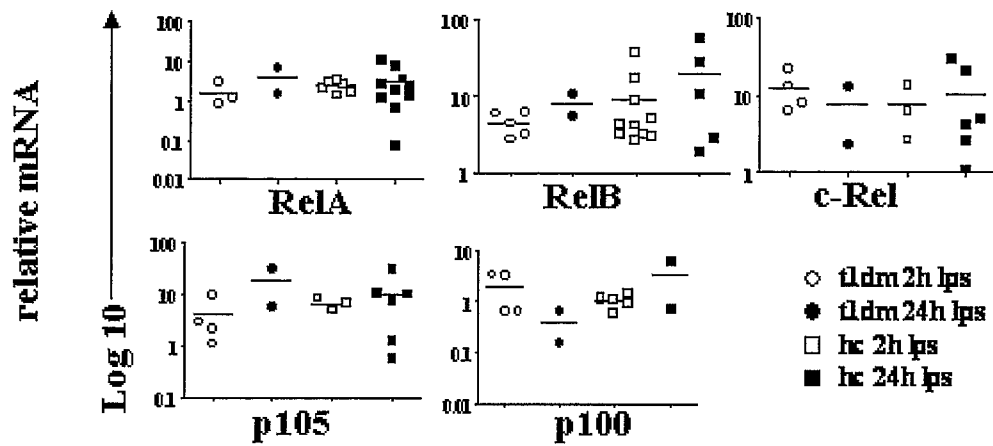


FIGURE 3

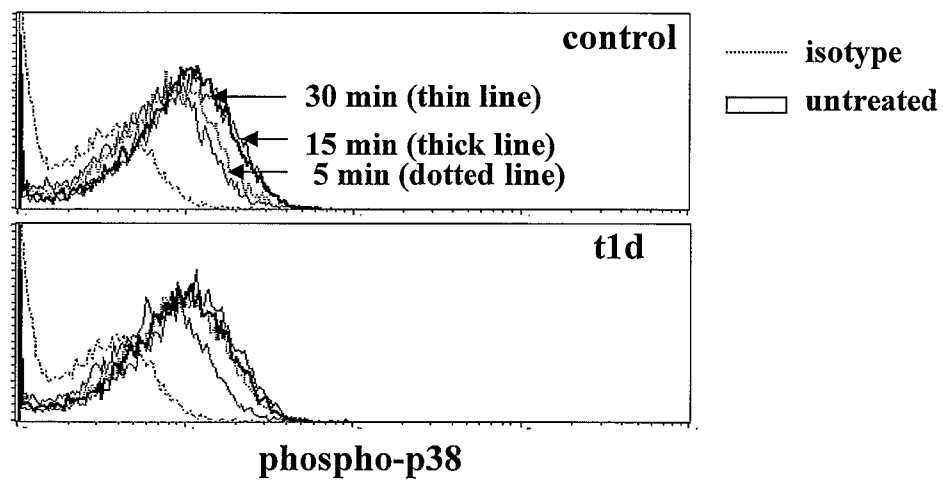


FIGURE 4

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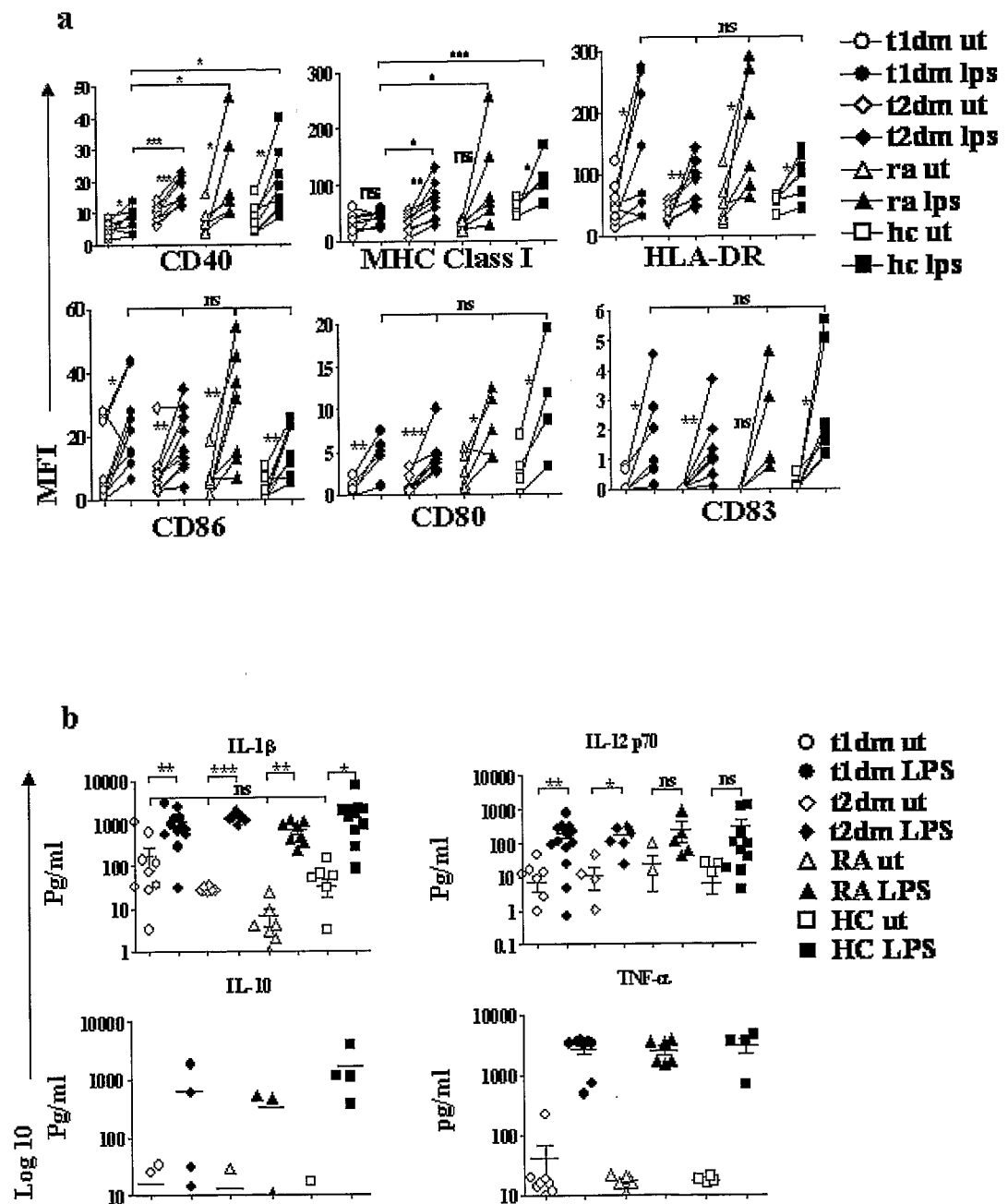


FIGURE 5

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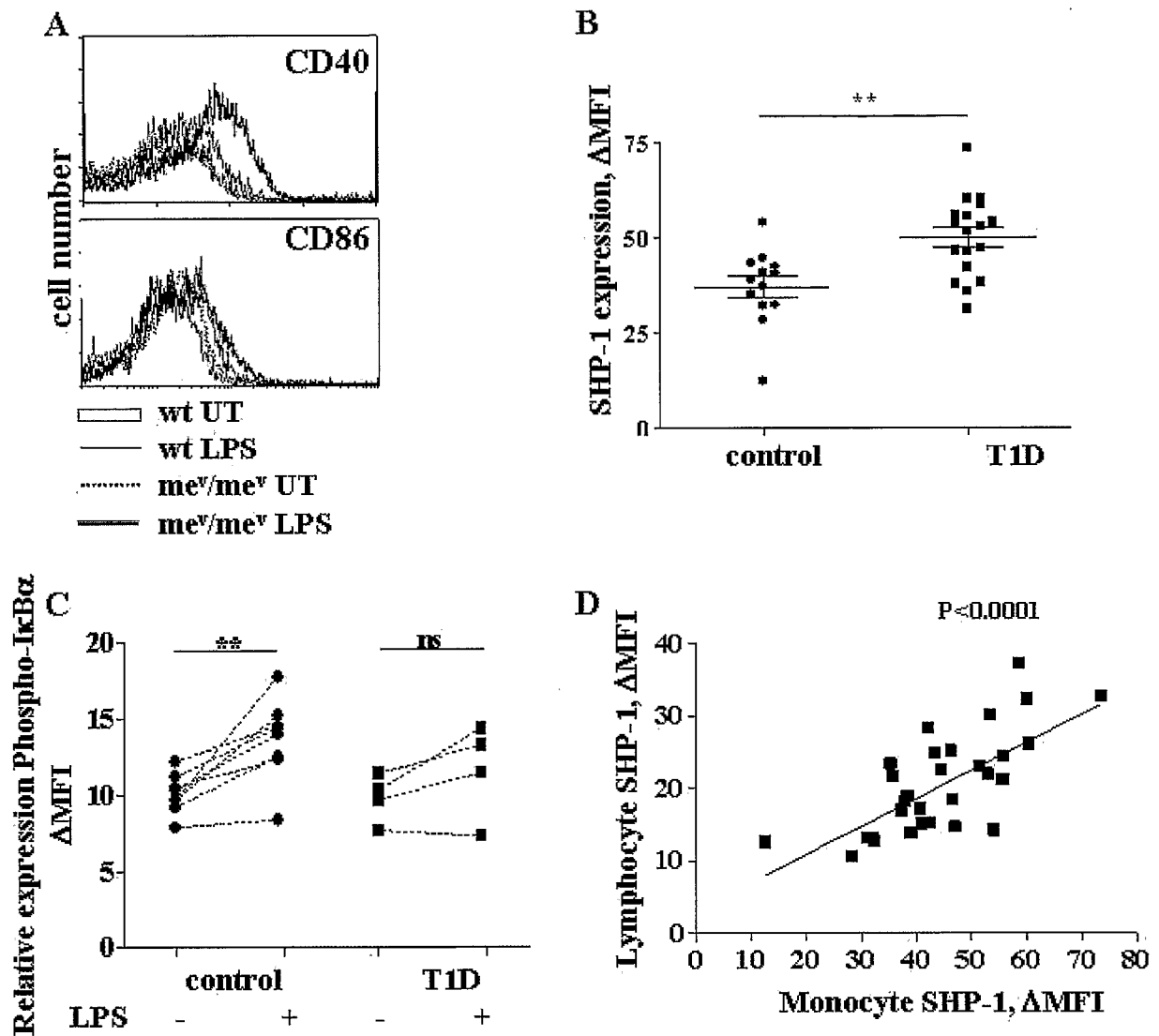


FIGURE 6

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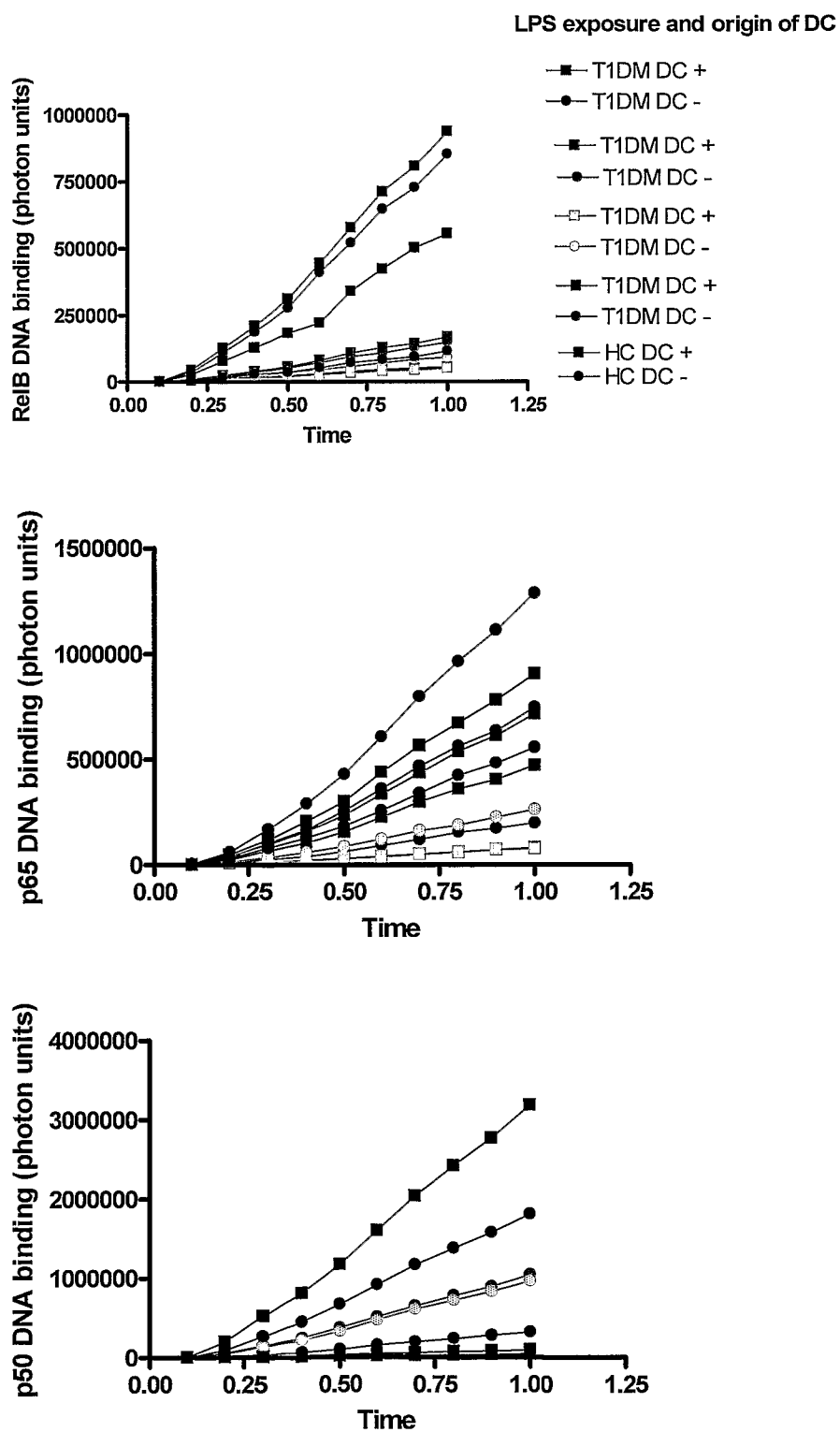


FIGURE 7

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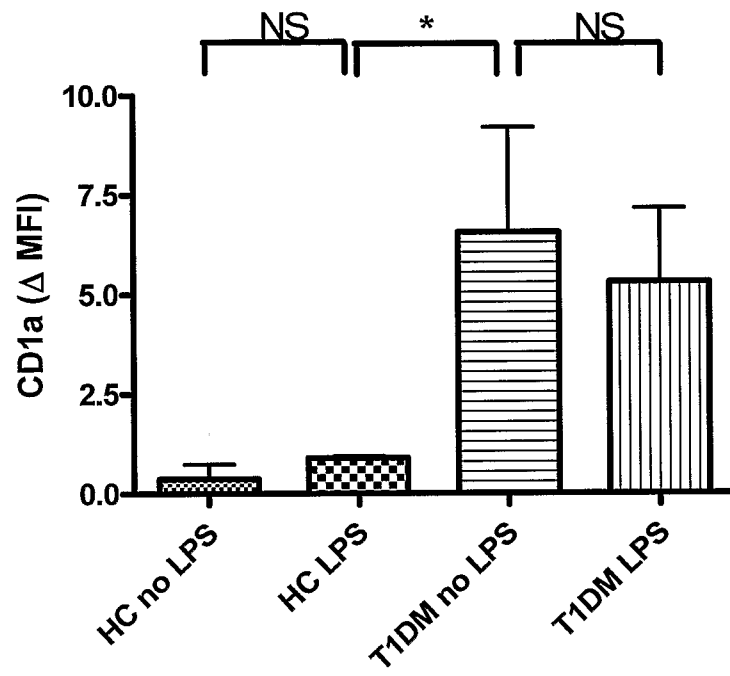


FIGURE 8

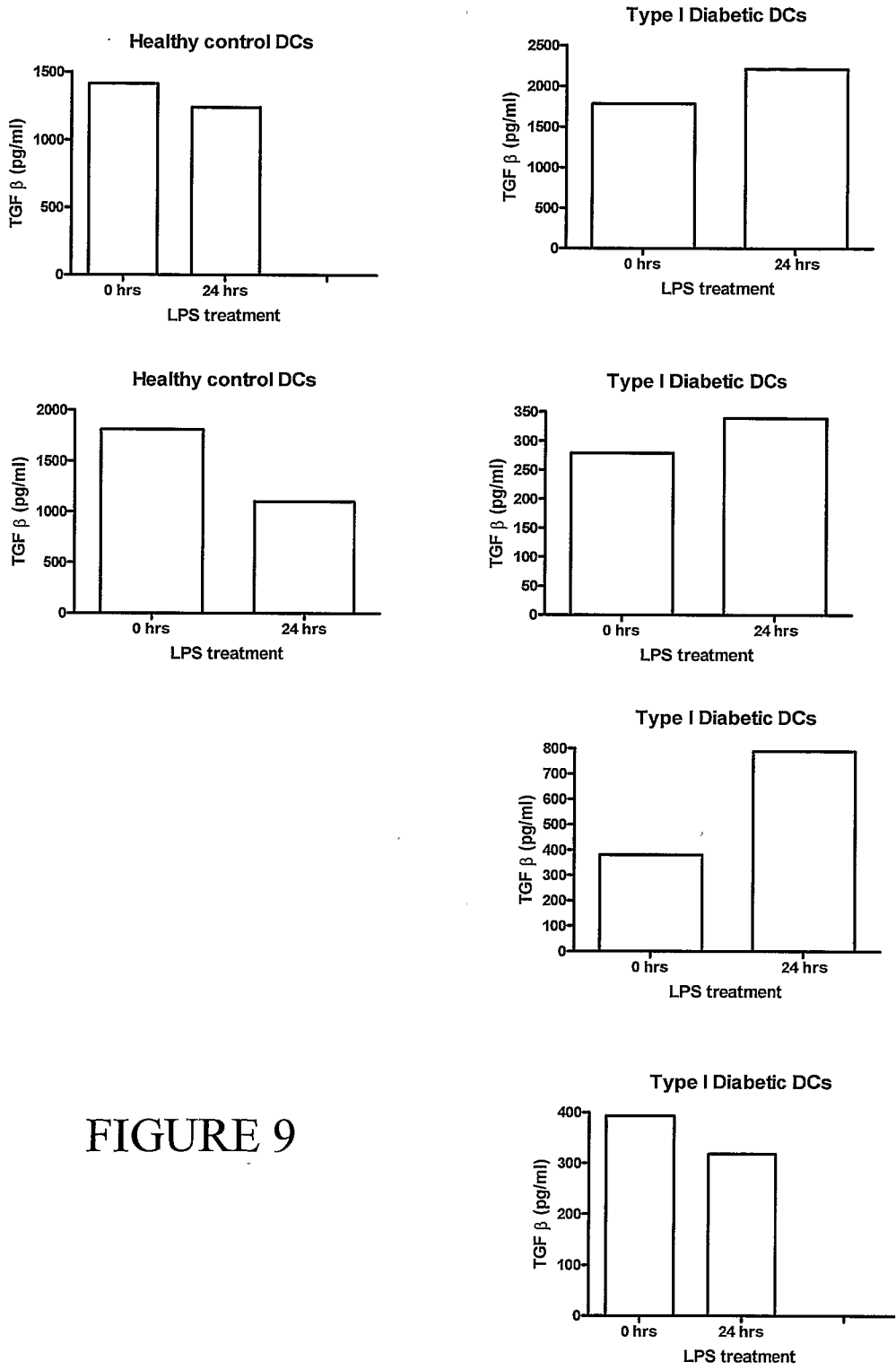


FIGURE 9

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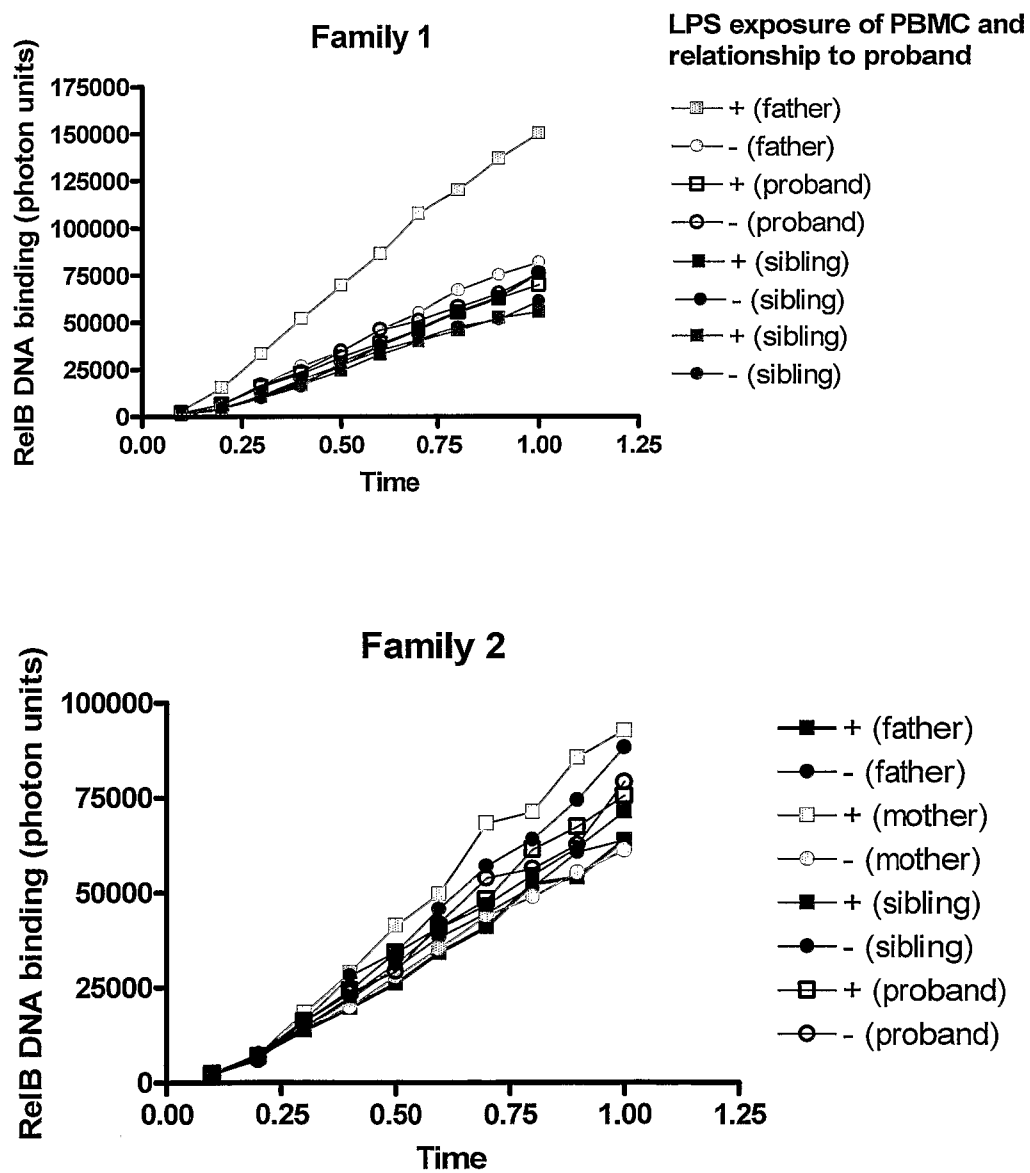


FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000692

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl.		
G01N 33/50 (2006.01) C12Q 1/68 (2006.01)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
Databases: MEDLINE CAPLUS WPIDS Keywords: type(w)1(s)(diabet?), nuclear(w)factor, NF?, NF(w)kappaB		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6410516 B1 (BALTIMORE ET AL.) 25 June 2002; Abstract, claims.	20-22
X Y	IMMUNOLOGY AND CELL BIOLOGY, February 2006, vol. 84, pp 20-27; Thomas, H.E. et al.; Perturbations in nuclear factor-κB or c-Jun N-terminal kinase pathways in pancreatic beta cells confer susceptibility to cytokine-induced cell death. Summary, Discussion pp25-27. Summary, Discussion pp25-27.	1-9, 12-18, 20 20-22
X	DIABETES, May 2006, vol. 55, pp 1252-1259; Starkey, J.M. et al.; Diabetes-Induced Activation of Canonical and Noncanonical Nuclear Factor-κB Pathways in Renal Cortex. Abstract, figures, Discussion pp 1256-1258. Earlier internet publication noted.	1-10, 12-19
☒ Further documents are listed in the continuation of Box C ☒ 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 8 August 2007		Date of mailing of the international search report 13 AUG 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer DAVID HENNESSY AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6222 3602

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000692

Ĉ (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEPHROLOGY DIALYSIS TRANSPLANTATION, 27 July 2004, vol. 19, pp2505-2512; Mezzano, S. et al.; NF- κ B activation and overexpression of regulated genes in human diabetic nephropathy. Abstract.	1,2, 5-7, 12, 14-17
X	DIABETES, December 2001, vol. 50, pp2792-2808; Bierhaus, A. et al.; Diabetes-Associated Sustained Activation of the Transcription Factor Nuclear Factor- κ B. Abstract, figures, Discussion pp2803-2805.	1-10, 12-17
X	CURRENT MOLECULAR MEDICINE 2001, vol. 1, pp287-296; Yamamoto, Y. and Gaynor, R.B.; Role of the NF- κ B Pathway in the Pathogenesis of Human Disease. Abstract, figure 1, Diabetes pp290-291, Inhibitors of the NF- κ B Pathway pp292-293.	1, 2, 4-7, 12, 13, 20
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, 29 April 2005, vol. 280 no. 17 pp17435-17448; Tian, B. et al.; Identification of Direct Genomic Targets Downstream of the Nuclear Factor- κ B Transcription Factor Mediating Tumor Necrosis Factor Signaling. Abstract, figures, tables, pp17446-17447.	
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, 28 December 2001, vol. 276 no. 52, pp 48879-48886; Cardozo, A.K. et al.; A Comprehensive Analysis of Cytokine-induced and Nuclear Factor- κ B-dependent Genes in Primary Rat Pancreatic β -Cells. Abstract, figures, tables.	1-7, 10, 12, 14-17
Y	Abstract, figures, tables.	1-7, 10, 12-17, 20
X	DIABETES, May 2001, vol. 50, pp 909-920; Cardozo, A.K. et al.; Identification of Novel Cytokine-Induced Genes in Pancreatic β -Cells by High-Density Oligonucleotide Arrays. Abstract, figures, table 1.	1-7, 10, 12, 14-17
Y	Abstract, figures, table 1.	1-7, 10, 12-17, 20
X	DIABETOLOGIA, 2001, vol. 44, pp2115-2133; Eizirik, D.L. and Mandrup-Poulsen, T.; A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis. Abstract, tables, figures.	1-8, 12-19
Y	Abstract, tables, figures.	1-8, 12-19
X	ENDOCRINOLOGY, April 2002, vol. 143 no. 4, pp1225-1234; Liu, D. et al.; Double-Stranded RNA Cooperates with Interferon- γ and IL-1 β to Induce Both Chemokine Expression and Nuclear Factor- κ B-Dependent Apoptosis in Pancreatic β -Cells: Potential Mechanisms for Viral-Induced Insulinitis and β -Cell Death in Type 1 Diabetes Mellitus. Abstract, figures.	1, 2, 4-7, 12, 14-17
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 280 no. 40, pp33984-33991; Rasschaert, J. et al.; Toll-like Receptor 3 and STAT-1 Contribute to Double-stranded RNA + Interferon- γ -induced Apoptosis in Primary Pancreatic β -Cells. Abstract, figures.	1,2, 5-7, 12, 14-17, 20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000692

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, 23 April 2004, vol. 279 no. 17, pp18091-18097; Miao, F. et al.; In Vivo Chromatin Remodeling Events Leading to Inflammatory Gene Transcription under Diabetic Conditions. Abstract, figures. Abstract, figures.	1-10, 12-17 1-10, 12-17
X	THE JOURNAL OF IMMUNOLOGY, November 2003, vol. 171, pp4886-4892; Lamhamedi-Cherradi, S. et al.; Transcriptional Regulation of Type I Diabetes by NF- κ B. Abstract, figures.	1-9, 12-18, 20-21
X	CELLULAR AND MOLECULAR LIFE SCIENCES, 2005, vol. 62, pp1850-1862; Kodama, S. et al.; The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis. Abstract, figures, pp1858 second column – pp1859 second column.	1, 2, 4-7, 20
Y	ANN. N.Y. ACAD.SCI., 2004, vol. 1037, pp41-58; Kutlu, B. et al.; New Approaches for in Silico Identification of Cytokine-Modified β Cell Gene Networks. Abstract.	1-10, 12-19
X	GENES AND IMMUNITY, 2002, vol. 3, pp430-432; Gylvin, T. et al.; Characterization of a nuclear-factor-kappa-B (NF κ B) genetic marker in type 1 diabetes (T1DM) families. Abstract.	1, 2, 5-6
X	GENES AND IMMUNITY, 2001, vol. 2, pp304-308; Hegazy, D.M. et al.; NF κ B Polymorphisms and susceptibility to type 1 diabetes. Abstract.	1, 2, 5-6
T	BMC BIOINFORMATICS, 2007, vol. 8, pp55-71; Naamane, N. et al.; In silico identification of NF-kappaB-regulated genes in pancreatic beta-cells. Abstract.	1-22
Y	PROC.NATL.ACAD.SCI. USA, 1994, vol. 91, pp 437-438; Tisch, R. and McDevitt, H.O.; Antigen-specific immunotherapy: Is it a real possibility to combat T-cell-mediated autoimmunity? Whole article	20-22
X	IMMUNOLOGY, 2003, vol. 108, pp263-273; Pritchard, N.R. and Smith, K.G.C.; B cell inhibitory receptors and autoimmunity. Page 269, second column.	1-8, 10-18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000692

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

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

A priori and a posteriori: 2 invention sets; (i) Claims 1-19; Methods for diagnosing the presence or risk of development of type 1 diabetes by detecting aberrant signalling through the NF- κ B pathway; (ii) Claims 20-22; Methods for treating, preventing or inhibiting the development of type 1 diabetes in a subject. As the detection of aberrant signalling through the NF- κ B pathway is disclosed in the specification at page 2 and the X documents listed, there is no special technical feature unifying the two sets of claims, and therefore 2 inventions are claimed.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2007/000692

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

Patent Document Cited in Search Report		Patent Family Member			
US 6410516		EP 0252146	EP 0407411	EP 0578065	
		JP 8275782	JP 10084974	US 5804374	
		US 6150090	US 2004214757	US 2006063709	
		WO 8704170	WO 8805083	WO 8907614	
		WO 8908147			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					