A Novel Small Molecule Drug Derived from Methimazole (Phenylmethimazole) that Targets Aberrant Toll-like Receptor Expression and Signaling for the Potential Prevention or Treatment of Diabetes Mellitus and Non-alcoholic Fatty Liver Disease

Kelly D McCall, PhD and Frank L Schwartz, MD, FACE

Department of Specialty Medicine, Heritage College of Osteopathic Medicine, Diabetes Institute, Ohio University, Athens, Ohio, US

Abstract

Toll-like receptors (TLRs) are pattern-recognition receptors located on the surface or within (endosome) immune cells (dendritic), whose function is to recognize pathogens from the environment and mediate both the innate and acquired immune responses. Environmental activation of TLRs in nonimmune cells is now recognized as a significant pathway that mediates the loss of self-tolerance in autoimmune diseases, as well as inflammation-induced cell damage in many chronic diseases. We have developed a small molecule drug derived from methimazole, phenylmethimazole (C10), which interferes with the environmental induction of TLR signaling in nonimmune cells and is an active inhibitor of pathologic inflammation in many animal disease models. This article has been written to introduce clinicians to TLR function and the potential therapeutic role that their inhibition could play in many inflammatory/autoimmune diseases, including diabetes and nonalcoholic fatty liver disease (NAFLD).

There are two major forms of diabetes mellitus, type 1 (T1DM) and type 2 (T2DM), with the latter being the most common form, accounting for over 90 % of cases.[1] Further, with the epidemic of obesity, T2DM can occur at any age, including children and adolescents,[1] affecting nearly 30 million in the US and 400 million worldwide.[2] Although there are multiple medications available to treat T2DM, overall success is poor given that no medication addresses the multiple abnormalities associated with T2DM and changes in diet and lifestyle are extremely difficult to achieve.[3] T1DM occurs more frequently in children, adolescents, and young adults and is caused by destruction of the insulin-producing beta cells of the pancreas.[4,5] Currently there is no cure for T1DM and it requires lifelong insulin replacement.[6] The prevalence of both forms of DM are increasing worldwide[1,4] and are associated with increased risk for multiple long-term diabetes complications, premature disability, and early death.[7–10] Nonalcoholic fatty liver disease (NAFLD) is even more common than T2DM affecting 75 % of all obese individuals, is the leading cause of cirrhosis, end-stage liver disease, and primary hepatocellular cancer in the US.[11,12] Treatment of NAFLD is also difficult due to similar lifestyle change requirements,[13]

and many of the medications used to reduce hepatic inflammation and fibrosis are potentially hepatotoxic themselves.[14–16] Thus, all three of these diseases (as well as atherosclerosis and many cancers) have a large inflammatory/immune component, which is driven by environmental insults to the body (viruses, dietary components such as high saturated fats, or overconsumption of high-fructose corn syrup [HFCS], etc.) Much contemporary research is directed at suppressing the various inflammatory pathways involved in these diseases without impairing the normal immune response or damaging the tissues we are attempting to protect.

Toll-like receptors (TLRs) are pattern-recognition receptors that were originally identified on the surface or within (endosome) immune cells (dendritic and monocytes/macrophages) that recognize various pathogen-associated molecular patterns (PAMPs) from the environment.[17,18] Recognition of these PAMPS by the various TLRs protect mammals from pathogenic organisms, such as bacteria, viruses, or protozoa and are important mediators of both the initial "innate" immune response to the molecular products of these organisms as well as the "adaptive" or memory immune response.[18] TLRs

Table 1: Toll-like Receptors, their Location, Specific Ligands, and Disease Associations

Human Toll-like Receptor	PAMPs/DAMPs/Other Ligands	Disease Associations
TLR1	Triacyl lipopeptides, modulin (phenol-solube)	Cancer, psoriasis, sepsis, leprosy
TLR2	Glycolipids, triacyl lipopeptides, heat shock proteins, high mobility group box 1 protein, rare LPS species (*Porphyromonas gingivalis*), lipopeptides, lipoteichoic acid, measles hemagglutinin, mannuronic acids, *Neisseria porins*, peptidoglycan, zymosan (Beta-glucan), bacterial fimbriae, Yersinia virulence factors, CMV virions, saturated fatty acids	Cancer, type 2 diabetes, psoriasis, pre-eclampsia, Alzheimer's, herpes simplex encephalitis, rheumatoid arthritis, acne vulgaris, acute rheumatic fever, asthma, atherosclerosis, chronic obstructive pulmonary disease, diabetic nephropathy, subhorrheic dermatitis
TLR3	dsRNA (self and viral), poly I:C	Cancer, type 1 diabetes, herpes simplex encephalitis, Hashimoto's thyroiditis, virus-associated autoimmune disease
TLR4	Lipopolysaccharides, saturated free fatty acids, fibrinogen, fibronectin, heat shock proteins, flavolipins, *Streptococcus pneumoniae pneumolysin*, heparin sulfate, hyaluronic acid, high mobility group box 1 protein, MMTV envelope proteins, nickel, paclitaxel, RSV fusion protein, respiratory syncytial virus coat protein, mannuronic acid polymers, teichuronic acids, bacterial fimbriae, surfactant protein A, b-defensin 2	Cancer, type 2 diabetes, toxic shock, ulcerative colitis, atherosclerosis, pre-eclampsia, alzheimer's, stroke, rheumatoid arthritis, chronic obstructive pulmonary disorder, Crohn's disease, periodontitis, peripheral arterial disease,
TLR5	Flagellin	Cancer, psoriasis, systemic lupus erythematosis
TLR6	Diacyl lipopeptides, bacterial cell wall components, modulin (phenol-soluble)	Cancer, leprosy (Hansen's disease), filarisis, asthma, hypersensitivity pneumonitis
TLR7	Self ssRNA, ssRNA, bropirimine, loxoribine, imidazoquinoline	Cancer, systemic lupus erythematosis, rheumatoid arthritis, arthritis
TLR8	Self ssRNA, small synthetic compounds, imidazoquinoline	Cancer, rheumatoid arthritis, viral keratitis, crimean-congohemorrhagic fever
TLR9	Self dsDNA, unmethylated CpG DNA	Allergies, asthma, cancer, multiple sclerosis, systemic lupus erythematosis, Graves' opthalmopathy, crimean-congohemorrhagic fever, arthritis
TLR10	Unknown	Cancer, asthma, nephropathy, viral keratitis

dsRNA = double-stranded RNA; CMV = cytomegalovirus; DAMPs = damage-associated molecular patterns; MMTV = mouse mammary tumor virus; PAMPs = pathogen-associated molecular patterns; poly I:C = polyinosinic-polycytidylic acid; RSV = respiratory syncytial virus; ssRNA = single-stranded RNA; TLR = toll-like receptor.

were consequently found to be expressed in multiple nonimmune cell types including the epithelial cells lining the gastrointestinal tract,[19] endothelium,[20] liver,[21,22] adipocytes,[23,24] and even pancreatic beta cells.[25] TLR also recognize endogenous ligands released by injured cells (from infection, inflammation, radiation, etc.), and these are termed damage-associated molecular patterns (DAMPs). Ten different TLRs have now been identified in humans, as well as many of their exogenous and/or endogenous ligands (except TLR 10 whose ligands are not yet known), as well as some of the diseases to which they are linked (see *Table 1*).

We first became interested in TLR function when attempting to determine the molecular mechanism of action of the drug phenylmethimazole (C10), which is a derivative of the antithyroid medication methimazole. Methimazole is commonly used to treat hyperthyroidism by blocking thyroxine biosynthesis in thyrocytes; however, methimazole was also shown to downregulate expression of the major histocompatibility (MHC) genes, I and II,[26,27] which suggested it might also possess anti-inflammatory and immunoregulatory activity. C10 (which does not have any effect on thyroxine biosynthesis) was subsequently found to regulate the expression of MHC genes I and II,[25] to block TLR3, TLR4/TLR2, and TLR9 expression, and suppress inflammation in a variety of diseases[25,28,29] including TLR3 in T1DM,[30,31] TLR4/TLR2 in T2DM,[24] and, most recently, TLR4/TLR2 (likely) in NAFLD.

Toll-like Receptor Function in the Normal Immune Response

TLRs are single, transmembrane, noncatalytic receptors, which are located either on the cell surface or internally on the endosome.[18,32] In general,

the TLRs on the cell surface, such as TLR2 and TLR4, recognize Gram-negative and Gram-positive PAMPs (lipoproteins, lipopolysaccharides, and peptidoglycans), while endosomal TLR3 functions to recognize injected viral nucleic acids. TLRs also recognize endogenous mammalian DAMPs, such as the intracellular lipoproteins, lipopolysaccharides, and peptidoglycans, or the self-DNA released following infection and/or other damaging environmental insults such as radiation, chemicals, etc.[19,20,33–37] Thus, TLR normally function to protect an organism from infections and help clear debris following tissue damage.

Toll-like Receptors in Autoimmune and Aberrant Inflammatory Responses

Chronic TLR activation and signaling in both immune and nonimmune cells by environmental antigens are now also thought to play critical roles in the induction of many chronic inflammatory diseases, the initiation and/or perpetuation of autoimmunity (loss of self-recognition), as well as contribute to oncogenesis, tumor growth, and spread.[38] Much of our recent research has focused on the roles of TLR3 and TLR2/TLR4 in the viral-induction of T1DM and high-fat diet (HFD) or free fatty acid (FFA) induction of obesity, insulin resistance (IR), T2DM, and, more recently, NAFLD, respectively. These three TLRs and their signaling pathways are depicted in *Figure 1*. Once TLRs bind to their respective ligands, they dimerize and intracellular adaptor molecules regulate their downstream signaling. The primary effector molecules of TLR2 and TLR4 are the transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and interferon regulatory factor (IRF)-5 while for TLR3 they are NFκB and IRF3. These transcription factors then enter the cell nucleus and activate

Figure 1: TLR2, TLR3, and TL4 Signaling Pathways
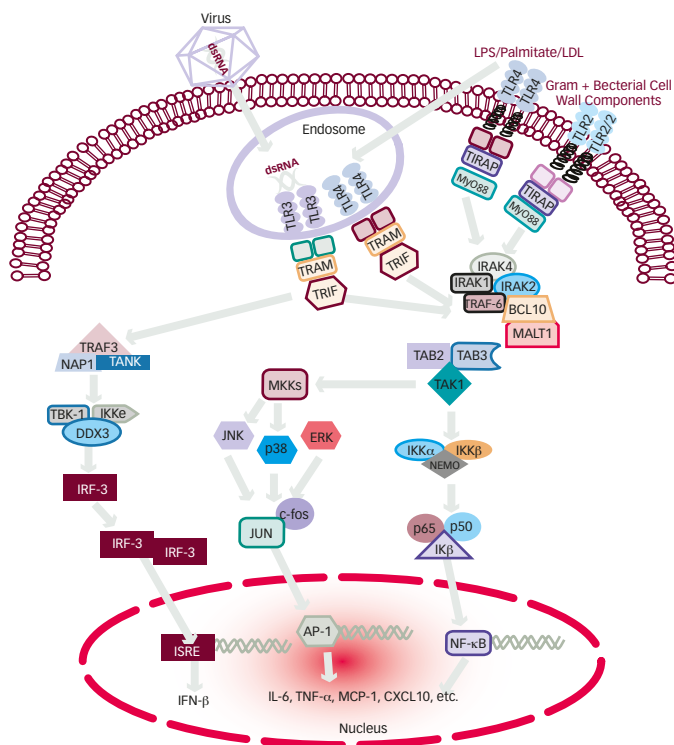
TLR2 and TLR4 are surface receptors while TLR3 is located on the endosome. Once activated, they dimerize and the primary effector molecules of TLR2 and TLR4 are the transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and interferon regulatory factor (IRF)-5, while NFκB and IRF-3 are activated by TLR3. NFκB, IRF-5, and IRF-3 are translocated to the nucleus and result in synthesis and release of their respective cytokines. AP-1 = activator protein 1; BCL10 = β-cell lymphoma/leukemia 10; CXCL10 = chemokine (C-X-C motif) ligand 1; DDX3 = (Asp-Glu-Ala-Asp) Box Helicase 3, X-linked; ERK = extracellular-signal-regulated kinases; IKKα = inhibitor of nuclear factor kappa-B kinase subunit alpha; Ikβ = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta; IKKβ = inhibitor of nuclear factor kappa-B kinase subunit beta; IL-1β = interleukin-1 beta; IL-6 = interleukin-6; IRAK = interleukin-1 receptor-associated kinase; ISRE = interferon-stimulated response element; JNK = Jun N-terminal kinases; JUN = c-Jun N-terminal; MALT1= mucosa-associated lymphoid tissue lymphoma translocation protein 1; MCP1 = monocyte chemoattractant protein-1; MKK = mitogen-activated protein kinase kinase; MyD88 = myeloid differentiation primary response gene 88; NAP1 = nucleosome assembly protein 1-like 1; NEMO = inhibitor of kappa light polypeptide gene; TAB2 = TGF-beta activated kinase 1/MAP3K7 binding protein 2; TAB3 = TGF-beta activated kinase 1/MAP3K7 binding protein 3; TANK = TRAF family member-associated NFκB activator; TAK1 = mitogen-activated protein kinase 7; TBK1 = TANK-binding kinase 1; TIRAP = toll-interleukin 1 receptor domain containing adaptor protein; TNF-α = tumor necrosis factor α; TRAF = TNF-receptor-associated factor; TRAM = TRIF-related adaptor molecule; TRIF = TIR-domain-containing adapter-inducing interferon-β.

genes responsible for the production of the type I interferons, and pro-inflammatory cytokines and chemokines involved in both the innate immune responses, as well as upregulate MHC genes I and II, which are critical for the development of antigen-specific adaptive immunity.[39,40]

The Role of Viruses in the Pathogenesis of T1DM

T1DM results from beta (β) cell destruction and the consequent loss of endogenous insulin secretion.[41–43] There are major genetic and environmental factors associated with the risk for T1DM. The vast majority of cases (85–90 %) are due to the initiation (environmental) of a predominately T-cell-mediated, gradual (months to years) autoimmune destruction of β cells (T1DM-A).[44] The etiology of the other 10–15 % cases, which are more fulminant in onset, is unknown (T1DM-B); however, viruses are suspected as the principle environmental trigger

## Figure 2: Role of Viruses and TLR3 in the Induction of Insulitis and T1DM
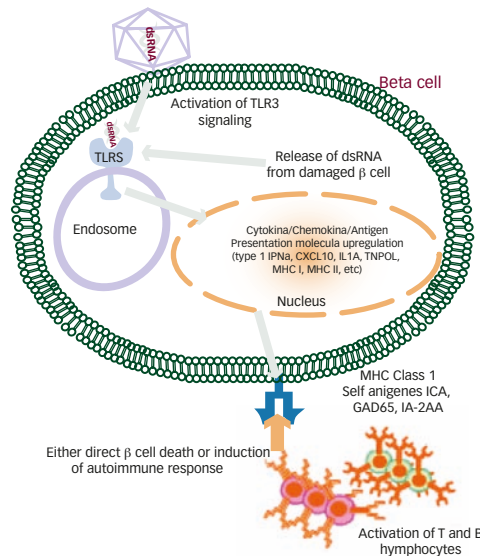
Viruses inject their nucleic acids into the cell to begin replication. There they are recognized by TLR3 on the endosome, initiate their downstream signaling, which mediates both the innate and acquired immune response via upregulation of major histocompatibility (MHC) class I antigens initially followed by self-antigens. Anti-GAD = anti-glutamic acid decarboxylase; CXCL10 = chemokine (C-X-C motif) ligand 1); ICA = islet cell antigen; IL-1β = interleukin-1 beta; insulinoma antibody = IA-2AA; type 1 IFNs = type I interferons.

of both forms.[44] The strongest genetic link to T1DM-A are the class II *HLA* genes that control antigen presentation within the innate and acquired immune systems, while the genes responsible for T1DM-B are poorly characterized (only single-gene polymorphism associations). The *HLA* haplotypes DR3,4DQ8 exhibit the highest predictive risk for T1DM-A, but the genetic component with this strongest haplotype is thought to be responsible for only 40–50 % of the total risk,[44–47] leaving environmental induction or acceleration as a major factor again in both forms of T1DM. While no single viral trigger for either form of T1DM has been clearly identified, the Coxsackie viruses (CBVs) are most closely associated with both forms, although influenza B, herpes simplex, and human herpes 6 virus have also been linked to both forms.[48–53] In one study of children with new-onset T1DM, 39 % demonstrated a CBV-specific immunoglobulin (Ig)-M response compared with 5 % in age-matched controls.[50] Some CVB strains have specific β cell tropism,[54] indicating that these cells may be specific targets for them, and a large research effort to develop a CBV-based vaccination for prevention of T1DM is underway. Finally, serum from patients with new-onset T1DM possess a subset of anti-CBV antibodies that recognize β-cell autoantigens and are able to induce β-cell apoptosis in a pancreatic beta cell line.[55]

The Role of TLR3 in the Pathogenesis of T1DM

Both mouse and human pancreatic islets express multiple TLRs, including TLR2, TLR3, TLR4, and TLR9.[56] TLR3 recognizes viral double-stranded RNA (dsRNA) while TLR9 recognizes self-DNA or unmethylated CpG DNA released from damaged β cells. Only TLR3 expression and signaling is upregulated in β cells by dsRNA, which is the intermediate nucleic acid generated during the life cycle of most enteroviruses[35,56,57] and an illustration of the mechanism by which viruses inject their nucleic acids into β cells, their recognition by TLR3 and subsequent inflammatory signaling pathways

Figure 3: Ectopic Fat and Pro-inflammatory Cytokines (TNF-α, IL-6, iNOS, etc.) Induce insulin Resistance and Local Inflammation
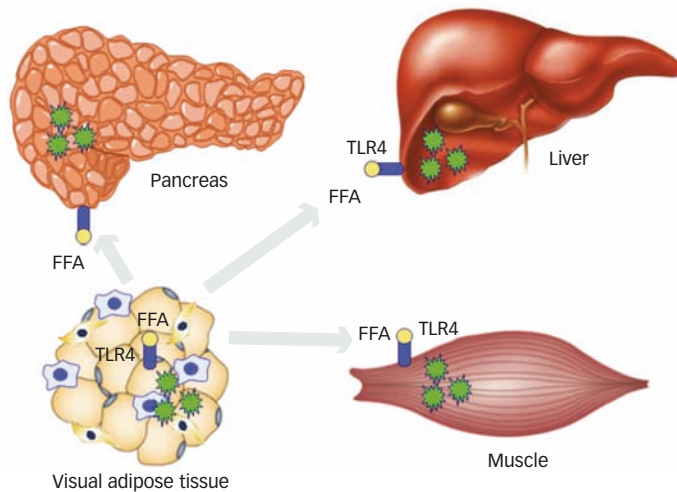
Free fatty acids (FFAs) released by saturated adipocytes or absorbed in the gut are dumped into the bloodstream where they then become ectopically deposited in insulin target tissues. In the adipocyte, FFAs bind to TLR4, activate the TLR4 signaling cascade, which leads to the production of pro-inflammatory adipokines. These adipokines then enter the bloodstream and travel to insulin target tissues where they cause insulin resistance in these tissues and upregulate TLR4 gene expression. FFAs can then bind TLR4 and trigger TLR4 signaling in these tissues, perpetuating this vicious cycle. IL-6 = interleukin 6; iNOS = nitric oxide synthase; TNF-α = tumor necrosis factor alpha; TLR4 = toll-like receptor 4.

involved in direct β cell destruction or the initiation of the autoimmune response directed at the β cell is depicted in *Figure 2*. Islets infected with CBV4 or treated with polyinosinic-polycytidylic acid (poly I:C) (a synthetic dsRNA that mimics host responses triggered by viral dsRNA) exhibit increased expression of TLR3.[58,59] Interferon (IFN)-α or IFN-γ and interleukin (IL)-1β expressed by (TH1) cells and activated macrophages also stimulate increased expression of TLR3 in the virus-damaged islet cells.[60–62] TLR9 are currently hypothesized to be involved in the recognition of self-antigens (self-DNA and unmethylated CpG DNA as well as intracellular proteins released from the damaged β cells) that then activate autoreactive B cells and promote the production of the islet cell autoantibodies (GAD65, I-A1, ZnT8, pre-proinsulin, and proinsulin), which are associated with T1DM-A.[57] Interestingly, increased TLR4 signaling has also been demonstrated in the NOD mouse model of T1DM and several studies suggest that high mobility group protein B1 (HMGB1), which is associated with several inflammatory diseases and released from damaged and necrotic β cells, is its probable ligand and correlates with β-cell loss and onset of diabetes.[63] Other studies have demonstrated that TLR4 deficiency accelerates T1DM in the NOD mouse.[64] Moreover, TLR4 agonists and probiotics are protective for T1DM in the NOD mouse, which suggests that certain gut microbes may offer protection from pathogenic enteroviruses.[65] Many studies have also reported increased TLR2 and TLR4 signaling in monocytes obtained from patients with T1DM. However, the increased monocyte signaling of TLR2 and TLR4 is probably a reflection of their upregulation from abnormal lipid metabolism rather than their involvement in the insulitis associated with progressive β-cell loss.

Pathogenesis of T2DM and NAFLD

T2DM and NAFLD are both chronic inflammatory diseases that are highly correlated with visceral obesity.[66–68] Diets from industrialized countries include large volumes of processed foods, saturated fats, simple carbohydrates, and HFCS, which, coupled with decreased physical activity, is largely responsible for the epidemic of obesity and obesity-induced chronic inflammatory diseases including T2DM and NAFLD throughout the world.[69] In the early stages of T2DM with weight gain and visceral obesity-induced IR, there is islet cell compensation with beta cell hyperplasia and hyperinsulinemia. Over time, however, this inevitably leads to progressive beta cell failure[70] and insulin deficiency.[71] The molecular pathogenesis of T2DM and NAFLD are depicted in *Figure 3*. Visceral adipocytes and associated macrophages are major contributors to these inflammatory processes via several signaling pathways. As visceral obesity increases (adipogenesis), both adipocytes and macrophages release increasing amounts of inflammatory adipokines/cytokines (TNF-α, IL-6, NF-κB, etc.)—a phenomenon that has been termed "low-grade sepsis" or "metabolic endotoxemia."[72] These adipokines/cytokines act locally to stimulate further adipogenesis as well as circulate to downregulate the insulin receptor (IR) function, which induces IR in the adipocyte as well as multiple other insulin target tissues (liver, skeletal muscle).[73,74] Once the storage capacity of visceral adipocytes is saturated, endogenous FFAs and triglycerides (TGs) are released into the portal and peripheral circulation and exogenous dietary FFAs and TGs can no longer be cleared from the blood following meals. Excess circulating FFAs and TGs are subsequently ectopically deposited in these insulin target tissues, where they trigger the local release of the same inflammatory cytokines and chemokines (lipotoxicity), further exacerbating IR.[75] The ectopic fat deposition in skeletal muscle results in the loss of mitochondrial function and consequent decreased oxidative phosphorylation, which also contributes to IR and interferes with weight loss.[76] The ectopic fat deposited in the pancreas also induces β cell autophagy contributing to accelerated β cell death and ultimately insulin deficiency.[77]

Role of the Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 in the Pathogenesis of T2DM and NAFLD

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a functional substrate of the IR in the liver and normally functions to promote insulin endocytosis and clearance from the circulation, occurring simultaneously with acquisition of visceral obesity and development of hyperinsulinemia from the pancreas. In the liver, which is the major regulator of glucose levels via hepatic storage (glycogen) or release (gluconeogenesis), there is also a major downregulation of CEACAM1.[78] Thus, downregulation of CEACAM1 contributes further to IR and hyperinsulinemia,[79] as well as NAFLD. Proof for the role of CEACAM1 in the pathogenesis of both T2DM and NAFLD was demonstrated in mouse studies using both a general CEACAM1 knockout and selective hepatic CEACAM1 knockout where the animals developed visceral obesity, IR, and steatosis.[80] Thus, ectopic fat deposition in the liver from excess portal (visceral) FFAs and TGs (lipotoxicity) and local CEACAM1 downregulation perpetuate a vicious cycle of inflammation, IR, and steatosis.[81]

The Role of TLR2/TLR4 Signaling in T2DM and NAFLD

Bacterial glycolipids and triacyl lipopeptides are the principle ligands for TLR2 while bacterial LPS is the principle exogenous ligand for TLR4. Their signaling pathways are demonstrated in *Figure 1*. Exogenous and endogenous FFAs (such as palmitate) induce pathologic TLR4 signaling in

adipocytes, resulting in the release of TNF-α and IL-6, and the induction of IR.[23,24] There is now increasing evidence that the pathologic expression of TLR2 and TLR4 in other insulin-sensitive tissues, including skeletal muscle, islets, and liver, are a major source of pro-inflammatory adipokine/cytokine (TNF-α, IL-6, NF-κB, etc.) production that leads to the "inflammatory storm" seen in T2DM and NAFLD.[82–84] These same processes also contribute to as much as 40 % of the total risk for the chronic inflammation-induced, and obesity-associated malignancies (esophagus, pancreas, liver, colon, gall bladder, endometrium, breast, kidney, and thyroid).[85]

Gut Flora, TLRs, and Chronic Inflammatory Diseases

Many interesting studies linking changes in intestinal (gut) flora, TLR function, and chronic diseases such as inflammatory bowel disease but also including T1DM, T2DM, and NAFLD, have been reported recently in the literature.[86,87] These studies suggest that different species of bacteria may contribute to the pathogenesis and/or protection from many diseases through activation and/or suppression of different TLR functions in gut epithelial cells and immune cells. Many early investigators suggested that the excessive TLR4 expression seen in obesity/T2DM was the result of gut bacteria-derived LPS and demonstrated that serum and tissue levels of LPS are higher in obesity/T2DM.[88] Although fascinating in nature, a discussion of the gut microbiome, their linkage to TLR function, and to these diseases is beyond the scope of this discussion.

TLR as Therapeutic Targets

As soon as the functional role of TLRs were appreciated, they became potential therapeutic targets for various infectious diseases, allergic, immunologic and inflammatory diseases, sepsis, as well as the similar signaling pathways associated with cancer development and spread.[89,90] The two principle sites for TLR targeting have been at their ligand binding sites or through inhibition of their downstream signaling pathways. Numerous strategies have been developed ranging from gene deletion to monoclonal antibodies. TLR-specific agonists and antagonists as well as inhibitors of the various TLR adaptor and regulatory molecules have also been described. A review of the current clinical trials using TLR-directed therapies highlighted at least eight compounds in 2014, wherein some used TLR3 and TLR9 agonists as vaccine adjuvants in HIV infections or chronic fatigue syndrome, while others used TLR7, TLR8, and TLR9 antagonists in various autoimmune diseases, and yet additional groups used TLR4 antagonists in sepsis trials.[89,90]

Novel Inhibitor of Pathologic TLR Expression and Signaling in T1DM, T2DM, and NAFLD

C10 is a derivative of the anti-thyroidal medication methimazole. Although it does not block thyroxine synthesis it does possess unique anti-inflammatory properties, many of which are mediated through suppression of TLR signaling pathways in both immune and nonimmune cells.[25] C10 was initially described when it was demonstrated that human thyrocytes from patients with Hashimoto's thyroiditis expressed a functional TLR3.[25] Moreover, TLR3 overexpression in these thyrocytes could be stimulated by viral infection (dsRNA), and reversed by C10 treatment.[25] This suggested that C10 might possess both immunoregulatory and anti-tumor properties especially given the role of chronic inflammation in the transformation of Hashimoto's thyroiditis into papillary thyroid cancer,[91] as well as the impact of the immune response on survival in multiple cancers.[92] C10 has now

Figure 4: C10 Treatment and TLR3 Deficiency Block CVB4-induced T1DM But Have No Effect on Spontaneous T1DM in NOD Mice
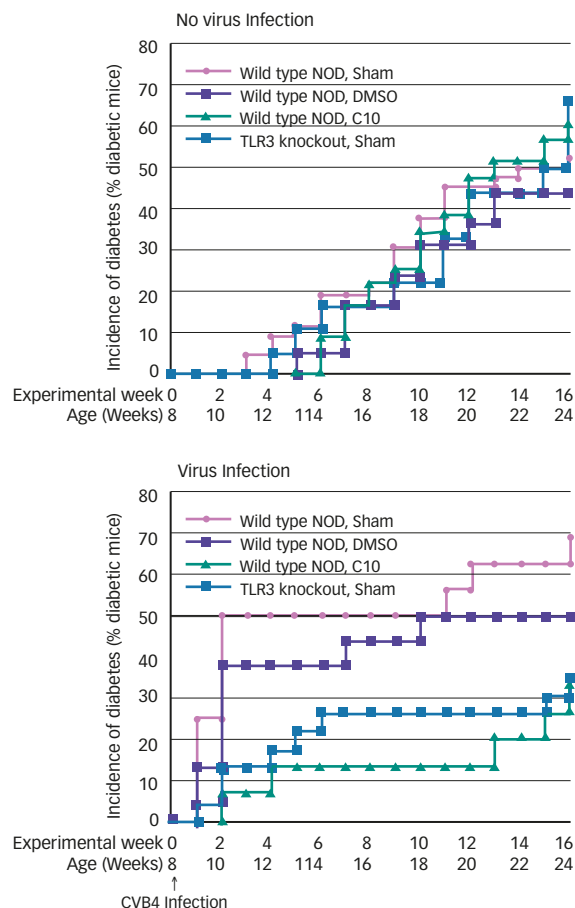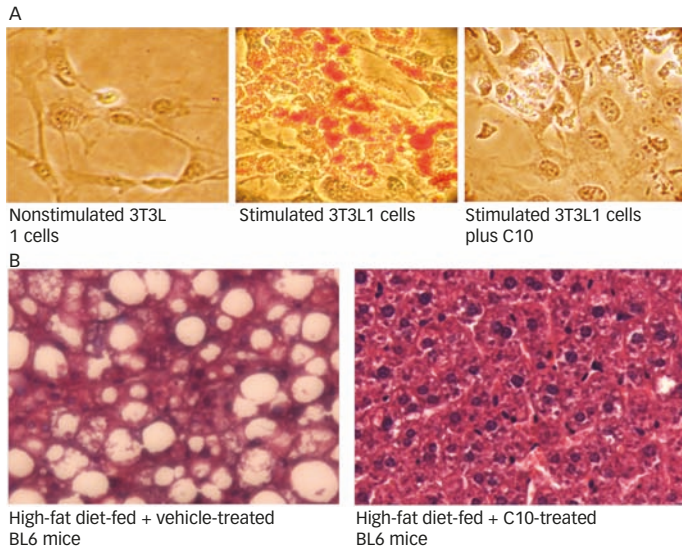

NOD mice (wild type) and TLR3-KO mice were studied to determine the effect of CBV4 infection. A) In nonvirus infected animals, neither C10 treatment nor TLR3-KO prevents the natural occurrence of type 1 diabetes mellitus (T1DM). (B) In CBV4-infected mice both C10 treatment and TLR3 KO mice are protected from the accelleration of T1DM. C10 = phenylmethimazole; CBVs = Coxsackie viruses; TLR = toll-like receptor.

been shown to block TLR signaling in a variety of immunologic diseases as well as the basal expression of TLR3 and TLR4 in multiple cancers,[24,28–31,93–95] suppressing tumor growth and spread in tissue cultures and tumor implants. Finally, in pancreatic cancer, C10 suppresses tumor growth and spread through the inhibition of TLR3-induced IRF3 dimerization, translocation to the nucleus, and gene transcription.[96]

Potential Use of C10 in T1DM

We have now demonstrated th at C10 prevents direct dsRNA poly I:C-induced cytotoxicity (apoptosis) in two β cell culture lines (TC-6 and NIT-1) through inhibition of poly I:C-induced TLR3 signaling and the consequent inhibition of many of the cytokines and chemokines associated with T1DM including: CXCL10, IFN-β, TNF-α, and MHC class I gene expression *in vitro*.[30] Pathologic IRF3 signaling via TLRs has previously been linked to both forms of diabetes. Next, we demonstrated that C10 protects 8 week-old NOD mice from CBV4 acceleration of diabetes, which usually occurs at 2 weeks post-infection.[31] We also demonstrated that 8-week-old TLR3 knockout (TLR3-/-) NOD mice, but not wild-type NOD mice, infected with CBV4 were also protected from the CBV4-induced insulitis and onset of diabetes at two

Figure 5: Effect of C10 on Adipogenesis (A) and Steatosis (B)

A

Nonstimulated 3T3L 1 cells | Stimulated 3T3L1 cells | Stimulated 3T3L1 cells plus C10

B

High-fat diet-fed + vehicle-treated BL6 mice | High-fat diet-fed + C10-treated BL6 mice

A: The differentiation of 3T3L1 pre-adipocytes into adipocytes was blocked by C10 treatment; B: High-fat diet-fed BL6 mice exhibit profound steatosis at 16 weeks, which is significantly inhibited by C10

weeks post-infection, confirming that this was a TLR3-mediated process.[31] These findings are summarized in *Figure 4*. We suspect that activation of TLR3 signaling in β cells occurs through stimulation of the programmed cell death-1 ligand 1 (PD-L1) or changes in T-regulatory cell (CD4+CD25+ Foxp3+) or effector T-cell (CD8) ratios in islets during early development.[97] Thus we are currently looking at the effects of C10 on TLR3 (+/+) and TLR3 (-/-) NOD mice to confirm this. We are also conducting studies to determine if C10 could preserve β cell function and insulin-secretory capacity following the initial onset of CVB4-induced hyperglycemia.[31]

Potential Use of C10 in T2DM

The observations that C10 (1) inhibits FFA (palmitate) induction of TLR signaling in 3T3 L1 pre-adipocytes; (2) inhibits palmitate-induced

TLR4 expression and inflammatory signaling (IL-6 and iNos) in 3T3L1 adipocytes; (3) inhibits LPS-stimulated RAW264[7] macrophages in culture;[24] (4) blocks palmitate-induced Socs-3 upregulation and IRS-1 serine 307 phosphorylation, which mediate IR in 3T3L1 cells;[24] and (5) prevents 3T3L1 cells from differentiating into adipocytes (*Figure 5A* [unpublished data]), suggested that it might be effective in reducing obesity-induced IR. To this end, we subsequently demonstrated that C10 treatment reversed IR, improved glucose intolerance wherein we observed improved area under the curve (AUC) in glucose tolerance tests (GTTs) as well as reduced elevated serum insulin, c-peptide, and leptin levels in high-fat diet-fed BL6 male mice, and if C10 is administered at the onset of a high-fat diet feeding (prevention study), animals gained less weight and it delayed the onset of T2DM in these mice (manuscript in preparation).

Potential Use of C10 in NAFLD

Anecdotally while conducting the T2DM prevention study using C10 in the high-fat diet-fed BL6 male mouse model of T2DM, we noted at necropsy that the C10-treated animals had normal-appearing livers while sham- and vehicle-injected animals exhibited enlarged, pale livers, which was consequently found histologically to be steatosis resulting from the high fat diet feeding (see *Figure 5B*). We are currently studying C10 and other small-molecule TLR inhibitors that we have developed, which also prevent high-fat diet-induced steatosis (unpublished data) in both high-fat diet and high-fructose diet prevention studies, to confirm that these compounds do indeed possess potential therapeutic action in NAFLD.

Conclusions

T1DM, T2DM, and NAFLD are all inflammatory diseases in which TLRs mediate important inflammatory pathways. We have developed a small molecule, prototypical drug (C10), which significantly inhibits many TLR-dependent inflammatory pathways and prevents viral-induction of T1DM in the NOD mouse model, and high-fat diet-induced visceral obesity, IR, and NAFLD in BL6 male mice fed a high-fat diet. Moreover, we have also developed an additional class of small molecule inhibitors of TLR signaling that may be more potent and have lower toxicities than C10, which we are currently testing for efficacy in preclinical animal models. ■

1. Dabelea D, Mayer-Davis EJ, Saydah S, et al., Prevalence of type 1 and type 2 diabetes among children and adolescents from 2001 to 2009, *JAMA*, 2014;311:1778–86.
2. Wild S, Roglic G, Green A, et al., Global prevalence of diabetes: estimates for the year 2000 and projections for 2030, *Diabetes Care*, 2004;27:1047–53.
3. Inzucchi SE, Bergenstal RM, Buse JB, et al., Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD), *Diabetes Care*, 2012;35:1364–79.
4. Atkinson MA, Eisenbarth GS, Michels AW, Type 1 diabetes. *Lancet*, 2014;383:69–82.
5. Soltesz G, Patterson CC, Dahlquist G, Worldwide childhood type 1 diabetes incidence-what can we learn from epidemiology?, *Pediatr Diabetes*, 2007;8(Suppl. 6):6–14.
6. Chiang JL, Kirkman MS, Laffel LM, Peters AL, Type 1 diabetes through the life span: a position statement of the American Diabetes Association, *Diabetes Care*, 2014;37:2034–54.
7. Podrouzkova B, [The effect of intensive insulin therapy on specific long-term complications in type II diabetes], *Vnitr Lek*, 1994;40:306–9.
8. Brun E, Nelson RG, Bennett PH, et al., Diabetes duration and cause-specific mortality in the Verona Diabetes Study, *Diabetes Care*, 2000;23:1119–23.
9. McBean AM, Li S, Gilbertson DT, Collins AJ, Differences in diabetes prevalence, incidence, and mortality among the elderly of four racial/ethnic groups: whites, blacks, hispanics, and Asians, *Diabetes Care*, 2004;27:2317–24.
10. The Diabetes Control and Complication Group: The effect of intensive treatment of diabetes on the developement and progression of long-term complications in insulin dependent diabetes mellitus, *N Engl J Med*, 1993;329:977–866.

11. Bellentani S, Scaglioni F, Marino M, Bedogni G, Epidemiology of non-alcoholic fatty liver disease, *Dig Dis*, 2010;28:155–61.
12. Browning JD, Szczepaniak LS, Dobbins R, et al., Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity, *Hepatology*, 2004;40:1387–95.
13. Huang MA, Greenson JK, Chao C, et al., One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis: a pilot study, *Am J Gastroenterol*, 2005;100:1072–81.
14. Chalasani N, Younossi Z, Lavine JE, et al., The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology, *Hepatology*, 2012;142:1592–609.
15. Bugianesi E, Gentilcore E, Manini R, et al., A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease, *Am J Gastroenterol*, 2005;100:1082–90.
16. Sanyal AJ, Chalasani N, Kowdley KV, et al., Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis, *N Engl J Med*, 2010;362:1675–85.
17. Takeda K, Akira S, Toll receptors and pathogen resistance, *Cell Microbiol*, 2003;5:143–53.
18. Takeda K, Kaisho T, Akira S, Toll-like receptors, *Annu Rev Immunol*, 2003;21:335–76.
19. Ortega-Cava CF, Ishihara S, Rumi MA, et al., Strategic compartmentalization of Toll-like receptor 4 in the mouse gut, *J Immunol*, 2003;170:3977–85.

20. Andonegui G, Bonder CS, Green F, et al., Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs, *J Clin Invest*, 2003;111:1011–1020.
21. Liu S, Gallo DJ, Green AM, et al., Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide, *Infect Immun*, 2002;70:3433–42.
22. Paik YH, Schwabe RF, Bataller R, et al., Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells, *Hepatology*, 2003;37:1043–56.
23. Shi H, Kokoeva MV, Inouye K, et al., TLR4 links innate immunity and fatty acid-induced insulin resistance, *J Clin Invest*, 2006;116:3015–25.
24. McCall KD, Holliday D, Dickerson E, et al., Phenylmethimazole blocks palmitate-mediated induction of inflammatory cytokine pathways in 3T3L1 adipocytes and RAW 264.7 macrophages, *J Endocrinol*, 2010;207:343–353
25. Harii N, Lewis C, Vasko V, et al., Thyrocytes express a functional toll-like receptor 3(TLR3): Overexpression can be induced by viral infection, reversed by Phenylmethimazole, and is associated with Hashimoto's autoimmune thyroiditis, *Mol Endo*, 2003;19:1231–50.
26. Montani V, Shong M, Taniguchi SI, et al., Regulation of major histocompatibility class II gene expression in FRTL-5 thyrocytes: opposite effects of interferon and methimazole, *Endocrinology*, 1998;139:290–302
27. Balducci-Silano PL, Suzuki K, Ohta M, et al., Regulation of major histocompatibility (MHC) class II human leukocyte antigen-DR alpha gene expression in thyrocytes by single strand binding protein-1, a transcription factor that also regulates thyrotropin

receptor and MHC class I gene expression, *Endocrinology*, 1998;139:2300–13.

28. McCall KD, Harii N, Lewis CJ, et al., High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration, *Endocrinology*, 2007;148:4226–37.

29. Schwartz AL, Malgor R, Dickerson E, et al., Phenylmethimazole decreases Toll-like receptor 3 and noncanonical Wnt5a expression in pancreatic cancer and melanoma together with tumor cell growth and migration, *Clin Cancer Res*, 2009;15:4114–22.

30. McCall KD, Schmerr MJ, Thuma JR, et al., Phenylmethimazole suppresses dsRNA-induced cytotoxicity and inflammatory cytokines in murine pancreatic beta cells and blocks viral acceleration of type 1 diabetes in NOD mice, *Molecules*, 2013;18:3841–58.

31. McCall KD, Thuma JR, Courreges MC, et al., Toll-Like Receptor 3 Is Critical for Coxsackievirus B4-Induced Type 1 Diabetes in Female NOD Mice, *Endocrinology*, 2015;156:453–61.

32. Gay NJ, Gangloff M, Structure of toll-like receptors, *Handb Exp Pharmacol*, 2008;181–200.

33. Nasu K, Narahara H, Pattern recognition via the toll-like receptor system in the human female genital tract, *Mediators Inflamm*, 2010:976024.

34. Yamasaki K, Kanada K, Macleod DT, et al., TLR2 expression is increased in rosacea and stimulates enhanced serine protease production by keratinocytes, *J Invest Dermatol*, 2010;131:688–697

35. Giarratana N, Penna G, Amuchastegui S, et al., A vitamin D analog down-regulates proinflammatory chemokine production by pancreatic islets inhibiting T cell recruitment and type 1 diabetes development, *J Immunol*, 2004;173:2280–7.

36. Schroder NW, Maurer M, The role of innate immunity in asthma: leads and lessons from mouse models, *Allergy*, 2007;62:579–90.

37. Ayari C, Bergeron A, LaRue H, et al., Toll-like receptors in normal and malignant human bladders, *J Urol*, 2011;185:1915–21.

38. O'Neill LA, Golenbock D, Bowie AG, The history of toll-like receptors – redefining innate immunity, *Nat Rev Immunol*, 2013;13:453–60.

39. Schnare M, Barton GM, Holt AC, et al., Toll-like receptors control activation of adaptive immune responses, *Nat Immunol*, 2001;2:947–50.

40. Akira S, Takeda K, Kaisho T, Toll-like receptors: critical proteins linking innate and acquired immunity, *Nat Immunol*, 2001;2:675–80.

41. Cnop M, Welsh N, Jonas JC, et al., Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities, *Diabetes*, 2005;54(Suppl. 2):S97–107.

42. Zipris D, Innate immunity and its role in type 1 diabetes, *Curr Opin Endocrinol Diabetes Obes*, 2008;15:326–31.

43. Wang PY, Ma W, Park JY, et al., Increased oxidative metabolism in the Li-Fraumeni syndrome, *N Engl J Med*, 2013;368:1027–32.

44. Bluestone JA, Herold K, Eisenbarth G, Genetics, pathogenesis and clinical interventions in type 1 diabetes, *Nature*, 2010;464:1293–300.

45. Rich SS, Akolkar B, Concannon P, et al., Current status and the future for the genetics of type I diabetes, *Genes Immun*, 2009;10(Suppl. 1):S128–31.

46. Noble JA, Valdes AM, Varney MD, et al., HLA class I and genetic susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium, *Diabetes*, 2010;59:2972–9.

47. Lipponen K, Gombos Z, Kiviniemi M, et al., Effect of HLA class I and class II alleles on progression from autoantibody positivity to overt type 1 diabetes in children with risk-associated class II genotypes, *Diabetes*, 2010;59:3253–6.

48. Stene LC, Oikarinen S, Hyoty H, et al., Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY), *Diabetes*, 2010;59:3174–80.

49. Yoon JW, Austin M, Onodera T, Notkins AL, Isolation of a virus from the pancreas of a child with diabetic ketoacidosis, *N Engl J Med*, 1979;300:1173–9.

50. King ML, Shaikh A, Bidwell D, et al., Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus, *Lancet*, 1983;1:1397–9.

51. Tapia G, Cinek O, Rasmussen T, et al., Longitudinal study of

parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study. *Pediatr Diabetes*, 2011;12:58–62.

52. Knip M, Veijola R, Virtanen SM, et al., Environmental triggers and determinants of type 1 diabetes, *Diabetes*, 2005;54(Suppl. 2):S125–36.

53. Filippi CM, von Herrath MG, Viral trigger for type 1 diabetes: pros and cons, *Diabetes*, 2008;57:2863–71.

54. Ylipaasto P, Klingel K, Lindberg AM, et al., Enterovirus infection in human pancreatic islet cells, islet tropism *in vivo* and receptor involvement in cultured islet beta cells, *Diabetologia*, 2004;47:225–39.

55. Bason C, Lorini R, Lunardi C, et al., In type 1 diabetes a subset of anti-coxsackievirus B4 antibodies recognize autoantigens and induce apoptosis of pancreatic beta cells, *PLoS One*, 2013;8:e57729.

56. Eizirik DL, Colli ML, Ortis F, The role of inflammation in insulitis and beta-cell loss in type 1 diabetes, *Nat Rev Endocrinol*, 2009;5:219–26.

57. Wen L, Peng J, Li Z, Wong FS, The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets, *J Immunol*, 2004;172:3173–80.

58. Rasschaert J, Ladriere L, Urbain M, et al., Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells, *J Biol Chem*, 2005;280:33984–91.

59. Ylipaasto P, Kutlu B, Rasilainen S, et al., Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets, *Diabetologia*, 2005;48:1510–22.

60. Liu D, Cardozo AK, Darville MI, Eizirik DL, Double-stranded RNA cooperates with interferon-gamma and IL-1 beta to induce both chemokine expression and nuclear factor-kappa B-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulitis and beta-cell death in type 1 diabetes mellitus, *Endocrinology*, 2002;143:1225–34.

61. Tanaka S, Nishida Y, Aida K, et al., Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes, *Diabetes*, 2009;58:2285–91.

62. Gur C, Porgador A, Elboim M,et al., The activating receptor NKp46 is essential for the development of type 1 diabetes, *Nat Immunol*, 2010;11:121–8.

63. Li M, Song L, Gao X, et al., Toll-like receptor 4 on islet beta cells senses expression changes in high-mobility group box 1 and contributes to the initiation of type 1 diabetes, *Exp Mol Med*, 2010;44:260–7.

64. Gulden E, Ihira M, Ohashi A, et al., Toll-like receptor 4 deficiency accelerates the development of insulin-deficient diabetes in non-obese diabetic mice, *PLoS One*, 2013;8:e75385.

65. Calcinaro F, Dionisi S, Murinaro M, et al., Oral probiotic admisitration induces interleukin-10 production and prevents spontaneous autoimmune diabetes in the non-obese diabetic mouse, *Diabetologia*, 2005;48:1565–75.

66. Boden G, She P, Mozzoli M, et al., Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver, *Diabetes*, 2005;54:3458–65.

67. Curat CA, Wegner V, Sengenes C, et al., Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin, *Diabetologia*, 2006;49:744–7.

68. Glass CK, Olefsky JM, Inflammation and lipid signaling in the etiology of insulin resistance, *Cell Metab*, 2012;15:635–45.

69. Sawada K, Ohtake T, Hasebe T, et al., Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice, *Hepatol Res*, 2014;44:920–34.

70. Marchetti P, Bugliani M, Boggi U, et al., The pancreatic beta cells in human type 2 diabetes, *Adv Exp Med Biol*, 2012;771:288–9.

71. Kahn SE, Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes, *J Clin Endocrinol Metab*, 2001;86:4047–58.

72. Kolyva AS, Zolota V, Mpatsoulis D, et al., The role of obesity in the immune response during sepsis, *Nutr Diabetes*, 2014;4:e137.

73. Paz K, Hemi R, LeRoith D, et al., A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to

undergo insulin-induced tyrosine phosphorylation, *J Biol Chem*, 1997;272:29911–8.

74. Ragheb R, Shanab GM, Medhat AM, et al., Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: evidence for PKC activation and oxidative stress-activated signaling pathways, *Biochem Biophys Res Commun*, 2009;389:211–6.

75. Kashyap SR, Belfort R, Berria R, et al., Discordant effects of a chronic physiological increase in plasma FFA on insulin signaling in healthy subjects with or without a family history of type 2 diabetes, *Am J Physiol Endocrinol Metab*, 2004;287:E537–46.

76. Frayn KN, Arner P, Yki-Jarvinen H, Fatty acid metabolism in adipose tissue, muscle and liver in health and disease, *Essays Biochem*, 2006;42:89–103.

77. Sharma RB, Alonso LC, Lipotoxicity in the pancreatic beta cell: not just survival and function, but proliferation as well?, *Curr Diab Rep*, 2014;14:492.

78. Poy MN, Yang Y, Rezaei K, et al., CEACAM1 regulates insulin clearance in liver, *Nat Genet*, 2002;30:270–6.

79. Najjar SM, Regulation of insulin action by CEACAM1, *Trends Endocrinol Metab*, 2002;13:240–5.

80. Lee SJ, Heinrich G, Fedorova L, et al., Development of nonalcoholic steatohepatitis in insulin-resistant liver-specific S503A carcinoembryonic antigen-related cell adhesion molecule 1 mutant mice, *Gastroenterology*, 2008;135:2084–95.

81. Zambo V, Simon-Szabo L, Szelenyi P et al., Lipotoxicity in the liver, *World J Hepatol*, 2013;5:550–7.

82. Glaros T, Chang S, Gilliam E, et al., Causes and consequences of low grade endotoxemia and inflammatory disease, *Front Biosci (Elite Ed)*, 2013;S5:754–65.

83. Lassenius MI, Pietilainen KH, Kaartinen K, et al., Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation, *Diabetes Care*, 2011;34:1809–15.

84. Pussinen PJ, Havulinna AS, Lehto M, Set al., Endotoxemia is associated with an increased risk of incident diabetes, *Diabetes Care*, 2011;34:392–7.

85. Gilbert CA, Slingerland JM, Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression, *Annu Rev Med*, 2013;64:45–57.

86. Sanz Y, Moya-Perez A, Microbiota, inflammation and obesity, *Adv Exp Med Biol*, 2014817:291–317.

87. Arslan N, Obesity, fatty liver disease and intestinal microbiota. *World J Gastroenterol* 201420:16452–63.

88. Kim KA, Gu W, Lee IA, et al., High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway, *PLoS One*, 2012;7:e47713.

89. Junquera EC, Mateos-Hernandez L, de la Fuente J, de la Lastra JM, Recent advances in the development of anti-infective prophylactic and/or therapeutic agents based on Toll-Like Receptor (TLRs). *Recent Pat Antiinfect Drug Discov*, 2014;9:14–24.

90. Zuany-Amorim C, Hastewell J, Walker C, Toll-like receptors as potential therapeutic targets for multiple diseases, *Nat Rev Drug Discov*, 2002;1:797–807.

91. Cunha LL, Ferreira RC, Marcello MA, et al., Clinical and pathological implications of concurrent autoimmune thyroid disorders and papillary thyroid cancer, *J Thyroid Res*, 2011;2011:387062.

92. Mantovani A, Allavena P, Sica A, Balkwill F, Cancer-related inflammation, *Nature*, 2008;454:436–44.

93. McCall K, Benencia F, Kohn L, et al., Toll-like receptors as novel therapeutic targets for the treatment of pancreatic cancer. In: Srivastava S, ed., *Pancreatic Cancer Molecular Mechanism and Targets*, Croatia: InTech, 2012;361–98.

94. Courreges MC, Kantake N, Goetz DJ, et al., Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization, *Molecules*, 2012;17:12365–77.

95. Benavides U, Gonzalez-Murguiondo M, Harii N, et al., Phenylmethimazole inhibits production of proinflammatory mediators and is protective in an experimental model of endotoxic shock*, *Crit Care Med*, 2011;40:886–94.

96. Courreges MC, Kantake N, Goetz DJ, et al., Phenylmethimazole blocks dsRNA-induced IRF3 nuclear translocation and homodimerization, *Molecules*, 2012;17:12365–77.

97. Shevach EM, Mechanisms of foxp3+ T regulatory cell-mediated suppression, *Immunity*, 2009;30:636–45.