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Reviews

E-mail id: daria.m.adamczak@gmail.com

The role of toll-like receptors and vitamin D in diabetes mellitus type 1 – a review.

Daria M. Adamczak[1,3], Jan K. Nowak[2], Magdalena Frydrychowicz[3*], Mariusz Kaczmarek[3*], Jan Sikora[3]

[1] Poznan University of Medical Sciences, Clinical Hospital No. 1, Poznan, Poland

[2] Poznan University of Medical Sciences, Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan, Poland

[3] Poznan University of Medical Sciences, Department of Clinical Immunology, Poznan, Poland

* – equal contribution

Abstract

It is widely accepted that type 1 diabetes mellitus (T1DM) is an autoimmune disease resulting from an interaction between immunologic, genetic, and environmental factors. However, the exact mechanism leading to the development of T1DM remain incomplete. There is a large body of evidence pointing towards the important role of toll-like receptor (TLR) activation and vitamin D deficiency in T1DM pathogenesis. In this article we review the available data on the influence of TLRs' level of activation and vitamin D status on the risk of the development of T1DM in humans and rodent models. We also summarized the current information regarding the interactions between TLRs' level of activation,

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vitamin D status, and various environmental factors, such as enteroviral infections, the gut microbiota, and breastfeeding substitution, among others. Our results stipulate that vitamin D seems to protect against T1DM by reducing the TLRs' level of activation.

Keywords: Coxsackie B virus, diabetes mellitus type 1, gut flora, toll-like receptors, vitamin D

Abbreviations

$1,25(OH)_2D$ – 1,25-dihydroxyvitamin D

25(OH)D – 25-hydroxyvitamin D

APC – Antigen presenting cell

B6/RIP-B7.1mouse – A mouse expressing RIP-B7.1 transgene

BBDP rat – Biobreeding diabetes-prone rat

BBDR rat – Biobreeding diabetes-resistant rat

CCL – C-C motif chemokine ligand

CD – Cluster of differentiation

CI – Confidence Interval

CL097 – A derivate of the imidazoquinoline compound R-848; TLR7/ TLR8 ligand

CMV – Cytomegalovirus

CpG – An unmethylated sequence of bacterial DNA recognized by TLR9

CV-B4 – Coxsackie virus B4

CXCL – C-X-C motif chemokine ligand

DAMP – Damage-associated molecular pattern

DC – Dendritic cell

dsRNA – Double-stranded RNA

EBV – Ebstein-Barr virus

HMGB1 – High-mobility group protein B1

ICA – Islet cell antibodies

IFN – Interferon

IL – Interleukin

KRV – Kilham rat virus

LCMV – Lymphocytic choriomeningitis virus

LCMV-GP – The envelope glycoproteins of LCMV

LPS – Lipopolysaccharide

LTA – Lipoteichoic acid

MAP kinase – Mitogen-activated protein kinase

MHC – Major Histocompatibility Complex

mRNA – Messenger RNA

MyD88 – Myeloid differentiation primary response gene (88)

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD mice – Non-obese diabetic mice

OR – Odds ratio

OVA – Ovalbumin

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononucleated cell

Poly(I:C) – Polyinosinic:polycytidylic acid

PRR – Pattern recognition receptor

R-848 – An imidazoquinoline compound; TLR7/ TLR8 ligand

SNPs – Single-nucleotide polymorphisms

ssRNA – Single-stranded RNA

STZ – Streptozocin

T1DM – Diabetes mellitus type 1

T2DM – Diabetes mellitus type 2

T-cell – T lymphocyte

TLR – Toll-like receptor

TIRAP – Toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TNF-α – Tumour necrosis factor α

TRAM – TRIF-related adaptor molecule

Treg – Regulatory T-cell

TRIF – TIR-domain-containing adapter-inducing interferon-β

VDR – Vitamin D receptor

VZV – Varicella zoster virus

Introduction

Diabetes mellitus type 1 is an autoimmune disease resulting from the destruction of insulin-producing β-cells within the islets of Langerhans in the pancreas. It has two distinguishable phases – insulitis and overt diabetes (1). The first is characterized by an infiltration of islets' interstitium by macrophages and CD 8+ T-cells (2). The islets of Langerhans undergo apoptosis and the residual β-cell mass is usually decreased by at least 90% at clinical onset of T1DM (3). The second phase is defined by an insufficient insulin production that results in impaired blood glucose regulation, which in turn leads to hyperglycemia. This ultimately causes both acute and chronic complications of disease.

The disease is generally believed to be a result of the interaction of the immune system with environmental and genetic factors (4). Studies conducted in the last two decades have suggested that the role of the humoral arm of the immune system is pivotal in T1DM pathogenesis. However, recent studies show that islet cell antibodies serve as mere markers of β-cell destruction. The crucial role of the cellular arm of the immune system in the development of disease has become more evident only recently (5).

Toll-like receptors

TLRs are pattern-recognition receptors that have changed little in the course of phylogenesis (6). There are 13 human TLRs known today, the first of which was discovered in 1997 (7). TLRs are mainly found on the surface of macrophages and dendritic cells, i.e. the sentinel cells. However, they are also expressed by tissue cells in the central nervous system, the kidneys, and in the liver (8). By recognizing molecular patterns, TLRs found the basis of the innate immune system's function of pathogen recognition. Since molecular patterns eliciting the inflammatory response can have their

sources not only in pathogens, but also in an organism's own cells (they are called DAMPs then), TLRs may mediate pathological cell death (9,10).Thus, TLRs are of particular interest in the study of autoimmune disease.

Different TLRs have the ability to recognize a wide variety of exogenous ligands from bacteria (flagellin, glycolipids, lipopeptides, lipoproteins, LTA and LPS), viruses (dsRNA, ssRNA, DNA), fungi (β-glucan), parasites (profilin), as well as endogenous ligands like fibrinogen, heat shock proteins, and HMGB1 (8). Exogenous ligands recognition is enabled by the presence of leucine-rich repeat motifs that facilitate protein to protein interactions. Activated TLRs trigger a signaling cascade that may use the adaptors MyD88 (the most important one), TIRAP, TRAM and TRIF, which results in the activation of NF-kB transcription (11). In fact, only TLR3 uses TRIF and not MyD88 (11,12). It should also be noted that MAP kinases may be activated as a consequence of ligand binding by TLRs. In general, these mechanisms lead to an increase in the microcellular environment concentration of proinflammatory cytokines, antimicrobial peptides, and type I INFs (11).

Vitamin D

Vitamin D is a name used to describe a group of fat-soluble secosteroids. Of these, cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) play the most important roles in human physiology. It is well known that vitamin D has other functions apart from regulating calcium homeostasis. Vitamin D influences the immune system, the central nervous system, and muscles (13). Therefore, consequences of vitamin D deficiency are not limited to bone disease, i.e. rickets, osteoporosis, and osteomalacia (14). A significant volume of data points towards a role of vitamin D deficiency in autoimmune, neurologic and cardiovascular disease (15). Vitamin D also seems to influence carcinogenesis.

The synthesis of vitamin D begins in the skin, where cholesterol is exposed to ultraviolet B photons. It should be stressed that this natural mechanism of vitamin D production is the most efficient source of vitamin D that the human organism possesses. This is well illustrated by the fact that at most latitudes, sunbathing for approximately 15 minutes between 10 a.m. and 3 p.m. provides sufficient amount of vitamin D (16). On the other hand, there are few nutritional sources of vitamin D, they are not well represented in the Western diet, and they contain insufficient amounts of the vitamin to meet demands. Oily fish, egg yolks, and artificially fortified, highly-processed foods are all examples of nutritional sources of vitamin D (14).

The actual product of biosynthesis of vitamin D in the skin from cholesterol is 7-dehydrocholesterol. It is metabolized to 25-hydroxyvitamin D by the liver. This form of vitamin D is the most commonly used marker of vitamin D status. 25-hydroxyvitamin D is further hydroxylated in the kidneys, yielding 1,25-dihydroxyvitamin D, the most potent form of naturally occurring vitamin D (17).

A commonly used measure of vitamin D deficiency is 25-hydroxyvitamin D serum concentration below 75 nmol/L (17). Although vitamin D deficiency is widespread and seems to be widely prevalent in healthy pediatric and adult populations (18), the recommendations on daily vitamin D intake are conflicting. They most often range from 400 IU daily for infants (United States Institute of Medicine) (19) to 2,000 IU daily for adults over 50 years old (Scientific Advisory Council of Osteoporosis

Canada) (20). It was shown that doses as large as 1,500 IU of vitamin D daily may be required to reach vitamin D adequacy. It is also recognized that vitamin D supplementation with doses up to 10,000 IU is safe (21). However, it should be noted that the evidence behind the definition of vitamin D deficiency and the recommendations of its supplementation still leaves many questions unanswered. A recent study by Autier et al. suggests that low 25-hydroxyvitamin D levels in various diseases result from associated chronic inflammation and may not play role in etiopathogenesis (22).

Epidemiology of diabetes mellitus type 1

T1DM constitutes 5-10% of all cases of diabetes (24). According to Diabetes Mondiale Project Group, the incidence of T1DM varies from 0.1/100,000/year in China and Venezuela to 36.5/100,000/year in Finland and 36.8/100,000/year in Sardinia. Twenty-one of thirty-nine European populations have a high or very high incidence of T1DM (10-19.99/100,000/year and ≥20/100,000/year, respectively). The incidence of T1DM among children increases with age and is highest among 10-14 years old in most populations worldwide (23). EURODIAB data in 17 European countries registered 29,311 new cases of T1DM in children aged under 15 between 1989 and 2003. The overall annual increase in incidence was 3.9% (95% CI 3.6-4.2). The predicted number of new cases in 2020 is 24,400. The incidence among children under 5 years old will double between 2005 and 2020. The prevalence among children younger than 15 years old will rise by 70% in this period (24).

The genetic factors of diabetes mellitus type 1

The cumulative significance of genetic factors is estimated to be as high as 50-65% according to twin studies (25). Approximately 70% of type 1 diabetics carry a HLA risk allele. However, only 3–7% of those carrying such a HLA haplotype will manifest T1DM (26). Individuals positive for HLA class II alleles – HLA-DRB1*03 (HLA-DR3), HLA-DRB1*04 (HLA-DR4) and DQB1*03:02 (HLA-DR8) have the highest risk of developing the disease (27). The link between HLA class II molecules and immune-mediated destruction of the pancreatic islets is still incomplete. The binding of crucial peptides from autoantigens such as GAD, insulinoma-associated 2 antigen, preproinsulin and zinc transporter ZnT8 in the thymus and peripheral organs of the immune system probably play an important role (28). Additionally HLA class I alleles, especially B*5701 and B*3906, are independently associated with the disease (29).

Beyond HLA genes, there are also other loci that are linked to T1DM. Current data point towards the following genes: *CD69, CTLA4, GLIS3, IL2Ra, IL10, IL19, IL20, IL27, PTPN22*, and *UBASH3A* (30–32). There is also an association between T1DM and single-nucleotide polymorphisms in the interferon-induced helicase 1 gene. This gene encodes melanoma differentiation-associated protein 5 – a cytoplasmic sensor for viruses, especially coxsackie B (33).

The environmental factors of diabetes mellitus type 1

Despite the large volume of research, the factor or factors triggering T1DM have not been established thus far. Findings obtained from many studies such as American DAISY (34), German BABYDIAB (35) and Finnish reports (36) are contradictory and do not give a satisfying answer to the T1DM problem. The traditional view of T1DM postulates that an environmental factor causes disease in genetically susceptible individuals, whereas newer theories indicate that the penetrance and the

expression of heritable immune aberrations, as well as the progression of initially inherited organ defects, are under the chronic influence of environmental factors (37).

Data concerning most investigated environmental risks in relation to TLRs and vitamin D are summarized in the table below (Table 1). Vitamin D deficiency, as it is presented in Table 2, is considered as an environmental risk factor as well.

Animal models of diabetes mellitus type 1

NOD mice and biobreeding rats are the most popular rodent models in T1DM research.

NOD mice

The strain was identified and bred in the late 1970s. NOD mice spontaneously develop T1DM that bears the molecular and clinical features of the disorder in humans, i.e. primary mediation by T lymphocytes and a chronic course. The inflammation of the islets that begins at the age of 4-5 weeks leads to a gradual loss of β-cells and ultimately results in insulin deficiency. Clinical symptoms of T1DM in NOD mice present at the age of 12-30 weeks. It is noteworthy that NOD mice do not require daily administration of insulin and do not present with ketoacidosis. The percentage of NOD mice that develop clinical T1DM is 90% in females and 60% in males. This animal model of T1DM seems to be as complex as T1DM in humans, in addition to its molecular basis also not being fully understood (1,79,80).

Biobreeding rats

The biobreeding rats (BB) were first recognized in the 1970s. There are two inbred strains of BB rats: diabetes-prone (BBDP) and diabetes-resistant (BBDR). In BBDP a T-cell deficiency resulting from a mutation in the gene *Ian4* that encodes a mitochondrial protein is at the core of the pathology that usually ensues at the age of 12 weeks. It includes polyuria with polydipsia, hyperglycaemia, weight loss, all linked with the lack of insulin. Unlike in NOD mice, the ketoacidosis is often fatal, and the mice may require insulin for survival. Development of T1DM in BBDR rats, who have normal immunologic phenotype, seems to be dependent on anti-viral antibodies (2,11,79).

As the animal models of T1DM are a result of inbred selection for hyperglycaemia, it is highly probable that not all amplified characteristics of the phenotype are related to the autoimmune processes that underlie T1DM (79).

The immune system in diabetes mellitus type 1

Although the exact mechanism leading to β-cell destruction and T1DM pathogenesis remains unclear, there are theories that seem to integrate the available data well.

In 2001, Mathis et al. proposed that T1DM has its roots in exposure of naive T-cells to islet antigens in lymph nodes. According to this theory, circulating naive T-cells encounter APCs of the pancreatic lymph nodes that carry islet antigens. The sources of the antigens may be diverse, and may include

products of cells constituting pancreatic islets and also the remains of apoptotic cells picked up by immature DCs. The T-cells activated after exposure to antigen peptides originating in β-cells may lead to a progressive loss of β-cells. It remains largely unknown in which circumstances the antigens of pancreatic islets could be picked up by the DCs (1).

Lien et al. proposed in 2009 an explanation of mechanisms leading to the development of T1DM in BBDR rats. It concentrates on the role of viruses and TLRs. It is postulated that DCs become activated in response to KRV and TLR ligands. The DCs would upregulate the expression of molecules of class II MHC and chemokines, leading to a proinflammatory state. Activation of signaling pathways linked with TLR9 in response to KRV infection results in inflammation in the lymph nodes of the pancreas. T-cells recruited to those lymph nodes become activated. Simultaneously, regulatory T-cells are systemically downregulated through exposure to cytokines and TLR activation. These events, involving infection and an increase in TLR activity, lead to a release of islet antigens, which are picked up by DCs and presented to T-cells (11). There are also other explanations, which put more stress on the role of an unknown environmental insult. They are based on research conducted with the use of STZ (81).

The "fertile field" hypothesis formulated in 2003 aimed at incorporating the information regarding the roles of various infectious agents in the development of T1DM. The "fertile field" itself is a period of time following a viral infection, during which autoreactive T-cells may expand, resulting in autoimmunity and ultimately T1DM. Therefore, β-cell death is a consequence of interaction between innate susceptibility and an environmental trigger (82). It is noteworthy that β-cells are especially prone to being exposed to pathogenic T-cells as they increase the production of IFN and MHC class I (83). The function of those effector T-cells is not sufficiently attenuated by regulatory T-cells because of their decreased population, altered reactions, and a proinflammatory environment (84). The antigens released from β-cells on their destruction are transferred to the pancreatic lymph nodes by APCs, possibly including immature DCs. Insulin autoantibody production begins after the conversion of B cells to plasma cells. This process occurs in the presence of β-cell antigens. This may in part be due to aberrations in the process of positive and negative T-cell selection in the thymus (85). The autoreactive CD8+ T-cells also play a role in the destruction of β-cells. These processes lead to a second wave of β-cell death. Local inflammation and stress stops insulin production in a large part of the remaining islets. The released antigens further stimulate the autoimmune response, thereby completing the vicious circle of T1DM etiopathogenesis. The proliferation of new pancreatic antigen-specific clones of immune cells is called "epitope spreading". The ensuing inflammation stimulates the proliferation of β-cells, which results in a temporary increase of their mass. The autoreactive process precipitates the onset of T1DM symptoms (82).

The enteroviruses can play a role in the pathogenesis of T1DM through several mechanisms that may yield synergistic effects, e.g. antibody-dependent enhancement of infection, bystander activation of T lymphocytes, IFN-α production by β-cells, molecular mimicry, thymic, pancreatic, and persistent β-cell infection (67).

The roles of toll-like receptors and vitamin D in diabetes mellitus type 1

According to the aforementioned hypotheses and data presented in the Table 2, TLRs play an important role in T1DM pathogenesis. However, the fact that hyperglycemia results in TLR's upregulation should be noted as well (86). The majority of studies indicates that TLR upregulation leads to T1DM development. A few studies described a reduction of risk of T1DM after TLR's early

stimulation. This is consistent with the hygiene hypothesis. Data concerning vitamin D's impact on T1DM development is also summarized in Table 2.

Vitamin D and toll-like receptors' effects on the immune system

Information regarding the contradictory effects of TLRs and vitamin D on the immune system, and the associations between them are collected in Table 3.

Conclusions

The current knowledge of TLRs and vitamin D involvement in T1DM points toward new and interesting aspects of autoimmunization and also sheds new light on the vast network of interdependent molecular processes underlying T1DM. The examples of particularly interesting and promising topics related to the role of TLRs in T1DM development are: the normal and pathological course of enteroviral infections, the relationship between the gut microbiota and the immune system, and the impact of substitution of breastfeeding on long-term predisposition to autoimmune disease. It is a matter of discussion whether currently available rodent models of T1DM will be sufficient to answer the arising questions.

Although there is abundant evidence for the role of TLRs and vitamin D in the pathogenesis of T1DM, the exact mechanisms remain elusive. It seems that TLRs exert their influence on the development of T1DM through the modulation of immune responses following β-cell destruction as well as to triggering factors, such as enteroviruses. As such, both TLRs and vitamin D are of particular interest in T1DM research that could potentially pave the way for new clinical interventions. It is noteworthy to mention that such interventions would be effective regardless of the nature of the triggering environmental factor. Supplementation of vitamin D, which is a multidirectional modulator of TLR function, is one such potential intervention, and initial results of trials assessing its efficacy are promising. However, future trials and observational studies are needed to confirm these findings.

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Table 1: The association of environmental factors with T1DM, TLR and vitamin D.

Potential causative factor	Association with T1DM	Association with TLRs or vitamin D
Climatic influence	Low exposure to sunlight and latitude deviation correlatee with the risk of developing T1DM (38), see also Fig. 1 in (39).	Low sunlight exposure is associated with vitamin D deficiency (15). Ultraviolet B radiation is necessary for precholecalciferol formation in the skin (40).
Gut microbiota	The lack of butyrate- and lactate-producing bacteria, along with increased proportion of *Bacteroidetes to Bifidobacterium* in gut microbiota are related to β-cell autoimmunity and T1DM, possibly through impairment of the function of the intestinal epithelial barrier (41). Small intestinal pathology observed in T1DM includes reduced integrity of the epithelial intestinal barrier leading to mucosal inflammation and decreased tolerance to food antigens. In BBDP and BBDR rats mucosal crypt depth and intestinal permeability are greater than in Wistar rats (42). There is evidence linking intestinal microbiota to intestinal epithelial barrier integrity (43,44). A metagenomic analysis in four children developing T1DM and four matched controls revealed that flagella synthesis and increased adhesion may stimulate autoimmunity leading to T1DM. In children with T1DM a deficiency of butyrate-producing bacteria was stated (45). MyD88 plays a role in reception of microbial stimuli by the intestinal epithelium. Its deficiency can result in alterations of the intestinal microflora. This is displayed with exposure of germ-free NOD mice to microflora of MyD88-negative NOD mice results in attenuation of T1DM (46). MyD88-negative NOD mice deprived of the intestinal microflora by regular sulfamethoxazole and trimethoprim use develop diabetes fast, whereas in MyD88-negative NOD mice harbouring microbiota of normal human intestinal flora, the development of T1DM is attenuated (46).	The homeostasis of the intestinal epithelium is dependent on TLR function, including: recognition of commensals and pathogens (47). TLRs are needed for both defense responses and epithelial barrier repair. The relationships between TLRs and intestinal microbiota are complex, as illustrated by the example of modulation of TLR4-dependent inflammation by *Lactobacillus jensenii* TL2937, an immunobiotic (48) and the impact of *Bifidobacterium breve* MCC-117 on TLR2 signaling with an anti-inflammatory effect (49). C57BL/6 mice are more susceptible to dextran sodium sulfate-induced colitis if unable to produce 1,25(OH)D or VDR-deficient. In this model vitamin D influences intestinal microflora homeostasis, with vitamin D deficiency resulting in dysbiosis (50).
Hygiene	In 220 children with T1DM and 433 matched controls odds ratio for T1DM development depending on frequency of daycare facilities attendance in infancy was 0.71 (95%CI: 0.51-1.00, p = 0.05), indicating a possibility that social mixing in infancy may help prevent T1DM (51). Epidemiological analyses point to a higher T1DM incidence in countries where standard of hygiene is better. In those industrialized countries T1DM incidence is increasing (52,53).	No data.

Psychological stress	Relative risk for T1DM development after highly stressful life events in children aged 5-9 years equals 1.82 (95%CI: 1.09-3.03) (54).	TLR4 may contribute to inflammation resulting from subacute stress. In TLR4-deficient rodents inflammatory and behavioral reaction to subacute stress are less pronounced than in normal mice (55). TNF-α and IL-6 production in response to TLR3, TLR4, and TLR9 activation in DCs from mice subjected to social disruption is greater than in DCs from non-stressed home cage mice (49).
Substitution of breast feeding	Starting cow's milk-based formula in infants younger than 3 months is a risk factor for T1DM (56). In children who are exposed to cow's milk before the 3rd month of life signs of an enteroviral infection in infancy are associated with autoimmunity (57). Human milk can neutralize CV-B4 (58). The incidence of autoantibodies associated with T1DM is 50% lower in children weaning to hydrolyzed milk (59). In 94 children at high risk of T1DM that were fed with cow's milk-based formula the development of T1DM (n = 8) was associated with increased levels of IgG to β-lactoglobulin, bovine insulin (60).	Analysis of serum 25(OH)D in 117 Korean infants aged 1 to 6 months revealed a lower mean concentration in breastfed vs. formula-fed infants (9 vs. 29 ng/mL) (61).
Toxins	Average daily ingestion of nitrate compounds was greater in 684 diabetic children and their mothers than in 595 children without T1DM and their mothers (0.9 mg vs. 0.8 mg, $p < 0.001$). A correlation between dietary nitrate and the risk of development of T1DM was found (62).	No data.
Vaccine administration	Risk ratio for T1DM development in children who received one dose of various vaccines compared with children who were unvaccinated was between 0.91 (*Haemophilus influenzae* type b) and 1.14 (measles, mumps and rubella). None of the relationships was statistically significant. Therefore, studies conducted to date do not support the hypothesis that vaccinations may trigger T1DM (63,64).	TLR agonists are investigated as vaccine adjuvants. Examples of research yielding promising results include TLR3 and TLR7 agonists (poly(I:C) and resiquimod) in a DNA vaccination against human papilloma virus 17 (65) and TriVax, which is a RSV vaccine containing poly(I:C) and anti-CD40 antibody (66).
Viral infections	There is convincing evidence that enteroviral infections (e.g. with CV-B4) can trigger T1DM (67,68). It is known that enterovirus infections are more common in children that will develop autoimmunity within 6 months than in children that will not (OR = 3.7, 95%CI: 1.2-11.4) (69). CV-B4 exhibits pancreatotropism *in vivo* (70). Infection of human islets of Langerhans with CV-B4 leads to upregulation of interferon induced with helicase C domain 1, CXCL10, CCL5, IFN-β, but not TLR3; the expression of insulin is reduced (71). Infection of human islets of Langerhans with CV-B3	An infection with CV-B4 may activate a TLR4-related signaling pathway and thus lead to an increase in cytokine production. TLR4 does not seem to directly bind CV-B4 and its activation is independent of CV-B4 internalization and replication (67).

	induces production of such proinflammatory factors, such as: IL-6, TNF-α, IL-8, IFN-γ-induced protein 10, and macrophage inflammatory protein-1α. CV-B3 replication is needed to elicit such a cellular response (72). A metaanalysis of 4448 cases revealed an association between enteroviral infections and the presence of biomarkers of autoimmunity related to T1DM (OR = 3.7 [95% CI: 2.1-6.8]). The association was also valid for clinical T1DM (OR = 9.8 [95% CI: 5.5-17.4]) (73). Coxsackie B virus was found in the pancreatic tissue of patients presenting with acute T1DM. The virus could induce diabetes in animal models (74). In NOD mice, coxsackie B virus infection of the islet cells contributes to the enhancement of a preexisting autoimmune process, inducing T1DM. However, although coxsackie B virus can hasten destruction of the β-cells, it does not initiate autoimmunity and the observed effect is dependent on preexisting autoreactive T-cells (75). Administration of CV-B4 to young NOD mice prevents T1DM. The effect is dependent on the timing of this intervention (76). On the other hand, previously uninfected NOD mice are susceptible to triggering of T1DM after exposure to CV-B4. Lymphocytes from NOD mice that were uninfected at early age may transfer the disease (77). T lymphocytes infiltrating the pancreatic islets of NOD and RIP-LCMV mice can be diverted to regional lymph nodes during a viral infection. In both models CXCL10 seems to play an important role in T lymphocyte recruitment (78).	

Table 2: Relations between TLR signaling, vitamin D, and the development of diabetes mellitus type 1 in humans and in animal models.

Area of research	Findings
Induction/acceleration of T1DM in relation to increased TLR signaling in humans	Incubation of PBMCs obtained in T1DM patients with R-848, an TLR7 and TLR8 agonist, results in an increase in percentage of IFN-α-producing plasmacytoid dendritic cells. This does not happen after PBMCs from healthy controls are subjected to the same treatment (87).
	The expression of TLR2 and TLR4 is increased in monocytes from patients with T1DM compared with healthy controls. This is associated with increased expression of downstream targets, such as NFκB, TRIF, and MyDF88. Monocyte production of TNF-α and IL-1b correlate with expression of TLR2 and TLR4 (88).
Induction/acceleration of T1DM in relation to increased TLR signaling in animal models	TLR4-deficient NOD mice develop T1DM faster (177 vs. 118 days, $p < 0.01$). TLR4 deficiency does not reduce Treg count, but impairs Tregs' T-cell proliferation-limiting activity (89).
	Stimulation of TLR7 and TLR8 with CL097 shortens the time of onset of T1DM in NOD mice and also in 8.3 NOD T-cell receptor transgenic mice, after addition of a CD40 agonist. The effect seen in 8.3 NOD mice was reduced after administration of IRS661, a TLR7 inhibitor (90).
	Pathways responding to TLR9 activation are involved in induction of T1DM by KRV in BBDR rats (91).
	LCMV-GP-expressing mice immunized with LCMV-GP do not develop T1DM until they are exposed to poly(I:C) and R-848, which are ligands for TLR3 and TLR7, respectively (92).
	Administration of poly(I:C), which is a TLR3 ligand, to BBDR rats during 3 days before KRV infection, increases the percentage of rats that develop T1DM to 100%. Other TLR (2, 4, 6, 7, 9) ligands, such as LPS, zymosan, R-848, and CpG also help induce diabetes in BBDR rats (93).
	Treatment with poly(I:C) leads to the development of T1DM in B6/RIP-B7.1 mice, probably through TLR3 activation (94).
Protection against T1DM in relation to TLR activity in animal models	TLR9 deficiency reduces susceptibility to T1DM in NOD mice. The effect results from an MyD88-independent increase in CD73+ cell count. TLR9 inhibition with chloroquine yielded similar effects (95).
	Peritoneal administration of TLR2, TLR3, TLR4, and TLR7 agonists for 10-20 weeks protects against T1DM in NOD mice. Probiotic microbiota reduce the risk of T1DM. This effect is TLR-dependent and T-cell-transferable (96).
	Chloroquine, which is a TLR9 antagonist, reduces IL-12p40 serum levels in BBDR rats infected with KRV (91).
Associations between T1DM and vitamin D	A meta-analysis of 8 studies revealed a possible association between higher vitamin D intake in early childhood and a reduced risk of T1DM (pooled OR = 0.71, 95%CI: 0.51-0.98). Maternal vitamin D intake does not modify the risk of T1DM in offspring (pooled OR from 3 studies was 0.95, 95%CI: 0.66-1.36) (97).
	25(OH)D serum concentrations did not differ in 907 children with newly diagnosed T1DM and in their 896 healthy siblings (98).

	Patients with T1DM and T1DM with microvascular complications are vitamin D deficient more often compared with control subjects (29% and 35%, respectively, vs. 17%, $p < 0.01$) (99).
	BXL-219, a vitamin D analog, reduces production of CXCL10, CCL2, and CCL5 by islet cells, thus inhibiting the development of T1DM. Treatment with BXL-219 does not result in TLR downregulation (100).
	Regular and irregular supplementation of vitamin D reduces the adjusted risk of T1DM development (risk ratio 0.12 [95%CI: 0.03-0.51] and 0.16 [95%CI: 0.04-0.74], respectively) (101).
Associations between TLR activity and vitamin D	TLR7 expression on monocytes, T-cells, and B-cells negatively correlates with serum 25(OH)D concentration. TLR7 activity in response to a specific agonist correlates with serum concentration of 25(OH)D, especially in study participants older than 60 years (102).
	There is a negative correlation between TLR4 expression and serum concentration of 25(OH)D. In monocytes pretreated for 24 hours with $1,25(OH)_2D$ (0.1 μM), the expression of TLR4 and cytokines in response to LPS is decreased (99).
	Eight-hourly treatment with 25(OH)D (1μM), $1,25(OH)_2D$ (0.1μM) reduces the expression of TLR4 in a human colon cancer cell line (HT29) (103).
	In monocytes from LADA patients, the reactivity to LTA and LPS, measured using assessment of TNF-α production and the level of phosphorylation of NF-κB-p65, is decreased in the presence of $1,25(OH)_2D$. A similar effect was found in monocytes from T2DM patients (104).
	The expression of TLR2 and TLR4 decreases with increasing 25(OH)D serum concentrations. The reduction in cellular concentrations of mRNA for TLR2 and TLR4 depends on the dose of $1,25(OH)_2D$. In monocytes treated with $1,25(OH)_2D$, activation of TLRs leads to a decrease in TNF-α synthesis (105).
	The expression of vitamin D receptor and vitamin D-1-hydroxylase is upregulated by activation of TLRs on the surface of macrophages. This results in an increased production of cathelicidin (106).

Table 3: Contradictory effects of toll-like receptors' and vitamin D receptor stimulation on the immune system.

	TLR stimulation	VDR stimulation
APC cells (especially DCs)	Activation by β-defensin 2 (107)	Suppression of maturation (108)
Autoreactive T lymphocytes ($CD8^+$)	Activation (109)	Inhibition of the recruitment to pancreatic cells (100)
Treg lymphocytes ($CD4^+$ $CD25^+$)	Reversal of function (110)	Enhancement (100)
NFκB transcription	Upregulation (111)	Downregulation (100)