

## **Genes Mediating Environment Interactions in Type 1 Diabetes**

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### ■ Abstract

The relative risk of type 1 (autoimmune) diabetes mellitus for a sibling of an affected patient is fifteen times that of the general population, indicating a strong genetic contribution to the disease. Yet, the incidence of diabetes in most Western communities has doubled every fifteen years since the Second World War - a rate of increase that can only possibly be explained by a major etiological effect of environment.

Here, the authors provide a selective review of risk factors identified to date. Recent reports of linkage of type 1 diabetes to genes encoding pathogen pattern recognition molecules, such as toll-like receptors, are discussed, providing a testable hypothesis regarding a mechanism by which genetic and environmental influences on disease progress are integrated.

**Keywords:** type 1 diabetes · genes · environment

### Introduction

Type 1 (autoimmune) diabetes (T1D) is an endocrine disease in which the body's immune system gradually destroys the insulin-producing  $\beta$ -cells in the pancreas, leading to a loss of insulin secretion and hyperglycemia. Progression from the pre-clinical stage of  $\beta$ -cell autoimmunity, known as insulinitis, to established diabetes can take up to a decade [1,2]. Globally, T1D affects between 10-20 million people. Forty percent of cases develop the onset of the disease before the age of 20, making it one of the most common severe chronic childhood illnesses [3-5]. T1D is the leading cause of end-stage renal disease, blindness, and amputation in many communities, and is a major cause of cardiovascular disease and premature death in the general population [6].

It is not clear what initiates the autoimmune cascade leading to T1D, but it is certain that both genetic

and environmental factors contribute to the risk of disease. Progress in the understanding of the pathogenesis of T1D has been complicated by the large number (>20) of genes involved. It appears that most linkage peaks located to date are the product of multiple linked loci, each of which confers relatively little risk. The limited effect of each locus has required very large sample sets to generate sufficient analytic power. Furthermore, many linkage peaks have not been replicated in subsequent studies, raising the possibility that they are dependent on local environmental conditions, or are affected by parental imprinting.

Animal models of human T1D, such as the NOD mouse [reviewed in 7] and BB rat [reviewed in 8], were originally applied to help develop paradigms and methodological tools for the dissection of this complex genetic trait. In several cases, correlation of syntenic linkage peaks has suggested, and in some cases molecular analysis has supported, the possibility that in

both the animal model and the human disease, similar polymorphisms in the same genes affect disease risk. The animal models have three other advantages in this context. Firstly, as they are inbred, large numbers of segregating progeny can be produced, greatly increasing the statistical power of genetic analyses. Secondly, mouse strains, and to a lesser extent rat strains, are highly amenable to recombinant genetic approaches, permitting allele exchange experiments to formally test hypotheses relating to causality. Finally, as the housing conditions of laboratory animals can be exactly controlled, these models provide a platform to test hypotheses relating to gene/environment interactions in determining the risk of disease. This review provides a selective summary of proposed genetic and environmental risk factors for type 1 diabetes.

### Monogenic autoimmunity

Valuable progress in the understanding of the pathogenesis of autoimmune disease (AID) has come with the recognition of several genes in humans that underlie Mendelian forms of the disease. In particular, two very rare syndromes are now genetically characterized: autoimmune polyendocrine syndrome type 1 (APS-1; also termed autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, APECED) and immune deregulation, polyendocrinopathy, enteropathy, X-linked (IPEX). Both syndromes are associated with the development of immune mediated diabetes - neonatal diabetes for the IPEX syndrome, and about 20% of patients with APS-I develop diabetes as children or young adults [9, 10].

#### *APS-1*

The APS-1 syndrome is inherited as an autosomal recessive disorder [11] and results from mutations of the *AIRE* (autoimmune regulator) gene [12] that is located on chromosome 21q22 [13, 14]. Through studies of targeted mutant *Aire*-deficient (knockout) mice, we know that *Aire*'s protein product acts as a thymic transcription factor for numerous genes [15], including some related to T1D, such as hormones (e.g. insulin) and growth factors (e.g. insulin-like growth factor 2). *Aire* knockout mice on a 129/C57BL/6 background develop high titres of autoantibodies reactive with multiple organs as well as T cell infiltrates of multiple organs, but do not develop diabetes. When backcrossed to the B10.Br background for 2 generations, *Aire* deficiency was associated with an increased incidence of diabetes in a model involving the transgenic expression of hen egg lysosyme (HEL) in the pancre-

atic islets and a HEL peptide-specific T cell receptor on T cells [16]. Thus, an attractive hypothesis is that mutations of the *AIRE* gene (e.g. APS-1 syndrome) in humans may cause reduced "peripheral antigen" expression in the thymus thereby decreasing the induction of thymic central tolerance to these antigens [17, 18].

#### *IPEX*

IPEX syndrome results from mutations of the *FOXP3* gene [19-21], which is located on chromosome Xp11.23. All disease-associated mutations appear to disrupt gene function causing a phenotype in hemizygous males but not in heterozygous females. *FOXP3* encodes the protein scurf, which belongs to the *Forkhead* family of winged-helix transcription factors [22, 23]. Its expression is largely restricted to T cells with a regulatory function (Treg cells), suggesting a role in the development of these cells [24-26]. Human Hassall's corpuscles express thymic stromal lymphopoietin (TSLP), which activates thymic CD11c-positive dendritic cells to express high levels of CD80 and CD86 molecules. These TSLP-conditioned dendritic cells are then able to induce the proliferation and differentiation of CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> thymic T cells into CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, which suppress autoimmune T cells and help to maintain tolerance to self [27].

At present, there is little or no indication that polymorphisms at either *AIRE* or *FOXP3* contribute to the common forms of T1D. While one small case-control study has reported an association of the *FOXP3* gene with T1D in Japanese patients [28], another study in Sardinian families found no evidence when compared with a case-control cohort [29].

### Polygenic type 1 diabetes

To date, there is at least suggestive evidence that more than thirty genes contribute to the susceptibility of T1D development. Loci for which reasonable confirmation has been obtained, or show significance in one of the two major publications reporting multiple genome-wide linkage scans ([30] and [31]; reporting data from 767 and 1,435 multiplex families respectively), include 6p21 (*IDDM1*), 11p15 (*IDDM2*), 2q31-q33 (*IDDM12*), 10p11-q11 (*IDDM10*), 6q25 (*IDDM5*), 1p13 and 16q22-24. The three chromosomal regions with consistently significant evidence of association with T1D are the *HLA* region at 6p21.3 (*IDDM1*) [32, 33], the *INS* region at 11p15 (*IDDM2*) [34, 35] and the *CTLA4/CD28* region at 2q31-q33 [36-39].

### *The major histocompatibility complex (MHC)*

The MHC region on human chromosome 6p21 has been identified as a critical susceptibility locus for many human AIDs, including T1D [40]. Through studies done on animals, it has been hypothesized that certain MHC alleles are less efficient at presenting self-peptides to the developing T cells in the thymus, so that negative selection fails [41-44]. Human genetic predisposition to T1D is strongly associated with *HLA-DQ $\beta$* , which is closely linked to *DR3* and *DR4* alleles [40]. T1D has a positive association with both *DR3* and *DR4*, where relative risk (RR) is 3 and 6, respectively; risk is the highest (RR=30) in individuals of the heterozygous *HLA-DR3/DR4* genotype. Susceptible *HLA-DQ $\beta$*  alleles comprise non-charged amino acids (valine, serine, or alanine) at position 57 (*HLA-DQ8 $\beta$* ) instead of the common aspartic acid residue at this site (*HLA-DQ7 $\beta$* ). The autoimmune feature of this single amino acid change may be explained by the loss of a salt bridge between  $\alpha$  and  $\beta$  chains making the MHC class II molecule less stable and affecting its peptide-binding affinity [45, 46]. On the other hand, resistance to disease is associated with *HLA DR2* and *DQB1\*0602*; people with these alleles rarely develop diabetes even if they express *DR3* and/or *DR4* [47].

### *INS-associated variable number of tandem repeats (VNTR)*

As HLA-mediated susceptibility does not explain all genetic susceptibility to T1D, it is evident that non-HLA loci must also be involved. There is a consistent association with T1D of the region near the insulin gene (*INS*) on chromosome 11p15, termed *IDDM2* [34, 48]. A major contributor to risk in this region seems to be a variable number of tandem repeats (VNTR) that is located within the regulatory sequences of the *INS* gene [49]. This polymorphism consists of a 14-15bp G-rich consensus sequence (ACAGGGGTC TGGGG) that clusters to 30-60 and 120-170 repeats in the class I and class III alleles, respectively. Intermediary class II alleles are uncommon in Caucasian populations [50]. A homozygous combination of class I alleles is found in about 85% of T1D patients in comparison to about 60% in the general population, suggesting it is an allele conferring susceptibility to T1D. Conversely, homozygosity for class III alleles is rarely found among diabetics [51, 50]. It is believed that T1D susceptibility and/or resistance associated with *IDDM2* may derive from the impact of VNTR alleles on insulin transcription in the thymus. An association between class III alleles and a higher steady-state level of (pro)insulin mRNA expression in the

thymus has been identified and correlated with increased preproinsulin expression, which could mediate improved induction of central tolerance [52-54].

### *IGF2*

Other loci in the 11p15 region may also contribute to *IDDM2*-encoded T1D susceptibility. Situated downstream from the *INS* VNTR, within the *IDDM2* region, is the *IGF2* (insulin-like growth factor 2) gene, which inhibits apoptosis and stimulates pancreatic  $\beta$ -cell proliferation [55]. *IGF2* may also act as a positive selecting peptide for insulin reactive T cells because of its sequence homology to preproinsulin [56, 57]. Paquette *et al.* [58] found an association between class I alleles of the *INS* promoter VNTR and increased expression of *IGF2* in placenta, and it has been suggested that *IGF2* expression may have some effect on intrauterine growth and birth size, both of which are known T1D risk factors [59]. However, this finding has not been confirmed in other studies [60, 61].

### *CTLA4*

The *CTLA4/CD28* region at 2q31-q33 contains genes encoding crucial T cell regulatory molecules, including *CTLA4* (encoding the cytotoxic T lymphocyte associated protein 4, CD152) and *CD28*. These proteins have the opposite roles of regulator, and enhancer, of T cell effector function, respectively. The human *CTLA4* gene may contain several polymorphisms, making it a candidate susceptibility gene for AIDs. At present, the most informative polymorphism is a single peptide dimorphism at position 49 (A49G) in exon 1 [62]. This substitution leads to the exchange of a threonine to alanine residue [63]. Most case-control studies confirm the link between the G allele of this SNP and several AIDs, including T1D in various populations [36-39, 64]. Interestingly, increased proliferation of T cells was found in G/G homozygotes [65, 66] but further functional studies are needed.

### *IDDM10*

A region on human chromosome 10 (10p11-q11; *IDDM10*) has been linked to T1D by the Type 1 Diabetes Genetics Consortium [31], supporting the prior association reported by Reed *et al.* [67] in an independent UK case control study. No candidate genes have been proposed.

### *SUMO4*

A non-synonymous coding SNP in the *SUMO4* gene within *IDDM5* on chromosome 6q25 encodes a

methionine to valine substitution at codon 55, which is also associated with T1D [68-70]. The intronless gene *SUMO4* belongs to the family of small ubiquitin-like modifiers (*SUMO* family) encoding post-translational modifying proteins [71]. *SUMO4* is believed to be involved in immune responses by modulating nuclear translation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), affecting the transcription of genes encoding pro-inflammatory cytokines [68, 71].

### *LYP/PTPN22*

Recently it was discovered that after *IDDM1* and *IDDM2*, the next gene which contributes the strongest effect of T1D risk is *LYP/PTPN22* mapping to chromosome 1p13.2 [72-74]. *PTPN22* codes for a key enzyme, protein tyrosine phosphatase N22, which associates with the molecular adaptor protein CBL and may be involved in regulating function in the T cell receptor (TCR) signaling pathway [75]. A functional single nucleotide polymorphism (SNP; C1858T) within *PTPN22* results in the exchange of arginine for a tryptophan residue at position 620 (R620W) in the proximal proline-rich SH3 domain of *PTPN22*. Bottini *et al.* [76] and Begovich *et al.* [77] showed that this substitution disrupts the interaction between *PTPN22* and an intracellular C-terminal Src protein tyrosine kinase (CSK), a molecule that down-modulates TCR signaling [78]. The T allele (encoding a tryptophan residue) has positive association with T1D at a relative risk of approximately 3 [79, 80].

### *Other loci*

The most recent data published by the Type 1 Diabetes Genetics Consortium did not find evidence for many of the previously published linkage regions (e.g. *IDDM3-9*, *IDDM11*, *IDDM13-18*, *PTPN22*) but found evidence suggestive of linkage at several other loci [31]. Given that some of the loci not identified have received significant prior support (such as *IDDM5* and *PTPN22*), local variations in allele frequencies or environmental conditions may play a significant role.

## Parent-of-origin effects

In addition to the sheer number of *IDDM* loci identified, the genetics of T1D is further complicated by the action of parental imprinting on their expression. Parent-of-origin effects have been most intensively studied for the HLA (*IDDM1*) and the *INS VNTR* (*IDDM2*), both with conflicting results. Excessive transmission of paternal HLA alleles compared

with maternal alleles have been observed by some groups [81-83], while others have either noted excessive transmission of the maternal alleles or identified no difference with respect to the parental origin of the alleles [84-86]. Similarly, paternal imprinting of *INS VNTR-IGF2* within *IDDM2* has been observed [87, 88], but this has also been disputed [89]. More recently, Bennett *et al.* proposed that the paternal effect was indeed present, but only when the father's untransmitted allele was of class III [90].

Other *IDDM* loci have been considered for effects of imprinting, including *IDDM5*, *IDDM8*, *IDDM10* and *IDDM15*. However, while Delepine and co-workers found no significant differences in the parental origin of alleles at *IDDM5*, *IDDM8* or *IDDM15* [91], Paterson *et al.* showed the paternal origin of *IDDM8* to be significant for disease incidence, while there was a maternal effect at *IDDM10* [92].

## Environment and T1D

The incidence of diabetes differs between different ethnic groups. Although this can partly be explained by differences in genetic predisposition between various populations, changes in diabetes incidence of migrating populations, as well as the rapidity of the increase in incidence of diabetes world wide [93] indicate that environmental factors play an important role.

### *Ante- and perinatal environment*

Advanced maternal age (> 35 years), excessive weight gain in pregnancy and amniocentesis have all been reported as risk factors in the pathogenesis of diabetes [94]. The possibility of the intrauterine environment having an effect on subsequent T1D development requires further investigation.

An association between increased weight (BMI) gain in the first 12 months after birth and T1D was first noticed by Baum *et al.* [95] and has been confirmed by others [96, 97]. This may be caused by excess fat cell accumulation affecting insulin resistance. An increased growth in length in the following two years was also observed, possibly leading to increased demands for insulin secretion. This pattern of increased growth was associated with the presence of autoantibodies to tyrosine phosphatase-like protein (IA-2), at clinical diagnosis many years later [97], suggesting that the increased growth in infancy could result in faster subsequent pancreatic islet destruction once IA-2 autoantibodies are present.

Kagohashi *et al.* transplanted preimplantation-stage mouse embryos from the diabetes-prone NOD strain

into the uterus of diabetes-resistant ICR and DBA/2J mice, to assess the role of maternal factors in the development of insulinitis and overt diabetes [98]. The NOD→ICR and NOD→DBA offspring were observed to develop insulinitis considerably earlier than NOD→NOD offspring but overt diabetes was significantly suppressed in these offspring in comparison to NOD→NOD offspring. Insulin autoantibodies (IAAs) were undetectable in ICR and DBA/2J surrogate mothers and in NOD→ICR and NOD→DBA offspring at the onset of insulinitis, suggesting that maternal factors other than transmitted IAAs induced the earlier onset. The authors concluded that this was an indication that altered maternal factors other than transmitted IAAs, such as hormone levels or viral infections and/or other infectious agents, could be transmitted vertically from the mother to the offspring during the perinatal period, modifying the immune response to islets, which in turn might affect the pathogenic course from insulinitis to overt diabetes.

## Dietary factors

### *Gluten*

The association between T1D and coeliac disease (CD), a condition affecting approximately 1 in 200 people [99] characterized by immune mediated damage to the jejunal mucosa, was first observed nearly 40 years ago [100, 101]. CD is an inflammatory disease triggered by gluten, a protein complex in wheat, rye and barley, and results in villous atrophy and crypt hyperplasia of the small bowel. As the symptoms of CD are often mild, atypical or absent, it often remains undiagnosed. Up until recently it was believed that the prevalence of CD in diabetics was similar to that of the general population [102], but with the advent of more sensitive screening methods, such as quantitation of anti-tissue transglutaminase (TTG) autoantibodies [103], it has become clear that the prevalence of CD in patients with T1D can be as high as 10% [104]. Furthermore, Bao *et al.* found that 33% of T1D patients homozygous for *DR3-DQ2* produce TTG autoantibodies [105]. Unstable diabetes and growth failure in children may be an indication of CD [106, reviewed in 107]. As complications of CD include development of lymphoma [108, 109] and osteoporosis [110], and the risk of these diseases can be reversed by eliminating gluten from the diet, screening and diagnosis of children with a high risk to diabetes is important.

Animal studies in BB rats in which gluten or the wheat protein gliadin were added to the diet, have

shown an increased risk of diabetes [111, 112]. Most studies in NOD mice support a causative role for gluten, with increased incidence of disease associated with increased consumption, and protection associated with exclusion of gluten from the diet [113-116]. Hummel *et al.* fed a gluten exclusion diet to patients who were positive for at least two T1D-associated autoantibodies, but did not find any consistent alteration in the subsequent titers of these antibodies over a 12 month period [117]. They therefore concluded that gluten does not directly drive islet autoantibody production in T1D as it does for TTG autoantibodies in CD. Pastore *et al.* found that although autoantibody titers were not influenced by a gluten free diet, insulin secretion in subjects at high risk for T1D was improved [118], thus indicating that eliminating gluten from the diet may nevertheless have a beneficial effect on the preservation of  $\beta$ -cell mass or function.

### *Cow's milk (CM) proteins*

An inverse correlation between breast-feeding frequency and T1D was observed in the early 1980s. Borch-Johnsen *et al.* reported that diabetic children were often either breast-fed for shorter periods of time, or not at all, in comparison to their non-diabetic siblings or the general population [119]. Several groups, working in both human and animal models, set about trying to confirm this finding with inconsistent results. Fort *et al.* could find no relationship between breast-feeding history and T1D [120] while others found that children with diabetes had been breast-fed over less time, on average, than non-diabetic children [121-123]. Similarly, a two-fold increase in diabetes incidence has been observed when the duration of breast-feeding was less than 3-4 months [reviewed in 124, 125, 107], or when the infant was exposed to CM formula before 2-3 months of age [126, 127]. More recently, the DIPP study (a Finnish birth-cohort study) observed an association between seropositivity for anti-IA-2 autoantibodies, or for all four diabetes-associated autoantibodies (anti-islet, anti-GAD65, anti-IA-2 and anti-insulin), with a breast-feeding time of less than 2 months [128], and an increased risk to T1D was also found to be associated with CM intake in children past the infancy stage [129, 130].

Patients with newly diagnosed T1D have been documented to have enhanced humoral and cellular immune responses to several CM proteins, including whey proteins, bovine serum albumin (BSA) [131, 132] and  $\beta$ -lactoglobulin (BLG) [133, 134]. Autoimmunity to ICA69 also occurs in some patients with other auto-

immune diseases such as rheumatoid arthritis, which shares some genetic background with T1D (i.e. *HLA DR0401* allele) [135]. While levels of antibodies to BSA and BLG decline with age in control subjects, patients with T1D showed no such reduction, indicating a failure of oral tolerance. Cytokines secreted by lymphocytes stimulated by BLG did not differ between T1D-patients and controls [136].

The immunogenic effect of dietary proteins such as CM has also been clearly demonstrated in studies in NOD mice [137] and BB rats [111, reviewed in 138]. Beppu *et al.* in 1987 reported elevated levels of antibodies to BSA (bovine serum albumin) in NOD mice compared to non-diabetic controls, indicating an exaggerated immune response to BSA [139]. It is possible that BSA mediates its effect on T1D through its structural similarity to an islet cell antigen termed ICA69 or p69 [132].

### Vitamin D

The risk of onset of T1D correlates to seasonal variation, with the largest proportion of cases being diagnosed during winter and autumn and the smallest in summer, especially in countries with extreme seasonal variations [140]. The levels of serum 25-hydroxyvitamin D<sub>3</sub> (25OHD<sub>3</sub>) shows reciprocal seasonal and geographic variations [141-145]. Vitamin D is not only essential for bone and mineral metabolism but also affects glucose metabolism and immune function.  $\beta$ -cell insulin synthesis and secretion are impaired in vitamin D-deficient animals [146] and glucose tolerance is restored when vitamin D levels are returned to normal. Human peripheral blood monocytes and activated T cells have high affinity receptors for vitamin D [147, 148] and NOD mice supplemented with the active form of vitamin D or its analogues do not develop insulinitis [146] or diabetes [149]. Human studies have shown that the incidence of T1D is reduced significantly with vitamin D supplementation of either infants at risk [150], or their mothers during pregnancy [151].

### Toxins

The  $\beta$ -cells of pancreatic islets are highly sensitive to metabolic stress, free radicals and cytokine exposure [152]. This issue was brought to the fore when a series of cases of attempted suicide with the rodenticide Vacor (containing N-3-pyridylmethyl-N'-p-nitrophenyl urea; RH-787) resulted in direct  $\beta$ -cell toxicity and onset of insulin-dependent diabetes [153]. Streptozotocin, derived from *Streptomyces spp.*, also induces T1D through

a direct toxic action on  $\beta$ -cells in certain inbred mouse strains [154]. A modification of the administration protocol was developed as a model of autoimmune diabetes, in which repeated, small doses of streptozotocin were administered, with a view to priming immune responses against chemically modified  $\beta$ -cell constituents released following tissue damage [155]. This model fell out of favor when Leiter *et al.* found that the resulting disease did not appear to be T cell-dependent, and it faced significant competition from the spontaneous autoimmune mouse model, the NOD mouse [156]. Ironically, results of recent experiments using anti-CD3 monoclonal antibodies in low-dose streptozotocin-treated mice suggest that islet destruction in this model is, at least in part, autoimmune [157].

Myers *et al.* found that nanogram quantities of the macrolide antibiotic bafilomycin A1 caused glucose intolerance and pancreatic islet disruption in mice [158]. This macrolide and others structurally similar are produced by the *Streptomyces* species ubiquitous in soil, which can infest tuberous vegetables, particularly potatoes and beet [124]. Myers *et al.* have proposed that dietary exposure to bacterial macrolides could therefore damage pancreatic islets, resulting in antigen shedding, priming autoimmune responses in susceptible individuals [158]. Consistent with this possibility is the high T1D incidence in Western countries where consumption of tuberous vegetables and sugar refined from beets is common.

### Microorganisms and T1D

In both humans and animal models, strong associations have been identified between infectious agents and T1D [159-160]. Viruses are regarded as a prime initiating factor because they may show tissue tropism for islets, can cause tissue damage and antigen shedding, and can prime strong immune responses leading to local inflammation.

#### *Congenital rubella infection*

Prenatal rubella infection in the first trimester is the best-established example of viral initiation of T1D [161]. Twenty percent of patients with congenital rubella develop the disease [161, 162] and animal studies in rabbits [161] and hamsters [163] confirm the association. Although the onset of infection clearly precedes the development of T cells in humans, it remains possible that the role of the virus is one of immunomodification, rather than initiation, as the virus maintains a productive infection of the pancreas throughout life [164]. Despite the major impact on the prevalence

of congenital rubella by vaccination programs in Western countries, T1D incidence continues to increase [165], suggesting that this is not a common cause of T1D in these communities.

### *Enteroviruses*

Enteroviruses are small (27 nm in diameter) viruses that are made of ribonucleic acid (RNA) and protein and include the polioviruses, coxsackieviruses, and echoviruses. There are 3 different polioviruses and more than 60 non-polio enteroviruses that can cause disease in humans, including 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 4 other enteroviruses. An association between enteroviruses and T1D was observed in both human [166, 167] and animal studies [168]. Prospective studies of enteroviruses in Finland, where diabetes incidence is high but enterovirus infections are uncommon, revealed that the viruses were more often detected in those children who later developed diabetes than in their siblings who did not [169-173] and Luppi *et al.* reported that host susceptibility to enteroviral infection may be influenced by *HLA* background [173] - potentially compounding any attempt at association studies. Despite this, a causal association is supported by the seroconversion to anti-islet antibodies after enterovirus infection found in children with the high risk *HLA-DQB1* genotype [170].

### *Coxsackie virus B4*

Gamble and colleagues [166, 167] described a seasonal variation in T1D incidence following enterovirus infection, as well as increased antibodies against Coxsackie virus B4 serotypes, in newly diagnosed patients when compared to control subjects. These findings were confirmed by subsequent studies [174-178]. Sequence similarity between the enterovirus 2C protein of Coxsackie virus B4 and the 65 kDa form of glutamate decarboxylase, (GAD65), a GABA-producing neuroendocrine enzyme present in  $\beta$ -cells [179], raises the possibility that an anti-viral T cell response may cross react with the native protein, inducing an autoimmune response [180, 181]. To date, attempts to demonstrate direct cross-reactivity at the T cell clonal level have failed despite the relevant peptides being highly immunogenic [182, 183].

Although great interest was generated by the report of Yoon *et al.* that Coxsackie virus B4, isolated from the pancreas of a child who died from diabetic ketoacidosis, transferred diabetes to diabetes-resistant SJL mice, this feat has never been replicated [168]. Horwitz

*et al.* revisited the issue by infecting recombinant mouse strains with Coxsackie virus B4 virus [184]. Although mice bearing the diabetes-associated MHC haplotype of NOD mice on a C57 strain background did not develop diabetes, and infected wild type NOD mice did not show evidence of exacerbated disease, infected NOD mice bearing a transgenic T cell receptor (BDC2.5), which was specific for an islet antigen, did. Serreze *et al.* subsequently found that infection with Coxsackie virus B4 can accelerate diabetes in NOD mice providing a certain level of autoreactive T cells have accumulated prior to infection [185].

### *Other viruses*

Infection with other viruses, such as mumps, human cytomegalovirus (CMV) and rotaviruses have also been suggested to be diabetogenic in susceptible individuals [169, 186, 171, 187-189]. Although the mumps epidemics have been associated with an increase in T1D incidence [190], the introduction of a vaccination for mumps has not proven an effective primary preventative. The association between CMV and T1D was implied by a case report of an infant with CMV who presented with T1D at 13 months of age [191]. Others tried to verify this association in T1D patients, but could not substantiate it [192].

Rotaviruses are double-stranded RNA viruses of the reovirus family and are a predominant cause of gastroenteritis in infants. They were suspected to have an association with T1D due to a strong sequence similarity between rotavirus VP7 and both GAD65 and IA-2 [193, 194]. In the longitudinal Australian BabyDiab study of at-risk children, Honeyman *et al.* revealed that islet antibodies appeared in 24 of 300 children within the same 6 month period in which they had significant rises in rotavirus-specific IgA and IgG [194]. This association of rotavirus infections with development of diabetes was not confirmed in a Finnish study [195].

## **Gene/environment interactions**

To date, most postulated mechanisms for environmental effects on the risk of T1D involve the initiation of disease; either due to cross reactivity of immune responses to food or microorganisms to  $\beta$ -cell constituents, or through tissue tropism and cytotoxicity of infectious agents, resulting in antigen shedding and priming of an autoimmune response. The data from the spontaneous animal models of T1D are partially consistent with such a mechanism. If protein antigens are excluded and the diet supplemented by protein hydro-

lysate as a source of amino acids, diabetes is prevented [137, 196].

As a generalization, these mechanisms do not appear to play a major role in the modulation of disease risk by infectious agents in these models, as the greater the microorganism burden of the mice, the lower the incidence of diabetes [197]. The exact nature of such interactions may be difficult to dissect as they could be due to multifaceted interactions [198]; several infections might have to act in concert to precipitate clinical autoimmunity and in some infections viruses may play a role in prevention rather than precipitation of disease [199]. Progress is unlikely to be achieved until the interactions between environmental component and genome are understood at the molecular level. For example, we have identified a molecular constituent of *Mycobacterium bovis* cell wall (mycolylarabino-galactan-peptidoglycan; MAPG) capable of preventing diabetes in NOD mice, providing it is administered after the onset of insulinitis, but before end-stage tissue destruction (PCT/AU97/00770). This appears to be the molecular basis of the diabetes protection attributed to complete Freund's adjuvant (CFA) [200] and whole *Mycobacterium bovis* [201] in this model. In contrast, exposure of intact skin to bacterial robosylating exotoxin can exacerbate disease in NOD mice [202]. Again, the effect appears to be a modulation of on-going inflammation, rather than due to conventional antigen priming.

#### *Toll-like receptors*

One potential mechanism of such interactions is via toll-like receptor (TLR) signaling [203, 198]. Toll-like receptors are a family of homologous proteins involved in the afferent limb of vertebrate innate immune responses to components of bacteria, viruses and parasites. Signaling via TLR has major effects on the activities of antigen presenting cells (APCs), including the production of inflammatory cytokines and the upregulation of MHC products and costimulator molecules. Although TLR4, the first identified member of the family, appears to act at the cell surface as the signal transduction component of a multimeric lipopolysaccharide (LPS) receptor [204], it is likely that some members operate within the cell. For example, TLR3 is necessary for responses to viral dsRNA; TLR9 is necessary for the immunopotentiating effects of unmethylated CpG DNA [205], which is presumably only exposed after bacterial lysis in phagolysosomes; and TLR2 is recruited to macrophage phagosomes following stimulation with a yeast cell wall preparation [206].

TLR2 is involved in responses to a broad range of constituents of pathogen cell walls, especially hydrophobic or lipid containing components. In many cases, it appears to act as a heterodimer with TLR1 or TLR6 or, based on strong sequence homologies, TLR-10. In contrast, TLR3, 7, 8 and 9 share relatively little sequence homology with the TLR2-associated members, respond to nucleic acids [207, 208] and all but TLR8 are known to mediate IFN- $\alpha$  and IFN- $\beta$  production. TLR4 lies somewhere between these two groups: it has intermediate sequence homologies, responds to the LPS of gram negative bacteria, but mediates IFN- $\beta$  secretion [209].

Like IL-1, ligated TLR interact with MyD88 through association of shared TIR domains. The death domain of MyD88 interacts with that of IRAK, a serine/threonine protein kinase, resulting in the activation and phosphorylation of IRAK, allowing it, in turn, to interact with TRAF6, an E3 ligase. TRAF6 then undergoes stimulus-dependent autoubiquitination, activating the kinase TAK1, which phosphorylates and activates the complex of IKK  $\alpha$  and  $\beta$  kinases, leading to I $\kappa$ B degradation [reviewed in 210]. The I $\kappa$ B protein family holds NF $\kappa$ B/Rel transcription factor dimers latently in the cytoplasm and their degradation results in NF $\kappa$ B/Rel translocation to the nucleus [reviewed in 211], resulting in the secretion of IL-1 $\beta$  and upregulation of the expression of CD80 and CD86. Although the pathway described is common to all TLR, the target specific differences in outcome of stimulation indicates additional regulatory mechanisms and signaling pathways. For example, TLR4 signaling also involves a novel TIR domain containing adapter protein, called TIRAP [212] and Nod2 modulates TLR-dependent responses to LPS and muramyl dipeptide [213].

The direct evidence for a role of TLR in mediating environmental effects on progression to T1D is currently scant. In mice bearing a transgene that induced expression of the costimulator molecule B7.1 (CD80) on their pancreatic  $\beta$ -cells, multiple injections of the TLR3 ligand polyinosinic-polycytidylic (poly(I:C)) precipitated diabetes [214]. Similarly, in a rat model of diabetes, in which disease is induced in BB-diabetes resistant rats by infection with Kilham rat virus, prior treatment with poly(I:C) increased the incidence of diabetes from 23% to 100% [215]. A suggestion of an association between T1D and TLR3 polymorphisms in South African blacks has also been reported [216], but as the gene encoding TLR3 does not map to a major linkage region for T1D in other populations, confirmation is needed.

The protective effect of both CFA and *Mycobacterium bovis* in NOD mice is mediated by IFN- $\gamma$  [217], which is likely to be a downstream consequence of IL-12 production by APC [218]. This, in turn, is known to be produced following TLR ligation by components of mycobacterial cell wall [219]. Consistent with this hypothesis is the claim that CFA mediated protection is dependent on an adjuvant action [200] and the dependence on MyD88 of the adjuvant action of CFA [220]. It is therefore interesting that the gene encoding TLR2, which plays a critical role in innate immune responses to mycobacterial wall (including to MAPG specifically) [221], maps to the same chromosome 3 region as the NOD mouse diabetes susceptibility gene *Idd17* [222]. Similarly, the gene encoding mouse TLR12, identified only through sequence homology at this stage, maps to the same genomic region of chromosome 4 as the NOD mouse diabetes susceptibility

gene *Idd25* [223].

Of greater clinical significance, is the colocalization of human genes. The gene encoding TLR4 maps to chromosome 9q33 [224], the same region Concannon *et al.* [31] mapped an unnamed T1D locus of suggestive genome wide significance. There is no clear published evidence of altered TLR4 function in diabetic patients. The gene encoding TLR5 in humans is located on chromosome 1q42, a T1D linkage region identified by Cox *et al.* [30] and subsequently confirmed by others [225, 226]. The systemic autoimmune disease systemic lupus erythematosus also maps to this locus in both humans [227, 228] and mice [229-232], including lupus induced in NOD mice by mycobacteria [233]. In this case, a polymorphism introducing a premature stop codon into the sequence of TLR5 in man has been associated with disease [234]. Clearly, in the case of diabetes, there is work to be done!

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