Persistent coxsackievirus B infection and pathogenesis of type 1 diabetes mellitus

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Abstract | Enteroviruses are believed to trigger or accelerate islet autoimmunity in genetically susceptible individuals, thereby resulting in loss of functional insulin-producing β-cells and type 1 diabetes mellitus (T1DM). Although enteroviruses are primarily involved in acute and lytic infections in vitro and in vivo, they can also establish a persistent infection. Prospective epidemiological studies have strongly associated the persistence of enteroviruses, especially coxsackievirus B (CVB), with the appearance of islet autoantibodies and an increased risk of T1DM. CVB can persist in pancreatic ductal and β-cells, which leads to structural or functional alterations of these cells, and to a chronic inflammatory response that promotes recruitment and activation of pre-existing autoreactive T cells and β-cell autoimmune destruction. CVB persistence in other sites, such as the intestine, blood cells and thymus, has been described; these sites could serve as a reservoir for infection or reinfection of the pancreas, and this persistence could have a role in the disturbance of tolerance to β-cells. This Review addresses the involvement of persistent enterovirus infection in triggering islet autoimmunity and T1DM, as well as current strategies to control enterovirus infections for preventing or reducing the risk of T1DM onset.

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disease that results from an autoimmune attack and loss of functional insulin-producing β-cells of the pancreas in genetically susceptible individuals. Predisposition to T1DM is influenced by HLA class II genes, in particular haplotypes *HLA-DRB1*03–DQA1*05–DQB1*02* (DR3–DQ2) and *HLA-DRB1*04–DQA1*03–DQB1*03:02* (DR4–DQ8) and HLA class I genes (*HLA-A*24*, *HLA-B*18* and *HLA-B*39* alleles) located on chromosome 6, as well as other genes identified outside the HLA region (such as *INS*, *PTPN22*, *IFIH1* and *CTLA4*)[1–3]. Genetic risk score (GRS) models derived from the combination of HLA and non-HLA T1DM-associated loci were designed to predict the progression of islet autoimmunity and clinical T1DM[4]. The performance of T1DM GRS models still needs to be improved based on racial and ethnic genetic differences[5]. However, genetic susceptibility is insufficient to explain the increasing annual incidence rate of T1DM, which in many locations has been 3–4% in children and adolescents over the past few decades[6]. For example, the most risky HLA DR3–DR4 genotype only accounts for a maximum of approximately 40% of T1DM cases[1,7]. Furthermore, the concordance rate in monozygotic twins is only 30–50%, and the risk of siblings of patients with T1DM developing the disease is 6% compared with 0.3% in the general population[1,7].

Epidemiological studies have shown seasonal and geographical variations in the incidence of T1DM[8], thus supporting the hypothesis that exogenous or environmental factors are involved in the development of the disease. Several exogenous factors have been implicated, such as drugs (for example, glucocorticoids, antihypertensive drugs, thiazide diuretics, β-blocking agents, antipsychotics, statins and immune checkpoint inhibitors)[9,10], nitrites, cow's milk proteins, gluten, vitamin D deficiency, gut microbiota and viral infections[11,12]. Some human or animal viruses can cause alterations in insulin-producing islet β-cells of the pancreas and induce the development of autoimmune T1DM[13–15]. In humans, several epidemiological and clinical studies, as well as experimental data, strongly support the involvement of enteroviruses, and in particular coxsackievirus B (CVB), in the pathogenesis of T1DM[2,16–18]. CVB are small, non-enveloped, positive-sense single-stranded RNA genome viruses that belong to the Enterovirus genus of the *Picornaviridae* family (FIG. 1). The genus Enterovirus includes seven species that infect humans: enteroviruses A–D and rhinoviruses A–C (BOX 1). CVB1–6 are classified among the enterovirus B species and are among the enteroviruses most likely to be involved in the pathogenesis of T1DM[19–21] (BOX 2). Despite the evolving knowledge on the subject, the pathological

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Key points

- Markers of enterovirus infection (protein, RNA or antibodies) in the saliva, serum, stool, monocytes, gut mucosa and pancreas are more often detected in patients with type 1 diabetes mellitus (T1DM) than in control individuals.
- Persistent or recurrent enteroviral infections occur over long periods before the first detection of the islet autoantibodies in at-risk individuals and have been strongly associated with islet autoimmunity and an increased risk of developing T1DM.
- Coxsackievirus B (CVB) can persist in vitro and in vivo in animal and human systems, especially in pancreatic cells, which leads to structural or functional alterations of these cells.
- Persistent CVB infections might promote or enhance islet autoimmunity through various mechanisms.
- Some antiviral strategies (vaccines and drugs) are currently under investigation to prevent or clear persistent CVB infection.
- These strategies against enteroviral infections could be relevant for preventing or reducing the risk of developing T1DM and/or preserving β-cell function in persistently infected at-risk individuals.

mechanisms that trigger the initiation and progression of CVB-induced autoimmunity against islet antigens in T1DM are not yet fully elucidated. Experimental data suggest various mechanisms to explain the initiation of autoimmunity by CVB; for example, molecular mimicry between the conserved enteroviral protein 2C and glutamic acid decarboxylase, or bystander activation of pre-existing autoreactive T cells through the initiation of inflammation[11,22].

CVBs are cytolytic viruses, but they are also able to establish a persistent infection in vitro in human primary pancreatic islets, ductal cells, thymic epithelial cells (TECs) and monocyte-derived macrophages or in human and mouse pancreatic cell lines and TEC lines[23–28] and in vivo in mice for several months[29]. Of note, persistent infection in vitro and in vivo in mice results in induction of structural or functional alterations in pancreatic and immune cells and development of autoimmunity towards islets[23–29]. Autopsy samples or biopsy samples of the pancreas of patients with newly diagnosed T1DM do not reveal acute lytic and extensive enteroviral infections, but rather suggest the presence of persistent enterovirus infections with a low proportion of infected cells and low viral replication[30–34]. Furthermore, prospective studies in young children genetically predisposed to T1DM support a strong association between the persistence of enteroviruses, particularly CVB, and the development of islet autoimmunity[35–38]. Enterovirus persistence has been suggested as a major mechanism in the enteroviral pathogenesis of T1DM and might be a target for therapeutic interventions.

In this Review, we focus on the role of persistent enterovirus infections, in particular CVB, in the initiation of islet autoimmunity and pathogenesis of T1DM. In addition, we discuss current strategies to control these viruses to prevent or treat T1DM.

Cytolytic viruses
Viruses whose replication induces cytolysis resulting in the death of infected host cells.

Enterovirus persistence in T1DM

Epidemiological and clinical evidence in favour of enterovirus-associated pathogenesis of T1DM have been previously discussed and are based on findings in many parts of the world that enteroviral components (VP1 capsid protein and/or RNA) in serum, monocytes, gut mucosa and pancreas, and circulating anti-enterovirus immunoglobulins (IgM, IgG and IgA), are more frequently detected in patients with T1DM than in healthy individuals[11,19,39–41]. The significance of the association between the presence of enteroviral infection markers in various human biological samples and the risk of developing islet autoimmunity or T1DM was confirmed in Europe, Africa, Asia, Australia, North America and South America in two case–control meta-analyses of 24 and 38 studies including 4,448 and 5,921 participants, respectively[16,42]. The selective impairment of β-cells in T1DM is a consequence of a progressive and slow autoimmune process that can occur for several years before the onset of overt disease. This observation implies that direct and extensive lysis of β-cells by viruses is not a plausible hypothesis for T1DM pathogenesis, except in the case of fulminant T1DM, in which the role of enteroviruses has been suggested[43–46].

Enterovirus infection of the pancreas in patients with T1DM. Morphological examinations of islets from patients with T1DM in which enteroviral capsid protein VP1 or RNA have been detected have not revealed evidence of extensive cell lysis[33,34]. However, lysis of a small number of islet cells could potentially occur early during infection or throughout the disease course. Studies of small intestine and pancreas biopsies or post-mortem pancreas samples from patients with newly or previously diagnosed T1DM revealed the presence of enteroviral RNA and VP1 capsid protein in a small proportion of intestinal and pancreatic cells (pancreatic ductal cells and islet β-cells)[30,32,47–52]. In addition, low levels of enteroviral RNA were detected (by a nested real-time PCR method or by real-time quantitative PCR that required as many as 40 cycles of amplification) in snap-frozen pancreas samples and in the medium harvested from the enriched islet preparations from patients with T1DM[32]. This finding argues in favour of low-grade enterovirus infection in the pancreas of patients with T1DM.

In a study from Norway (the Diabetes Virus Detection (DiViD) study), enterovirus B RNA was predominantly detected in purified pancreatic islets and duodenal mucosa from biopsy samples, peripheral blood mononuclear cells and stool samples from six adult patients with T1DM[37]. However, no islet-resident enteroviruses could be isolated using permissive cell lines[37], which suggests defective or slowed viral replication of these viruses[53]. This finding is consistent with the idea of persistent infection in the pancreas of patients with T1DM[32].

Enterovirus infection of the gut of patients with T1DM. In studies involving sequencing of the virome in stool samples from children with a genetic risk of T1DM, enteroviruses A and B were detected in the gut (several months before the detection of anti-islet autoantibodies) more frequently than in samples from control children[35,36,54]. This finding suggests that long-term or repeated infections with enteroviruses might influence the long-term risk of developing islet autoimmunity[35,36,54]. Indeed, in a large birth cohort prospective study (the Environmental Determinants of Diabetes in the Young (TEDDY)), enterovirus circulation was evaluated in

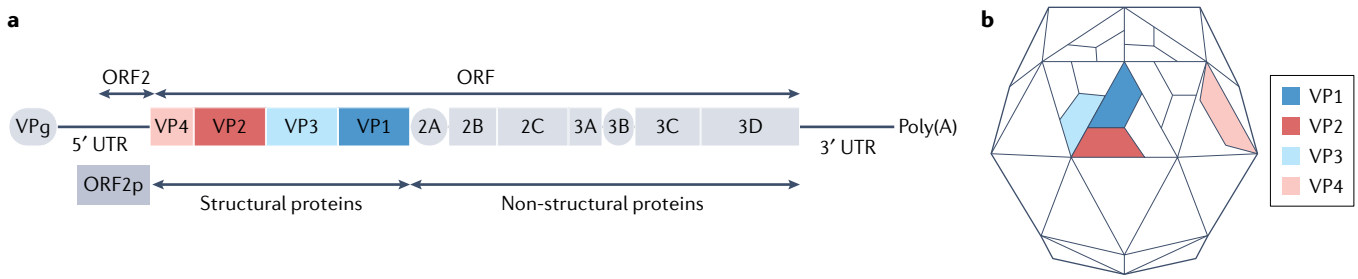

Fig. 1 | **The genome and capsid of enteroviruses.** The enteroviruses (viruses of the genus Enterovirus) are small (25–30 nm diameter) non-enveloped viruses with an icosahedral capsid that belong to the *Picornaviridae* family. **a** | The genome of enteroviruses (~7,400 bases) is a positive-sense single-stranded RNA genome, which contains a large open reading frame (ORF) flanked by a 5′-untranslated region (UTR) linked to the VPg (a viral non-structural protein also known as 3B) and 3′-UTR terminated with a poly(A) tail. A shorter ORF2 is located upstream from the main ORF[229,230]. The ORF encodes a polyprotein that is processed into four capsid proteins (VP1–VP4) (structural proteins) and seven other proteins, 2A, 2B, 2C, 3A, 3B, 3C and 3D (non-structural proteins), which are involved in viral replication. The ORF2 is translated into a single protein, ORF2p, which is involved in the infection of intestinal cells[229,230]. **b** | The icosahedral capsid consists of an arrangement of 60 protomers each consisting of four structural proteins (VP1, VP2, VP3 and VP4). VP4 is located on the internal side of the capsid according to studies based on X-ray diffraction carried out with virus particles frozen at −196°C.

young children recruited at six study centres (Georgia (USA), Washington (USA), Colorado (USA), Finland, Sweden and Germany). Coxsackievirus A (CVA) was the most frequently detected enterovirus in stool samples from children with a genetic risk of T1DM (61.7%), followed by echoviruses (19%) and CVBs (16.1%)[54]. Moreover, CVA4, echovirus 18 and echovirus 25 were shed for longer than other enterovirus types in these children[54]. Furthermore, in the Australian Viruses in the Genetically at Risk prospective birth cohort study, enterovirus A (CVA2) and enterovirus B (echovirus 30 and CVB3) were the most frequent and abundant enteroviruses in stool samples from children with islet autoimmunity[36]. In this study, a notable association was found between islet autoimmunity and increased gut enterovirus A abundance[36]. Finally, in a prospective study from Finland (the Type 1 Diabetes Prediction and Prevention (DIPP) study), virome analysis of stool samples from children indicated that an excess of enterovirus infections occurs more than 1 year before the first detection of islet autoantibodies. The most frequent enteroviruses detected were CVA4 (28% of genotyped viruses), CVA2 (14%), CVA16 (11%), CVB (11%) and echoviruses (10%)[35].

The primary replication of enteroviruses takes place in the enteric mucosa before the virus spreads to other target organs, such as the pancreas, via the lymphatic and blood systems[55]. The presence of enteroviruses in the gut can result in specific IgA antibodies in both the intestinal mucosa and saliva, as all inducer and effector sites of the mucosa-associated lymphoid tissue are functionally interconnected[56]. For example, in studies from several countries (France, Republic of the Congo, Lebanon and Benin), high titres of neutralizing IgA1 anti-CVB4 antibodies were detected in the saliva more often in patients with T1DM than in control individuals[57,58]. Interestingly, this salivary anti-CVB4 activity was maintained throughout a 4-year follow-up period in patients with T1DM but not in control individuals[57]. This finding is in agreement with the results of previous studies that demonstrate persistence of enteroviruses associated with increased inflammatory activity in the intestinal mucosa of patients with T1DM over a 12-month follow-up period[49,50]. Of note, the high enteroviral load observed in stool samples of patients with T1DM[36,37] suggests active enteroviral replication takes place within the gut.

Together, these observations suggest that the intestinal mucosa is an important viral reservoir from which enteroviruses can participate in the development of T1DM, by activating islet autoimmunity from this site and/or by spreading to the pancreas to initiate autoimmune processes[50]. This hypothesis is also supported by the results of TEDDY, in which 8,676 children with a genetically high risk of T1DM (HLA-DR–DQ genotype) were followed from the age of 3 months. Indeed, in these children, the presence of persistent enterovirus B, and in particular CVB, in stool samples was associated with a significantly higher risk of initiation and acceleration of islet autoimmunity (OR 3.7) but not T1DM than in control children positive for enteroviruses but with no evidence of persistent shedding of these viruses in stool[38].

Further evidence of persistent enterovirus infection in T1DM. In three studies from Finland (the Childhood Diabetes in Finland Study[59], the Trial to Reduce IDDM in Genetically at Risk[60] and the DIPP study[61–64]), elevated levels of anti-enterovirus antibodies and/or enteroviral RNA were often detected in the serum of patients who went on to develop T1DM, on average 6 months before the onset of islet autoantibodies and the clinical phase of the disease. In the DIPP study, the risk of developing islet autoimmunity and T1DM in genetically predisposed children (haplotype HLA-DQB1) was also statistically significantly associated with CVB1 infections occurring within 1 year before the first detection of anti-insulin antibodies[19,64]. In patients with T1DM, enteroviral RNA has been detected more frequently in peripheral blood (leukocytes and mononuclear cells) than in serum or plasma, which suggests long-term infection[65–68]. The hypothesis of persistence of enterovirus infection in patients with newly diagnosed T1DM is also supported by the fact that enteroviral RNA was

Box 1 | **Human enteroviruses**

The genus Enterovirus consists of 15 species, seven of which infect humans (enteroviruses A–D and rhinoviruses A–C) and over 250 serotypes. Human enteroviruses A–D include more than 110 serotypes, the best known of which are the polioviruses (PV1–3), enterovirus A71, echoviruses (28 serotypes), coxsackievirus A (21 serotypes), and coxsackievirus B (CVB1–6).

These viruses are ubiquitous worldwide and are transmitted mainly through the faecal–oral and respiratory routes following a seasonal pattern, but vertical transmission might also occasionally occur. They replicate in the gut mucosa or upper respiratory tract and can spread through the lymphatic system into the bloodstream and reach various target organs.

Most enterovirus infections are asymptomatic or induce subclinical or mild symptoms, such as common cold, fever, mild respiratory symptoms or cutaneous manifestations not requiring hospitalization. However, enterovirus infections can provoke severe acute diseases such as: hand, foot and mouth disease; pericarditis; meningitis; pancreatitis; encephalitis; myocarditis; neonatal sepsis; and acute flaccid paralysis. Furthermore, enteroviruses are responsible for chronic pathologies such as chronic meningoencephalitis in agammaglobulinaemia and dilated cardiomyopathy, and have been associated with initiation and/or aggravation of type 1 diabetes mellitus.

detected in peripheral blood mononuclear cells but not in throat and stool samples of these patients, thus arguing against an acute enterovirus infection at the time of T1DM diagnosis[67]. Furthermore, $CD14^+$ monocytes are the main cells that harbour enterovirus RNA in the peripheral blood of patients with newly or previously diagnosed T1DM, suggesting that enteroviruses might persist in these immune cells beyond the acute infection stage and throughout the course of T1DM[69]. Studies supporting the persistence of enteroviruses in patients with islet autoimmunity and/or with T1DM are summarized in BOX 3.

Negative data. Some studies investigating the association between persistence of enteroviruses and islet autoimmunity or T1DM found no significant differences between patients and control individuals (TABLE 1). The discrepancy between these studies[70–74] and those in which an association was observed[19,20,38,64] can be explained by the small numbers of patients and long sampling interval in the negative studies that did not allow the identification of all systemic enterovirus infections. In addition, a low virus titre in samples does not allow complete characterization of all viruses by next-generation sequencing or by quantitative PCR protocols[70].

Box 2 | **Rationale for the involvement of coxsackievirus B in the pathogenesis of T1DM**

Coxsackievirus B (CVB) is among the enterovirus species most likely to spread from the gut mucosa to the pancreas and to be involved in the pathogenesis of type 1 diabetes mellitus (T1DM). First, the coxsackievirus and adenovirus receptor, the major receptor for CVBs (not used by other enterovirus types) is strongly expressed in human pancreatic islets[47,86,132] and mainly in insulin secretory granules[21]. Epidemiological studies have suggested that CVB, and particularly CVB1, is associated with an increased risk of β-cell autoimmunity and clinical T1DM in several European countries[19,20,64]. Metagenomic sequencing performed on stool samples suggested that persistence of enterovirus B and in particular CVB is statistically significantly associated with the risk of initiation and acceleration of islet autoimmunity[38]. However, the role of other enteroviruses cannot be excluded. The enteroviral genome found in the pancreas of patients with T1DM has been sequenced. Sequences of enteroviruses other than CVB were identified in some patients, but due to the low level of viral RNA, the exact genotype of the virus was not identified[37].

Experimental evidence for CVB persistence

Persistent CVB infections have been reported experimentally in several human and mouse cell types in vitro[75–77] and in various organs such as the heart, skeletal muscle, central nervous system and pancreas in vivo. Furthermore, persistent CVB infections have been linked to chronic pathologies in humans and in experimental models such as chronic myocarditis[78,79], dilated cardiomyopathy[79], T1DM[51] and chronic fatigue syndrome[80,81]. Here, we focus on cellular and viral changes related to CVB persistence in vitro and in vivo that might contribute to the pathogenesis of T1DM (FIG. 2).

Persistence of CVB in vitro. The tropism of CVB for pancreatic islets and preferentially for β-cells[82–84] has been reported in vitro and in vivo and is explained in part by the expression of the CVB receptor coxsackievirus and adenovirus receptor (CAR) and/or CVB co-receptors such as complement decay accelerating factor[85–87]. Acute infection of human and mouse primary pancreatic islets and pancreatic β-cell lines by CVB can lead to alteration of the Golgi apparatus, decreased insulin secretion, increased expression of interferon-stimulated genes and cell death[88–90]. Various strains of CVB (CVB3 Nancy, CVB4 E2 (isolated from a patient with T1DM and able to induce T1DM in mice[91]), CVB4 JVB and CVB4 VD2921) were able to establish persistent infection without obvious cytolysis in human pancreatic islets obtained from brain-dead organ donors and induced sustained IFNα production only by β-cells in these islets in culture[23]. The CVB4 VD2921 strain also disrupted glucose-induced insulin secretion in human pancreatic islets[92].

Two types of persistent infections have been described for enteroviruses, especially for CVB infection in vitro: steady-state persistence and carrier-state persistence. The steady-state persistent infection is characterized by a large proportion of infected cells without a lytic viral replication cycle[76]. By contrast, in the carrier-state persistent infection, only a small proportion of the cells are considered to be infected but high titres of virus particles are produced[76,77]. Carrier-state CVB persistent infection has been reported in human pancreatic islets[23] and in pancreatic cell lines[26–28,93,94]. Enteroviruses and especially CVBs were thought to be released from infected cells by cell lysis; however, evidence indicates that these viruses can use autophagosome-like vesicles, cellular protrusions or extracellular vesicles for non-lytic viral egress[95,96]. These non-lytic viral release mechanisms can contribute to dissemination of CVB in persistently infected β-cells by cell-to-cell transmission via membrane fusion[97] and by avoiding neutralizing antibodies.

The presence of CVB outside the pancreatic islets in the exocrine pancreas of patients with T1DM, specifically in the ductal epithelium cells, has occasionally been reported[30,47,52,98]. CVB4 E2 strain can also persistently infect the human pancreatic ductal cell line PANC-1 and alters the synthesis of the transcription factor PDX1 (required for endocrine pancreas formation), thus disrupting differentiation into islet-like cell aggregates (ICAs)[26,93]. In addition, insulin and C-peptide production are inhibited by persistent CVB4 E2 infection of human primary pancreatic ductal cells differentiated

Central tolerance
A selection process during lymphocyte development that results in the deletion of self-reactive B and T cells in the central lymphoid organs.

into ICAs[99]. These observations are important insofar as CVB persistent infection of pancreatic ductal cells might impair the trans-differentiation process of these cells[100] and thus compromise their role in the replacement of impaired β-cells. Other cellular changes were observed in PANC-1 cells persistently infected with CVB4 E2, which included decreased CAR expression with consequent resistance to lysis during reinfection[93]. Persistent CVB infection of primary human pancreatic ductal cells and PANC-1 cells was productive, with continued release of infectious virus particles despite the low proportion of infected cells[26,93,94,99]. These findings suggest that pancreatic ductal cells that are persistently infected with CVB are reservoirs from which the virus can spread to β-cells.

Enterovirus infections have been reported to deregulate microRNAs (regulators of cellular gene expression) in β-cells, thereby inducing an altered cell cycle, cytokine secretion and apoptosis[101,102]. Of note, PANC-1 cells persistently infected with CVB4 E2 have a very different microRNA profile from uninfected cells[93,103]. A role of microRNAs in viral persistence and enteroviral pathogenesis of T1DM cannot be excluded[102,104]. Inhibition of PCSK2 (an enzyme involved in the maturation of proinsulin to insulin) and induction of DNA hypermethylation have been reported in an insulin-producing rat β-cell line (INS-1), during persistent infection with CVB4 E2 (REF.[28]).

Non-structural proteins of picornaviruses (such as 3A, 2B and 2BC) can disrupt the structure and function of the Golgi apparatus, which leads to altered expression of HLA class I molecules on the surface of infected cells[105–109]. Along these lines, persistent CVB4 infection of the human pancreatic β-cell line 1.1B4 has been shown to cause inhibition of HLA class I molecule cell surface expression[27]. These persistently CVB4-infected cells are targeted for cytotoxic cell death by primary human natural killer cells when they are added into culture[27]. Of note, however, is the fact that some stocks of the 1.1B4 cell line might contain a mixture of rodent and human cells and therefore might not retain characteristics expected of primary β-cells[110]. Thus, the persistence of enteroviruses in β-cells can lead to structural or functional cellular alterations and might have a role in the development of T1DM.

Alterations in the T cell repertoire in patients with T1DM have been described[111,112], which suggests that an alteration in central tolerance to β-cell antigens following CVB infection of the thymus cannot be excluded. In the thymus, TECs have a crucial role in the establishment of central tolerance, through education of T cells to tolerate autoantigens and elimination of self-reactive T cells. Interestingly, CVB4 E2 can replicate and persist in primary cultures of human TECs, which leads to production of IL-6, leukaemia inhibitory factor and granulocyte–macrophage colony-stimulating factor[24]. Furthermore, persistent infection of a mouse TEC line (MTE4-14) was characterized by decreased *Igf2* transcription and reduced production of insulin-like growth factor 2 (IGF2), a protein involved in central tolerance to islet β-cells[25]. This effect might lead to disruption of negative selection of autoreactive thymocytes and decreased generation of regulatory T cells[113].

Persistence of CVB in vivo. Most of the knowledge about the persistence of CVB in vivo comes from studies on dilated cardiomyopathy. In vivo persistence of CVB3 in the heart of experimentally inoculated mice and naturally infected humans was associated with a deletion at the 5′ end of the viral RNA genome[78,114–116]. Of note, this deletion has also been reported in a mouse model during CVB3 persistence in the pancreas[117]. In orally inoculated Swiss albino mice, CVB4 E2 viral RNA persists more than 70 days after infection in the heart, blood

Box 3 | Markers of enterovirus infection in samples from patients with islet autoimmunity or T1DM

Studies support the notion of persistence of enteroviruses in various clinical samples from patients with islet autoimmunity or with type 1 diabetes mellitus (T1DM). Markers of enterovirus infection (protein, RNA or antibodies) are detected in the saliva, serum, monocytes, gut mucosa, pancreas and stool of patients with T1DM or over a long period of time before the appearance of T1DM-associated autoantibodies.

Saliva

- Higher and persistent anti-CVB4 neutralizing activity in the salvia of patients with T1DM than in control individuals during a 4-year follow-up period[57,58].

Pancreas

Biopsies

- Enteroviral VP1 and RNA detected with a low-grade infection and overexpression of class I HLA molecules in pancreatic islets in patients with newly diagnosed T1DM[32].
- Enterovirus B (CVB3, CVB4, CVB5, and echoviruses 5, 7, 9, 11, 13 and 25) was detected in biopsy samples obtained from live adult patients with newly diagnosed T1DM. Islet-resident enteroviruses might have been present in a double-stranded form[37].

Necropsies

- Enteroviral RNA was detected in both the endocrine and exocrine pancreas of organ donors with preclinical and diagnosed T1DM[3,52].

Blood

Serum

- Detection of enteroviral RNA and circulating anti-enterovirus antibodies in serum samples obtained from children genetically predisposed to T1DM frequently precedes the appearance of T1DM-associated autoantibodies and are associated with T1DM risk[19,20,59–64].

Monocytes

- Enteroviral RNA detected in $CD14^+$ monocyte-enriched peripheral blood mononuclear cells from patients with newly diagnosed and long-term T1DM[69].

Gut biopsies

- Positive-strand enteroviral RNA detected only in biopsy samples obtained from live adult patients with newly diagnosed T1DM and echovirus 30 identified[37].
- Enteroviral VP1 and RNA detected in patients with long-standing T1DM[49].
- Frequent detection of enteroviral RNA associated with increased inflammatory activity in the small intestine of patients with T1DM over a 12-month follow-up period[50].

Stool

- Enterovirus A (CVA2, CVA5, CVA6, CVA8 and CVA14) and enterovirus B (echovirus 30 and CVB3) are more abundant in samples collected before or at seroconversion from children with islet autoimmunity[36].
- Frequent detection of CVA2, CVA4, CVA16, CVB and echoviruses in stool samples obtained from children with a genetic risk for T1DM more than 1 year before the first detection of islet autoantibodies[35].
- Statistically significant association between consecutive shedding of enterovirus B, particularly CVB, and an increased risk of islet autoimmunity in children with a genetically high risk of T1DM[38].

Table 1 | **Studies finding no association between enteroviral persistence and T1DM**

Study (COHORT); study location	Cases; controls	Samples	Virus detection strategy
Mercalli et al.[71]; Italy	25 individuals at various clinical stages of T1DM; 21 healthy individuals	Small-intestine biopsy	In situ hybridization; RT–PCR; immunohisto-chemical staining (VP1, VP2, VP3 and VP4)
Lee et al.[72] (TEDDY); Finland, Germany, Sweden and USA	14 children with rapid-onset T1DM and high-risk HLA haplotype; 14 matched control individuals	Plasma collected prior to and at the appearance of islet autoimmunity	Next-generation sequencing
Kramná et al.[70] (DIPP); Finland	19 children with at least two anti-islet antibodies and a high-risk HLA haplotype; 19 matched control individuals	Stool collected 3, 6 and 9 months before the onset of islet autoimmunity	Next-generation sequencing; virus-specific RT–PCR
Zhao et al.[73] (DIABIMMUNE); Finland and Estonia	11 children with at least two anti-islet antibodies and a high-risk HLA haplotype; 11 matched control individuals	Stool collected once a month starting from the age of 1 month up to the age of 36 months	Next-generation sequencing
Cinek et al.[74]; Nigeria, Sudan, Azerbaijan and Jordan	73 children with new-onset T1DM and a high-risk HLA haplotype; 105 matched controls	One stool sample collected on average 64 days after the clinical onset of diabetes mellitus	Next-generation sequencing; virus-specific RT–PCR

DIABIMMUNE, pathogenesis of type 1 diabetes – testing the hygiene hypothesis; DIPP, Type 1 Diabetes Prediction and Prevention; RT–PCR, real-time PCR; T1DM, type 1 diabetes mellitus; TEDDY, The Environmental Determinants of Diabetes in the Young.

and lymphoid organs (spleen, thymus) and more than 90 days after infection in the pancreas and small intestine[118]. The long-term presence of viral RNA in the pancreas of CD-1 mice infected with CVB4 E2 was associated with chronic islet inflammation, increased islet autoantibodies and development of diabetes mellitus 6–12 months after infection[29]. The infection of Swiss albino mice with CVB4 alters *Igf2* expression in TECs, which leads to a decrease in pro-IGF2 protein[119]. Furthermore, deficient *Igf2* expression in the thymus has been implicated in the development of autoimmune diabetes mellitus in BBDP rats[120]. Together, these findings support the hypothesis that CVB infections, through the inhibition of *Igf2*, might play a part in the decrease of central tolerance to insulin due to defective negative selection of autoreactive T cells[121].

Maternal–fetal transfer of CVB infection in the development of diseases affecting the fetus, neonates and young infants has been described[122–124]. In this light, CVB4 E2 can infect the mouse fetal thymus in utero and disrupt the homeostasis of thymic T cell subpopulations[125–127], which might have a role in the development of autoimmune processes[128].

CVB persistence in T1DM pathogenesis

CVB persistence in humans results from co-evolution between viral factors such as genomic RNA changes (mutations and/or deletions) and cellular factors including the cell cycle, metabolism and the activation state of virus-hosting cells[76,129]. Viral factors involved in CVB persistence in human and mouse heart or in mouse pancreas include a multi-nucleotide deletion in the 5′ non-coding region of viral RNA[115–117,130]. In addition, the formation of an atypical and stable form of enteroviral double-stranded RNA (dsRNA) was found in muscle biopsy samples from patients with chronic fatigue syndrome[80] and in CVB-persistent infection of mouse myoblasts and myocardial tissues[78,131]; this dsRNA could restrict viral RNA replication and reduces the cytopathic effect of the virus[78,132]. The frequent detection of enteroviruses in small-intestine biopsies and stool samples from patients with T1DM are in favour of viral persistence[36–38,49]; however, to our knowledge the molecular form in which enteroviruses can persist in the intestine has not yet been described. Recognition of viral dsRNA by cytoplasmic sensors such as Toll-like receptor 3, MDA5 (encoded by *IFIH1*), antiviral innate immune response receptor RIG-I (encoded by *DDX58*), and protein kinase R (PKR; encoded by *EIF2AK2*) leads to the activation of transcription factors IRF3, IRF7 and NF-κB[133], which result in synthesis of type I interferons in the serum and pancreas of mice infected with CVB[134] and pro-inflammatory cytokines by a CVB-infected insulin-producing rat β-cell line[135]. Of note, blockade of chronic type I interferon signalling has been shown to control persistent lymphocytic choriomeningitis virus infection in mice[136,137]. In addition, interferon immunotherapy is considered to be associated with many autoimmune diseases in humans[138].

The interferon response in T1DM. The role of type I interferons in the pathogenesis of T1DM has been highlighted by the detection of high IFNα expression in pancreatic islets isolated from patients with T1DM[139,140]. Furthermore, children who are genetically predisposed to T1DM show a strongly increased type I interferon signature in whole-blood and peripheral blood mononuclear cells before the onset of disease-associated autoantibodies[141,142], as do some patients with T1DM[143,144]. The antiviral innate immune response and inflammatory mediators such as interferons induced by the long-term presence of enteroviral components seem to be an important factor in the initiation of the autoimmune process during the pre-diabetic phase of T1DM pathogenesis[145,146].

Treatment with exogenous type I interferons confers on human and mouse pancreatic islets a decreased

permissiveness to CVB infection and a strong protection against CVB replication[147,148]. However, treatment in vitro with exogenous IFNα can also induce overexpression of HLA class I molecules, endoplasmic reticulum (ER) stress, inhibition of PCSK1 and PCSK2, impairment of insulin secretion and apoptosis in human pancreatic β-cells[145,149–151]. In animal models, treatment with exogenous rat interferon can also lead to decreased insulin secretion in primary rat β-cells[152]. Moreover, blockade of IFNα receptor 1 with a neutralizing antibody delayed the onset of T1DM in non-obese diabetic (NOD) mice[153].

The overexpression of islet HLA class I molecules often observed in patients with T1DM[154–157] is consistent with the hypothesis of IFNα secretion that could occur during a persistent CVB infection[139,140]. Furthermore, increased expression of intracellular sensor genes for viral RNA such as *TLR3*, *DDX58*, *IFIH1* and *EIF2AK2* has been observed in human islets infected with CVB5 or exposed to cytokines such as IL-1β and IFNγ[158]. PKR protein is also selectively overexpressed in β-cells of human pancreatic islets obtained from organ donors with T1DM, that are positive for the enteroviral capsid protein VP1 (REF.[31]). According to some authors, PKR expression in the pancreas should not be considered as a marker of a viral footprint[159]. However, in the pancreas of patients with newly diagnosed T1DM and donors without T1DM with islet autoantibodies, other markers of an interferon response (such as myxovirus resistance protein 1 and HLA-I molecules) are overexpressed and co-expressed in islets that still contain insulin and in islets with insulitis, and these markers correlate with the presence of enteroviral protein VP1 (REF.[160]). In addition, expression of these markers is associated with downregulation of several genes in the insulin secretory pathway[160].

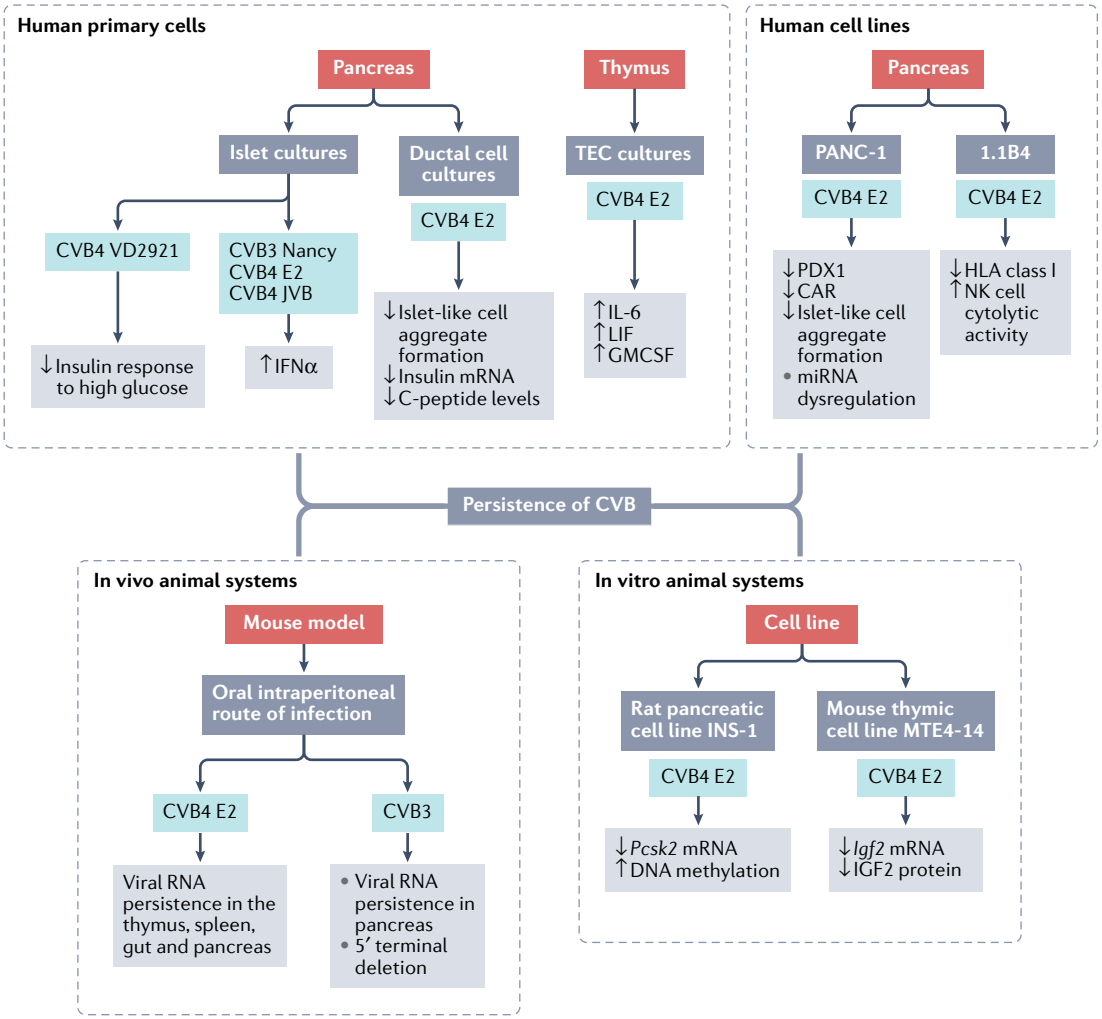

Fig. 2 | **Persistence of CVB in vitro and in vivo.** Coxsackievirus B (CVB) can persist in vitro in human and mouse pancreatic ductal and β-cells and thymic cells (primary cells and in cell lines) and lead to various structural or functional alterations of these cells. CVB persists in vivo in the gut mucosa, heart, thymus and pancreas of orally or intraperitoneally infected mice, weeks after the acute infectious period. 1.1B4, β-cell line derived from electrofusion of primary human β-cells with PANC-1; CAR, coxsackievirus and adenovirus receptor; GMCSF, granulocyte–macrophage colony-stimulating factor; INS-1, β-cell line derived from a rat insulinoma; LIF, leukaemia inhibitory factor; MTE, murine thymic epithelial cell line; NK, natural killer; PANC-1, human pancreatic ductal carcinoma cell line; PDX1, pancreatic duodenal homeobox factor 1; TEC, thymic epithelial cell.

These observations suggest that persistent enterovirus infections of pancreatic islets can induce a chronic antiviral response, which leads to the dysfunction of β-cells mediated by inflammatory mediators[161–164]. Furthermore, overexpression of HLA-I molecules mediated by local IFNα production enhances the presentation of viral antigens and/or islet autoantigens, which could promote the recruitment and activation of pre-existing specific autoreactive T cells in genetically predisposed individuals, as well as β-cell destruction[145,165–168]. The mechanism by which CVB and β-cells achieve a balance to establish a non-cytolytic persistent infection is not fully understood. IFNα has been observed to have a role in the persistence of CVB4 in β-cells of human islets in vitro. Indeed, the addition of anti-IFNα neutralizing antibodies to human islet cultures persistently infected with CVB4 resulted in the lysis of islets[23]. However, the presence of enteroviral RNA (especially dsRNA) that was sensed by MDA5, leads to a strong antiviral response in human islets, which might result in viral clearance. Thus the persistence of enteroviral RNA in human islets indicates that there are complex interactions between the virus and β-cells[33].

A role for lymphocytes. Autoimmunity towards pancreatic islets can also be initiated both by β-cell apoptosis and autoantigen release[169,170] caused by the cytolytic activity of natural killer cells towards β-cells that are persistently infected with CVB4 E2 (REF.[27]). In addition, the production of IFNγ by natural killer cells causes the activation of autoreactive T cells[147,169,171–174]. However, several studies have shown in patients with T1DM, a low frequency of natural killer cells[173,174], aberrant signalling of their activation receptor NKG2D[175,176] and impaired cytolytic activity[173,174], especially towards pancreatic β-cells persistently infected with CVB4 E2 (REF.[27]), might contribute to the persistence of CVB. Defective viral clearance by natural killer cells from patients with T1DM might be owing to the exhaustion of these cells. For example, the exhaustion of natural killer cells was observed during HIV-1 infection, which is another persistent viral infection[177].

Chronic inflammation in the intestinal mucosa due to long-term or repeated enterovirus infections might also promote islet autoimmunity without direct viral infection of the pancreas through 'bystander activation' of pre-existing autoreactive T cells, which can have a role in the pathogenesis of T1DM[50]. Furthermore, impaired innate or adaptive immunity against enteroviruses might potentially explain the long-term enterovirus shedding in the stool of children who are predisposed to T1DM before the first detection of islet autoantibodies[38]. Risk genes for T1DM can influence innate or adaptive immune responses against enteroviruses and make the intestine of patients with T1DM particularly susceptible to enteroviral replication and persistence[178]. Further studies are needed to elucidate this open question.

Enhancing antibodies in CVB infection. Neutralizing antibodies specific to surface epitopes of CVB capsid proteins are produced by the immune system following infection. However, non-neutralizing antibodies can also be generated. For example, non-neutralizing anti-CVB4 E2 immunoglobulins isolated from human serum can facilitate or enhance infection of human peripheral blood mononuclear cells by CVB4 in vitro, which is followed by elevated IFNα and inflammatory cytokine production[179–182]. Furthermore, CVB4 E2 infection in vitro in human and mouse monocytes[179,181–184] and in vivo in the ICR-CD1 mouse strain[183] as well as in vitro in human monocyte-derived macrophages and in mouse bone-marrow-derived macrophages[183,185] is facilitated by antibodies directed against the viral capsid protein VP4, through interactions between the virus and surface receptors of cells (Fcγ RII and Fcγ RIII and CAR). In human monocytes and monocyte-derived macrophages, the enhanced infection results in production of IFNα and pro-inflammatory cytokines IL-6 and tumour necrosis factor[179–182,184,185].

Of note, antibodies that enhance CVB infection have been detected more frequently in the blood of children and adults with newly and previously diagnosed T1DM than in control individuals[180]. In addition, high levels of IFNα were found in the plasma of 75% of patients with T1DM at various stages of the disease (of whom 50% were infected with CVB), but not in the plasma of control individuals[65]. In a 2020 study in patients prior to the development of autoantibodies and T1DM, patient serum showed a predominant enhancing activity, as opposed to neutralizing activity, against CVB3, CVB5 and CVA4 strains, which represent viruses identified in patient stool samples[186]. Interestingly, young mice infected with CVB4 E2 for the first time generated enhancing antibodies against CVB4 (REFS[187,188]); reinfection with the same virus at a later age caused pancreatic tissue damage accompanied by hyperglycaemia and a high viral load in the pancreas. However, control mice of the same age that were not infected with CVB4 at the young age and were exposed to the virus only once at the later age were less susceptible to infection, with very low or null levels of viral RNA in organs and did not show hyperglycaemia[188]. These results suggest that the presence of enhancing antibodies that are produced following primary CVB infection is a risk factor that might contribute to the pathogenesis of T1DM in individuals who experience recurrent homologous or heterologous CVB infections or in persistently infected individuals.

Endogenous human retroviruses. A role of human endogenous retrovirus-W (HERV-W) has also been suggested in the pathogenesis of autoimmune diseases, especially the HERV-W envelope protein (HERV-W Env) given its immunopathogenic properties[189]. HERV-W *ENV* mRNA and the protein itself were expressed in serum, exocrine pancreas and peripheral blood mononuclear cells in a significantly higher proportion of patients with T1DM than control individuals[190,191]. Of note, in vitro infection of human primary pancreatic ductal cells and macrophages with CVB4 has been shown to activate HERV-W *ENV* gene transcription[192]. Pathogenic effects of HERV-W Env protein have been reported and probably contribute to the pathophysiology of T1DM. For example, HERV-W *ENV* transactivation and expression can induce inhibition of insulin secretion by β-cells and superantigen-like activity of HERV-W Env

Human endogenous retrovirus-W
A family of human endogenous retroviruses that are ancestral viral sequences (representing about 8% of the human genome) integrated in primate germinal cells and vertically transmitted across generations over the course of evolution.

could exacerbate the immune response against pancreatic cells[190,191,193]. The possible mechanisms discussed in this section by which the persistence of CVB might have a role in the pathogenesis of T1DM are summarized in FIG. 3.

Prevention and treatment of T1DM

Knowledge of the mechanisms and consequences of enteroviral persistence in the initiation and progression of T1DM opens perspectives for developing pharmacological approaches that target these viruses to combat T1DM. Clinical trials with vaccines and drugs that target enteroviruses and demonstrate their efficacy in preventing or curing T1DM would be definitive proof of the causal role of these viruses in T1DM pathogenesis. Effective and safe vaccines against enteroviruses such as poliovirus and enterovirus 71 have been developed[194–197]. Effective antivirals against chronic viral infections such as hepatitis B virus and hepatitis C virus have also been

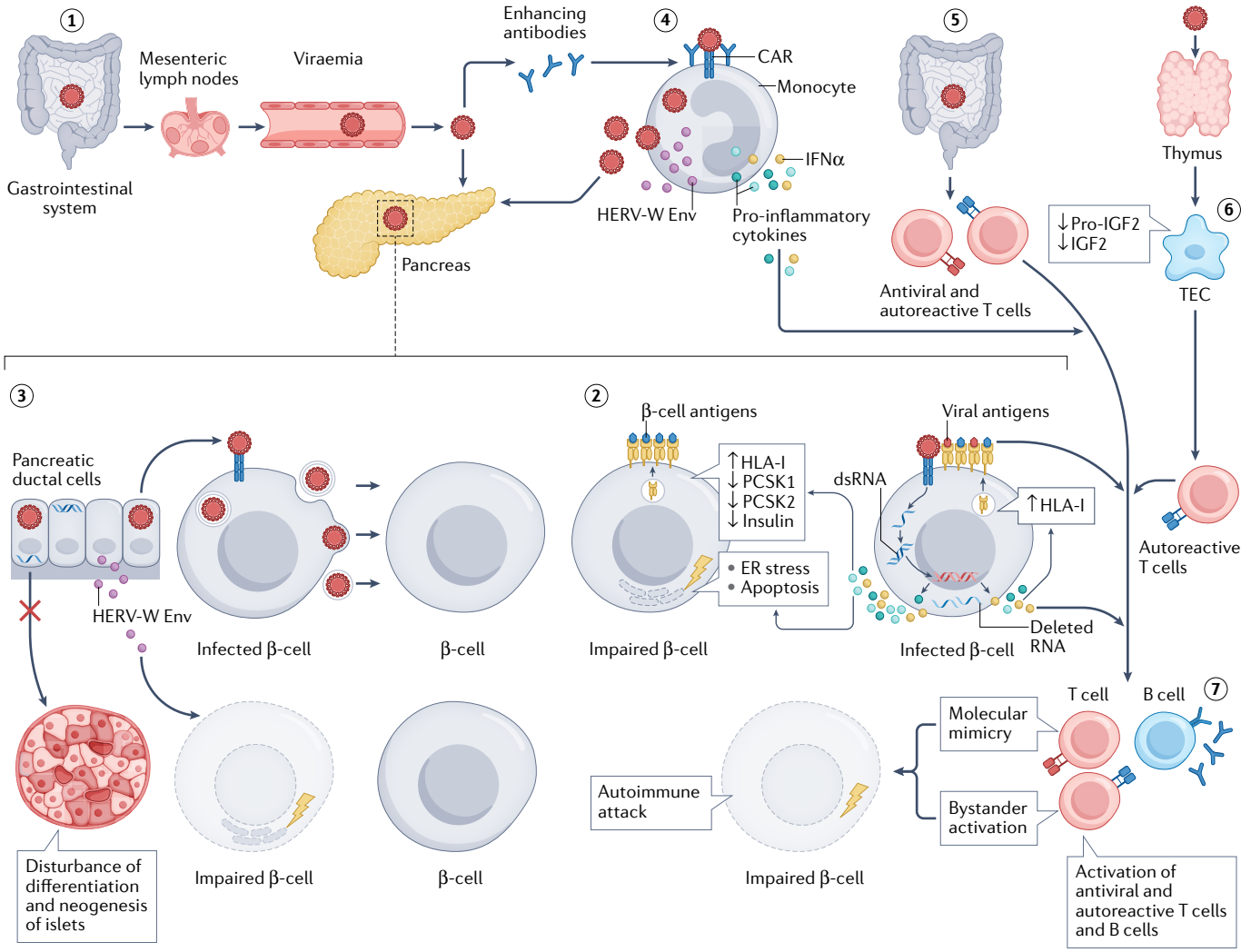

Fig. 3 | **Persistence of CVB and pathogenesis of T1DM.** (1) Coxsackievirus B (CVB) spreads through the gastrointestinal tract and possibly the oropharyngeal mucosa, and then to the pancreas via the lymphatics and the bloodstream. (2) CVB can persist in β-cells through 5′-UTR deletions of the viral RNA genome and the formation of double-stranded RNA (dsRNA), which activates host pathogen recognition receptors. This activation results in the expression of pro-inflammatory cytokines and upregulation of interferon response genes, resulting in production and release of type I interferons. These induce overexpression of HLA class I antigens on the β-cell surface (including neighbouring cells around infected cells), leading to enhanced presentation of β-cell and viral antigens. IFNα also causes endoplasmic reticulum (ER) stress, inhibition of PCSK1 and PCSK2, impaired insulin secretion and apoptosis in β-cells. CVB uses autophagosome-like vesicles, cellular protrusions or microvesicles to spread to β-cells by cell–cell transmission via membrane fusion. (3) CVB can also persist in pancreatic ductal cells, potentially spreading to β-cells, and can alter the differentiation of pancreatic ductal cells into insulin-producing cells. CVB4 activates the expression of human endogenous retrovirus-W envelope protein (HERV-W Env) in pancreatic ductal cells, which could have deleterious effects on β-cells. (4) CVB infection can be maintained in monocytes and macrophages via a mechanism involving enhancing antibodies, so that these cells behave as reservoirs for spreading of the virus to pancreatic cells. The persistent infection of immune cells could result in activation of HERV-W Env expression and a chronic inflammatory state, which can activate autoimmune T lymphocytes. (5) CVB persistence in the intestine might increase the number and activation of antiviral and autoreactive T lymphocytes. (6) CVB persistence in the thymus, especially in thymic epithelial cells (TECs), can disturb self-tolerance to β-cells, resulting in release of autoimmune T cells from the thymus. (7) CVB persistence results in the activation of antiviral T cells and might induce and/or aggravate autoimmune reactions against β-cells through various mechanisms, such as molecular mimicry and bystander activation. CAR, coxsackievirus and adenovirus receptor; IGF2, insulin-like growth factor 2; PCSK, pro-hormone convertase.

developed[198,199]. However, anti-enterovirus vaccines and drugs currently available are still in the experimental phase or in clinical trials.

CVB vaccines. Vaccines against CVB could be effective for the primary prevention of T1DM if they are administered early to children before exposure to these viruses. In addition, such vaccines would need to elicit adequate levels of neutralizing but not enhancing antibodies. Candidate children for vaccination could be selected using a GRS for T1DM[4,5].

Two studies found that a monovalent, formalin-inactivated CVB1 vaccine without an adjuvant was well tolerated, highly immunogenic (inducing efficient production of neutralizing antibodies) and protective against acute CVB1 infection in NOD mice[200,201]. Furthermore, the vaccine protected against CVB1-induced insulin-dependent diabetes mellitus in transgenic mice that expressed suppressor of cytokine signalling 1 (REF.[201]).

Of note, infection of children at genetic risk of T1DM with CVB3 or CVB6 early in life confers protection against appearance of islet autoimmunity and T1DM, whereas CVB1 infection is associated with an increased risk of islet autoimmunity[19]. In addition, seasonal and geographic variations in enteroviral infections suggest that the development of a multivalent inactivated vaccine that includes all six CVB serotypes (CVB1–6) would be relevant, as is the case for the polio vaccine (PV1–3)[195,202]. In 2020, a phase I randomized clinical trial (NCT04690426)[203] of a hexavalent vaccine made from whole formalin-inactivated CVB1–6 viruses (PRV-101 vaccine) was designed to evaluate its immunogenicity and safety in healthy adults. In preclinical testing, this vaccine demonstrated good safety and strong neutralizing antibody production in C57BL/6J and NOD mouse models as well as in rhesus macaque primates[204]. This hexavalent vaccine also induced immunity to acute CVB infections in mice and prevented the development of CVB-induced myocarditis and T1DM in mouse models[204].

Owing to safety concerns with live virus vaccines and the limited and expensive production of inactivated vaccines, an alternative vaccine strategy has been developed based on the use of virus-like particles that lack the infectious genome. Such vaccines were developed for CVB1, CVB3 and CVB4, and these were shown to induce a strong immune response in C57BL/6J and BALB/c mice[205–208]. Other enteroviruses cannot be excluded from being associated with islet autoimmunity and T1DM. However, clinical trials with CVB vaccines will reveal whether they are protective and whether or not to develop vaccines against other enteroviruses.

Antiviral therapy. For secondary prevention, antiviral treatment could be administered to individuals who are already exposed to enteroviruses and potentially carry a persistent infection. A randomized clinical trial (NCT04838145)[209] evaluated the effect of 6 months of treatment with the combination of pleconaril (a capsid binding drug) and ribavirin (a nucleoside analogue) on the persistence of enterovirus infection in the pancreas of patients with newly diagnosed T1DM. These molecules, as well as others such as hizentra (a human immunoglobulin concentrate), enviroxime (a kinase inhibitor) and favipiravir (a viral polymerase inhibitor) have shown efficacy against CVB in vitro and within their recommended therapeutic serum concentrations[210]. Various molecules that act at different stages of the viral cycle such as pirodavir (a capsid binding drug) and fluoxetine (a selective serotonin reuptake inhibitor used as an antidepressant drug and targeting non-structural viral 2C protein) have demonstrated efficacy in reducing enterovirus replication in vitro[211–217]. For further details about these antiviral molecules see REF.[218].

CVB4 replication was inhibited by fluoxetine in vitro in an acute infection model of human PANC-1 cells and mouse insulin-secreting Min-6 cells[219,220]. In vivo, fluoxetine reduced the level of infectious virus particles in organs of CD-1 mice infected with CVB4 E2 (REF.[220]). Furthermore, in PANC-1 cells persistently infected with CVB4, no infectious particles were found following 21 days of fluoxetine treatment[219]. However, the cellular changes (for example, reduced CAR expression) that are induced by persistent CVB4 infections were maintained even after virus elimination[93]. This finding suggests the hypothesis of a lasting impact of persistent CVB4 infections in pancreatic cells. Other antivirals such as enviroxime, pleconaril and hizentra have also shown promising results in inhibiting persistent CVB1 infections in PANC-1 cells[221]. However, the emergence of fluoxetine-resistant viral variants was observed during treatment of PANC-1 cell cultures that were persistently infected with CVB4 (REF.[222]). Treatments based on natural products derived from bacteria and/or their metabolites and plant extracts have shown anti-CVB4 potential in vitro[223–226] and might be an alternative solution to synthetic molecules.

Baricitinib, an oral inhibitor of the tyrosine protein kinases JAK1 and JAK2 approved by the FDA, has shown promising results in the treatment of rheumatoid arthritis in humans[227]. In a 2020 study, this molecule was able to substantially reduce in vitro hyperexpression of HLA class I molecules, ER stress and apoptosis of human β-cells and islets that was initiated by IFNα treatment[151]. The antagonistic effect of baricitinib on interferon response markers[151] that are often identified in islets of patients with T1DM following enterovirus infection[145,150,157,160] opens up prospects for secondary prevention of T1DM. Thus, a randomized clinical trial (NCT04774224) investigating the efficacy of baricitinib in slowing the progressive loss of insulin-producing β-cells in patients with newly diagnosed T1DM is underway in Australia[228].

The development of antiviral therapy capable of eliminating persistent enterovirus infection that can be administered before the development of overt clinical T1DM, combined with molecules that inhibit the adverse effects of CVB infection, could lead to treatments able to combat inflammation and limit the risk of worsening the autoimmunity that leads to T1DM.

Conclusions

Epidemiological and experimental evidence supports the role of enteroviruses, especially CVB, in the pathogenesis of T1DM. Enteroviruses can cause acute and lytic

infections, but they can also establish persistent infections in various cellular or tissue models in vitro and in vivo. Persistence of CVB in the pancreas promotes chronic inflammation, which results from activation of innate immunity. In genetically predisposed individuals, this process could lead to insulitis and progressive autoimmune destruction of β-cells by pre-existing autoreactive cytotoxic T lymphocytes. In addition, persistence of CVB in other sites, such as the gut, blood cells and the thymus, might serve as a reservoir for infection or reinfection of the pancreas, or result in disturbance of central tolerance that could lead to islet autoimmunity and T1DM. Maintaining optimal anti-enterovirus immunity in at-risk populations through vaccination or by blocking viral replication with antivirals might be effective in preventing T1DM and an effective early treatment for T1DM.

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