

# Enterovirus infection and type 1 diabetes: unraveling the crime scene

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## Summary

Enteroviruses (EV) have been historically associated to type 1 diabetes. Definitive proof for their implication in disease development is lacking, but growing evidence suggests that they could be involved in beta cell destruction either directly by killing beta cells or indirectly by creating an exacerbated inflammatory response in the islets, capable of attracting autoreactive T cells to the 'scene of the crime'. Epidemiological and serological studies have been associated with the appearance of islet autoimmunity and EV RNA has been detected in prospective studies. In addition, the EV capsid protein has been detected in the islets of recent-onset type 1 diabetic donors, suggesting the existence of a low-grade EV infection that could become persistent. Increasing evidence in the field shows that a 'viral signature' exists in type 1 diabetes and involves interferon responses that could be sustained during prolonged periods. These include the up-regulation of markers such as protein kinase R (PKR), melanoma differentiation-associated protein 5 (MDA5), retinoic acid inducible gene I (RIG-I), myxovirus resistance protein (MxA) and human leukocyte antigen-I (HLA-I) and the release of chemokines able to attract immune cells to the islets leading to insulinitis. In this scenario, the hyperexpression of HLA-I molecules would promote antigen presentation to autoreactive T cells, favoring beta cell recognition and, ultimately, destruction. In this review, an overview of the standing evidence that implicates EVs in beta cell 'murder' is provided, the time-line of events from EV entry in the cell to beta cell death is investigated and possible accomplices that might be involved in beta cell demise are highlighted.

**Keywords:** beta cell destruction, interferon response, type 1 diabetes, virus infection

## Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by the loss of insulin-producing beta cells in the pancreas. The trigger(s) is still unknown. Genetic predisposition and environmental factors play an important role, acting as candidate triggers of islet autoimmunity. Viruses, and especially enteroviruses (EV), have been examined for their potential implication in T1D

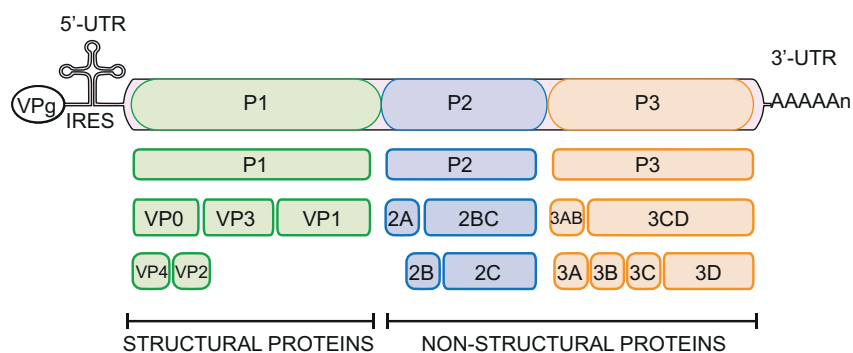
pathogenesis. The association between EVs and T1D has, on many occasions, been a matter of debate for the scientific community. This division between supporters and opponents has pushed the field forward, and interesting studies looking at the possible presence of EVs in the pancreas have been published during recent years. EVs are known to infect beta cells, which situates them as 'possible suspects in beta cell murder'. However, other

factors, such as the host-immune response to the infection, could play an important role in beta cell demise through the stimulation of immune autoreactivity, first triggered and then maintained by potential persistent infections in the pancreas [1]. Thus, EVs might not act alone and the establishment of an inflammatory environment could contribute to beta cell destruction. A careful examination of the 'scene of the crime' might therefore be necessary to uncover hidden evidence. Despite the cumulative proof of an association of EVs with T1D, causal evidence is still lacking and remains a challenge for the field. Access to human samples at different stages of the disease as well as the use of new technologies to study them will be fundamental in order to find it. Co-ordinated efforts carried out by specialized working groups such as the Network for Pancreatic Organ Donors with Diabetes (nPOD)-Virus group or the Persistent Virus Infection in Diabetes Network (PEVNET) are currently investigating the possible presence or absence of EVs in human samples as well as the host-immune response, and are already performing groundbreaking research in the field. In this review we will focus on the most recent findings pertaining EV infections, the host response to a potential infection and possible interventions aimed to find definitive proof implicating these pathogens in T1D development.

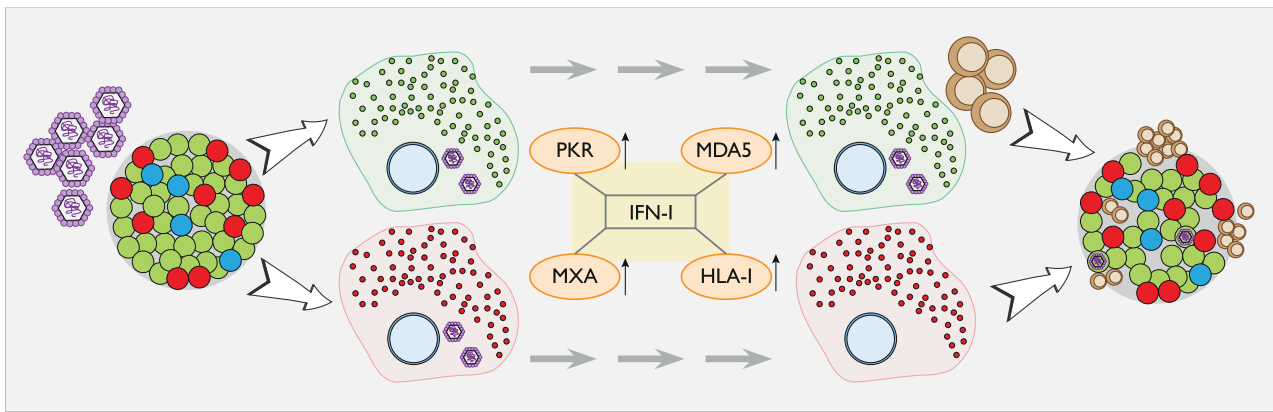
### There has been a murder: can we find the weapon?

Enteroviruses are non-enveloped single-stranded, positive sense RNA viruses that belong to the *Picornaviridae* family. Their genome has a 5'-untranslated region (5'UTR), a coding region that is translated into a single polyprotein, and a 3'UTR with a polyadenylated tail (3'UTR) [2] (Fig. 1). The coding region is translated into 11 proteins

comprising the structural proteins (VP1-VP4, for capsid formation), the polymerase (for replication) and proteases (for polyprotein cleavage) (Fig. 1) [3,4]. These viruses are transmitted by the fecal-oral and respiratory routes, with an initial replication phase in the gastrointestinal or the upper respiratory tract, respectively [4]. After this initial replication, and if the appropriate cellular receptors are present, they can spread to other organs. EVs enter the cell through the decay accelerating factor (DAF) [5] and the Coxsackie-adenovirus receptor (CAR) [6]. The former has not been detected in the pancreas, but CAR is expressed in islet cells [7], representing a potential entrance for EVs to beta cells. Recent studies suggest that both alpha and beta cells express CAR but that EV replication is less efficient in alpha cells due to a more efficient blocking of protein translation [8], which stops viral production. In addition, interferon (IFN) and signal transducer and activator of transcription 1 (STAT-1)-dependent genes are expressed at a higher level in alpha cells compared to beta cells, allowing them to rapidly eliminate the virus (Fig. 2). Conversely, beta cells present a more sustained EV protein and IFN-induced markers expression, reflecting a more chronic and non-cytolytic infection [8]. These differences in viral clearance between alpha and beta cells correlate well with the absence of VP1 positivity in alpha cells in the pancreas of patients with T1D [9]. In an analysis of the distribution of VP1-positive cells between insulin-deficient islets (IDIs) and insulin-containing islets (ICIs) in 10 patients with frequent VP1-positive endocrine cells, Richardson and colleagues reported that 78.7% of the ICIs were found to be positive for VP1. By contrast, only 2.6% of the IDIs showed evidence of VP1 [10], suggesting that beta cells might fail to control viral replication and could harbor virus particles



**Fig. 1.** Schematic representation of the enterovirus genome. At the 5' end the RNA contains an internal ribosomal entry site for cap-dependent translation (IRES) and is covalently linked to the tyrosine-3 residue of a small virus-encoded peptide (VPg), which is used as a primer for RNA replication. The 3' end has a polyadenylated tail [poly(A)] and contains an untranslated region (UTR). The genome encodes a single polyprotein that is cleaved into P1, P2 and P3. From the precursor P1, the capsid proteins VP0, VP3 and VP1 are generated. The non-structural proteins 2A (protease) and 2BC are formed from P2. Finally, P3 yields the production of 3AB and 3CD. The final processing steps lead to the production of VP4 and VP2 (capsid proteins), 2B (increases membrane permeability and inhibits cellular secretory pathways), 2C (vesicle formation), 3A (inhibits intracellular transport), 3B (VPg, primes RNA synthesis), 3C (protease) and 3D (polymerase).



**Fig. 2.** Beta cells might fail to control enterovirus (EV) replication. EVs are able to replicate in both alpha and beta cells. Once the virus has entered the cells through its interaction with Coxsackie-adenovirus receptor (CAR), viral RNA is sensed by protein kinase R (PKR) and melanoma differentiation-associated protein 5 (MDA5), which induce the production of interferon (IFN)-I and the creation of an anti-viral state in the cell. This includes the up-regulation of myxovirus resistance protein (MxA) (increasing IFN production) and islet human leukocyte antigen-I (HLA-I) expression. Due to a more efficient blocking of protein translation [8], alpha cells are able to stop viral production. In addition, IFN and other IFN-related molecules are expressed at a higher level in alpha cells compared to beta cells, allowing them to rapidly eliminate the virus. Conversely, beta cells present a more sustained EV protein and IFN-induced markers expression, reflecting a more chronic and non-cytopathic infection [8]. Additionally, beta cells can harbor persistent infections [11] and non-cytopathic slow replicating viruses have been detected in the pancreas of persistently infected NOD mice [12], suggesting that beta cells might fail to control viral replication. Lastly, a sustained IFN-stimulated HLA-I hyperexpression could enhance beta cell antigen presentation, potentially leading to beta cell destruction by autoreactive CD8 T cells. Alpha cells are shown in red, beta cells in green, delta cells in blue, EVs in purple and CD8 T cells in brown.

for a long time. Additionally, beta cells can sustain a persistent infection for up to 30 days post-infection in intact human islets [11] and non-cytopathic slow replicating viruses harboring a deletion at the 5' terminus region have been detected in persistently infected non-obese diabetic (NOD) mice [12]. Therefore, after an initial replication phase, a non-cytopathic, persistent infection could be established in some beta cells.

While the detection of VP1 in the islets of diabetic donors suggests that EV capsid antigens are present in positive cells, it does not provide information regarding the EV type that infected them. The widely used Dako 5D8/1 antibody, originally generated using CVB5 as immunogen, reacts with most of the enterovirus strains of the Coxsackie, echo and poliovirus groups [13], leaving a long list of potential suspects. Investigators are focusing on producing more suitable reagents to specifically detect EVs, which might have important consequences for vaccine development. A good example is the recent study by Laitinen *et al.* [14], in which new monoclonal antibodies to the CVB-encoded proteases 2Apro and 3Cpro were generated. These antibodies were able to detect the vast majority of EV-B species in EV-A-, B- and C-infected cells. In addition, the authors reported their suitable use for Western blotting, immunocyto- and immunohistochemistry and flow cytometry. Although this is just a small step towards EV identification in human samples, ongoing efforts are generating new reagents with increased specificity. In addition, alternatives to the Dako 5D8/1

have been produced recently. The new 3A6 antibody is able to detect all CVBs but has no reactivity against EV-A species, providing a new tool for EV identification [15]. Moreover, this antibody can be used in Western blotting, peptide immunofluorescence, immunotransmission electron microscopy, immunohistochemistry and immunofluorescent assay, and constitutes a complementary option to the use of the commercial 5D8/1 or the previously reported 5D9 antibody for the detection of EVs in the pancreas of diabetic donors [13].

Despite these difficulties, several studies in the field have presented a large body of evidence that proves that an association between certain EVs serotypes and T1D exists. Epidemiological studies [16], detection of RNA [17–19] and serological analyses [20,21] have confirmed a link between different CVB infections and T1D. In a recent report, stool samples were analyzed to evaluate whether the presence of EVs was associated with the appearance of islet autoimmunity in the Type 1 Diabetes Prediction and Prevention study (DIPP). Children with multiple autoantibodies had more EV infections than control children, and this enhanced level of infection occurred more than 1 year before the first detection of islet autoantibodies [19]. Several serotypes have been identified as potential triggers of the disease [19]. CVB1 infections have been associated with the first appearance of insulin autoantibodies [21]. CVB5 tended to have a similar association, while none of the CVBs were associated with the appearance of GAD autoantibodies [22].

In an interesting study, antibody responses to CVB capsid proteins were evaluated in sera and cord blood serum from 440 children or adolescents enrolled in the prospective German BABYDIAB, BABYDIET, DiMelly and the TEENDIAB studies [23]. Children who developed early insulin autoimmunity had response profiles associated with weak protection against CVB infection, whereas competent responses were observed in children who developed autoimmunity against GAD [23]. There was also a clear association between the production of VP1 antibodies and the induction of neutralizing antibodies, suggesting an important link between VP1 responses and protection [23]. Similarly, children who had islet autoimmunity before 1 year of age or had multiple autoantibodies by 9 months had defective humoral responses after tetanus toxoid booster vaccination, while no differences were found for rubella between autoantibody-positive and -negative children [24]. These studies therefore suggest an impaired ability to mount effective humoral immune responses to exogenous antigens and raise the possibility that viral clearance may be impaired in children with early islet autoimmunity [23,24].

Coxsackieviruses have also been detected in pancreatic biopsies obtained from six individuals with recent-onset T1D, providing further evidence of the possible association of EV with T1D in individuals very close to disease onset [25]. VP1 was detected in the islets of all patients and in two of nine controls. Enterovirus-specific RNA sequences were detected in four of six patients and in none of the six controls. All patients and controls were polymerase chain reaction (PCR)-negative for rhinovirus, norovirus, rotavirus and parechovirus. The CVBs isolated from these samples failed to efficiently amplify *in vitro*, suggesting a poor replicative capacity and pointing to a replication-deficient virus that could have persistently infected the pancreas. Persistent infections can generate replication deficient viruses with naturally occurring 5' terminus deletions [26]. This has been proved in NOD murine pancreas, where the generation of these terminally deleted viruses was associated with persistence in the absence of cytopathic effect [12]. This could be favored by a deficient humoral response in genetically predisposed individuals. As described above, children who develop early insulin autoimmunity had an incomplete antibody response to CBV [23].

### The scene of the crime: what is the evidence telling us?

Increasing evidence in the field shows that a 'viral signature' exists in T1D, one that could be expected to appear after an EV infection. Viral sensors appear at the 'scene of the crime' when viruses manage to enter the cells and initiate their replication cycle. Protein kinase R (PKR) is a double stranded (dsRNA)-dependent protein kinase that plays a critical role in anti-viral defense [27]. Its activation leads to the impairment of eukaryotic initiation factor

2 (eIF-2a), which results in the inhibition of protein synthesis [28]. It also has a role in cellular signal transduction and transcriptional control. As a consequence of dsRNA accumulation in infected cells, PKR is activated to inhibit the translation of viral mRNAs [27] in an attempt to stop viral replication. In the pancreas of diabetic patients, PKR has been detected in cells that also express VP1 [29], providing additional proof of the existence of an EV infection in these individuals. The presence of long-length dsRNA is sensed by the RNA-helicase melanoma differentiation-associated protein 5 (MDA5) [30]. It is encoded by the IFN induced with helicase C domain 1 (IFIH1) gene, in which various single nucleotide polymorphisms (SNPs) have been found to confer a higher risk of T1D development, increasing inflammation and IFN responses [31]. MDA5 is expressed in isolated human islets in response to EV infection [32] and IFN production [33]. However, its pancreatic expression has not been fully investigated in prediabetic and diabetic donors. The myxovirus resistance protein (MxA) is a GTPase induced exclusively by IFN production that inhibits RNA virus replication. Studies on a very limited number of samples have shown that its expression is elevated in the islets of T1D and autoantibody-positive donors [34]. Lastly, retinoic acid inducible gene I (RIG-I) is a cytosolic pattern recognition receptor (PRR) involved in sensing RNA, virus infection and in inducing IFN-I production [35]. Human islets infected with CVB3 express high levels of IFN-I, IFN-III, MDA5, RIG-I and Toll-like receptor (TLR)-3, along with a variety of inflammatory cytokines [36]. Studies in patients with fulminant T1D potentially infected with EVs reported a strong expression of MDA5, RIG-I and VP1 in the islets compared to controls [37] and significantly increased numbers of alpha cells expressing RIG-I and IRF3 [38].

The interaction of these viral sensors with its ligand activates downstream signaling cascades leading to the production of IFNs, which results in the activation of an anti-viral state and enables the detection of viral signatures [39]. It has been proposed that EVs could be inducing interferonopathy-like conditions within the islet micro-environment [40]. In isolated islets and EndoC- $\beta$ H1 cells IFN- $\alpha$  is capable of inducing ER stress, reducing insulin content, increasing the proinsulin to insulin ratio and decreasing the expression of the prohormone convertases PC1 and PC2, responsible for the processing of proinsulin to insulin [41]. Accordingly, increases in the proinsulin to insulin ratio in the pancreas of prediabetic and recent-onset donors have been reported [42]. Thus, IFN- $\alpha$  produced locally in the islets could alter the functional state of beta cells.

IFN production induces the expression of other gene products, such as STAT-1, which binds to the IFN-regulatory factor 9 (IRF9) and ultimately induces the

transcription of IFN-stimulated genes (ISGs) aimed to inhibit viral transcription, translation and replication [43]. STAT-1 is present at low levels in the islets of non-diabetic donors and in IDIs of T1D donors [44]. However, its expression is elevated in ICIs and is higher in beta cells in diabetic donors. In addition, laser-microdissected islets also present higher STAT-1 mRNA levels, especially close to disease onset [44]. Viral infections or exposure to IFN- $\gamma$ /IL-1 $\beta$  or IFN- $\alpha$  increase the islet expression of ISGs [45]. Similarly, islets of recent-onset T1D patients express high levels of ISGs [46]. Quantitative mass spectrometry analysis on cultured primary human islets infected with CVB4 revealed a considerable decrease in insulin and an increase in proteins that participate in the host anti-viral response, such as MxA, ISG15, IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), IFIT2, IFIT3, STAT-1 and 2'-5'-oligoadenylate synthase 3 (OAS3) [47]. IFN-I signaling culminates in the induction of an anti-viral response which aims to interfere at various steps with the viral cycle [45]. These studies suggest that an anti-viral signature exists in the islets of T1D patients and highlights the need to characterize these responses in the human pancreas, especially during the prediabetic phase and close to onset of disease.

IFN-I can also induce HLA-I hyperexpression, a hallmark of T1D [48,49]. Gian Franco Bottazzo and colleagues, 35 years ago, were the first to discuss the possibility that insults (like viruses) could induce the aberrant expression of human leukocyte antigen (HLA) molecules, leading to organ-specific autoimmunity [50,51]. In 1985, the careful examination of the pancreas from a diabetic child who had died at onset of T1D revealed the hyperexpression of HLA-I molecules in the islets [52]. During recent decades, HLA-I expression has become a hallmark of T1D and multiple studies have reported its elevated expression in the islets of prediabetic and diabetic donors, especially in ICIs, at the protein and mRNA level [44,53–58]. In addition, Eizirik and colleagues have recently shown that the hyperexpression of HLA-I at the cell surface of beta cells remains even 7 days post-IFN- $\alpha$  stimulation, while other markers of inflammation and ER stress rapidly return to baseline [59]. Moreover, continuous IFN- $\alpha$  stimulation is not required for persistent HLA-I hyperexpression at the cell surface, indicating that its prolonged expression could be due to the abundant presence of antigenic peptides generated in response to IFN- $\alpha$  [59]. Alternatively, the presence of a small amount of persistently infected cells in the islets could be sufficient to maintain a low-level IFN response, which could also prolong the hyperexpression of HLA-I molecules years after onset, as suggested by studies of pancreas sections from long-standing T1D donors (up to 12 years post-onset) [44]. As EVs can inhibit HLA-I antigen presentation in infected cells through blockage of the secretory pathway in order to avoid recognition by the immune system [60,61], IFN- $\alpha$ -stimulated

HLA-I hyperexpression could enhance beta cell antigen presentation early in the disease process and extend it for a long period of time after onset.

In the context of an enhanced antigen presentation, cytosolic peptides are transported into the ER lumen via the transporter associated with antigen processing (TAP) [62], which is part of the HLA-I peptide loading complex. The ER aminopeptidase 1 (ERAP1) cuts these peptides into a suitable length for loading into the HLA-I [63]. Peptide–HLA complexes are transported to the cell surface for immune recognition. IFN-I exposure induces the increase of TAP1, TAP2, TPBP, chaperones and the editing enzyme ERAP1, suggesting an increase in transport, stable processing and loading of peptides onto HLA-I within the ER [45,64]. The resulting augmented autoantigen presentation could potentially lead to beta cell destruction by autoreactive CD8 T cells. TAP and ERAP1 have been shown to potentially contribute to the generation of preproinsulin (PPI) epitopes that target beta cells for killing by PPI-specific cytotoxic CD8 T cells [65]. Accordingly, TAP expression was elevated in ICIs of T1D donors compared to IDIs or islets from non-diabetic donors. Similarly to HLA-I expression, TAP was highly expressed in beta cells but was also present in other islet cells, while ERAP1 was not different in non-diabetic and diabetic donors [65]. This suggests that, in the context of an EV infection, IFN would induce the expression of HLA-I and could create a fertile field for the presentation of beta cell antigens to autoreactive T cells, attracted to the islets by the inflammatory environment.

#### The investigation: did it act alone?

Based on this evidence, one could envision a 'crime scene' or scenario in which viral infections might induce the production of IFN-I, increase the expression of HLA-I molecules and the presentation of antigens on the surface of beta cells. IFN-I also influences the islet microenvironment and modulates both the innate and the adaptive immune responses by activating resident monocytes and plasmacytoid dendritic cells (pDCs), which can produce large amounts of IFNs themselves [66]. This local signature creates a positive feedback loop of inflammation where cytokines and chemokines may recruit both anti-viral and autoreactive T cells [40]. Once T cells have reached the islet, IFN-I provides the necessary signals to complete CD8 T cell expansion and activation, increasing their cytotoxic capacity [67]. In addition, IFN-I could also promote cross-presentation of antigens from DCs directly to CD8 T cells [68]. We have reported the presence of high numbers of CD11c<sup>+</sup> cells in the pancreas of autoantibody-positive and T1D donors [69]. In addition, it has been shown that CVB3 infection drastically reduces the capacity of DCs to prime naive CD8 T cells *in vitro* and *in vivo* [70]. This might have implications for the potential

detection of anti-viral T cell responses in individuals with T1D who have been exposed to EVs.

An important missing link between EV infections and T1D is the detection of circulating or infiltrating EV-reactive T cells. Predicted CVB epitopes and *in-vitro* peripheral blood mononuclear cell (PBMC) stimulation assays have tried to analyze T cell responses against EV [71]. Peakman and colleagues have recently reported a novel strategy for the study of CVB epitopes by using *in-silico* and *ex-vivo* approaches [72]. By combining conventional major histocompatibility complex (MHC)-I-binding algorithms with phylogenetic approaches, they identified regions of the CVB genome evolving under positive selection in order to identify epitopes from immunogenic regions. Interestingly, the 2A protein presented the highest variation within each serotype. The most conserved region between different CVB serotypes was the 3C protease. Conversely, the most variable regions were the capsid proteins, but they were also the most immunogenic across serotypes. PBMCs were stimulated *ex vivo* with pools of synthetic peptides based on the *in-silico* predictions. Positive responses to CVB peptide pools were low, broad and not different between T1D and non-diabetic patients [72]. However, only adults and long-standing T1D patients were included in this study. As EV infections are very common early in life and viral signatures have been found mainly in prediabetic and recent-onset T1D donors, studies that target these populations are necessary to fully characterize T cell responses against EV and evaluate their possible role in disease pathogenesis.

The existence of molecular mimicry reflecting potential cross-reactivity between EV protein epitopes and host islet proteins that are recognized by autoreactive T cells has been debated for a long time. Although many studies have attempted to prove this hypothesis, the evidence to support it is not strong. Technical challenges and restricted knowledge on the nature of the infiltrating cells have influenced the outcome of these studies. The majority reported possible cross-reactivity between EV and GAD or IA-2 epitopes [73,74]. A potential cross-reactivity between the 2C of CVB4 and GAD<sub>247-266</sub> at the TCR level in memory CD4 T cells has also been reported in one patient [23]. This suggests that exposure to CVB4 could indeed activate GAD-specific CD4 T cells via molecular mimicry. However, these findings will have to be confirmed in larger populations. An interesting hypothesis is that molecular mimicry does not act as a disease trigger and, rather, it contributes to disease pathogenesis [75]. In this context, the expansion of previously primed autoreactive T cell populations via heterologous virus infections and molecular mimicry could lead to the acceleration of the disease in already prediabetic hosts [75]. This brings to the table the concept that islet inflammation is a pre-existing condition for the contribution of viral infections to molecular mimicry or to disease

pathogenesis in general. For example, the presence of a persistent infection creates a chronic inflammatory environment in which autoreactive T cell populations could be activated and expanded [75]. In this context, the careful examination of the clinical history of the patient becomes critical in order to identify if multiple infections could have contributed to disease development by increasing the inflammatory milieu and attracting immune cells, both anti-viral and autoreactive, to the islets.

### Innocent until proved guilty

With or without molecular mimicry, the reality is that there is a gap in our knowledge on the nature of the islet infiltrating antigen-specific T cells (autoreactive and/or virus-reactive). Only a small number of self-reactive cells have been detected in the periphery or in the islets of T1D patients [53,76] and the majority of infiltrating cells are considered of unknown reactivity or bystanders. Bystander activation can be caused by sensing of viral RNA and activation of the anti-viral response through molecules such as PRK, MDA5, RIG-I or MxA, among others, with the subsequent production of IFN-I. A virus infection that kills a limited amount of beta cells and generates the activation of an anti-viral state could create a fertile field for autoreactive T cells. Interestingly, CVB4 infection accelerates diabetes only if a minimum number of autoreactive T cells are present [77]. In addition, studies in mice have shown that the timing of the infection is also a critical factor for the development of T1D. Mice that were infected when low levels of insulinitis (or autoreactive T cells) were present were protected from developing the disease through a non-specific immunostimulatory mechanism [77]. Studies in mice have shown that islets that have a high level of non-islet specific T cells do not develop diabetes due to bystander suppression of antigen-specific T cells. The authors hypothesized that the non-specific T cells restrict the access of the antigen-specific cells to their cognate antigen at the inflammatory site [78]. Whether this scenario occurs in human T1D needs further investigation, but suggests that weak adaptive immune responses against viruses could also be detrimental for the host, if circulating autoreactive T cells are present and a predisposition to T1D exists.

### Calling for reinforcements, co-operation is the key

Many laboratories have investigated the role of EVs during the recent decades. The difficult timing and the heterogeneity of T1D has made data collection and interpretation extremely challenging. The nPOD-Virus group was created a few years ago in an attempt to coordinate studies regarding the role of EVs in disease

pathogenesis in human samples, bringing together investigators with different expertise. This collaborative effort has a strong focus on EVs, with the aim to fill many of the critical knowledge gaps about their role in the disease. By gaining access to innovative and powerful technologies that had not been used previously, the group aims to find definitive proof for the association of EV and T1D. In addition, the ability to gain access and to analyze samples from the same donors in multiple laboratories by using different techniques provides an excellent opportunity to generate robust data. Ongoing analyses will provide strong evidence for the association of EV with T1D by not only identifying viral protein in the pancreas but also viral RNA and viral indicators of infection, such as the expression of HLA-I, MxA (in humans), PKR, MDA5 and dsRNA. In addition, the group is also looking at the presence of CD45, CD3 and CD8, as well as virus-specific T cells, in an effort to provide strong evidence for the presence or absence of a viral signature in T1D. These efforts can lead to a better characterization of the virus–host interaction and the link between EV infections and pancreas pathology. If EVs play a role in T1D, the nPOD-Virus group could provide enough evidence for the development of a vaccine or the use of anti-virals that could prevent or delay the disease. An optimized and scalable protocol for the development of a CVB1 vaccine has been already tested in murine models [79]. The vaccine was able to induce a strong, virus-neutralizing antibody response, protected against a CVB1 challenge and did not accelerate diabetes in NOD mice. In a follow-up study, the vaccine was tested on suppressor of cytokine signaling transgenic (SOCS1-tg) mice, in which beta cells are unable to respond to IFNs and have an increased susceptibility to EV infection [80]. Similarly to NOD mice, SOCS1-tg mice developed a robust antibody response, were protected from a CVB1 challenge and from diabetes and had normal pancreas morphology [80].

The studies exposed above and the identification of different EVs serotypes with possible roles in T1D suggest that the development of an EVs vaccine for T1D could have great benefits, but could be challenging to produce. Prospective studies such as DIPP [81] and the Environmental Determinants of Diabetes in the Young (TEDDY) Study [82] could be very important for the identification of diabetogenic viruses and could provide a valuable platform to test the efficacy of vaccination strategies in preventing the disease. As several serotypes have been associated to T1D, a hexavalent vaccine including all six CVB serotypes seems to be the best approach [80]. In addition, this vaccine would be able to prevent other diseases associated with CVB infection, such as myocarditis and septic meningitis [80]. However, if EVs other than CVBs are implicated, the vaccine might not

be able to halt the disease, and other non-CVB EVs might have to be considered. Although the scientific community has recently increased its efforts to identify specific EV species by virus amplification and sequencing techniques [83], the hostile environment of the pancreas is prone to RNA degradation [84], which does not facilitate such a quest. While this continues to limit the identification of EVs in the target organ, it is expected that new technologies and improved sensitivity and specificity of current techniques will overcome this challenge.

Based on the efficacy and safety observed in mice, investigators in Finland will begin clinical trials to study the use of a CVB vaccine in humans [85]. In Phase I the vaccine will be tested for safety in adult healthy volunteers. In Phase II, it will be administered to children in order to test its safety and efficacy against EV infections. In Phase III the investigators will test if the vaccine is effective in preventing the onset of T1D [85]. Parallel efforts by investigators in Norway will study the safety and efficacy of anti-virals to successfully reduce virus amplification in recent onset T1D patients. This is also of interest in the context of persistent viral infections, as it could reduce pancreatic inflammation and recover insulin secretion.

## Conclusions

In summary, a high number of studies have reported the association of EV infection with the development of islet autoimmunity and clinical onset, but have failed to find definitive proof for their implication as triggers of T1D. In humans, the heterogeneous disease development and the infection dynamics make EV detection very challenging. Longitudinal studies could provide a definitive answer but frequent sample collection and exhaustive follow-ups seem necessary to catch EVs at the ‘scene of the crime’. In addition, an extensive characterization of the host response to infections prior to disease development and the access to human pancreata from individuals with two or more autoantibodies and at onset of disease will be crucial to investigate if an interferonopathy-like condition exists in T1D. Whether beta cells are more susceptible to viral infections, EVs are directly responsible for beta cell death, or it is the host response which creates a fertile field for autoreactivity, a vaccine against EVs could provide definitive proof for the role of EV as triggers of T1D.

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## Disclosures

T. R.-C. declares no conflicts of interest.

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