Endocrine Reviews, 2023, **44**, 737–751
https://doi.org/10.1210/endrev/bnad007
Advance access publication 8 March 2023
Review

Coxsackievirus and Type 1 Diabetes: Diabetogenic Mechanisms and Implications for Prevention

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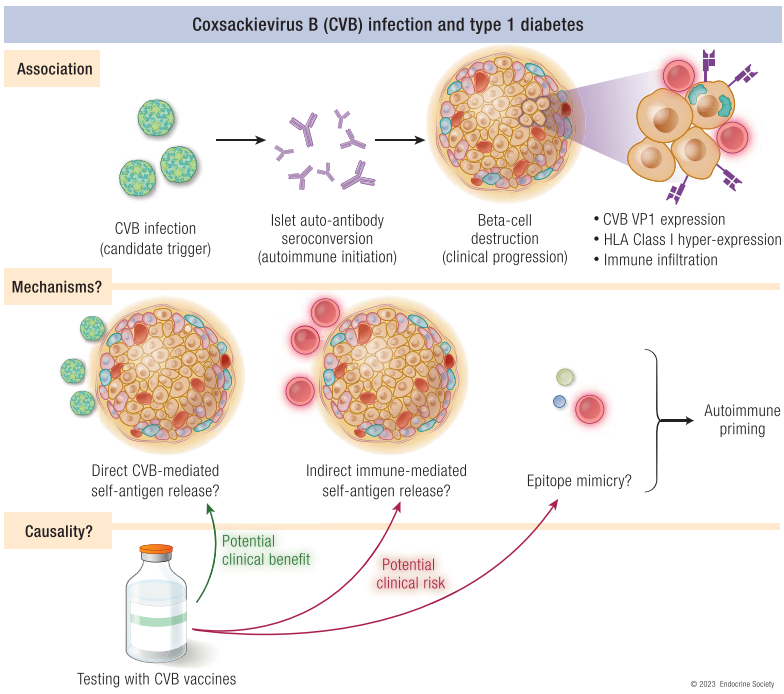
Abstract

The evidence for an association between coxsackievirus B (CVB) infection, pancreatic islet autoimmunity, and clinical type 1 diabetes is increasing. Results from prospective cohorts and pancreas histopathology studies have provided a compelling case. However, the demonstration of a causal relationship is missing, and is likely to remain elusive until tested in humans by avoiding exposure to this candidate viral trigger. To this end, CVB vaccines have been developed and are entering clinical trials. However, the progress made in understanding the biology of the virus and in providing tools to address the long-standing question of causality contrasts with the scarcity of information about the antiviral immune responses triggered by infection. Beta-cell death may be primarily induced by CVB itself, possibly in the context of poor immune protection, or secondarily provoked by T-cell responses against CVB-infected beta cells. The possible involvement of epitope mimicry mechanisms skewing the physiological antiviral response toward autoimmunity has also been suggested. We here review the available evidence for each of these 3 non-mutually exclusive scenarios. Understanding which ones are at play is critical to maximize the odds of success of CVB vaccination, and to develop suitable tools to monitor the efficacy of immunization and its intermingling with autoimmune onset or prevention.

Graphical Abstract



Received: 5 November 2022. **Editorial Decision:** 22 February 2023. **Corrected and Typeset:** 22 March 2023

Key Words: beta cells, *Enterovirus*, immune evasion, mimicry, T cell, vaccine

Abbreviations: aAb, autoantibody; Ab, antibody; APC, antigen-presenting cell; CAR, Coxsackievirus and Adenovirus Receptor; CVB, Coxsackievirus B; DC, dendritic cell; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; GAD, glutamic acid decarboxylase; HLA, human leukocyte antigen; IFN, interferon; MHC, major histocompatibility complex; NOD, nonobese diabetic; OR, odds ratio; PBMC, peripheral blood mononuclear cell; PRR, pattern recognition receptor; T1D, type 1 diabetes; TCR, T-cell receptor; TEDDY, The Environmental Determinants of Diabetes in the Young study; TNF, tumor necrosis factor; UPR, unfolded protein response; VP, viral protein.

ESSENTIAL POINTS

- Infections by enteroviruses, particularly *Coxsackievirus B* (CVB), have been associated with type 1 diabetes (T1D) development and share some key features: peak incidence during infancy, high prevalence, oro-fecal transmission, and beta-cell permissiveness
- The evidence for such association comes from prospective cohort and histopathological studies and has been linked to autoimmune initiation (ie, islet autoantibody seroconversion) rather than to clinical progression
- CVB infections may become persistent (chronic) in susceptible individuals and sustain a prolonged immune activation favoring loss of tolerance, possibly through re-iterative priming of autoimmune T cells
- A first diabetogenic mechanism of CVB infection may rely on a direct pathogenic effect on beta cells, releasing self-antigens in an inflammatory tissue environment
- A second diabetogenic mechanism may rely on the indirect pathogenic effect of antiviral immune responses mounted against infected beta cells, similarly leading to self-antigen release and autoimmune priming
- A third mechanism may involve epitope mimicry and cross-priming of islet-reactive T cells initially recruited by viral antigens
- Distinguishing between these 3 non-mutually exclusive mechanisms has implications for vaccination strategies aimed at preventing T1D by protecting against CVB infections

The most outstanding question in the field of type 1 diabetes (T1D) concerns the identification of environmental triggers (1), a conundrum shared with many other autoimmune diseases. These environmental triggers are gaining importance, as the steady progression of T1D incidence in the last 50 years cannot be accounted for by a genetic drift of the human population. Rather, the relative weight of predisposing gene variants (mostly mapping to human leukocyte antigen [HLA] class II loci) is decreasing. Indeed, T1D patients diagnosed in more recent years carry HLA haplotypes considered protective to a larger extent than patients diagnosed decades ago, while high-risk haplotypes are becoming less common (2). This observation indirectly suggests that environmental pressure is increasing. Moreover, these environmental factors exert their role very early in life, as the majority (~64%) of children that later develop T1D display their first seroconversion for islet autoantibodies (aAbs) during the first 2 years of life (3, 4).

Given the heterogeneity of disease, mostly related to age (5), a universal environmental trigger underlying all T1D cases is unlikely to exist. Nonetheless, the strongest evidence for an association with T1D points to enterovirus infection, particularly to coxsackieviruses B (CVBs) (6–8). This echoes the robust evidence linking Epstein-Barr virus infection with multiple sclerosis (9–12). We will discuss current evidence for the association between CVB and T1D, and the possible pathogenic mechanisms at play. We contend that a better knowledge of such mechanisms is essential to inform the design of T1D prevention trials based on CVB vaccination (13, 14).

Common Features of Putative Diabetogenic Viruses

Many different viruses have been implicated as candidate triggers for T1D. These include *Enterovirus* (both CVB and echoviruses), *Parechovirus*, and *Rotavirus*. The strength of evidence for each of those has been recently reviewed (15), but it is important to note that they share 4 key features:

1. They are responsible for infections during the first years of life, which may be in line with the early timing of aAb seroconversion in most pediatric T1D progressors (3, 4). CVB infections show some seasonal trends, being more common during the summer and early fall.
2. These infections are highly prevalent, with >95% of the general population being enterovirus-seropositive. It thus needs to be explained how such prevalent infections may trigger T1D in only few individuals. As discussed, genetic susceptibility, infection timing, viral clearance vs persistence, and antiviral immune responses may be at play.
3. They are transmitted mainly through the oro-fecal route and, to a lesser extent, through the respiratory route. This feature is relevant because pancreatic lymph nodes drain not only the pancreas itself but also parts of the intestinal tract (16), thus providing an ideal crossroad for immune cells and viruses to reach the pancreas.
4. They can infect beta cells. They can thus exert their pathogenic mechanisms on the same cells that are targeted by T1D autoimmunity.

CVBs are classified into 6 serotypes (1 to 6) and belong to the family Picornaviridae and the genus *Enterovirus*, which also includes *Poliovirus* and echoviruses. They are small nonenveloped single-stranded RNA viruses that initially replicate in the submucosal lymph tissue of the distal small bowel, and of the upper respiratory tract when transmitted by aerosols. Although they usually cause asymptomatic infections, further dissemination to target organs can occur following a secondary viremia. This can occasionally result in severe diseases, such as meningitis, encephalitis, myocarditis, and systemic neonatal infections. Some of these clinical conditions, eg,

myocarditis, exemplify the capacity of CVBs to cause persistent (chronic) infections (8, 17–19).

Association Between CVB Infection and T1D: Prospective and Histopathology Studies

The association between CVB infection and T1D is supported by temporal correlations from prospective cohorts and by spatial correlations documented in histopathological studies.

Temporal Correlations

A 2011 meta-analysis of 26 case-control studies reported a significant association between enterovirus infection and islet autoimmunity (odds ratio [OR] 3.7) or clinical T1D (OR 9.8) (20), although some publication bias in favor of positive results is likely. The CVB serotypes more frequently associated with T1D are CVB1 (21) and CVB4 (22), while some others, such as CVB3 and CVB6, have occasionally been suggested to be protective (21). Associations between CVB exposure (ie, detection of CVB RNA in stools) and seroconversion and T1D progression were provided by prospective cohorts, such as the Finnish T1D Prediction and Prevention (DIPP; https://dipp.fi) and The Environmental Determinants of Diabetes in the Young (TEDDY; https://teddy.epi.usf.edu) studies. Based on serological evidence, ie, neutralizing antibody (Ab) titers against different CVB serotypes, Laitinen et al, working in the group of H. Hyöty (21), reported that the prevalence of anti-CVB1 Abs was higher in children later seroconverting for islet aAbs, while that of anti-CVB3 and anti-CVB6 Abs was lower. When considered altogether, a positive CVB1 serology and a negative CVB3/CVB6 serology conferred a 2.5 OR for subsequent islet aAb seroconversion. A protective role for maternal Abs was further suggested, as a negative CVB1 serology in cord blood and subsequent CVB1 seroconversion by 18 months of age was associated with a similar OR of 2.6. Given its focus on preclinical stage 1 (ie, asymptomatic islet autoimmunity with normal insulin secretion), this study suggested that CVB exposure may be associated with aAb seroconversion rather than clinical progression. This is in line with previous studies from Cuba reporting an association between epidemic echovirus infection and aAb seroconversion in the convalescent phase (23–25). This possibility was confirmed by a recent TEDDY study based on the longitudinal analysis of the fecal viral metagenome enhanced through a preliminary culture step (22). The risk of future aAb seroconversion was not associated with short and independent infections, but rather with prolonged infections with the same enterovirus B (mostly CVB4) serotype, with a risk increase of ~20% at each positive stool sample.

Spatial Correlations

A spatial correlation between CVB infection and insulitis (ie, the immune infiltration of islets) has been highlighted by immunohistochemistry (26–29), reverse-transcription polymerase chain reaction (29), and in situ hybridization (30), using tissue specimens available through the network for Pancreatic Organ Donors with T1D (nPOD; https://www.jdrfnpod.org), from brain-dead organ donors; the UK Exeter Archival Diabetes Biobank (EADB; https://pancreatlas.org/datasets/960/overview), from autopsy material; and the Norwegian Diabetes Virus Detection Study (DiViD; https://www.oslodiabetes.no/diabetes-virus-detection-study-divid), from living donors (29, 31). Immunohistochemistry for the enterovirus viral protein (VP)1 revealed a colocalization with T-cell infiltration and/or HLA class I hyper-expression, which are the 2 hallmarks of insulitis. When considering these immunohistochemistry studies altogether (26–29), VP1+ islets were detected in ~70% of T1D cases and 14% of nondiabetic controls. All these studies also conclude that only a fraction (7%-29%) of islets with residual insulin-containing cells are VP1+, suggesting that persistent low-grade rather than short-lived high-grade infections may occur, probably long before T1D clinical onset (13).

Possible Mechanisms of Persistent CVB Infection

As discussed above, the small fraction of VP1+ cells in the pancreas of T1D patients (corresponding to the late stage 3 disease in natural history) together with the temporal association between prolonged fecal CVB shedding and aAb seroconversion (ie, early stage 1 disease) suggest a scenario of prolonged CVB infections. On the other hand, the few cases of aAb+ organ donors analyzed to date did not show evidence of acute, extensively lytic CVB infection (5), although also in this case we only have a "snapshot" available, which may date well beyond the time of CVB encounter. It should be noted that this does not exclude the possibility that a more limited lysis of CVB-infected beta cells may occur. The TEDDY stool virome study (22) further indicates that these prolonged CVB infections are the result of viral persistence (ie, chronic infection following a single viral encounter) rather than recurrence (ie, multiple viral encounters).

The viral mechanisms leading to persistent CVB infections have been recently reviewed (8). A first mechanism involves repeated CVB seeding from infection reservoirs in the gut (mostly duodenal epithelial cells), pancreas (ductal and beta cells), and blood cells (mostly antigen-presenting cells, APCs) (32, 33), through infections that can be either cytolytic (with release of new virions) or not (with virions released through other mechanisms, eg, extracellular vesicles) (34). A predisposing allele of the T1D risk gene *IFIH1* (interferon induced with helicase C domain 1; coding for the melanoma differentiation associated protein 5 MDA5) may favor CVB persistence in the blood (33). Reservoirs of viral persistence are also established in microvascular endothelial cells, without causing overt cytopathic effects but inducing the upregulation of adhesion molecules that may contribute to leukocyte recruitment (35).

A second mechanism exploits a naturally occurring deletion in the 5′ noncoding region of the viral genome, which leads to reduced viral replication and persistent low-grade infection (36, 37). Such deletions might be favored by the high mutation rate of CVBs, due to the lack of proofreading in their RNA-dependent RNA polymerase (38).

A third mechanism may be a non-lytic CVB egress pathway active in beta cells. This is mediated by extracellular vesicles, which also protect CVB from neutralizing Abs, thus also providing an immune evasion mechanism (34). Another immune-evading CVB transfer mechanism via cell protrusions has been described in other cells (39).

Immune mechanisms may also participate, and include innate type I interferon (IFN, mainly IFN-α) responses (40) (another hallmark of T1D, starting from its early preclinical stages) (41, 42) limiting viral replication and beta-cell lysis (43); and insufficient adaptive antiviral responses that do

not clear the virus, as discussed below. These mechanisms may also engage a vicious cycle, with persistent infection promoting a prolonged immune activation favoring loss of tolerance, possibly through re-iterative priming of autoimmune T cells. Figure 1 summarized the timeline of the immune responses triggered by CVB infection.

From Association to Causality: Possible Diabetogenic Mechanisms of CVB Infection

With these points in mind, 3 non-mutually exclusive mechanisms can be proposed to explain the triggering effect of CVB infection on islet autoimmunity (Fig. 2):

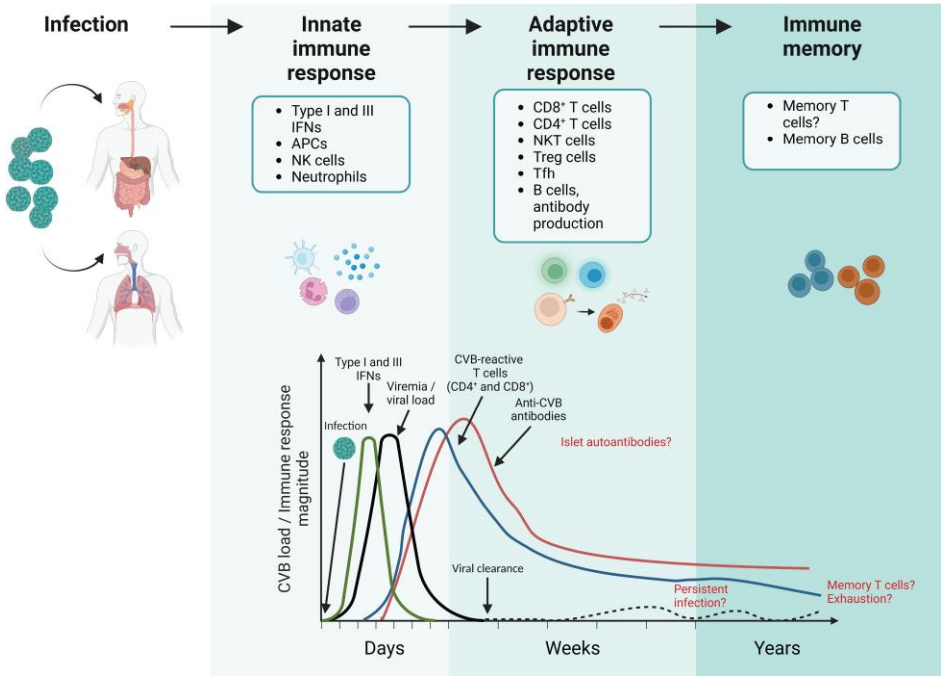


Figure 1. Timeline of the immune responses triggered by CVB infection. CVB infection through the digestive or respiratory route first leads to the activation of innate immune responses, including the production of type I and III interferons. CVB-reactive T and B cells are subsequently activated along with other adaptive immune cell subsets. Finally, the formation of an immune memory is well documented for B cells (as neutralizing Abs persist for decades after CVB encounter), but not for T cells, where exhaustion mechanisms may also be at play. The predicted time course of CVB load and its associated immune responses is shown in the bottom graph. Abbreviations: APCs, antigen-presenting cells; NK, natural killer; NKT, natural killer T; Treg, T regulatory; Tfh: T follicular helper.

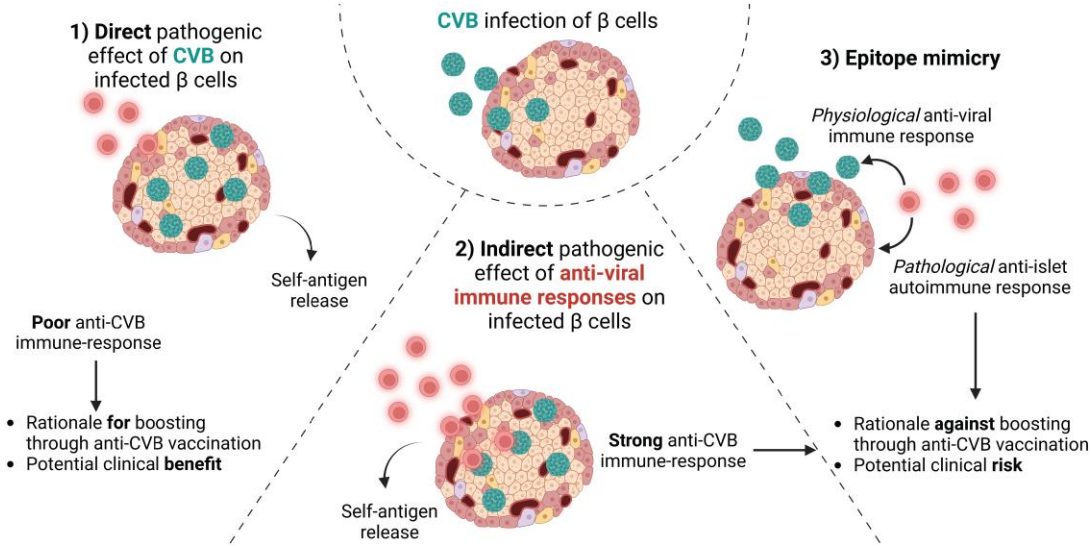


Figure 2. Three hypothetical, non-mutually exclusive mechanisms leading to beta-cell autoimmunity, and implications for T1D prevention by means of CVB vaccination. The direct (primary) pathogenic effects of CVB on infected beta cells are detailed in Fig. 3. The indirect (secondary) pathogenic effects of antiviral immune responses on infected beta cells, including epitope mimicry mechanisms, are detailed in Fig. 4.

1. *Direct* (primary) pathogenic effects of CVB on infected beta cells. These may imply *poor* immune responses unable to efficiently clear the virus.
2. *Indirect* (secondary) pathogenic effects of *antiviral immune responses* on infected beta cells, eg, involving cytotoxic CD8+ T-cell-mediated beta-cell lysis, among others. These may imply *strong* immune responses leading to viral clearance.

Both mechanisms would lead to the release of beta-cell antigens in a proinflammatory environment, which may subsequently trigger islet autoimmunity.

1. *Epitope mimicry*. The physiological antiviral immune response may turn into a pathological autoimmune response against beta cells due to T-cell cross-reactivity between homologous CVB and beta-cell epitopes.

In the following sections, we will review the level of evidence for each of these mechanisms.

Direct Pathogenic Effects of CVB on Infected Beta Cells.

CVB enters cells primarily by binding to surface Coxsackievirus and Adenovirus Receptor (CAR). Both human and murine alpha and beta cells express CAR and are CVB-permissive (43). Moreover, beta cells selectively express a CAR isoform (CAR-SIV), which is localized mainly in secretory granules (44). Hence, these granules may be hijacked by CVB during exocytosis and subsequent recycling, possibly contributing to the high sensitivity of human beta cells to CVB infection. An increased secretory demand on beta cells (eg, during growth spurts) may thus increase their susceptibility to infection, and the infectious and metabolic stress may synergize toward beta-cell demise.

Mouse Studies

Several in vivo experimental models document the lytic effect of CVB on beta cells and its capacity to trigger diabetes without engaging potent antiviral T-cell responses. These effects are dependent on the CVB serotype and the genetic background of the murine host. For instance, infection with CVB3/4 in C57BL/6 mice does not induce diabetes, while it does in 25% of infected SJL and CD1 hosts, independently of T cells (45, 46). This variable susceptibility is reminiscent of that observed with experimental CVB3-induced myocarditis (47). Most strains develop a severe acute myocarditis, but completely recover; few others develop chronic myocarditis associated with anti-cardiac myosin heavy chain aAbs that are also found in humans. This disease can be recapitulated by immunizing mice with cardiac myosin.

A direct lytic effect is also suggested in the multiple low-dose streptozotocin (LD-STZ) model, which triggers autoimmunity by inducing a moderate beta-cell lysis that releases autoantigens in an inflammatory environment. While a single LD-STZ injection was not diabetogenic, its combination with CVB3/4/5 inoculation induced hyperglycemia in CD1 mice (48, 49). Moreover, only a peri-islet insulitis pattern was observed (49), suggesting an accessory role for T cells in mediating beta-cell destruction.

Humanized immunodeficient mouse models further highlighted the beta-cell-lytic effect of CVB infection. These models are based on NOD/*scid*/*gamma* (NSG) mice deprived of endogenous islets by high-dose STZ or diphtheria toxin (DT) treatment (via a rat insulin promoter/DT-receptor transgene) and grafted with human islets (50, 51). Subsequent CVB4 infection led to hyperglycemia in 50% of animals 28 days later. Hyperglycemia was associated with CVB4 RNA and protein persistence in transplanted islets, increased endoplasmic reticulum (ER) stress and a type I IFN gene signature. Notably, pancreas histopathology revealed reduced insulin content but no significant islet destruction.

In spontaneously diabetes-prone nonobese diabetic (NOD) mice, CVB1/CVB3/CVB4 inoculation precipitates diabetes only when applied at the prediabetic stage—starting at 9 weeks of age, when invasive insulitis is already present (52–55). At this stage, CVB replicates efficiently in islets (56). On the contrary, CVB3 or CVB4 inoculation of young (<8 weeks old) NOD mice with minimal insulitis has a preventative effect (52, 53), which is associated with a defective ability of CVB to replicate in islets, despite the detection of high viral titers in islets 1 to 2 days after inoculation and the expression of the CVB receptor CAR (53, 57). This CVB failure to replicate in islets has been associated with the induction of beta-cell-intrinsic IFN responses and HIF-1α expression. Indeed, NOD mice with a beta-cell-specific HIF-1α deficiency display accelerated diabetes upon CVB1/CVB4 infection, accompanied by increased pancreatic viral loads (58). This provides a mechanistic link between the susceptibility or resistance of beta cells to CVB infection and their capacity to sense viral RNA and induce intracellular antiviral, IFN-mediated defense mechanisms. Accordingly, the inhibition of IFN responses with a beta-cell-specific transgene for the suppressor of cytokine signaling-1 (*socs-1*) rapidly induces diabetes after CVB1/CVB3/CVB4 infection, with very few residual insulin-positive cells (59–61). Also in this case, disease onset does not require T cells, as the outcome is similar in immunodeficient *socs-1*-transgenic NOD/*scid* mice (60).

The diabetes protection in young NOD mice vs acceleration at an older age seem at variance with the notion that, in humans, CVB infection may be an early trigger that precedes islet autoimmunity (21, 22). Possible explanations include the fact that the persistent CVB infections that may release a critical self-antigen load in humans are difficult to reproduce in NOD mice, and that the magnitude and/or quality of anti-CVB immune responses may be different. In NOD mice, the requirement for persistent CVB infection may be bypassed by the critical threshold of beta-cell destruction already achieved by autoimmune mechanisms on their own. The relatively high CVB doses used in these infection models, which are administered through the intraperitoneal rather than the natural oral route, are another confounder.

Human Studies

Lytic effects on beta cells

An early study assessed the lytic effect of several CVB and A strains on islet cells (62). The infected outer islet cells died over a few days and detached from islets, leading to smaller structures. No features of apoptosis were observed, rather suggesting necrosis with early chromatin condensation (pyknosis). Nonetheless, some beta cells in close proximity to infected and damaged ones remained virtually intact. This

underlines the heterogeneous outcome of CVB infection (62), possibly reflecting different levels of basal ER stress across beta cells and/or preferential CVB replication in dividing cells and latent infection of quiescent cells (63), a feature shared by many viruses (64, 65).

Non-lytic effects on beta cells

CVB infection impacts beta cells on many other levels. First, the double-stranded (ds)RNA replicative intermediate is recognized by pattern recognition receptors (PRRs) such as toll-like receptor 3 (TLR3), retinoic-acid-inducible gene I (RIG-I) and MDA5/IFIH1 that trigger the production of proinflammatory cytokines, notably type I IFNs. Indeed, children sampled at the time of enteroviral RNA appearance in the blood display an IFN response gene signature, similar to that of peripheral blood mononuclear cells (PBMCs) or islets exposed in vitro to enteroviruses (40). Accordingly, infection of human islets with CVB3, CVB4, or CVB5 induces type I IFN (mostly IFN-β) expression (43, 66). These results suggest that the early blood type I IFN signature of T1D (41, 42) may reflect an antiviral response (40). While this IFN response limits CVB replication and spreading, it also enhances beta-cell apoptosis (43). Of note, 4 rare single-nucleotide polymorphisms that reduce MDA5 function provide T1D protection (67). Additional effects of dsRNA on beta cells have been observed in an in vitro model using the synthetic dsRNA mimic polyinosinic-polycytidyilic acid (polyI:C) on a beta-cell line and on primary human islets (68). PolyI:C downregulated beta-cell-specific genes (eg, *INS*, *G6PC2*, *SLC30A8*, *MAFA*), induced de novo expression of the progenitor-like transcription factor *SOX9* and impaired glucose-stimulated insulin secretion. This gene expression pattern suggests a dedifferentiation process and was recapitulated upon CVB5 infection. This dedifferentiated phenotype seems beta-cell-specific, as glucagon mRNA levels were unaffected in infected human islets (69). These polyI:C effects were triggered by the NF-κB and IFN regulatory factor pathways, and by the secretion of their downstream cytokines IFN-α and tumor necrosis factor (TNF)-α. Indeed, the use of IFN-α, alone or in combination with TNF-α, led to a similar *SOX9* expression (68), suggesting that IFN-α may trigger a vicious cycle by disrupting the identity of neighboring cells in a paracrine fashion.

Other outcomes of CVB infection on different cells include intensive viral protein production, inhibition of host cell protein translation, impaired cellular calcium homeostasis, and ER membrane modifications (70–72). Altogether, these alterations potentiate ER stress, therefore activating the unfolded protein response (UPR). The UPR is a natural cellular response to stress which aims at decreasing the translation rate, increasing the biosynthesis of protein-folding chaperones, and inducing the degradation of misfolded proteins. If the UPR fails to resolve cellular stress, it triggers apoptosis. Given their high insulin synthesis, beta cells have high basal levels of ER stress and naturally adjust the UPR to survive (73). This situation is easily decompensated by triggers such as CVB infection (74). Of further note, the anterograde vesicular trafficking is reduced, concomitantly with increased retrograde trafficking (75). In the highly secretory beta cells, this translates into reduced secretory activity and insulin stores (76), likely imposing an additional stress. The disruption of protein trafficking induced by CVB infection extends to autophagy (77), which is vital for beta cells to dispose of unused insulin granules (78). Like other enteroviruses, CVB3 hijacks the autophagy pathway to replicate into vesicles (77, 79–81). Yet, the step at which CVB disturbs the autophagic processes remains controversial. Indeed, CVB has alternatively been reported to enhance the autophagic flux (80) or to drive autophagosome accumulation by inhibiting the fusion of autophagosomes with lysosomes and endosomes (77, 79, 81). These discrepancies possibly reflect methodological differences, including cell models, infection conditions, and autophagy readouts.

Summing Up

Both mouse and human studies suggest that direct cytopathic effects of CVB infection are major contributors to beta-cell demise. Besides cytolysis, the strategies used by CVBs to hijack cellular pathways and favor its own replication impact beta-cell survival, identity, and insulin secretion. The same is true for the proinflammatory cytokine release triggered by PRRs, which enhances beta-cell apoptosis. These direct, immune-independent lytic and non-lytic effects on beta cells may secondarily trigger islet autoimmunity and are summarized in Fig. 3.

Effects of CVB on Other Cell Types Relevant to T1D

Effects on Other Islet Cells

In alpha cells, several T1D candidate genes regulating antiviral responses display higher expression than in beta cells (82), notably *IFIH1* (83) and its protein product, the dsRNA sensor MDA5 (84). Moreover, IFN-α signaling in alpha cells leads to higher expression of other antiviral factors, eg, *GBP1/3*, *OAS2*, *TRIM22*, *XAF1* (82). Altogether, these gene signatures may explain the observation that alpha cells can clear CVB more efficiently (85).

Effects on the Exocrine Pancreas

A recent report (86) employed a highly sensitive single-molecule-based fluorescent in situ hybridization method to clarify the localization of infected cells in the pancreas. Although enterovirus-positive beta cells were found at higher densities in T1D vs control donors, they were rare and outnumbered by the infected cells found scattered in the exocrine pancreas. Moreover, the exocrine pancreas harbored more infected cells in both T1D and aAb+ donors than in controls. Morphological signs of plasma membrane disintegration were noted in virus-containing cells, suggesting a lytic infection.

Effects on Immune Cells

The above report (86) also documented that most of the scattered infected cells in the exocrine pancreas were of hematopoietic origin (CD45+) or in close proximity to CD45+ cells (possibly suggesting a combination of antiviral immune responses and viral transfer to immune cells), and that infected (CD45+) cells were largely more abundant in the spleen than in the pancreas, and in T1D donors. Other reports previously documented enteroviral RNA in PBMCs (32, 33), mostly localized in APCs (B cells, monocytes, dendritic cells [DCs]) (33, 87), but not in the plasma (33). Interestingly, the prevalence of enterovirus-positive PBMCs was not different between T1D and control adults, while it was higher in

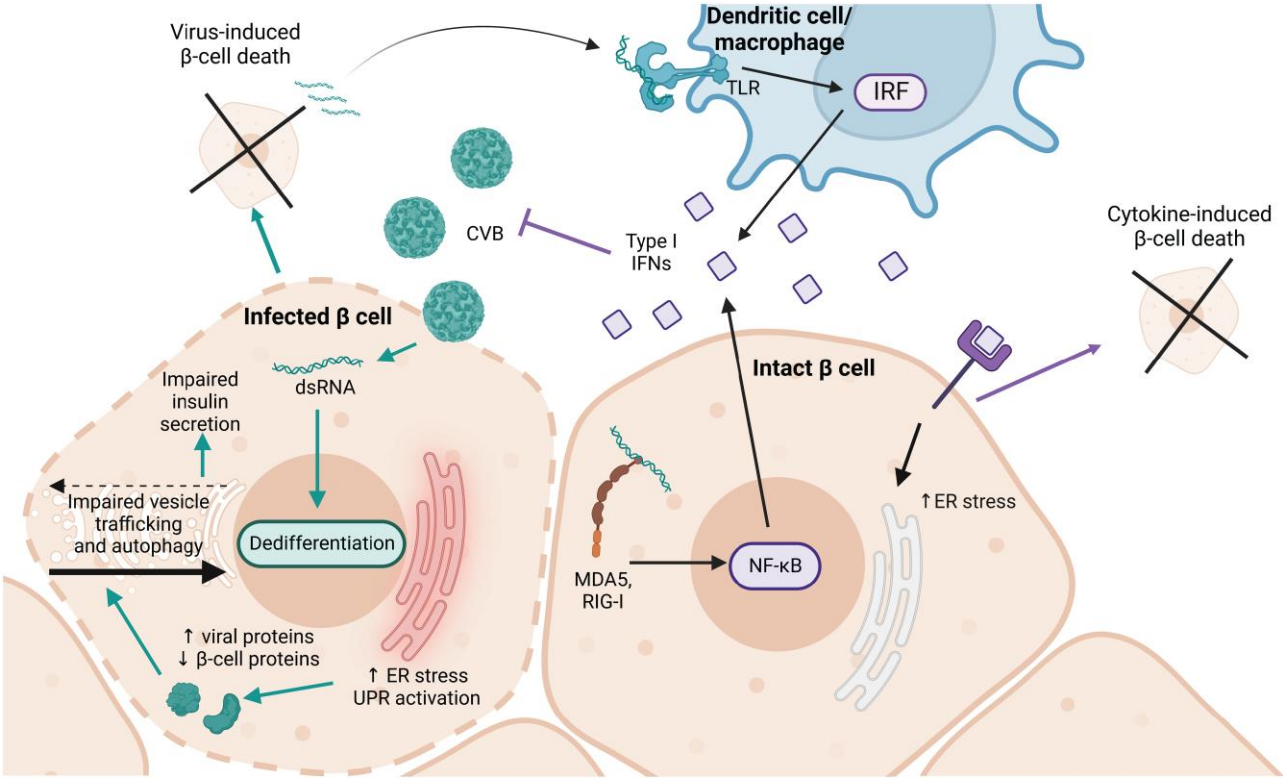


Figure 3. Direct pathogenic (lytic and non-lytic) effects of CVB on infected and neighboring beta cells. Infected beta cells increase the synthesis of viral proteins at the expense of endogenous proteins, resulting in a dedifferentiated phenotype and impaired insulin secretion. Insulin secretion is further impacted by a decreased anterograde and increased retrograde vesicular trafficking. Enhanced endoplasmic reticulum (ER) stress leads to the activation of the unfolded protein response (UPR). Significant beta-cell death ensues, leading to release of dsRNA and viral particles. In antigen-presenting cells like DCs and macrophages, dsRNA sensing through toll-like receptors (TLRs) leads to the activation of IFN response factors (IRF) and to type I IFN release, which inhibits viral replication. Type I IFNs are also secreted by noninfected beta cells sensing dsRNA through intracellular sensors such as MDA5 and RIG-I that activate the NF-κB pathway. The binding of type I IFNs on surface receptors of beta cells enhances ER stress and apoptosis independently of CVB infection.

multiple-aAb+ children considered altogether (ie, with or without stage 3 clinical T1D). The effects elicited by enteroviral infections on immune cells are largely unknown. A mouse study (88) reported that CVB3, although marginally infective on DCs both in vitro and in vivo, diminished their capacity to prime naïve CD8+ T cells in vivo. This correlated with a surge in spleen plasmacytoid DCs and the loss of spleen and pancreatic lymph node conventional DCs, notably of the cross-presenting CD8α+ subset, with neither downregulation of surface major histocompatibility complex (MHC) class I/II and co-stimulatory molecules nor reduced T-cell stimulatory capacity in vitro on a per cell basis. This DC-depleting effect of CVB is more profound than for other viruses and, given the DC resistance to infection, must be indirect, likely mediated by type I IFNs, as described for lymphocytic choriomeningitis virus (89). Together with the lack of infectious permissiveness in DCs, it may represent another efficient immune escape mechanism. In human monocyte-derived DCs, phagocytosis of CVB3-infected islet cells was shown to induce IFN-stimulated genes without ensuing viral replication (90). CVB3 infection in mice further induces a transient T and B lymphopenia, which is also partly mediated by type I IFNs (91).

Effects on Enterocytes

The gut, mostly duodenal enterocytes, is a major entry site for enteroviruses, and provides a viral reservoir that may contribute to persistent infections. Indeed, duodenal biopsies from living T1D patients yielded higher enteroviral titers than PBMCs or pancreas tissues (32). Moreover, enteroviruses are more frequently found in duodenal biopsies from T1D patients (92, 93), although this finding has been questioned (94). Using stem-cell-derived human small intestine enteroids infected with different enteroviruses, including CVB3, a study (95) documented that epithelial cells were infected by CVB3 without inducing either significant cell lysis or antiviral responses, as assessed by the lack of cytokine, chemokine, and IFN-stimulated gene transcripts. Thus, the effects induced by CVB infection at the intestinal entry site are quite different than those induced on beta cells.

Summing Up

Alpha cells are more resistant to CVB infection than beta cells. Enteroviral RNA-positive cells, largely CD45+, are instead abundant in the exocrine pancreas, and more so in T1D and aAb+ donors. In human circulating immune cells, enteroviral RNA is mostly detected in APCs, despite the fact that DCs are poorly permissive to CVB infection. The effects of CVB on human immune cells have not been investigated, although infection in the mouse boosts plasmacytoid DCs and depletes conventional DCs, thus favoring immune escape. Human enterocytes are readily infected but do not undergo lysis nor mount antiviral responses.

Indirect Pathogenic Effects of Antiviral Immune Responses on Infected Beta Cells

Mouse Studies

Innate antiviral responses

CVB3 was unable to precipitate diabetes in NOD mice deficient for the NADPH oxidase, which display reduced superoxide production and impaired M1 (proinflammatory) responses by macrophages (96). This underlines the importance of the inflammatory microenvironment driven by CVB3, independently of the direct, viral-mediated lysis. Conversely, diabetes protection may result from innate immune responses triggered by invariant natural killer T cells (97), which may limit viral spreading.

Ab-mediated antiviral responses

Antiviral neutralizing Abs are important for CVB clearance. Mirroring data in the human (21), CVB infection induces neutralizing Abs in NOD mice (98). These Abs are transferred to the offspring of CVB-infected NOD mice and protect the offspring from infection, and from diabetes development in *socs-1*-transgenic NOD mice (98). As discussed below, an increased risk of developing T1D may be linked to a weak anti-CVB Ab response and lack of protection by maternally transferred neutralizing Abs.

Effects of CVB infection on antigen processing and presentation

Beta-cell and immune-derived IFN responses increase surface MHC class I expression and upregulate several genes of the antigen processing and presentation pathway. This results in an increased beta-cell visibility to islet-reactive, and possibly viral-reactive, CD8+ T cells. Indeed, CVB3 inoculation failed to exacerbate T-cell insulitis and diabetes onset in *tlr3*$^{-/-}$ NOD mice that mount impaired IFN responses (99), despite higher viral titers than in wild-type mice (100).

Following CVB4 infection, while necrosis occurs in neighboring acinar cells but not in islets, beta-cell engulfment by resident APCs is observed in diabetes-resistant immunodeficient NOD/*scid* mice, and these APCs can prime diabetogenic islet-reactive BDC2.5 T cells in vitro (101). In addition, adoptive transfer of macrophages from CVB4-infected NOD/*scid* mice into NOD/BDC2.5 T-cell receptor (TCR)-transgenic animals triggers diabetes (101). This suggests that diabetes may result from the uptake of infected beta cells by APCs and subsequent presentation of islet antigens.

T-cell-mediated antiviral responses

The mechanisms underlying diabetes protection in younger NOD mice following CVB infection are unclear, but increased TGF-β-producing regulatory T cells (Tregs) have been reported (102). In older NOD mice, data point to a role of CVB as a diabetes accelerator through bystander activation of islet-reactive effector T-cell responses and autoimmune beta-cell lysis. Only indirect clues are instead available on whether CVB-reactive T-cell responses are required to trigger diabetes. For instance, CVB4-infected NOD/*scid* mice develop high CVB4 titers but no diabetes (103), suggesting that CVB-mediated beta-cell lysis alone is not sufficient to trigger disease without downstream autoimmune priming. Diabetes is instead triggered when CVB4-infected NOD/*scid* mice are adoptively transferred with islet-reactive BDC2.5 TCR-transgenic T cells (101), suggesting a requirement for islet-reactive but not CVB-reactive T cells. Similarly, CVB4 induces diabetes in BDC2.5 TCR-transgenic NOD mice that are otherwise diabetes-resistant (104).

Collectively, the diabetes acceleration induced by CVB infection in NOD mice does not seem to exclusively rely on a direct, viral-mediated lytic effect on beta cells, but also on the presence of autoreactive T cells and the enhanced presentation of islet antigens by APCs through phagocytosis of infected beta cells. Anti-CVB T-cell responses seem instead dispensable and even protective.

Human Studies

Innate antiviral immune responses

Apart from the beta-cell-autonomous proinflammatory response triggered by CVB infection through PRRs, direct evidence about the innate immune responses that may be triggered by CVB infection in human T1D is very limited. This likely reflects experimental challenges: the question could be addressed either with in vitro coculture systems of human islets and relevant immune subsets or with histopathological studies, but neither approach has been reported to date. Most histopathological studies have rather sought evidence of antiviral responses in beta cells. A recent report from the DiViD study (105) documented that the pancreas from newly diagnosed living T1D donors harbored a small subset of VP1+ beta cells with markedly increased expression of the viral response protein kinase R. An increased islet expression of other viral response proteins, ie, MDA5 and MxA, relative to nondiabetic controls was also noted. A colocalization of IFN response markers (MxA, protein kinase R, and HLA class I) and VP1, together with downregulation of genes in the insulin secretion pathway, was also reported in aAb+ donors (106). An IFN response gene signature coincident with enteroviral RNA detection has also been reported in the blood of children (40). Of note, enteroviruses can mount mechanisms to limit such innate responses, including the formation of replication organelles derived from the host cell membranes that protect viral RNA from sensing by PRRs and viral protease-mediated cleavage of PRRs and their downstream signaling molecules (72, 107).

Ab-mediated antiviral responses

CVB serology has often been used as an indirect readout of viral exposure (21). A recent work by Ashton et al in the group of E. Bonifacio measured neutralizing anti-VP1 Abs to look at the magnitude of response according to islet aAb status (108). The results were striking: anti-VP1 Abs against all 6 major CVB serotypes were absent in children later developing early anti-insulin aAbs, which represent a fast-progressing T1D endotype (5, 109) preferentially associated with CVB1 infection in another study (110). This contrasted with the detection of anti-VP1 Abs in children without islet aAb seroconversion and in those positive for anti-glutamic acid decarboxylase (GAD) aAbs at a later age. A caveat of this study is that, although anti-VP2 Abs were detected in anti-VP1 Ab-negative children, no direct evidence of prior CVB infection was provided. Hence, the lack of anti-VP1 Abs could also reflect an absence of viral exposure. Nonetheless, these results may suggest that weak anti-CVB Ab responses predispose to islet autoimmunity. This scenario would help explain the epidemiological paradox that T1D incidence is higher where

CVB exposure is lower. Indeed, CVB exposure drastically dropped over the last 40 years, while T1D incidence steadily increased (111). Also, geographically (111, 112), the world highest T1D incidence of Finland contrasts with its very low CVB circulation. This paradox has led to the "poliovirus hypothesis" (113, 114), postulating that this scenario may be similar to that of the beginning of the twentieth century, when reduced poliovirus circulation due to improved sanitary conditions was paralleled by an increased incidence of its severe form, poliomyelitis (115). The explanation proposed is that a low frequency of CVB infection in the background population leads to decreased herd immunity and transmission of protective maternal Abs. Children are thus less protected during the first years of life and tend to develop higher-titer CVB infections, which favor viremia and viral spreading to vulnerable organs such as the heart or the pancreas (or motor neurons in the case of poliovirus). It would be informative to know whether the incidence of CVB myocarditis has also increased during the last decades, but the improved diagnostic workup, which identified CVB as a major etiologic agent (116), introduces a major bias.

Effects of CVB infection on antigen processing and presentation

The CVB-induced disruption of membrane trafficking (decreased anterograde flux and increased endocytosis) that perturbs insulin secretion also represents an immune evasion mechanism. This disruption results in the internalization of surface MHC class I molecules and limits presentation of newly formed complexes, notably of viral peptides (75). It is plausible that internalized complexes are returned to the cell surface, circumventing natural killer cell detection (117). This mechanism likely involves the internalization of other surface molecules such as cytokine receptors.

Moreover, CVB infection distinctively alters the antigen processing and presentation pathway, as observed in CVB3-induced myocarditis models (118). Also in this case, self-limited viral myocarditis can lead to loss of tolerance to cardiac antigens and autoimmunity (47). Infection induces IFN-α expression, which upregulates MHC class I and promotes the switch from the constitutive proteasome to immuno-proteasome (118). The immuno-proteasome has different cleavage preferences to process peptides for the MHC class I pathway, thus generating more antigenic peptides for cell surface presentation. In vivo studies revealed that CVB3-susceptible mouse strains display a longer and higher immuno-proteasome expression postinfection (118) along with higher expression of genes of the antigen processing and presentation pathway. However, most proteasomal enzymatic activities were reduced upon infection (118), suggesting the existence of other immune escape mechanisms to counteract the IFN-α effects. Similar processes might be at play in infected beta cells to limit antiviral T-cell responses.

T-cell-mediated antiviral responses

A major gap in knowledge concerns the features of anti-CVB T-cell responses, which is largely due to the lack of information about the viral epitopes recognized. A first report by M. Atkinson in 1994 (119) identified an antigenic CVB region by first analyzing T-cell responses against GAD in unfractionated PBMCs stimulated with overlapping peptides. While these responses were detected in both T1D and healthy donors, a $GAD_{247\text{-}279}$ region was preferentially recognized in T1D and at-risk individuals compared to healthy controls. This region harbors a significant homology with the P2C protein of CVB (the so called PEVKEK region), and this homologous CVB sequence was recognized by the same donors. Although this suggests the possibility of epitope mimicry and T-cell cross-reactivity, formal proof at the single T-cell level and whether of the CD4+ or CD8+ subset was not provided. Indeed, a subsequent study by Schloot et al (120) using CD4+ T-cell clones dismissed this possibility.

Anti-CVB CD4+ T-cell responses are likely to be elicited upon CVB infection, as they are required to drive B-cell activation and Ab production. Using individual recombinant CVB proteins, Varela-Calvino et al (121) reported that the structural proteins VP1, VP2, and VP3 were preferentially targeted by T cells (likely CD4+ T cells in the unfractionated PBMCs used in these assays), and that T1D patients harbored lower frequencies of proliferative responses against VP2 but stronger IFN-γ responses against VP3 and P2C. Another study (122) identified human CD4+ T-cell responses against poliovirus VP2 and VP3 peptides selected from regions conserved across enteroviruses.

CVB infection is also likely to recruit CD8+ T cells, as they are key players of viral clearance via the cytotoxic destruction of infected cells. Using HLA-A2 binding prediction algorithms, Varela-Calvino et al (123) identified a $CVB4_{1137\text{-}1145}$ epitope (EVKEKHEFL) located in the same P2C region described by Atkinson et al. This peptide was naturally processed and presented by protein-pulsed APCs and recognized by IFN-γ-producing CD8+ T cells from 2 of 3 HLA-A2+ healthy donors tested. While T-cell lines raised against this epitope were cytotoxic against peptide-pulsed target B cells, there was no evidence of cross-reactivity with the homologous $GAD_{261\text{-}269}$ sequence (EVKEKGMAA). Another study (124) identified an HLA-A2-restricted epitope (ILMNDQEVGV) largely conserved across serotypes that was naturally processed and presented by CVB3-infected PBMCs and recognized by 25% of tested donors. Responses against an EVREKHEFL variant of the epitope previously described by Varela-Calvino et al were not detected. CD8+ T-cell responses, quantified by either IFN-γ ELISpot or tetramer/IFN-γ staining, were overall weak, requiring a prior 12-day in vitro PBMC sensitization. Using more sophisticated in silico approaches, additional HLA-A2-restricted CVB epitopes were proposed, but cognate CD8+ T-cell responses detected by IFN-γ ELISpot were marginal (125).

Finally, like other viruses, CVB can also mount mechanisms to escape T-cell recognition, such as downregulating surface HLA class I expression (75, 117). It is unknown whether HLA class II expression is also downregulated. This is part of a more general immune evasion mechanism by which CVB inhibits protein trafficking (126), thus limiting the routing toward the cell surface of multiple immune receptors and the secretion of soluble immune mediators (127, 128). Another open question is whether CVB persistence may favor the induction of CD8+ T-cell exhaustion and, ultimately, poor antiviral memory (129–131), and whether viral persistence is a common outcome following an acute CVB infection.

Summing Up

There is very limited information about the anti-CVB CD4+ and CD8+ T-cell responses mounted upon infection that

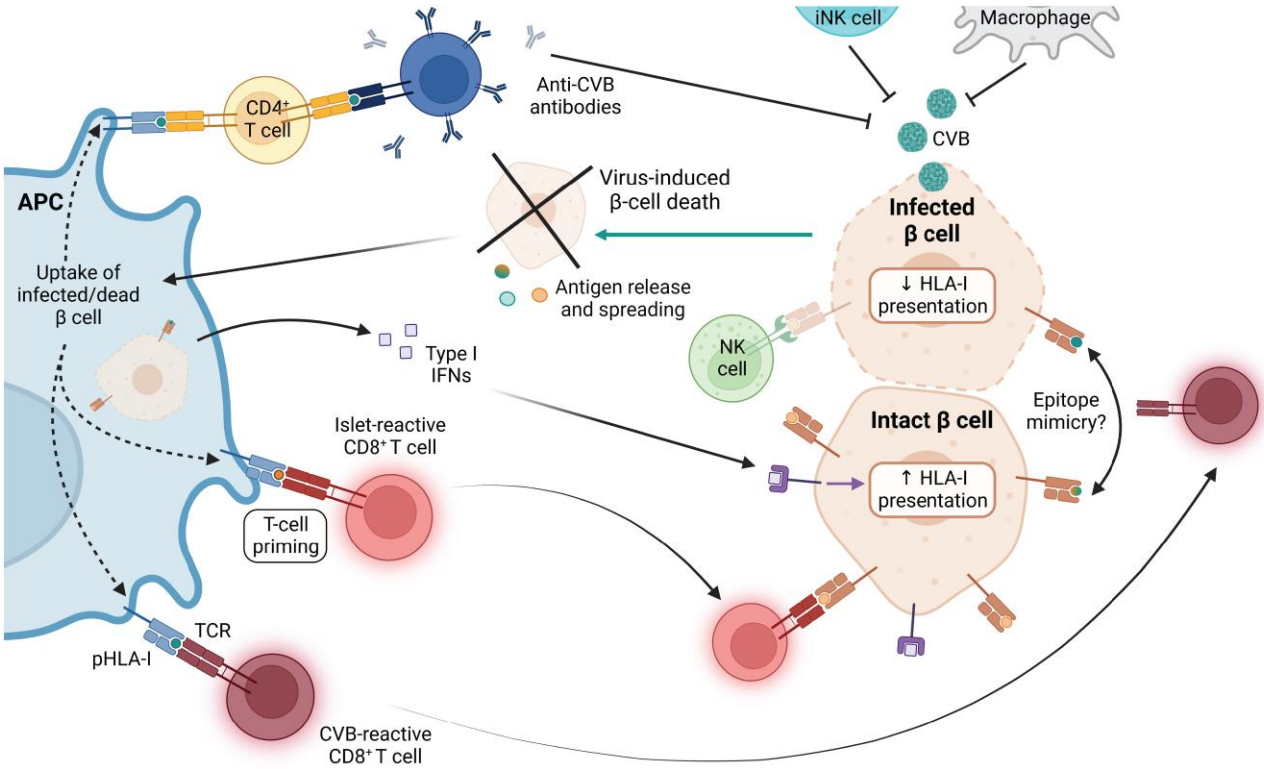


Figure 4. Indirect immune-mediated pathogenic effects of CVB infection on beta cells. Infected beta cells downregulate surface HLA class I (HLA-I) expression, thus providing an immune escape mechanism by limiting recognition by antiviral CD8+ T cells and, possibly, enhancing recognition by natural killer (NK) cells. Infected beta cells are lysed and antigens are taken up by antigen-presenting cells (APCs), thus favoring priming of both islet-reactive and CVB-reactive CD4+ and CD8+ T cells. CD4+ T-cell activation provides help to B cells for anti-CVB antibody production, which inhibits viral replication along with invariant (i)NK cells and macrophages. APC activation also leads to type I IFN secretion, which upregulates surface HLA-I presentation on beta cells, favoring recognition by islet-reactive CD8+ T cells of beta-cell endogenous epitopes, including neo-epitopes generated under these inflammatory conditions. CVB-reactive CD8+ T cells may also be diverted toward recognition of noninfected beta cells if epitope mimicry mechanisms are at play.

may secondarily cause beta-cell damage. This gap in knowledge reflects the lack of reliable epitopes to track them. Mouse studies support a role for autoimmune T cells, while antiviral T cells might be protective rather than harmful. The overall indirect, immune-mediated effects targeting beta cells that may secondarily trigger islet autoimmunity are summarized in Fig. 4.

Epitope Mimicry

Mouse Studies

CVB/GAD homologous peptides can be presented by the diabetes-predisposing I-A^{g7} MHC class II allele of NOD mice (132). Although T-cell cross-reactivity was demonstrated by in vitro functional assays, in vivo evidence that CVB-reactive T cells can also recognize GAD peptides and amplify autoimmune anti-GAD T-cell responses or transfer diabetes is lacking. Indeed, T cells isolated from CVB4-infected NOD mice neither exhibited increased proliferation to GAD protein or homologous GAD/CVB peptide, nor triggered diabetes (104). Molecular similarities were also identified between the VP1 capsid protein of CVB and the T1D autoantigens tyrosine phosphatases IA-2 and IAR, and sera from NOD mice inoculated with CVB4 showed some cross-reactivity with a IAR peptide (133). However, whether such mechanism can trigger autoreactive T cells to attack beta cells has not been demonstrated.

Human Studies

Despite some initial enthusiasm on the possibility of T-cell cross-reactivity between CVB and GAD peptides mapping to the PEVKEK region (119), subsequent reports dismissed this possibility for both CD4+ (120) and CD8+ T cells (123). Cross-reactive responses between a Rotavirus $VP7_{16\text{-}49}$ region and sequences from the IA-2 and GAD islet antigens encompassing both CD4+ and CD8+ epitopes have also been described (134).

Summing Up

Although molecular mimicry is an intriguing scenario, the evidence for a CVB cross-reactivity with islet antigens that may underlie this hypothetical mechanism is limited and conflicting. Moreover, the evidence for a causal role of this cross-reactivity in triggering islet autoimmunity is missing altogether, in both humans and mice. These putative molecular mimicry mechanisms are summarized in Fig. 4 together with the other immune-mediated effects of CVB infection on beta cells.

Implications for T1D Prevention Strategies

Despite the strength of evidence for an association between CVB infections and islet autoimmunity, demonstration of a cause-effect relationship is lacking, and is likely to remain elusive until tested in the human by removing this candidate

environmental trigger (13). The most effective way to achieve this would be through vaccination against CVB. This could allow to prevent the most common manifestations of this infection (common cold) and, more importantly, its rare but severe complications (myocarditis, encephalitis, meningitis). This is similar to what has been achieved by Rotavirus vaccination in several countries. Following this rationale, a formalin-inactivated nonadjuvanted CVB vaccine has been developed. Preclinical studies using a prototype CVB1 vaccine were performed in BALB/c and NOD mice (54). High titers of neutralizing Abs were induced. While nonimmunized NOD mice displayed accelerated diabetes after CVB1 infection, this was not the case in vaccinated animals, suggesting that the vaccine itself does not accelerate diabetes development. A subsequent study in CVB1-infected *socs-1*-transgenic NOD mice, which harbor beta cells that are unable to respond to IFNs and thus develop diabetes due to massive beta-cell destruction, documented protection against both CVB1 infection and subsequent CVB1-induced diabetes (61). A hexavalent version of the vaccine comprising the 6 CVB serotypes was subsequently shown to induce strong neutralizing Ab responses without adjuvant in both mice and nonhuman primates, and it provided immunity and protection against CVB-induced myocarditis and diabetes (135). In NOD mice, the vaccine did not accelerate spontaneous diabetes, while it delayed diabetes acceleration upon CVB1 infection (55).

On these grounds, a formalin-inactivated nonadjuvanted pentavalent intramuscular vaccine comprising the 5 most common serotypes (CVB1 to CVB5, ie, barring the less prevalent CVB6) is undergoing a phase I safety trial in CVB-seronegative and -seropositive healthy adults (NCT04690426). Early timing of vaccination will be critical for subsequent trials in order to intervene before CVB exposure, eg, starting at 2 months of age as for the inactivated polio (Salk) vaccine. This will also require an excellent safety profile. In this perspective, it is important to gain further insights into the mechanisms by which CVB infection may trigger beta-cell autoimmunity. Such mechanisms hold implications for the clinical benefit that may be expected (Fig. 2). If beta-cell destruction is mainly provoked by the secondary pathogenic effect of antiviral immune responses on infected beta cells, vaccination may increase such responses and accelerate beta-cell destruction. The same could be true if epitope mimicry mechanisms contribute to this destruction, unless the viral sequences at play are excised from the vaccine constructs. While this possibility assumes that CVB may still reach the pancreas in vaccinated individuals, preclinical studies demonstrate that the vaccine efficiently prevents infection ab initio, including viremia and systemic spreading (54, 55, 135). Conversely, the scenario of a primary pathogenic effect of CVB on infected beta cells leading to self-antigen release and autoimmune priming, possibly associated with poor anti-CVB immune responses, would lend a strong rationale for boosting these responses by vaccination.

Conclusions and Perspectives

Recent advances in our understanding of the association between CVB infections and islet autoimmunity, and between Epstein-Barr virus and multiple sclerosis, invite us to move one step further to definitely prove or disprove causality and explore preventative interventions through antiviral vaccination. Several lines of evidence support the possibility of persistent CVB infection in individuals later developing islet autoimmunity. This persistent infection status is located in beta cells, in the exocrine pancreas as well as in the gut, and is favored by multiple immune escape mechanisms mounted in different immune and non-immune cell types. While this provides a strong rationale for preventing such infections ab initio, it might also encourage efforts to eradicate them by antiviral treatment. In both cases, the timing of intervention will be critical, since association studies highlight a temporal sequence where CVB infection is an early event preceding aAb seroconversion and autoimmune initiation. Whether autoimmune progression can be halted by viral eradication once initiated remains uncertain. A better knowledge of the immune responses elicited by CVB infections and vaccines is vital to optimize vaccination strategies and their risk/benefit ratio. On one hand, available evidence suggests that direct CVB-induced cytopathic effects are major contributors to beta-cell demise, which may be favored by poor immune responses unable to efficiently clear the virus. On the other hand, there is very limited information about the anti-CVB T-cell responses mounted and about the epitopes targeted upon infection. These responses may secondarily cause damage by recognizing viral antigens exposed by infected beta cells and, possibly, homologous self-antigens on noninfected beta cells. The magnitudes of these antiviral responses and whether they are eventually protective or harmful for beta cells deserve further scrutiny. Elucidating their dynamics will also provide immune monitoring tools and surrogate markers to predict vaccination efficacy and, hopefully, protection from T1D.

Acknowledgments

Figures were created with BioRender.com.

Funding

Work performed in the Laboratory of R.M. and M.F.T. was supported by JDRF nPOD-Virus grant 3-SRA-2017-492-A-N. The Laboratory of R.M. and S.Y. acknowledges the support of grants from Agence Nationale de la Recherche (ANR-19-CE15-0014-01), Fondation pour la Recherche Medicale (EQU20193007831), and from The Leona M. and Harry B. Helmsley Charitable Trust to INSERM; and from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreements 115797 and 945268 (INNODIA and INNODIA HARVEST), which receive support from the EU Horizon 2020 program, the European Federation of Pharmaceutical Industries and Associations, JDRF, and The Leona M. & Harry B. Helmsley Charitable Trust. The laboratory of M.F.T. is supported by The Swedish Child Diabetes Foundation, The Swedish Diabetes Foundation, The Swedish Research Council (#2020-02969), The Swedish Heart and Lung Foundation (#20200682), Novo Nordisk Foundation, Denmark, and Karolinska Institutet, including the Strategic Research Program in Diabetes.

Disclosures

A.C., F.V., and S.Y. have nothing to declare. M.F.T. consults for the scientific advisory board of Provention Bio Inc. R.M. receives research service support (April 15, 2022 to April 14, 2024) from Provention Bio Inc.

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