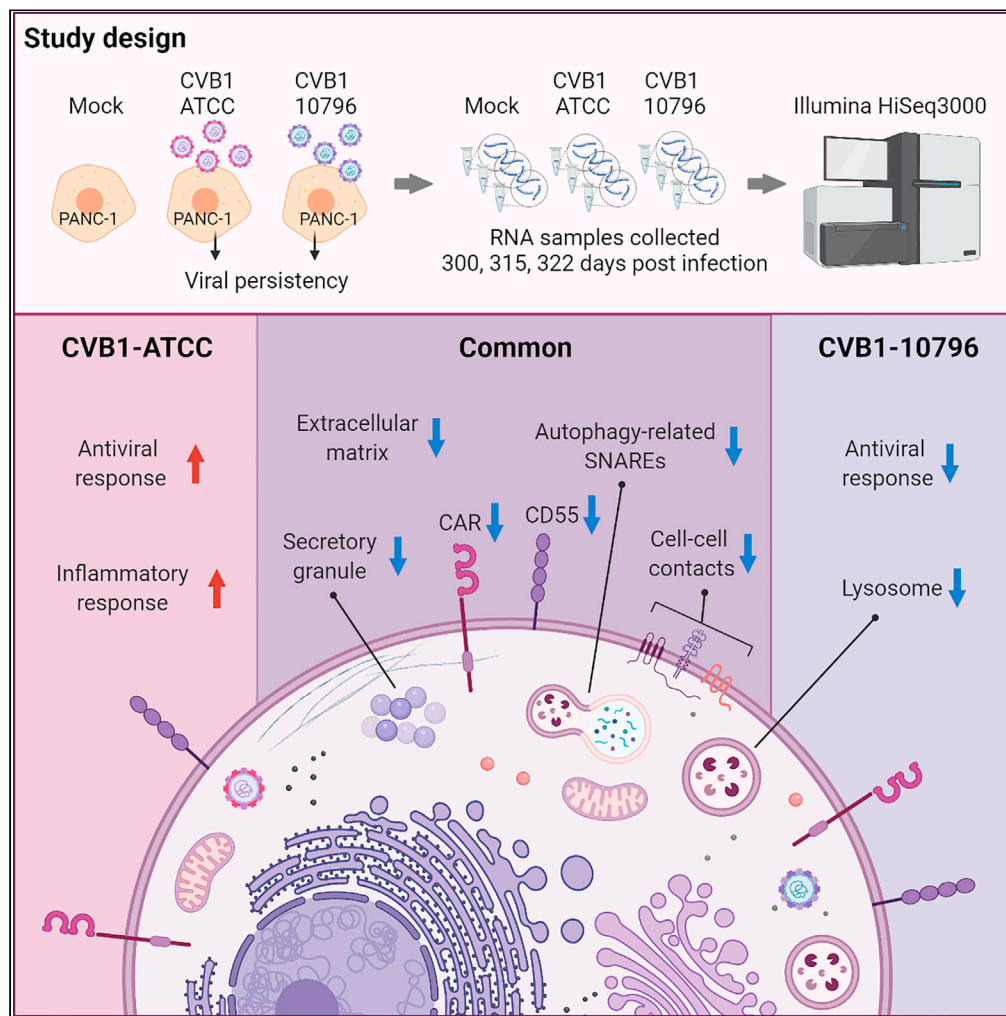


Article

Persistent coxsackievirus B1 infection triggers extensive changes in the transcriptome of human pancreatic ductal cells



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Highlights

Establishment of persistent CVB1 infection in PANC-1 cells using two CVB1 strains

Extensive transcriptional responses in persistently CVB1-infected pancreatic cells

Changes in pancreatic microenvironment, secretory pathway, and lysosomes

Antiviral immune response was activated differently by the two CVB1 strains

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Article

Persistent coxsackievirus B1 infection triggers extensive changes in the transcriptome of human pancreatic ductal cells

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SUMMARY

Enteroviruses, particularly the group B coxsackieviruses (CVBs), have been associated with the development of type 1 diabetes. Several CVB serotypes establish chronic infections in human cells *in vivo* and *in vitro*. However, the mechanisms leading to enterovirus persistency and, possibly, beta cell autoimmunity are not fully understood. We established a carrier-state-type persistent infection model in human pancreatic cell line PANC-1 using two distinct CVB1 strains and profiled the infection-induced changes in cellular transcriptome. In the current study, we observed clear changes in the gene expression of factors associated with the pancreatic microenvironment, the secretory pathway, and lysosomal biogenesis during persistent CVB1 infections. Moreover, we found that the antiviral response pathways were activated differently by the two CVB1 strains. Overall, our study reveals extensive transcriptional responses in persistently CVB1-infected pancreatic cells with strong opposite but also common changes between the two strains.

INTRODUCTION

Enteroviruses are among the most common human pathogens worldwide. They are non-enveloped viruses with a positive single-stranded RNA genome. The Group B coxsackieviruses (CVBs) belong to the *Enterovirus* genus and consist of six different CVB serotypes (CVB1–6) (Tuthill et al., 2010). The life cycle of the CVBs depends entirely on the host cell system and begins with virus attachment to host cell receptors. Upon entry into the cytoplasm, the viral genome is transcribed and translated, followed by the release of mature virions via host cell lysis or non-lytic pathways, such as egress in vesicular structures or directly via cellular protrusions (Laitinen et al., 2016; Netanyah et al., 2020; Paloheimo et al., 2011; Robinson et al., 2014; Wernersson et al., 2020).

CVB infections are typically acute, ranging from the common cold to severe diseases, such as encephalitis, meningitis, myocarditis, hepatitis, hand-foot-and-mouth disease, and pancreatitis (Tapparel et al., 2013). Especially, young children are at the highest risk of developing severe CVB diseases (Tebuegge and Curtis, 2009). However, CVBs also establish persistent infections (Chapman and Kim, 2008; Pinkert et al., 2011; Sane et al., 2013) that have been linked to the pathogenesis of certain chronic diseases, including chronic cardiomyopathies and type 1 diabetes (T1D) (Hyöty, 2016; Massilamany et al., 2014; Nurminen et al., 2012). The mechanisms leading to CVB persistence are not understood, but it most likely involves co-evolutionary development of the host cell and the virus (Alidjinou et al., 2017; Pinkert et al., 2011).

Based on *in vitro* and *in vivo* studies, there are two proposed types of persistent enterovirus infections, a steady-state and a carrier-state persistency. Steady-state persistency is associated with non-lytic replication in which nearly all the cells are infected (Frisk, 2001). In a carrier-state infection, high titers of virus are produced from only a small subpopulation of infected cells (Alidjinou et al., 2017; Pinkert et al., 2011). Carrier-state persistent CVB4 infections have been established in pancreatic duct cells (PANC-1). In these models, mutations in the viral genome and the downregulation of the major CVB receptor, coxsackievirus and adenovirus receptor gene (CXADR), possibly contribute to establishment of persistency

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(Alidjinou et al., 2017; Sane et al., 2013). Furthermore, CVB strains differ in their ability to induce innate immune responses (Hämäläinen et al., 2014; Lietzén et al., 2019; Ylipaasto et al., 2012) that might also influence viral persistence in infected cells.

T1D is one of the most common autoimmune diseases among children. Complex interactions of genetic and environmental factors trigger the onset of autoimmune mechanisms responsible for the development of autoimmunity to beta cell antigens and subsequent selective destruction of insulin-producing beta cells in the pancreas. Enterovirus infections may contribute to islet autoimmunity, potentially via molecular mimicry, inflammation, endoplasmic reticulum stress, and host immune responses, such as T cell activation or suppression, which are detrimental to beta cell function and survival (Dunne et al., 2019). Several epidemiological studies suggest that CVBs contribute to the development of T1D (Dahlquist et al., 1989; Frisk et al., 1985; Graves et al., 2003; Green et al., 2004; Hyoty et al., 1995). In addition, high expression of CXADR in insulin-producing beta cells may facilitate the entry of CVBs into these cells (Ifie et al., 2018). Both enteroviral RNA and viral capsid protein VP1 have been detected at low levels in beta cells of patients with T1D (Krogvold et al., 2015; Richardson et al., 2009, 2013). The low amount of virus in the pancreatic islets of patients with T1D implies a low-grade enteroviral persistent infection rather than acute infection (Krogvold et al., 2015). This was further supported by our recent study that revealed low levels of enterovirus strains in the purified islets that were different from the strains simultaneously detected in stools, blood, duodenal biopsy, and/or the whole pancreas tissue in the same patient with T1D (Oikarinen et al., 2021). The association between CVBs and T1D has been emphasized by prospective birth cohort studies (Laitinen et al., 2014; Oikarinen et al., 2014; Sioofy-Khojine et al., 2018; Vehik et al., 2019). This association was linked to CVB infections that occurred before the appearance of islet autoimmunity, suggesting their contribution to the initiation of islet autoimmunity. A recent prospective study showed that the risk of islet autoimmunity was associated with a prolonged course of these infections, again suggesting that viral persistence is an important factor in the pathogenesis of T1D (Vehik et al., 2019). Nevertheless, the exact mechanism of how CVB persistent infection triggers damage to the insulin-producing pancreatic beta cell is still to be clarified.

Here we studied the transcriptome of human pancreatic cells during persistent CVB1 infections to reveal CVB-triggered changes in cell physiology. For this purpose, a carrier-state-type persistent infection model was established in PANC-1 cells with two strains of CVB1. CVB1 is one of the enterovirus serotypes most commonly linked to the development of T1D (Laitinen et al., 2014; Oikarinen et al., 2014; Sioofy-Khojine et al., 2018). We previously observed that various CVB1 strains differ in their ability to trigger antiviral responses *in vitro* (Hämäläinen et al., 2014) and thus hypothesized that the “viral immunophenotype” influences the diabetogenicity of individual virus strains. In our recent study, we reported significant alterations in the proteome and secretome of PANC-1 cells during persistent CVB1 infections (Lietzén et al., 2019). In addition, CVB1 adapted to the carrier-state persistence, as reflected by accumulation of mutations in the viral genome, with one dominant mutation in the VP1 protein detected in all persisting CVB1 strains (Honkima et al., 2020). Here, we used the transcriptomics approach to identify infection-associated molecular pathways in persistently CVB1-infected pancreatic cell line. We observed extensive changes in the gene expression of factors associated with the pancreatic microenvironment, the secretory pathway, and lysosomal biogenesis, during persistent CVB1 infections. In accordance with our recent proteomics study (Lietzén et al., 2019), we gained deeper insights into immune-related genes that are differently affected by the two CVB1 strains. Overall, our study reveals extensive transcriptional responses in pancreatic cells persistently infected with CVB1 that could be important for the development of viral persistence and T1D.

RESULTS

Carrier-state-type persistent coxsackievirus B1 infection changes the transcriptome of pancreatic cell line

Two carrier-state-type persistent CVB1 infection models were established in human pancreatic ductal cell lines (PANC-1) with two virus strains, the ATCC strain Conn-5 and the wild-type strain 10796, as reported recently (Lietzén et al., 2019). RT-qPCR showed constant existence of viral RNA in cell culture supernatants. The presence of viral capsid protein VP1 in PANC-1 cells was shown by IHC, and viral peptides were also detected by mass spectrometry one year after the initial infection (Lietzén et al., 2019). Here we also show the detection of the viral capsid protein VP1 by immunoblot analysis in both persistent infection models at three time points at 300–322 days after the initial infection (Figure 1A). Immunofluorescence staining confirmed the establishment of a carrier-state-type persistent CVB1 infection in PANC-1 cells, by showing a small subpopulation of infected cells strongly positive for VP1 protein, thus indicating active replication of the virus (Figure 1B).

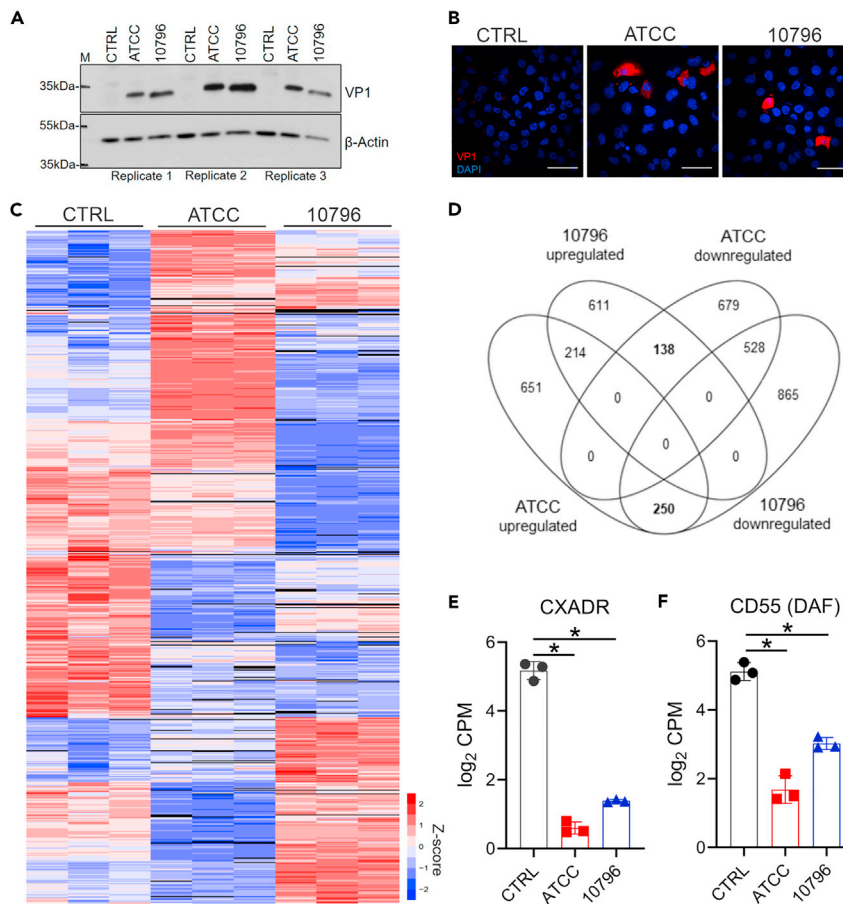


Figure 1. Persistent carrier-state-type CVB1 infection revealed changes in the transcriptome of pancreatic cell line

(A and B) Detection of viral capsid protein VP1 in non-infected PANC-1 cells and cells with persistent CVB1-ATCC and 10796 infections by immunoblotting at 300, 315, and 322 days post infection with β -Actin as loading control (M, molecular weight marker) and (B) by immunofluorescence. Representative cell images show VP1 protein in red and nuclei in blue (DAPI); scale bar, 50 μ m.

(C) Heatmap of Z score standardized expression for the 3,936 differentially expressed genes in the persistent CVB1 infection models with false discovery rate (FDR) of <0.05 and fold-change (FC) ≥ 2 .

(D) Venn diagram demonstrating the number of overlapping up- and downregulated genes in cells with persistent CVB1-ATCC and 10796 infections.

(E and F) The \log_2 transformed counts per million (CPM) expression of CXADR and CD55 are shown as mean \pm SD for three biological replicates (* FDR <0.05).

To assess global changes in host gene expression during persistent CVB1 infection, RNA samples for sequencing were also collected within the same time window. Persistent CVB1 infections were associated with changes in the expression of 3,936 genes using a false discovery rate (FDR) of <0.05 and fold-change (FC) ≥ 2 (Figures 1C and 1D). Among these, 1,864 genes were upregulated, including 214 genes that were commonly upregulated in both persistent CVB1 infection models. Moreover, persistent CVB1 infection was associated with the downregulation of 2,460 genes with 528 genes suppressed in both persistent CVB1 infection models. Interesting, the expression of 388 genes changed in the opposite direction between the two persistent CVB1 infection models (Figure 1D), which is in line with the differences observed at the protein level between these persistent infection models (Lietzén et al., 2019).

In our earlier proteomics study, the protein expression of the coxsackievirus and adenovirus receptor (CAR), a tight junction protein that facilitates CVB entry into host cells, was only detected in the non-infected PANC-1 cells and was below the detection limit in the PANC-1 cells with persistent CVB1 infection (Lietzén et al., 2019). The current RNA sequencing data confirm the strong downregulation of

coxsackievirus and adenovirus receptor gene (CXADR) in both persistent infection models (Figure 1E). Downregulation of the CXADR is a common feature of carrier-state persistent CVB infections (Alidjinou et al., 2017; Pinkert et al., 2011). We also showed the downregulation of GPI-anchored protein decay-accelerating factor (DAF or CD55), another host cell-surface receptor utilized by some of the CVBs as a co-receptor molecule (Figure 1F). Besides the detection of the viral VP1 protein, active replication of the virus was further supported by the significant upregulation of genes associated with lipid metabolic and cholesterol biosynthetic processes in both persistent infection cell models (Table S1). Together, these results indicate that the successful establishment of a carrier-state-type persistent CVB1 infection model in PANC-1 cells led to extensive changes in the transcriptome of persistently infected PANC-1 cells.

Persistent infections by two strains of CVB1 have contrasting effects on cell's innate immune system

In our recent proteomics study, we showed that the expression of antiviral immune response proteins clearly differed between these two persistent CVB1 infection models (Lietzén et al., 2019). In particular, persistent infection with the CVB1 strain ATCC induces, whereas strain 10796 shuts down, the host antiviral immune responses in persistently infected PANC-1 cells. In the current study, we confirmed clear differences in the innate immune system activation by the two CVB1 strains during persistent infection. Furthermore, gene expression profiling provided deeper insights into innate immunity-related factors that were strongly modulated during persistent CVB1 infections.

Gene ontology (GO) enrichment analysis showed that many of the genes associated with the inflammatory response and virus replication were upregulated during persistent infection with the ATCC strain of CVB1, whereas CVB1-10796 significantly suppressed the expression of these genes (Figure 2A, Table S2). Genes with opposite expression patterns between the two strains formed a highly interconnected network of antiviral immune response, including the cytosolic viral ssRNA/dsRNA sensors IFIH1 and DDX58, the IFN-induced antiviral effectors (e.g., DDX60, MX1, TMEM173), interferon-stimulated genes (ISGs), and the members of the oligoadenylate synthase family (OAS) (Figure 2B).

Furthermore, members of the poly(ADP-ribose) polymerases (PARP) superfamily genes, including PARP9 and PARP14, together with the Deltex E3 ubiquitin ligase 3L (DTX3L), were strongly upregulated in the persistent CVB1-ATCC infection model but downregulated in the CVB1-10796 model (Figure 2B). Based on Ingenuity Pathway Analysis (IPA), PARP9 and its downstream targets, which are associated with antiviral host defense (e.g., IFIT1, IFIT2, IFIT3, IFI44, ISG15, and OAS2) were also significantly enhanced in the CVB1-ATCC model (Figure 2C), but suppressed in the CVB1-10796 model (Figure 2D). These antiviral genes were shared with the downstream targets of interferon lambda-1 (IFNL1). IFNL1 and 23 of its downstream targets were upregulated in PANC-1 cells persistently infected with the CVB1-ATCC strain (Figure 2C), whereas 29 target genes were suppressed in the CVB1-10796 infection model, including IFNL1 itself (Figure 2D).

Moreover, several other genes associated with innate immunity and inflammatory responses were regulated in opposing directions in the two persistent CVB1 infections (Figure S1). In particular, persistent CVB1-ATCC infection in PANC-1 cells induced the transcriptional activation of several genes belonging to tumor necrosis factor responses (TNFRSF9, TNFRSF18, TNFRSF1B), early components of the complement activation pathway (C1R, C1S, C1RL, CFB), and endoplasmic reticulum aminopeptidase 2 (ERAP2) involved in trimming of MHC Class I-presented peptides (Figure S1). On the contrary, these genes were significantly downregulated during persistent CVB1-10796 infection (Figure S1). In addition, proinflammatory cytokines CCL5, IL32, and IL34 were significantly increased in the CVB1-ATCC infection model, whereas significantly lower levels of CCL5, IL34, and IL18 were observed during persistent CVB1-10796 infection, as compared with control cells (Figure 2E). Of note, the chemokine CXCL5 that recruits immune cells to an inflammatory site was upregulated in CVB1-10796, whereas it was downregulated during persistent CVB1-ATCC infection (Figure 2E). The macrophage colony stimulating factor 1 (CSF1), a cytokine that has been reported in the acute state of CVB3-induced myocarditis in mouse (Meyer et al., 2018), was enhanced in both persistent CVB1 infection models (Figure 2E).

Genes associated with pancreatic beta cell communication and function are strongly affected in persistent CVB1 infection

Cell adhesion is the primary feature of the architecture of many tissues and performed via cell-cell and cell-matrix junctions. These specialized junctions consist of clustered cell-adhesion proteins on the cell surface.

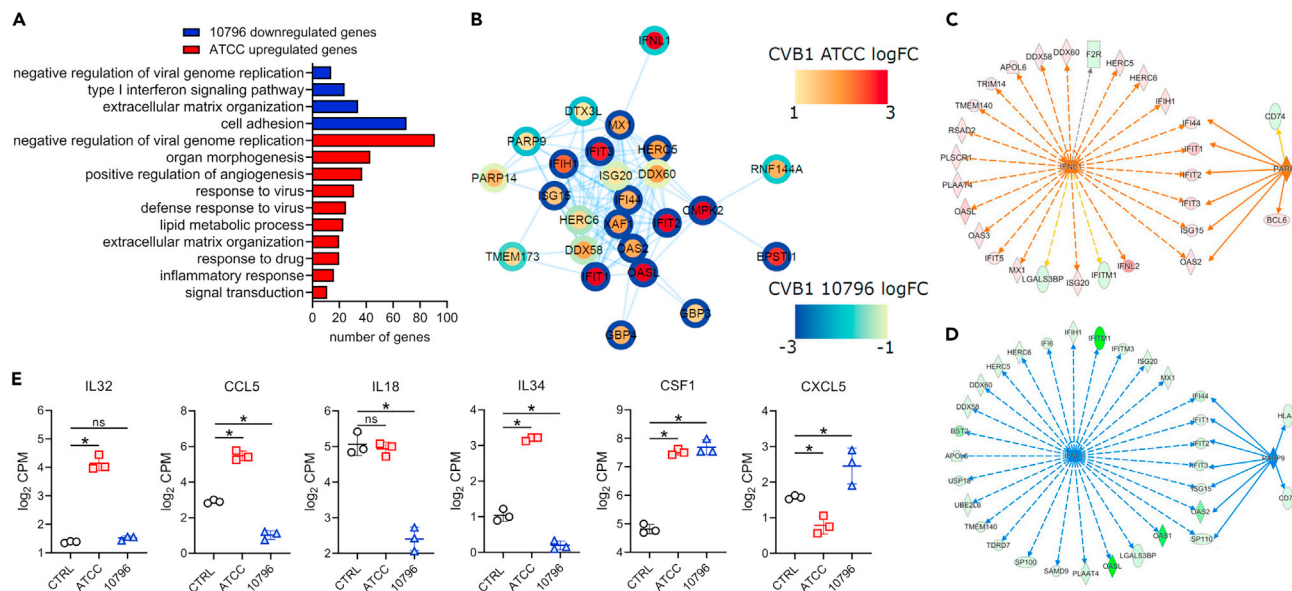


Figure 2. Persistent CVB1 infection modulates innate immune response

(A) Significantly enriched GO biological processes, based on the DAVID database tool, are shown with FDR <0.05 for the upregulated differentially expressed genes in the persistent CVB1-ATCC infection model and the downregulated differentially expressed genes in persistent CVB1-10796 infection. (B) A signature of 25 genes with opposite expression pattern between the two CVB1 strains reveals an interconnected cluster with central role in antiviral immune response. Protein-protein interactions were downloaded from the STRING database (only high-confidence interactions with a combined interaction score of ≥ 0.7 are included). The color of the nodes in the network represents the expression of the genes as logFC: downregulation (blue) or upregulation (red) of persistent CVB1-10796 (outer circle) and CVB1-ATCC (inner circle) infections. (C and D) Differently expressed PARP9 and IFNL1 downstream target genes in cells with persistent CVB1-ATCC (C) and CVB1-10796 infection (D) are depicted based on IPA analysis (red, significantly upregulated; blue, significantly downregulated). (E) The log₂ transformed CPM expression of proinflammatory cytokines IL32, IL18, CCL5, IL34, CSF1, and CXCL5 in both persistent CVB1 infection models are shown as mean \pm SD for three biological replicates (* FDR <0.05; ns, not significant).

Cell-cell and cell-matrix junctions maintain barrier function of most tissues and enable cells in tissues to function in an integrated manner and mediate direct communication between cells, thus supporting correct organ function. In the current study, GO analysis of all the differentially expressed genes in the persistent CVB1 infection models revealed the enrichment of genes involved in extracellular matrix organization and cell adhesion and migration, suggesting that persistent CVB1 infection clearly influences cell contacts and communication (Figures 3A and 3B, Table S3). Indeed, genes with the strongest persistent infection-associated changes have also been linked to cell-cell communication and function. Among the top downregulated genes in both persistent CVB1 infection models was the gap junction associated protein 1 (GJA1) (Figure 3C). Based on *in vitro* and *in vivo* models, GJA1 supports differentiation of embryonic stem cells into pancreatic cell lineages, and it is required for control of secretory function and survival (Carvalho et al., 2010; Klee et al., 2011; Serre-Beinier et al., 2002; Yang et al., 2019). Cell-adhesion molecule 1 (CADM1), the predominant CADM isoform in human islets binds essential components of the cell secretory machinery, thereby influencing insulin secretion (Zhang et al., 2016). In the current study, CADM1 was significantly downregulated in persistent CVB1 infections, with the strongest effect in cells persistently infected with the CVB1-10796 strain (Figure 3C). Among the common downregulated genes in persistent CVB1 infection models, we also observed members of the claudin family (CLDN3, CLDN4, CLDN10, CLDN11), which are crucial structural and functional components of tight junctions, and the ephrin receptor type A tyrosine kinases, including EPHA3, EPHA4, EPHA10, and receptor type B (EPHB1, EPHB6). EPHA5, reported to a play role in beta cell-to-cell communication and hormone secretion (Konstantinova et al., 2007), was among the top five downregulated genes in persistent CVB1-ATCC infection (Figure 3C).

In addition, network analysis, utilizing high-quality interactions from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, revealed that several interconnected transcripts with central roles in constitutive and regulatory secretion pathways were downregulated during persistent CVB1 infections (Figure 3D). In line with our earlier secretome data (Lietzén et al., 2019), members of the granin family

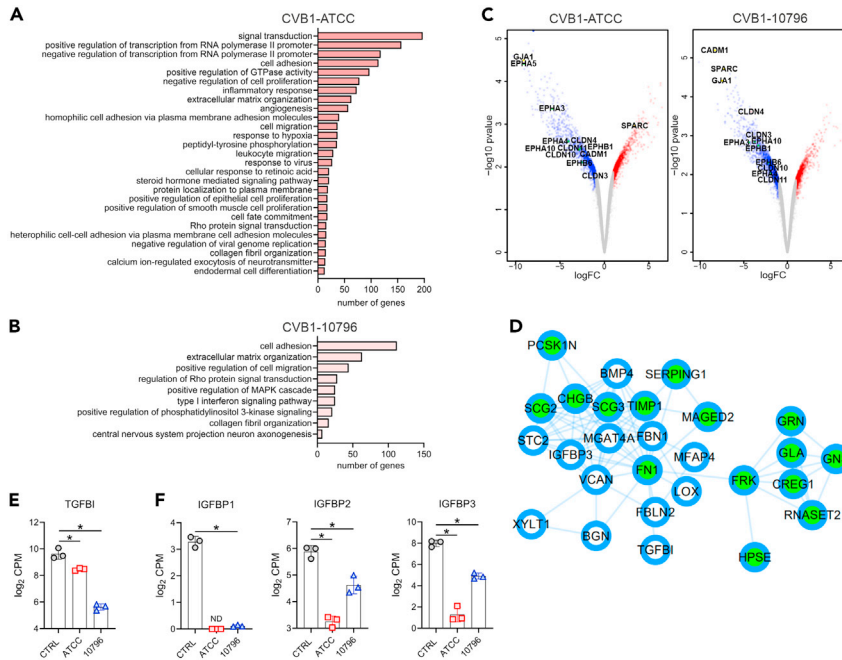


Figure 3. Genes associated with beta cell communication and function are strongly affected in persistent CVB1 infection

(A and B) Significantly enriched GO biological processes, based on the DAVID database tool, are shown with FDR <0.05 for the 2,460 and 2,606 differentially expressed genes in persistent CVB1-ATCC and 10796 infection models, respectively. (C) Persistent CVB1 infections induced changes in the gene expression of GJA1, CADM1, SPARC, CLDNs, EPHAs, and EPHBs (red color means upregulated, blue downregulated, and gray non-differentially expressed).

(D) Common downregulated genes in both persistent CVB1 infection models form an interconnected cluster related to secretion. All genes in the network are included in the gene ontology term GO:0030141~secretory granule and/or in the UniProt keyword KW-0964~secretory. Network nodes included in the GO:0030141 term are indicated with a green inner color.

(E and F) The log₂ transformed CPM expression of TGFBI (E), IGFBP1-3 (F) in both persistent CVB1 infection models are shown as mean ± SD for three biological replicates (* FDR <0.05; ND, not detected).

(CHGB, SCG2, SCG3, and PCSK1N), which are stored in secretory granules and released on demand in response to stimulus (Bartolomucci et al., 2011), were strongly downregulated during persistent CVB1 infections. Within the same cluster, additional genes relevant for beta cell function and glucose homeostasis were suppressed during persistent infection. These included the bone morphogenetic protein 4 (BMP4) (Goulley et al., 2007) and the glycosyltransferase MGAT4A (Ohtsubo et al., 2011) (Figure 3D), as well as the transforming growth factor β induced (TGFBI) (Figures 3D and 3E) and the insulin-like growth factor binding protein-3 (IGFBP3) (Figures 3D and 3F). Other IGFbps, such as IGFBP1 and IGFBP2, were also downregulated or not detected in persistent CVB1 infection models, compared with control cells (Figure 3F). Fibronectin (FN1), fibrillin-1 (FBN1), and fibulin-2 (FBLN2), which constitute the interstitial matrix that underlies the peri-islet basement membrane, were also detected within this cluster and downregulated in both persistent infection models (Figure 3D). The secreted matricellular protein SPARC, a negative regulator of beta cell growth and survival (Ryall et al., 2014), was the third most-downregulated gene in persistent CVB1-10796 infection, whereas it was significantly upregulated in persistent CVB1-ATCC infection (Figure 3C). This finding agrees with our earlier protein expression data (Lietzén et al., 2019), showing that SPARC was among the most strongly upregulated proteins in ATCC infected cells, but it was not detected in the 10796 infection.

Persistent CVB1 infection alters lysosomal biogenesis and impairs expression of autophagy-related SNAREs

In this study, one of the main features of persistent CVB1-10796 infection was the significant downregulation of genes involved in lysosomal biogenesis (Table S3). Lysosomes are the main proteolytic

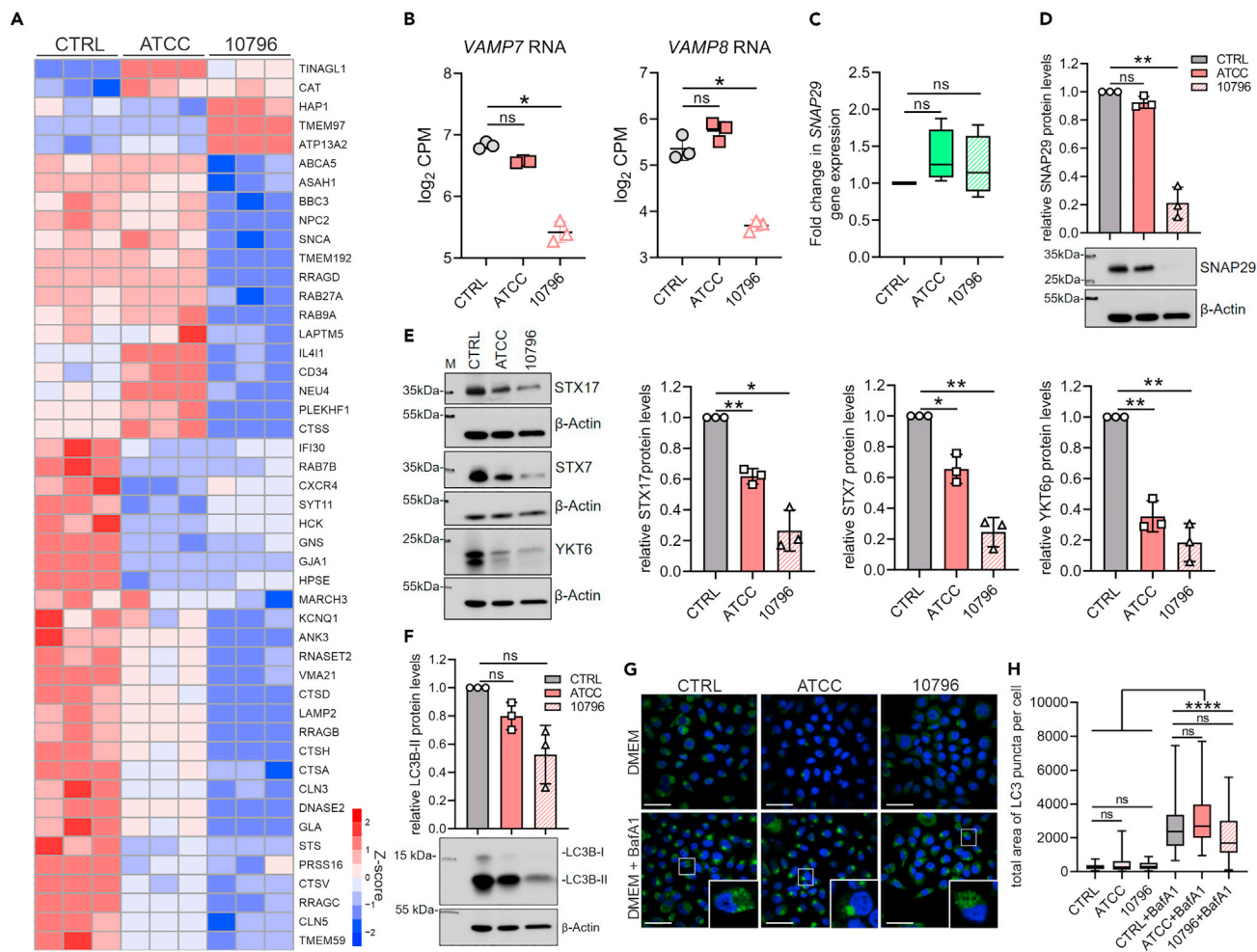


Figure 4. Persistent CVB1 infection impairs the expression of lysosome genes and autophagy-related SNAREs

(A) Heatmap of Z score standardized expression of differentially expressed genes in the cellular components GO: 0005764 (lysosome) of persistent CVB1-10796 infection.

(B) The log₂ transformed CPM expression of VAMP7 and VAMP8 in both persistent CVB1 infection models are shown as mean ± SD for three biological replicates (* FDR < 0.05; ns, not significant).

(C and D) SNAP29 gene expression was validated by real-time qPCR (C) and immunoblotting, with β-Actin as a loading control. Quantification of SNAP29 protein was normalized to loading control (D).

(E and F) Autophagy-related SNARE proteins (STX7, STX17, and YKT6) (E) and LC3B expression (F) was analyzed in both CVB1 infection models by immunoblotting, with β-Actin as a loading control (M, molecular weight marker). Band intensities of target proteins were normalized to loading control.

(G) For autophagic flux analysis, CVB1-infected PANC-1 cells were treated for 2 h with bafilomycin A1 (BafA1) and immunofluorescence stained with anti-LC3 (green) and DAPI (blue). The cells were analyzed with Nikon A1R + laser scanning confocal microscope; scale bar, 50 μm (representative images).

(H) Immunofluorescence images were analyzed using ImageJ Fiji and the total area of LC3 puncta per cell was calculated using 3D object counter analysis tool; (n = 30 cells per group). Data are represented as mean ± SD from three biological replicates; statistical analysis: (C, D, E, F, and H) Student's t test (unpaired, two-tailed, unequal variance), ns, not significant; *p < 0.05; **p < 0.01; ****p < 0.0001.

compartments in mammalian cells, providing a complex signaling platform that regulates transcriptional activity and autophagy. During persistent CVB1-10796 infection, we observed the downregulation of cathepsins, which are the most abundant lysosomal proteases (CSTA, CSTD, CSTD, CSTV, CTSS), the lysosomal membrane protein LAMP2, and small Rag GTPases (RRAGB, RRAGC, RRAGD) (Figure 4A). Rag GTPases facilitate the recruitment of mTORC1 to the lysosomal surface and regulate its activation (Sancak et al., 2008), and inhibition of mTORC1 leads to increased autophagy activity. During acute infection, CVB3 induces the autophagy pathways for viral replication, but autophagic cargos escape lysosomal degradation through viral protease 3C-induced cleavage of SNAP29, a soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) (Mohamud et al., 2018). SNAP29 interacts with the

endosomal/lysosomal SNARE protein VAMP8 and STX17, which is recruited to the membrane of autophagosomes to mediate fusion of lysosomes with the autophagosomes (Itakura et al., 2012).

In the current study, VAMP7 and VAMP8 transcripts were clearly downregulated during persistent CVB1-10796 infection, whereas no changes were observed during persistent CVB1-ATCC infection (Figure 4B). Transcripts of other autophagy-related SNAREs, such as STX17, YKT6, and STX7 (Figure S2), and SNAP29 (Figure 4C) remained unchanged in both persistent CVB1 infection models as validated by real-time qPCR. At the protein level, however, we found that SNAP29 was significantly decreased in persistent CVB1-10796 infection, whereas its expression was not altered in cells persistently infected with the CVB1-ATCC strain, compared with uninfected control (Figure 4D). STX17 and other SNARE proteins STX7 and YKT6 required for fusion of autophagosomes with lysosomes independently of STX17 (Matsui et al., 2018) were clearly downregulated at a protein level in both persistent CVB1 infection models (Figure 4E). Nevertheless, downregulation of lysosomal genes and autophagy-related SNAREs did not lead to an increase in protein levels of the autophagosomal marker LC3BII during persistent CVB1 infections, as shown by immunoblotting and immunofluorescence (Figures 4F and 4G). LC3BII protein remained unchanged in persistent CVB1-ATCC infection, but it was slightly downregulated during persistent CVB1-10796 infection when compared with controls (Figure 4F). To investigate the rate of autophagic degradation, we performed autophagy flux analysis and monitored LC3BII levels by immunofluorescence. Bafilomycin A1-mediated inhibition of lysosome function caused a significant increase in LC3BII, compared with untreated cells, in non-infected and persistently infected PANC-1 cells (Figures 4G and 4H). In persistent CVB1 infection, however, we observed no increase in LC3BII upon Bafilomycin A1 treatment, indicating no virus-induced accumulation in autophagosomes (Figures 4G and 4H). These data indicate that persistent CVB1 infection, especially by the 10796 strain, altered the expression of lysosomal genes and autophagy-related SNAREs in persistently infected PANC-1 cells but did not impair autophagic flux.

DISCUSSION

In the current study, two carrier-state-type persistent CVB1 infection models were established in human pancreatic PANC-1 cells, and virus-induced transcriptional changes in cells were explored in detail. The models were established with two different CVB1 strains that, based on our previous studies, clearly differ in their abilities to induce innate immune responses (Hämäläinen et al., 2014; Lietzén et al., 2019). In the present study, we observed broad changes in the gene expression profiles of these persistently infected PANC-1 cells and identified gene expression pathways whose activation differed markedly between cell models persistently infected by either of these two CVB1 strains.

Carrier-state persistent enterovirus infection is characterized by relatively high titers of virus produced by the infected cells while only a subpopulation of cultured cells are infected (Alidjinou et al., 2017; Pinkert et al., 2011). The current persistent CVB1 infection models show clear features of carrier-state-type persistence. Evidence of active replication of the virus in the model was supported by the presence of the viral protein VP1 and the upregulation of genes associated with lipid metabolic and cholesterol biosynthetic processes. Lipids are known to facilitate CVB replication, and it has been shown that CVBs redirect the host cell cholesterol to viral replication organelles for efficient replication (Illytska et al., 2013; Li et al., 2020b). The establishment of the carrier-state-type persistent infection can be partly explained by downregulation of the CVB receptor gene CXADR. The downregulation of CXADR has also been shown earlier *in vitro* in CVB3 and CVB4 persistent infection models (Alidjinou et al., 2017; Pinkert et al., 2011). Another co-receptor DAF that is used by some of the CVBs, including CVB1 (Shafren et al., 1995), was downregulated in both CVB1 persistent infection models. These findings suggest that the virus may adapt to use other cellular receptors (Zhao et al., 2019) or may hitchhike in extracellular vesicles that are exported from the cell and that may transport the virus into uninfected cells without the receptor-binding step (Netanyahu et al., 2020; Robinson et al., 2014). This type of spreading of the virus could explain both the lack of extensive cell lysis and the occurrence of viral infection despite the extensive downregulation of its commonly used receptors.

Recent studies have also reported that enteroviruses exploit the autophagy pathway, a cell's self-degradation system, for viral replication and non-lytic egress (Robinson et al., 2014; Wernersson et al., 2020). However, the exact mechanism of how enteroviruses utilize the autophagy pathway is still poorly understood. It has been reported that enteroviruses induce autophagy and the formation of double-membrane vesicles that resemble autophagosomes, followed by their fusion with lysosomes, to support viral replication (Lin

and Huang, 2020; Shi et al., 2016). In addition, several studies have shown that enteroviruses inhibit lysosomal degradation of autophagosomes (Corona et al., 2018; Mohamud et al., 2018; Tian et al., 2018). CVB3 induces autophagosomes that escape lysosomal fusion through viral protease 3C-induced cleavage of SNAP29 and downregulation of STX17 (Mohamud et al., 2018). Moreover, enterovirus-induced inhibition of autophagosome fusion with lysosome serves as a source of extracellular membrane vesicles for non-lytic egress (Kemball et al., 2010; Mohamud et al., 2018; Robinson et al., 2014). In the current study, SNAP29 and STX17 protein levels together with lysosomal genes were clearly downregulated during persistent CVB1 infection. However, autophagic flux analysis revealed no increase in the autophagosomal marker LC3BII, suggesting autophagosomes did not accumulate during persistent CVB1 infections. One explanation could be that, in carrier-state-type persistent infection, only a proportion of cells are infected and the status of the infection varies over time between infected cells, which is different from synchronized acute and short-term infections. This probably makes it more challenging to detect autophagy-related markers and carry out flux analysis during persistent CVB1 infection. Single-cell approaches could contribute to a better understanding of how autophagy is utilized by persistent CVB1 infection.

Persistent CVB4 infection in PANC-1 cells has been shown to result in downregulation of PDX1 (Sane et al., 2013), a core transcription factor responsible for pancreatic development and maturation of beta cell function. However, in the present study, PDX1 gene expression was slightly upregulated in both persistent CVB1 infection models at day 322, whereas Sane et al. showed that PDX1 mRNA was no more detected after day 36 post CVB4 infection (Sane et al., 2013). Thus, it is possible that enterovirus-induced changes in cellular gene expression depend on CVB serotype or strain.

Within the cell, CVBs are recognized by cellular RNA sensors, ultimately leading to the transcription of the genes encoding type I IFN and IFN-stimulated genes that orchestrate antiviral functions. Since enteroviruses are among the potential environmental triggers for the development of T1D, and an interferon-related gene signature was reported in individuals at risk of T1D before the appearance of T1D autoantibodies (Ferreira et al., 2014; Kallionpaa et al., 2014) and in blood transcriptomes of a few children with genetic susceptibility to T1D (Lietzen et al., 2018), viral interactions with the innate immune system is an important research target.

In the current study, gene expression profiling of CVB1-ATCC-infected PANC-1 cells revealed the upregulation of virus response genes including the cytosolic viral receptors IFIH1 and DDX58 and several genes of type I IFN signaling pathways, indicating that the infected cells actively sense the presence of virus. Persistent CVB1-ATCC infection also resulted in the induction of the type III interferon IFNL1 and many of its downstream targets, along with inflammatory genes. These findings are in line with previous acute infection models, showing that various CVB strains trigger innate immune responses (Lind et al., 2013; Nyalwidhe et al., 2017; Ylipaasto et al., 2012). Increased type III interferon response in CVB-infected pancreatic islets has also been associated with a SNP within the T1D associated gene IFIH1 (Domsgen et al., 2016). Besides IFIH1, several other T1D gene risk variants regulate the immune response and type I IFN production, which could influence the development of viral persistence (Blanter et al., 2019). Moreover, upregulation of PARP9, PARP14, and DTX3L transcripts was induced by the immunogenic CVB1-ATCC, and several downstream target genes of PARP9 associated with antiviral host defense including IFIT1, IFIT2, IFIT3, IFI44, ISG15, and OAS2 were activated. PARPs are known ISGs and have shown to possess antiviral properties (Zhu and Zheng, 2021). PARP9 together with DTX3L promotes ISG expression during viral infection to enhance the immune response (Zhang et al., 2015), and it has been suggested that PARP9 serves as a non-canonical sensor for RNA virus to initiate and amplify type I IFN production in dendritic cells (Xing et al., 2021).

Inversely, in PANC-1 cells persistently infected by the less immunogenic strain CVB1-10796, nearly all genes of the type I IFN signaling pathway together with the upstream regulators IFNL1 and PARP9 that were detected during persistent CVB1-ATCC infection were downregulated. Similar findings were reported in our previous proteomics study demonstrating clear differences in the expression of antiviral immune response proteins between cells persistently infected by these two CVB1 strains (Lietzen et al., 2019). Taken together, our results indicate that persistent CVB1 infection interferes with the cell's antiviral machinery not only at the level of host proteins but also at the transcriptional level. A recent study has shown that enterovirus strains isolated from the spleen of T1D donors slowly replicate and no cytopathic effect was observed, while viral genome and antigens were consistently detected in cell cultures. Moreover, these enterovirus

strains shut down the IFN pathways in acutely infected endothelial cells, when compared with the replication-competent prototype strain CVB3 (Poma et al., 2020). Apart from antiviral factors, persistent CVB1-10796 infection also represses the gene expression of proinflammatory cytokines including IL18, IL34, and CCL5, which coordinate immune cells to sites of inflammation and thus might support persistence and make virus less visible to the immune cells.

The regulated secretory pathway is important to maintain beta cell function. Studies have shown that enterovirus infections can interfere with the regulated secretory pathway in both pancreatic cell lines and beta cells (Richardson et al., 2013; Wernersson et al., 2020). Transcriptomics analysis of laser captured islets of autoantibody-positive T1D donors has recently demonstrated that the expression of at least one IFN response marker correlated with the presence of the enteroviral VP1 protein in the same islet and decreased expression of genes important for islet function, insulin secretion, and components of vesicles or membrane fusion machinery (Apaolaza et al., 2021). These findings are in line with a previous study demonstrating decreased insulin expression and secretion in islets infected with CVBs *in vitro* (Hodik et al., 2016). Lower levels of insulin were also confirmed in beta cells positive for enterovirus VP1 protein in pancreatic tissue samples from T1D organ donors than in controls (Richardson et al., 2013). We recently reported reduced secretion of granins from persistently CVB1-infected PANC-1 cells (Lietzén et al., 2019). Granin family members are key players of regulated secretory pathway and responsible for insulin processing and secretion. Here we show that also mRNAs of granins were clearly decreased in both persistent infection models. In addition, insulin-like growth factor binding proteins (IGFBP1-3), which are associated with T1D (Ding and Wu, 2018), were significantly downregulated in persistent CVB1 infections. Secreted IGFBPs bind to and regulate the biological activity of insulin growth factors (IGFs), which are structurally and functionally similar to insulin and play important roles in growth and development (Ding and Wu, 2018). IGFBPs can also act independently of IGFs, affecting cell adhesion and migration, cell growth, and apoptosis (Firth and Baxter, 2002). Interestingly, treatment of enterovirus-infected cells *in vitro* with drug vemurafenib successfully inhibited viral replication therefore retained IGFBP2 secretion (Ianevski et al., 2020). Overall, these data suggest that the molecular machinery involved in secretion might be disrupted with sustained enteroviral infection.

Beta cells are coordinated within individual islets with constant interaction and support from the extracellular matrix, neighboring cells, and several chemical, metabolic, and physical cues. Loss of such interaction and support is detrimental to islet function and viability (Kelly et al., 2011). The strongest changes observed in the current persistent CVB1 infection models involved genes that have an important role in beta cell contacts and communication and are required for the control of secretory function. Among these common downregulated genes, we detected the cell adhesion protein CADM1 (Zhang et al., 2016), gap junction proteins GJA1 (Carvalho et al., 2010; Klee et al., 2011; Serre-Beinier et al., 2002), members of claudins (Li et al., 2020a), and ephrin A receptors (Konstantinova et al., 2007; Volta et al., 2019). We therefore hypothesize that CVB infection might cause changes in the same genes in beta cells, which finally could result in changes that compromise beta cell function. Moreover, persistent CVB1 infection resulted in strong downregulation of human peri-islet basement membrane genes FN1 and FBN1 that are involved in cytoskeletal remodeling, differential cell adhesion, and beta cell survival (Llacua et al., 2018). The gene expression of TGFBI, a secreted extracellular matrix protein found in pancreatic islets, was also significantly reduced in both persistent CVB1 infection models. Based on mouse and human genetic studies, TGFBI is a risk factor for T1D (Han et al., 2014) and serum levels of TGFBI are significantly decreased in patients with T1D (Zhi et al., 2011). Its role in promoting islet survival, function, and regeneration has been supported by transgenic TGFBI overexpressing and TGFBI gene knockout mouse model studies (Han et al., 2011, 2014). A previously described inhibitory function of TGFBI in diabetogenic T cell activation and production of cytotoxic molecules via interfering with early factors of the TCR signaling pathway emphasizes the critical component of beta cell matrix in autoimmune response (Patry et al., 2015). Defects in cell adhesion factors and basement membrane integrity suggest that persistent CVB1 infection may disturb the islet microenvironment, which in turn could facilitate immune cell infiltration and beta cell destruction.

The current study provides a detailed profiling of the transcriptomic changes induced by persistent CVB1 infection in human pancreatic ductal-like cell line. Two persistent infection models using two CVB1 strains with distinct immunogenic properties revealed strong opposite but also common changes in gene expression of infected cells. Part of these changes are similar to our previously reported proteomics studies on persistently CVB1-infected cells (Lietzén et al., 2019). In addition, the current study showed clear changes

in the gene expression of factors associated with the pancreatic microenvironment and lysosomal biogenesis and gained deeper insights into immune-related genes that are affected during persistent CVB1 infection of pancreatic cell line. Further studies are needed to fully understand the mechanisms by which enteroviruses inhibit or activate cellular IFN and immune response and the strategies by which host cells adopt a defense system during persistence. Overall, the current study reveals new insights on persistent enteroviral-triggered changes in the transcriptome of pancreatic cell line and opens new avenues for studying potential mechanisms regulating the development of enteroviral persistency in human pancreatic cells and tissue samples.

Limitations of the study

Persistent CVB infection models have been established in pancreatic cell lines to study the outcomes of chronic CVB infections in human pancreas. One of the limitations of the current study is the use of pancreatic ductal cell line compared with an insulin-producing human beta cell line. Establishing long-term CVB persistency in primary human pancreatic islets is challenging and has not yet been published. Another limitation in carrier-state-type persistent infection is that only a small percentage of cells are infected, and the status of infection can vary between infected cells, compared with acute and short-term infections. This makes it more challenging to track the course of persistent infection. Monitoring persistently infected cells over time combined with single-cell sequencing approaches could provide important additional insights into host-virus interplay during prolonged CVB infection of pancreatic beta cells and allow detailed dissection of the infection-induced responses observed in the current study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Persistent enterovirus infection in PANC-1 cells
- METHOD DETAILS
 - Transcriptomics data analysis
 - Quantitative real-time PCR
 - Western blot
 - Autophagy flux
 - Immunofluorescence and confocal microscopy analyses
- QUANTIFICATION AND STATISTICAL ANALYSES

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

T.B. designed and performed the transcriptomics and immunoblotting experiments, analyzed data, interpreted results, prepared figures, and wrote the manuscript. A.H. and A.-B.S.-K. established the persistent infection model. A.H., A.-B.S.-K., and J.E.L. performed autophagic flux experiments and immunofluorescence staining and provided the samples. A.-B.S.-K. and J.E.L. critically revised the manuscript. A.H. was responsible for the imaging experiments and wrote part of the manuscript. T.V. performed major part of the computational analysis, prepared figures, and wrote part of the methods. N.L. and M.K.H. provided expertise and carefully revised the manuscript. E.-L.E. provided expertise in autophagy assessment, provided guidance, and critically revised the manuscript. L.L.E. provided expertise and critically revised the manuscript. H.H. initiated the study, provided expertise and supervision, and critically revised the manuscript. R.L. initiated and supervised the study, provided expertise and critically revised the manuscript. All authors have contributed to the final version of the manuscript.

DECLARATION OF INTERESTS

H.H. is a shareholder and chairman of the board of Vactech Ltd (<http://www.vactech.fi/en/>), which develops vaccines against picornaviruses. H.H. serves on the scientific advisory board of Provention Bio Inc., which is developing a clinical CVB vaccine in collaboration with Vactech Ltd.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-VP1	Agilent	Cat#M7064; RRID:AB_2118128
Rabbit monoclonal anti-SNAP29	Abcam	Cat#ab181151; RRID:AB_2687668
Rabbit polyclonal anti-STX7	Bethyl	Cat#A304-512A; RRID:AB_2620706
Rabbit polyclonal anti-STX17	Sigma-Aldrich	Cat#HPA001204; RRID:AB_1080118
Mouse monoclonal anti-YKT6	Santa Cruz Biotechnology	Cat#sc-365732; RRID:AB_10859388
Rabbit polyclonal anti-LC3	Novus	Cat#NB100-2220; RRID:AB_10003146
Mouse monoclonal β -Actin	Sigma Aldrich	Cat#A5441; RRID:AB_476744
Rabbit polyclonal anti-LC3 (immunofluorescence)	MBL International	Cat#PM036; RRID:AB_2274121
Rat monoclonal anti-VP1 (clone 3AG) (immunofluorescence)	(Saarinen et al., 2018)	N/A
Mouse IgG κ BP-HRP	Santa Cruz Biotechnology	Cat#sc-516102; RRID:AB_2687626
Goat polyclonal anti-rat IgG (H+L) Alexa Fluor 555	Thermo Fisher Scientific	Cat#A-21434; RRID:AB_2535855
Goat polyclonal anti-rabbit IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11034; RRID:AB_2576217
Bacterial and virus strains		
Coxsackievirus B1 strain Conn-5	ATCC	Cat#ATCC-VR-28
Coxsackievirus B1 strain 10796	Centers for Disease Control and Prevention	Clinical isolate CVB1 10796
Chemicals, peptides, and recombinant proteins		
Bafilomycin A1	Sigma-Aldrich	Cat#B1793
Critical commercial assays		
AllPrep DNA/RNA/miRNA universal kit	Qiagen	Cat#80224
SuperScript TM II reverse transcriptase	Invitrogen	Cat#18064022
TruSeq [®] stranded mRNA library prep	Illumina	part#15031047
SNAP29 Taqman gene expression assay	Applied Biosystems	Hs00191150_m1
Deposited data		
RNA-Sequencing data set	This paper	GEO database: GSE184831
Experimental models: Cell lines		
PANC-1	ATCC	Cat#CRL-1469; RRID:CVCL_0480
Oligonucleotides		
GAPDH Probe FAM/TAM 5'-ACCAGGCGCCCAATACGA CCAA-3'	(Hamalainen et al., 2000)	N/A
Fw 5'-GTTTCGACAGTCAGCCGCATC-3'		
Rw 5'-GGAATTTGCCATGGGTGGA-3'		
Software and algorithms		
GraphPad Prism8	GraphPad	https://www.graphpad.com/
DAVID 6.8	(Huang et al., 2009)	https://david.ncifcrf.gov/
ImageJ Fiji	(Schindelin et al., 2012)	https://imagej.net/software/fiji/
UCSC human reference genome GRCh38	(Schneider et al., 2017)	https://genome.ucsc.edu/cgi-bin/hgGateway
STAR aligner 2.5.2b	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
FeatureCounts 1.5.1, Subread software package	(Liao et al., 2014; Liao et al., 2013)	http://subread.sourceforge.net/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TMM normalization, Bioconductor package edgeR 3.32.1	(McCarthy et al., 2012; Robinson et al., 2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Voom transformation, Bioconductor package limma 3.46.0	(Law et al., 2014; Ritchie et al., 2015)	https://bioconductor.org/packages/release/bioc/html/limma.html
Reproducibility Optimized Test Statistic, ROTS Bioconductor package 1.10.1	(Suomi et al., 2017)	https://bioconductor.org/packages/release/bioc/html/ROTS.html

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and code should be directed to the lead contact, Riitta Lahesmaa (riitta.lahesmaa@utu.fi).

Materials availability

The study did not generate new unique materials.

Data and code availability

- RNA-Sequencing data sets have been deposited in the Gene Expression Omnibus (GEO) database: GSE184831. The accession number is listed in the [key resources table](#).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Persistent enterovirus infection in PANC-1 cells**

Pancreatic ductal cells (PANC-1) were obtained from American type culture collection (ATCC) and cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% v/v of FBS and 0.5% penicillin-streptomycin (Pen-Strep, Gibco). PANC-1 were infected with Coxsackievirus B1 (CVB1) prototype strain obtained from ATCC (American type culture collection, strain Conn-5) and a wild type CVB1 strain (strain 10796) (Hämäläinen et al., 2014) which was obtained from a case of acute flaccid paralysis Argentine and purchased from Centers for Disease Control and Prevention (CDC). Persistent infection was established as described by Sane et al. (Sane et al., 2013) and as in our previous studies (Honkima et al., 2020; Lietzén et al., 2019). Briefly, PANC-1 cells were infected using a low titer of the virus which led to a strong cytopathic effect and cell death. Cells were washed regularly with Hank's balanced salt solution (Sigma-Aldrich) and passaged by scraping using Grainer-Bio cell scraper. After the initial infection, cells started to grow and the virus propagated in the cell culture for over 300 days (Lietzén et al., 2019) before sample collection. Persistent CVB1 (ATCC and 10796) infection samples were collected by scraping and dry cell pellets were quick frozen in liquid nitrogen and stored at -80°C for further analysis.

METHOD DETAILS**Transcriptomics data analysis**

RNA isolation and sequencing. RNA sequencing was performed on the three biological replicates of persistently infected cells and non-infected controls collected 300–322 days post-infection. Total RNA was purified using the Allprep DNA/RNA/miRNA Universal kit (Qiagen) and treated in-column with DNase for 15 min, according to the manufacturer's instructions. The quality of the total RNA was ensured with Advanced Analytical Fragment Analyzer (Advanced Analytical Technologies, Heidelberg, Germany). Libraries were prepared, according to Illumina TruSeq® Stranded mRNA sample protocol. The high quality of the libraries was confirmed with Advanced Analytical Fragment Analyzer, and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantification kit (Life Technologies). The sequencing was performed using Illumina HiSeq3000 Next Generation Sequencing platform at the Finnish Functional Genomics Centre (FFGC).

Transcriptomics data processing. The quality of the raw RNA-seq reads was evaluated using the high-throughput quality-control tool FastQC (Andrews et al., 2015). The raw reads were observed to be of high quality with no need for additional trimming. The raw RNA-seq reads were mapped to the University of California, Santa Cruz (UCSC) human reference genome Reference Consortium Human Build 38 (GRCh38) (Schneider et al., 2017) using the STAR aligner version 2.5.2b (Dobin et al., 2013). The featureCounts tool (Liao et al., 2014) version 1.5.1 included in the Subread (Liao et al., 2013) software package was applied to generate the read counts using the uniquely mapped reads. Prior to further analysis, the lowly expressed genes were filtered out. Genes with more than one count per million (cpm) expression in at least three samples, corresponding to the size of the smallest experimental group, were retained in the analysis (Robinson et al., 2010). The trimmed mean of M-values (TMM) normalization from the Bioconductor package edgeR (McCarthy et al., 2012; Robinson et al., 2010) was used to normalize the filtered gene counts between the samples. For visualization, the raw count data were transformed to counts per million (cpm), offsetted by 1 and log₂-transformed.

Differential expression analysis. For determining the differentially expressed genes between the different CVB virus strains and the controls, the data were first transformed using the voom (Law et al., 2014) transformation from the limma package (Ritchie et al., 2015) and then further analyzed with the Reproducibility Optimized Test Statistic (ROTS) (Suomi et al., 2017), which performs well in RNA-seq data (Seyednasrollah et al., 2016). Differential expression was examined pairwise between different experimental groups (CVB strains and controls). In each pairwise comparison, genes with no expression in either group were removed prior to the ROTS analysis and investigated separately. Genes with false discovery rate (FDR) of < 0.05 and fold-change of ≥ 2 were considered as differentially expressed.

Network analysis. For defining protein-protein interaction networks related to specific gene sets the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk et al., 2019) was used to query the known and predicted high-confidence protein-protein interactions (combined interaction score ≥ 0.7). For visualization of the acquired networks, Cytoscape (Shannon, 2003) version 3.7.2 was utilized. Protein-protein interactions were examined from genes upregulated in persistent CVB1-ATCC but downregulated in CVB1-10796 when compared to the uninfected control, and from all genes commonly downregulated in both persistent infection models (CVB1-ATCC and CVB1-10796), which were included in the terms "GO:0030141-secretory granule" and "KW-0964 secreted (UniProt keyword, obtained from STRING)". Only the discovered clusters of interacting genes related to antiviral immune response and secretion are shown in Figures 2B and 3D, respectively.

Functional data analysis. Functional enrichment analysis was performed using the database for Annotations, Visualization and Integrated Discovery (DAVID) version 6.8 (Huang et al., 2009) and Ingenuity Pathway Analysis (Qiagen). The GO DIRECT terms (not including the parent terms) were utilized in the enrichment analysis. Gene ontology classes with FDR < 0.05 were considered to be significantly enriched.

Quantitative real-time PCR

For quantitative real-time PCR total RNA was purified using the Allprep DNA/RNA/miRNA Universal kit (Qiagen) and treated with DNase I (Invitrogen) to ensure complete removal of genomic DNA. RNA concentration was measured by NanoDrop (Thermo). Single-stranded cDNA was synthesized with the SuperScript II Reverse Transcriptase (Invitrogen). Quantification of SNAP29 mRNA was performed using the TaqMan Gene Expression Assay Hs00191150_m1 (Applied Biosystems). GAPDH gene was used as endogenous control (Hamalainen et al., 2000). The qPCR runs were analyzed using 7900HT Fast Real-time PCR System (Applied Biosystems). Relative quantification of gene expression values was calculated using the ddCt method.

Western blot

Cell samples were lysed in cell lysis buffer (CST) supplemented with cOmplete EDTA-free Protease inhibitor cocktail and phosphatase inhibitors (Roche). Cell lysates were sonicated on a Bioruptor (Diagenode) and cleared by centrifugation at 18,000 \times g for 10 min. Protein concentration was determined using DC Protein assay (Biorad). After boiling in 6X loading dye (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β -ME; 170 mM bromophenol blue; 30% glycerol), 20 μ g of protein were separated on 8–16% or 4–20% Mini-PROTEAN TGX gels (BioRad Laboratories), and transferred to nitrocellulose membranes (Trans-blot Turbo

Transfer Packs, BioRad Laboratories). Membranes were blocked with 5% BSA-TBST (Tris-buffered saline and 0.1% Tween 20) and incubated with primary antibody overnight at 4°C. Following antibodies were used anti-VP1 (mouse monoclonal, M7064, Dako), anti-SNAP29 (rabbit monoclonal, ab181151, abcam), anti-STX7 (rabbit polyclonal, A304-512A, Bethyl Laboratories), anti-STX17 (rabbit polyclonal, HPA001204, Sigma), anti-YKT6 (mouse monoclonal, sc-365732, Santa Cruz), LC3B (rabbit polyclonal, NB100-2220, Novus Biologicals) and β -Actin (mouse monoclonal, A5441, Sigma Aldrich). After primary antibody incubation, membranes were washed four times with TBST and incubated with alkaline phosphatase coupled secondary antibodies diluted (1:5000) in 5% BSA-TBST at room temperature for 1 h. Membranes were either cut into sections and probed with antibodies, or the membranes were stripped with stripping buffer (25 mM glycine and 1% SDS, pH 2.5), blocked with 5% BSA-TBST and re-probed with antibodies. The band intensity of each target was quantified using ImageJ (NIH) and normalized to loading control band intensity in each lane.

Autophagy flux

To monitor the autophagy flux, persistently infected PANC-1 cells and non-infected control cells were cultured in complete DMEM (10% FBS) and treated with lysosomal inhibitor Bafilomycin A1 (Sigma-Aldrich) at a final concentration of 100 nM for 2 h.

Immunofluorescence and confocal microscopy analyses

Cells were plated on cover slips and fixed in 4% paraformaldehyde for 15 min at room temperature (RT). After fixation, cells were washed and permeabilized using 0.2% saponin in PBS (Sigma-Aldrich) for 10 min at RT, followed by blocking in 3% BSA in 0.2% saponin-PBS. Cells were incubated with primary antibodies for 1 h at RT. Autophagosomes were visualized using anti-LC3 (rabbit polyclonal antibody, #PM036, MBL International). The virus was stained with anti-VP1 (clone 3A6, rat monoclonal antibody, (Saarinen et al., 2018)). Secondary antibodies (anti-rat Alexa Fluor 555 and anti-rabbit Alexa Fluor 488, Invitrogen) were incubated on cells for 30 min at RT. After washing, cover slips were embedded with Prolong Diamond (Invitrogen) containing DAPI. The samples were imaged using Nikon A1R + Laser Scanning Confocal Microscope. Immunofluorescence images were analyzed using ImageJ Fiji (Schindelin et al., 2012). The total area of LC3 puncta per cell was calculated using 3D object counter analysis tool (Bolte and Cordelières, 2006).

QUANTIFICATION AND STATISTICAL ANALYSES

Quantitative real-time PCR, western blotting and imaging data are represented as the means \pm SD of at least three experiments. Statistical analyses were performed with GraphPad Prism8 software. Statistical significance was calculated using Student's two tailed unpaired t test. Statistical significance was concluded when a probability value (p value) was lower than 0.05, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Statistical analyses and figures were performed using GraphPad Prism8 software (GraphPad Software, Inc.).