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ARTICLE

Enterovirus-induced gene expression profile is critical for human pancreatic islet destruction

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Abstract
Aims/hypothesis Virally induced inflammatory responses, beta cell destruction and release of beta cell autoantigens may lead to autoimmune reactions culminating in type 1 diabetes. Therefore, viral capability to induce beta cell death and the nature of virus-induced immune responses are among key determinants of diabetogenic viruses. We hypothesised that enterovirus infection induces a specific gene expression pattern that results in islet destruction and that such a host response pattern is not shared among all enterovirus infections but varies between virus strains.
Methods The changes in global gene expression and secreted cytokine profiles induced by lytic or benign enterovirus infections were studied in primary human pancreatic islet using DNA microarrays and viral strains either isolated at the clinical onset of type 1 diabetes or capable of causing a diabetes-like condition in mice.
Results The expression of pro-inflammatory cytokine genes (IL-1-α, IL-1-β and TNF-α) that also mediate cytokine-induced beta cell dysfunction correlated with the lytic potential of a virus. Temporally increasing gene expression levels of double-stranded RNA recognition receptors, antiviral molecules, cytokines and chemokines were detected for all studied virus strains. Lytic coxsackievirus B5 (CBV-5)-DS infection also downregulated genes involved in glycolysis and insulin secretion.
Conclusions/interpretation The results suggest a distinct, virus-strain-specific, gene expression pattern leading to pancreatic

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islet destruction and pro-inflammatory effects after enterovirus infection. However, neither viral replication nor cytotoxic cytokine production alone are sufficient to induce necrotic cell death. More likely the combined effect of these and possibly cellular energy depletion lie behind the enterovirus-induced necrosis of islets.

Keywords Coxsackievirus · Echovirus · Enterovirus · Interleukin 1 · Microarray analysis · Pancreatic beta cells · Pancreatic islets · Tumour necrosis factor · Type 1 diabetes

Abbreviations

AP-1	Activator protein 1
CVB	Coxsackievirus B
dsRNA	Double-stranded RNA
E	Echovirus
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
GSEA	Gene set enrichment analysis
HEV	Human enterovirus
iNOS	Inducible form of nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain containing
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
p38	P38 mitogen-activated protein kinases
PARP	Poly(ADP-ribose) polymerase
p.i.	Post infection
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
ssRNA	Single-stranded RNA
TRAIL	TNF-related apoptosis-inducing ligand

Introduction

The rapid worldwide increase in incidence of type 1 diabetes cannot be explained by a change in genetic predisposition, suggesting that environmental factors play an important role [1, 2]. In this respect viral infections have been mentioned and, according to cross-sectional and prospective studies, human enteroviruses (HEVs) are the strongest candidates (reviewed in [3]). Furthermore, HEV genomes, proteins and/or infections have been reported in pancreatic islets of deceased patients with type 1 diabetes [4–6], indicating that HEVs are capable of reaching the islets during infection.

Direct cytolysis and/or virus-targeted innate immune responses are held responsible for some cases of fulminant type 1 diabetes without evidence for autoimmunity [7–10]. However, it is possible that virus-induced host responses culminate in autoimmune reactions that are involved in type 1 diabetes in the majority of type 1 diabetes patients. In one possible scenario, virus-induced beta cell damage would result in the presentation of autoantigens to the immune system in a local inflammatory milieu with upregulation of MHC molecules and secretion of pro-inflammatory cytokines. Uptake and presentation of autoantigens by antigen-presenting cells would promote further beta cell damage by augmenting the innate antiviral responses and by recruiting and activating autoreactive T cells [11, 12]. Thus, beta cell tropism and virus-induced cytolysis, combined with host-cell responses to infection (e.g. inflammatory responses), are likely to determine the diabetogenic potential of a virus.

HEVs are a large and genetically diverse group of RNA viruses with over 100 known serotypes [13]. Although HEVs are often implicated as triggers of type 1 diabetes, it is not known precisely which of the HEVs are involved in type 1 diabetes development. In-vitro studies with human islets suggest that enterovirus strains differ in their capability to induce islet destruction [14–17]. Some HEV strains are highly cytolytic in vitro whereas others replicate without apparent islet destruction [15–17]. The ability to induce islet destruction is not determined solely by the virus serotype, but strain-specific differences exist within some serotypes (e.g. echovirus 9 [E-9] and 30 [E-30]) [15]. This suggests a role for viral genetic factors in beta cell dysfunction and death. Host-cell factors have a role in the process since there is a significant delay between viral growth and islet destruction, even for highly destructive virus strains [14, 15]. Therefore, pancreatic beta cell tropism and the ability to induce beta cell dysfunction and death depends both on the genetic properties of the virus and on the host-cell response to the infection.

The outcome of islet infection (i.e. destructive vs benign) may have implications on type 1 diabetes pathogenesis, since lytic infection (with necrotic cell death) is likely to induce a strong inflammatory response that, together with presentation of beta cell autoantigens, could induce autoimmunity. Benign infection, on the other hand, may induce persistent infection [18] and thus lead to sustained stimulation of autoimmunity.

In a previous study, a complex network of hundreds of genes participating in islet responses to a viral infection was identified [19]. In addition to upregulation of multiple inflammatory mediators probably contributing to the homing and activation of immune cells in the islets during infection and/or autoimmune response, HEV infection is capable of changing expression of several cytokines that directly contribute to islet destruction [19]. However, a prolonged exposure of human beta cells to cytokines and/or double-stranded RNA (dsRNA) culminates in apoptosis [19–21] whereas most coxsackievirus B5 (CVB-5)-infected human

pancreatic islet cells die by necrosis [14]. The pathways leading to beta cell apoptosis have been studied extensively [22], but the molecular networks leading to necrosis after HEV infection are not known.

We hypothesised that: (1) HEV infection induces a specific gene expression pattern that results in islet destruction and (2) that the ability to induce such a pattern is not common for all HEVs, but that different HEV strains may trigger distinct gene expression profiles leading to either a destructive or a merely benign outcome.

To identify a 'destructive' gene expression pattern, human pancreatic islets were infected in vitro with beta cell tropic HEV strains that induce varying degrees of necrosis, ranging from highly lytic to benign. Since the outcome of infection may depend on the timing of the expression changes, the dynamics of human pancreatic islet responses to infection were tested for a highly cytolytic CVB-5-DS strain leading to islet necrosis using two time points, representing the time of maximum virus concentration and the time before apparent cellular damage. A 'destructive gene expression profile' was further assessed by comparing the gene expression patterns induced by beta cell destructive and benign E-9 clones, one leading to necrosis, the other replicating with no apparent damage to islets. These E-9 clones differed only by two amino acids, thereby minimising the effect of viral genetics.

Methods

Human pancreatic islets Pancreatic islets were isolated and purified, as described previously [23], at Uppsala University Hospital, Sweden, or Islet Cell Resource (ICR) Center Basic Science Islet Distribution Program, with the consent of corresponding ethics committees.

Pancreatic islets from four donors (three men, one woman; age 50–64 years) were used for microarray analysis (electronic supplementary material [ESM] Table 1). The cold ischaemia time was between 5 and 11 h. The mean purity of the islets was 80±8% (mean ± SD). For viral replication and viability analyses six additional preparations (four men, two women; age 19–63 years; purity 50–85%) were used.

The islets were maintained in non-adherent culture plates (Falcon; Becton Dickinson Lincoln Park, NJ, USA) in Hams F-10 cell culture medium (Sigma-Aldrich, St Louis, MO, USA) containing 2% FBS, 25 mmol/l HEPES (pH7.4), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The culture medium was changed twice a week.

Viruses CVB-5-DS [24] and molecular clones derived from echovirus 9 strain DM (E-9-DM) [9, 17] were used in the study. The infective clones were constructed as described previously [25]. The E-9 clones have been characterised at the molecular and biological levels (A. Paananen, P. Ylipaasto, T. Smura, O. Korsgren, J. Galama and M. Roivainen, unpublished data).

Infection and viral growth The pancreatic islets were infected as described previously [14]. For viral growth determination, islets were harvested at different intervals and freeze–thawed four times. The infectivity of each sample was determined by endpoint titration in 96-well plates of green monkey kidney (GMK) cells [14].

Immunocytochemistry The islet samples were stained using antivirus and anti-insulin antibodies (The Binding Site, Birmingham, UK), as described previously [14]. Slides were analysed using a confocal microscope (Leica TCS SPE; Wetzlar, Germany)

Cell viability The viability of islets was monitored using Live/Dead assay (Invitrogen, Carlsbad, CA, USA) as described previously [14].

RNA extraction Total RNA of the islets was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. The RNA was cleaned using RNeasy (Qiagen, Hilden, Germany) cleanup protocol with DNA digestion treatment (Qiagen).

Hybridisation The quality and quantity of RNA was controlled with electrophoresis (Biorad Laboratories, Hercules, CA, USA) and spectrophotometer (Nanodrop ND-100; Thermo Fisher Scientific, Waltham, MA, USA), respectively. Affymetrix HG U133A chips or Illumina Sentrix HumanHT-12 Expression BeadChips were hybridised with the samples according to manufacturers' protocols.

Gene expression analysis The mRNA expression levels of infected islets and mock-infected control islets were compared. For Illumina probes the gene expression levels were considered to be changed if the mean fold change in expression signal between infected islets and mock-infected control islets was equal to or more than twofold or statistically significant. For Affymetrix probes, manufacturer's recommendations were used, as described previously [19].

Gene set enrichment analysis Gene set enrichment analysis using GSEA 2.06 and Molecular Signature Database (MSigDB) was performed to detect statistically significant and concordant changes of genes belonging to known gene sets [26, 27]. Data from Affymetrix gene chip were analysed using a pre-ranked list based on the signal log ratio. The data from Illumina arrays were analysed using signal to noise ratio as the metric for ranking genes. Gene set permutations were used to determine the statistical significance of the enrichment score of pathways.

Cytokine assay Multiplex bead-based assays based on xMAP technology (Bio-Plex; Biorad Laboratories) were used to assay cytokine/chemokine concentrations in the culture-medium supernatant fractions of the islets. ESM Table 2 contains the limits of detection and the coefficients of variability of these assays. Statistical significance was evaluated using Student's *t* test.

Lipidomics analyses The islet samples were analysed for lipids and small polar metabolites by applying the methods described previously [28, 29]. For more details, please refer to the ESM text.

Statistical analysis The statistical significance of difference between means in the quantification of mRNA was calculated with paired two-tailed Student's *t* test. A p value less than 0.05 was considered statistically significant.

Results

Pancreatic islet gene expression profile induced by beta cell tropic CBV-5-DS strain In search of HEV-induced gene expression patterns that result in islet destruction, the dynamics of islet mRNA expression and cytokine secretion were measured after infection with the beta cell tropic and highly cytolytic CVB-5-DS strain.

The pancreatic islets of three donors were either infected with CVB-5-DS or were mock infected. Virus titre reached its maximum 1 day post infection (p.i.) (Fig. 1a). Concurrently, beta cell infection was confirmed by co-staining of insulin and viral capsid (Fig. 1b). Despite proven infection of the beta cells, the viability of infected islets remained equivalent to that of mock-infected islets for 3 days p.i. Partial destruction of islets was observed 4 days p.i., and the majority of islets were destroyed by 1 week p.i. (Fig. 1c). The viability of mock-infected control islets remained high throughout the experiments (Fig. 1c).

Global mRNA expression and cytokine secretion were measured 1 and 3 days p.i, representing the time of maximum virus concentration and the time before apparent cellular damage. GSEA analysis indicated significant upregulation in the pathways related to inflammation, cytokines, innate immune response, antigen processing and presentation, cell death (both apoptosis and necrosis) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK) and nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB) signalling cascades, whereas the metabolic pathways (including glycolysis and oxidative phosphorylation) were downregulated (ESM Fig. 1).

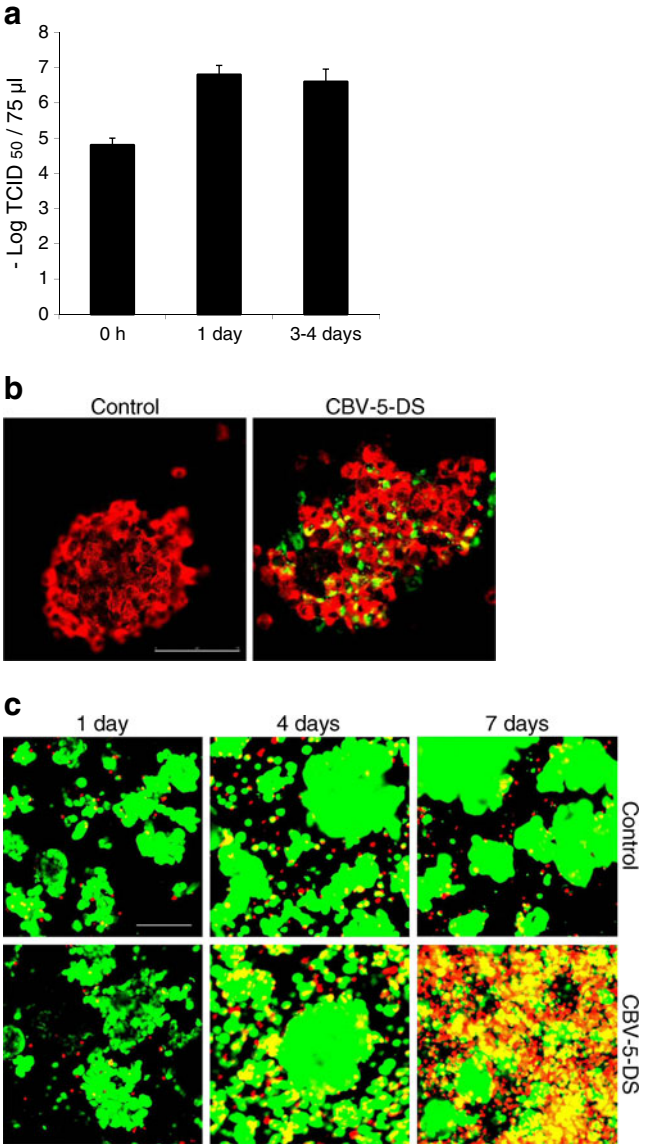


Fig. 1 (**a**) CBV-5-DS infectious progeny production in human pancreatic islets. Subsets of islets derived from three donors were infected with an apparently high multiplicity of infection and the samples were collected immediately (0 h), 1 day and 3–4 days after infection. The total infectivity (50% tissue culture infectious dose, $TCID_{50}$) of each sample was determined by endpoint titration in green monkey kidney (GMK) cells. Data are means ± SD. (**b**) Co-staining of enterovirus-specific polyclonal rabbit antiserum (green) and insulin-specific polyclonal sheep antiserum (red) 24 h after infection with CBV-5-DS. The yellow colour indicates co-staining of insulin and virus. Scale bar, 75 µm. **c** Viability of pancreatic islets 1, 4 and 7 days after infection with CBV-5-DS. The viability was assessed using Live/Dead assay (Molecular Probes). Because of their esterase activity, live cells are stained green by calcein, while nuclei of dead cells are stained red by ethidium homodimer-1. Scale bar, 100 µm

Immunoregulatory gene expression pattern after CVB-5-DS infection Host-cell responses against RNA virus infections begin with the activation of pattern recognition receptors (PRRs) culminating in the production of type I IFNs (IFN-α

and IFN-β). CVB-5-DS induced temporally increasing expression of type I (*IFNB*) and type III (*IL28A*, *IL28B* and *IL29*) IFNs (Fig. 2a). The effects of both type I and type III IFNs are mediated via *STAT1*, *STAT2* and *IRF9*, all of which were upregulated after infection. No mRNA upregulation for IFN-α and IFN-γ was detected. However, high concentrations of IFN-γ protein were measured in tissue culture media after infection (ESM Table 2).

Corresponding to increased IFN production, the expression of several genes coding for IFN-inducible antiviral defence molecules were upregulated (ESM Table 3). Likewise, upregulation of intracellular (viral) dsRNA and single-stranded RNA (ssRNA) receptors *DDX58* (*RIG-I*), *IFIH1* (*MDA-5*), *DHX58* (*LGP2*) toll-like receptor 3 (*TLR3*) and nucleotide-binding oligomerisation domain containing (NOD)-like receptor 2 (*NOD2*) was detected (Fig. 2a).

The effects of PRR activation are mediated mainly via three signal transduction cascades–JAK/STAT-, MAPK- and NFκB-mediated pathways, all of which showed upregulation in GSEA analysis (ESM Fig. 1). For NFκB and MAPK, upregulation of inhibitors were also detected (Fig. 2a). The expression of genes for three MAPKs, extracellular-signal-regulated kinase (ERK) (*MAPK3*), c-Jun N-terminal kinase (JNK) (*MAPK10*) and p38 (*MAPK11*), were downregulated. MAPKs regulate the activity of the activator protein-1 (AP-1) transcription factor complex. AP-1 transcription factor members *ATF3*, *ATF4* and *ELK4* as well as AP-1 complex suppressors *BATF2* (*SARI*) and *BATF3* (*JDP1*) were upregulated after CBV-5-DS infection.

The expression of multiple cytokine and chemokine genes were upregulated by CVB-5-DS infection (Fig. 2b). Furthermore, most of the observed upregulations increased from 1 to 3 days p.i. and were also detected in the tissue culture medium after infection (ESM Table 2).

The expression of several genes involved in antigen processing and presentation were upregulated after CVB-5-DS infection. These included regulatory members of immunoproteasome complex (*PSMB8*, *PSMB9*, *PSMB10*, *PSME2*), antigen peptide transporters (*TAP1* and *TAP2*), as well as several MHC class I molecules (Fig. 2a).

Beta cell function-related gene expression after CVB-5-DS infection In addition to its immunomodulatory effects, CVB-5-DS infection changed the expression of several genes important for beta cell function and metabolism (ESM Fig. 1), including genes associated with glycolysis and insulin secretion.

The signalling cascade for insulin secretion in pancreatic beta cells begins with the closure of ATP-dependent K^+ channels due to an increased intracellular ATP/ADP ratio. This leads to cell membrane depolarisation, opening of voltage-sensitive Ca^{2+} channels and release of insulin stored in secretory vesicles due to increased cytosolic Ca^{2+} concentration. GSEA analysis suggested downregulation of glycolysis and oxidative phosphorylation after CVB-5-DS infection (Fig. 2c, ESM Fig. 1). Likewise, genes for a modulator of ATP-dependent K^+ channels (*ABCC8* [*SUR1*]) and voltage-dependent Ca^{2+} channel subunits (*CACNB2* and *CACNA2D2*) as well as *ATP2A3* (*SERCA*), an endoplasmic reticulum (ER) Ca^{2+} pump that translocates calcium from the cytosol to the ER lumen were downregulated (Fig. 2c, ESM Table 3). The expression of several genes coding for other (K^+, Na^+ and Cl^-) ion channel subunits and modulators were also downregulated after CVB-5-DS infection (ESM Table 3).

In addition to apparently impaired intracellular Ca^{2+} homeostasis and membrane potential regulation, the expression of several genes involved in insulin production and secretion were changed after CVB-5-DS infection (Fig. 2c). These included genes associated with insulin secretory granule assembly, hydrolases responsible for synthesis of insulin from proinsulin, enzymes required for acidification of the granule lumen and molecules associated with granule traffic and release, as well as granule cargo proteins co-secreted with insulin. These results are in line with previous studies that indicate impaired beta cell insulin secretion after CBV-5 infection [14, 15].

Pancreatic islet gene expression profiles induced by lytic vs non-lytic virus strains The gene expression pattern induced by CBV-5-DS suggested that the excess innate immune response and changes in beta cell metabolism might cause the delayed destruction of pancreatic islets. To test this hypothesis, we used two molecular clones of echovirus 9 (E-9-M2-2 and E-9-M1-12). These clones were produced from an E-9-DM strain isolated from a 6-week-old child at the clinical onset of type 1 diabetes [9, 17]. The two clones differed only by two amino acids but had a substantial phenotypic difference in human pancreatic islets. E-9-M1-12 induced more pronounced necrosis (Fig. 3a) and had a slight replication advantage compared with E9-M2-2 (Fig. 3b). The E-9 microvariants have been phenotypically characterised (A. Paananen, P. Ylipaasto, T. Smura, O. Korsgren, J. Galama and M. Roivainen, unpublished data).

Global mRNA expression was measured 48 and 96 h p.i., representing the time of maximum virus concentration and the time before visible cellular damage. The numbers of both upregulated and downregulated genes were higher for the lytic E-9-M1-12 strain than for the non-lytic E-9-M2-2 strain. Furthermore, the number of regulated genes increased from 48 to 96 h for the lytic E-9-M1-12 strain, whereas for the non-lytic strain E-9-M2-2 the number of regulated genes remained constant (Fig. 4a, b).

Several genes were similarly upregulated after infection with any of the enterovirus strains (i.e. CVB-5-DS vs both E-9 strains). These included genes encoding PRRs (*DDX58*,

a

Interferons		
IFNB1	2.9	26.1
IL28A	2.8	8.0*
IL28B	1.4	2.8*
IL29	1.9	4.5*

Pattern recognition receptors		
DDX58	5.0*	7.6*
DHX58	2.8**	4.6***
IFIH1	3.2*	5.8**
NOD2	1.5	3.1*
TLR3	2.0*	3.2

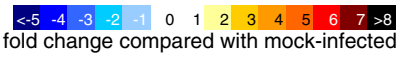
MAPK signalling pathway		
ATF3	1.8*	2.1*
ATF4	1.3	1.7*
BATF2	2.4***	3.5**
BATF3	1.4*	2.1
DUSP5	1.6*	1.8***
DUSP6	1.3*	1.4
DUSP10	1.4**	1.7
DUSP19	1.6***	2.4
ELK4	1.3***	1.9**
FLNB	-4.4*	-7.0***
JUN	1.6*	1.7
JUNB	1.4*	1.2
MAP2K3	1.5	1.8*
MAP3K8	2.3	3.7***
MAPK3	-1.1*	-1.3
MAPK4	-1.3*	-2.1
MAPK10	-1.2	-1.8*
MAPK11	-1.4	-2.4*
MAPK8IP3	-1.2	-2.0

STAT-mediated signal transduction		
IRF9	2.2*	2.8*
STAT1	3.5***	6.4***
STAT2	1.8*	2.5***

NFkB-mediated signal tranduction		
NFKB1	1.5*	1.8
NFKB2	1.3*	1.5
NFKBIA	1.7*	2.3*
NFKBIE	1.5*	1.8*
TNFAIP3	1.8**	2.3*
TNIP1	1.5*	1.9***

MHC-I mediated antigen presentation and proteasome complex		
HLA-B	1.3	3.1*
HLA-C	1.1	2.1
HLA-E	1.2	2.0*
HLA-F	1.4	2.6*
HLA-H	1.3	2.4**
PSMA4	1.3*	1.8*
PSMA6	1.2**	2.5***
PSMB8	1.7*	3.1**
PSMB9	2.7*	5.7**
PSMB10	1.4*	2.0***
PSMC4	1.3*	1.7*
PSME2	1.4*	2.3*
TAP1	2.5***	4.3***
TAP2	2.1*	4.4*

MHC-II mediated antigen presentation		
CD74	1.2	2.5*
HLA-DQA1	1.1	1.9*
HLA-DRA	1.3	2.1*



Mean fold change compared with mock-infected control

b

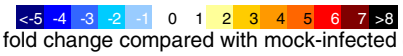
CC chemokines and receptors		
CCL2	1.5*	1.6
CCL3	3.9*	18.5
CCL3L1	2.7	12.6
CCL3L3	4.0	19.8*
CCL4L2	5.1*	15.5
CCL5	10.8*	42.0
CCL7	1.2*	1.6
CCL8	13.5	32.2*
CCR7	1.0	1.7*
CCL20	2.2	4.3*
CCL22	1.3*	2.5
CCL28	1.1	2.2

CX3C chemokines		
CX3CL1	5.1***	5.4***

CXC chemokines and receptors		
CXCL1	2.2*	3.9*
CXCL2	2.5	7.6
CXCL5	1.8	11.1***
CXCL6	1.7	3.2*
CXCL9	4.0	2.5
CXCL10	80*	127.9**
CXCL11	1.7	3.3
CXCL16	1.2	1.9*
CXCL17	1.1	2.2
CXCR4	1.3*	-1.4

Interleukins and receptors		
IL1A	2.7	4.8*
IL1B	2.2	8.7*
IL1F9	2.3	8.1***
IL1RN	2.9***	3.9
IL6	2.8*	5.1**
IL11	1.8**	3.6*
IL12A	1.5**	2.5
IL15	1.6***	1.9**
IL23A	1.3	5.9
IL24	1.9	4.6
IL33	2.0	2.9
IL7R	1.7***	3.3
IL13RA1	-1.4*	-1.7
IL15RA	1.8*	2.4
IL20RA	-1.3	-1.7*
IL20RB	1.4*	1.7

TNF-superfamily and receptors		
TNF	2.4*	4.9*
TNFSF10	2.6*	3.9*
TNFSF13B	2.3*	4.1*
TNFRSF11B	1.6*	1.7
TNFRSF12A	1.4*	1.8*



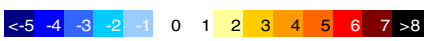
Mean fold change compared with mock-infected control

c

Glycolysis		
ALDOA	-1.1	-2.0
ALDOB	-1.2	-1.9
ALDOC	-1.3	-2.2
ENO2	-1.3*	-3.3
GAPDH	-1.3*	-1.7
GCK	-1.4	-1.9
HK2	1.4	2.1
PFKFB2	-1.5*	-1.9*
PFKL	-1.0	-1.5
PFKP	-1.1	-1.8
PGK1	-1.1	-1.6
SLC2A3	-1.1	-1.8
SLC2A6	1.5*	1.9

Insulin transcription factors		
ISL1	-1.2	-2.1*
MAFA	2.3*	7.0***
NEUROD1	1.1	-2.0**
STAT5B	-1.3	-1.8
USF1	1.3	1.9*

Insulin secretion		
CDK5R2	-1.3	-1.9
CHGA	-1.1	-1.3
CHGB	-1.2	-2.0*
CPE	-1.5	-3.5**
CPLX1	-1.2	-1.9
CPLX2	-1.2*	-1.4
PAM	-1.3	-2.3*
PCSK1	-2.0**	-4.4**
PTPRN	-1.6	-5.0
PTPRN2	-1.3	-2.1
RAB3B	-1.3*	-1.8**
RAB3C	-1.3	-1.9***
RPH3AL	-1.5*	-3.3
SCG3	-1.5*	-2.0*
SCG5	-1.2***	-1.7
SLC30A8	-2.0***	-6.3*
SNAP25	-1.2	-2.1*
STX1A	1.1*	-2.0
STXBP1	-1.3*	-2.1***
STXBP5	-1.1	-1.5
SYT4	-1.4	-2.3*
SYT7	-1.5	-3.7*
SYT11	-1.3	-1.8
SYT13	-1.4	-2.7
SYT17	-1.3	-1.9*
SYTL1	-1	-1.7
VAMP2	-1.1	-1.9
VGF	-1.2	-1.7



Mean fold change compared with mock-infected control

◀ **Fig. 2** (**a**–**c**) Mean fold changes of expression signals between islets infected with CBV-5-DS and mock-infected control islets (n=3). Heatmaps with statistical significances of selected genes are shown. *$p \leq$ 0.01, **$p \leq 0.005$, ***$p \leq 0.001$ compared with mock-infected control (Student's t test)

IFIH1 and *NOD2*), IFNs (*IFNB1*, *IL28* and *IL29*), antiviral molecules, STAT1, the regulatory members of immunoproteasome complex (*PSMB8*, *PSMB9*, *PSMB10* and *PSME2*) and antigen peptide transporters (*TAP1* and *TAP2*).

Also differences between lytic and non-lytic strains were detected (Fig. 4c, ESM Fig. 2, ESM Table 4). Upregulation of chemokine gene expression was stronger after lytic E-9-M1-12 infection than after benign E-9-M2-2 infection. The expression of genes encoding pro-inflammatory cytokines and beta cell apoptosis inducers IL-1α, IL-1β, TNF-α and tumour necrosis factor superfamily 10 (TNFSF10; TRAIL) were upregulated by cytolytic virus strains CVB-5-DS and E-9-M1-12, but not by the benign strain E-9-M2-2 (Fig. 2b, Fig. 4c, ESM Fig. 2). Furthermore, TRAIL receptor gene *TNFRSF10D* was downregulated after infection with the benign E-9-M2-2 strain.

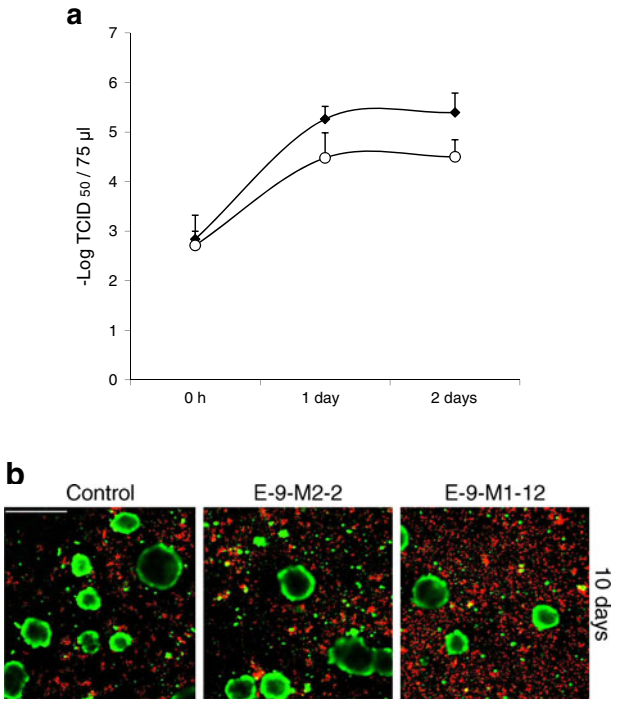


Fig. 3 (**a**) Infectious progeny production of E-9-M1-12 (diamonds) and E-9-M2-2 (circles) in human pancreatic islets. Subsets of islets derived from seven donors were infected with an apparently high multiplicity of infection and the samples were collected immediately (0 h), 1 day and 2 days after infection. The total infectivity (50% tissue culture infectious dose, $TCID_{50}$) of each sample was determined by endpoint titration in green monkey kidney (GMK) cells. (Data are means ± SD). (**b**) Viability of pancreatic islets 10 days after infection with E-9-M1-12 or E-9-M2-2. The viability was assessed using Live/Dead assay (Molecular Probes). Because of their esterase activity, live cells are stained green by calcein, while nuclei of dead cells are stained red by ethidium homodimer-1. Scale bar, 250 µm

Both apoptotic and anti-apoptotic genes were upregulated after infection with the cytolytic virus strains. These included genes associated with IAP (inhibitor of apoptosis) protein family members (*BIRC3*), X-linked inhibitor of apoptosis (*XIAP*, *BIRC4*) and XIAP antagoniser (*XAF1*, XIAP associated factor 1) as well as genes for B cell lymphoma-2 protein family members, such as *PMAIP1* (*NOXA*) and *MCL1* (Fig. 4c). Benign E-9-M2-2 infection induced downregulation of genes encoding a cell-cycle and apoptosis regulator, tumour protein p53 (*TP53*), and p53 activator ataxia telangiectasia mutated (*ATM*) as well as upregulation of p53 inhibitor gene *MDM4* (Fig. 4c).

Out of the genes involved in cellular responses to reactive oxygen species (ROS), all three virus strains induced upregulation of mitochondrial superoxide dismutase 2 (*SOD2*). After CVB-5-DS infection the expression of genes coding for glutathione peroxidase 3 (*GPX3*), peroxiredoxin 2 (*PRDX2*) and sestrin 1 (*SESN1*) (that regenerates overoxidised peroxiredoxins), were downregulated, whereas E-9-M1-12 infection induced downregulation of the gene for catalase (*CAT*) (Fig. 4c, ESM Table 3).

ROS-induced DNA damage is counteracted by DNA repair enzymes such as poly-(ADP-ribose) polymerase (PARP). The expression of genes encoding several members of the PARP family was upregulated by all of the HEV strains, although *PARP1* was downregulated after CVB-5-DS infection (Fig. 4c, ESM Table 3).

The ESM text discusses changes observed in metabolomics studies after infection of islets with CVB-5-DS.

Discussion

Innate immunity and inflammatory mediators have a major role in the development of type 1 diabetes [30]. The potential pathogenetic link between HEV infection and type 1 diabetes can therefore be the linkage of virus infection in beta cells with an inflammatory response. Here we studied the connection between HEV-induced islet destruction, viral genetics and host-cell response to infection and detected a highly pro-inflammatory islet response to the infection. Furthermore, the expression of pro-inflammatory cytokine genes (*IL1A*, *IL1B*, *TNF* and *TRAIL*) correlated with the lytic potential of a virus.

The outcome of islet infection (i.e. destructive vs benign) and inflammatory response are probably interconnected, since the major mechanism of islet destruction after HEV infection is necrosis [14]. This is likely to have a profound effect on the local inflammatory milieu at the site of infection, since necrosis represents a severe form of death where

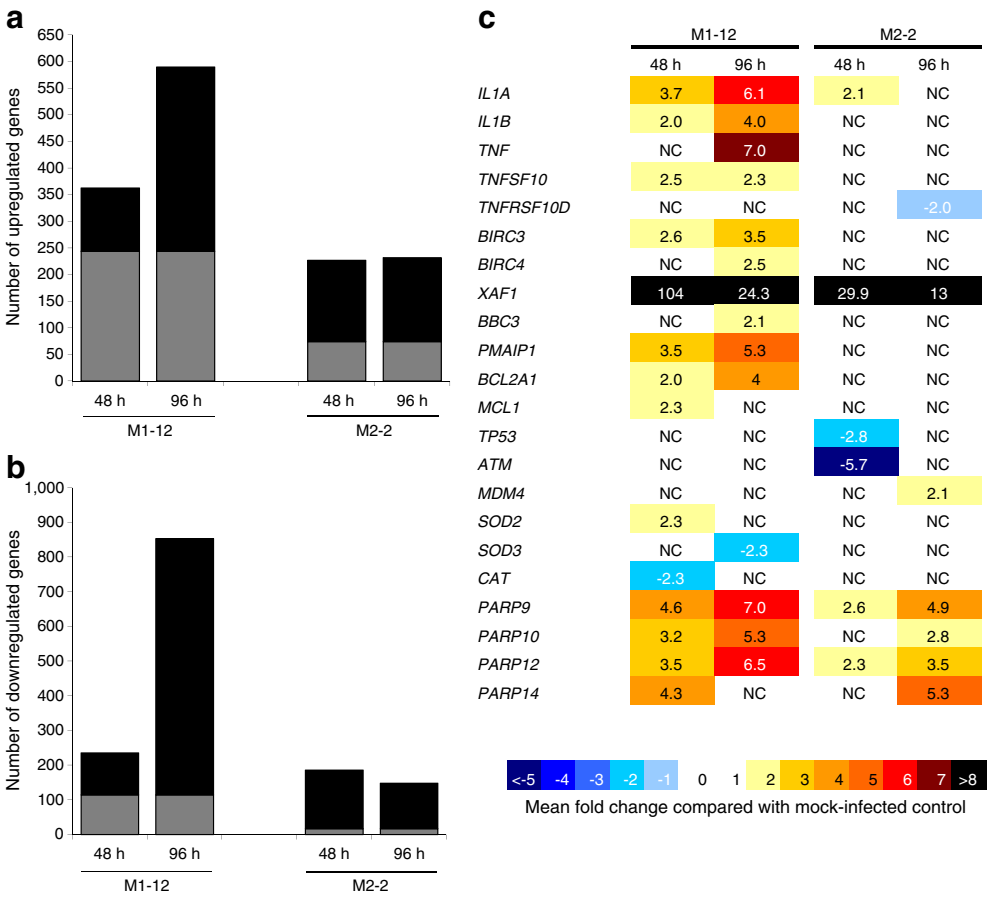


Fig. 4 The number of upregulated (**a**) and downregulated (**b**) genes 48 and 96 h after infection with E-9-M1-12 or E-9-M2-2. Grey bars indicate the number of genes regulated at both time points and black bars indicate the number genes regulated at one time point only. (**c**) Fold changes of expression signals between islets infected with E-9-M1-12 or E-9-M2-2 and mock-infected control islets. Heatmap of selected genes are shown. NC, not changed according to Affymetrix criteria (i.e. not detected or less than twofold change)

the cell content leaks into the surrounding tissue causing damage to bystander cells and inducing strong inflammatory response [31]. Thus, in addition to impaired beta cell function, islet infection with a lytic HEV strain may lead to autoantigen presentation in an inflammatory milieu, which may induce autoimmune reactions.

Some HEV strains are highly cytolytic in pancreatic islets whereas others replicate with no apparent islet destruction [15–17]. This indicates that viral strain-specific genetic factors partly determine the outcome of infection. Since there is a significant delay between viral growth and islet destruction [14, 15], we hypothesised that lytic HEV strains induce a distinct host response profile leading to islet destruction, a profile that differs from that caused by other strains having a more benign effect. This hypothesis was tested by comparing the gene expression patterns after lytic and 'benign' HEV infections.

Complete islet preparations were used in this study. Thus, the gene expression patterns monitored reflect the combined effects of virus and virus-induced factors on all of the cell types present. This is likely to mimic the situation in vivo, where the fate of a beta cell is affected by responses of neighbouring non-beta cells. Since the islet response to HEV infection involves multiple interconnected networks of genes, the experiments were designed to reveal large-scale patterns of changes in gene expression over a prolonged period rather than the impact of a single or few pathways. Therefore, the conclusions deduced from this study need to be further elucidated with more detailed experiments.

In a previous study, a complex network of genes participating in islet responses to a viral infection was identified [19]. In the present study, the changes in gene expression were measured at two time points to gain information about temporal changes in gene expression patterns. The number of regulated genes increased from the first to the second time point for the lytic strains, but remained constant for the non-lytic strain (Fig. 4a, b, ESM Table 3). This suggests that the dynamics of the changes in gene expression is associated with islet destruction. Furthermore, the changes induced by CVB-5 were compared with those of lytic and non-lytic E-9 infections to study which of the genes are regulated by HEV

infection in general and which of the gene expression changes are specific for lytic infections.

The benign HEV strain induced fewer changes in gene expression than the lytic HEV strains. All of the virus strains upregulated the expression of genes associated with key mediators of the innate immune response, including type I and type III IFNs, dsRNA receptors, antiviral molecules and corresponding signal transduction cascades; this may have implications on the outcome of infection. IFNs upregulate the expression of genes encoding PRRs in human pancreatic islets [19, 32], thus forming a positive feedback loop that has to be tightly regulated. The lack of accurate negative regulation of PRRs would lead to overt innate immune reactions, whereas lack of accurate positive regulation would lead to an impaired antimicrobial response. The temporally increasing upregulation of genes involved in the innate immune response suggests that a lytic HEV infection may drive this positive feedback loop towards overt innate immune response, whereas for the benign strains the innate immune response is weaker, potentially providing a possibility for establishment of chronic infection.

Many of the PRR- and IFN-elicited responses in beta cells are mediated by NFκB [33], STAT-1 [34] and MAPK [22] signal transduction pathways, all of which showed upregulation after infection. However, NFκB and MAPK pathways also showed increased inhibition (both pathways) and/or downregulation of the signal cascade molecules (MAPK) after CVB-5-DS infection. This may reflect negative feedback mechanisms aimed at inhibiting overt activation of these pathways.

PRR-mediated induction of IFNs and transcription factors leads to the expression of pro-inflammatory cytokines, chemokines and adhesion molecules, which collectively regulate the recruitment of immune cells to sites of infection and modulate the magnitude and duration of the immune response. The expression of genes associated with pro-inflammatory cytokines IL-1α, IL-1β and TNF-α were upregulated by lytic strains (CBV-5-DS and E-9-M1-12) only. Furthermore, consistent with previous studies [19, 32, 35–37], the cytolytic enterovirus strains induced strong stimulation of genes involved in chemokine secretion, whereas after infection with the benign (E-9-M2-2) strain, the chemokine response was weaker. These results suggest that lytic HEV strains are more potent inducers of a pro-inflammatory micro-environment and probably recruit immune cells to the site of infection more efficiently.

IL-1α, IL-1β and TNF-α induce cellular death in pancreatic islets [38, 39]. However, cytokine production induced by HEV infection cannot be the sole factor leading to destruction of pancreatic islets. Exposure of human islets to these cytokines induces cell death by apoptosis [21, 40, 41], whereas HEV infection induces secondary necrosis after initial nuclear pyknosis [14]. Only a small fraction of beta cells show signs of apoptosis after infection [14, 42]. Accordingly, the GSEA analysis indicated upregulation of apoptosis and necrosis pathways together with shutdown of metabolic pathways. The reduction of phosphatidylcholines, the major constituents of biological membranes, revealed by the lipidomics, may be a result of cell lysis resulting from necrosis.

The switch between apoptosis and necrosis may be determined by the availability of ATP [43, 44]. Downregulation of glycolysis and oxidative phosphorylation by CVB-5 infection might lead to intracellular depletion of ATP and NADH and, thereby, combined with IL-1, TNF-α and TRAIL expression, induce secondary necrosis. In this respect HEV-infected human islets may resemble rodent islets, which, when exposed to IL-1β, IFN-γ and TNF-α, die predominantly by necrosis [45], possibly due to depletion of cellular ATP and NAD^+ [22, 46]. However, in murine beta cells the ATP and NAD^+ depletion is dependent on nitric oxide produced by the inducible form of nitric oxide synthase (iNOS) while in human beta cell death the role of iNOS is controversial [19, 22].

The ATP levels may be further lowered by PARP family members [46, 47] that are involved in ROS-induced DNA strand-break repair. Excessive poly-(ADP-ribose) formation by PARP may deplete cellular NAD^+ pools and therefore induce necrosis in several cell types, including pancreatic beta cells [48]. Consistently, several members of the PARP family were upregulated by HEV infection.

These results suggest that (analogous to a hypothesis by Eizirik and Mandrup-Poulsen [22]) HEV-induced islet cell necrosis might result from a combined effect of cytotoxic cytokines and ATP depletion due to downregulated glycolysis and oxidative phosphorylation and possibly via PARP overactivation.

In conclusion, the results support the hypothesis that a distinct gene expression profile correlates with HEV-induced human pancreatic islet necrosis. This profile includes temporally increasing gene expression levels of pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α and TRAIL), which are considered to be key mediators of cytokine-induced beta cell dysfunction. This virally induced destructive gene expression profile may be linked to generation or maintenance of autoimmune reactions since it combines pro-inflammatory cytokine/chemokine expression with beta cell destruction.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement PY, PG, AP, SK, HA analysed and interpreted data and edited the manuscript. TS analysed and interpreted data and drafted the manuscript. OK, JRTL, RL, TSL, MO, LP and JG designed experiments and revised the manuscript. MR is the principal investigator, conceived and designed experiments, revised the manuscript and critically reviewed the manuscript for intellectual content. The final version of the manuscript was approved by all the authors.

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