Phagocytosis of enterovirus-infected pancreatic beta-cells triggers innate immune responses in human dendritic cells

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Running title: uptake of β-cells results in human DC activation

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Background. Type 1 diabetes is a chronic endocrine disorder, in which enteroviruses, such as coxsackie B viruses and echoviruses, are possible environmental factors that can trigger or accelerate disease. The development or acceleration of type 1 diabetes depends on the balance between autoreactive effector T cells and regulatory T cells. This balance is particularly influenced by dendritic cells (DCs).

Objective. To investigate the interaction between enterovirus-infected human pancreatic islets and human DCs.

Research Design and Methods. In vitro phagocytosis of human or porcine primary islets or Min6 mouse insuloma cells by DCs was investigated by flow cytometry and confocal analysis. Subsequent innate DC responses were monitored by qPCR and western blotting of interferon-stimulated genes (ISGs).

Results. We show that both mock- and coxsackievirus B3 (CVB3)-infected human and porcine pancreatic islets were efficiently phagocytosed by human monocyte-derived DCs. Phagocytosis of CVB3-infected, but not mock-infected human and porcine islets resulted in induction of ISGs in DCs, including the RIG-I-like helicases (RLHs) RIG-I and Mda5. Studies with murine Min6 insuloma cells, which were also efficiently phagocytosed, revealed that increased ISG-expression in DCs upon encountering CVB-infected cells resulted in an antiviral state that protected DCs from subsequent enterovirus infection. The observed innate antiviral responses depended on RNA within the phagocytosed cells, required endosomal acidification and were type I interferon (IFN)-dependent.

Conclusions. Human DCs can phagocytose enterovirus-infected pancreatic cells and subsequently induce innate antiviral responses, such as induction of RLHs. These responses may have important consequences for immune homeostasis in vivo and may play a role in the etiology of type 1 diabetes.
Type 1 diabetes mellitus, or insulin-dependent diabetes, is a chronic endocrine disorder characterized by the progressive loss of insulin-producing \( \beta \)-cells. In the majority of cases, type 1 diabetes is associated with an autoimmune reaction against \( \beta \)-cell constituents. Genetic predisposition is a major risk factor for the acquisition of type 1 diabetes but the pairwise concordance between monozygotic twins is limited (< 40%), which indicates that other, environmental, factors are involved (1). Also other observations (a gradual rise of the incidence and a 10-fold difference in occurrence of type 1 diabetes in different parts of Europe) point to a significant contribution of the environment (1).

Enteroviruses of the Human Enterovirus B (HEV-B) species of the Picornaviridae, such as coxsackievirus B (CVB) and echovirus (EV), have since long been associated with type 1 diabetes (2-4). These small, non-enveloped single-stranded RNA viruses are widespread. Infection usually remains limited to the gastrointestinal tract and causes mild disease or even remains asymptomatic. However, during severe infections it can spread to secondary target organs such as the pancreas, brain and heart (5). CVB has been implicated in type 1 diabetes on the basis of (i) isolation of virus from patients with acute diabetes, (ii) detection of viral RNA in blood at onset, (iii) epidemiological surveys, and (iv) prospective studies (reviewed in (6; 7)).

Importantly, several recent studies reported detection of HEV-B in the pancreatic islets of type 1 diabetes patients at autopsy, providing evidence that these viruses are able to infect beta cells in vivo (8-10). Inflammation in pancreatic islets is common in type 1 diabetes patients, and several immune cells can be detected in the islets of type 1 diabetes patients. Among these are phagocytes, as well as different subsets of T cells, such as effector CD4\(^+\) and CD8\(^+\) T cells and in some cases regulatory T cells (T\(_{\text{reg}}\)) (8; 11; 12). The development or acceleration of type 1 diabetes might depend on the balance between autoreactive effector T cells and T\(_{\text{reg}}\) (13); a balance which is predominantly decided by dendritic cells (DCs).

The importance of antigen-presenting cells (APCs, such as macrophages and DCs) in type 1 diabetes development has been shown in vivo in mouse studies, where phagocytosis of CVB-infected islet cells was crucial for development of auto-immune type 1 diabetes. Resident APCs were shown to engulf CVB-infected \( \beta \)-cells, and subsequently stimulated antigen-specific T cell proliferation and induced diabetes upon adoptive transfer (14). Furthermore, mouse studies have revealed that influx of macrophages and DCs precedes that of effector T cells in the islets and aberrantly express pro-inflammatory cytokines (15). In human pancreatic islets DCs are present in low numbers under steady-state conditions; however, as \( \beta \)-cell destruction ensues leading to diabetes there is an increase in DC-infiltrate (16; 17), strengthening the hypothesis that these cells likely play an important role in the progression of human type 1 diabetes. To our knowledge, no studies have been performed that examine the interaction between CVB-infected human islets and human DCs.

DCs are the professional APCs of the immune system and play a decisive role in initiating immune responses and maintaining self tolerance. They do not only participate in innate immunity, but also initiate and control adaptive immunity (18). DCs continuously sample their microenvironment; they phagocytose pathogens or pathogen-associated products such as immune complexes, yet also apoptotic cells are taken up (19). This results in the induction of immunity against invading pathogens and
tolerance to self-antigens, respectively (20). Induction of an immune response occurs when DCs detect pathogen-associated “danger signals” or pathogen-associated molecular patterns (PAMPs), through specialized receptors, known as pattern recognition receptors (PRRs). These PRRs include several receptors that sense the presence of viral RNA, such as Toll-like receptors (TLR) 3, 7 and 8 and the RIG-I-like helicases (RLHs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (Mda5) (21; 22). Polymorphisms and mutations in PRR family members have been associated with disease, including autoimmune diseases such as type 1 diabetes (23-25). The interaction between PRRs and PAMPs facilitates DC maturation, after which the DCs migrate to draining lymph nodes where they present antigen to T lymphocytes (18). During viral infection, triggering of these receptors induces type I interferons (IFNs). The IFNs are crucial for a first innate line of defense against invading viruses, yet they also influence adaptive immunity by affecting DC cytokine production and maturation (26) and autoimmunity (27).

In this study, we set out to investigate the interaction between human DCs and CVB-infected islets and the immunological consequences that follow. For this, we used monocyte-derived DCs from healthy blood donors and CVB-infected pancreatic islet preparations or CVB-infected Min6 cells, a murine insuloma cell line. We show that both human islets and Min6 cells are phagocytosed by human DCs. CVB-infected cells, but not mock-infected cells, induced DC activation as indicated by the expression of interferon-stimulated genes (ISGs) resulting in protection of the DCs from subsequent enteroviral infection. These innate DC responses depended on the recognition of RNA in the CVB-infected cells and additionally required IFNs produced by the DC itself, but did not depend on soluble factors secreted by the infected cells. These virus-induced alterations in DCs may have important consequences for immune homeostasis in vivo and may play a role in the etiology of type 1 diabetes.

**RESEARCH DESIGN & METHODS**

**Virus stocks and purification.** Reference strain Echovirus 9 Hill (EV9) was obtained from the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands). CVB3 Nancy (CVB3) was kindly provided by R. Kandolf (University of Tübingen, Germany). Production of virus stocks and virus titrations were performed on buffalo green monkey cells as described previously. Serial 10-fold dilutions were tested in 96-well microtiter plates and 50% Tissue Culture Infective Doses (TCID<sub>50</sub>) were calculated as described before (28).

**Islet and Min6 cell culture.** Human and porcine pancreatic islets were isolated in Pittsburgh as described before (29). Human islets were obtained from deceased anonymous donors procured by CORE (Center for Organ recovery and Education, Pittsburgh), and islets were isolated using a modification of the semi-automated method described by Ricordi (29; 30). Islets batches used in this study were obtained from 4 adult human pancreas and 3 porcine donors, cultured for a minimum of 3 to 6 days in CMRL-1066 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100U/ml penicilin, 0.1mg/ml streptomycin (Complete CMRL), at 37°C in an atmosphere of 5% CO<sub>2</sub>. Islet viability was estimated by dual fluorescence viability dyes (Calcein-AM and Propidium Iodide, Invitrogen, Eugene, OR) and was higher than 80% in all batches. Glucose stimulated insulin release was carried out by dynamic perifusion (31). After culture for 3 to 6 days the islets were sent to Nijmegen as free-floating islets and cultured in Complete CMRL in ultra low attachment culture plates (Corning) at 37°C in 5% CO<sub>2</sub>. Islets were cultured in Nijmegen for
maximum 2 days before start of the experiments. Min6 cells (32) were a kind gift from Dr. Merja Roivainen and Dr. Per Bendix Jeppesen, and were cultured in DMEM (Gibco) supplemented with 15% FCS, ciproxin, 50µM β-mercaptoethanol at 37°C in 5% CO₂. Medium was refreshed every other day.

**Infection of islets and Min6 cells.** Islets and Min6 cells were infected in a small volume of CMRL-1066 or DMEM, respectively, at indicated multiplicity of infection (MOI) for 1 h at 37°C. Subsequently, cells were washed and viral titers were determined at different time points as described above. In some experiments supernatant from infected cultures was harvested at indicated time post infection (p.i.) and cleared from cell debris by centrifugation before stimulation of other cells.

**Stimulation of monocyte-derived DCs.** Monocyte-derived DCs were generated as described previously (28). Mature DCs were obtained by stimulating cells with poly(I:C) (20 µg/ml). Stimulation of DCs with supernatant from Min6 cells or from DC/Min6 co-cultures was performed using a 1:2 dilution of supernatant. To block the actions of type I interferons (IFNs), cells were stimulated in presence or absence of neutralizing anti-human IFN antibodies (1:75, Iivari, Kaaleppi or bovine serum, courtesy of Dr Julkunen, National Public Health Institute, Helsinki, Finland) (33). For infection, stimulated or unstimulated DCs were harvested using cold PBS and infected in RPMI. After 60 min incubation at 37°C, cells were washed and viral titers were determined as described above.

**Uptake of human pancreatic islets and Min6 cells.** Human and porcine pancreatic islets or Min6 cells were labeled using PKH26 (Sigma-Aldrich) according to manufacturer’s instructions. PKH-labeled cells were added to CFSE-labeled DC cultures at a ratio of 1:1. Alternatively, labeled, infected Min6 cells were incubated for 48h and subsequently harvested and resuspended in fresh medium at a density of 5x10⁶ cells/mL prior to placing them at -20°C until further use. Freeze-thawed cell preparations were subsequently used in DC co-cultures at a 1:1 ratio and resulted in similar inductions of ISGs and innate responses compared to co-culture with viable cells (Supplementary figure 1 in the online appendix at http://diabetes.diabetesjournals.org and (34)). These cell preparations were also used for RNase-treatment prior to DC stimulation. For that purpose Min6 cell preparations were exposed to a mixture of RNaseA, RNase VI and RNasel (all Ambion) or an equal volume of PBS for a period of 30 min at 37°C prior to addition to DCs. Uptake of islets or Min6 cells by DCs was analyzed using flow cytometry and confocal microscopy. In some experiments phagocytosis was inhibited using cytochalasin D (CytD) (2.5µg/ml), or endosomal acidification was inhibited using chloroquine (CQ) (10µM). DCs were pretreated for 30 min with CytD or CQ, and subsequently stimuli were added. Both treatments had no effect on cell viability as assessed by trypan blue exclusion 8h after stimulation.

**Confocal microscopy.** Staining and visualization of DCs has been described before (34). Human islets were adhered onto fibronectin-coated coverslips for 2h at 37°C in islet medium and subsequently fixed in 2% PFA. Cells were blocked in PBS containing 100 mM glycine and 2% goat serum, permeabilized using TX-100 and stained with mouse anti-VP1 (Dakocytomation) and rabbit anti-3A (35) followed by incubation with goat anti-mouse IgG alexa 488 and goat anti-rabbit
IgG alexa 594. Cells were analyzed using a Leica DMR microscope.

**RNA isolation, quantitative PCR (qPCR), western blot and flow cytometry.** These techniques have been described before (34)

**Statistical analysis.** Statistical analysis was performed using Student’s *t* test (2-tailed distribution). A P-value < 0.05 was considered a significant difference.

**RESULTS**

**Human dendritic cells phagocytose human pancreatic islets and induce innate immune responses.** Human pancreatic islets were found to be susceptible to infection by CVB3 as indicated by the profound increase in virus titer, cytopathic effects and immunofluorescence staining against viral proteins 3A and VP1 (Fig. 1A-C). The latter revealed that only a small number of cells were infected. Infected cells mainly resided at the outer layers of the islets, where cells might be more accessible for the virus (Fig. 1C). Previously it was shown that such a productive infection results in impaired islet function (36).

To investigate whether human DCs can phagocytose human pancreatic islets, PKH-labeled human islets were mock- or CVB3-infected for 48 hours and subsequently co-cultured with CFSE-labeled DCs. CVB3 has no direct effect on human DCs and is incapable of infecting DCs (28). Mock- and CVB3-infected islets were taken up with equal efficiency, as indicated by the number of DCs that became PKH-positive (Fig. 1D). Confocal analysis confirmed uptake of human pancreatic islets material by DCs. The DC plasma membrane was stained with CD86 specific antibodies (green), and PKH-positive islet cells (red) were observed within the DCs (Fig. 1E).

To examine whether and how DCs respond to virus-infected human islets we studied activation of innate immune response pathways by measuring levels of ISGs, such as RIG-I, Mda5, PKR and IRF-7 following phagocytosis of infected cells. qPCR revealed induction of ISGs after engulfment of CVB3-infected islets; moreover, increased expression was observed for all ISGs tested (Fig. 1F). Importantly, stimulation of DCs with mock-infected human islets alone did not induce any ISGs (Fig. 1F). Furthermore, also stimulation with CVB3 alone did not induce ISGs (28), suggesting that the induction depends on the presence of virus or viral products within phagocytosed cells. CVB3 was not able to replicate in DCs upon entry via phagocytosis, as determined by endpoint titration at several times after start of co-cultures (data not shown). In some experiments, freeze-thawed mock- or CVB-infected cells were used. This had no effect on either phagocytosis or ISG-induction (see below).

At this stage we were unable to discriminate whether the ISGs were upregulated in DCs or in the islets themselves. Therefore, we performed experiments using porcine islets, which have been shown a useful model for studies on the interaction of CVBs with β-cells (37). To discriminate between ISGs in porcine islets and human DCs, primers were used that specifically recognize human, but not porcine ISG sequences. Infection and uptake of porcine islets by DC was similar as described above for the human islets (data not shown). Phagocytosis of CVB-infected porcine islets resulted in a profound increase in human ISGs (Fig. 1G). Thus, using porcine islets we confirmed induction of ISGs in human DCs upon uptake of CVB3-infected human islets, and, additionally revealed that ISG-expression is increased in the human DC-population.

Phagocytosis of CVB3-infected Min6 cells by human DCs results in an antiviral state that protects DCs from subsequent enterovirus infection. To further investigate the underlying mechanism of DC responses upon uptake of islets, we selected the murine
Min6 cell line as a model for pancreatic β-cells. Min6 cells retain the physiological characteristics of normal β-cells, and respond to glucose within the physiological range (32). These islet-like cells grow in patches (Fig. 2B, left panel) and were confirmed to express several β-cell associated mRNAs (e.g. GAD65, IGRP, IA2, insulin, data not shown). Analogous to human and porcine islets, Min6 cells were susceptible to infection with CVB3 as shown by the increase in virus titer (Fig. 2A). Cytopathic effects were observed in nearly 100% of the cells (Fig. 2B), suggesting that the majority of cells were infected. Uptake of mock- and CVB3-infected Min6 cells by DCs was very efficient (up to 75%) as shown by flow cytometry and confocal analysis (Fig. 2C and 2D).

Upon engulfment of CVB-infected Min6 cells, IFN-β and ISGs were strongly induced in DCs (Fig. 2 E and F), reaching a greater than 100-fold increase compared to unstimulated DCs in the case of RIG-I, while Mda5 and PKR showed increases of up to 20- and 10-fold, respectively (Fig. 2F). The induction of ISGs following uptake of infected Min6 cells was confirmed at the protein level by western blotting (Fig. 2G). No ISG signal was observed in mock- or CVB3-infected Min6 cells alone (data not shown), excluding that the observed protein induction in DC/Min6 co-cultures is due to detection of Min6 cell proteins. In some experiments, freeze-thawed mock- or CVB-infected cells were used. This had no effect on either phagocytosis or ISG-induction (Supplementary Fig 1).

IFNs and ISGs induce an antiviral state that restricts virus replication (38). To determine whether upregulation of ISGs following uptake of CVB3-infected Min6 cells protects DCs from subsequent infection, DCs that had taken up Min6 cells were infected with EV9, an enterovirus strain that does replicate in DCs (28), but not in Min6 cells (data not shown). Uptake of mock-infected cells had no influence on replication of EV9 in DCs. In contrast, phagocytosis of CVB-infected Min6 cells strongly protected the DCs from EV9 replication (Fig. 2H).

**Innate responses in DC that phagocytosed CVB3-infected Min6 cells depend on autocrine IFN-α/β signaling.** ISGs can be upregulated through both IFN-dependent and IFN-independent pathways (39; 40). To investigate whether secreted cytokines like IFN-α/β were involved in ISG induction, we stimulated fresh DCs with cell-free supernatant from DC/Min6 co-cultures. An increase in ISGs was observed upon stimulation of DCs with supernatant from co-cultures of DCs plus CVB-infected Min6 cells, but not of DCs plus mock-infected Min6 cells (Fig. 3A). Moreover, the induction of ISGs after uptake of CVB-infected cells was markedly reduced in the presence of neutralizing IFN-α/β antibodies, at both the mRNA level (Fig. 3B) and protein level (Fig. 3C compare lanes 6 and 7), implying that ISG induction strongly depends on IFNs.

To determine whether IFNs produced by DCs or the infected Min6 cells were responsible for ISG upregulation, supernatants from infected Min6 cultures were added to DCs, after which ISG-induction was monitored. Although recombinant murine IFNα induced ISG expression in DCs, supernatant of either mock- or CVB-infected Min6 cells did not (Fig. 3D). Thus cytokines potentially produced by infected Min6 cells are not responsible for ISG expression in DCs.

Collectively, these data imply that DCs induce an innate immune response upon uptake of CVB3-infected Min6 cells and that this induction depends on IFN-induction by DCs themselves.

**Induction of ISGs in DCs following uptake of CVB3-infected Min6 cells require endosomal acidification and recognition of viral RNA.** Both PRRs located on the cell surface and those with intracellular
localization may mediate the observed responses in DCs. To investigate whether phagocytosis of cells is required for the induction of ISGs, or whether extracellular exposure to infected cells might initiate ISG responses in DCs (e.g. via TLRs on the plasma membrane), phagocytosis was inhibited using Cytochalasin D (CytD). DCs pretreated with CytD and subsequently stimulated with CVB3-infected Min6 cells did not show any induction of ISGs, indicating that phagocytosis is required for ISG-induction. Similar decreases in ISG induction were observed when using CVB-infected primary porcine islets (data not shown). Poly I:C-induced ISGs were also reduced upon CytD-treatment, as has been reported before (41). Importantly, LPS induced responses were not abrogated in the presence of CytD, proving that CytD has no adverse effects on DC viability or ISG-induction (Fig 4A) (42).

Phagocytosed cells are localized to so-called phagosomes, which subsequently “mature” via fusion with the endosomal/lysosomal compartments, followed by progressive decrease in pH (20; 43). Intracellular TLRs (TLR3, 7, 8, 9) are also recruited to these compartments, enabling interaction with potentially released PAMPs, like dsRNA, which occurs in a pH-dependent fashion (44). Alternatively, phagocytosed material might be translocated into the cytoplasm, where for example the RLHs (RIG-I, Mda5) can interact with viral RNA. To determine if endosomal acidification is required for the induction of ISGs following uptake of infected cell preparations, we pretreated DCs with chloroquine (CQ), a chemical that blocks acidification of these compartments. Pretreatment of DCs with CQ markedly decreased CVB-infected Min6-induced mRNA expression of RIG-I, Mda5 and PKR (Fig 4B), suggesting that endosomal acidification is critical for DC responses upon phagocytosis of infected cells. Similar decreases in ISG induction were observed when using CVB-infected primary porcine islets (data not shown). FACS analysis showed that phagocytosis of Min6 cells was not inhibited due to CQ treatment (data not shown). Poly I:C induction, known to require endosomal acidification, was also decreased (41; 44). LPS-induced responses do not require endosomal acidification (41; 44). As expected, LPS-induced ISG and IL-6 expression was not decreased, indicating that viability and intracellular signaling to induce cytokines was unaltered upon CQ-treatment (Fig 4B and data not shown).

Using RNases, we investigated the contribution of (viral) RNA in our CVB-infected cells to DC responses. For this, freeze-thawed Min6 cell preparations were used, as viable cells with an intact plasma membrane will make degradation of intracellular RNA impossible. RNase-treatment of freeze-thawed Min6 cell preparations prior to addition to DCs reduced upregulation of RIG-I, Mda5 and PKR at both mRNA and protein level (Fig. 4C and Fig. 4D compare lane 4 and lane 6), demonstrating the important role of viral RNA present in infected cells for the induction of innate immunity. Together, our data show that phagocytosis of CVB-infected cells is required, and that subsequent signaling requires endosomal acidification and depends on the presence of viral RNA.

**DISCUSSION**

DCs play a critical role in inducing immunity and preventing auto-immunity. Although diabetes pathogenesis and the possible role of APCs such as DCs therein has been investigated in mice (14; 15), to our knowledge, no studies have been performed that examined the interaction between islets and DCs in humans. Here, we show for the first time that CVB-infected human islets are efficiently phagocytosed by human DCs
resulting in a rapid RNA- and IFN-dependent innate antiviral response by the DCs.

The response of DCs was further characterized with use of porcine islets and murine Min6 cells. Mock-infected cells did not induce innate responses, even though, surprisingly, their phagocytosis was as efficient. The reason for equal uptake of mock- and CVB-infected cells is unknown; islets/β-cells may display enhanced molecular signals that mediate phagocytosis (“eat-me signals”, such as phosphatidyl serines (PS)) (45), possibly caused by ER-stress inherent to massive insulin production (46). Preliminary data revealed that PS are higher expressed on the outer cell surface of steady-state insulin-producing Min6 cells when compared to other steady-state cell lines that are not efficiently phagocytosed (e.g. HeLa, L929, BGM, Vero) (data not shown). Whether PS or other “eat-me signals” are present on isolated primary human pancreatic islets, and facilitate their engulfment requires further investigation. Nevertheless, equal uptake of mock- and CVB-infected cells enabled us to make a good comparison of DC-responses, because only the infection status of islets or Min6 cells or islets differed. Phagocytosis of CVB-infected islets and Min6 cells invariably resulted in an increase in ISGs. Importantly, this IFN-dependent ISG-increase was sufficient to completely protect DCs from subsequent EV9 infection. This reveals a mechanism by which DCs may protect themselves when attracted to an environment with ongoing infection, ensuring their functional integrity.

Viral RNA is known to be an important inducer of antiviral immunity by triggering PRRs from the TLR and RLH family. Here we show that induction of innate responses in DCs requires endosomal acidification and is largely dependent on the presence of RNA within the infected cells. Some residual ISG induction is still observed after RNase treatment. Possibly, part of the RNA might be shielded within intact virus particles, and therefore be inaccessible for RNases. This residual viral RNA may have triggered ISG induction, when recognized in endosomes and/or lysosomes following phagocytosis. Although we favor the idea that viral RNA present in CVB-infected cells triggers antiviral immunity, we can not exclude that viral proteins or modified host proteins contribute to the observed antiviral responses.

Interestingly, engulfment of CVB-infected cells resulted in the development of type 1 diabetes in a susceptible mouse model (14). In that study, resident APCs in the pancreas were shown to engulf CVB-infected β-cells, and these APCs were capable to subsequently stimulate antigen-specific T cell proliferation and trigger diabetes, demonstrating that the (infectious) microenvironment may drive innate, as well as adaptive and autoimmune responses in vivo. We studied human DC maturation in vitro and our preliminary data on the induction of costimulatory molecules showed no consistent upregulation, even though ISG-induction was consistently observed. In some donors upregulation of CD80 and CD86 and production of TNF-α and IL-12 were observed in DCs upon uptake of CVB3-infected Min6 cells; however, in the majority of DC-donors no increases were observed. The reason for these inconsistent outcomes is unknown and requires further investigation.

Obviously, during phagocytosis of infected cells by DCs in vivo, other cell populations are present that can interact with DCs. These cells, including macrophages, plasmacytoid DCs and NK cells, and the cytokines they produce, can greatly influence the microenvironment and DC responses. For instance, IFNs can, besides their function in innate immunity, also influence adaptive immunity. The amount of IFNs, timing of encountering IFNs, but also possible synergy with other PRR-stimuli can greatly influence DC maturation (47; 48). Moreover, other
pancreas-constituents, such as duct cells may influence DC function. For example, duct cells produce TNF-α upon CVB3 infection (G. Vreugdenhil, F. van Kuppeveld J. Galama and D. Pipeleers, unpublished observation), and duct cell-derived TNF-α has been shown to influence DC maturation (49). Further in depth investigation of adaptive immune responses in DCs from healthy controls upon encountering CVB-infected islets and the interplay with other cell types and cytokines would be extremely valuable.

Most human infections with enteroviruses are efficiently controlled due to adequate antiviral immune responses. Prolonged or successive enterovirus infections have been suggested to play a role in the development of type 1 diabetes (50; 51), raising the possibility that individuals susceptible for type 1 diabetes may have impaired anti-viral defense. The genetic background of susceptible individuals greatly influences disease development. For example, polymorphisms in Mda5, the RNA sensor that recognizes picornavirus RNA (52), have been associated with type 1 diabetes development (25; 53). Alterations in Mda5 function may affect adequate sensing of viral infections, and thus hamper antiviral immunity. CVB-infection in a genetically susceptible individual might therefore progress to chronic inflammation in the pancreas. In this pro-inflammatory environment, where self-antigens and viral antigens are encountered by DCs, autoimmunity might develop or accelerate, ultimately resulting in type 1 diabetes.

In conclusion, we show that CVB-infected human islets, porcine islets and Min6 cells are phagocytosed by human DCs and that this results in an RNA- and IFN-dependent antiviral state in DCs. These events may alter programming of DCs and thus influence the development of $T_{reg}$ and/or effector T cell populations. These novel findings provide important new insights in the possible role of DCs during human type 1 diabetes development.

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FIGURE LEGENDS

Figure 1. CVB replicates in human and porcine pancreatic islets, and DC can phagocytose islets, resulting in induction of ISGs. (A and B) Human islets cells were infected with CVB3 at an MOI of 10 and (A) at indicated time points replication was analysed and (B) at 48h p.i. images were taken. (C) Human islets were infected with CVB3 at an MOI of 10. After 24 h incubation, islets were adhered onto fibronectin coated coverslips and stained using 3A (red) and VP1 (green) specific antibodies. Hoechst stain is included to visualize cell nuclei (D) Human islets were PKH-labeled and infected with CVB3 at an MOI of 10 and cultured for 48h before addition to CFSE-labeled DC. Uptake of islets either mock-infected (M) or CVB-infected (CVB) was analyzed by flow cytometry 24h after co-culture. (E) Human islets were PKH-labeled and infected as in (D) and co-cultured with unlabeled DC for 24h. Subsequently, DCs were harvested, stained using CD86-specific antibodies (green), and analyzed using confocal microscopy. (F) Porcine islets were prepared and co-cultured as in (F) and at indicated times ISG-induction was analyzed. In some experiments (F, G) freeze-thawed preparations of islets were used, but these yielded similar results compared to using viable cells. Experiments are representative of 2, 3 or more than 3 independent experiments. Med = medium, unstimulated DCs; Islet/M = mock-infected human islets; Islet/CVB = CVB-infected human islets; porc Islet/M = mock-infected porcine islets; porc Islet/CVB = CVB-infected porcine islets.

Figure 2. Min6 cells are a good model for primary pancreatic cells, are phagocytosed by DCs and induce innate antiviral immune responses. (A and B) Min6 cells were infected with CVB3 at an MOI of 10 and (A) at indicated time points replication was analyzed and (B) at 48h p.i. images were taken. (C) Min6 cells were PKH-labeled and infected with CVB3 at an MOI of 10. After 24h incubation cells were harvested and added to CFSE-labeled DCs at a 1:1 ratio. 24h after co-culture cells were harvested and uptake was determined using flow cytometry. (D) Min6 cells were prepared as in (C) and co-cultured with unlabeled DCs for 24h, after which DCs were harvested, stained as for 1E and analyzed using confocal microscopy. (E and F) Min6 cells were prepared as in (C), and after 48h incubation, cells were harvested and added to DCs at a 1:1 ratio, or DCs were stimulated with 20µg/ml poly I:C or left untreated. At (E) 5h after addition, or (F) 5h and 8h after addition, mRNA induction of ISGs was determined as described. (G) Protein expression of RIG-I, Mda5, and PKR was analyzed by western blot 24h after stimulation of DCs as described for panel (E). (H) DCs were stimulated as in (E) and after 24h co-culture cells were harvested and infected with EV9 at an MOI of 1. At indicated times p.i. EV9 replication was analysed. Poly I:C was used as a positive control (34). In some experiments freeze-thawed cell populations were used but these yielded similar results compared to using viable cells (Supplementary figure 1). Data shown are representative (A-D, G, H) or averages (E, F) of at least 3 independent experiments. Med = medium, unstimulated cells; M6/M = mock-infected Min6 cells; M6/CVB = CVB-infected Min6 cells. * = p < 0.05; n.s. = not significant.

Figure 3. Type I IFNs produced by DC themselves are required for ISG induction. (A) DCs were stimulated with cleared supernatants from stimulated DC and DC/Min6 co-cultures (harvested 24h after co-cultured started and used at a 1:2 dilution) and 8h after stimulation mRNA induction of RIG-I, Mda5, and PKR were determined using qPCR. (B) Min6 cells were
infected with CVB3 at an MOI of 10 and after 48h incubation cells were harvested and added to DCs at a 1:1 ratio. Stimulations were performed in the absence or presence of neutralizing antibodies (livari, Kaaleppi, and bovine anti-IFN-α/β; see Materials and Methods). After 8h, mRNA expression levels of RIG-I, Mda5, and PKR were determined using qPCR. (C) DC were treated as in (B) and after 24h protein expression of RIG-I, Mda5, and PKR was analyzed by western blotting. (D) DCs were stimulated with 100U/ml mIFNaA or cleared supernatants from Min6 cells (harvested 48h p.i. and used at a 1:2 dilution) and after 8h ISG mRNA induction was determined. Data shown are representative of 2 (C), or average of 3 (A, B, D) independent experiments. Sup = supernatant; Med = medium, unstimulated cells; IC = poly I:C; M6/M = mock-infected Min6 cells; M6/CVB = CVB-infected Min6 cells; w/o Ab or w Ab = without or with neutralizing anti IFN-α/β antibodies, respectively; mIFNa = murine recombinant IFN-α. * = p < 0.05; n.s. = not significant.

Figure 4. Induction of innate immune responses in DC after Min6/CVB uptake requires (viral) RNA within Min6 cells. (A) DCs were pretreated with Cytochalasin D for 30 minutes prior to stimulation with TLR ligands or Min6 cells. After 8h, mRNA expression levels of RIG-I, Mda5, and PKR were determined using qPCR. (B) DCs were pretreated with chloroquine for 30 minutes, stimulated as in (A) and mRNA expression was determined as in (A). (C) Min6 cell preparations were exposed to a mixture of RNase A, RNase VI and RNase I prior to addition to DC cultures as described. Expression of RIG-I, Mda5 and PKR in DCs was analyzed using qPCR 5h after addition of Min6 cell preparations. (D) DCs were co-cultured with Min6 cell preparations as described for panel A, and protein expression of RIG-I, Mda5, and PKR was analyzed by western blotting 24h after the start of co-culture. Data are representative of 2 (D) or average of 2 (A, B, C) independent experiments. In these experiments freeze-thawed cell populations were used. Med = medium, unstimulated cells; IC = poly I:C; M6/M = mock-infected Min6 cells; M6/CVB = CVB-infected Min6 cells; w/o RNase or w RNase = without or with RNase-treatment of Min6 cell preparations prior to co-culture. * = p < 0.05; n.s. = not significant.
Figure 1

1A  Titer increase (delta (Log TCID50/100µl))

Time (h p.i.)

1B  Mock

CVB3

1C  hoechst VP13A

CVB3

1D  CFSE

DC

Islet/M

Islet/CVB

DC + Islet/M

DC + Islet/CVB

PKH

1E

DC + Islet/M

DC + Islet/CVB

1F  mRNA expression (AU)

RIG-I

Mda5

PKR

IRF-7

Time (h p.i.)

1G  mRNA expression (AU)

RIG-I

Mda5

porc Islet/M

porc Islet/CVB

PKR

IRF-7

Time (h p.i.)
Figure 2

2A

![Mock CVB3 Titer increase](image)

Time (h p.i.)

2B

Mock

CVB3

2C

![CFSE expression](image)

DC

Min6

DC + M6/M

DC + M6/CVB

2D

DC + M6/M

DC + M6/CVB

2E

![mRNA expression](image)

IFN-β

RIG-I

Mda5

PKR

2F

![mRNA expression](image)

2G

![Western blot](image)

2H

![Titer increase](image)

Time (h p.i.)
Figure 3

3A

RIG-I

mRNA expression (AU)

med
sup IC
sup M6/M
sup M6/CVB

n.s.

n.s.

n.s.

n.s.

Mda5

mRNA expression (AU)

med
sup IC
sup M6/M
sup M6/CVB

n.s.

n.s.

n.s.

n.s.

PKR

mRNA expression (AU)

med
sup IC
sup M6/M
sup M6/CVB

n.s.

n.s.

n.s.

n.s.

3B

RIG-I

mRNA expression (AU)

Med
M6/CVB

n.s.

n.s.

Mda5

mRNA expression (AU)

Med
M6/CVB

n.s.

n.s.

PKR

mRNA expression (AU)

Med
M6/CVB

n.s.

n.s.

w/o Ab
w Ab

3C

Medium
Poly I/C
Poly I/C + anti-IFN
Min6/M
Min6/CVB
Min6/CVB + antiIFN

RIG-I
PKR
Mda5
Actin

3D

RIG-I

mRNA expression (AU)

med
mRNA
M6/M
M6/CVB

n.s.

n.s.

Mda5

mRNA expression (AU)

med
mRNA
M6/M
M6/CVB

n.s.

n.s.

PKR

mRNA expression (AU)

med
mRNA
M6/M
M6/CVB

n.s.
Figure 4

4A

RIG-I

Mda5

PKR

mRNA expression (AU)

4B

RIG-I

Mda5

PKR

mRNA expression (AU)

4C

RIG-I

Mda5

PKR

mRNA expression (AU)

4D

Medium

Poly I:C

M6(CVB) + R

RIG-I

PKR

Mda5

Actin

1 2 3 4 5 6