Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12779

Distinct activation of primary human BDCA1$^+$ dendritic cells upon interaction with stressed or infected β cells

B. M. Schulte,* E. D. Kers-Rebel,* R. Bottino,† J. D. Piganelli,† J. M. D. Galama,‡ M. A. Engelse,§ E. J. P. de Koning§¶** and G. J. Adema*

*Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands, †Department of Pediatrics, Diabetes Institute, University of Pittsburgh, Pittsburgh, PA, USA, ‡Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, §Department of Nephrology, ¶Department of Endocrinology, Leiden University Medical Center, Leiden, and **Hubrecht Institute, Utrecht, the Netherlands

Accepted for publication 14 February 2016
Correspondence: G. J. Adema, Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein 28, 6525 GA, Nijmegen, the Netherlands.
E-mail: gosse.adema@radboudumc.nl

Summary

Derailment of immune responses can lead to autoimmune type 1 diabetes, and this can be accelerated or even induced by local stress caused by inflammation or infection. Dendritic cells (DCs) shape both innate and adaptive immune responses. Here, we report on the responses of naturally occurring human myeloid BDCA1$^+$ DCs towards differentially stressed pancreatic β cells. Our data show that BDCA1$^+$ DCs in human pancreas-draining lymph node (pdLN) suspensions and blood-derived BDCA1$^+$ DCs both effectively engulf β cells, thus mimicking physiological conditions. Upon uptake of enterovirus-infected, but not mock-infected cells, BDCA1$^+$ DCs induced interferon (IFN)-α/β responses, co-stimulatory molecules and proinflammatory cytokines and chemokines. Notably, induction of stress in β cells by ultraviolet irradiation, culture in serum-free medium or cytokine-induced stress did not provoke strong DC activation, despite efficient phagocytosis. DC activation correlated with the amount of virus used to infect β cells and required RNA within virally infected cells. DCs encountering enterovirus-infected β cells, but not those incubated with mock-infected or stressed β cells, suppressed T helper type 2 (Th2) cytokines and variably induced IFN-γ in allogeneic mixed lymphocyte reaction (MLR). Thus, stressed β cells have little effect on human BDCA1$^+$ DC activation and function, while enterovirus-infected β cells impact these cells significantly, which could help to explain their role in development of autoimmune diabetes in individuals at risk.

Keywords: β cells, BDCA1$^+$ myeloid DC, DC maturation, enterovirus, human, islets of Langerhans

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which a progressive autoreactive immune response selectively destroys the insulin-producing beta cells of the pancreas; however, the mechanism(s) that result in loss of tolerance and infiltration of islets of Langerhans with autoreactive T cells remain poorly understood. Viral infections, particularly with human enterovirus B (HEV-B) such as Coxsackie B viruses (CVB), have been linked to the development of autoimmune diabetes [1–4]. HEV-Bs are RNA viruses that usually cause mild or asymptomatic infections in the upper respiratory and/or gastrointestinal tract, yet severe infections can result in, e.g. myocarditis or encephalitis [5]. The hypothesis that virus infections cause or contribute to autoimmune T1D is supported strongly by a significant association between enterovirus infection and T1D-related autoimmunity as summarized recently in a meta-analysis and by two recent studies investigating gene expression in individuals at risk for diabetes of whom part seroconverted and/or developed clinical diabetes [1,6–8]. Multiple not-mutually exclusive mechanisms exist by which viruses may induce diabetes. They can directly infect and kill target cells or, alternatively, can induce inflammation and autoimmunity through indirect mechanisms, e.g. via bystander immune activation.

Dendritic cells (DCs) shape both innate and adaptive immune responses. They continuously sample their microenvironment via phagocytosis. Depending on the presence of either exogenous pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular

patterns (DAMPs) that bind to pattern recognition receptors [PRRs, e.g. Toll-like receptors (TLRs) or retinoic acid-inducible gene 1-like receptors (RLRs)] DCs induce inflammatory or more tolerogenic responses [9,10]. Presentation of β cell-derived antigens from islets of Langerhans occurs constitutively under physiological conditions [11], and DCs are involved in the development of autoimmune diabetes (reviewed in [12]).

In-vitro-generated monocyte-derived DC are used most frequently as the DC source in experiments. However, various distinct subsets of DCs are present in human blood and lymph nodes (LN) [13–15], of which the blood-derived cell antigen 1 (BDCA1$^+$) are the most prevalent myeloid DC subset [14,15]. BDCA1$^+$ DCs express various TLRs and are known to produce chemotactic proteins [16] and both blood-derived and LN-derived BDCA1$^+$ DCs (cross)present antigens to T cells [17,18]. BDCA1$^+$ DCs have been described in various human LN, although the exact subpopulations can differ per LN [13,15].

Inflammation in islets of Langerhans causes β cell stress leading to cell death, e.g. by proinflammatory cytokine exposure [19,20], and subsequent release of endogenous 'danger' signals such as heat shock proteins that may subsequently induce activation of immune cells [19,21]. In addition, stressed β cells are also likely to play an important role in the acute and chronic islet loss that occurs after islet transplantation [22–25].

We have reported previously on the activation of human naturally occurring DC subsets upon encounter of enterovirus-infected, but not mock-infected β cells [26]. Strikingly, these naturally occurring DCs reacted more strongly to enterovirus-infected cells than *in-vitro*-generated monocyte-derived DC. How primary human DCs respond to differentially stressed or infected β cells is largely unknown, and may provide important insight in the onset and progression of autoreactive responses in genetically susceptible individuals.

In this study, we therefore compared the activation and functional status of fresh human DCs upon encounter of 'stressed' β cells, uninfected β cells or CVB-infected β cells and expanded our studies using human pancreas-draining LN material. Lastly, we extended our findings using mock- or CVB-infected primary human islets of Langerhans and showed that CVB-infected, but not mock-infected islets strongly activate BDCA1$^+$ DCs.

Materials and methods

Isolation and culture of cells

Buffy coats were obtained via Sanquin Blood Bank (Nijmegen, the Netherlands) from healthy volunteers after written consent according to institutional guidelines and the Declaration of Helsinki. Blood products were released anonymized to laboratory personnel. BDCA1$^+$ DCs were isolated and cultured as described [27] and were routinely up to 90–95% pure, assessed by double-staining BDCA1/CD11c. Min6 cells [28] were cultured as described previously [29].

Human islets of Langerhans that could not be used for clinical application were obtained from the Human Islet Isolation Laboratory at the Leiden University Medical Center (LUMC) or at the Center for Organ Recovery and Education (CORE) (Pittsburgh, PA, USA), as described previously [29]. Islets were cultured as described previously [30]. Human pancreas-draining lymph nodes (pdLNs) were recovered from the peri-pancreatic tissue that is discarded during the organ preparation phase before islet isolation at the Human Islet Isolation Laboratory. If they could be identified as the superior and inferior pancreatic LNs, splenic LNs, pancreaticoduodenal LNs were recovered. Similar to the donor organ, the pdLNs were flushed during the organ procurement procedure with University of Wisconsin solution. After recovery, the LNs were preserved in University of Wisconsin solution until dissociation into single-cell suspensions using 1 mg/ml collagenase A (Roche, Basel, Switzerland) and 0·1 mg/ml DNaseI (Invitrogen, Carlsbad, CA, USA). Subsequently, LN cells were cultured in X-Vivo15 supplemented with 10% fetal calf serum (FCS) or frozen in human serum albumin (HSA)/10% dimethylsulphoxide (DMSO) for future use.

Virus propagation, purification and infection of β cells

CVB3 Nancy (CVB3) and GFP-CVB were produced as described previously [31,32]. When used for flow cytometry or confocal microscopy, cells were labelled with PKH67, PKH26 (both Sigma Aldrich, St Louis, MO, USA) or CFSE (Invitrogen) prior to infection [multiplicity of infection (MOI) 15], as described previously [29].

pdLN and BDCA1$^+$ DC stimulation

pdLN single-cell suspensions were co-cultured with labelled, mock- or CVB-infected β cells and after 18 h cells were analysed by flow cytometry. Blood BDCA1$^+$ DCs were stimulated with poly I:C (Enzo Life Sciences, Exeter, UK) (20 μg/ml) or co-cultured in a 1 : 1 ratio with mock- or CVB3-infected human islets or Min6 cells or exposed to CVB3 (MOI 50). Stress was induced in Min6 cells by exposure to murine interleukin (IL)−1β, tumour necrosis factor (TNF)-α and interferon (IFN)-γ at 250, 1000 and 1000 U/ml, respectively, for 3 or 24 h prior to co-culture with DCs. This cytokine mixture is known to induce β cell stress and apoptosis [33]. Alternatively, cells were ultraviolet (UV)-irradiated (50 mJ) 48 h before co-culture with DCs or kept in serum-free medium prior to co-culture. RNase treatment of Min6 cells was performed as described previously [34].

RNA isolation, quantitative PCR (qPCR) and Western blot

These techniques were performed as described previously [27,29].

Flow cytometry

Cells were stained with fixable Viability Dye (eBioscience, San Diego, CA, USA), followed by staining as described previously [27]. Antibodies against CD11c, Lineage1, CD80, CD86, programmed death ligand 1 (PD-L1) and PD-L2 were all from BD Pharmingen (San Diego, CA, USA), human leucocyte antigen D-related (HLA-DR), human CD1c (BDCA1) and CD123 were from Biolegend (San Diego, CA, USA), Miltenyi Biotec (Bergisch Gladbach, Germany) and eBioscience, respectively. Cells were analysed on a CyAn flow cytometer (Beckman Coulter) and data were analysed using FlowJo software (TreeStar, Inc., Ashland, OR, USA).

Confocal microscopy

DCs were harvested and stained as described previously [34]. Cell surface stain was performed using HLA-DR/DP/DQ and goat anti-mouse Alexa647-conjugated secondary antibodies (Invitrogen). Analysis was performed on an Olympus FV-1000 and FV10-ASW 1·6 viewer software.

ELISA/bead array

TNF-α and IL-6 were analysed as described previously [27]; IL-10 production was analysed using a human IL-10 enzyme-linked immunosorbent assay (ELISA kit) (eBioscience). IL-12p70 production was measured with standard sandwich ELISA (Pierce Endogen, Inc., Rockford, IL, USA). Other cytokines and chemokines were assessed using Millipore multiplex and analysed with Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA, USA).

Allogeneic mixed lymphocyte reaction (MLR)

MLR and T cell proliferation were performed as described previously [26]. Supernatant was analysed for T helper cytokines using Flowcytomix kits (BenderMed Systems, Wien, Austria).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test or *t*-test as indicated. A P-value $< 0{\cdot}05$ was considered significantly different.

Results

Pancreas-draining lymph node (pdLN) and blood BDCA1$^+$ DCs phagocytose Min6 β cell material

To detect BDCA1$^+$ DCs in human pdLNs, single-cell suspensions from pdLN were analysed using flow cytometry. As shown in Fig. 1a, DCs (lineage-negative, HLA-DR-positive) comprise $< 1\%$ of total viable pdLN cells. Depending on the donor and pdLN-station, 0·3–1·1% of all viable cells are DCs (not shown). Further analysis of these DCs defined clear populations of CD11c$^+$/BDCA1$^+$ myeloid DCs and CD11c$^-$/CD123$^+$ plasmacytoid DCs (Fig. 1a, right panel). The percentage of DCs within total DCs was 7–25% BDCA1$^+$ DCs and 2–24% pDCs (not shown). Initial experiments using blood DCs revealed that blood pDCs did not become activated strongly upon co-culture with CVB-infected Min6 cells (data not shown), whereas BDCA1$^+$ and BDCA3$^+$ DCs did [26]. BDCA3 is not a specific marker in human LN suspensions, as LN-BDCA1$^+$ DCs also express BDCA3 ([15] and data not shown). Thus, although additional DC subsets exist, here we will focus upon the relatively abundant BDCA1$^+$ subset. To determine whether the pdLN BDCA1$^+$ DCs engulf β cell material, total pdLN suspensions were co-cultured with PKH-labelled Min6 insulinoma cells (β cells) that were either mock- or CVB3-infected 48 h prior to co-culture. Both mock-infected and CVB-infected Min6 cells were engulfed, as indicated by the appearance of PKH$^+$/BDCA1$^+$ DCs (up to 28% of total BDCA1$^+$ DCs) (Fig. 1b, upper panels). Control incubations kept at 4°C showed little or no uptake of material by BDCA1$^+$ DCs, indicating that the uptake is an active process (Fig. 1b). Next, we aimed to study DC activation in total pdLN suspensions. To prove feasibility of these studies, pdLN suspensions were stimulated using a combination of poly I:C and R848, which is known to strongly activate human myeloid DCs. Our data suggested an increase in CD80 expression on pdLN BDCA1$^+$ DCs; however, analysis was very challenging due to changes in the markers used to identify the DC subsets and the relative small number of BDCA1$^+$ DCs within total LN suspension (data not shown). Therefore, we decided to focus on blood BDCA1$^+$ DCs. We investigated whether freshly isolated blood BDCA1$^+$ DCs phagocytose β cells and confirmed that both mock- and CVB3-infected Min6 cells were phagocytosed, although uptake of CVB3-infected cells was somewhat more efficient (73 *versus* 51%) (Fig. 1c). We expanded our studies to stressed Min6 cells, i.e. cytokine-stressed, serum-starved cells or UV-irradiated cells. UV-irradiated and serum-starved cells showed decreased viability comparable to CVB-infected cells. Cytokine exposure resulted in even lower viability upon 24 or 48 h culture (Supporting information, Fig. S1A). Efficient uptake was observed for all stimulations (Supporting information, Fig. S1B). Uptake of β cells by BDCA1$^+$ DCs was confirmed using confocal

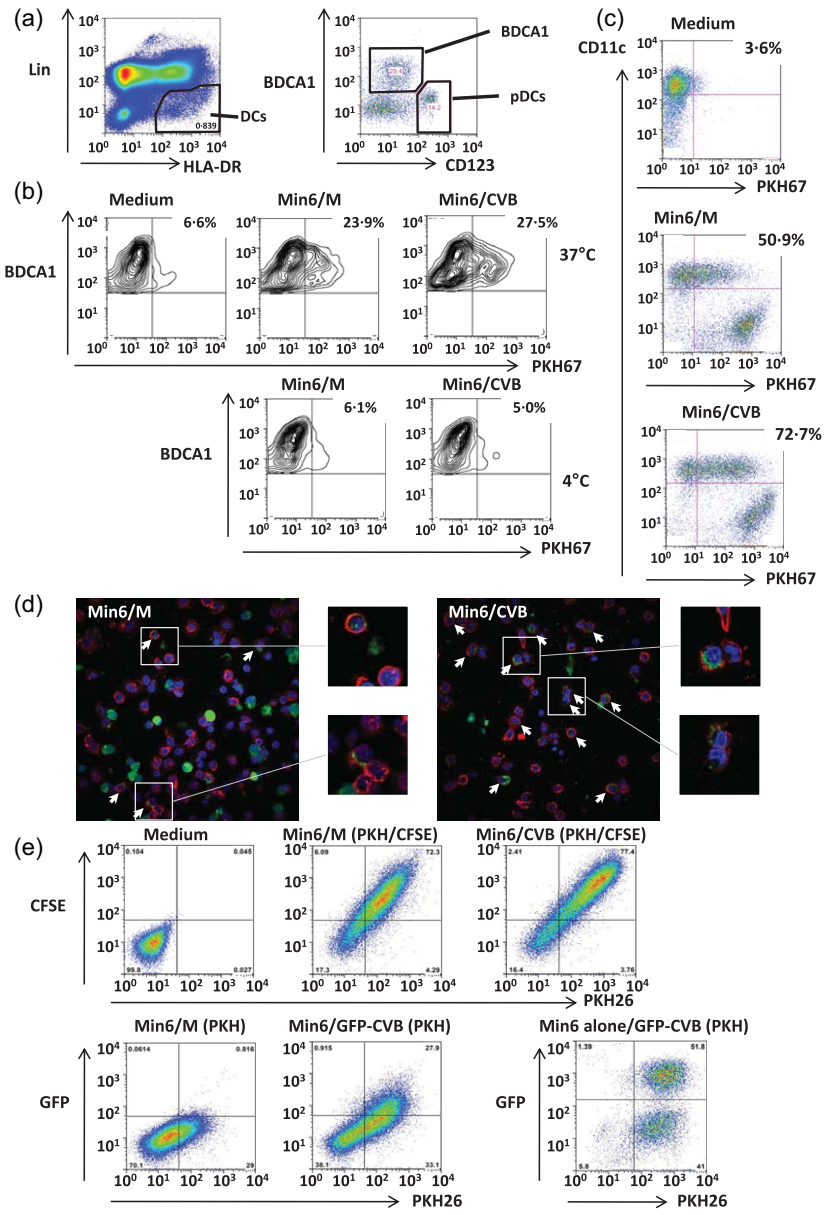


Fig. 1. Blood-derived cell antigen 1 (BDCA1)$^+$ dendritic cells (DCs) are present in pancreas-draining lymph nodes (pdLNs) and pdLN BDCA1$^+$ DCs and blood BDCA1$^+$ DCs phagocytose Min6 material. (a) pdLN single-cell suspensions were stained as described and analysed using flow cytometry. Dot-plot of superior pancreatic lymph node suspension is shown. (b) pdLN cell suspensions were co-cultured with PKH-labelled Min6 cells overnight at 4°C or 37°C and analysed subsequently using flow cytometry. Percentages in the upper right corner represent the percentage of DCs that have engulfed β cell material. Contour-plot of superior pancreatic lymph node (co-)culture is shown. (c) Blood-derived BDCA1$^+$ DCs were co-cultured with PKH67-labelled mock- or Coxsackie B virus (CVB)-infected Min6 cells, or left unstimulated (medium) overnight and were subsequently harvested, stained and analysed with flow cytometry. Percentages in the upper right corner represent the percentage of DCs that have engulfed β cell material. (d) As for (c), cells were harvested, adhered onto poly-L-lysine-coated coverslips, stained and analysed using confocal laser scanning microscopy. White arrows indicate DCs that have phagocytosed Min6 material. Squares represent enlarged areas. (e) As in (c), using PKH26 (PKH), carboxyfluorescein succinimidyl ester (CFSE) or double-labelled (PKH/CFSE) Min6 cells, as indicated between brackets. CD11c-high expressing DCs were analysed for CFSE, PKH or green fluorescent protein (GFP), except for the lower right panel (Min6 alone), where all cells were analysed. Note that GFP-expressing virus was used in combination with PKH26 (red) and not CFSE. M6/M = mock-infected Min6 cells; M6/CVB = CVB-infected Min6 cells; Lin = lineage; pDC = plasmacytoid DC; Min6/GFP-CVB = GFP-CVB-infected Min6 cells. Representative of three (a), two (b,d,e) or more than three (c) experiments.

microscopy (Fig. 1d). Furthermore, using Min6 cells stained with PKH and carboxyfluorescein succinimidyl ester (CFSE) dyes that label lipid and proteins, respectively, we readily detected both PKH- and CFSE-positive DCs in DC/Min6 co-cultures. This indicates that DC engulf both lipid material as well as proteins from β cells (Fig. 1e, upper panels). To assess directly whether viral material was engulfed by DC, we made use of green fluorescent protein (GFP)-expressing CVB, which produces GFP in addition to all other viral proteins [32]. Approximately half the Min6 cells produced GFP upon infection with this virus (Fig. 1e, lower right panel). Moreover, virus-derived GFP was detected in > 25% of BDCA1$^+$ DCs, indicating that viral proteins are scavenged by the BDCA1$^+$ DCs (Fig. 1e, lower middle panel).

Uptake of CVB3-, but not of mock-infected or stressed β cells, induced type I IFN responses in BDCA1$^+$ DC

Type I IFNs (IFN-α/β) are indispensable during the anti-viral immune responses. We determined IFN-α production to assess innate IFN responses and detected no IFN-α in unstimulated DC cultures, or when co-cultured with

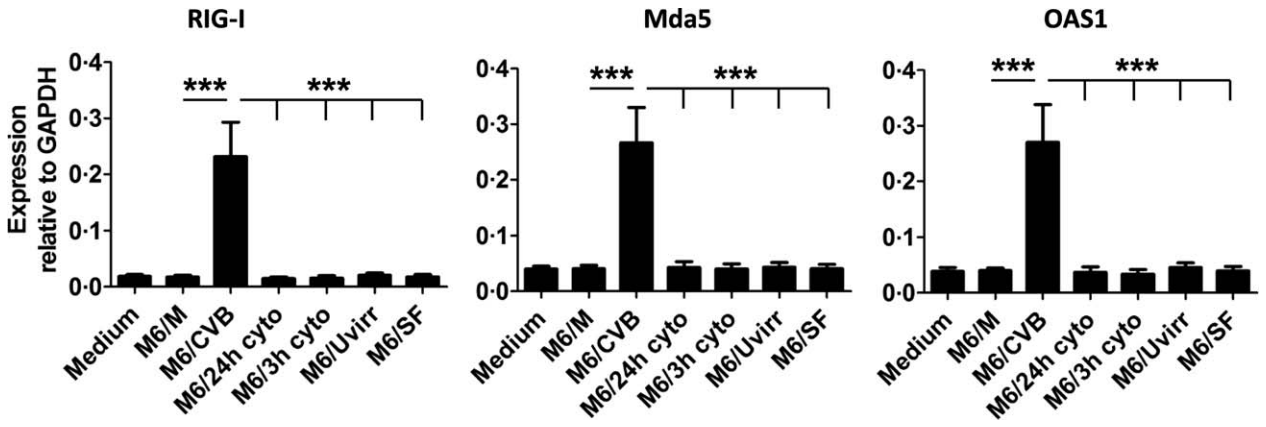


Fig. 2. Blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) that encounter Coxsackie B virus (CVB)-infected, but not mock-infected or stressed Min6 cells, induce type I interferon (IFN) responses. DCs were co-cultured with mock- or CVB-infected Min6 cells, exposed to stressed Min6 cells as indicated or left unstimulated (medium) and mRNA expression was analysed after 6 h. M6/cyto = cytokine-stimulated Min6 for *n* hours; UVirr = ultraviolet-irradiated Min6 cells; M6/SF = serum-starved Min6 cells. Average ± standard error of the mean (s.e.m.) of three experiments. ***$P < 0{\cdot}001$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

mock-infected β cells. However, strong IFN-α induction was observed upon co-culture with CVB-infected β cells or with poly I:C, a double-stranded RNA mimic serving as positive control (Supporting information, Fig. S2A). We used qPCR to assess the downstream induction of IFN-stimulated genes (ISGs), melanoma differentiation-associated protein 5 (MDA5), RIG-I and OAS1. The use of human mRNA-specific primers enabled us to determine gene expression in human mDCs specifically, while not amplifying the corresponding murine Min6 cDNAs. As we have shown previously, low basal expression of ISGs was observed in unstimulated DC (medium), which was not enhanced upon co-culture with mock-infected cells (Supporting information, Fig. S2B), whereas co-culture with CVB3-infected Min6 cells resulted in major increase of the ISGs tested. Importantly, our previous studies showed that CVB3 is unable to infect BDCA1$^+$ DCs productively [27]; however, ISGs were induced modestly when DCs were exposed to CVB3, but at much lower levels compared to stimulation with CVB3-infected Min6 cells. Next we investigated whether uninfected, cytokine-stressed, serum-starved or apoptotic cells similarly induce innate ISG expression in BDCA1$^+$ DCs. No ISG induction whatsoever was observed upon exposure to cytokine-stressed, or otherwise stressed Min6 cells, whereas CVB-infected Min6 cells induced ISGs efficiently (Fig. 2). Western blot analysis confirmed our qPCR data and showed clear increases in Mda5, RIG-I and PKR protein expression upon stimulation with CVB3-infected Min6 cells. Co-culture with mock-infected Min6 cells did not result in increased ISG protein expression, whereas exposure to CVB3 induced very modest Mda5, RIG-I and PKR protein expression (Supporting information, Fig. S2C). No ISGs were detected in mock- or CVB3-infected Min6 cells, confirming that the observed ISG expression originated from BDCA1$^+$ DC (Supporting information, Fig. S2C). Thus, despite efficient uptake of mock-, stressed or CVB-infected β cells by BDCA1$^+$ DCs, type I IFN responses are only induced efficiently upon encountering the infected cells.

Uptake of CVB-, but not mock-infected or stressed β cells, results in DC maturation and production of various cytokines and chemokines

To investigate whether BDCA1$^+$ DCs matured, the expression of co-stimulatory molecules (CD80, CD86) and co-inhibitory molecule PDL1 after co-culture with Min6 cells was analysed. Co-culture with CVB3-infected Min6 cells resulted in a significant increase of CD80, CD86 and PDL1 expression (Fig. 3a). In accordance with our previous findings [26], co-culture with mock-infected Min6 cells or stimulation with CVB3 had no effect on BDCA1$^+$ DCs on any of the cell surface markers tested (Figs 3a and Supporting information, S3A). Co-culture with cytokine-stressed, UV-irradiated or serum-starved cells did not induce CD80 or CD86 expression strongly either (Fig. 3a). The cytokine-stressed Min6 cells, however, modestly induced the co-inhibitory molecule PDL1.

Cytokine analysis revealed that co-culture with stressed Min6 cells or mock-infected Min6 cells did not induce expression of any of the proinflammatory cytokines or chemokines tested, nor the anti-inflammatory IL-10 (Fig. 3b). In contrast, co-culture with CVB3-infected β cells induced the production of proinflammatory cytokines TNF-α, IL-6 and IL-12p70, as well as IL-10 (Fig. 3b) and various chemokines (Fig. 3c), indicating that the virus or virus-induced alterations in the β cell are responsible for DC activation. Exposure to virus alone did not induce cytokines or chemokines (Supporting information, Fig. S3B,C). Remarkably, most chemokines were induced to a similar extent by CVB-infected Min6 cells and our positive control

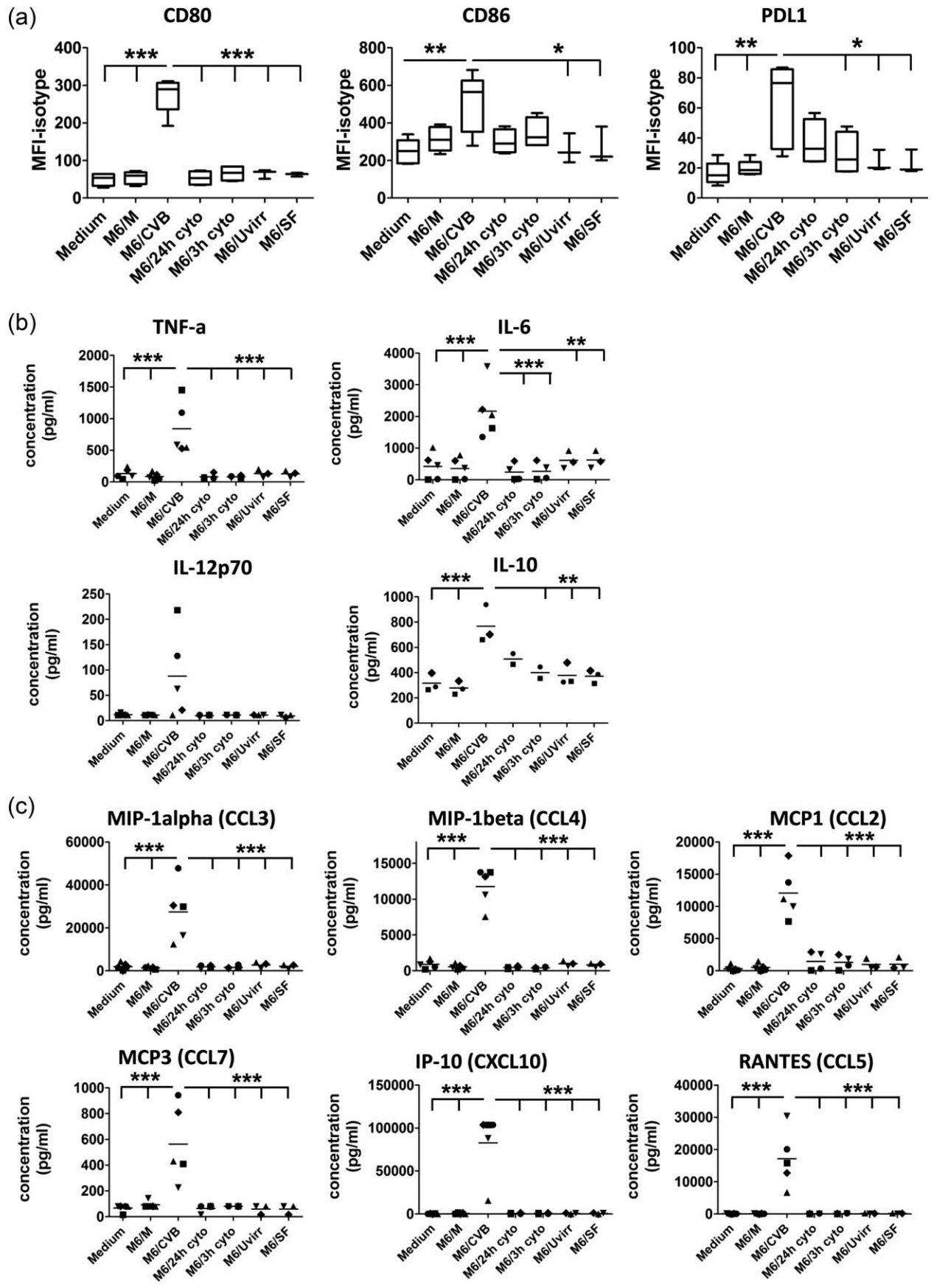


Fig. 3. Phenotypical dendritic cell (DC) maturation and production of proinflammatory cytokines and chemokines by blood-derived cell antigen 1 (BDCA1$^+$) DCs that engulf Coxsackie B virus (CVB)-infected, but not mock-infected or stressed Min6 cells. (a) DCs cultured as in Fig. 2a were analysed for indicated cell surface markers after overnight culture. (b,c) Supernatant of cells cultured in (a) is analysed for indicated cytokines and chemokines. Whisker plot for more than three experiments (a) or column scatterplot from five different donors (b,c). Corresponding symbols represent the same donor within a figure (b,c). $^*P < 0{\cdot}5$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

poly I:C; however, monocyte chemoattractant protein (MCP1)/chemokine (C-C motif) ligand (CCL)2 and MCP3/CCL7 showed higher induction upon encounter of CVB-infected β cells, indicating that differences exist in response to physiological stimuli such as virally infected cells and synthetic TLR ligands such as poly I:C (Supporting information, Fig. S3C). Mock or CVB3-infected Min6 cells alone did not produce any cytokines, excluding that the observed effect are from Min6-derived cytokines (not shown).

Virally infected β cells induce DC activation in a dose-dependent and RNA-dependent manner

Virus-derived RNA can activate various PRRs and induce immune responses. To assess whether (viral) RNA is required for BDCA1$^+$ DC maturation, frozen and thawed 'lysate' of CVB-infected β cells was exposed to RNases prior to co-culture with BDCA1$^+$ DCs. Our results show clearly that CD80 and PDL1 induction is decreased upon co-culture with RNase-treated β cells, indicating that (viral) RNA is at least partially required for induction of CD80 and PDL1 (Fig. 4a).

To gain detailed insight into the requirements for efficient DC activation, β cells were infected using different MOIs and by applying different time-periods before co-culture with DCs. These experiments revealed that induction of CD80, CD86 and PDL1 and cytokines were observed even when 1000-fold less virus was used to infect the β cells (Fig. 4b,c). The strength of DC activation depended upon virus dose, as lower responses were observed when using lower MOIs. Shortening infection-time (i.e. 5 h instead of 48 h) before co-culture resulted in decreased CD80 and PDL1 induction and very modest IL-6 induction, yet no CD86 or IL-10 were induced. At this time, no obvious cytopathic effects were observed in the infected Min6 cells. When combining short infection time and lower MOI (5 h, MOI 1·5), no increase in co-stimulatory molecules or cytokine levels was observed. This indicates that the strength of DC activation correlates with the amount of virus and/or virus replication intermediates present within infected β cells.

BDCA1$^+$ DCs that encountered CVB-, but not mock-infected or stressed β cells, yield T helper type 1 (Th1) responses and suppress Th2 cytokines in allogeneic MLR

To study whether T cell activation occurred by our stimulated BDCA1$^+$ DCs they were co-cultured with allogeneic peripheral blood leucocytes. As expected, in this allogeneic setting unstimulated BDCA1$^+$ DCs (medium, Med) induced robust proliferation of T cells, which was not enhanced further by any of the stimuli tested (Fig. 5a). To assess the profile of stimulated T cells IFN-γ, IL-5 and IL-13 were determined and revealed that IFN-γ, IL-13 and IL-5 were produced at low levels by unstimulated DCs, which was not altered upon co-culture with mock-infected Min6 cells or CVB stimulation (Supporting information, Fig. S4). Strikingly, BDCA1$^+$ DCs co-cultured with CVB-infected β cells induced IFN-γ production and clearly suppressed IL-13 and IL-5, indicating that a predominant Th1 response was induced (Fig. 5b). BDCA1$^+$ DCs exposed to cytokine-stressed or UV-irradiated Min6 cells did not induce IFN-γ strongly and did not reduce IL-13 and IL-5 production (Fig. 5b).

CVB-, but not mock-infected human islets of Langerhans, induce BDCA1$^+$ DC activation

To extend our findings to a more physiological setting, we assessed the response of BDCA1$^+$ blood DCs to allogeneic mock- or CVB-infected human islets of Langerhans. Both mock- and CVB-infected islet material was engulfed by BDCA1$^+$ DCs (Fig. 6a). CVB-infected islets of Langerhans induced IFN-α in DCs (Fig. 6b) and induction of ISGs at the protein level was apparent (Fig. 6c). The latter analysis is, however, complicated by low expression of ISGs in mock-infected human islets of Langerhans, which is enhanced further upon CVB infection (Supporting information, Fig. S5). CD80 and PDL1 were induced strongly in DCs co-cultured with CVB-infected, but not mock-infected, islets of Langerhans. CD86 was induced upon co-culture of BDCA1$^+$ DCs with mock-infected islets of Langerhans, but expression was enhanced even further when CVB-infected islets were encountered (Fig. 6d). Proinflammatory cytokines were induced by CVB-infected islets of Langerhans, although analysis of some cytokines was complicated by their production by human islets of Langerhans themselves (Fig. 6e,f). To circumvent this problem, DCs were exposed to frozen and thawed 'lysates' of human islets, excluding cytokine production by islets themselves. This yielded a very similar picture compared to using fresh islets and, importantly, showed induction of all cytokines and chemokines tested upon exposure to CVB-infected islets lysate, with the exception of IL-12 (Supporting information, Figs S6 and S7). Allogeneic MLR showed that T cell proliferation induced by

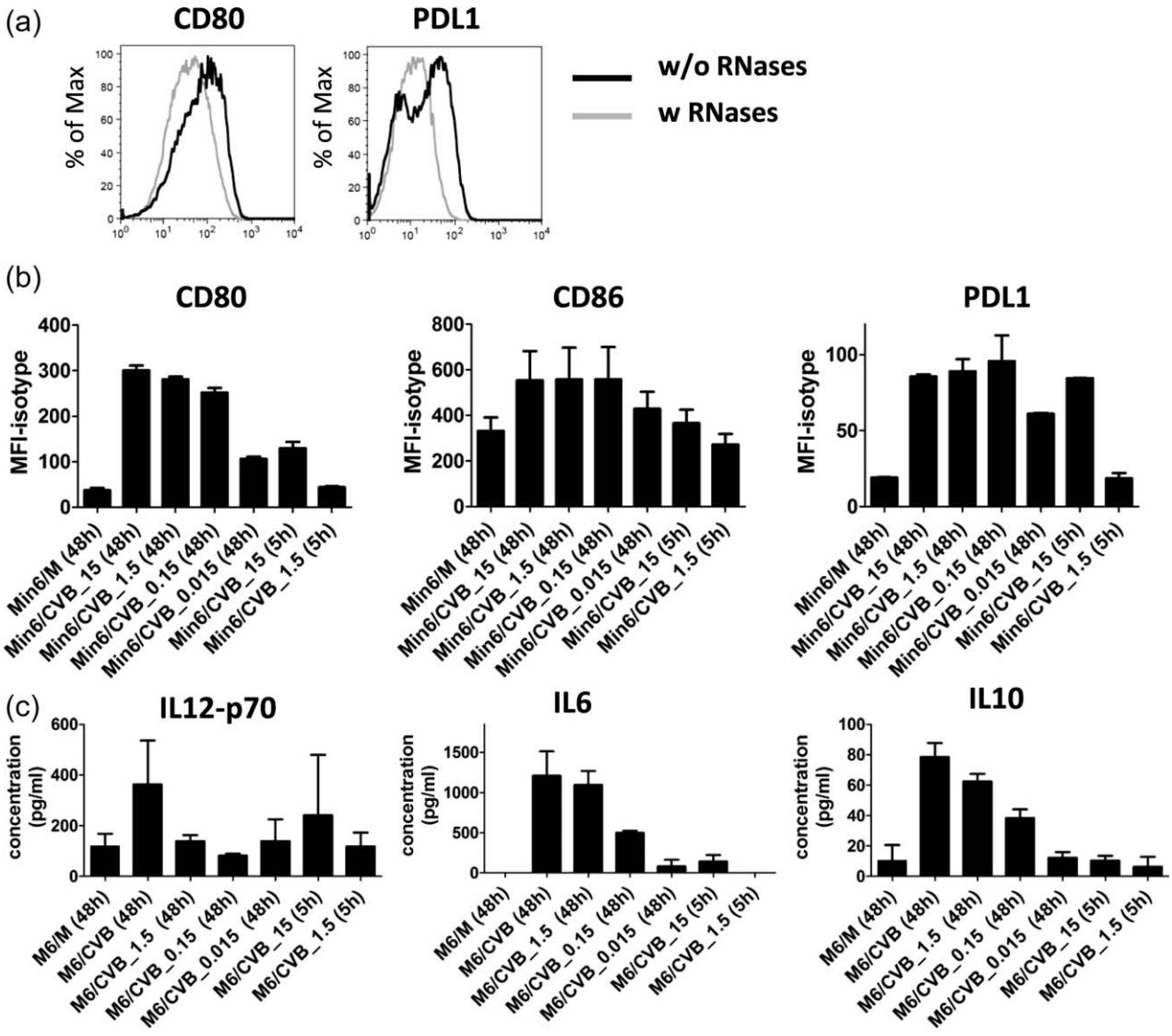


Fig. 4. Activation of blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) by Coxsackie B virus (CVB)-infected Min6 cells is RNA- and multiplicity of infection (MOI)-dependent. (a) Frozen and thawed 'lysate' of CVB-infected Min6 cells is treated with (w) RNases or not (without, w/o) and co-cultured subsequently with BDCA1$^+$ DCs. After overnight co-culture cell surface marker expression was determined. (b,c) DCs were co-cultured with Min6 cells infected with different MOIs and infected at different times prior to co-culture with DCs as indicated. After overnight culture cell surface marker expression (b) and cytokine analysis (c) was determined. Shown are representative of two (a) or average of two (b,c) different experiments.

unstimulated DCs was not enhanced further upon co-culture with mock- or CVB-infected human islets (Fig. 6g), similar to results obtained with Min6 cells. We assessed the induction of T cell-derived cytokines by these T cells using readily available frozen and thawed lysates of mock- and CVB-infected human islets. In contrast to Min6 cells, no IFN-γ induction was observed in DCs stimulated with lysates of CVB-infected human islets compared to mock (Fig. 6h), which may be caused by the absence of IL-12 induction when using frozen and thawed lysates of islets (Supporting information, Figs S6 and S7). Th2-associated cytokine IL-5 was suppressed potently by DCs exposed to frozen and thawed lysates of CVB-infected islets of Langerhans, which was also the case for IL-13 in four of six donors. To exclude that the lack of IFN-γ induction was due to the use of frozen and thawed islets, we investigated whether BDCA1$^+$ DCs exposed to live, CVB-infected islets of Langerhans induced IFN-γ. This revealed that BDCA1$^+$ DCs from one of four donors stimulated with CVB-infected islets showed a modest increase of IFN-γ compared to mock-infected islets (Fig. 6I, filled squares) and confirmed the potent reduction in Th2-associated cytokines upon encounter of CVB-infected islets (Fig. 6i).

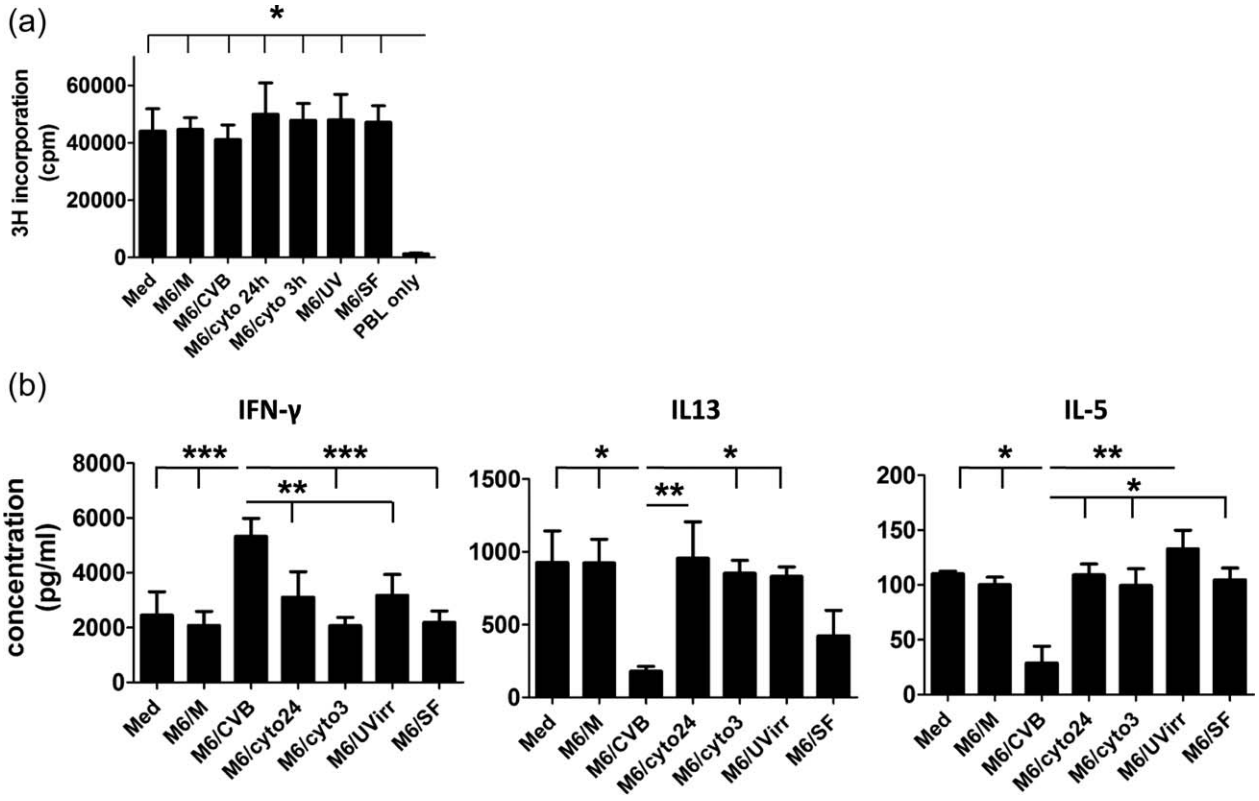
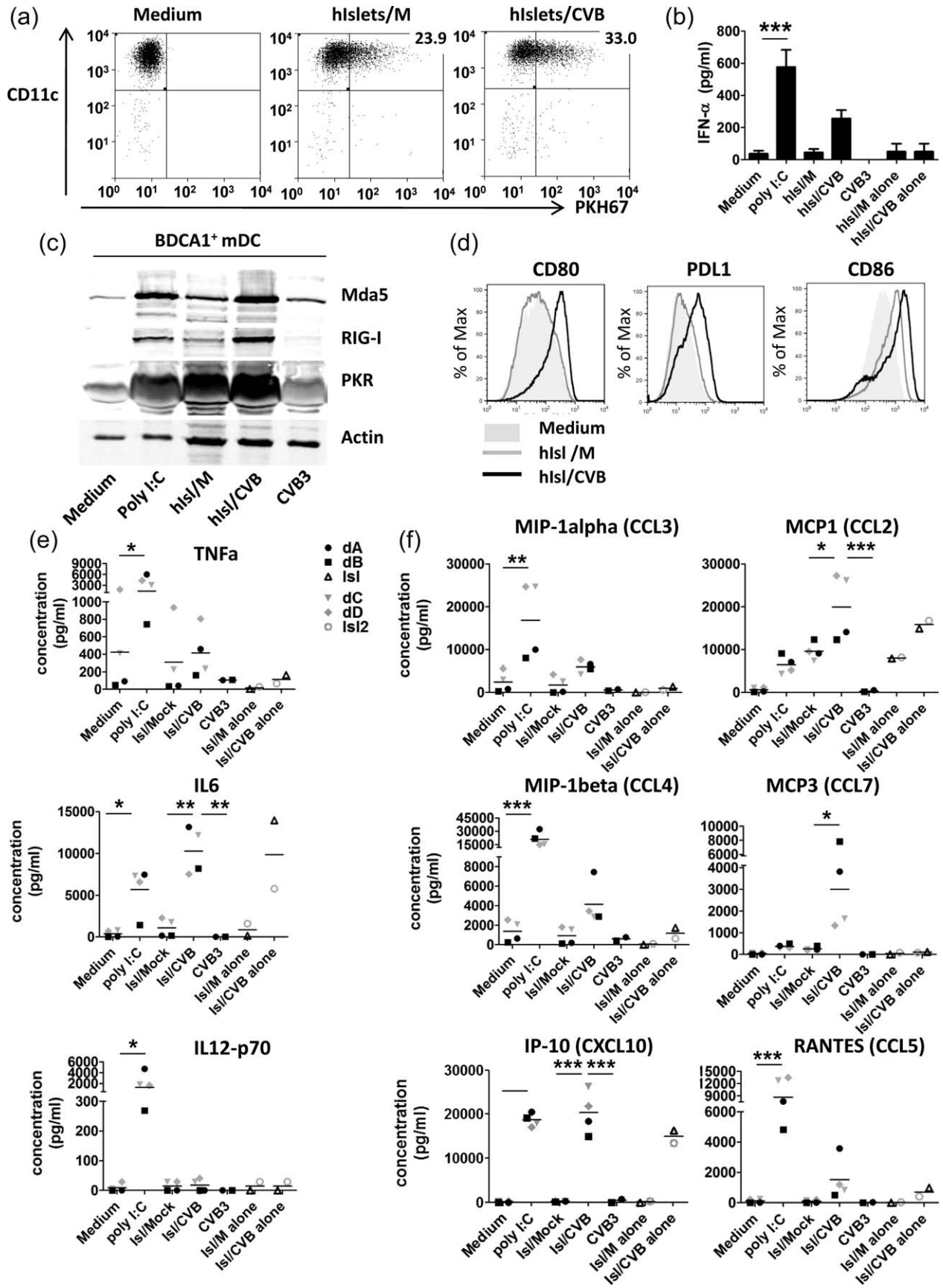


Fig. 5. Blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) stimulated with Coxsackie B virus (CVB)-infected, but not mock-infected Min6 cells induce T cells with T helper type 1 (Th1) phenotype while suppressing Th2 responses. (a) BDCA1$^+$ DCs were stimulated overnight as in Fig. 2a and co-cultured subsequently with allogeneic peripheral blood leucocytes (PBLs) in a 1 : 10 DC : PBL ratio using 10^5 peripheral blood leucocytes. Proliferation was assessed after 4 days. (b) Supernatant from mixed lymphocyte reaction (MLR) cultures using indicated stimuli was analysed for cytokine production 48 h after start of MLR. Shown is average ± standard error of the mean (s.e.m.) of 3 different donors. *$P < 0{\cdot}5$; **$P < 0{\cdot}01$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

Discussion

DCs play a crucial role in balancing our immune system, including the prevention of autoaggressive responses. The role of antigen-presenting cells (APCs) such as DCs in diabetes development is based largely on studies in mouse models [35–38], yet very limited knowledge exists on the early development of autoimmune diabetes in humans and the role of pancreas draining LN-derived and BDCA1$^+$ DCs therein. In this study we investigated the interplay between human primary BDCA1$^+$ DC and either stressed or enterovirus-infected β cells/human islets of Langerhans. We show that although DCs phagocytose material efficiently from mock or cytokine-stressed, UV-irradiated or serum-starved β cells, they only become activated significantly upon uptake of CVB-infected β cells. CVB-infected β cells/human islets induce induction of IFN-α/β, co-stimulation molecules and a variety of proinflammatory cytokines and chemokines in DCs which result in T cell proliferation, suppression of Th2-type T cell responses and, under particular conditions, induction of IFN-γ.

Our studies revealed that in human pancreas-draining LNs ≤ 1% of all viable cells comprise HLA-DR$^+$/Lin$^-$ DCs. To our knowledge, this is the first study reporting on DCs within human pancreas-draining LNs. From these DCs, 7–25% is BDCA1$^+$; the remainder of the cells are pDCs and other populations of DCs that require further characterization. We attempted to study the activation of BDCA1$^+$ DCs within total LN suspensions, although this proved to be challenging due to alterations in the markers used to define DC subsets upon culture. Additionally, a large proportion of the DCs die upon culture, particularly in unstimulated conditions. We cultured pdLN suspensions without addition of cytokines to minimize 'skewing' of DCs; however, this may influence DC viability as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-3 are added to purified myeloid DC and plasmacytoid DC cultures, respectively, to maintain viability. Future studies investigating and optimizing the requirements to maintain DC viability (e.g. by addition of cytokines) without skewing DC responses are warranted. Additionally, whether CVB can infect pdLN cells including DCs productively needs to be elucidated. Furthermore, the similarities and differences between blood DC and pdLN DCs await identification. These studies, however, are challenging, as



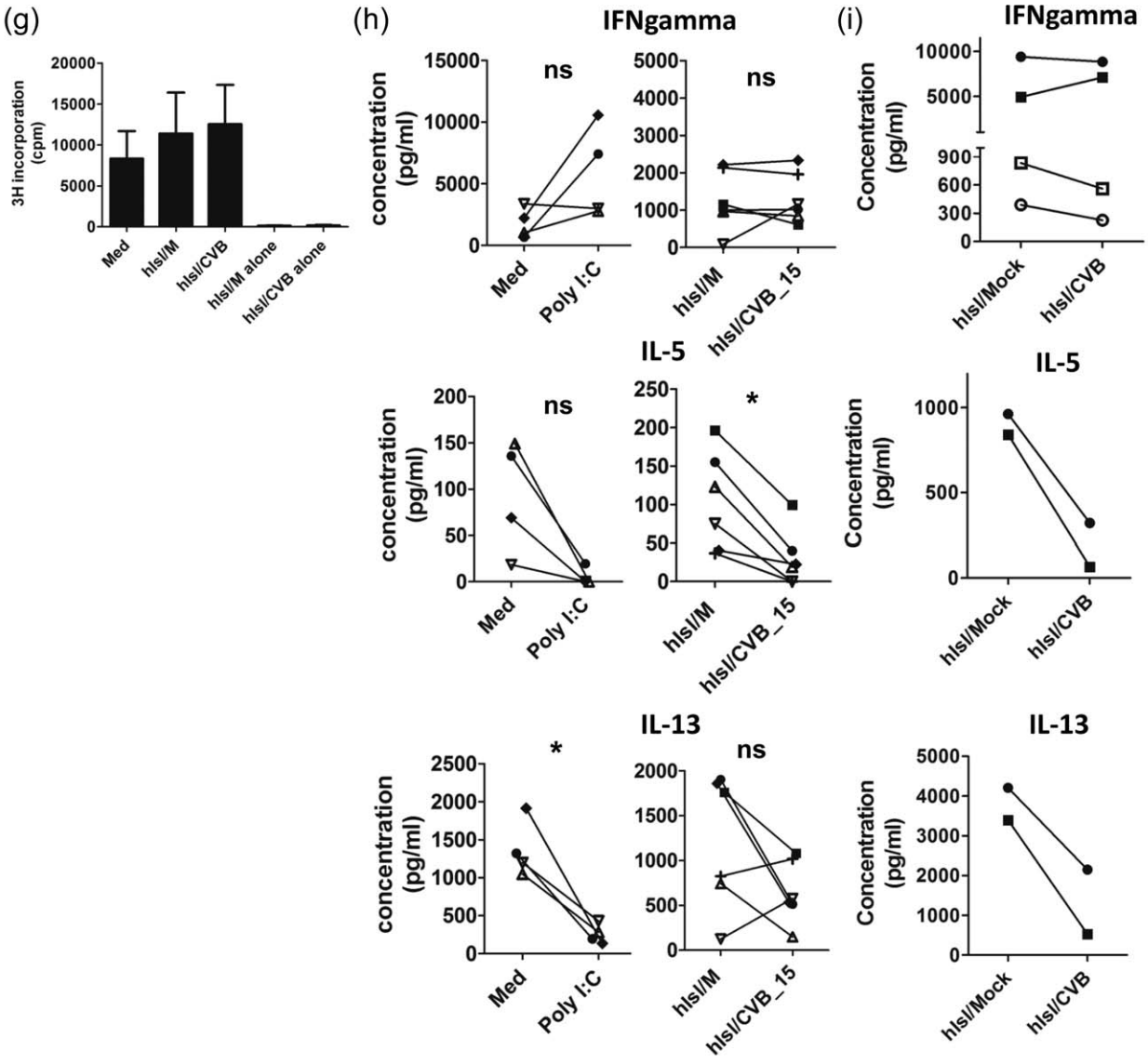


Fig. 6. Coxsackie B virus (CVB)-infected, but not mock-infected, human islets of Langerhans induce dendritic cell (DC) activation, type I interferon (IFN) responses and proinflammatory cytokine production, and these DCs suppress T helper type 2 (Th2) responses. (a) PKH-labelled human islets of Langerhans were mock- or CVB-infected for 48 h, co-cultured with blood-derived cell antigen 1 (BDCA1$^+$) DCs and after overnight culture, uptake of islet material was determined. (b) BDCA1$^+$ DCs were stimulated as indicated or co-cultured with mock- or CVB-infected human islets of Langerhans and after overnight culture, interferon (IFN)-α production was determined. (c) Protein expression or (d) cell surface marker expression of BDCA1$^+$ DCs cultured as in (b) was analysed. (e,f) Cytokine and chemokine production was determined in cultures stimulated as in (b). (g) BDCA1$^+$ DCs stimulated as in (b) were co-cultured with PBLs and analysed as in Fig. 5a. (h,i) BDCA1$^+$ DCs were stimulated with frozen and thawed lysates (h) or live cultures (i) of mock- or CVB-infected human islets of Langerhans, used in mixed lymphocyte reactions (MLRs) as in Fig. 5a and T cell-derived cytokines were determined 48 h after start of MLR. Shown are representative of four (a,d) or two (c) different experiments, average ± standard error of the mean (s.e.m.) of four (b) or two (g) or column scatterplot from two to six different donors (e,f,h,i). Corresponding symbols represent the same donor within a figure. *$P < 0{\cdot}5$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$; n.s. = not significant, as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis (b,e,f) or paired *t*-test (h). hIsl/M = mock-infected human islets of Langerhans; hIsl/CVB = CVB-infected human islets of Langerhans.

pdLN of healthy individuals and their freshly isolated blood DCs are not readily available.

Throughout our studies we used human islets of Langerhans or, alternatively, Min6 insulinoma cells. DC responses to CVB-infected islets or Min6 cells are similar in most readouts, although the CVB-infected Min6 cells induced somewhat stronger responses in most instances compared to CVB-infected human islets, and differed on the induction of IL-12 in DCs and production of IFN-γ by T cells. Preliminary experiments revealed that IL-12p35 mRNA is

induced at low levels in co-cultures of DCs and CVB-infected human islets (not shown); thus, low levels of IL-12 may be produced and could be consumed by other cells within these cultures. The reason for a lower magnitude of the responses when using human islets may be that Min6 cells grow in smaller patches where most cells are exposed to virus during infection. Isolated islets of Langerhans are present in larger units that are more spherical, limiting the number of cells susceptible for initial infection, which is also supported by previous studies from our group [29]. To investigate whether an increase in virus-infected cells would induce more potent responses we (i) infected islets at higher MOI (i.e. 150), (ii) increased infection time in islets (120 h *versus* 48 h), (iii) partly dissociated islets using trypsin-ethylenediamine tetraacetic acid (EDTA) before infection to make more cells available for the virus or (iv) used higher amounts of virus material (i.e. 5 : 1 *versus* 1 : 1 ratio islet material : DCs). None of these, however, induced stronger responses consistently in DC activation or IL-12 production. Additionally, the amount of replicating virus was not enhanced when using higher MOI, longer infection time or trypsin-EDTA dissociation (not shown), indicating that anti-viral responses in human islets [39–41] may prevent more potent infection. Alternatively, unknown factors within human islets of Langerhans may prevent stronger DC responses, induction of IL-12 and Th1-type T cell reactivity. Furthermore, variation in DC donors, as well as human islet donors, may influence the strength of observed responses. Future studies using DCs from T1D patients or individuals at risk will be particularly interesting to compare to DCs from healthy individuals.

Islet inflammation may contribute to diabetes pathogenesis [21] and the loss of islets after islet transplantation [22]. Thus, we studied DC responses upon encountering cytokine-stressed and otherwise stressed β cells. None of the conditions tested induced strong DC activation. However, cytokine-stressed Min6 cells modestly induced the co-inhibitory molecule PDL1, indicating that immunosuppressive pathways are activated under these conditions. Our data suggest that β cell stress *per se* is not sufficient for DC activation, but additional triggers such as viral infection are required for strong DC activation. Our finding that DC activation depends (partially) upon RNA within the infected cells supports this concept, and confirms our previous studies using moDCs [29,34]. In our setting, no or very little DC activation is observed upon exposure of DCs to cytokine-stressed β cells *in vitro*. The finding that encounter with cytokine-stressed β cells alone is insufficient for proper DC activation cannot be extrapolated simply, as *in-vivo* additional signals may be present that affect DC behaviour.

We used poly I:C, a double-stranded RNA mimic that can activate TLR-3 and MDA5, as a mimic of virus infection. Similar trends were obtained for most readouts, although responses to the synthetic poly I:C were stronger in most cases. However, for particular chemokines, i.e. MCP1/CCL2, MCP3/CCL7, stronger responses were observed for virus-infected cells compared to poly I:C. MCP3/CCL7 was induced particularly strongly by virus-infected β cells, but not by poly I:C or exposure to stressed β cells. Furthermore, combined stimulation of poly I:C and R848 (a single-stranded RNA mimic activating TLR-7/8) did not result in strong MCP3 induction (not shown), indicating that this chemokine is induced preferentially upon encounter of virus-infected cells and not by stimulation of TLRs recognizing 'viral PAMPs'.

MCPs are pluripotent chemokines which activate multiple leucocytes by binding to multiple different chemokine receptors and attract monocytes, lymphocytes, granulocytes, natural killer (NK) cells and DCs [42]. MCP3 is increased in serum of autoantibody-positive children [43] as well as in monocytes from patients with T1D [44] and individuals at risk for autoimmune diabetes [45]. Additionally, MCP1 and MCP3 are suggested to be involved in mouse models of autoimmune diabetes, particularly in infiltration of Th1 cells in the pancreas [46], and MCP1 was detected recently in the islets of Langerhans from a donor who recently developed T1D, but not a control donor [47], showing that MCPs may be involved actively in the development of T1D. The exact role of MCPs in development of autoimmune diabetes in general and virus-induced autoimmune responses in particular remains to be determined.

To conclude, our data show that pdLN-derived BDCA1$^+$ DCs and blood-derived BDCA1$^+$ DCs engulf β cell material. Uptake of CVB-infected, but not mock-infected, β cells or islets of Langerhans resulted in strong blood-derived BDCA1$^+$ DC activation and induced suppression of Th2 cytokines in MLRs. Priming of the immune system by early events upon HEV-B infection through, e.g. high expression of co-stimulatory molecules, production of type I IFNs and the induction of high levels of CCL2 and CCL7 may trigger expansion of anti-viral and possibly autoimmune reactions. Our results aid in understanding the pathways activated in primary DCs from healthy individuals upon encounter of enterovirus-infected β cells. Ultimately, this will provide the opportunity to compare the quality of these responses pathways in healthy individuals with those of individuals at risk for immune-mediated destruction of insulin-producing β cells.

Acknowledgements

This work was supported by grant 24-2008-949 from the Juvenile Diabetes Research Foundation (to G. J. A.) and Ruby grant 2011.80.1427 from the Dutch Diabetes Research Foundation (to B. M. S. and G. J. A.). Funding agencies had no role in conducting research and/or preparation of the paper. We thank Dr Per Bendix Jeppesen for providing Min6 cells and Dr Marije Koenders and Richard Huijbens for help

with 22-plex analysis. Additionally, we thank Professor Dr Cees J. Tack and Dr Lammy D. Elving for assistance and revision of the manuscript and Professor Dr Frank J. M. van Kuppeveld, Dr Jurjen Tel and Dr Gerty Schreibelt for useful discussions and suggestions.

Disclosure

The authors declare no disclosures.

Author contributions

B. M. S. designed and performed the research, analysed and interpreted the data and wrote the manuscript; E. D. K.-R. designed and performed the research, analysed and interpreted the data; R. B. and J. D. P. provided human islets of Langerhans; J. M. D. G. designed the research and wrote the paper, E. J. P. d. K. and M. A. E. provided human islets of Langerhans and human pancreas-draining lymph nodes and wrote the paper; G. J. A. designed the research, interpreted the data and wrote the paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Efficient uptake of mock-, virus-infected and stressed β cells by blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs). (a) Min6 cells were mock- or Coxsackie B virus (CVB)-infected or exposed to indicated stress and Min6 viability was determined prior to co-culture with BDCA1$^+$ DCs using viability dye. (b) Blood-derived BDCA1$^+$ DCs were co-cultured with PKH67-labelled mock- or CVB-infected Min6 cells, stressed Min6 cells, were stimulated with poly I:C or left unstimulated, and uptake was determined as in Fig. 1c.

Fig. S2. Blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) that encounter Coxsackie B virus (CVB)-infected, but not mock-infected Min6 cells induce type I interferon (IFN) responses. (a) DCs were co-cultured with mock- or CVB-infected Min6 cells, stimulated with poly I:C or left unstimulated (medium) and after overnight culture supernatant was harvested and assessed for IFN-α. (b) DCs were stimulated as in (a) or exposed to CVB3 and after 6 h RNA was harvested and mRNA expression was analysed. (c) DCs were stimulated as in (b) and protein expression was analysed after overnight culture. Average ± standard error of the mean (s.e.m.) three (a), seven (b) experiments or representative of three experiments (d). * $P < 0{\cdot}5$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

Fig. S3. Phenotypical dendritic cell (DC) maturation and production of proinflammatory cytokines and chemokines by blood-derived cell antigen 1 (BDCA1$^+$) DCs that engulf Coxsackie B virus (CVB)-infected, but not mock-infected Min6 cells. (a) DCs cultured as in Fig. S2b were analysed for indicated cell surface markers after overnight culture. (b,c) Supernatant of cells cultured in (a) is analysed for indicated cytokines and chemokines. Whisker plot for more than 16 experiments (a) or column scatterplot from nine different donors (b,c). Corresponding symbols represent the same donor in within a figure (b,c). * $P < 0{\cdot}5$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

Fig. S4. Blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) stimulated with Coxsackie B virus (CVB)-infected, but not mock-infected Min6 cells, induce T cells with T helper type 1 (Th1) phenotype while suppressing Th2 responses. Supernatant from mixed lymphocyte reaction (MLR) cultures using indicated stimuli was analysed for cytokine production 48 h after start of MLR. Shown is average ± standard error of the mean (s.e.m.) of five different donors. ** $P < 0{\cdot}01$ as determined by one-way analysis of variance (ANOVA) and *post-hoc* Tukey analysis.

Fig. S5. Induction of interferon (IFN)-stimulated genes in Coxsackie B virus (CVB)-infected human islets of Langerhans. Human islets of Langerhans were mock- or CVB-infected and protein expression was analysed after 48 h. hIsl/M = mock-infected human islets of Langerhans; hIsl/CVB = CVB-infected human islets of Langerhans.

Fig. S6. Cytokine and chemokine production within one blood-derived cell antigen 1 (BDCA1$^+$) dendritic cell

(DC) donor upon co-culture with Min6 cells or frozen and thawed lysate of islets of Langerhans. DCs from one donor were cultured as in Fig. 3a or co-cultured with frozen and thawed lysate of mock- or Coxsackie B virus (CVB)-infected human islets of Langerhans. Cytokines and chemokines were analysed as for Fig. 3b,c.

Fig. S7. Cytokine and chemokine production upon co-culture of blood-derived cell antigen 1 (BDCA1$^+$) dendritic cells (DCs) with frozen and thawed lysates of mock- or Coxsackie B virus (CVB)-infected human islets of Langerhans. DCs were cultured and analysed as in Fig. S6.