

# Susceptibility of Human Pancreatic $\beta$ Cells for Cytomegalovirus Infection and the Effects on Cellular Immunogenicity

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**Objectives:** Human cytomegalovirus (HCMV) infection has been suggested to be a causal factor in the development of type 1 diabetes, posttransplantation diabetes, and the failure of islet allografts. This effect of CMV has been interpreted as an indirect effect on the immune system rather than direct infection-induced cell death. In the present study, we investigated (i) the susceptibility of  $\beta$  cells to HCMV infection, (ii) regulation of immune cell-activating ligands, (iii) release of proinflammatory cytokines, and (iv) the effects on peripheral blood mononuclear cell (PBMC) activation.

**Methods:** CM insulinoma cells and primary  $\beta$  cells were HCMV-infected in vitro using a laboratory and a clinical HCMV strain. The susceptibility to infection was measured by the expression of viral genes and proteins. Furthermore, expression levels of Major Histocompatibility Complex I, Intracellular Adhesion Molecule-1, and Lymphocyte Function Associated Antigen-3 and the release of proinflammatory cytokines were determined. In addition, PBMC activation to HCMV-infected  $\beta$  cells was determined.

**Results:**  $\beta$  Cells were susceptible to HCMV infection. Moreover, the infection increased the cellular immunogenicity, as demonstrated by an increased MHC I and ICAM-1 expression and an increased proinflammatory cytokine release. Human cytomegalovirus-infected CM cells potentially activated PBMCs. The infection-induced effects were dependent on both viral "sensing" and viral replication.

**Conclusions:** In vivo  $\beta$ -cell HCMV infection and infection-enhanced cellular immunogenicity may have important consequences for native or transplanted  $\beta$ -cell survival.

**Key Words:** cytomegalovirus,  $\beta$  cells, immunogenicity, immune cell activation

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Human cytomegalovirus (HCMV) may contribute to  $\beta$ -cell dysfunction in new-onset type 1 diabetes mellitus,<sup>1–5</sup> the development of diabetes mellitus after solid-organ transplantation,<sup>6–8</sup> and islet allograft failure.<sup>9–11</sup> Because direct CMV infection of the pancreatic islets was never indisputably

proven, the diabetogenic potential of CMV still remains a subject of debate and the exact mechanism of CMV-induced  $\beta$ -cell dysfunction remains to be clarified. In most studies addressing the effect of CMV infection on the development of diabetes, the accelerating effect of CMV has been interpreted as a CMV-induced reinforcement of existing autoimmune responses,<sup>1,5</sup> rather than direct cytolytic infection of  $\beta$  cells.<sup>1–4</sup> However, knowledge on the cellular and molecular basis of these observations is largely lacking.

Cytomegalovirus belongs to the family of  $\beta$ -herpes viruses and is the largest and most complex member of this family.<sup>12</sup> Owing to several immunoevasive strategies, the virus establishes a symptomless but persistent infection in healthy individuals. However, in immunocompromised individuals, owing to the lack of immunologic control, the virus is able to reactivate and to cause severe CMV disease. Viral activity can be observed in all organs, including the pancreas,<sup>13</sup> demonstrating that the virus has a broad cellular tropism. This broad cellular tropism is because widely spread receptors, such as integrins and the epidermal growth factor receptor, serve as entry receptors.<sup>14–16</sup> These are also found on pancreatic  $\beta$  cells,<sup>17</sup> making them putative targets for CMV infection.

Studies addressing the cellular effects of CMV largely focus on the infection of fully permissive fibroblasts. In fibroblasts, the infection has a number of immunologic consequences, both immunostimulatory to enhance viral replication and viral spread, but also immunoevasive to prevent immune recognition and elimination of the host cells. The immunostimulatory effects, which are usually observed during the (immediate) early stages of fibroblast CMV infection, include the release of proinflammatory cytokines and type 1 interferons<sup>18,19</sup> and the up-regulation of the T-cell stimulatory adhesion molecules ICAM-1 and LFA-3.<sup>20</sup> The immunoevasive effects, which are usually observed during the early to late stages of infection, include the down-regulation of the cellular MHC I expression,<sup>21</sup> as well as the expression of virally encoded anti-inflammatory cytokines and chemokine decoy receptors.<sup>12</sup> Recently, we have demonstrated that rat  $\beta$  cells show a marked increase in the cellular immunogenicity after rat CMV infection, as demonstrated by the increased expression of adhesion molecules ICAM-1 and LFA-3, classic and nonclassic MHC I molecules, and Toll-like receptor 2 (TLR-2).<sup>22</sup> In the light of CMV-reinforced immune responses, these results suggest that  $\beta$ -cell CMV infection and subsequently increased immunologic recognizability may be a mechanism of immune-mediated  $\beta$ -cell dysfunction in vivo.

As a model for HCMV-induced  $\beta$ -cell pathology, we investigated the susceptibility of human pancreatic  $\beta$  cells to HCMV infection and the cellular immunologic consequences of the infection in vitro. Subsequently, we determined the mechanism by which HCMV infection changes  $\beta$ -cell immunogenicity. Finally, we evaluated whether HCMV-infected  $\beta$  cells are able to activate peripheral blood mononuclear cells (PBMCs).

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## MATERIALS AND METHODS

### Study Design

The susceptibility of  $\beta$  cells to HCMV infection was determined using human CM insulinoma cells (CM cells;  $n = 7$ ) and primary human  $\beta$  cells isolated from cadaveric pancreata ( $n = 3$ ). Although the insulin release of this CM line is not as in native  $\beta$  cells,<sup>23,24</sup> with respect to surface marker expression the cell line demonstrates many similarities to native  $\beta$  cells.<sup>25</sup> Further, the cell line has been proven to be predictive for immunologic consequences of viral infection in vitro<sup>26</sup> and for the susceptibility to autoimmune processes in type 1 diabetes.<sup>27</sup> All cell types were infected with the laboratory HCMV strain *AD169* at a multiplicity of infection (MOI) of 0.01, 1, or 10 plaque-forming units per cell (PFUs/cell). Infected permissive primary human fibroblasts served as controls ( $n = 6$ ). The susceptibility of  $\beta$  cells to HCMV infection was determined by assessing the expression of viral UL111 and US28 transcripts and immediate early-1 (IE1) and pp65 proteins. Viral protein expression was analyzed at 24 to 96 hours after infection, whereas the expression of UL111 and US28 was determined at 72 hours after infection.

To study the immunogenicity of the cells after infection, we infected CM cells with *AD169* at an MOI of 0.01, 1, or 10 PFUs/cell ( $n = 7$ ). This study was performed on CM cells because the amount of cells required for these studies could not be obtained from cadaveric pancreata. In accordance with our study in rat  $\beta$  cells,<sup>22</sup> we quantified the expression of the major histocompatibility complexes HLA-A/B/C, the adhesion molecules ICAM-1 and LFA-3, and the TLR-2 in response to *AD169* infection. These molecules were quantified in response to *AD169* infection at 24 to 96 hours after infection. Furthermore, the release of proinflammatory and viral infection-associated IL-1 $\beta$ , IL-6, IL-8, IL-15, interferon- $\alpha$ , and Monocyte Chemoattractant Protein-1 by CM cells was quantified in response to *AD169* infection. Because the virus-induced effects were most prominent at 72 hours after infection, the release of cytokines to infection at an MOI of 1 and 10 PFUs/cell was determined at this time point.

To study the mechanism of *AD169*-induced changes in cellular immunogenicity and cytokine release, several experiments were performed using virus particles which were UV-inactivated before infection ( $n = 5$ ). The UV inactivation of virus particles completely abolishes viral replication. This allows us to discriminate between cellular effects induced by viral replication or protein expression and effects induced by viral binding and cellular entry only.

Because the laboratory HCMV strain *AD169* is adapted to replication in fibroblasts,<sup>28,29</sup> it may be argued that infection using this HCMV strain in  $\beta$  cells is not fully representative for clinical HCMV infection. Therefore, key findings were repeated using the clinical HCMV isolate *Merlin* at an MOI of 0.01 PFU/cell or 1 PFU/cell ( $n = 5$ ). Low propagation rates of this HCMV strain limited us to infections at an MOI of 0.01 and 1 PFU/cell in most experiments.

Finally, the immune stimulatory capacity of *AD169*-infected, *Merlin*-infected, noninfected CM cells, and CM cells inoculated with UV-inactivated virus particles was analyzed in a <sup>3</sup>H-thymidine PBMC proliferation assay ( $n = 5$  PBMC donors). For this, CM cells were infected with infectious or UV-inactivated *AD169* virus particles at an MOI of 0.01 or 1 PFU/cell or with infectious or UV-inactivated *Merlin* at an MOI of 0.01 PFU/cell for 72 hours.

### Cell Lines, Primary Human $\beta$ Cells, and PBMCs

Human islets were isolated from cadaveric pancreata in the Leiden University Medical Center, as previously described.<sup>30</sup>

The Leiden University Medical Center has permission from the Dutch government to act as an organ bank for human islets. The organs, for which consent for research was obtained, were allocated by the Eurotransplant Foundation (Leiden, The Netherlands). Use of anonymous donor material was acted on in accordance with the Code of Proper Secondary Use of Human Tissue in The Netherlands as formulated by the Dutch Federation of Medical Scientific Societies. In The Netherlands and some other countries in the Eurotransplant zone, permission for organ donation is obtained after checking a national database of people who have consented to organ donation during life and/or interviews by the organ procurement team with relatives after the diagnosis of brain death. Consent for research is obtained verbally from relatives by the organ procurement team and put in writing on organ donor reports. All donor organs are coded (anonymized) when they arrive at the human islet isolation unit at the Leiden University Medical Center. Primary human  $\beta$  cells were isolated from dispersed islets, based on cellular flavin adenine dinucleotide fluorescence.<sup>31</sup>

Peripheral blood mononuclear cells were isolated from the blood of 5 healthy volunteers using a Lymphoprep density gradient according to standard protocols (1.078 g/mL; Axis-Shield, Oslo, Norway).

CM cells<sup>25</sup> and primary human  $\beta$  cells were cultured in RPMI (Gibco, Breda, The Netherlands) containing 60  $\mu$ g/mL gentamicin and 10% heat-inactivated fetal calf serum (FCS).

Human fetal lung fibroblasts were prepared from fetal lungs in 1992. The necessary material was obtained from the women's clinic after approval of the Groningen University Medical Ethical Committee and oral informed consent of both parents. Human fetal lung fibroblasts (FLF92)<sup>32</sup> were cultured in Dulbecco modified Eagle medium (Gibco) containing 60  $\mu$ g/mL gentamicin and 10% FCS. Cell lines (CM and FLF92) were always used between passage numbers 5 and 20.

### Human Cytomegalovirus Infection

Three days before infection, primary  $\beta$  cells, CM cells (both 300,000 cells per well), and FLF92 (250,000 cells per well) were seeded in 6-well plates. One day before infection, cells were serum-starved in culture medium containing 3% FCS (infection medium). Thereafter, cells were infected with infectious HCMV particles (the laboratory strain *AD169* or the clinical strain *Merlin* [kindly provided by Prof Puchhammer-Stöckl, Medical University of Vienna, Vienna, Austria]) at MOIs ranging from 0.01 to 10 PFUs/cell. Noninfected cell cultures in infection medium served as controls. *AD169* or *Merlin* infection was performed for 1 hour in 1 mL of infection medium, after which 2 mL of infection medium was added. Infected and noninfected cells were harvested using 0.05% Trypsin 0.5 mM EDTA.

Virus particles were UV-irradiated for 10 minutes at room temperature. UV-inactivated virus particles were used immediately. Complete inactivation of the particles was confirmed by the absence of viral protein expression after infection of FLF92.

### Immunocytochemistry

Cells (50,000) were spotted on glass slides, air-dried, fixed, and permeabilized using a CMV Brite Turbo Kit (IQ products, The Netherlands). Subsequently, cells were stained for the cellular expression of IE1 (clone E13; AbD Serotec, Düsseldorf, Germany) and pp65 (clone C10-C11; Hybridoma derived) according to standard protocols, using a horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine tetra HCl for staining.

TABLE 1. Primer Sequences

	UL111	US28
Forward	5'-cattgaggagatctgcatgaagt-3'	5'-tgtctgctggcggagt-3'
Reverse	5'-atccacactaggagagcagact-3'	5'-gacgcgaaaagctcatgct-3'
Probe	5'-fam-cttggccagctacattct-tamra-3'	5'-fam-cccgcgatgatctg-tamra-3'

### Viral Genes

Total RNA was extracted using TRIzol reagent (Gibco). After DNase treatment (DNase-free; Ambion, Nieuwerkerk a/d IJssel, The Netherlands) complementary DNA was synthesized (SuperScript III and OligodT primers; Invitrogen, Breda, The Netherlands). Cellular viral transcripts UL111 and US28 were detected on a ABI7900 TaqMan (Applied Biosystems, Carlsbad, Calif) using gene-specific primers (Table 1). Relative gene expression was normalized for the cellular expression of GAPDH and depicted as the  $\Delta C_t$  value.

### Flow Cytometry

Cells were counted and fixed in ice-cold phosphate-buffered saline (PBS) containing 0.5% paraformaldehyde. Before staining, the cells were washed and incubated with 10% rabbit serum for 30 minutes. Cells were rinsed in PBS containing 1% bovine serum albumin (BSA) and incubated with the primary antibody for 60 minutes. Primary antibodies used were antihuman ICAM-1 (Hu5/3-2.1; kindly provided by Dr M.A. Gimbrone, Harvard Medical School, Boston, Mass), anti-LFA-3 (TS2/9.1.4.3; ATCC, Manassas, Va), and anti-MHC I (W6/32; ATCC), all undiluted, and biotin-conjugated anti-TLR-2 (1:50; BioLegend, Uithoorn, The Netherlands). Secondary antibodies used were fluorescein isothiocyanate-conjugated rabbit antimouse (1:50; Dako, Glostrup, Denmark) and fluorescein isothiocyanate-conjugated streptavidin (1:100; Dako). Cells were incubated with the secondary antibody for 30 minutes, and all incubations were carried out on ice and in the dark. Phosphate-buffered saline/BSA was used instead of the primary antibody as a negative control.

At least 10,000 cells were analyzed by flow cytometry. The percentage of positive cells was determined based on the sample stained with PBS/BSA instead of the primary antibody. Data were analyzed using Winlist 5.0 software (Verity Software House, Topsham, Me). Protein expression levels of HCMV-infected cells were compared with those of noninfected cells, and relative expression levels were calculated. Because 100% of the cells were positive for MHC class I, ICAM-1, and LFA-3,<sup>26</sup> the mean fluorescence intensity (MFI) levels were used to calculate the relative expression levels. The cells demonstrated very low levels of TLR-2, therefore the percentage of positive cells was used to calculate these relative expression levels. Relative expression levels are indicated as follows: expression level of the HCMV-infected cells (MFI or %) / expression level of the noninfected cells (MFI or %).

### Cytokine Production

Culture supernatants were harvested, and cellular debris was removed by centrifugation. The culture supernatants were stored at  $-20^{\circ}\text{C}$  until further use. A Biorad 6-plex luminex kit was used to detect the presence of IL-1 $\beta$ , IL-6, IL-8, IL-15, IFN- $\alpha$ , and MCP-1 (Biorad, Venendaal, The Netherlands). The assay was performed according to the kit protocol.

### <sup>3</sup>H-Thymidine Incorporation Assay

Human cytomegalovirus-infected and noninfected cells were used as stimulator cells. After 72 hours of infection, the

cells were harvested, washed, and fixed in PBS containing 0.5% paraformaldehyde for 5 minutes on ice. Fixed cells were stored in liquid nitrogen until further use.

Peripheral blood mononuclear cells, at a concentration of  $2 \times 10^5$ , were cocultured with  $2 \times 10^4$  stimulator cells (ratio, 10:1) for 72 hours in 96-well round-bottom plates at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  according to standard protocols.<sup>33</sup> Peripheral blood mononuclear cells incubated in RPMI 10% FCS (medium) served as negative controls, whereas PBMCs incubated in medium containing 5  $\mu\text{L}/\text{mL}$  concanavalin A (ConA) served as positive controls. Peripheral blood mononuclear cells were labeled with 1  $\mu\text{Ci}$  <sup>3</sup>H-thymidine 16 hours before analysis. <sup>3</sup>H-Thymidine incorporation was measured using liquid scintillation.

### Statistics

Results are expressed as the mean  $\pm$  SEM. Normality of the data sets was determined using the Kolmogorov-Smirnov test. If the data sets were not normally distributed, they were log-transformed and reanalyzed using the Kolmogorov-Smirnov test. Normally distributed data sets were analyzed using the (paired) 2-tailed Student *t* test or the 2-tailed 1-sample *t* test. When data sets were not normally distributed, differences were calculated using the Mann-Whitney *U* test or the Wilcoxon signed-rank test. *P* values less than 0.05 were considered to be statistically significant.

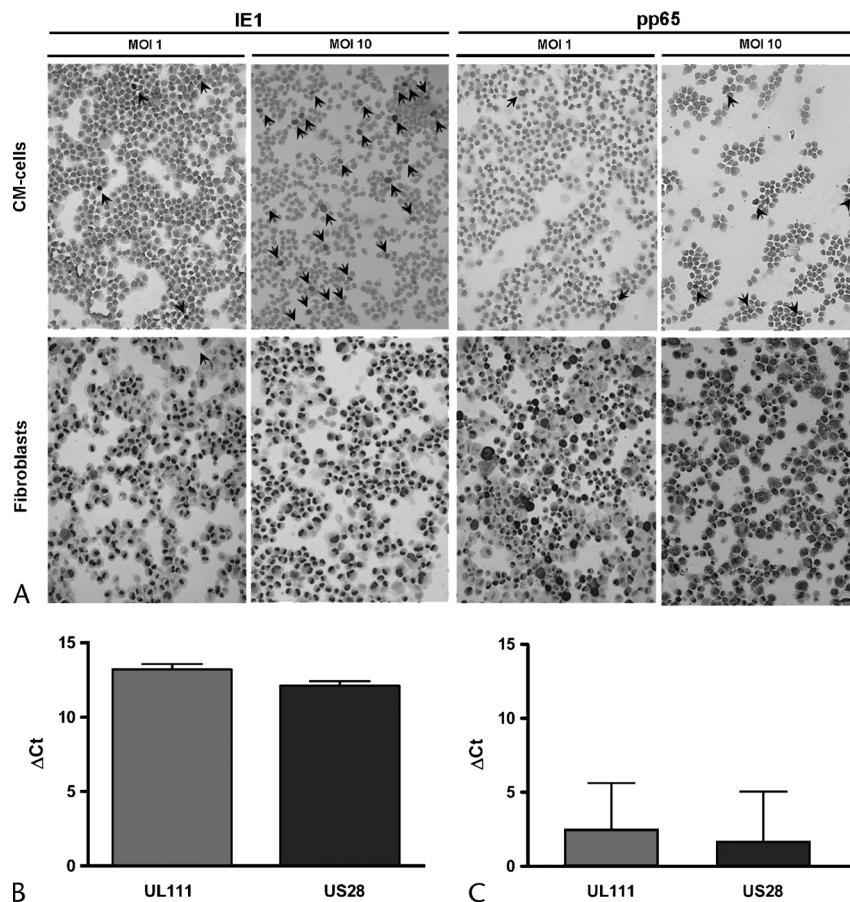
## RESULTS

### Human $\beta$ Cells and CM Cells Are Susceptible to HCMV Infection

The susceptibility of human  $\beta$  cells to HCMV infection was investigated in an in vitro infection assay. To this end, CM cells were infected with the HCMV strain *AD169* at an MOI of 0.01, 1, and 10 PFUs/cell. The cells were harvested 24 to 96 hours after infection and stained for the IE1 protein and the early-late pp65 protein. Noninfected CM cells and *AD169*-infected fully permissive fibroblasts served as controls.

At 72 hours after infection, viral protein-expressing CM cells were observed in a dose-dependent manner, that is, MOI 0.01 PFU/cell, no IE1- or pp65-expressing cells detected (not shown); MOI 1 PFU/cell, less than 1% IE1- and pp65-expressing cells detected (Fig. 1A); and MOI 10 PFUs/cell, 4% IE1-expressing CM cells and less than 1% pp65-expressing CM cells detected (Fig. 1A). Compared with *AD169*-infected fibroblasts, the number of *AD169*-infected CM cells was profoundly lower than the number of infected fibroblasts, which demonstrated 50% to 80% IE1-expressing cells (MOI 1 and 10 PFUs/cell), and 10% to 40% pp65-expressing cells (Fig. 1A). This difference between the 2 cell types is further substantiated by the observation that the number of IE1- and pp65-expressing CM cells remained constant over time (up to 96 hours after infection), whereas the number of *AD169*-infected fibroblasts increased over time, reaching an infection grade of almost 100% in 96 hours after infection (data not shown). The susceptibility of human CM cells to HCMV infection was confirmed on mRNA level. Viral immediate-early UL111 and early US28 transcripts were detected in the cell cultures infected with *AD169* for 72 hours but not in the noninfected cultures (Fig. 1B).

To demonstrate the relevance of these findings for native pancreatic  $\beta$  cells, the infectability of primary human  $\beta$  cells, isolated from cadaveric pancreata, was studied. The cells were infected with *AD169* at an MOI of 1 PFU/cell for 72 hours and analyzed for HCMV infection on an mRNA level. Human cytomegalovirus infection of primary human  $\beta$  cells was demonstrated by the presence of viral UL111 and US28 transcripts in



**FIGURE 1.** Human  $\beta$  cells and CM cells are susceptible to HCMV infection. A, Immunohistochemical staining of CM cells and fibroblasts. Cells were *AD169*-infected at an MOI of 1 and 10 PFUs/cell. At 72 hours after infection, the cells were harvested. Subsequently, the expression of viral IE1 and pp65 protein was determined by immunohistochemistry.  $\beta$ -Cells expressing IE1 or pp65 are depicted by the arrows. B and C, Viral gene expression was analyzed by quantitative polymerase chain reaction. Human CM cells (B) and primary human  $\beta$  cells (C) were *AD169*-infected. Gene expression of viral UL111 and US28 was detected 72 hours after infection. Noninfected cells served as controls. Viral transcripts were not detected in noninfected controls and therefore not depicted ( $\Delta C_t$  value > 40). Transcriptional levels were corrected for the transcription of GAPDH ( $\Delta C_t$  value).

the cells infected with *AD169* but not in the noninfected cells (Fig. 1C).

### CMV Infection Increases the Cellular Immunogenicity

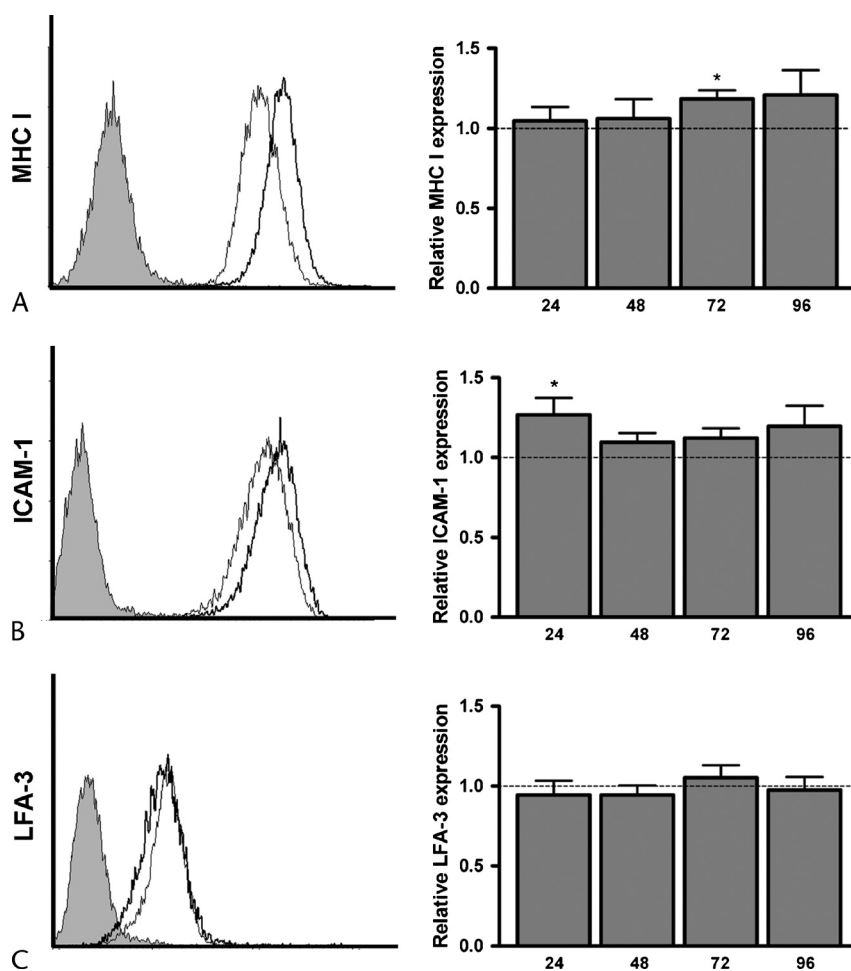
Next, we investigated the immunologic consequence of the  $\beta$ -cell HCMV infection by measuring the cellular expression of MHC I, ICAM-1, LFA-3, and TLR-2. Because this study required large amounts of cells, we used CM cells rather than primary human  $\beta$  cells. CM cells were *AD169*-infected at an MOI of 0.01, 1, and 10 PFUs/cell and analyzed at 24 to 96 hours after infection. Noninfected CM cells and infected fibroblasts served as controls.

*AD169* infection of CM cells using an MOI of 0.01 PFU/cell did not result in alterations in MHC I, ICAM-1, LFA-3, or TLR-2 expression (data not shown). *AD169* infection at an MOI of 1 or 10 PFUs/cell induced a significant increase in MHC class I expression compared with noninfected CM cells (Fig. 2A, only MOI 1 is presented). This increase was significant at 72 hours after infection. Further, the infection induced an increase in the expression of ICAM-1 at 24 hours after infection (Fig. 2B), whereas the cellular LFA-3 expression remained unaffected on

*AD169* infection (Fig. 2C). The expression of TLR-2 was low on CM cells and was not affected by the *AD169* infection (data not shown). The expression of MHC I and adhesion molecules in *AD169*-infected CM cells was differently regulated compared with *AD169*-infected fibroblasts. Fibroblasts infected with *AD169* at an MOI of 0.01 PFU/cell did not demonstrate any alterations in the MHC I, ICAM-1, LFA-3, or TLR-2 expression (data not shown). Fibroblasts infected at an MOI of 1 or 10 PFUs/cell demonstrated decreased MHC I expression and increased ICAM-1 and LFA-3 expression (Supplemental Figure 1, Supplemental Digital Content 1, <http://links.lww.com/MPA/A75>, only MOI 1 is presented). Fibroblast infection at an MOI of 10 PFUs/cell further decreased the cellular MHC I expression but did not further increase the cellular ICAM-1 expression (data not shown).

### CMV Infection Induces the Release of Proinflammatory Cytokines

We also studied the release of proinflammatory cytokines from *AD169*-infected CM cells. Because *AD169* infection at an MOI of 0.01 PFU/cell did not result in viral protein expression or alterations in CM cell immunogenicity, we only analyzed CM cells that were infected with *AD169* at an MOI 1 and 10 PFUs/



**FIGURE 2.** HCMV infection increases the cellular immunogenicity. Human CM cells ( $n = 7$ ) were *AD169*-infected at an MOI of 1 PFU/cell. Subsequently, the cellular expression of MHC class I (A), ICAM-1 (B), and LFA-3 (C) was determined by flow cytometry from 24 to 96 hours after infection. All graphs show the mean  $\pm$  SEM of relative expression levels of *AD169*-infected cells compared with uninfected control cells. All panels show representative flow cytometry graphs. The graphs show cells stained with the secondary antibody only (filled gray), uninfected cells stained with both primary and secondary antibodies (thin black line), and HCMV-infected cells stained with both primary and secondary antibodies (bold black line). \* $P < 0.05$ . \*\* $P < 0.01$ .

cell. At 24 to 96 hours after infection, the presence of IL-1 $\beta$ , IL-6, IL-8, IL-15, IFN- $\alpha$ , and MCP-1 was determined in the culture supernatant of infected and noninfected CM cells. Infected fibroblasts served as controls.

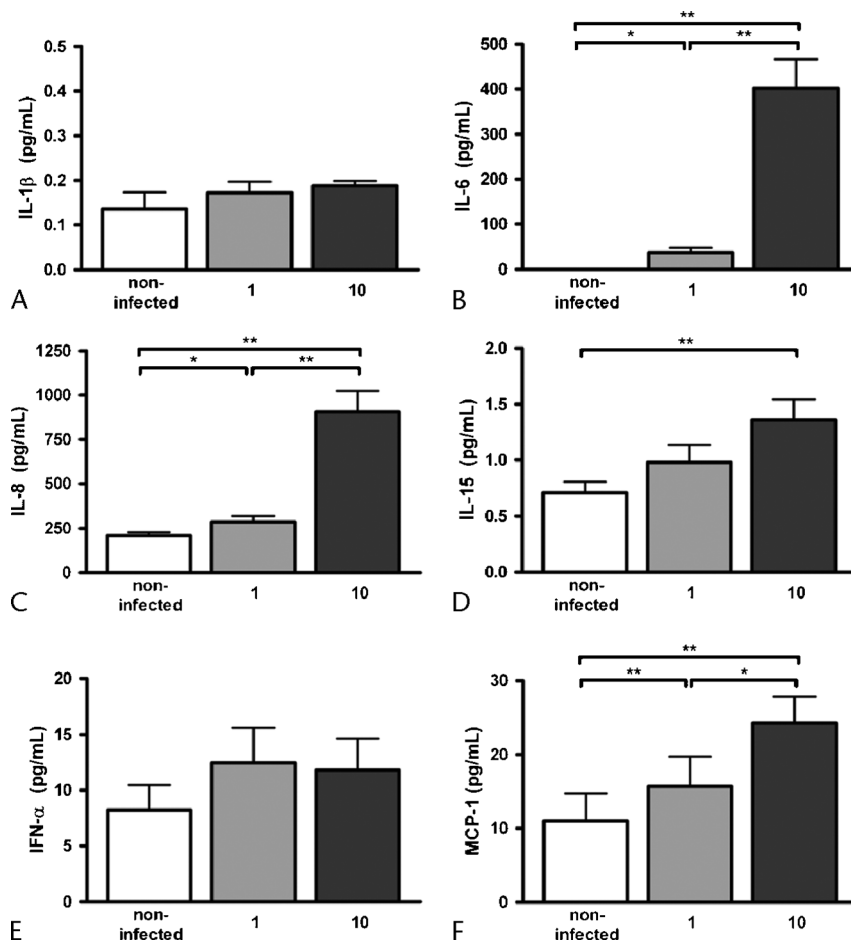
After *AD169* infection, the CM cells released IL-6, IL-8, some IL-15, and MCP-1 in the culture supernatant, in a dose-dependent manner (Figs. 3B–E). These cytokines were detected in a similar pattern at all time points analyzed. Therefore, we only show cytokine release at 72 hours after infection. The release of IL-1 $\beta$  (Fig. 3A) and IFN- $\alpha$  (Fig. 3E) was not affected by the *AD169* infection. The release of cytokines from infected CM cells was different compared with infected fibroblasts, which released IL-1 $\beta$ , IL-6, IL-8, IL-15, and some IFN- $\alpha$  after *AD169* infection (Supplemental Figure 2, Supplemental Digital Content 2, <http://links.lww.com/MPA/A76>). *AD169* infection significantly decreased the fibroblast MCP-1 release (Supplemental Figure 2, Supplemental Digital Content 2, <http://links.lww.com/MPA/A76>).

### Viral Binding Versus Viral Replication

Although only a few CM cells demonstrated viral gene expression, the increase in immunogenicity was observed in a

large proportion of the CM cells. This suggests that binding of the virus particles to the cells, or viral entry alone, is enough to increase the cellular immunogenicity. To test this hypothesis, CM cells and fibroblasts were incubated with UV-inactivated virus particles at an MOI of 1 and 10 PFUs/cell. Because our previous results showed that virus-induced effects were most prominent at 72 hours after infection, the cells were harvested and analyzed at this time point.

The effect of *AD169* infection on CM-cell MHC I up-regulation was abrogated when the virus particles were UV-inactivated (Fig. 4A). In contrast to the MHC I expression, UV-inactivated virus particles increased the CM cell ICAM-1 expression in a similar manner compared with infectious virus particles (Fig. 4B). In fibroblasts, infection with UV-inactivated virus completely abrogated the infection-induced down-regulation of MHC I and even increased the expression of MHC I in a dose-dependent manner (Supplemental Figure 3, Supplemental Digital Content 3, <http://links.lww.com/MPA/A77>). Similar to infected CM cells, the infection-induced up-regulation of ICAM-1 was still observed despite UV inactivation (Supplemental Figure 3, Supplemental Digital Content 3, <http://links.lww.com/MPA/A77>).



**FIGURE 3.** HCMV infection induces the release of proinflammatory cytokines. Human CM cells (A–F) were *AD169*-infected at a concentration of 1 or 10 PFUs/cell (CMV 1 or 10, respectively). At 72 hours after infection, the culture medium was harvested, and the concentration of IL-1 $\beta$  (A), IL-6 (B), IL-8 (C), IL-15 (D), IFN- $\alpha$  (E), and MCP-1 (F) was quantified by Luminex. All graphs show the mean  $\pm$  SEM. \* $P$  < 0.05. \*\* $P$  < 0.01.

After incubating CM cells with UV-inactivated virus particles, CM cells released similar quantities of IL-6, IL-8, IL-15, and MCP-1 compared with infectious virus particles (Figs. 4C–H compared with Fig. 3). Also in fibroblasts, the release of cytokines in response to UV-inactivated virus was similar to the release profile observed after inoculating with infectious virus particles (Supplemental Figure 3, Supplemental Digital Content 3, <http://links.lww.com/MPA/A77>). However, UV inactivation of *AD169* abrogated the fibroblast MCP-1 down-regulation (Supplemental Figure 3, Supplemental Digital Content 3, <http://links.lww.com/MPA/A77>).

### Clinical Isolates Versus Laboratory Strains of CMV

Key findings of CM cell infection with *AD169* were repeated using the clinical HCMV isolate *Merlin*. Low propagation rates of this virus strain limited us to infections at an MOI of 0.01 PFU/cell in most of the experiments. Again, noninfected CM cells and *Merlin*-infected fibroblasts served as controls.

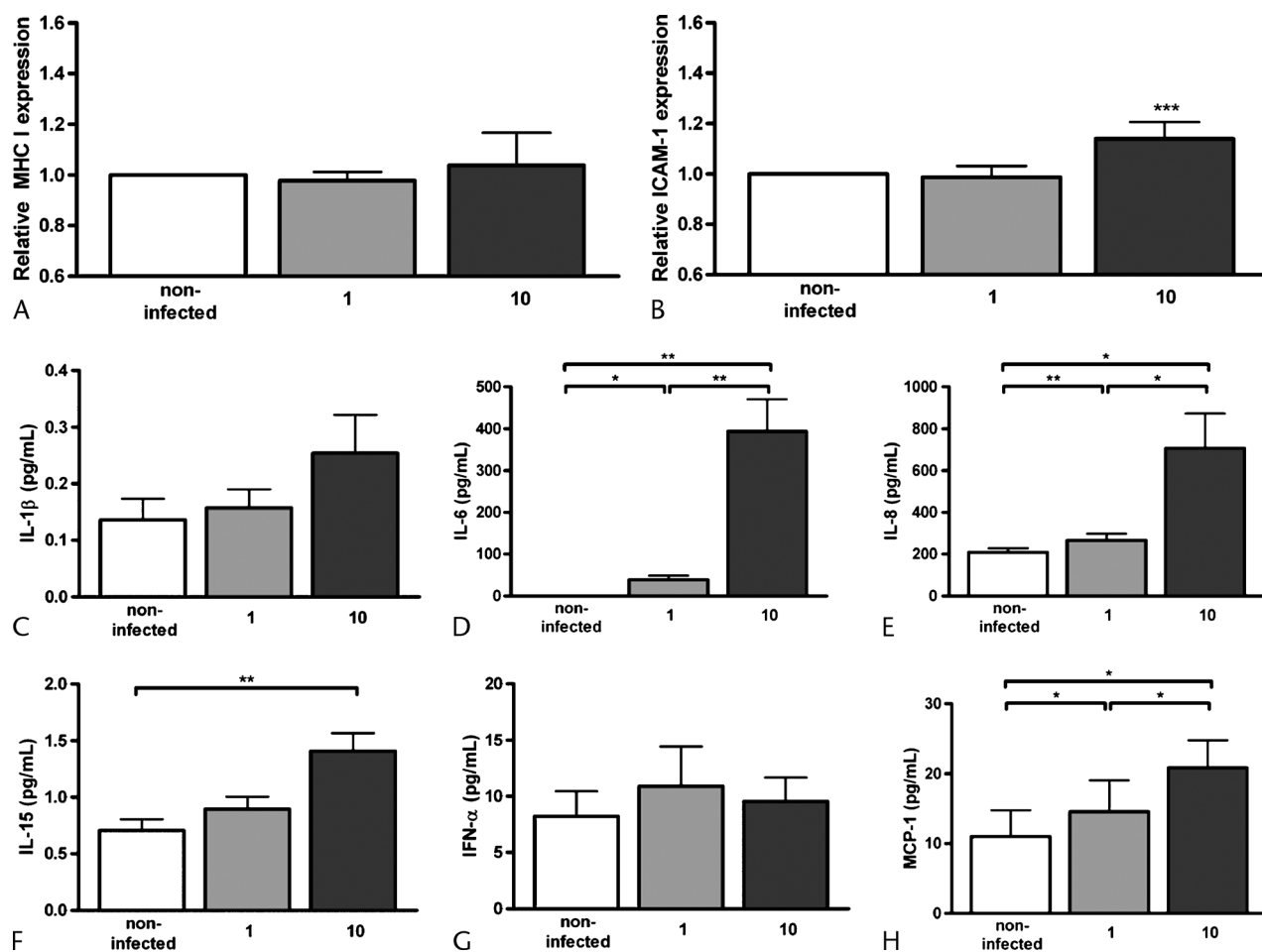
*Merlin* infection of CM cells resulted in similar findings compared with *AD169*; at 72 hours after infection, less than 1% of IE1- and pp65-expressing CM cells were found using an MOI of 1 PFU/cell, whereas infection at an MOI of 0.01 PFU/cell did not demonstrate any IE1- or pp65-positive cells (Fig. 5A). Similar to *AD169*-infected fibroblasts, *Merlin* infection of fibroblasts

resulted in large numbers of IE1- (10% and 80%) and pp65-expressing cells (5% and 30%) (Fig. 5A).

Comparable to *AD169*-infected CM cells, *Merlin* infection increased the cellular expression of MHC I at 48 to 72 hours after infection (Fig. 5B). The ICAM-1 expression was increased at 24 to 48 hours after infection (Fig. 5C). CM cell LFA-3 expression was not affected by *Merlin* infection (not shown). In contrast to *AD169*-infected fibroblasts, *Merlin* infection increased the cellular expression of MHC I, as well as the ICAM-1 and LFA-3 expression (Supplemental Figure 4, Supplemental Digital Content 4, <http://links.lww.com/MPA/A78>).

### CMV-Infected $\beta$ Cells Potently Activate Human PBMCs

The previously mentioned results indicate that  $\beta$  cells may not only be infected by HCMV, but they may also activate the immune system because of their increased immunogenicity. To test this hypothesis, we determined the immunostimulatory capacity of HCMV-infected  $\beta$  cells. For this, we quantified the  $^3\text{H}$ -thymidine incorporation in PBMCs on exposure to noninfected CM cells, 72-hour (UV-inactivated) *AD169*-infected CM cells (MOI 0.01 and 1 PFU/cell), (UV-inactivated) *Merlin*-infected CM cells (MOI 0.01 PFU/cell), medium alone, or



**FIGURE 4.** Viral binding versus viral replication. Human CM cells were *AD169*-infected with UV-inactivated nonreplicating virus at a concentration of 1 or 10 PFUs/cell (CMV 1 or 10, respectively). At 72 hours after infection, the cellular expression of MHC I (A) and ICAM-1 (B) was determined by flow cytometry. In addition, culture medium was harvested for quantification of IL-1 $\beta$  (C), IL-6 (D), IL-8 (E), IL-15 (F), IFN- $\alpha$  (G), and MCP-1 (H) by Luminex. All graphs show the mean  $\pm$  SEM. \* $P < 0.05$ . \*\* $P < 0.01$ .

ConA. Peripheral blood mononuclear cell proliferation to *AD169*- and *Merlin*-infected fibroblasts served as a control. The PBMC proliferation rates to infected and noninfected cells were calculated as a percentage of the PBMC proliferation observed after ConA stimulation ( $10,026 \pm 1865$  disintegrations per second, which was set to 100% for each individual experiment).

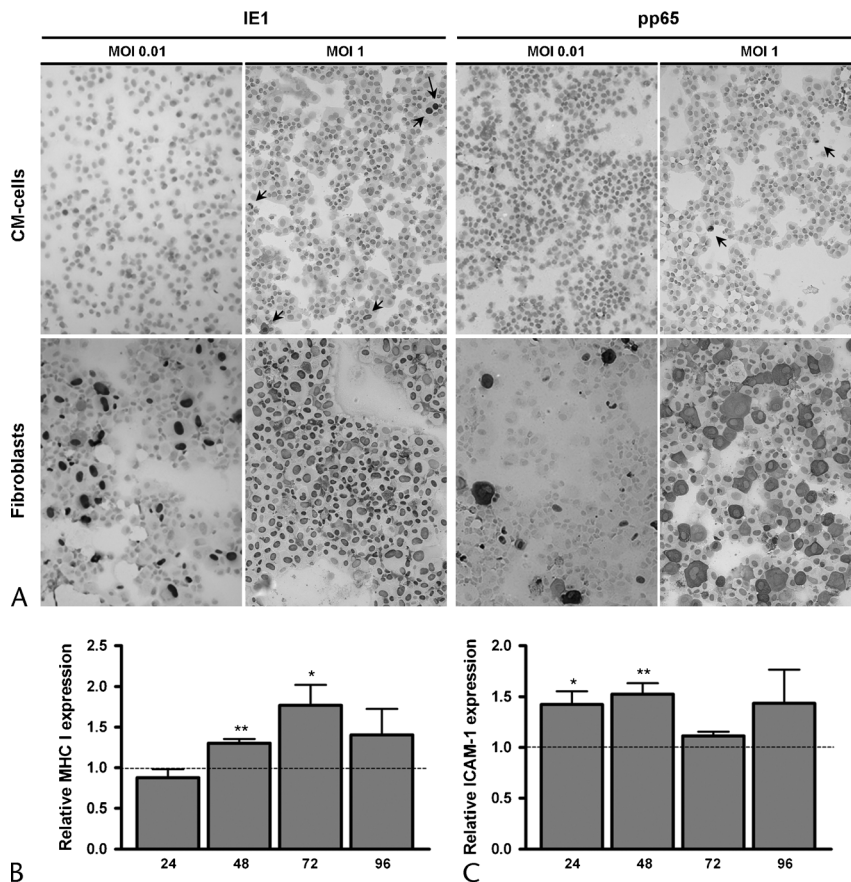
CM cells infected with *AD169* at an MOI of 1 PFU/cell induced 7.7% of the maximal proliferation, which was significantly higher than PBMC proliferation to noninfected CM cells (3.4% of maximal proliferation; Fig. 6). CM cells infected with *Merlin* at an MOI of 0.01 PFU/cell induced 7.5% of the maximal proliferation, which was significantly higher than the PBMC proliferation to noninfected CM cells (3.4% of maximal proliferation; Fig. 6). Peripheral blood mononuclear cell proliferation to *Merlin*-infected CM cells was significantly higher than PBMC proliferation to CM cells infected with *AD169* at a similar MOI of 0.01 PFU/cell (3.6% of maximal proliferation; Fig. 6). Peripheral blood mononuclear cell proliferation to *Merlin*-infected CM cells decreased when the virus particles were UV-inactivated before infection (5.1% of maximal proliferation). However, CM cells inoculated with UV-inactivated *Merlin* still increased PBMC proliferation above the level observed after stimulating with noninfected CM cells (Fig. 6). CM

cells inoculated with UV-inactivated *AD169* at an MOI of 1 PFU/cell induced similar PBMC proliferation rates as CM cells inoculated with infectious *AD169* particles (Fig. 6).

Only fibroblasts infected with *AD169* at an MOI of 1 PFU/cell were able to increase PBMC proliferation (10.1% of maximal proliferation), whereas *Merlin*-infected fibroblasts failed to increase the PBMC proliferation above the level of PBMC proliferation to noninfected fibroblasts (6.4% of maximal proliferation; Supplemental Figure 5, Supplemental Digital Content 5, <http://links.lww.com/MPA/A79>).

## DISCUSSION

In the present study, we investigated the susceptibility of human pancreatic  $\beta$  cells to HCMV infection together with the associated immunologic consequences of the infection. We show that human pancreatic  $\beta$  cells, as demonstrated by both primary  $\beta$  cells as well as CM insulinoma cells, are susceptible to HCMV infection, replication, and viral protein expression. The infection had large immunologic consequences, which were demonstrated by an infection-induced increase in the cellular immunogenicity and the release of proinflammatory cytokines. Although virus-specific responses are observed, a similar increase in  $\beta$ -cell MHC



**FIGURE 5.** Human CM cells are susceptible to *Merlin* infection. Human CM cells were *Merlin*-infected at a concentration of 1 or 0.01 PFU/cell. At 72 hours after infection the cellular expression of IE1 and pp65 (A) was determined as a measure for *Merlin* infection. In addition, the expression of MHC I (B) and ICAM-1 (C) was determined in  $\beta$  cells infected with 0.01 PFU/cell *Merlin*. All graphs show the mean  $\pm$  SEM. \* $P < 0.05$ . \*\* $P < 0.01$ .

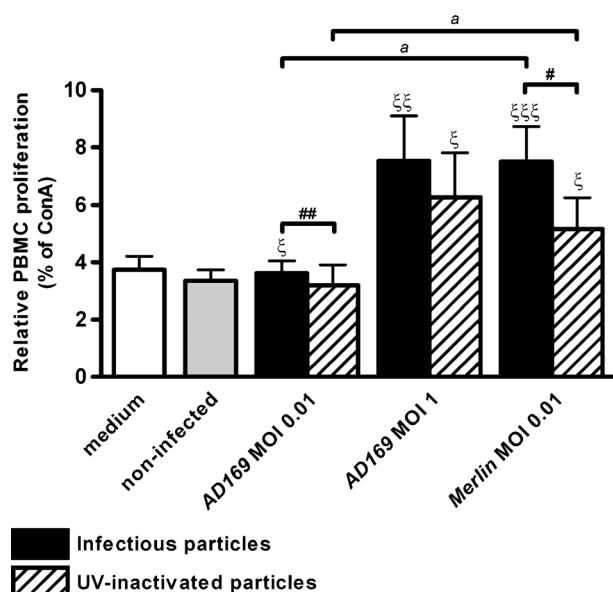
I expression and proinflammatory cytokine and chemokine release was observed after infection with other diabetes-associated viruses, such as measles, mumps, rubella,<sup>26</sup> or coxsackie virus.<sup>34</sup> Our results are in line with the fact that HCMV-infected  $\beta$  cells increased their capacity to activate immune cells. Our results furthermore demonstrated that parts of the  $\beta$ -cell response to HCMV are due to binding of the virus or cellular entry only, that is, ICAM-1 up-regulation and cytokine release, whereas for some molecules, such as MHC I, regulation is dependent on viral replication. Next to the laboratory HCMV strain *AD169*, we also tested the infectability, effects on immunogenicity, and activation of immune cells after infection of  $\beta$  cells with the clinical HCMV strain *Merlin*. We found results similar to the effects of *AD169*. That the immunologic consequences of cellular HCMV infection are highly cell type-specific was shown by fibroblasts, which demonstrated a rather different regulation of immune cell activating ligands and cytokines compared with HCMV-infected  $\beta$  cells.

Although the presence of IE1- and pp65-expressing  $\beta$  cells clearly demonstrated that pancreatic  $\beta$  cells are directly susceptible to HCMV infection, the replication process proceeds much slower in  $\beta$  cells than in fibroblasts. That this mode of HCMV replication is cell type-specific, rather than dependent on adaptations of the laboratory strain *AD169*, was demonstrated by a similar infection grade using the clinical HCMV strain *Merlin*. This strengthens the hypothesis that pancreatic  $\beta$  cells

do support HCMV infection and replication, similar to several other CMV-supporting cell types in humans<sup>35–44</sup> and  $\beta$  cells in rats.<sup>22</sup> This is further substantiated by two other findings. First, the presence of active viral replication in  $\beta$  cells was demonstrated by the regulation of MHC I in the presence of infectious virus particles but not when the viral replication was abolished by UV inactivation. Second, we observed a marked activation of immune cells in response to HCMV-infected CM cells. Because PBMC activation and proliferation are restricted to the presentation of viral antigens on MHC I, these results suggest that, although viral protein expression in infected  $\beta$  cells may be hard to detect using conventional laboratory techniques, the cells do allow viral replication and protein expression, which in turn can be detected by the immune system. This observation is of clinical importance because persistent low-grade infection of native pancreatic  $\beta$  cells may explain the absence of overt  $\beta$ -cell CMV infection in vivo, despite clear cut effects of the CMV infection on  $\beta$ -cell function and survival.<sup>1,45</sup>

We demonstrate not only that human pancreatic  $\beta$  cells are susceptible to HCMV infection but also that the virus induced the release of proinflammatory cytokines and increased the cellular immunogenicity. This increased cellular immunogenicity may enhance the intrinsic capacity of pancreatic  $\beta$  cells to activate T cells, making HCMV-infected  $\beta$  cells even more prone to (auto- and/or allo-) immune-mediated destruction. If the production of proinflammatory cytokines is also observed in vivo,





**FIGURE 6.** HCMV-infected  $\beta$  cells potently activate human PBMCs. Human cytomegalovirus–infected or noninfected  $\beta$  cells (CM insulinoma cells) were coincubated with PBMCs from healthy donors for 72 hours. CM cells were inoculated with either infectious (black bars) or UV-inactivated nonreplicating (dashed bars) virus of the *AD169* strain (MOI 0.01 PFU/cell and MOI 1 PFU/cell) or the *Merlin* strain (MOI 0.01 PFU/cell) for 72 hours. Peripheral blood mononuclear cells incubated with medium alone were included as negative controls, whereas PBMCs incubated with ConA served as positive controls. Peripheral blood mononuclear cell proliferation was determined by measuring  $^3\text{H}$ -thymidine incorporation. Peripheral blood mononuclear cell proliferation was determined as the percentage of the total proliferative capacity of the cells as observed after ConA stimulation (100%). All graphs show the mean  $\pm$  SEM.  $\xi, \xi, \xi, \#P < 0.05$ .  $\xi\xi, \#\#P < 0.01$ .  $\xi\xi\xi P < 0.001$ .  $\xi$ Statistical significance compared with proliferation to noninfected cells.  $a$ Statistical significance between different viral strains.  $\#$ Statistical significance between infectious and UV-inactivated virus.

a CMV-induced local proinflammatory cytokine environment in the native pancreas, or in islet grafts, may further contribute to T-cell activation and proliferation.<sup>46</sup> In fact, proinflammatory cytokine release *in vivo* has been demonstrated to directly compromise  $\beta$ -cell function and viability<sup>34</sup> and to recruit and activate auto-specific or allo-specific immune cells.<sup>47–51</sup> In addition, inhibition of islet cytokine release has been associated with decreased insulinitis<sup>52</sup> and improved islet graft function.<sup>53,54</sup> Furthermore, the association between up-regulation of ICAM-1 and MHC-I and the destruction of islet cells have been demonstrated in several studies. For example, administration of blocking antibodies directed against ICAM-1 significantly improved islet allograft survival.<sup>55</sup> Also, it has been demonstrated that increased  $\beta$ -cell antigen presentation by MHC I is associated with autoimmune destruction of native  $\beta$  cells.<sup>56,57</sup> Together, this suggests that an increased cellular immunogenicity, combined with an enhanced immune cell–activating capacity of the infected  $\beta$  cells, may have direct deleterious consequences *in vivo*.

The clinical relevance of our study is further substantiated by the findings using the clinical HCMV isolate *Merlin*. Infection of CM cells with this clinical HCMV strain enhanced the cellular immunogenicity and induced immune cell activation compared with noninfected and *AD169*-infected cells. The time frame in which the immunologic changes occurred was similar to that of the laboratory strain *AD169*. However, the clinical isolate induced approximately 2.5 times more MHC I and ICAM-1 expression than the laboratory strain. This is remarkable because the clinical isolate was used in a 100-fold lower concentration than the laboratory *AD169* strain. This not only suggests that genetic differences between the clinical and laboratory strain<sup>58</sup> are responsible for differentially affecting  $\beta$ -cell immunogenicity but also strongly underlines the deleterious

potential of clinical HCMV infection for pancreatic  $\beta$ -cell survival *in vivo*.

Most immunologic effects in  $\beta$  cells, for example, proinflammatory cytokine release and ICAM-1 up-regulation, are also observed after UV inactivation of the virus. This suggests that sensing of the virus via viral binding or viral entry is an important mechanism in HCMV-enhanced  $\beta$  cells immunogenicity. It is therefore tempting to speculate that similar to fibroblasts, these effects may be mediated through interactions between the virus and pattern recognition receptors on the cellular surface.<sup>19</sup> Alternatively, the  $\beta$ -cell response may also be mediated through intracellular sensing of unmethylated CpG regions in the viral DNA or sensing of viral RNA.<sup>59–61</sup> The interactions between HCMV and pattern recognition receptors are well described and are thought to be necessary for nuclear factor  $\kappa\text{B}$  activation and subsequent expression of the viral immediate early genes.<sup>62,63</sup> Another consequence of this nuclear factor  $\kappa\text{B}$  activation is the expression and release of proinflammatory factors, such as IL-6 and IL-8,<sup>19,62</sup> and the up-regulation of ICAM-1.<sup>64</sup> This mechanism of action may also hold true for HCMV-infected  $\beta$  cells as well.

A marked difference between HCMV-infected fibroblasts and  $\beta$  cells was the regulation of MHC I. On infection, fibroblasts demonstrated down-regulation of MHC I, whereas infected  $\beta$  cells demonstrated a marked up-regulation of MHC I. The regulation of MHC I after HCMV infection has been demonstrated before to be highly cell type–specific<sup>42,65–69</sup> and may be explained by cell-specific differences in the course of the infection and thus the expression of viral proteins. In fibroblasts, the down-regulation of MHC I is dependent on the cellular expression of viral immediate-early protein US3 and early-late proteins US2, US6, US10, and US11 and serves to prevent immune recognition of

infected cells.<sup>21</sup> In case of low-grade HCMV infection in  $\beta$  cells, the absence of US3, US2, US6, US10, and/or US11 protein expression may therefore prevent MHC I down-regulation. The observed up-regulation of MHC I in infected  $\beta$  cells may be the result of immediate-early viral gene expression because UV inactivation abolished the increase in the cellular MHC I expression and demonstrated the dependence on (parts) of the viral replicative cycle.

In addition to immunogenicity studies on HCMV-infected  $\beta$  cells, we also demonstrated that HCMV infection of the  $\beta$  cells increases the cellular capacity to activate T cells. The HCMV-induced increased expression of MHC I and adhesion molecules may further add to antigen presentation and T-cell costimulation. By using UV-inactivated virus, we demonstrate the importance of this increased cellular immunogenicity. Because viral replication and protein expression is abolished after UV inactivation, only low amounts of viral proteins can be presented on MHC I (only proteins from viruses that entered the cell). Although activation of PMBCs is significantly decreased after stimulation with  $\beta$  cells coincubated with UV-inactivated virus particles compared with  $\beta$  cells coincubated with infectious virus particles, the PBMC proliferation was still significantly increased compared with noninfected  $\beta$  cells. Thus, although UV inactivation strongly decreased the level of viral antigen presented on MHC I, the HCMV-induced increased immunogenicity may still provide enough costimulation to induce a potent immune activation.

In conclusion, we demonstrate that, using primary  $\beta$  cells and the CM insulinoma model, human  $\beta$  cells are directly susceptible to low-grade HCMV infection, as demonstrated by infection with the laboratory strain *AD169* and the clinical isolate *Merlin*. Moreover, we demonstrate that HCMV sensing together with low-grade replication and viral gene expression has large immunologic consequences. Both viral infection and increased immunogenicity may lead to potent stimulation of immune cells. We propose a model in which in vivo low-grade viral infection of the native or transplanted  $\beta$  cells, combined with an enhanced cellular immunogenicity and a local proinflammatory cytokine environment provokes a strong and destructive immune response against the insulin producing  $\beta$  cells. This might be a mechanism responsible for HCMV-induced  $\beta$ -cell dysfunction in new-onset type 1 diabetes mellitus,<sup>1–5</sup> the development of posttransplantation diabetes mellitus,<sup>6–8</sup> and islet allograft failure.<sup>9–11</sup>

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