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Differential Initiation of Innate Immune Responses Induced by Human Cytomegalovirus Entry into Fibroblast Cells

Laura K. Juckem,* Karl W. Boehme,* Adam L. Feire,*† and Teresa Compton2*†

Infection of permissive fibroblasts with human CMV (HCMV, AD169) is accompanied by a robust activation of innate immune defense. In this study, we show that inflammatory cytokine (IC) secretion and activation of the type I IFN pathway (αβ IFN) are initiated through distinct mechanisms. HCMV is recognized by TLR2 leading to the NF-κB activation and IC secretion. However, the IFN response to HCMV is not a TLR2-dependent process, as a dominant negative TLR2 does not affect the antiviral response to infection. Additionally, baflomycin, an endosomal acidification inhibitor, has no effect on HCMV-induced IFN responses suggesting that IFN signaling is independent of endosomal resident TLRs. By contrast, disruption of lipid rafts by depletion of cellular cholesterol inhibits both HCMV entry as well as IFN responses. Cholesterol depletion had no effect on the induction of ICs by HCMV, illustrating a biological distinction at the cellular level with the initiation of innate immune pathways. Furthermore, HCMV entry inhibitors block IFN responses but not IC signaling. In particular, blocking the interaction of HCMV with β1 integrin diminished IFN signaling, suggesting that this virus-cell interaction or subsequent downstream steps in the entry pathway are critical for downstream signal transduction events. These data show that HCMV entry and IFN signaling are coordinated processes that require cholesterol-rich microdomains, whereas IC signaling is activated through outright sensing via TLR2. These findings further highlight the complexity and sophistication of innate immune responses at the earliest points in HCMV infection. The Journal of Immunology, 2008, 180: 4965–4977.

Human CMV (HCMV), a member of the Herpesviridae family, is a ubiquitous opportunistic pathogen. The outcome of HCMV infection typically correlates with the immune status of the host. Infection of healthy individuals is often asymptomatic; however, infection of immunocompromised hosts, including organ and stem cell transplant recipients and AIDS patients, can be devastating (1, 2). Neonates are also susceptible to HCMV infection results in the secretion of inflammatory cytokines (ICs) and type I IFNs from host cells (7–9). Both classes of molecules are hallmarks of innate immunity that contribute significantly to control infections (10, 11). ICs, such as TNF-α, IL-1, IL-6, IL-8, IL-12, and IL-18, have a wide range of biological effects on tissues and cells and are critical for the recruitment and activation of phagocytic leukocytes (12). Type I IFNs consist of IFN-β and multiple forms of IFN-α and are produced in response to many viruses and intracellular bacterial infections (13). IFNs elicit the expression of IFN-stimulated genes (ISGs), a subset of cellular factors that inhibit viral replication (7, 8, 11, 14). Together these responses serve to limit viral replication and spread at early times following infection and also activate and promote adaptive immune responses that will ultimately contain or clear the infection. HCMV does not require virus replication or cellular protein synthesis for the robust initial induction of innate immune responses, suggesting that structural components of the virus are responsible for the up-regulation of these genes during virus entry into cells (7, 9).

The IC branch of innate immunity is defined by activation of NF-κB, which is responsible for the transcription of genes encoding many proinflammatory cytokines and chemokines (15). In response to stimuli such as cytokines or viruses, activation of the canonical NF-κB pathway occurs via signal transduction cascades that promote the phosphorylation and degradation of IκBs by ubiquitination, thereby releasing the NF-κB heterodimer. Once released, the activated heterodimer comprised of p50 and p65/RelA is able to translocate to the nucleus and drive expression of target genes (16). Fibroblasts and monocytes infected with HCMV exhibit activated NF-κB as evidenced by its nuclear translocation and increased DNA binding activity (17, 18).

One means by which viruses elicit ICs and IFNs is through TLRs that detect and initiate innate immune response to microbial pathogens. TLRs are a class of pattern recognition receptors (19). To date, 12 members of the TLR family have been identified in humans (20). TLRs are expressed at high levels on phagocytic cells such as dendritic cells and macrophages. However, all cell types appear to express at least a subset of these receptors (21, 22). TLRs recognize microbial pathogens on the basis of structural motifs, termed pathogen-associated molecular patterns (PAMPs), which differ from those found in host cells (23). Examples of
PAMPs include dsRNA (TLR3), LPS (TLR4), ssRNA (TLR7/8), and unmethylated CpG DNA (TLR9). A growing body of evidence indicates that viral envelope glycoproteins can trigger TLR-mediated innate immune signaling. For example, the fusion protein from respiratory syncytial virus and the envelope protein of mouse mammary tumor virus are sensed by TLR4 (24, 25). Similarly, HCMV envelope glycoprotein B (gB) and glycoprotein H (gH) elicit IC secretion through TLR2 (26, 27).

Induction of type I IFN responses to virus infection can be divided into two phases, the activation phase and the amplification phase. Virus infection activates an initial activation phase through a key regulatory transcription factor, IFN regulatory factor 3 (IRF3). Incompletely characterized signal transduction pathways lead to virus-induced phosphorylation of IRF3 on C-terminal serine residues by related kinases TANK-binding kinase 1 and IκB kinase ε (28–30). This results in the homodimerization and translocation of IRF3 to the nucleus where it can interact with the co-activators, CREB binding protein and p300, and form a complex that drives the transcription of IFN-β and a subset of ISGs (31).

The nascent IFN is then able to act in an autocrine and paracrine manner to initiate signaling through the cellular αβ IFN receptor leading to activation of a JAK and STAT signal transduction cascade. This amplification phase induces the robust expression of a broad panel of ISGs, which further assists the cell in establishing an antiviral state (11).

The mechanisms by which HCMV activates IFN responses remain largely undefined. HCMV and a soluble version of gB are able to activate IRF3 and the transcription of ISGs (32–34). Recent evidence using several strategies to deplete cellular IRF3 confirms its requirement and proposes that IRF3 is the primary transcription factor mediating HCMV-induced IFN signaling (35). The ability of a soluble version of gB to activate IFN signaling suggests that gB binding to a cell surface receptor during virus entry is sufficient for antiviral responses. Interestingly, a small molecule HCMV entry inhibitor that targets gB, as well as neutralizing Abs to both gB and gH, inhibit ISG accumulation (36). These studies suggest that glycoprotein-cell interactions during the entry process are important for HCMV-induced IFN activation.

HCMV has evolved many mechanisms to attenuate the host immune response to create an environment that is conducive to virus replication and persistence. The means by which HCMV modulates host immune responses is largely attributed to newly synthesized viral proteins. The immediate early gene product 1 (IE1) (IE72) is rapidly translated and plays a major role in evading host immune responses (31). It has been shown to prevent the association of STAT1, STAT2 and STAT3 with the interferon receptor (38). HCMV IE1 associates with NF-κB to the IFN-β promoter and interferes with NF-κB binding to the IFN-β promoter (38). HCMV also evades host immune responses through the membrane mimicry of many immune molecules including, but not limited to, the expression of viral chemokines, a MHC class I homolog, chemokine binding proteins, and virus encoded G protein-coupled receptors (39–43). Due to the ability of live HCMV to modulate the host innate immune responses, UV-inactivated virus was used in the current study to focus on the initiation of HCMV-induced innate immune responses, whereas live virus was used to study virus entry through tegument delivery and the initiation of viral gene expression.

Understanding the coordination of innate immune activation and virus entry is a daunting task due in part to the complexity associated with HCMV entry. Multiple envelope glycoprotein complexes decorate HCMV virions and interact with several cellular receptors to mediate entry into cells. HCMV glycoprotein M and gB interact with cell surface heparan sulfate proteoglycans to tether the virion to the cell surface (44). This allows for more stable docking with additional cellular receptors including β1 and β2 integrins that interact with gB and gH, respectively (45, 46). Epidermal growth factor receptor has also been proposed as an HCMV entry receptor, although its role remains controversial (47–49). HCMV entry may occur in a pH neutral or pH-dependent manner depending on the cell type (50, 51). The envelope glycoproteins gB, gH, and glycoprotein L are essential for entry and critical in mediating fusion (52, 53). It is during this entry process that the cell gets its first glimpse at the invading pathogen and the first opportunity to contain it.

We report that HCMV activates IFN and IFN responses through discrete mechanisms during virus entry. HCMV activates IC responses via TLR2 apparently independent of events needed for productive infection. By contrast, HCMV entry and IFN signaling are intimately linked processes critically dependent on the organization of cholesterol-rich microdomains. These findings enhance our understanding of the complex interplay between virus entry and host detection.

**Materials and Methods**

**Cell lines, reagents, and viruses**

Normal human dermal fibroblasts (NHDFs; Clonetics) were grown at 37°C in 5% CO₂, in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone Laboratories), 1% glutamine, and 1% penicillin-streptomycin-amphotericin B (Fungizone; BioWhittaker). The NHDF cells were serum starved for 24 h before all treatments. The AD169 strain of HCMV was propagated in NHDF cells and titered by immediate early gene product 1 (IE1, IE72) and gene product 2 (IE2, IE86) expression by indirect immunofluorescence (54). HCMV AD169 IE2-GFP virus was provided by D. H. Spector (University of California, San Diego, CA) (55). Where noted, purified AD169 virions isolated by density gradient centrifugation were used (50). UV inactivation of HCMV was performed as previously described (26). Replication competent recombinant vesicular stomatitis virus (VSV) expressing the enhanced green encoded gene GFP was provided by J. Yin (University of Wisconsin, Madison, WI) and propagated in baby hamster kidney BHK cells.

**Reagents and Abs**

NHDFs were transduced with retroviral vectors containing a TLR2 dominant negative construct with Tol/IL-1R domain cytoplasmic deletions as previously described (27). IFN treatments were performed using a combination of α and β recombinant human IFNs (BioSource International). Recombinant human IL-1β was obtained from R&D Systems. Pam3CSK₄ was obtained from EMC Microcollections and polyinosinic-polycytidylic acid potassium salt (poly(I:C)) was obtained from Sigma-Aldrich. A mAb to pp65 was obtained from the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, FL). A mAb to IRF3 was obtained from Active Motif. Rabbit polyclonal Abs to 1xβ- and p65 (RelA) were obtained from Santa Cruz Biotechnology. The anti-p56 Ab was a gift from G. Sen (The Cleveland Clinic Foundation, Cleveland, OH) (56). The second generation β-peptide 19, a potent inhibitor of HCMV entry (57), is designated β-peptide (+) in this study, and a control β-peptide that does not block HCMV entry is designated β-peptide (−).

**VSV plaque reduction assay**

Subconfluent NHDFs were washed with PBS, mock infected, treated with αβ IFNs (100 IU/ml), or infected with HCMV as indicated. All treatments were performed in serum-free medium. Following a 6-h incubation, the cells were washed once with PBS and infected with 100 PFU/ml VSV (New Jersey strain). VSV was absorbed for 1 h at 37°C; the inoculum was removed, and the cells overlaid with 2 ml of a 60/40 mixture of 2% Eagle’s MEM (BioWhittaker) and 1% agarose. The cells were incubated at 37°C, and plaques were visualized by crystal violet staining (0.5% PBS, 0.07% crystal violet, 5.5% formaldehyde) at 48 h of postinfection.

**Inhibition of endosome acidification**

NHDF cells were pretreated for 30 min with bafilomycin (1 μM). HCMV AD169 IE2-GFP at a multiplicity of infection (MOI) of 1 was directly added to the bafilomycin-containing medium and adsorbed for 2 h. Nonpenetrated virus was inactivated with a low pH citrate buffer wash (40 mM citric acid, 10 mM KCl, 135 mM NaCl (pH 3.0)) (58). The medium was replaced, and cells were incubated in the presence of bafilomycin at 37°C.
Cholesterol depletion and replenishment treatments

NHDFs were incubated with 8 mM methyl-β-cyclodextrin (MβCD; Sigma–Aldrich), as determined by a dose-response curve, and diluted in serum-free medium for 2 h at 37°C. After two washes with serum-free medium, the cellular cholesterol was replenished by incubating with water-soluble cholesterol (2.5–20 μg/ml; Sigma–Aldrich) or serum-free medium alone for an additional 2 h. Cells were washed an additional two times with serum-free medium and infected with HCMV (MOI = 1) for 2 h. Cells were washed with citrate buffer and incubated in serum-free medium.

Flow cytometry

At 18–24 h postinfection, cells were released from the tissue-culture plate with trypsin, resuspended in complete medium, pelleted, and suspended in PBS. Propidium iodide (Molecular Probes) was added and samples were analyzed using a FACScan flow cytometer (BD Biosciences) using a standard filter set. Samples were gated for propidium iodide exclusion (live cells) and assayed for GFP expression.

Immunofluorescence

NHDF cells were depleted of membrane cholesterol as described and cooled at 4°C for 30 min. HCMV (MOI = 2) was incubated at 4°C for 2 h. Cells were washed three times in cold serum-free medium and harvested in lysis buffer. DNA was extracted using QIAamp Mini Elute Virus Spin kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 50 μl of nuclease free water (Ambion). Viral genomes were quantitated using the primer pair, pp549s and pp821as, and UFL3 FAM-TAMRA probe as previously described (59). Unknown samples were determined based on a standard curve of known UFL3 copy numbers using pCGNAHA-pp65, which was a gift from R. Kalejta (University of Wisconsin, Madison, WI) (60). PCR contained 2.5 μl of 50 μl extracted DNA, 50 nM primers and probe, 12.5 μl of TaqMan Universal PCR Master mix (Applied Biosystems), and nuclease free water to a total volume of 25 μl. Real-time PCR was run on an ABI 7900HT, and data were analyzed using the SDS 2.2.1 program.

Detection of RNA accumulation of ISGs, ICs, and TLRs by RT-PCR

Total cellular RNA was harvested at 6 h postinfection using RNA-STaT 60 (“B”; Tel-Test), according to the manufacturer’s instructions. Briefly, cells were lysed by addition of guanidinium thiocyanate–phenol and chloroform extracted, and RNA was isopropyl alcohol precipitated. RT-PCR was performed on 200 ng of recovered total cellular RNA using Recombinant Thermus thermophilus rTth DNA Polymerase (Applied Biosystems). The primer pairs used were as follows: IFN-β sense 5’-CACCAGACGTCCTT TCCATGA, antisense 5’-AGGATTCCTACTGACTGATATG GTCC; ISG56 sense 5’-CAT CAG GTC AAG GAT AGT CTG GAG C, antisense 5’-GGA ATT CCA TAT GGT AAC GTC TTC TGA AGC C-3’; IFN-α sense 5’-TTGTTGAGCAGCAAAGGAGC, antisense 5’T-TGGGCTAAGGGGTT GTCC; ISG56 sense 5’-CAT CAG GTC AAG GAT AGT CTG GAG C, antisense 5’-GGA ATT CCA TAT GGT AAC GTC TTC TGA AGC C-3’ and the induction of IFN responses (65). However, this hypothesis was challenged by the finding that HCMV-IE2-GFP (MOI = 1) for 2 h. Cells were washed with citrate buffer and incubated in serum-free medium.

Expression and purification of gB-DLD and gB-651 soluble proteins

A fragment corresponding to aa 57–146 of gB (gB-DLD) was amplified from pCAGGS-gB (61) by PCR with the following primer: gB-DLD sense 5’-GGA ATT CCA TAT GGT AAC GTC TTC TGA AGC C-3’, antisense 5’-CGG GAT CCT TAA ACC TTT TGG TAG ACC CG-3’. The amplified fragment was cloned into the NeoI and BamHI sites of the bacterial expression vector pET-28a (Novagen) with an N-terminal His-6 tag fragment corresponding to aa 651–718 of gB (gB-651) was also amplified from pCAGGS-gB by PCR with the following primers: OML30 5’-GGA ATT CCA TAT GGT AAC GTC TTC TGA AGC C-3’, antisense 5’-CGG GAT CCT TAA ACC TTT TGG TAG ACC CG-3’. The amplified fragment was cloned into the NeoI and BamHI sites of the bacterial expression vector pTriEx-1.1 (Novagen) with a C-terminal His-6 tag. Both vectors were transformed into Escherichia coli DH5α. To produce recombinant protein, gB-DLD and gB-651 plasmids were isolated by FastPlasmid Mini per the manufacturer’s instructions (Eppendorf) and transformed into E. coli BL21 (DE3) strain for protein expression. E. coli containing the pet-28a-gB-DLD was grown at 37°C in Luria-Bertani medium containing kanamycin (50 μg/ml) and pTriEx-1.1-gB-651 in Luria-Bertani medium containing ampicillin (50 μg/ml) to an A560 of 0.6. To induce recombinant protein, isoprropyl β-n-thiogalactopyranoside (Roche) was added to a final concentration of 1 mM and incubated for 4 h at 37°C. The cells were chilled on ice and harvested by centrifugation at 4000 rad/min for 10 min at 4°C. Protein was isolated from inclusion bodies as previously described (62). Protein was then solubilized in 8 M urea/300 mM NaCl/10 mM imidazole/50 mM Tris (pH 7.9). Affinity purification of the proteins was accomplished through Ni-NTA agarose columns (Qiagen) per the manufacturer’s instructions. Eluate from the Ni-NTA column was placed onto an S-200 sizing column, and 1-ml fractions collected. Fractions containing protein were determined by measuring the absorbance of each fraction at 214 nm using a SpectraMax 190 spectrophotometer (Molecular Devices). Fractions that corresponded to absorption peaks were analyzed by SDS-PAGE to determine the size of the protein. The absorption peak fractions that contained a protein of the same size as the gB-DLD or gB-651 were pooled and concentrated using a Ni-NTA column we described. The concentrated fractions of gB-DLD were dialyzed extensively against 55 mM MES (pH 5.5) and 300 mM NaCl, whereas gB-651 fractions were dialyzed against 55 mM Tris (pH 8.3) and 300 mM NaCl.

Results

HCMV induces IFN responses in a TLR2-independent manner

HCMV activates TLR2 through a physical interaction with envelope glycoproteins gB and gH, allowing the cell to mount an IC response (26, 27). TLR2 has also been shown to mediate IC signaling in response to HSV type 1 (HSV-1) and varicella-zoster virus (63, 64). To date, no link has been established between TLR2 and the induction of IFN responses (65). However, this hypothesis has not been tested using viral ligands for TLR2. To determine whether TLR2 can activate the IFN pathway following HCMV infection, we used a signaling defective, dominant negative TLR2 construct (TLR2ΔC) that lacks the cytoplasmic Toll/IL-1R domain.
common to all TLRs. NHDFs were transduced with a bicistronic retrovirus vector encoding TLR2/H9004C and GFP. GFP-positive cells were collected by FACS. To confirm that TLR2-dependent processes were inhibited by TLR2/H9004C, IL-8 secretion was measured by ELISA (Fig. 1A). The IL-8 response to the TLR-independent cytokine IL-1β was unaffected by the expression of TLR2/H9004C. However, the cytokine responses to the TLR2-specific ligand Pam3CSK4 and UV-inactivated HCMV (UV-HCMV) were markedly reduced in the TLR2/H9004C-expressing cells (Fig. 1A). The incomplete block of IL-8 secretion in response to UV-HCMV suggests that TLR2 is not the only mechanism by which UV-HCMV can activate NF-κB. These data confirm that TLR2/H9004C specifically inhibits TLR2-dependent IC responses.

To test the hypothesis that TLR2 mediates type I IFN responses to HCMV, we determined the capacity of UV-HCMV to elicit a functional antiviral response from the TLR2/H9004C-expressing NHDF cells. Plaque formation by vesicular stomatitis virus (VSV), a virus sensitive to the effects of IFNs, was assessed following treatment with type I IFNs or infection with UV-HCMV (34). As shown in Fig. 1B, both control and TLR2ΔC-expressing NHDFs were protected from VSV infection by recombinant type I IFNs or HCMV virions. These data indicate that HCMV can induce a functional antiviral state in the absence of a signaling-competent TLR2 molecule. Furthermore, these data suggest that HCMV initiates IFN and IC responses through distinct cellular mechanisms.

Endosomal acidification is not required for HCMV-induced IFN responses

The observation that TLR2 function does not mediate HCMV-induced IFN signaling does not rule out other members of the TLR family from serving in this capacity. TLR3, TLR7, TLR8, and TLR9 are a subset of TLRs known to activate IFN responses and are localized to endosomal compartments (66–68). This subset of intracellular localized TLRs requires a low pH trigger to initiate signaling and allow for the rapid recognition of viral ligands during uncoating or degradation processes (69–71). Interestingly, several members of the Herpesviridae family are reported to induce IFN responses through TLR9, which senses unmethylated CpG 2′-deoxyribo dinucleotides in DNA (72). The CpG rich genome of HSV-1 and HSV type 2 and mouse CMV are recognized by TLR9 in plasmacytoid dendritic cells or dendritic cells in vitro (73–75). NHDF cells express high levels of TLR3 and TLR8, whereas there is a very low level of TLR7 and no detectable expression of TLR9 (Fig. 2C).
To determine whether this subset of TLRs activate the IFN response to HCMV, we examined the effect of bafilomycin, a macrolide antibiotic that specifically inhibits the vacuolar type H\textsuperscript{+}-ATPase and prevents the acidification and maturation of endosomal compartments (76). In the presence of bafilomycin, the ability of UV-HCMV to activate IRF3, as evidenced by its translocation to the nucleus, was unaffected as compared with the response in the untreated control (Fig. 2\textit{A}, panel \textit{d}). In addition, the accumulation of representative ISG transcripts, IFN-\(\beta\), ISG56, IL-6, and GAPDH transcript levels were determined by RT-PCR. PCR products were analyzed on a 1\% agarose gel and visualized by ethidium bromide staining. C, Endosomal TLR transcript levels were determined by RT-PCR from RNA isolated from NHDF cells. PCR products were analyzed on a 1\% agarose gel and visualized by ethidium bromide staining.

FIGURE 2. Endosomal maturation is not required for HCMV-induced IFN signaling. NHDF cells were incubated with bafilomycin (1 \(\mu\)M) for 30 min before infection with UV-HCMV (MOI = 1) or live VSV (MOI = 150). \textit{A}, Cells were fixed at 6 h postinfection and assayed for IRF3 localization by immunofluorescence assay. Cell nuclei were stained with DAPI. \textit{B}, RNA was isolated at 6 h postinfection, and IFN-\(\beta\), ISG56, IL-6, and GAPDH transcript levels were determined by RT-PCR. PCR products were analyzed on a 1\% agarose gel and visualized by ethidium bromide staining.

HCMV entry is dependent on cholesterol-rich microdomains

Lipid rafts are cholesterol rich, detergent insoluble microdomains within the plasma membrane hypothesized to serve as platforms for molecules involved in cell signaling, cell adhesion, and membrane trafficking (80, 81). It is becoming increasingly evident that many viruses, including HCMV, use lipid rafts as portals for virus entry (46, 82–86). To determine whether lipid rafts are required for HCMV entry, NHDFs were treated with the cholesterol-depleting reagent, M\textsubscript{B}CD, to disrupt lipid rafts before infection with a recombinant HCMV with GFP fused to the viral immediate early protein 2 (HCMV IE2-GFP) (87, 88). Live HCMV was used to
measure virus entry, and the GFP-positive cells were scored at 24 h postinfection as a surrogate marker of infection (Fig. 3A). The average total percentage of cells infected for the three independent experiments conducted was 67% at MOI = 1. In cells pretreated with MβCD, viral gene expression was reduced by approximately 90% compared with expression in untreated control; the effects of

FIGURE 3. HCMV entry is dependent on cholesterol-rich microdomains. NHDF cells were pretreated with 8 mM MβCD to deplete membrane cholesterol, and indicated wells were subsequently incubated with watersoluble cholesterol. Cells were washed and infected with live HCMV (MOI = 1). A, At 18 h postinfection, GFP expression was assessed by flow cytometry as a surrogate marker of infection. No cellular toxicity was observed by propidium iodide staining (data not shown). Data represent the average of three independent experiments. B, Delivery of the HCMV tegument protein pp65 was measured by indirect immunofluorescence at 1 h postinfection. At least six random fields were counted per treatment. Graph is representative of two independent experiments. C, Bound HCMV genomes was measured by quantitative PCR in mock NHDF cells, infected with live HCMV (MOI = 2), pretreated with 8 mM MβCD and infected with live HCMV or with live HCMV treated with soluble heparin (30 μg/ml). Error bars represent SDs.
MβCD could be reversed in a dose-dependent manner with the addition of water-soluble cholesterol (Fig. 3A).

Due to the important role that lipid rafts play in cell signaling, it is possible that the depletion of lipid rafts disrupts signal transduction cascades required for the initiation of viral gene expression. Therefore we assessed virus payload delivery by measuring the nuclear localization of the tegument protein pp65, which traffics to the nucleus almost immediately upon infection with HCMV. Similar to the results obtained with the IE2-GFP reporter virus, MβCD pretreatment resulted in a 70% decrease in the number of pp65-positive nuclei compared with untreated cells (Fig. 3B). This result suggests that the block to infection is at or before fusion. Notably, pretreatment with MβCD did not alter the binding of HCMV to cells (Fig. 3C). Together these data suggest that lipid rafts are essential for HCMV entry.

**HCMV-induced IFN responses are dependent on cholesterol-rich microdomains**

We next assessed whether disruption of lipid rafts by cholesterol depletion interferes with the induction of the IFN response to HCMV infection. Nuclear translocation of IRF3 induced by UV-HCMV infection was inhibited by pretreatment of cells with MβCD as compared with the infection of untreated cells (Fig. 4A, panels b and d). Activation of IRF3 was restored by the addition of water-soluble cholesterol (Fig. 4A, panel c). Neither MβCD nor cholesterol treatment alone activated IRF3 (Fig. 4A, panels e and f). Consistent with the lack of IRF3 nuclear translocation, the accumulation of representative ISG transcripts, IFN-β and ISG56, in response to UV-HCMV were inhibited by pretreatment with MβCD (Fig. 4B, lane 4). ISG transcript levels were restored when membrane cholesterol was replenished. MβCD and cholesterol treatments alone did not result in the accumulation of ISG mRNA. Consistent with our transcriptional studies, MβCD pretreatment diminished induction of the ISG56 protein (P56) by UV-HCMV. Densitometric analysis revealed a 5- to 6-fold decrease in P56 levels with MβCD pretreatment compared with P56 levels in untreated controls; P56 protein levels were restored by the addition of water-soluble cholesterol (Fig. 4C). Again, treatment with MβCD or water-soluble cholesterol alone did not induce P56 accumulation.

To test the specificity of MβCD pretreatment on the HCMV-mediated IFN response, we also measured the accumulation of P56 following treatment with recombinant type I IFNs. No difference was observed upon IFN treatment (Fig. 4C). To further verify the specificity of MβCD pretreatment, RT-PCR analysis of IFN-β and IL-6 induction was examined in response to additional innate immune ligands. VSV, which enters cells through the classical clathrin-coated pit pathway in a lipid raft-independent manner was unaffected by MβCD pretreatment (Fig. 4D) (78). Similarly, treatment with poly(I:C), a TLR3 specific ligand, induced IFN-β in the presence and absence of MβCD pretreatment (Fig. 4D). Consistent with a previous report (65), the TLR2-specific ligand PamCSK did not cause the accumulation of IFN-β transcripts (Fig. 4D). Together these data suggest that virus entry and activation of the host IFN responses in fibroblasts are dependent on cholesterol-rich microdomains and are specific to HCMV.

**HCMV activates IC secretion independent of the organization of cholesterol-rich microdomains**

We next assessed the role of cholesterol-rich microdomains in the activation of NF-κB and the induction of ICs by UV-HCMV. NF-κB activation was determined by monitoring the degradation of IκBα, a protein that binds and retains the NF-κB dimer in an inactive state in the cytoplasm of cells (89). In response to stimuli such as cytokines or viruses, signal transduction cascades promote the phosphorylation and degradation of IκBα through ubiquitination, thereby releasing NF-κB to translocate to the nucleus where it drives target gene expression (16). We measured the degradation of the NF-κB inhibitory subunit, IκBα, as an indirect measure of NF-κB activation. In cells pretreated with MβCD and infected with gradient purified UV-HCMV, IκBα was degraded to a similar extent as UV-HCMV alone at both 1 and 3 h postinfection (7 and 12% of mock cells, respectively) (Fig. 5A and data not shown). IκBα degradation following UV-HCMV infection was slightly affected by treatment of cells with MβCD or 20 μg/ml water-soluble cholesterol (90% and 85% of mock cells, respectively) (Fig. 5A). Following IκBα degradation, NF-κB heterodimers composed of p50 and p65/RelA rapidly translocate from the cytosol to the nucleus (16). Using indirect immunofluorescence with Abs to RelA as well as the HCMV tegument protein pp65, we can simultaneously visualize HCMV-induced NF-κB activation and virus entry (Fig. 5B). At 2 h postinfection both RelA and pp65 can be visualized in the nucleus of cells infected with live HCMV. MβCD pretreatment blocks pp65 uptake but not RelA nuclear localization (Fig. 5B, panel b). Finally, IL-6 induction by UV-HCMV infection was unaffected following cholesterol depletion or replenishment as measured by ELISA (Fig. 5C). Treatment with MβCD or cholesterol alone did not affect IL-6 secretion. These results suggest that, unlike the IFN response, activation of NF-κB and subsequent production of ICs is independent of organized cholesterol-rich microdomains. These results also indicate that HCMV-induced IC activation is not dependent on virus entry into cells, as cholesterol depletion blocks virus entry (Fig. 3).

**HCMV entry inhibitors block HCMV-induced IFN signaling but not IC signaling**

The observation that cholesterol depletion inhibits HCMV entry, as well as the induction of the IFN response (Figs. 3 and 4), suggests that 1) lipid rafts serve as a platform on which cellular machinery act to induce the IFN response, or 2) the process of HCMV entry is an important factor for activation of IFN signaling. To further probe the role of HCMV entry in HCMV-induced innate immune responses, we used two inhibitors of HCMV entry. The protein, gB-DLD, contains a highly conserved region of gB encompassing an integrin binding motif, the disintegrin-like domain. Protein gB-DLD acts as a ligand mimic and interacts with β1 integrin to potently block HCMV entry at a postattachment step (45 and A. L. Feire and T. Compton, manuscript in preparation). As a control, we used a protein termed gB-651 derived from a region of gB that contains no recognizable receptor binding motifs. Next, we used β-amino acid oligomers (β-peptide) designed to mimic the heptad repeat region of gB that block HCMV entry at a step before fusion. A β-peptide that does not block HCMV infection was used as a control. The inhibitory β-peptide displays a high degree of specificity for HCMV, as it does not block entry of mouse CMV or HSV-1 (57). Pretreatment of cells with gB-DLD resulted in a 92% decrease in virus infection as assessed by the HCMV-IE2-GFP reporter virus (Fig. 6A). The gB-651 protein did not block HCMV IE2-GFP infection (107% of control infection) (Fig. 6A). The β-peptide inhibitor, β-peptide (+), was preincubated with live HCMV before infection of NHDFs and caused a 89% decrease in HCMV IE2-GFP infection; whereas the negative control peptide, β-peptide (−), had little effect, resulting in a 12% reduction in virus infection (Fig. 6A).

P56 protein accumulation was used as a measure of IFN activation following treatment with entry inhibitors. Preincubation of cells with gB-DLD prevented P56 induction after infection with UV-HCMV (33% of UV-HCMV alone). P56 levels were...
unaffected by the control gB-651 protein (Fig. 6B). Accumulation of P56 protein was not detected with gB-DLD or gB-651 pretreatments alone. Similarly, UV-HCMV-mediated induction of P56 was diminished when virus was treated with β-peptide (+), but not when treated with β-peptide (−) (60% and 114% of UV-HCMV alone, respectively) (Fig. 6B).

Next, the effect of HCMV entry inhibitors on NF-κB activation was assessed. Pretreatment with gB-DLD or gB-651 resulted in similar levels of IκBα degradation as UV-HCMV alone (14% and 4% of the mock IκBα level, respectively, compared with 15% for HCMV alone) (Fig. 6C). The β-peptide (+) slightly inhibited IκBα degradation and the β peptide (−) did not affect IκBα degradation by UV-HCMV (15% and 35% of mock IκBα level, respectively) (Fig. 6C). Interestingly, pretreatment with the gB-DLD peptide alone resulted in IκBα degradation (11% of the mock IκBα level) (Fig. 6C). These data further support the hypothesis
FIGURE 5. HCMV-induced IC signaling is unaffected by cholesterol depletion. NHDF cells were pretreated with 8 mM MβCD to deplete membrane cholesterol, and indicated wells were subsequently incubated with water-soluble cholesterol. Cells were washed with PBS and either mock-infected, infected with UV-HCMV (MOI = 1), or treated with IL-1β (100 pg/ml) or Pam3CSK4 (20 μg/ml). A, Gradient purified UV-HCMV was used, and whole cell lysates were prepared at 1 h postinfection. IκBα and actin protein levels were assessed by Western blot analysis. IκBα protein levels were measured by densitometry and standardized to actin levels. Values indicated (+) represent the percentage of mock-infected IκBα levels. B, Cells were fixed at 2 h postinfection and the nuclear localization of the NF-κB subunit, p65/RelA, and the tegument protein, pp65, was determined by indirect immunofluorescence. Nuclei were stained with DAPI. C, NHDF cells were pretreated as described for this experiment and stimulated with UV-HCMV (MOI = 0.1) or treated with IL-1β (100 pg/ml) or Pam3CSK4 (20 μg/ml). At 18 h postinfection, supernatant IL-6 levels were determined by ELISA.
that UV-HCMV-induced innate immune responses are initiated through distinct mechanisms.

Discussion

The goal of this study was to define the innate immune pathways elicited by HCMV and determine their relationship with virus entry. These experiments were performed in subconfluent, serum-starved secondary strains of human fibroblasts. Similar levels of IC and IFN induction are observed in the presence or absence of serum following HCMV infection (data not shown) (90). However, serum starvation reduces the basal level of some cytokines. In addition, serum starvation may increase the sensitivity of cells to ligands, such as IFN, because inhibitors have been found in serum (91). In addition, more robust innate immune signaling is observed following infection with UV-HCMV because replication-dependent counter-measures are not used (7). In this study we show that the IFN response to HCMV is independent of TLR2 because an antiviral state is intact in cells expressing a dominant negative construct of TLR2. We also did not find a role for intracellular localized TLRs, as inhibition of endosome acidification had no effect on HCMV-induced IFN responses. It has been reported that TLR3 can be found on both the cell surface and in intracellular compartments in fibroblast cells (92). However, a signaling defective, dominant negative construct of TLR3 also failed to block HCMV-induced IFN signaling (data not shown). In addition, we further define the mechanisms of activation for the HCMV-induced IC and IFN responses. Dissociating lipid rafts using cholesterol depletion greatly diminished IFN signaling, but the IC response was intact. Interestingly, depletion of cholesterol also inhibited HCMV entry suggesting that the process of IFN induction and virus entry are linked. These data also indicate that entry is not required for TLR2-dependent IC secretion. Finally, we used two HCMV-specific entry inhibitors to further explore the relationship between IC and IFN responses in regard to HCMV infection. Both entry inhibitors prevented IFN signaling, whereas the IC response was unaffected. Therefore we propose that the host cell has developed a multifaceted innate immune defense mechanism to recognize and battle infection at the earliest points in the virus entry process.

Lipid rafts are multifunctional assemblies involved in diverse processes including signal transduction, endocytosis, and cholesterol trafficking (87). These microdomains are enriched in cholesterol and glycosphingolipids, which facilitate protein-protein and lipid-protein interactions on the cell surface (93). The only other time that lipid rafts have been implicated in HCMV entry is in a report by Wang and colleagues (46). They found that lipid rafts are important for the coordination of signaling complexes within minutes of HCMV entry. In addition, two proposed HCMV coreceptors, α,β, and EGFR, localize to lipid rafts along with the signaling molecules, Src and the p85 subunit of PI3K (46). The exact mechanism by which lipid rafts initiate signal transduction has not been defined, nor is the role of lipid rafts in TLR signaling fully understood. TLR2 is reported to reside in lipid rafts on the apical surface of epithelial cells after bacterial infection (94), and clustering of TLR2 and GM1 ganglioside is detected in HEK293 cells expressing TLR2 and CD14 following treatment with lipoteichoic acid (95). Disruption of lipid rafts normally inhibits the function of raft-associated receptors, therefore we hypothesized that HCMV-induced IC responses would be diminished upon cholesterol depletion. In contrast, we found that the IC response remains intact when lipid rafts are disrupted. Under conditions in which HCMV entry is inhibited by cholesterol depletion, IC responses are unaffected suggesting that entry is not required for the initiation of IC secretion in response to HCMV. However, cholesterol depletion inhibits HCMV-mediated IFN signaling.
suggesting that it is a process that is independent of IC activation and reliant on the events that occur during the entry process.

Next, we tested two inhibitors of HCMV entry. β1 integrin, a HCMV cellular receptor, was blocked using a small protein fragment of gB that contains the disintegrin like domain integrin binding motif (A. L. Feire and T. Compton, manuscript in preparation). The gB-DLD effectively inhibited HCMV entry and IFN signaling but not IC activation. This result suggests that the interaction between β1 integrin and gB or subsequent steps after binding in the HCMV entry process are important determinants for IFN activation. Integrins have been shown to have immunomodulatory roles in promoting adaptive immune responses but to our knowledge there has been no link to innate immune activation. In addition, the gB-DLD protein appears to be functioning as a ligand mimic and is able to activate IC signaling alone (Fig. 6C), suggesting that the gB-DLD protein may contain a PAMP from gB that is recognized by TLR2. The inhibitory β-peptide, designed to mimic the fusogenic heptad repeat region within gB, was also used to impede HCMV entry (57). It is hypothesized that the β-peptide (+) forms a stable 12-helical conformation that physically interacts with gB of HCMV and blocks a step before fusion. The β peptides alone are not immunogenic (data not shown) (96, 97), likely due to their unnatural amino acid composition. The β peptide (+) effectively blocked both HCMV entry and IFN signaling. Activation of the IC response was observed with both β-peptide treatments and slight variations in the activation are likely due to alterations in the PAMP recognition of gB by TLR2.

These findings support a model in which virus binding promotes multiple receptor-ligand interactions between viral envelope glycoproteins and one or more cellular receptors. The IC response appears to be activated irrespective of events needed for a productive infection through outright sensing by TLR2. In contrast, the IFN response occurs by a postbinding or fusion-dependent mechanism. We hypothesize that the congregation of stable docking receptors promotes the activation of cellular IFN responses through one or more of the following events: 1) stable binding to cellular receptors; 2) the physical fusion event; or 3) delivery of virion contents into the cytoplasm. Our findings illustrate a clear distinction in the initiation of IFN and IC signal transduction cascades by UV-HCMV. RelA translocates to the nucleus in the absence of detectable pp65 localization, a measure of HCMV content delivery (Fig. 5B). However, type I IFN signaling is blocked in situations in which HCMV entry is impeded, suggesting a correlation between virus entry and IFN activation. The contribution of NF-κB to HCMV-induced IFN signaling has not been addressed in these experiments because it is activated before virus entry and IFN signaling. A recent report studying the contribution of NF-κB to type I IFN signaling following RNA virus infection found that the NF-κB subunits p50, RelA, and cRel play a minor role (98). Although the initiation of IFN signaling by RNA viruses and DNA viruses appear to be distinct, the RNA virus VSV was unaffected by cholesterol depletion (Fig. 4D). It is likely these viruses, at least partially, share downstream signaling pathways. An additional study by Wittek et al. (99) found that IRF3-mediated activation of the IFN stimulation response element by TLR4, but not TLR3, required the RelA subunit of NF-κB, suggesting that two parallel pathways may exist. Additional studies examining the degree of cross-talk between these two pathways following DNA virus infection are required. Because IL-8 levels are diminished but not eliminated in NHDF cells expressing the TLR2 dominant negative construct (Fig. 1A), we cannot rule out that other mechanisms of NF-κB activation coincide with later steps in the entry process. This model of innate immune activation is restricted to fibroblasts because HCMV entry can occur through alternate pathways in different cell types.

This report illustrates the existence of multiple mechanisms used by cells to detect HCMV and elicit distinct innate immune responses. In this study, we were able to dissect early events in the HCMV entry process and determine an order to the innate immune induction. It appears that the IC response is initiated via TLR2 before IFN activation and virus entry. The immediate activation of the IC response allows the cell to get a jump-start on the beneficial effects associated with NF-κB activation, such as the infiltration of professional immune cells. Interestingly, HCMV may also benefit from rapid IC induction. HCMV contains multiple NF-κB elements in its major immediate early promoter region, although the exact role of these elements in the initiation of viral gene expression remains controversial (100, 101). Postbinding or fusion-dependent steps trigger the IFN response, promoting an antiviral state to protect neighboring cells from the infection. The rapid recognition of HCMV by multiple pattern recognition receptors and the activation of both branches of the innate immune response are advantageous for the cellular defense mechanism. Increasing our understanding of HCMV-induced innate immune activation allows for a more comprehensive view of virus-host interactions and will aid in the identification of new targets for therapeutic intervention.

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Disclosures
The authors have no financial conflict of interest.

References
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