A Prominent Role of the Human Cytomegalovirus UL8 Glycoprotein in Restraining Proinflammatory Cytokine Production by Myeloid Cells at Late Times during Infection

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ABSTRACT Human cytomegalovirus (HCMV) persistence in infected individuals relies on a plethora of mechanisms to efficiently reduce host immune responses. To that end, HCMV uses a variety of gene products, some of which have not been identified yet. Here we characterized the *UL8* gene, which consists of two exons, sharing the first with the HCMV RL11 family member *UL7*. *UL8* is a transmembrane protein with an N-terminal immunoglobulin (Ig)-like domain in common with *UL7* but with an extended stalk and a distinctive cytoplasmic tail. The *UL8* open reading frame gives rise to a heavily glycosylated protein predominantly expressed on the cell surface, from where it can be partially endocytosed and subsequently degraded. Infections with *UL8*-tagged viruses indicated that *UL8* was synthesized with late-phase kinetics. By virtue of its highly conserved Ig-like domain, this viral protein interacted with a surface molecule present on activated neutrophils. Notably, when ectopically expressed in THP-1 myeloid cells, *UL8* was able to significantly reduce the production of a variety of proinflammatory cytokines. Mutations in *UL8* indicated that this functional effect was mediated by the cell surface expression of its Ig-like domain. To investigate the impact of the viral protein in the infection context, we engineered HCMVs lacking the *UL8* gene and demonstrated that *UL8* decreases the release of a large number of proinflammatory factors at late times after infection of THP-1 cells. Our data indicate that *UL8* may exert an immunosuppressive role key for HCMV survival in the host.

IMPORTANCE HCMV is a major pathogen that causes life-threatening diseases and disabilities in infected newborns and immunocompromised individuals. Containing one of the largest genomes among all reported human viruses, HCMV encodes an impressive repertoire of gene products. However, the functions of a large proportion of them still remain unknown, a fact that complicates the design of new therapeutic approaches to prevent or treat HCMV-associated diseases. In this report, we have conducted an extensive study of *UL8*, one of the previously uncharacterized HCMV open reading frames. We found that the *UL8* protein is expressed at late times postinfection and utilized by HCMV to reduce the production of proinflammatory factors by infected myeloid cells. Thus, the work presented here points to a key role of *UL8* as a novel HCMV immune modulator capable of restraining host antiviral defenses.

KEYWORDS human cytomegalovirus, immune evasion, proinflammatory cytokine, virus-host interaction

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Human cytomegalovirus (HCMV), the prototypic member of the betaherpesvirus subfamily, is a widely disseminated opportunistic pathogen that rarely leads to noticeable clinical symptoms in healthy individuals. Following primary infection, HCMV achieves a homeostatic balance with the immune system and establishes a lifelong latency predominantly in cells of the myeloid lineage. However, uncontrolled HCMV viremia and severe complications occur frequently in immunosuppressed populations, including transplant recipients, AIDS and cancer patients, and in the immature host where HCMV infection represents the leading cause of congenital infections worldwide (1, 2). A stunning hallmark of the immunobiology of this pathogen is its rich repertoire of immune evasion strategies, which deeply interfere with both the innate and adaptive arms of the host immunity, precluding viral clearance (3–5).

The large double-stranded DNA genome (around 230 kb) of HCMV contains 15 gene families (6, 7). Among them, the rapidly evolving RL11 gene family is composed of 14 members (*RL5A*, *RL6*, *RL11-13*, *UL1*, and *UL4-11* genes) that are located in close proximity on the left extremity of the HCMV genome. The RL11 genes were originally grouped together because they shared a motif conserved between some members of the immunoglobulin (Ig) superfamily (6). Subsequently, the RL11 core domain (RL11D), comprising a region of variable length (65 to 82 amino acids) containing three characteristic conserved residues (a tryptophan and two cysteines) and several potential N-linked glycosylation sites, was defined as the common feature of this gene family (8). Interestingly, the RL11D domain shares homology with the CR1 domain of the E3 immunomodulatory proteins of adenoviruses (8). All members of this HCMV gene family are dispensable for virus growth in cultured fibroblasts, and most of them are predicted to encode type I transmembrane glycoproteins (9, 10). With these characteristics it is reasonable to assume that RL11 members act as immune modulators; however, to date the precise biological functions of the majority of the individual RL11 genes remain elusive. Consistent with this notion, *TRL11/IRL11* encodes a structural and functional homologue of Fc γ receptors able to bind to the Fc portion of all four human IgG subclasses (11–13). In a similar way, RL12 and RL13 were subsequently shown to bind to the Fc region of human IgG, preferentially from IgG1 and IgG2 subclasses (14). UL11 has been reported to interact with the receptor tyrosine phosphatase CD45, impairing T cell proliferation and inducing T cell interleukin-10 (IL-10) secretion (15, 16). RL13, UL1, and UL4 are constituents of the virion envelope, UL1 being implicated in cellular tropism (17) and *RL13*, a so-called temperance gene, involved in cell type-specific virus growth inhibitory functions (9, 18, 19). Our group reported that the protein encoded by the *UL7* gene contains an Ig-like domain that structurally resembles the N-terminal Ig variable domain of CD229 (Ly9), a cell surface molecule of the signaling lymphocyte activation molecule (SLAM) family. The N-terminal Ig domain of CD229 mediates homophilic interactions that trigger T and B cell signaling (20). We demonstrated that UL7 was unable to bind to any of the SLAM family receptors but instead interacts with a molecule which is present on the surface of dendritic cells (DCs) (21). Remarkably, we found that UL7 is a highly glycosylated transmembrane protein secreted from the cell surface. Furthermore, we showed that, when ectopically expressed, UL7 attenuates the production of tumor necrosis factor alpha (TNF- α), IL-8, and IL-6 in primary monocyte-derived dendritic cells. Subsequent studies by MacManiman and coworkers (22) have indicated that soluble UL7 could be involved in inducing endothelial cell angiogenesis. Lastly and more recently, another RL11 gene family member, the *UL10* gene, also has been reported to encode a soluble protein able to interfere with the activation, proliferation, and cytokine production of T cells (23).

In recent years, genome-wide studies (24, 25) have unveiled the dramatic complexity of the coding capacity of HCMV, indicating that far from the approximately 200 open reading frames (ORFs) previously estimated (8, 26, 27), the virus has the potential to express more than 700 protein-coding transcripts during infection. In this regard, instead of the originally predicted *UL8* ORF located downstream of *UL7* (6), Gatherer and colleagues detected a new spliced transcript arising from a putative new member of the RL11 gene family, designated UL8, which has the 5' end in common with the UL7

FIG 1 UL8 transcript characterization and detection of its expression in HCMV-infected cells. (A) Diagram of the HCMV (TB40/E strain) genome showing the localization of the DNA sequences that code for UL7 and UL8. Below the complete genome (top thick black line) and a closeup view of the UL7/UL8 genomic region (second thick black line), the UL7 and UL8 coding sequences (including their transcription orientations) are represented (gray arrows). 5' and 3' untranslated regions of UL8 are shown (thin black line). Exon start and end nucleotide positions are indicated above the line symbolizing the genome. UL8 transcript start and end nucleotide positions are indicated too. Arrows below transcripts correspond to the primer pairs used for detection of the UL7 and UL8 transcripts. Sizes of the two exons and the intron of UL8 are indicated below the scheme. (B) Expression of UL8 and UL7 mRNA during HCMV infection. HEL299 cells were mock infected or infected with HCMV-GFP (TB40/E strain) at a multiplicity of infection of 1. Whole-cell RNA was harvested at 72 hpi, treated with DNase, and reverse transcribed using oligo(dT). PCRs were performed using primer sets specific for UL8 (UL7ForEco and UL8spBamRev) as depicted in panel A, for IE1, and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control reactions in which the reverse transcriptase (RT) was not added during the reverse transcription reaction were included (lanes 1 and 3). The amplified products were separated on 1% agarose gel and visualized by RedSafe nucleic acid staining solution. The size of the amplified products is indicated in bp. (C) Annotated UL8 genomic sequence. The two exons of UL8 are shown in uppercase letters shaded gray, and the start and stop codons of the coding sequence are highlighted in boldface. Upstream, downstream, and intron sequences of UL8 are displayed in lowercase letters. The TSS is indicated by a bent arrow. Nucleotides corresponding to promoter motifs (TATA box, Inr, and DPE) and polyadenylation motifs [USE, poly(A) signal, and DSE] are underlined and labeled. Numbers on the left indicate the genome position of the first nucleotide. Coordinates in panels A and C refer to TB40/E BAC (GenBank accession number [AF999921](https://www.ncbi.nlm.nih.gov/nuccore/AF999921)).

transcript (24). In this study, we have characterized the protein encoded by this novel UL8 transcript. We report that the UL8 protein, independently of whether it is expressed in isolation or in the context of the infection, exerts immunosuppressive effects by hampering proinflammatory cytokine production in myeloid cells.

RESULTS

Transcription of UL8 in HCMV-infected cells. We first sought to confirm, by reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) assays, the presence of the UL8 transcript that Gatherer and coworkers (24) identified through deep-sequencing approaches in HCMV (Merlin strain)-infected cells. We assessed this in HEL299 cells infected with the TB40/E strain of HCMV. To verify UL8 splice junctions, RNA was extracted from cells infected for 72 h postinfection (hpi), and cDNA was generated by reverse transcription and PCR amplified with primers specific for UL8 (Fig. 1A shows a schematic representation of the UL8 transcript and the location of the primers used). Sequence analysis of the PCR-amplified product obtained (991 bp; Fig. 1B, lane 4) resulted in the expected 975-bp spliced UL8 transcript, with the exon-intron

boundaries at nucleotide positions 48905 and 49008 (Fig. 1A). A similar spliced UL8 transcript of 975 bp was also found in HEL299 cells infected with the AD169 strain of HCMV (data not shown). As expected, when using a primer within the UL8 intron, at the 3' end of UL7, a shorter PCR-amplified product (685 bp) originating from the UL7 transcript was observed (Fig. 1A and B, lane 4). We next examined the 5' and 3' ends of the UL8 mRNA transcript by RACE experiments. Sequencing of the major amplified cDNA product retrieved from the 5'- and 3'-RACE PCRs (data not shown) revealed that the UL8 transcript starts at nucleotide position 48307 of the HCMV genome and terminates at nucleotide position 49412 (Fig. 1A and C).

An inspection of the *UL8* gene and its flanking regions unveiled the presence of a number of regulatory elements, further supporting the existence of the UL8 transcript. A polyadenylation signal, AATATA (nucleotides 49396 to 49401), an upstream sequence element (USE), and a downstream sequence element (DSE) were found at the usual positions and distances with respect to the 3' end of the transcript (Fig. 1C) (28, 29). In addition, three core promoter elements could be distinguished near the transcription start site (TSS; the determined 5' transcript end): a potential TATA box and an initiator (Inr) motif upstream of TSS and a downstream promoter element (DPE) downstream of the TSS (Fig. 1C) (30). Taken together, our results demonstrate that the *UL8* gene was transcribed during HCMV infection.

Structure and diversity of the UL8 protein among HCMV strains. The *UL8* gene is predicted to encode a type I transmembrane protein of 324 amino acids (aa), comprising a 35-aa signal peptide, a 102-aa IgV-like domain, a 142-aa stalk, a 23-aa transmembrane region, and a 22-aa cytoplasmic tail (Fig. 2A and B). As UL8 shares with UL7 the first 192 residues of its ectodomain, these two viral proteins contain a common signal peptide, the IgV-like domain, and 55 aa of the stalk region (Fig. 2A to C shows a comparison between UL8 and UL7 structures). However, UL8 has an extended stalk and a longer and distinctive cytoplasmic tail compared to those of UL7. The extracellular region of UL8 contains 12 potential N-linked glycosylation motifs, 6 in the Ig domain and 6 in the stalk region, and 16 residues predicted to be O-glycosylated, located in the stalk (Fig. 2A and B). The cytoplasmic domain of UL8 lacks the immunoreceptor tyrosine-based switch motifs (ITSM) present in SLAM receptors but bears several remarkable sequence motifs, including two potential tyrosine-based endocytic motifs and a PDZ-binding domain at the carboxy terminus (Fig. 2A).

A search of orthologs of *UL8* among the genomes of the different CMV species completely sequenced to date and available at the GenBank database (31) indicated that it is only found in chimpanzee CMV (CCMV) but is not present in other primate or more distantly related CMVs (data not shown). In CCMV, the corresponding ORF, also designated *UL8*, is located at an analogous position in the viral genome. In order to assess the sequence variation of protein UL8, we compared its amino acid sequence among 214 HCMV strains published in GenBank, including some unpassaged and low-passage-number clinical isolates, bacterial artificial chromosome (BAC)-cloned isolates, and laboratory-adapted strains (see Fig. S1 in the supplemental material). All HCMV strains examined have the potential to express the *UL8* ORF, encoding a protein of 324 aa in most of them (161 strains). Fifty-two HCMV strains encode a shorter UL8 protein (323 aa) that lacks a threonine in the stalk. Finally, there is a low-passage-number clinical isolate, JER847, that encodes a UL8 protein of 321 aa with a deletion of three amino acids (PKP) in the boundary between the Ig and stalk domains. The degree of amino acid sequence identity of UL8 found among HCMV strains ranged from 79.9% to 100%. However, as indicated in Fig. 2C, most of the UL8 sequence variation occurred in the signal peptide and stalk region (81.7% and 82.6% amino acid identity, respectively), with the predicted Ig domain and transmembrane segment being highly conserved (95% and 96.9% amino acid identity, respectively), consistent with the importance of these later regions for maintaining protein structure and function. As already indicated for UL7 (21), the Ig-like domain and the majority (29 of 32) of amino acids conserved by SLAM family members remain intact among all of the different UL8

FIG 2 Protein domains of UL8 and differences among the HCMV strains. (A) Amino acid sequence and predicted domains of the UL8 protein encoded by AD169. The N-terminal region underlined corresponds to the signal peptide. The IgV-like domain is boxed and shaded dark blue. The transmembrane region is underlined in gray. The putative O-glycosylation sites are marked in boldface, and the N-glycosylation sites are underlined in black. In the cytoplasmic region, the two potential tyrosine-based endocytic motifs and the C-terminal PDZ-binding domain are boxed. The sequence region shared with UL7 is shaded blue. (B) Graphical illustration of UL8. The extracellular IgV-like domain is represented by a dark blue open oval. The stalk region shared between UL8 and UL7 is indicated in blue. Putative N- and O-glycosylation sites are indicated. The plasma membrane is depicted in gray. (C) Schematic representation of the UL8 and UL7 protein domains and the interstrain amino acid differences inside UL8. The number of amino acids (aa) in each protein domain and the total lengths of UL8 and UL7 are shown. Blue shading indicates the region shared between UL8 and UL7 (195 aa), including the IgV-like domain in dark blue. The number of HCMV strains with amino acid changes (with respect to Merlin) in each position along the UL8 sequence is plotted over the UL8 protein domain diagram.

sequences. In addition, whereas most N- and O-linked glycosylation sites predicted in the stalk region show variation between the different HCMV strains, the six potential N-linked glycosylation sites of the IgV-like domain are conserved among the strains, with only a loss of one of these sites observed in one strain (Fig. S1). Finally, the cytoplasmic tails exhibit an amino acid identity of 84.8%, with the predicted AP-2- and PDZ-binding motifs completely preserved among all strains (Fig. S1).

It is of interest that, at the nucleotide level, splice site features were highly conserved within the predicted *UL8* intron in all viral strains analyzed, including the canonical 5' donor (GT) and 3' acceptor (AG) splice sites and their boundary motifs, the

typical adenine nucleotide in the branch point, and the polypyrimidine tract adjacent to the 3' splice site (data not shown). Hence, these data indicate that *UL8* has the potential to be expressed upon infection with all these HCMV strains.

UL8 is a heavily glycosylated cell surface protein. In order to analyze the cellular expression of UL8, we cloned the full-length ORF in a eukaryotic expression vector and transfected COS-7 cells. We first explored its cellular localization by immunofluorescence microscopy using an anti-UL7/UL8 monoclonal antibody (MAb; MAb UL7.1.87) (21) that specifically recognizes the Ig-like domain shared by the UL8 and UL7 proteins. As shown in Fig. 3Aa to c, in permeabilized cells the UL8 protein was found distributed throughout the cytoplasm, producing a diffuse staining pattern and, frequently, a strong juxtanuclear signal. Although under these conditions only some weak plasma membrane staining could be distinguished, cell surface labeling of UL8 was clearly observed in nonpermeabilized cells (Fig. 3Ad to f). Moreover, the presence of the UL8 protein at the cell surface was confirmed by flow cytometry (Fig. 3B). To determine the molecular mass of the UL8 protein we constructed a plasmid, termed HA-UL8, encoding a hemagglutinin (HA)-tagged UL8 fusion protein, with the HA tag positioned immediately after the signal peptidase cleavage site. COS-7 cells transfected with HA-UL8 were analyzed by Western blotting using the anti-HA specific antibody. Two bands of apparent masses of 90 to 114 kDa and 47 to 70 kDa, higher than the theoretical molecular mass of 35.6 kDa predicted from the UL8 amino acid sequence, were detected, pointing to extensive posttranslational modification of UL8 (Fig. 3C, left, lane 2). Accordingly, an increase in the electrophoretic mobility of both forms of the protein after treatment with N-glycosidase showed that UL8 was highly N-glycosylated, consistent with the presence of 12 potential N-linked glycosylation sites in its ectodomain (Fig. 3C, left, lane 3). Immunoprecipitation of biotinylated HA-UL8 from lysates of transfected cells with the anti-HA MAb demonstrated that the larger UL8 species corresponded to the one present at the cell surface (Fig. 3C, middle). The occurrence of two distinct polypeptides after N-glycosidase F digestion may be due to differential degrees of O-glycosylation, a fact sustained by the existence of a number of potential O-glycosylation sites in the stalk region of UL8. Supporting this notion, the slower migrating 90- to 114-kDa HA-UL8 band modestly shifted to an 86- to 103-kDa band following digestion with O-glycosidase and neuraminidase (Fig. 3C, right, compare lanes 2 and 3). Moreover, combined treatment with N-glycosidase, O-glycosidase, and neuraminidase led to a further reduction, of approximately 12 kDa, of the UL8 slower migrating band detected after N-glycosidase digestion (Fig. 3C, right, compare lanes 4 and 5), demonstrating that cell surface UL8 is, in addition, O-glycosylated.

Since we have previously reported that UL7 is shed to the extracellular milieu (21), we explored whether UL8 is cleaved from the plasma membrane to a certain extent and its ectodomain secreted. Thus, the potential release of soluble UL8 was evaluated by a sandwich enzyme-linked immunosorbent assay (ELISA) using an anti-HA MAb, and the anti-UL7/UL8 MAb in tissue culture supernatants was collected at various time points from COS-7 cells transfected with HA-UL8. As illustrated in Fig. 4A, constitutive shedding of UL8 was evident after 3 h, with the soluble viral protein accumulating over time. Furthermore, stimulation with the protein kinase C agonist phorbol-12-myristate-13-acetate (PMA) resulted in a marked enhancement of protein released (Fig. 4B). When we compared the shedding of UL8 with that of UL7 under the same conditions, we clearly observed that the extent to which UL8 was released was considerably lower (Fig. 4C).

Ectodomain shedding of transmembrane proteins generally occurs by proteolytic cleavage at sites located in close proximity to the outer surface at the cell membrane. To map the region of the putative cleavage site within UL8, two plasmids encoding the HA-tagged UL8 with deletions that eliminate a stalk sequence immediately juxtaposed to the transmembrane domain (plasmid HA-UL8 Δ Q-H) or adjacent to the previous one (plasmid HA-UL8 Δ E-V) were produced (Fig. 4D). COS-7 cells were transiently transfected with wild-type (WT) HA-UL8, HA-UL8 Δ Q-H, or HA-UL8 Δ E-V, and the effects of the removed amino acid stretches were quantitatively assessed by measuring UL8

FIG 3 Localization of UL8 in transfected cells. (A) COS-7 cells were transiently transfected with the pCIneo UL8 construct expressing full-length UL8. After 24 h, the cells were fixed with 4% formaldehyde (d to f) or fixed and permeabilized with acetone (a to c) and stained with the anti-UL7/UL8 MAb followed by anti-mouse IgG-Alexa Fluor 555. The cells were examined under a microscope at 405 nm (DAPI) and at 510 to 560 nm (Alexa Fluor 555). Shown are representative cells from cultures stained for UL8 (a and d), nuclei stained with the DAPI reagent (b and e), and overlaid images (c and f). Magnification, $\times 40$. (B) Flow cytometry analysis of COS-7 cells transfected with the pDisplay UL7 (HA-UL7) or the pDisplay UL8 (HA-UL8) construct and stained with the anti-UL7/UL8 MAb (solid line) or an isotype control (dashed line) followed by anti-mouse IgG-PE. The percentage of positive cells and the mean fluorescence intensity (MFI) are indicated in the histograms. (C) Untransfected COS-7 cells (CTL) or COS-7 cells transiently transfected with the pDisplay UL8 construct (HA-UL8) were lysed (left and right) or were surface labeled with biotin, lysed, and then immunoprecipitated with an anti-HA MAb (middle). When indicated, cell lysates were treated with N-glycosidase F (N-Gly F), O-glycosidase, and neuraminidase (O-Gly + Neu) or a combination of them (N-Gly F, O-Gly + Neu). Samples were subjected to SDS-PAGE and subsequent Western blot analysis using an anti-HA MAb, followed by anti-rabbit IgG-horseradish peroxidase (HRP) (left and right) or streptavidin-peroxidase (middle). An anti- β -actin MAb followed by anti-mouse IgG-HRP was used as an internal control. Molecular masses (in kilodaltons) are indicated.

surface expression using fluorescence-activated cell sorting (FACS) analysis. As shown in Fig. 4E, a slight increase of surface staining with the anti-UL7/UL8 MAb was observed in cells transfected with HA-UL8 Δ E-V compared to that of HA-UL8-transfected cells. However, surface expression of UL8 was strongly augmented after transfection of

FIG 4 Shedding of UL8. (A) COS-7 cells were transiently transfected with the pDisplay UL8 construct (COS-7 HA-UL8) or left untransfected (CTL). Supernatants were harvested at the indicated time points and assessed by sandwich ELISA for soluble UL8 (using the biotinylated anti-HA and the anti-UL7/UL8 MAbs). Measurements (optical density [OD] 450 to 470 nm) were performed in triplicate and a representative experiment is shown. (B) COS-7 cells transiently transfected with the pDisplay UL8 construct were incubated with or without PMA (50 ng/ml) for 24 h at 37°C. Subsequently, supernatants were collected and assessed for soluble UL8 as indicated in panel A. One representative experiment is shown. (C) COS-7 cells were transiently transfected with either the pDisplay UL7 (HA-UL7) or the pDisplay UL8 (HA-UL8) construct. After 24 h, supernatants were harvested and cell lysates were prepared. Samples were analyzed by sandwich ELISA as indicated in panel A. Measurements were performed in triplicate, and the supernatant/cell lysate ratio from one representative experiment is shown. (D) Schematic representation of UL8 mutants bearing deletions in the membrane-proximal residues. Amino acid sequence of the membrane-proximal and first five residues of the transmembrane region of UL8, gray shaded, in the pDisplay UL8 (HA-UL8), pDisplay UL8 ΔE-V (HA-UL8 ΔE-V), and pDisplay UL8 ΔQ-H (HA-UL8 ΔQ-H) constructs, with the corresponding deleted residues in boldface and underlined. (E) COS-7 cells were transiently transfected with the pDisplay UL8 (HA-UL8), the pDisplay UL8 ΔE-V (HA-UL8 ΔE-V), or the pDisplay UL8 ΔQ-H (HA-UL8 ΔQ-H) construct. After 24 h, transfected cells were analyzed by flow cytometry staining with the anti-UL7/UL8 MAb or an isotype control followed by anti-mouse IgG-PE. The percentage of positive cells and the MFI value for each sample are indicated. (F) COS-7 cells were transiently transfected with the same constructs as those described for panel E. After 24 h, supernatants were harvested and cell lysates were prepared and analyzed by ELISA as indicated in panel A. Measurements were performed in triplicate, and the supernatant/cell lysate ratios from one representative experiment are shown. **, *P* < 0.01; ***, *P* < 0.001. Values in panels A, B, C, and F represent the means ± SEM.

HA-UL8 ΔQ-H. Consistent with these data, when analyzed by ELISAs, smaller amounts of soluble UL8 were detected after removal of both types of sequences, being significantly more pronounced in the case of deletion of the amino acid residues immediately juxtaposed to the transmembrane domain (Fig. 4F). Altogether, the results indicate that a fraction of UL8 is proteolytically cleaved, and that sequences in its stalk juxtaposed to the plasma membrane region play a key role in this process.

UL8 undergoes endocytosis, and the tyrosine-based motifs are involved in this process. As indicated before (Fig. 2A), the conserved cytoplasmic domain of UL8 bears two putative tyrosine-based internalization YXXΦ motifs (where X stands for any amino acid and Φ represents an amino acid with a bulky hydrophobic side chain): YDRW (aa 305 to 308) and YGQV (aa 314 to 317). These motifs are responsible for mediating

endocytosis of proteins from the plasma membrane into endosomes (32). Therefore, we decided to investigate whether cell surface UL8 undergoes endocytosis by directly examining the potential functional relevance of its tyrosine-based motifs in this process. To this end, we generated HA-UL8-Y305/314A, an HA-UL8 construct containing mutations that abrogate both motifs by replacing the critical tyrosine residues in positions 305 and 314 with alanines (Fig. 5A). 300.19 cells were then stably transfected with HA-UL8 or HA-UL8 Y305/314A and FACS sorted to isolate populations of cells that expressed comparable levels of UL8 on their surface (Fig. 5B). Sorted cells were then subjected to antibody uptake experiments. As illustrated in Fig. 5C, a time-dependent increase of both HA-UL8 and HA-UL8 Y305/314A internalization could be observed. However, HA-UL8 Y305/314A internalization was significantly impaired compared with that detected for HA-UL8. Therefore, we concluded that UL8 can undergo endocytosis and that the tyrosine-based motifs in the cytoplasmic tail of UL8 are involved in this process. Notably, the two tyrosine-based YXX Φ motifs are highly conserved between CMV strains (Fig. S1).

Once internalized, proteins containing tyrosine-based motifs can be sorted to either a recycling or a lysosomal degradative pathway. To address whether UL8 was degraded in the endosomal/lysosomal compartment, we tested the effect of leupeptin, an inhibitor of cysteine and serine proteases, on UL8 expression through analysis by confocal microscopy. Figure 5D (compare –Leu and +Leu images) shows that treatment of HA-UL8-transfected COS-7 cells with leupeptin resulted in increased cytoplasmic accumulation of UL8, leading to a more prominent vesicular staining. Consistent with these results, immunoblot analysis of HA-UL8-transfected COS-7 cells exposed for different times to both leupeptin and bafilomycin, an inhibitor of vacuolar H⁺-ATPase, revealed higher levels of UL8 compared to those of untreated cells (Fig. 5E). Taken together, these data suggest that once internalized, UL8 is proteolytically degraded in an endosomal/lysosomal compartment.

UL8 is able to selectively bind to the surface of activated neutrophils. We have previously shown that the UL7 Ig domain, common to UL8, was able to bind to dendritic cells when it was anchored as a chimeric molecule to the cell surface (21). Here, we assessed the capacity of full-length UL8 to interact with several leukocyte types. Thus, DCs, monocytes, NK cells, and T and B lymphocytes isolated from peripheral blood were tested. In addition, in these assays we also included neutrophils before or after lipopolysaccharide (LPS) stimulation. No significant binding to COS-7 cells expressing full-length UL8 was detected for monocytes, NK cells, neutrophils, and T and B lymphocytes (data not shown). Consistent with our previous data (21), specific binding of UL8 to DCs was also observed (data not shown). Notably, as illustrated in Fig. 6A, a strong binding of LPS-activated neutrophils to COS-7 expressing UL8, but not to control COS-7 cells, could be appreciated. Moreover, pretreatment of UL8-transfected COS-7 cells with the anti-UL7/UL8 MAb that recognizes the Ig-like domain of UL8 blocked these cellular interactions. This result showed the specificity of the binding of the viral UL8 protein through its N-terminal Ig-like domain, shared with UL7, to a molecule present in DCs and induced after neutrophil activation (Fig. 6A).

We reasoned that mutations in UL8 that impair its internalization or its shedding properties, thereby allowing a large proportion of the viral protein to localize at the plasma membrane, would result in an enhanced binding to activated neutrophils. To examine this issue, we transfected COS-7 cells with plasmids expressing HA-UL8 versions in which its cytoplasmic tail was completely removed (HA-UL8 Δ tail) or it lacked sequences immediately juxtaposed to the transmembrane domain (HA-UL8 Δ Q-H) and compared their ability to interact with activated neutrophils with that of HA-UL8-transfected cells. As expected and illustrated in Fig. 6B, both the resection of the UL8 tail as well as the elimination of stalk sequences, which reduced shedding of the viral protein, led to increased levels of activated neutrophil binding to the corresponding transfected cells.

FIG 5 Internalization of UL8. (A) Amino acid sequences of the UL8 cytoplasmic tail in the pDisplay UL8 (HA-UL8) and pDisplay UL8 Y305/314A (HA-UL8 Y305/314A) constructs. The tyrosine-based motifs are underlined. The residues that were mutated to alanine are marked in red in HA-UL8 Y305/314A. (B) Flow cytometry analysis of 300.19 cells stably transfected with HA-UL8 (black line histogram) or HA-UL8 Y305/314A (green line histogram), FACS sorted, and stained with the anti-UL7/UL8 MAb or an isotype control (dashed line histogram) followed by anti-mouse IgG-PE. (C) The tyrosine-based motifs are relevant for the internalization of UL8. 300.19 cells stably transfected with the HA-UL8 or the HA-UL8 Y305/314A construct were labeled with the anti-UL7/UL8 MAb at 4°C. Subsequently, cells were incubated at 4°C or 37°C for the indicated time points and stained with anti-mouse IgG-PE. The percentage of anti-UL7/UL8 MAb internalization was calculated as indicated in Materials and Methods. Each data point represents the means $\pm$ SEM from three separate samples. *, $P < 0.05$; **, $P < 0.01$. (D) COS-7 cells were transiently transfected with the pDisplay UL8 construct. After 24 h, cells were

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We then sought to establish a quantitative assay suitable to measure these cellular interactions. For this purpose, and to ensure high levels of expression of the IgV-like domain of UL8 at the cell surface, we used a chimeric construct (UL8-ch) that anchors this viral domain at the plasma membrane. LPS-activated neutrophils were labeled with calcein before being incubated with COS-7 cells transfected with UL8-ch or control COS-7 cells. After allowing cellular binding, cultures were washed to remove unbound activated neutrophils, trypsinized, and analyzed by flow cytometry. Based on the calcein labeling, the number of bound activated neutrophils was determined for each culture and normalized to the number of COS-7 cells (Fig. 6C). Using this assay, the effect of the anti-UL7/UL8 MAb on these cellular interactions could be quantitatively measured, resulting in a dose-dependent block that reached 70% inhibition at the 10- μ g/ml MAb concentration (Fig. 6D).

Identification of key amino acid residues of the Ig-like domain of UL8 that are involved in cell interactions. Previous studies have shown an involvement of specific surface-exposed charged amino acids, within the N-terminal V-like domain of SLAM receptors, in their homophilic interactions (20, 33). Thus, to identify critical amino acid residues of UL8 that contribute to binding of this viral protein to activated neutrophils, a number of point mutations in its Ig-like domain were introduced by site-directed mutagenesis. After aligning the Ig-like domains of UL8 and CD229, UL8-charged amino acids R29, R32, D34, K45, E48, R78, E82, R86, E89, and E91 (Fig. 7A) were mutated to alanine in the UL8-ch plasmid. The binding of activated neutrophils was tested in COS-7 cells transfected with parental and mutated UL8-ch plasmids. All mutant UL8-ch molecules were expressed at levels approximately equal to those of parental UL8-ch when analyzed by flow cytometry (Fig. 7B and data not shown). In addition, the absence of significant alterations in the tertiary structure of mutant UL8-ch molecules, which could affect their binding properties, was confirmed by testing their immunoreactivity with two MAbs, the anti UL7-UL8 MAb and the MAb UL7.1400, which recognize two distinct conformational epitopes within the UL7/UL8 Ig-like domain (data not shown). As can be seen in Fig. 7B, the results of the analysis revealed that mutation of R29 and E91 of the N-terminal IgV-like domain caused a dramatic decrease in binding (72% in the case of R29 and 94% in the case of E91), whereas the rest of the mutants had no significant disruptive effects on the binding of activated neutrophils. Interestingly, one mutation, K45, resulted in a significant increase in binding to activated neutrophils (Fig. 7B). To better understand the structural aspects of the IgV-like domain of UL7/UL8, as well as the location of the mutations that we introduced, we built a three-dimensional (3D) molecular model of this domain based on the known crystal structures of SLAM family members. The location of these three key residues in the molecular model is shown in Fig. 7C.

UL8 interferes with the production of IL-8 and TNF- α in THP-1 cells. As UL7 was capable of reducing the production of IL-8, IL-6, and TNF- α in myeloid cells, we subsequently investigated whether cytokine release also could be modulated by UL8. Thus, using ELISAs, we quantitatively measured IL-8 and TNF- α secretion in supernatants from THP-1 cells transfected with either HA-UL8 or the control empty plasmid 24 h after incubation with medium or with LPS (1 μ g/ml). As shown in Fig. 8A, both

FIG 5 Legend (Continued)

left untreated (–Leu) or incubated with 50 μ M leupeptin (+Leu) for an additional period of 24 h. Cells were then permeabilized with acetone and stained with the anti-UL7/UL8 MAb followed by anti-mouse IgG-Alexa Fluor 555. Nuclei were counterstained with DAPI reagent. The cells were examined under a microscope at 405 nm (DAPI) and at 510 to 560 nm (Alexa Fluor 555). Shown are the overlaid images of representative cells. Magnification, $\times 40$. Arrows indicate the UL8 accumulations detected in leupeptin-treated cells. (E) Untransfected COS-7 cells (CTL) or COS-7 cells transiently transfected with the pDisplay UL8 construct (HA-UL8) were, after 24 h, incubated (+) with 50 μ M leupeptin (Leu) and 20 nM bafilomycin A1 (B-A1) or left untreated (–). Cells were then lysed at the different time points after treatment as indicated, and collected samples were subjected to SDS-PAGE and subsequent Western blot analysis using an anti-HA MAb followed by anti-rabbit IgG-HRP. An anti- β -actin MAb followed by anti-mouse IgG-HRP was used as an internal control. Molecular masses (in kilodaltons) are marked. A representative Western blot, out of three, is shown. The bar graph on the right indicates the relative density ratios of HA-UL8/ β -actin taking into consideration the results obtained in the three different Western blots. Values represent the means $\pm$ SEM. Significant differences in relative density ratios are shown as one ($P < 0.05$) or two ($P < 0.01$) asterisks.

FIG 6 UL8 interacts with a molecule on the surface of activated neutrophils. (A) COS-7 cells were nontransfected (CTL) or transiently transfected with pCneo UL8 (UL8). After 16 h, cells were incubated for 1 h with neutrophils previously activated with LPS at 100 ng/ml for 30 min. When indicated, cells were preincubated for 30 min with 10 μ g/ml of the anti-UL7/UL8 MAb (UL8 + anti-UL7/UL8 MAb) before the addition of activated neutrophils. Cultures were examined under an inverted microscope (10 $\times$ magnification), and neutrophil attachment was assessed by counting the number of neutrophils bound per field (bar graph on the left). For each sample, a total of 5 fields were counted. One representative image (20 $\times$ magnification) of each culture is shown (three panels on the right). The results are representative of those obtained in at least three independent experiments. (B) COS-7 cells were nontransfected (CTL)

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unstimulated and LPS-stimulated HA-UL8-transfected THP-1 cells secreted IL-8 levels that were significantly lower than those of the corresponding THP-1 control cells (44.3% and 42.3% reduction, respectively). Moreover, the presence of UL8 markedly hindered the secretion of TNF- α , as judged by a comparison with the control THP-1 cells, both under unstimulated and LPS-stimulated conditions (47.1% and 68.3% reduction, respectively).

We then sought to address how the alteration of cytokine production by UL8 correlated with its structural properties. To this end, we analyzed the secretion of IL-8 and TNF- α in LPS-induced THP-1 cells expressing HA-UL8 mutated versions. First, we examined whether the modulatory effect was dependent on the tyrosine-based YXX Φ motifs embedded in the cytoplasmic domain of UL8 by analyzing cells transfected with HA-UL8 Y305/314A or HA-UL8 Δ tail. Figure 8B shows that after mutating the two Y residues (Y₃₀₅ and Y₃₁₄) or deleting completely the UL8 cytoplasmic tail, an increase in the cell surface expression of the viral protein was observed. Notably, IL-8 and TNF- α levels were further diminished in LPS-treated THP-1 cells transfected with HA-UL8 Y305/314A or HA-UL8 Δ tail compared to those seen in UL8 transfectants (Fig. 8C and D). Given that sequences within the stalk region of UL8 mediate some shedding of the viral protein when exposed at the cell surface, we assessed the effect of the sequences immediately juxtaposed to the transmembrane domain (QYQRTKTH), shown to play a more prominent role in this process, on cytokine production by transfecting THP-1 cells with the mutant HA-UL8 Δ Q-H. Concomitantly with the expected increase of the viral protein at the surface of the stimulated THP-1 cells (Fig. 8B), a significant effect of this mutant was observed, resulting in a pronounced reduction of IL-8 and TNF- α release (Fig. 8C and D). Finally, to investigate whether mutations that disrupted the binding properties of UL8 affected the cytokine profile, LPS-treated THP-1 cells were transfected with HA-UL8 E91A. As seen in Fig. 8C and D, the single mutation of this charged E91 residue within the IgV-like domain of UL8 yielded a substantial increase of the levels of both IL-8 and TNF- α released by transfected cells. Thus, taking these findings together we can conclude that UL8 exerts its immunomodulatory activities when located at the cell surface via its IgV-like domain.

Expression of the UL8 protein during HCMV infection. We next examined UL8 expression in the context of the infection. To this end, we engineered a recombinant HCMV (strain TB40/E) where UL8 was selectively tagged. This was achieved by fusing the HA epitope tag at the N terminus of UL8 (four amino acids after its signal peptidase cleavage site) and removing the UL8 intron by linking its first and second exons, thereby abrogating UL7 expression (Fig. 9A, diagram of the viral recombinant). The recombinant genome was generated in *Escherichia coli* by mutagenesis of CMV BACs employing a markerless homologous recombination-based technique (34, 35). The viral recombinant recovered after transfer of the UL8-tagged CMV BAC to permissive cells was termed HCMV HA-UL8. We then performed Western blot analysis on lysates from HEL299 cells left uninfected or infected with either HCMV WT or HCMV HA-UL8, using the MAb directed against the HA epitope. As shown in Fig. 9B (lane 3), two UL8-specific bands were detected in HCMV HA-UL8-infected cells, with apparent molecular masses

FIG 6 Legend (Continued)

or transiently transfected with pDisplay UL8 (HA-UL8), pDisplay UL8 Δ tail (HA-UL8 Δ tail), or pDisplay UL8 Δ Q-H (HA-UL8 Δ Q-H). As in panel A, after 16 h, cells were incubated with activated neutrophils, cultures were examined under the microscope, and the number of neutrophils bound per field (5 fields, $\times 10$ magnification) was determined (bar graph on the left). One representative image ($\times 20$ magnification) of each culture is shown (four panels on the right). The results are representative of those obtained in at least three independent experiments. The values in panels A and B represent the means $\pm$ SEM of activated neutrophils attached per field. Significance in number of bound neutrophils is shown as one ($P < 0.05$) or three ($P < 0.001$) asterisks. (C) COS-7 cells, nontransfected (COS-7 CTL) or transiently transfected with the UL8-ch construct (COS-7 UL8-ch) for 24 h, were incubated for 1 h with neutrophils previously activated with LPS (100 ng/ml for 30 min) and stained with calcein. Cells then were detached with trypsin and analyzed by flow cytometry. Neutrophils and COS-7 cells were gated based on forward and side scatter (upper) and analyzed for calcein expression (bottom). The numbers of COS-7 cells (COS CTL or COS UL8-ch) and calcein-stained neutrophils are indicated in brackets. One representative experiment is shown. (D) As described for panel C, except that COS-7 cells (CTL or UL8-ch) were preincubated for 30 min with the indicated concentration of anti-UL7/UL8 MAb or the control anti-CD66 MAb before the addition of the calcein-labeled activated neutrophils. The percentage of binding of activated neutrophils is shown. One representative experiment is shown.

FIG 7 Identification of the key residues involved in the interaction between the IgV-like domain of UL7 and UL8 and activated neutrophils. (A) Alignment between the predicted IgV-like domain of UL7 and UL8 and the N-terminal Ig domain of human CD229. Identical and conservative substitutions (+) between the two sequences are indicated. The residues that were mutated to alanine are underlined, and the position in the IgV-like domain is indicated. The position of the β -sheets in the IgV-like domain predicted for CD229 is shown above the alignment. (B) Site-directed mutagenesis dissection of the interaction between the IgV-like domain of UL7 and UL8 and activated neutrophils. COS-7 cells were transiently transfected with parental (–) and mutant UL8-ch constructs (UL8-ch R29, UL8-ch R32, UL8-ch D34, UL8-ch K45, UL8-ch E48, UL8-ch R78, UL8-ch E82, UL8-ch R86, UL8-ch E89, and UL8-ch E91). After 24 h, transfected cells were incubated for 1 h with previously activated (LPS 100 ng/ml for 30 min) and calcein-stained neutrophils. Subsequently, cells were detached with trypsin and analyzed by flow cytometry, and the percentage of binding of activated neutrophils to each mutant compared to that of the parental UL8-ch was calculated. Surface expression of parental and mutant UL8-ch was also measured by staining transfected COS-7 cells with anti-UL7/UL8 MAb or an isotype control followed by anti-mouse IgG-PE. Only mutations that caused a substantial alteration in the interaction between the IgV-like domain of UL7 and UL8 and activated neutrophils are included in the table. The relative surface expression of each of these mutants and the percentage of activated neutrophils binding to them compared to parental UL8-ch are indicated. One representative experiment is shown. (C) Molecular model of the UL7/UL8 IgV-like domain. Ribbon diagram of the UL7/UL8 N-terminal IgV-like domain with residues that modify the interaction between this viral domain and activated neutrophils marked and highlighted in red on the model. β -sheets A', B, C, C', D, E, F, and G are shown in yellow.

of approximately 70 kDa and 110 to 127 kDa. We found that these two bands coincide with the ones detected in lysates of HEL299 cells transfected with HA-UL8 (Fig. 9B, lane 5), corroborating the specificity of the findings. The expression of IE1 was assessed as a control of infectivity in these assays, allowing us to confirm that infection with parental and recombinant viruses was comparable. To ascertain whether UL8 was also glycosylated in HCMV-infected cells, as found in transfected cells, samples were subjected to N-glycosidase F treatment. Accordingly, after treatment with N-glycosidase F, the two bands corresponding to UL8 shifted (from infected and from transfected cells) (Fig. 9D, left, lanes 3 and 4, and right, lanes 2 and 3). As was found when the viral protein was ectopically expressed in COS-7 cells, two distinctive bands were observed, of approximately 43 kDa and 78 to 95 kDa in this case, likely due to their differential

FIG 8 Effects of UL8 on IL-8 and TNF- α production in LPS-treated THP-1 cells. (A) THP-1 cells were transiently transfected with the pDisplay (CTL) or pDisplay UL8 (HA-UL8) construct. After 16 h, live cells were sorted and either left untreated (unstimulated) or stimulated with LPS (1 μ g/ml) for 24 h. Culture supernatants were assessed by ELISA for IL-8 and TNF- α . (B) Flow cytometry analysis of THP-1 cells transfected with pDisplay (CTL), pDisplay UL8 (HA-UL8), pDisplay UL8 Y305/314A (HA-UL8 Y305/314A), pDisplay UL8 Δ tail (HA-UL8 Δ tail), pDisplay UL8 Δ Q-H (HA-UL8 Δ Q-H), or pDisplay UL8 E91A (HA-UL8 E91A). After 16 h, live cells were sorted and stained with anti-HA mAb (shaded histogram) or isotype control (open histogram) followed by anti-mouse IgG-PE. The mean fluorescence intensity (MFI) value is indicated in each histogram. (C and D) THP-1 cells were transfected and sorted as indicated in panel B and incubated for 24 h with LPS (1 μ g/ml). Culture supernatants were assessed by ELISA for IL-8 (C) and TNF- α (D), as indicated. Measurements were performed in triplicate. Values in panels A, C, and D represent the means $\pm$ SEM. Significant differences in cytokine levels are shown as one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) asterisks.

degrees of O-glycosylation and additional posttranslational modifications. These results confirmed that in the context of the infection UL8 is expressed as two heavily glycosylated forms.

Further corroboration of UL8 expression in the context of the infection was obtained by using an HCMV recombinant, termed HCMV HA-UL7/UL8, in which both UL7 and UL8 were HA tagged at the N terminus (with the HA tag inserted four amino acids after their signal peptidase cleavage site) (Fig. 9A). Immunoblot analysis of the HA epitope of whole-cell

FIG 9 Expression of UL8 in the context of the HCMV infection. (A) The scheme depicts the HCMV WT (TB40/E strain) and the HA-tagged HCMV mutants used in this study. Below the genome (black line) the UL6, UL7, UL8, and UL9 transcripts are represented (gray arrows). The black square indicates the HA tag. (B) UL8 expression during HCMV infection. HEL299 cells were mock infected (MOCK) or infected with HCMV WT, HCMV HA-UL8, or HCMV HA-UL7/UL8 at an MOI of 2, and at 72 hpi total protein cell extracts were prepared. In addition, cell extracts were obtained from HEL299 cells left untransfected (CTL) or transfected with HA-UL8 (HEL HA-UL8) or HA-UL7 (HEL HA-UL7). Samples were subjected to SDS-PAGE (40 μ g on lanes 1, 2, 3, and 6, or 1 μ g on lanes 4, 5, and 7) and subsequent Western blot analysis using anti-HA, anti- β -actin, or anti-IE1 MAbs. The expression levels of β -actin and IE1 were assessed as controls for loading and infection, respectively. (C) UL7 expression during HCMV infection. HEL299 cells were mock infected (MOCK) or infected with HCMV WT or HCMV Δ UL8 (also named HCMV HA-UL7; Fig. 10A) at an MOI of 2, and at 72 hpi cell culture supernatants were collected, concentrated, and subjected to SDS-PAGE and subsequent Western blot analysis using the anti-HA MAb. (D) N-glycosidase F treatment of HA-UL8. Total protein cell extracts were prepared from HEL299 cells either mock infected (MOCK) or infected with HCMV HA-UL8 for 72 h at an MOI of 2 (left), or HEL299 cells were nontransfected (CTL) or transfected with HA-UL8 (right). When indicated, cell lysates were treated with N-glycosidase F (N-Gly F). Samples were then subjected to SDS-PAGE and subsequent Western blot analysis using anti-HA MAb. The anti-IE1 and anti- β -actin MAbs were used as controls. An overexposure of part of the Western blot indicated in the figure is shown below. (E) Expression kinetic class of UL8. HEL299 cells were mock infected (MOCK) or infected with HCMV HA-UL8 at an MOI of 2 in the absence (lane —) or presence of CHX and actinomycin D (lane CHX) or PPA (lane PPA). Cell lysates were prepared at 72 hpi, except for the CHX-treated samples that were collected at 16 hpi, and were subjected to SDS-PAGE and subsequent Western blot analysis using anti-HA MAb. The anti- β -actin, anti-IE1, and anti-pp28 MAbs were used as controls. High- and low-molecular-mass UL8 bands are indicated with the pound sign and asterisks, respectively. Molecular masses are marked in kilodaltons.

protein extracts prepared from HEL299 cells infected with HCMV HA-UL7/UL8 yielded the expected 70-kDa and 110- to 127-kDa bands (Fig. 9B, lane 6). In these assays, however, we were unable to detect additional bands that could correspond to the UL7 protein, which migrates with apparent molecular masses of about 78 to 90 kDa and 58 to 67 kDa when

FIG 10 UL8 reduces IL-8 production during HCMV infection of THP-1 cells. (A) The scheme depicts the HCMV WT or HCMV-GFP WT (TB40/E strain) and the HCMV Δ UL8 (also named HCMV HA-UL7) and HCMV-GFP Δ UL7/UL8 deletion mutants. Below the genome (black line) the UL6, UL7, UL8, and UL9 transcripts are represented (gray arrows). The deleted regions are shown in white. (B) HCMV-GFP Δ UL7/UL8 displays viral replication kinetics similar to those of HCMV-GFP WT in THP-1 cells. THP-1 cells were infected at an MOI of 1 with HCMV-GFP WT or HCMV-GFP Δ UL7/UL8. At the indicated days after infection, the amount of cell-associated infectious virus present in the cultures was determined. Each data point represents the means $\pm$ SEM from three separate cultures. The limit of detection is indicated by the dotted line. (C) Kinetics of IL-8 secretion in THP-1 cells infected with the HCMV Δ UL8 deletion mutant. THP-1 cells were stimulated with PMA (50 ng/ml) for 24 h and then were mock infected (MOCK) or infected with HCMV WT or HCMV Δ UL8 at an MOI of 2. Twenty-four hours later, cells were washed and fresh culture medium containing PMA (50 ng/ml) was added. Culture supernatants were collected at different time points after infection as indicated and assessed by ELISA for IL-8. (D) As described for panel C, but cells were mock infected (MOCK) or infected with HCMV-GFP WT or HCMV-GFP Δ UL7/UL8. Measurements were performed in triplicate. Significant differences in cytokine levels between HCMV WT and HCMV Δ UL8 or HCMV-GFP WT and HCMV-GFP Δ UL7/UL8 are shown as three asterisks ($P < 0.001$). Values in panels C and D represent the means $\pm$ SEM.

expressed in isolation in HEL299 cells (Fig. 9B, lane 7). The lack of detection of the UL7 protein when using the HCMV recombinant with the HA tag at the N terminus of both UL7 and UL8 (Fig. 9B, lane 7) most likely reflects the presence of limited cellular amounts of the UL7 protein in infected cells as a result of most of it being shed to the extracellular milieu during productive HCMV infection. Consistent with this notion, a band of 68 to 87 kDa corresponding to soluble UL7 could be detected by Western blotting in the supernatants of HEL299 cells infected with an HCMV recombinant lacking the UL8 gene and with the UL7 gene HA tagged at the N terminus (named HCMV Δ UL8 or HCMV HA-UL7; see next section) (Fig. 10A) using the anti-HA MAb (Fig. 9C, lane 3).

We next explored the temporal kinetic class of the *UL8* gene during the HCMV replicative cycle. For this purpose, we examined the expression of UL8 after infection of

HEL299 cells treated with either the translation inhibitor cycloheximide (CHX), which selectively restricts viral gene expression to immediate-early genes, or the viral DNA replication inhibitor phosphonoacetic acid (PPA), which precludes late viral gene expression. As shown in Fig. 9E, UL8 was not detected after release from the CHX block in the presence of actinomycin D, whereas under these conditions the major immediate-early HCMV protein IE1 was, as expected, robustly expressed. Similarly, UL8 was not recovered after PPA treatment, being found only in the absence of inhibitors, in consonance with the pattern of expression presented by the true late gene pp28. These data indicate that the *UL8* gene is expressed with late kinetics during productive infection of human fibroblasts.

Deletion of *UL8* from the HCMV genome results in an exacerbated production of proinflammatory cytokines during infection of THP-1 cells. To ascertain the impact of UL8 on proinflammatory cytokine modulation during HCMV infection of myeloid cells, we employed a recombinant HCMV lacking this gene (named HCMV Δ UL8) (Fig. 10A). In this recombinant virus, sequences downstream of the *UL7* ORF, containing part of the intron and the second exon of *UL8*, were removed from the TB40/E strain. Note that in this recombinant virus the *UL7* ORF is intact. Therefore, in addition, due to the involvement of the N-terminal Ig-like domain of UL8 shared with UL7 in the cytokine modulatory activity of UL8, we also constructed a recombinant HCMV null for expression of both the *UL7* and *UL8* genes (HCMV-GFP Δ UL7/UL8) (Fig. 10A). While these ORFs have been shown not to impact fitness of the virus in fully permissive human fibroblasts or human microvascular endothelial (HMVEC) or epithelial (RPE) cell lines, some members of the RL11 family have been reported to be involved in tissue tropism (9, 17). Therefore, we first examined growth kinetics of these two recombinant viruses in the myelomonocytic cell line THP-1. THP-1 cells were treated with PMA for 24 h and infected with HCMV Δ UL8 or HCMV-GFP Δ UL7/UL8 and their corresponding parental wild-type HCMV at a multiplicity of infection (MOI) of 1. At different days after infection, cell extracts were harvested and titrated. Growth curves showed that deletion of either *UL8* or *UL7* and *UL8* ORFs does not adversely affect viral replication kinetics (Fig. 10B and data not shown).

THP-1 cells, treated with PMA, were then mock infected or infected at an MOI of 2 with parental HCMV or mutant viruses, and the production of IL-8 was measured at different time points. As seen in Fig. 10C and D, higher levels of IL-8 were released from THP-1 cells infected with HCMV Δ UL8 or HCMV-GFP Δ UL7/UL8 at days 3 and 4 postinfection compared with those infected with HCMV WT or HCMV-GFP WT. These results were consistent with the late kinetics of *UL8*. Of note, it is worth mentioning that similar results were also obtained in U373 cells, where increased IL-8 production was observed after infection with HCMV mutants lacking *UL8* or both *UL8* and *UL7* compared to infections with the parental virus (data not shown). Importantly, these results indicate that *UL8* is capable of exerting its expected immunomodulatory function during HCMV infection of myeloid cells.

To further assess whether the effects of UL8 during HCMV infection could be extended to a number of other cytokines, we performed Luminex assays that enable simultaneous quantification of a range of analytes from tissue culture supernatants. To this end, supernatants from PMA-treated THP-1 cells either mock infected or infected with the parental or UL8-defective HCMV and collected at day 4 after infection were analyzed using the cytokine human 30-plex panel (Life Technologies) that permits measuring 22 cytokines, including 7 chemokines, 2 cytokine receptors, and 6 growth factors. As illustrated in Fig. 11, compared with mock-infected cells, HCMV infection caused a markedly reduced secretion of nearly all cytokines, cytokine receptors, and growth factors examined, except for IL-6, monocyte chemoattractant protein 1 (MCP-1), and IL-8. Notably, significantly increased levels of several cytokines, i.e., alpha interferon (IFN- α), IFN- γ , MIG, MCP-1, MIP-1 β , IL-1 β , IL-6, IL-12, RANTES, and IL-8, the IL-2 receptor, and the growth factors granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and hepatocyte growth factor (HGF) were detected in cells infected with HCMV Δ UL8 compared to those of parental

FIG 11 UL8 alters the cytokine profile during HCMV infection of THP-1 cells. THP-1 cells, previously stimulated with PMA (50 ng/ml) for 24 h, were mock infected (MOCK) or infected with HCMV WT or with HCMV Δ UL8 at an MOI of 2. Twenty-four hours later, cells were washed and fresh culture medium containing PMA (50 ng/ml) was added. Culture supernatants were collected at 96 hpi and assessed by multiplex Luminex assay. Measurements were performed in triplicate. Values represent the means $\pm$ SEM. Significance in cytokine levels between HCMV WT and HCMV Δ UL8 are shown as one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) asterisks. IL-2, IL-5, IL-10, IL-17, EOTAXIN, and IP-10 values were below the detection limit.

HCMV-infected cells (Fig. 11). It must also be appreciated that the levels of other cytokines tended to be higher in the absence of UL8; however, they did not achieve statistical significance. Furthermore, these results largely coincide with those obtained after analyzing supernatants from cells infected with HCMV-GFP Δ UL7/UL8 (Fig. 12), thus reinforcing our observations and demonstrating the ability of UL8 to attenuate proinflammatory cytokine production following HCMV infection.

Finally, to determine if UL8, when ectopically expressed, was also capable of altering the cytokine profile beyond IL-8 and TNF- α , we carried out Luminex assays on super-

FIG 12 Cytokine profile during infection of THP-1 cells with an HCMV mutant lacking UL7 and UL8. The depictions are similar to those in Fig. 11, except that stimulated THP-1 cells were mock infected (MOCK) or infected with HCMV-GFP WT or with HCMV-GFP ΔUL7/UL8. Significance in cytokine levels between HCMV-GFP WT and HCMV-GFP ΔUL7/UL8 are shown as one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) asterisks. IL-2, IL-5, IL-10, IL-17, EOTAXIN, and IP-10 values were below the detection limit.

natants from THP-1 cells transfected with HA-UL8 or the control empty plasmid for 16 h and subsequently treated with LPS. As shown in Fig. 13, THP-1 cells released significantly less IL-6, TNF- α , IFN- α , IFN- γ , IL-2R, MIP-1 α , MIP-1 β , RANTES, IL-8, MCP-1, and vascular endothelial growth factor (VEGF) after transfection with HA-UL8, thus mirroring to a large extent the results obtained from infected cells. Of interest, the fact that the altered secretion of TNF- α , MIP-1 α , and VEGF observed in the transfection assays was not evidenced in the infection context suggests that additional viral proteins besides UL8 contribute to block their production during HCMV infection.

FIG 13 Ectopically expressed UL8 decreases cytokine production in THP-1 cells. THP-1 cells were transiently transfected with the pDisplay HA (CTL) or HA-UL8 constructs. After 16 h, live cells were sorted and stimulated with LPS (1 μ g/ml) for 24 h. Culture supernatants were assessed by multiplex Luminex assay. Measurements were performed in triplicate. Values represent the means $\pm$ SEM. Significance in cytokine levels are shown as one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) asterisks. IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-13, IL-15, IL-17, IL-18, EOTAXIN, MIG, G-CSF, GM-CSF, EGF, and FGF values were below the detection limit.

DISCUSSION

Much progress has been made over recent years in the understanding of the lytic and latency-associated HCMV transcriptomes. However, the precise number and nature of the HCMV gene products expressed during infection of a given cell type remain to be determined. Moreover, taking into consideration the immense coding capacity now attributed to HCMV, a large number of potential novel protein-coding genes require a detailed investigation and a functional assignment (2, 36). In the present study, we have characterized the HCMV RL11 family member UL8 and demonstrated an immunomodulatory role of this late viral protein.

To maximize its information content, HCMV utilizes, among other strategies, overlapping genes that give rise to different proteins as the result of splicing events. Indeed, this mechanism has been recently shown to be more frequent than previously recognized (24, 25). Here, by performing RT-PCR and RACE assays from HCMV strain TB40/E-infected fibroblasts, we demonstrate the expression of the novel *UL8* spliced gene. This is the only case of the RL11 family of HCMV originated from a splicing event described so far. Furthermore, based on the conservation of the splicing site sequences of *UL8* in different HCMV strains, we postulate that the viral protein should be expressed by all of them. We showed that the *UL8* gene consists of two exons, with the 5' part of the first exon shared with the HCMV RL11 member *UL7*, and gives rise to a type I transmembrane protein with the Ig domain in common with *UL7* but with a longer stalk and a distinctive cytoplasmic tail.

In contrast to *UL7*, which we found to be shed when ectopically expressed (21), *UL8* was predominantly localized at the cell surface. We also observed, however, that the ectodomain of *UL8* can be released to a certain extent from the plasma membrane to the extracellular milieu, with the proteolytic cleavage mainly depending on the region immediately juxtaposed to the plasma membrane. It is interesting that to date only a small number of HCMV-encoded proteins have been identified in the secretome of infected cells (37) and thereby have the potential to function in an autocrine/paracrine

manner. To our knowledge, besides some HCMV-encoded homologs of cytokines, chemokines, and their receptors, just two additional viral gene products, UL7 and UL10, both belonging to the RL11 family, have been described to be released to the extracellular space (21, 23). Thus, shedding of selective RL11 members probably constitutes a viral mechanism HCMV uses to tactfully interfere with specific components of the immune system of the local environment.

Since UL8 contains in its cytoplasmic tail two highly conserved tyrosine-based YXXØ endocytosis motifs, we explored the possibility that part of the UL8 protein could be internalized from the plasma membrane into endosomes. Through antibody uptake experiments and the use of a UL8 mutant of the two tyrosine-based YXXØ motifs, we showed that these motifs play a relevant role in the endocytosis of the viral protein. Consistent with this observation, the removal of the complete cytoplasmic tail resulted in enhanced UL8 cell surface retention. After endocytic uptake, vesicles mature and cargo ultimately can be delivered to lysosomes for its degradation or to the *trans*-Golgi network for utilization in the secretory pathway. Treatment with leupeptin, a serine and cysteine protease inhibitor, and bafilomycin A1, an inhibitor of vacuolar type H⁺-ATPases that prevents acidification of endosomes and lysosomes, resulted in significantly increased UL8 cellular levels. Moreover, after leupeptin exposure, UL8 was detected by immunofluorescence microscopy at substantially larger amounts in internal vesicles. Hence, these findings suggest that UL8 is partially targeted for lysosomal degradation. It is known that viruses, including CMV, have evolved proteins that bind immunologically relevant cargo molecules at the cell surface for their downmodulation by internalization (38, 39). By means of the YXXØ motifs in their cytoplasmic tails, these viral molecules connect the bound cell surface protein to the adapter protein 2 (AP-2), directing it to the clathrin-dependent endocytic pathway. We speculate that in a similar way, the UL8 protein at the plasma membrane, through recruitment of AP-2 by the YXXØ motifs, targets selective cellular molecules to direct them to the lysosome for degradation. While this aspect would require further investigation, in support of this hypothesis, in pulldown assays from myelomonocytic U937 cell extracts using the cytoplasmic tail of UL8 as bait, AP-2 and lysosomal proteins, such as AP-3 and LAMP-1, were identified among others as potential UL8 association partners (data not shown).

Our results indicate that UL8 gets abundantly glycosylated. Two products of different sizes are synthesized from the *UL8* ORF, with the higher molecular mass species being the one reaching the cell surface. Both forms of the protein are extensively N-glycosylated, consistent with the presence of 12 predicted N-glycosylation sites scattered along its Ig-like domain and stalk region. In addition, O-glycosylations, and possibly other posttranslational modifications, take place in the larger UL8 form that reaches the cell surface. In this sense, in a recent O-glycoproteomic analysis of HCMV-infected cells, UL8 was identified among the 28 HCMV proteins carrying O-glycans (40). It is possible that the high level of N-glycosylation contributes to modulate its selective binding properties and to hinder unwanted interactions. Likewise, O-glycans attached to the UL8 stalk residues might be critical to ensure a proper extended conformation of the mature UL8 at the cell surface that could facilitate the establishment of cell-cell interactions or to mask particular regions from unspecific proteolytic cleavage (41, 42). O-linked glycans may also participate in the transport and stable cell surface expression of the viral protein.

A prominent feature of UL7 and UL8 is their Ig-like domain, which we have previously reported to display a significant homology with the membrane-distal Ig domain of the SLAM family member CD229, sharing with it characteristic key structural residues. Ig domains are one of the most common and widely disperse structural units of human proteins and are involved in many sorts of different interactions (43). Mostly found in membrane proteins, they usually participate in cell-cell recognition activities, playing essential roles in immune responses, cell adhesion, and many other processes. Ig domains are also present in virally encoded proteins, although their prevalence is much lower (44, 45). In HCMV, Ig domain-containing proteins have been involved in a wide range of interactions with uninfected immune cells, including with T lymphocytes

and NK cells. This is the case, for example, with the RL11 family proteins UL10 and UL11, which are capable of altering T cell functions (23, 46). In our earlier study analyzing UL7, we showed that the UL7/UL8 N-terminal Ig domain was capable of binding to dendritic cells. Here, we have further expanded the adhesion properties of this viral Ig domain, demonstrating that the UL8 protein interacts with activated neutrophils. Of note, the viral protein was not able to bind to unstimulated neutrophils or to other immune cell populations, such as T, B, or NK cells. Blockade of the activated neutrophil adhesion with a MAb directed against the UL7/UL8 Ig-like domain demonstrated the specificity of this interaction. Further, through site-directed mutagenesis of specific charged amino acids of the UL7/UL8 Ig domain, we delineated the amino acid residues essential for ligand interaction. We identified R29 and E91 as key residues involved in ligand binding. Interestingly, mutagenesis of residue K45 caused a significant increase of the interaction. To gain insights into the structure of the IgV-like domain of UL7/UL8, we built the 3D structure of this region by molecularly modeling it on the basis of the known crystal structures of SLAM family members. The model revealed that the two amino acids shown to be relevant for optimal interaction of UL8, R29, and E91 were at exposed sites in the edge of the predicted C and G strands, respectively, and largely coincide with residues E29 and R89, reported to be relevant in CD229 homophilic interactions (Fig. 7C) (20). Also, the model predicted an exposed position of residue K45 within the C'-C'' loop. Of note, mutation of the residue placed at the same site in CD229 (R44) also resulted in enhanced binding of CD229 to itself (20).

It is intriguing that HCMV would express two proteins, UL7 and UL8, with a common N-terminal Ig domain that would dictate identical binding specificities. However, while UL7 tends to be actively secreted and barely detectable at the cell surface, UL8 remains mostly cell associated. Thus, a plausible explanation is that depending on their final location, these two viral proteins may have evolved different means to target their ligand. UL7 may be better suited to function as a decoy in the extracellular environment. On the other hand, UL8 could act predominantly exposed at the cell surface of the infected cell, serving to interact with neighboring cells and/or contributing to drive a particular molecule to degradation. While the original hypothesis we postulated was that UL7 would bind to CD229, we showed that this viral Ig-like domain did not interact with this SLAM receptor (21). Moreover, no binding of UL7 to CEACAM family members or additional CEACAM ligands was reported in the study by MacManiman and coworkers (22). Our attempts to determine the interaction partner(s) of UL7/UL8 using a phage display technique led to the identification of matrix metalloproteinase 9 (MMP-9) as a potential cellular molecule binding to the Ig-like domain of these viral proteins (data not shown). However, we have been unable, so far, to demonstrate a direct interaction between these molecules. Thus, the ligand(s) interacting with the Ig-like domain of UL7/UL8 has yet to be uncovered.

In general, the detection of HCMV RL11 family members during the course of the infection has proven challenging due to the extremely low levels at which they are produced, and this is the reason why the majority of investigations of these proteins have focused on their expression in isolation in transfection assays. In this study, to analyze UL8 in the context of the infected cell we constructed two recombinant HCMVs containing UL8 with an HA epitope tag at the N terminus, one of them tagging uniquely UL8 and the other one tagging simultaneously both UL8 and UL7. Importantly, we found that the *UL8* ORF gives rise to two broadly glycosylated proteins during the late phase of the viral replication of HEL299 cells. These two forms of the protein fully coincide with those observed when the viral protein was individually expressed, and therefore we can infer that during HCMV infection the larger UL8 form is present at the cell surface.

The biological significance of UL8 was approached by expressing it in isolation as well as in the context of HCMV infection. In both situations, we show that UL8 lessens the production of a variety of proinflammatory cytokines. When measuring a panel of cytokines, chemokines, and some growth factors in the culture supernatant of LPS-treated THP-1 cells expressing UL8, we observed that this viral protein was able to

significantly diminish the release of TNF- α , IL-6, IL-8, IFN- α , IFN- γ , MIP-1 α , MIP1- β , RANTES, MCP-1, and VEGF. Moreover, our studies demonstrate that alterations of structural elements of UL8, such as the tyrosine-based YXX Φ endocytosis motifs or the stalk sequences implicated in the shedding, which resulted in its increased cell surface expression, provoked a larger reduction of the two cytokines analyzed, IL-8 and TNF- α . Thus, the data pointed out that this viral protein exerts its effects on cytokine production primarily when located at the plasma membrane. In addition, we determined that these UL8 activities were dependent on the Ig-like domain of UL7/UL8, since mutation of the conserved E91 residue, key for cell interactions, largely abrogated its inhibitory effects.

Different studies have shown that, at the very early stages of the life cycle, the simple cellular contact of the HCMV virion triggers signaling events that result in a profound release of proinflammatory cytokines and IFN-stimulated genes characteristic of innate immune detection (47–49). This may facilitate the recruitment of neutrophils and monocytes to the site of infection that will disseminate the virus throughout the infected host. In contrast, late in the infection, HCMV suppresses the production of proinflammatory cytokines that can induce the attraction of adaptive immune cells, which in turn would kill the infected cells and control HCMV infection. However, there is limited information regarding the gene products and mechanisms for this late reduction in proinflammatory cytokine production. Very recently, HCMV microRNAs miR-US55-1 and miR-UL112-3p have been reported to diminish, late in the lytic infection, the production of IL-6, RANTES, and TNF- α by negatively regulating the NF- κ B response through direct targeting of the IKK complex kinases IKK- α and IKK- β (50). To ascertain whether UL8 was also able to alter the proinflammatory milieu in the infection context, we used two mutant HCMVs, one that was unable to express UL8 and another that was unable to express both UL7 and UL8. Taking IL-8 as an example, we analyzed the production of this particular cytokine in time course experiments after infection of differentiated THP-1 cells with both recombinant viruses. The results demonstrated that starting at day 3 postinfection, the levels of this particular cytokine were significantly enhanced compared to those released after wild-type HCMV infections, concordant with the late kinetics of *UL8*. Furthermore, when we expanded the scrutiny at these late times postinfection to the larger panel of cytokines and growth factors, it could be clearly observed that both the single deletion of *UL8* as well as the combined deletion of *UL7* and *UL8* from the HCMV genome resulted in an elevated production of a variety of these molecules, mostly numerous proinflammatory cytokines, IL-2R, and some growth factors. Thus, these observations mirrored to a large extent the findings obtained with UL8 ectopically expressed. We must note, however, the inability of the HCMV mutants to increase the production of two cytokines, TNF- α and MIP-1 α , that were reduced by UL8 when expressed individually or to completely restore the levels of a number of the UL8-altered cytokines to the levels released from mock-infected cells. These observations are consistent with the concerted action of UL8, with additional viral products already reported to participate in the regulation of some of the specific proinflammatory cellular factors examined. Even though, setting aside these specific considerations, our results overtly illustrate that in the infectious context, UL8 plays a prominent role in suppressing most of the proinflammatory cytokines and growth factors under study. Thus, altogether our data clearly support that UL8 is necessary and sufficient to dampen proinflammatory cytokine production during HCMV infections of differentiated myeloid cells. The exact mechanism by which UL8 exerts these activities remains to be defined, although its broad effects on numerous cytokines, chemokines, and growth factors suggest that they are mediated via NF- κ B. In addition, previous work has indicated that an unidentified HCMV protein with late kinetics is involved in limiting NF- κ B signaling during infection (47, 50).

Supporting the biological relevance of UL8 during HCMV infection and in contrast to the impressive variability displayed by other genes of the RL11 region among HCMV strains, we did not find ORF-disrupting mutations inside this viral protein in any of the 214 HCMV strains that we examined (51). Moreover, the functional IgV-like domain and

its predicted N-linked glycosylation sites are nearly conserved, and in the cytoplasmic tail, the two tyrosine-based endocytic motifs appeared totally preserved. Altogether these observations suggest that the structure and interaction capabilities of UL8 are maintained among HCMV strains. Importantly, Ross and coworkers recently have presented evidence indicating that polymorphisms in the *UL8* coding region are associated with CMV-related hearing loss in congenitally infected infants (52). These observations illustrate the importance of UL8 in HCMV pathogenesis *in vivo* and are compatible with a key role of this viral protein contributing to limiting the overall proinflammatory cytokine milieu during infection.

MATERIALS AND METHODS

Cell culture and viral infections. The cell lines HEL299 (human embryonic lung fibroblast), MRC-5 (fetal human lung fibroblast), HFF (human foreskin fibroblast), COS-7 (green monkey fibroblast), 300.19 (mouse pre-B), THP-1 (human monocytic), and RPE-1 (human retinal pigment epithelial) were obtained from the American Type Culture Collection. HEL299, MRC-5, HFF, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 U of penicillin per ml, 50 μ g of streptomycin per ml, and 10% fetal bovine serum (FBS). THP-1 and 300.19 cells were cultured in RPMI 1640 medium, supplemented as indicated above, and 0.05 mM 2-mercaptoethanol. RPE-1 cells were grown in DMEM-Ham's F-12 medium supplemented with 2 mM glutamine, 50 U of penicillin per ml, 50 μ g of streptomycin per ml, 0.25% sodium bicarbonate, and 5% FBS. Neutrophils were isolated by Ficoll density gradient centrifugation from fresh blood samples obtained from healthy human donors and purified by dextran sedimentation followed by hypotonic lysis of erythrocytes. Neutrophils were resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 U of penicillin per ml, 50 μ g of streptomycin per ml, and 10% FBS. When indicated, neutrophils were activated with 100 ng/ml of LPS (Sigma-Aldrich) for 30 min. Monocytes, dendritic cells, and T and B cells were obtained as described previously (21). Human cells and tissues were obtained in accordance with the protocols approved by the Ethics Committee of the Hospital Clinic of Barcelona (Barcelona, Spain) and according to the principles of the Declaration of Helsinki. The HCMV strains AD169 and TB40/E were used in the study. Viral stocks were prepared by infecting HEL299 or MRC-5 cells at a low multiplicity of infection using DMEM supplemented as stated above, except that 3% FBS was used. Infected cell supernatants were recovered when maximum cytopathic effect was reached and were then cleared of cellular debris by centrifugation at $1,750 \times g$ for 10 min. Viral titers were determined by standard plaque assay. For assays measuring cytokine levels, viral preparations were layered and pelleted through a sorbitol cushion (20% sorbitol and 100 μ g/ml bacitracin in Tris-buffered saline [TBS]) by ultracentrifugation at $34,500 \times g$ for 90 min. When performing RT-PCR assays and cytokine quantifications, infections included a centrifugal enhancement-of-infectivity step (53). For THP-1 infections, THP-1 cells were activated for 24 h with 50 ng/ml PMA (Sigma-Aldrich).

Generation of HCMV recombinants. The HCMV mutants used in this study are based on the BAC-cloned HCMV genome of the TB40/E strain (54) with or without GFP, here termed HCMV-GFP WT or HCMV WT, respectively. HCMV-GFP Δ UL7/UL8 was generated by homologous recombination between a linear DNA PCR fragment and TB40/E-derived BAC4 in *Escherichia coli* as previously described (55). A PCR fragment containing the KanR cassette flanked by minimal FLP recombination target (FRT) sites was amplified from the pOri6k-F5 plasmid with the following oligonucleotides: UL7KO6KFor (5'-TGTTTGAG CATGACTGTTTCCAAACCGTAACGTGGTAAATAAATCGAAAAGTGCCACCTGCAGAT-3') and UL7KO6KFor (5'-AGTCATTGTTGGCACAACCTTCTCCTGATAAAAAGCACATCACAGGAACACTTAACGGCTGA-3'). HCMV-GFP Δ UL7/UL8 was reconstituted by transfecting MRC-5 fibroblasts with BAC DNA isolated from *Escherichia coli* cultures together with a plasmid expressing HCMV pp71. HCMV HA-UL7/UL8, HCMV HA-UL8, and HCMV Δ UL8 were generated using homologous recombination as described previously (34, 35). For HCMV HA-UL7/UL8, the KanR cassette was amplified from the pOri6K-RIT vector with the following primers: AA_HAF (5'-GTTGATTTAGCTTTGTTGCCCCGTGATTCTGGAATCCTACCCTTATGACGT GCCAGACTATGCCGACGCATCGTGGCCGGATCTC-3') and AA_HAR (5'-GGGTAACTTCCCTCTGTGGCC GGACACGTAGATAACGGCATAGTCTGGCACGTCATAAGGGTAGGATTCCAGAATCGTGACCACGTCGTGGAA TGC-3'; the HA tag sequence, positioned four amino acids after the UL7/UL8 signal peptidase cleavage site, is underlined). The KanR cassette was subsequently removed in the second recombination step, and the resulting construct, HCMV HA-UL7/UL8, was further used as a parental BAC for HCMV HA-UL8 and HCMV Δ UL8. For the HCMV HA-UL8 construct, the intron containing the stop codon for the *UL7* ORF was replaced by the KanR cassette through homologous recombination with the fragment amplified with the following primers: AA_UL8F (5'-CAATCAGACGCTTTGGAAAACAACACGCACATGGCCTTGGGAGAAGGTTT TGTACCAACGACGCATCGTGGCCGGATCTC-3') and AA_UL8R (5'-ATAGCCCCGATGTAGTCATTGTTGGTACA AACCTTCTCCCAAGGCCATGTGCGTGTGTGACCACGTCGTGGAATGC-3'). For the HCMV Δ UL8 construct, which can be considered HCMV HA-UL7, the part of the ORF downstream of the stop codon was replaced by a KanR cassette via recombination with the fragment obtained by amplification with the primers AA_UL7F (5'-GGGGTGGTGAAGTTGTTGTGTAGTAAACCAAAGTTATAGTATACTATGATGTCTAAGAA GACGCATCGTGGCCGGATCTC-3') and AA_UL7R (5'-ATAGAAATAACCGCAACACTTCTTAGACATCATAGTA TACTATACTTGTGTTTACTACGTGACCACGTCGTGGAATGC-3'). For all HCMV-BAC constructs the regions comprising the introduced changes were analyzed by sequencing. HCMV HA-UL7/UL8, HCMV HA-UL8, and HCMV Δ UL8 were reconstituted by transfection of RPE-1 cells with BAC DNA isolated from *Escherichia coli* cultures as described previously (56).

Plasmid constructions. The full-length UL8 was obtained by PCR using as a template cDNA obtained from RNA of MRC-5 cells infected with HCMV (strain AD169) and primers UL7ForEco (5'-ATCGAATTCATGGCTCCGACGTG-3') and UL8EndRev (5'-CATTGTAGTTCATCACAGCTCCGT-3'). Subsequently, the PCR product was inserted into the pGEM-T vector to produce the pGEMT UL8 plasmid. To construct pCneo UL8, expressing full-length UL8, pGEMT UL8 was used as a template for the generation of a PCR product employing primers UL7ForEco and UL8spNotRev (5'-CTTGCGGCCGCTCACAGCTCCGTGTCCGT-3'). The resulting PCR product was inserted into the pGEM-T vector, and the EcoRI-NotI fragment was excised and cloned into the EcoRI-NotI-digested pCneo. pDisplay UL8 (also named HA-UL8 in this study), expressing UL8 with an N-terminal HA tag positioned immediately after the signal peptidase cleavage site, was constructed as follows: a PCR product was generated using pGEMT UL8 as a template and primers BglUL7 (5'-CAAGATCTGAATCCATCATCTACGTC-3') and PSTUL8sp (5'-CTTCTGCAGTCACAGCTCCGTGTCCGT-3'). The resulting PCR product was introduced into the pGEM-T vector, and subsequently a BglII-PstI fragment, corresponding to the UL8 gene without its signal peptide sequence, was excised and fused in frame with the HA tag at the N-terminal end by inserting it into the BglII-PstI-digested pDisplay. pDisplay UL8 Δ E-V, expressing UL8 with a deletion in residues 265 to 271, was constructed with the following method: two independent PCR products were generated using pDisplay HA-UL8 as a template and the primers BglUL7 and UL7/8-828Rev (5'-CGTTCTTTGATATTGCACAGTTGTGGAGT-3') for one PCR product and UL7/8-778For (5'-ACTCCACAACCTGTGCAATATCAAAGAACG-3') and PSTUL8sp for the other. Splicing by overhang extension (SOE)-PCR was then performed to join the two PCR fragments using the external primers BglUL7 and PSTUL8sp. This new PCR product was inserted into the pGEM-T vector, and then the BglII-PstI fragment was excised and cloned into the BglII-PstI-digested pDisplay. pDisplay UL8 Δ Q-H, expressing UL8 with a deletion of residues 272 to 279, was generated as indicated for pDisplay UL8 Δ E-V but using primers BglUL7 and UL7/8-852Rev (5'-TAGCATCCAAGAATATACTTCCCCAGCTGG-3') for one PCR product and UL7/8-799For (5'-CCAGCTGGGGAAGTATATTCTTGGATGCTA-3') and PSTUL8sp for the other. pDisplay UL8 Y305/314A, expressing UL8 with the tyrosines in position 305 and 314 exchanged for alanines, was generated by employing a PCR product using pDisplay-UL8 as a template and primers BglUL7OK (5'-AGATCTGAATCCATCATCTACGTGTCTGGGCCA-3') and PstUL8Y305/314A (5'-CTGCAGTCACAGCTCCGTGTCCGCATAAATACTTGTCCGGCCTGTTATTGTCTTCCAGCGATCGGCGACTTTTGAGG-3'). The PCR product was inserted into the pGEM-T vector, and the BglII-PstI fragment was excised and cloned into the BglII-PstI-digested pDisplay. pDisplay UL8 Δ tail, expressing UL8 without the last 20 residues of the cytoplasmic tail, was generated as indicated for the construct described above but using the primers BglUL7OK and PSTUL8Y305ST (5'-CTGCAGTCAGACTTTTTGAGGTGCTCGCAG-3'). pDisplay UL8 E91A, expressing UL8 with the glutamic acid in position 91 exchanged for alanine, was generated as follows: two independent PCR products were generated using pDisplay-UL8 as a template and primers UL7Bgl and E91ANLRev (5'-CAGGAACGTTGCATTTTCGCC-3') for one PCR product and E91ANLFor (5'-GGCGAAAATGCAA CGTTCCTG-3') and PSTUL8sp for the other. SOE-PCR was then performed to join the two PCR products using the external primers BglUL8 and PSTUL8sp. This new PCR product was inserted into the pGEM-T vector, and then the BglII-PstI fragment was excised and cloned into the BglII-PstI-digested pDisplay. pDisplay UL7, expressing UL7 with an N-terminal HA tag (also named HA-UL7), full-length UL7 (pCneo UL7), and pCneo UL7-CD229 (named UL8-ch in this study), expressing a molecule in which the N-terminal Ig domain of CD229 was swapped for the Ig domain of UL7 and UL8, were previously described (21). Construction of UL8-ch R29A, UL8-ch R32A, UL8-ch D34A, UL8-ch K45A, UL8-ch E48A, and UL8-ch R78A, expressing the chimeric UL8-ch with the indicated mutations, was performed by generating two independent PCR products using pCneo UL7 as a template and the primer UL7LD1For (5'-CAACTCGAGATGGCTTCCGACGTGAGC-3') and an antisense primer containing the mismatched base for one PCR product or a sense primer that contained the mismatched base plus the primer UL7LD1Rev (5'-CCGGAATTCAGGAACGTTTCATTTC-3') for the other PCR product. The sets of sense and antisense primers containing the appropriate mutations were the following: R29ARev (5'-GCGAAACACGCTGCGCTGATG-3') and R29AFor (5'-CATCAGCGCAGCGTG GTTTCGC-3'), R32ARev (5'-TTGCCGTCCAGGCAAAACACCGT-3') and R32AFor (5'-ACGGTGGTTTGCC TGGGACGGCAA-3'), D34ARev (5'-ATCGTTTCCGGCCAGCGAAAC-3') and D34AFor (5'-GTTTCGCTGG GCCGGC AACGAT-3'), K45ARev (5'-CTCTCCACGTGCGTAAAGCAAA-3') and K45AFor (5'-TTTGCTTT TACGCACGTGGAGAGG-3'), E48ARev (5'-TAGAAAGACCGCTCCAGTTTG-3') and E48AFor (5'-CAAACG TGGAGCGGGTCTTTCTA-3'), and R78ARev (5'-CGACTCCGGTGTACGAGCA-3') and R78AFor (5'-TGCTC CGTAAGCACCGGAGTCG-3'). SOE-PCR was then performed to join the two PCR products using the external primers UL7LD1For and UL7LD1Rev. This new product was inserted into the pGEM-T vector, and then the XhoI/EcoRI fragment was excised and cloned into the XhoI/EcoRI UL8-ch plasmid. UL8-ch E82A, UL8-ch R86A, UL8-ch E89A, and UL8-ch E91A were constructed, generating a PCR product using pCneo UL7 as a template and primer UL7LD1For and an antisense primer containing the mismatched base. Primers containing the appropriate mutations were E82ARev (5'-CCCGAATTCAGGAACGTTTCATTTCGCG CGTTCCTGTAACCTGCTGCTCC-3'), R86ARev (5'-CCCGAATTCAGGAACGTTTCATTTCGCGCGTTCGCGT AAA-3'), E89ARev (5'-CCCGAATTCAGGAACGTTTCATTTCGCGCGTTCCT-3'), and E91ARev (5'-CCCGAAT TCAGGAACGTTGCATTTTCGCGCT-3'). The resulting PCR products were inserted into the pGEM-T vector, and then the XhoI/EcoRI fragments were excised and cloned into the XhoI/EcoRI UL8-ch plasmid.

RT-PCR. Total RNA was isolated from HEL299 either mock-infected or infected with HCMV-GFP at an MOI of 1 for 72 h by the TRIzol method (Invitrogen). RT-PCR was then carried out using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's protocol. Briefly, RNA samples were treated with RNase-free DNase I (Promega) for 30 min at 37°C, and the DNase was inactivated at 65°C for 10 min. The RNA was reverse transcribed using oligo(dT) primers at 50°C for 50 min, and reactions were terminated by heating at 85°C for 5 min. The reverse-transcribed products were treated with RNase H for 20 min at 37°C and amplified with specific primers. The primers used for the

different genes and the sizes of the amplified fragments were the following: primers UL7ForEco and UL8spBamRev (5'-CTCAGGATCCCAGCTCCGTGTCCTCATAAA-3') for the UL8 gene (991-bp fragment), primers UL7ForEco and UL7BamRev (21) for the UL7 gene (685-bp fragment), primers IEP4BII (5'-CAATACACTTCATCTCCTCGAAAGG-3') and IEP3C (5'-CAACGAGAACCCCGAGAAAGATGTC-3') for the HCMV *ie1* gene (321-bp spliced fragment and 391-bp unspliced fragment), and primers GAPDHFor and GAPDHRev (57) for the *GAPDH* gene (101-bp fragment). PCRs were performed under the following conditions: 1 cycle at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C; and 1 cycle at 72°C for 10 min. Control reactions carried out in the absence of RT were used to assess the specific detection of RNA. Amplified products were separated on a 1% agarose gel and visualized by RedSafe nucleic acid staining solution (iNTRON Biotechnology Inc.). The RT-PCR-amplified fragment corresponding to the UL8 gene was cloned in the pGEMT vector and its identity confirmed by DNA sequencing.

5'- and 3'-RACE analysis. HFFs cells were mock infected or infected with HCMV (TB40/E) at an MOI of 1 for 96 h, and total RNA was isolated using an RNeasy minikit (Qiagen). 5'- and 3'-RACE analysis was performed using the FirstChoice RLM-RACE kit (Invitrogen) according to the manufacturer's instructions. Briefly, for 5'-RACE, an adapter was added to the 5' end and the RNA reverse transcribed with random decamer primers. The resulting cDNA was amplified with a 5' RACE outer primer and NP_Rev2 (5'-TGAAGATGGTGATCTGGTA-3'), and the obtained product was amplified again with the 5' RACE inner primer and NP_Rev1 (5'-CCTTCTCCAGCTTTGTAAA-3'). For 3'-RACE, the RNA was reverse transcribed with an oligo(dT) primer adapter. The resulting cDNA was amplified with a 3'-RACE outer primer and NP_For3 (5'-ACGCATTACTCTTGGATGCT-3'), and the obtained product was amplified again with the 3'-RACE inner primer and NP_For4 (5'-CACCTCGAAAAGTTTATGAT-3'). The final products were purified from a 1% agarose gel and their identities verified by direct DNA sequencing.

Transient and stable transfections. COS-7 cells were transiently transfected with 5 µg of the corresponding plasmid using the Amaxa Cell Line Nucleofector kit R according to the manufacturer's protocol. Likewise, THP-1, HEL299, and 300.19 cells were transfected using the Cell Line Nucleofector kit V. To generate 300.19 stable cell lines, transfections were followed by G418 selection.

Antibodies. The anti-UL7/UL8 monoclonal antibody (clone UL7.1.87, previously named anti-UL7 MAb) has been described before (21). The MAb UL7.1.400, recognizing a different epitope of the Ig-like domain of UL7 and UL8 that was used to assess the absence of alterations in the tertiary structure of the mutant UL8-ch molecules, and the anti-HA MAb (employed for flow cytometry) were generated in our laboratory. The goat anti-mouse IgG (H+L)-Alexa Fluor 555 was from Life Technologies, and the anti-mouse IgG labeled with phycoerythrin (PE) was from Jackson ImmunoResearch. The mouse biotinylated anti-HA (employed for the ELISAs) and the rabbit anti-HA (used for Western blot assays) were purchased from Sigma-Aldrich and Cell Signaling, respectively. The anti-rabbit Ig labeled with peroxidase (POD) was from Promega, and the anti-mouse IgG labeled with POD was from Sigma-Aldrich. The anti-β-actin MAb was purchased from MP Biomedicals. The anti-IE1 MAb recognizing the HCMV IE1 protein was from Merck Millipore. The anti-pp28 MAb recognizing the HCMV pp28 protein was purchased from Abcam.

Immunofluorescence microscopy. Transiently transfected COS-7 cells were cultured on glass coverslips in 24-well tissue cultures plates. When indicated, 24 h after transfection cells were incubated with 50 µM leupeptin (Sigma) for a period of 24 h. Cells were fixed with 4% formaldehyde and, when indicated, permeabilized with 0.05% Triton X-100 for 5 min. Alternatively, cells were fixed and permeabilized in ice-cold acetone. Subsequently, cells were blocked with 3% bovine serum albumin. Samples were incubated with the anti-UL7/UL8 MAb and goat anti-mouse IgG (H+L)-Alexa Fluor 555. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) reagent (Life Technologies). The samples were mounted in ProLong gold antifade reagent (Invitrogen). Fluorescence images were obtained using a Nikon Optiphot-2 microscope (Nikon Corp.).

Flow cytometry analysis. Flow cytometry analysis was performed using standard procedures. Cells were stained with the corresponding MAbs, followed by anti-mouse IgG labeled with PE. Cell viability was measured using the LIVE/DEAD fixable violet dead cell stain kit (Invitrogen) according to the manufacturer's instructions. Sortings were performed using a FACSAria cell sorter (BD Biosciences). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar Inc.).

Western blot analysis. For selective expression of viral immediate-early proteins, cells were incubated from 30 min prior to infection to 4 h postinfection in the presence of CHX (100 µg/ml; Sigma-Aldrich), followed by incubation in the presence of actinomycin D (10 µg/ml; Sigma-Aldrich) for another 12 h, as previously described (58). Selective expression of early genes was carried out by treatment of cells with PPA (250 µg/ml; Sigma-Aldrich) for 72 h (58). When specified, cells were incubated with 50 µM leupeptin (Sigma) and 20 nM bafilomycin A1 (Sigma) during 24 h. For the analysis of the supernatants from mock-infected or infected cells, cell monolayers were washed three times with phosphate-buffered saline (PBS) after viral adsorption and incubated for 72 h in serum-free DMEM. Subsequently, supernatants were clarified to remove virus particles and concentrated 20-fold using an Amicon Ultra-15 centrifugal filter unit with an Ultracel-30 membrane (Millipore). For Western blot analysis, samples were lysed and quantified by a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). When indicated, an N-glycosidase F deglycosylation kit (Roche Diagnostics GmbH) and/or an O-glycosidase and neuraminidase bundle (New England BioLabs) were used by following the manufacturer's instructions. Samples were subjected to SDS-PAGE in 10% acrylamide gels, except in the assay shown in Fig. 3C (right), where an 8% acrylamide gel was used, and subsequently transferred to nitrocellulose membranes (Protran; Whatman Schleicher & Schuell). The membranes were incubated with rabbit anti-HA Ab followed by anti-rabbit POD and MAbs anti-IE1, anti-pp28, and anti-β-actin, with an

anti-mouse POD as a secondary antibody. Blots were developed using the SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's protocol.

ELISA. Detection of the protein HA-UL8 or HA-UL7 in the supernatants was performed by sandwich ELISA using the anti-UL7/UL8 MAb as capturing Ab and biotinylated anti-HA, followed by incubation of streptavidin-POD conjugate (Roche Diagnostics GmbH).

Internalization assays. The stably transfected mouse 300.19 cell lines were sorted in order to obtain cells expressing similar amounts of UL8 protein at the cell surface. Subsequently, cells were incubated with 1 μ g/ml of the anti-UL7/UL8 MAb at 4°C for 45 min. Afterwards, cells were washed with PBS and incubated at 4°C or 37°C for the indicated time points, followed by anti-mouse IgG-PE, and analyzed using a FACSCalibur flow cytometer and FlowJo software. The percentage of internalization was calculated using mean fluorescence intensity (MFI) values of PE as determined with the following formula: $100 - [(MFI \text{ at } 37^\circ\text{C}/MFI \text{ at } 4^\circ\text{C}) \times 100]$.

Adhesion assay. Transfected COS-7 cells were cultured on glass coverslips in 24-well tissue culture plates. After 24 h, COS-7 cells were washed with PBS and maintained at 4°C for 20 min. In some cases, COS-7 cells were preincubated with the anti-UL7/UL8 MAb at the indicated concentrations for 30 min at 4°C. Afterwards, LPS-activated neutrophils were added to wells for 1 h at 4°C at a concentration of 1×10^6 /well. When indicated, activated neutrophils were previously stained with calcein (1 μ M; Life Technologies) for 30 min. Subsequently, coverslips were washed by repeated immersion, and the adhesion of activated neutrophils was examined by observing cellular rosettes around transfected COS-7 cells. Fluorescence images were obtained using an inverted Leica DMI600 B microscope (Leica Microsystems, Germany). When specified, the adhesion of calcein-stained activated neutrophils was quantified by flow cytometry. In these cases, after performing the adhesion assay, cells were trypsinized and analyzed using a FACSCalibur flow cytometer and FlowJo software.

Cytokine determination. Transfected THP-1 live cells were sorted and seeded to a final concentration of 5×10^5 /well and were either left untreated or treated with LPS (1 μ g/ml). After 24 h, cell-free culture supernatants were harvested. For infection assays, THP-1 cells were previously stimulated with PMA (50 ng/ml) for 24 h. Cell-free culture supernatants were collected at different time points after infection. The concentration of IL-8 or TNF- α in these supernatants was measured by high-sensitivity human ELISA sets (ImmunoTools). A cytokine human magnetic 30-plex panel for the Luminex platform (Invitrogen) was used to assess the concentration of GM-CSF, TNF- α , IL-1 β , IL-4, IL-6, RANTES, MIG, VEGF, HGF, EGF, IL-8, IL-17, MIP-1 α , IL-10, IL-1R α , IL-12, IL-13, fibroblast growth factor (FGF), IFN- γ , G-CSF, MCP-1, IL-7, IL-15, IFN- α , IL-2R, IP-10, MIP-1 β , EOTAXIN, IL-5, and IL-2 by following the manufacturer's instructions. Data for the cytokine human magnetic 30-plex panel were analyzed as recommended by the manufacturers. The concentration of each analyte was obtained by interpolating fluorescence intensity to a 14-point dilution standard curve supplied by the kit and calculated by xPONENT software.

Sequence analysis. Genomic and UL8 protein sequences of all of the HCMV sequenced strains were downloaded from GenBank (31). For the strains without annotation of the correct UL8 gene, the UL8 Merlin annotation of NCBI's blastn (<http://blast.ncbi.nlm.nih.gov>) and Expasy Translate (<http://web.expasy.org/translate/>) were employed to obtain the UL8 coding sequence and the corresponding protein, respectively. For those strains with more than one sequence available in GenBank (Merlin, AD169, TB40/E, Towne, Toledo, and TR), no differences in the UL8 region were observed among the sequences. Thus, only one sequence for each strain was considered. Multiple-sequence alignments of DNA and amino acid sequences were obtained using MAFFT (<http://mafft.cbrc.jp/alignment/software/>). Amino acid differences among the HCMV strains were calculated with respect to Merlin, the usual reference strain, and plotted along the UL8 sequence protein using Microsoft Excel. The 5' and 3' genomic regions of UL8 were manually scanned in order to identify promoter and polyadenylation motifs according to well-documented regulatory elements (28–30).

Protein domain and motif prediction. The signal peptide and the IgV-like domain are the same for UL8 and UL7, as both proteins share the first 195 aa. Thus, previous characterization of these domains was used (21). The UL8 transmembrane region was predicted using the TMHMM v2.0 software, whereas protein N-glycosylation and O-glycosylation sites were identified by using NetNGlyc v1.0 and NetOGlyc v3.1, respectively; these three prediction programs are available at CBS Prediction Servers (<http://www.cbs.dtu.dk/services/>). The two tyrosine-based endocytic motifs and the C-terminal PDZ-binding domain were predicted using MiniMotifs Miner 3.0 (59).

Statistical analyses. Analyses were performed with GraphPad Prism software (version 5.0) and Microsoft Excel (2010). Results are given as means $\pm$ standard errors of the means (SEM), and statistical significances were determined with the Student *t* test (two-tailed). *P* values less than or equal to 0.05 (*), 0.01 (**), and 0.001 (***) were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.02229-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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