SAPIENZA
Università di Roma
Facoltà di Scienze Matematiche Fisiche e Naturali

DOTTORATO DI RICERCA
IN BIOLOGIA CELLULARE E DELLO SVILUPPO

XXXI Ciclo
(A.A. 2017/2018)

Functional characterization of the newly identified HCMV envelope glycoprotein UL116

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“L’uomo non smette di giocare perché invecchia, ma invecchia perché smette di giocare.”

(George Bernard Shaw)

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SUMMARY

Human Cytomegalovirus (HCMV) is a β-herpesvirus, whose infection can cause serious diseases in immunocompromised adults and in utero infected fetuses. The gH/gL/gO and gH/gL/UL128/UL130/UL131 (Pentamer) complexes play a key role in HCMV entry and cell tropism. The variability of the relative amount of the two complexes on the virion surface is reflected in different cell tropism and it appears to be regulated by UL148 viral protein interaction with gH favoring the formation of the gH/gL/gO complex. Recently it has been shown that the *UL116* gene encodes a previously unknown protein that forms a heterocomplex with gH on the surface of HCMV in a gL-independent manner and by unknown function. In a preliminary experiment, the recombinant gH/UL116 complex was purified and used as a probe on a microarray representing a human protein chip searching interactors and leading to the identification of the putative cellular receptors TREM-1, IL-6R and TARM-1.
In this work we verified that there are no interactions between the recombinant gH/UL116 complex and its putative cellular receptors TREM-1, IL-6R and TARM-1 on transfected cells and not even on the surface of different cell lines used in binding assays. Furthermore, it seems that in the absence of gL protein, therefore without formation of gH/gL/gO complexes and Pentamer, gH/UL116 complex is not sufficient for virus regeneration and propagation.
However, here, we show that the absence of UL116 protein compromises virus infection and spreading in epithelial cells as well as it causes the incorporation reduction of gH/gL-based complexes into the virion. Furthermore, we prove that UL116 interacts directly with UL148, suggesting an involvement of UL116 in the correct assembly of gH/gL-based complexes. Finally, although it is yet to be verified, the absence of UL116 seems to

compromise the presence of pp71 viral protein in virions, which could influence a correct HCMV replication.

RIASSUNTO

Human Cytomegalovirus (HCMV) è un β-herpesvirus, la cui infezione può causare gravi patologie negli adulti immunocompromessi e nei feti infetti in utero. I complessi gH/gL/gO e gH/gL/UL128/UL130/UL131 (Pentamero) svolgono un ruolo chiave nell'ingresso e nel tropismo cellulare del HCMV. La variabilità della quantità relativa dei due complessi sulla superficie del virione si riflette nel diverso tropismo cellulare, e sembra essere regolata dall'interazione della proteina virale UL148 con gH favorendo la formazione del complesso gH/gL/gO. Recentemente è stato dimostrato che il gene *UL116* codifica una proteina, precedentemente sconosciuta, che forma un eterocomplesso con gH sulla superficie di HCMV in maniera gL-indipendente, e dalla funzione ignota. In un esperimento preliminare, il complesso ricombinante gH/UL116 è stato purificato ed utilizzato come sonda su un microarray rappresentante un chip di proteine umane alla ricerca di interattori, portando all'identificazione dei putativi recettori cellulari TREM-1, IL-6R e TARM-1.
In questo lavoro abbiamo verificato che non vi sono interazioni tra il complesso ricombinante gH/UL116 ed i suoi putativi recettori cellulari TREM-1, IL-6R e TARM-1 su cellule trasfettate e neanche sulla superficie di diverse linee cellulari utilizzate in saggi di binding. Inoltre, sembra che in assenza della proteina gL, essenziale per la formazione dei complessi gH/gL/gO e Pentamerico, il complesso gH/UL116 non sia sufficiente per la rigenerazione e propagazione del virus.
Tuttavia, qui mostriamo che l'assenza della proteina UL116 compromette l'infezione e la diffusione del virus in cellule epiteliali, oltre a ridurre l'incorporazione dei complessi gH/gL-based nel virione. Inoltre, proviamo che UL116 interagisce direttamente con UL148, suggerendo un coinvolgimento di UL116 nel corretto assemblaggio dei complessi gH/gL-based. Infine,

sebbene sia ancora da verificare, l'assenza di UL116 sembra compromettere la presenza della proteina virale pp71 nei virioni, il che potrebbe influenzare una corretta replicazione di HCMV.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus whose infection concerns about half of the world population. Normally, HCMV infection is asymptomatic in healthy individuals, but the virus persists in a latent state. Primary infection or reactivation of latent virus in immunocompromised individuals can lead to severe disease including gastroenteritis, encephalitis, retinitis, pneumonitis, and, in the case of transplant recipients, graft rejection. In pregnant women, HCMV can cross the placental barrier and is associated with several types of birth defects (Britt and Alford, 1996). This wide manifestation of HCMV-associated disease likely relates to the ability of the virus to infect a diverse range of cell types, including epithelial and endothelial cells, fibroblasts, monocyte/macrophages, dendritic cells, hepatocytes, neurons, and leukocytes (Plachter et al., 1996). These observations suggest that tropism is unlikely dictated by a restricted cell surface receptor although it is believed that dissemination of cytomegaloviruses in all hosts, but the most in immunocompromised individuals, occurs through cell-associated virus and not free virus (Britt, 2010).

The HCMV cell tropism may reflect the relative abundance of distinct glycoprotein complexes in the virion envelope. Together with glycoprotein B (gB), the gH/gL dimer comprises the "core membrane fusion machinery" conserved among all herpesviruses, where the prevailing view is that gH/gL complexes regulate the fusogenic activity of gB (Heldwein and Krummenacher, 2008). However, like most of the herpesviruses, HCMV encodes a set of proteins that bind alternatively to gH/gL and modify or regulate the activity of the gB-gH/gL core fusion machinery. These alternative complexes arise in different tropism during the virus spreading in host cells (Zhou et al., 2013). In particular, in HCMV, gH/gL exists on the viral surface as part of a trimeric complex with gO (gH/gL/gO) or a pentameric complex with UL128, UL130 and

UL131A (Pentamer) (Ryckman et al., 2007). Recent evidences suggest that gH/gL/gO complex may be required for entry into all cell types (Zhou et al., 2015), and its interaction with platelet-derived growth factor receptor alpha (PDGFR-α), recently identified as host cell receptor, is essential for entry into fibroblasts (Kabanova et al., 2016; Yurochko et al., 2017). By contrast, Pentamer is required to infect most cell types, including epithelial, endothelial and myeloid cells, but is dispensable for infection of fibroblasts (Wang and Shenk, 2005), and neuropilin-2 (Nrp2) has been very recently identified as the receptor for Pentamer during HCMV infection in epithelial/endothelial cells (Martinez-Martin et al., 2018).

It has been found that the relative abundance of gH/gL/gO and Pentamer complexes on the virus surface vary depending on the HCMV strain-specific properties of the constituents of the multimeric gH/gL complexes (Zhang et al., 2018). The mechanism underlying the switch of complexes formation received a solid contribution by the identification of an ER-resident viral protein encoded by the *UL148* gene (UL148) that influences the ratio of gH/gL/gO to gH/gL/UL128/130/131 and the cell tropism of HCMV virions (Li et al., 2015). In particular, if *UL148* is deleted from the viral genome, the incorporation of the trimeric gH/gL/gO complex into virions results strongly impaired, leading to a reduced capacity of viral particles to establish infection in fibroblast cultures and an increased capacity to establish infection in epithelial cell cultures. Although different models have been proposed on the basis of the data showed in *Li et al*., to date the mechanistic underpinnings that explain how UL148 influences the maturation of gH/gL complexes remain unknown or unclear (Li and Kamil, 2016).

Recently, HCMV gH has been shown to form a dimeric complex with an alternative components of unknown functions (Caló et al., 2016). In particular, it has been demonstrated that the product of the HCMV *UL116* gene forms a noncovalent heterodimer with gH independently from gL. In infected cells, UL116 shows late

kinetics of expression, existing in two different glycosylated forms: a heavily glycosylated mature form carrying Golgi-type carbohydrate chains, and an immature ER-type glycosylated UL116 of lower mass. The high molecular weight UL116 mature form is part of the viral envelope while the ER species is not. Formation of a heterodimeric gH/UL116 complex has been confirmed by experiments in HCMV-infected cells suggesting that UL116 is an HCMV envelope glycoprotein that forms a novel gH-based complex alternative to gH/gL and potentially plays a role in viral infection and/or dissemination. Transient expression experiments of UL116 alone or in combination with gH reveal that the two proteins need co-expression to be targeted to the secretory pathway, while as single proteins both are retained in the ER. Among the HCMV envelope proteins, gH is the only protein identified so far that interacts with UL116 and that is able to promote UL116 release from ER in transfected cells. Furthermore, UL116 is the only protein among all herpesviridae able to interact and promote ER exit of gH in absence of gL. Intriguingly, the restoration of Pentamer expression in the AD169 laboratory strains induces the increased expression of UL116 (Freed et al., 2013), leading to the hypothesis that the increased expression of UL116 somehow is linked to the acquisition of a functional Pentamer and the concomitant expansion of cellular tropism.

Indeed, the function role of UL116 is still to be elucidated and, while its presence on the viral envelope suggests an implication in cellular tropism determination, it cannot be excluded a function related to complex switch in the ER compartment.

In this work we have tried to explore both aspects of the possible UL116 function. In an attempt to identify a potential cellular receptor for gH/UL116 heterodimer, the recombinant soluble gH/UL116 complex has been purified and used as probe on immobilized human extracellular protein microarray searching for

cellular interactors. These preliminary experiments led to the identification of a number of putative receptors, many of them cell-type restricted. The most promising candidates were selected as TREM1, IL6R and TARM1.
Here, we report that the gH/UL116 was not able to interact neither with the surface of non-permissive transfected cells with plasmids expressing each above-mentioned putative receptor or with the surface of different tested cell lines.
Studies on recombinant TR strain HCMV revealed that gH/UL116 is not sufficient to replace gH/gL/gO complex and Pentamer in a gL-null genetic background. However, it seemed that gH/UL116 is needed for propagation of infection in epithelial cells and for gH/gL-based complexes inclusion in virions. Moreover, co-immunoprecipitation analyses showed an interaction between UL116 and UL148 both in infected and in transfected cells. Finally, preliminary results conducted on UL116-null virions proteome through two-dimensional electrophoresis and mass spectrometry showed a loss of pp71 tegument protein.

AIMS OF THE WORK

This project is aimed to investigate on the functional role of UL116 HCMV envelope protein. As previously reported by *Caló et al.,* UL116 localizes on the viral envelope forming a complex with the glycoprotein gH, alternative to gH/gL-based complexes.
To verify the role of gH/UL116 complex in cell-specific interactions and its involvement in the HCMV tropism, various approaches have been followed.
First, validation experiments on the three putative selected human receptors, TREM-1, IL-6R and TARM-1, were performed, evaluating their ability to specifically bind gH/UL116 on the surface of non-permissive cells. In parallel, the recombinant gH/UL116 complex was used in protein binding assays on the surface of different cell lines.
Another approach was the generation of different recombinant viruses, carrying knock-out mutations and tagged viral protein. Hence, we tried to generate viruses lacking of the gL glycoprotein, essential for the formation of the gH/gL/gO and gH/gL/UL128/UL130/UL131 envelope complexes, to the aim of understanding if the presence of UL116 could make a compensative complex on the viral surface.
In addition, viruses knocked-out of UL116 have been characterized in order to verify any phenotypic variation. Finally, viruses expressing tagged viral proteins have been generated to assess the hypothesis of interaction between UL116 and UL148, involved in the switching of the gH/gL/gO and gH/gL/UL128/UL130/UL131 envelope complexes hence regulating the viral tropism; for this purpose UL148-tagged and UL148 knock-out viruses were generated.

RESULTS

Validation analysis of gH/UL116 cell-specific interactions

As previously described, the glycoprotein complexes involved in the early stages of entry of the virus into human cells are very important for tropism and HCMV infection. Therefore, these complexes are considered concrete targets for vaccine development. The gH/gL/gO complex has been shown to be essential for infection in all cell types by specifically interacting with PDGFR-α while a cell receptor for the Pentamer has been very recently identified as Nrp2 and found to be essential for HCMV infection in epithelial/endothelial cells.
The presence of the gH/UL116 complex on the viral envelope led us to hypothesize that this heterodimer may be involved in host receptor(s) recognition aimed at conveying HCMV infection. Consequently, similar to the gH/gL/gO and Pentameric complexes, gH/UL116 could play a role in cell-specific interactions during the early stages of infection and in virus tropism.
With the aim to understand if UL116 was involved in cell-specific interactions, the recombinant soluble gH/UL116 complex, that include an HIS-tag on gH, has been purified and used as probe in a human protein microarray searching for cellular interactors. In this preliminary experiment, conducted on a protein array of a human "surfome" library including about 7,000 soluble or plasma membrane exposed protein, a strong signal was obtained for 3 proteins: TREM-1, IL-6R and TARM-1 (data not shown).
TREM-1 is member of the triggering receptors expressed on myeloid cells (TREM) family and plays an important role in the amplification of inflammation modulating the intensity of innate immune responses, crosstalk with other pattern recognition receptors (PRR) pathways and activation of antigen-presenting cells (APC). TREM-1 signaling is linked to several diseases, such as polymicrobial septic shock and inflammatory bowel disease, and

in animal models of pneumonia and asthma (Ford and McVicar, 2009; Klesney-Tait et al., 2013). IL-6R is the Interleukin-6 (IL-6) membrane-bound receptor, which is only expressed on hepatocytes and certain subpopulations of leukocytes. In a second pathway, named trans-signaling, IL-6 binds to soluble forms of the IL-6R (sIL-6R), and this agonistic IL-6/sIL-6R complexes can in principle activate all cells. Most pro-inflammatory roles of IL-6 have been attributed to the trans-signaling pathway, whereas anti-inflammatory and regenerative signaling, including the anti-bacterial acute phase response of the liver, is mediated by IL-6 classic signaling (Wolf et al., 2014). TARM-1 (T cell-interacting, activating receptor on myeloid cells-1) is a novel receptor encoded within the human Leukocyte Receptor Complex (LRC), and it is expressed constitutively by bone marrow granulocytes, monocytes and neutrophils that home to sites of inflammation. A preliminary study shows that a putative T cell ligand can interact with TARM1 receptor resulting in bi-directional signaling, raising the T cell activation threshold whilst co-stimulating the release of pro-inflammatory cytokines by macrophages and neutrophils (Radjabova et al., 2015).
All three proteins were tested for the binding to the gH/UL116 recombinant protein.

The ectopic expression of candidate receptors TREM-1, IL-6R or TARM-1 do not bind the recombinant soluble gH/UL116 on the surface of transfected cells.

Plasmids expressing either TREM-1, IL-6R or TARM-1 were generated and transfected in CHO-K1 non-permissive hamster cells in order to verify if their ectopic expression resulted in the binding of gH/UL116 complex on the surface of the transfected cells. The expression of TREM-1, IL-6R or TARM-1 was verified after transfection on the surface of CHO-K1 cells by FACS, using anti-TREM1 or anti-IL6R antibodies, and expression of TARM1

only by Western Blotting (WB) (Figure 1A and B). Anti-His antibody was used to detect binding of gH/UL116 at the cell surface (Figure 1C). Although transfected cells expressed the desired molecule, no signal for the binding of the recombinant soluble gH/UL116 complex could be revealed (Figure 1C).

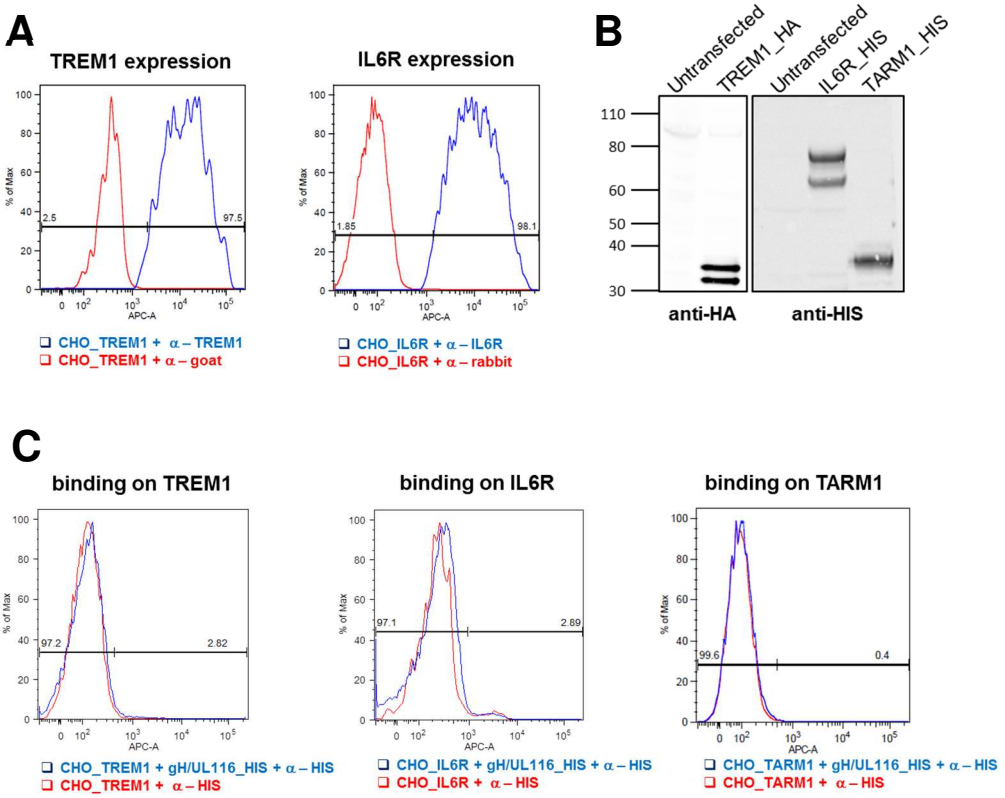

Figure 1. The recombinant gH/UL116 complex does not bind transfected cells expressing candidate receptors.
(A) FACS analyses on CHO-K1 cells transfected with plasmid expressing TREM1 or IL6R: blue signals revealed the presence of each candidate receptor on cells surface. (B) Immunoblotting on transfected CHO-K1 cells lysates: the expression of TREM1 was evaluated using anti-HA tag; the expression of IL6R and TARM1 was evaluated using anti-HIS tag. (C) FACS analyses to evaluate the gH/UL116 binding on transfected cells: no signal for the recombinant protein was detected on the surface of transfected CHO-K1 for each of tested putative receptors. The experiment was repeated three times.

FACS analysis reveals no binding of gH/UL116 for each tested cell lines.

Failing to identify putative receptors by protein array analysis, we wanted to verify whether gH/UL116 was involved in cell-specific interactions by adopting a different approach. Therefore, a gH/UL116 binding assay to different cell types was performed. In particular, the recombinant soluble gH/UL116 complex was incubated with HFF-1 (human fibroblasts), ARPE-19 (human epithelial), CALU-3 (human epithelial), THP-1 (human monocyte-like), JURKAT (human T-like cells), and HL-60 (human neutrophilic) cells. The recombinant gH/gL/gO complex was used as positive control. Both gH complexes used were HIS-tagged at gH C-TERM. FACS analysis revealed that gH/gL/gO bound to the surface of all tested cell lines, with the exception of HL-60 cells (Figure 2A); conversely, we could not detect binding of gH/UL116 to any of the tested cell lines (Figure 2B).

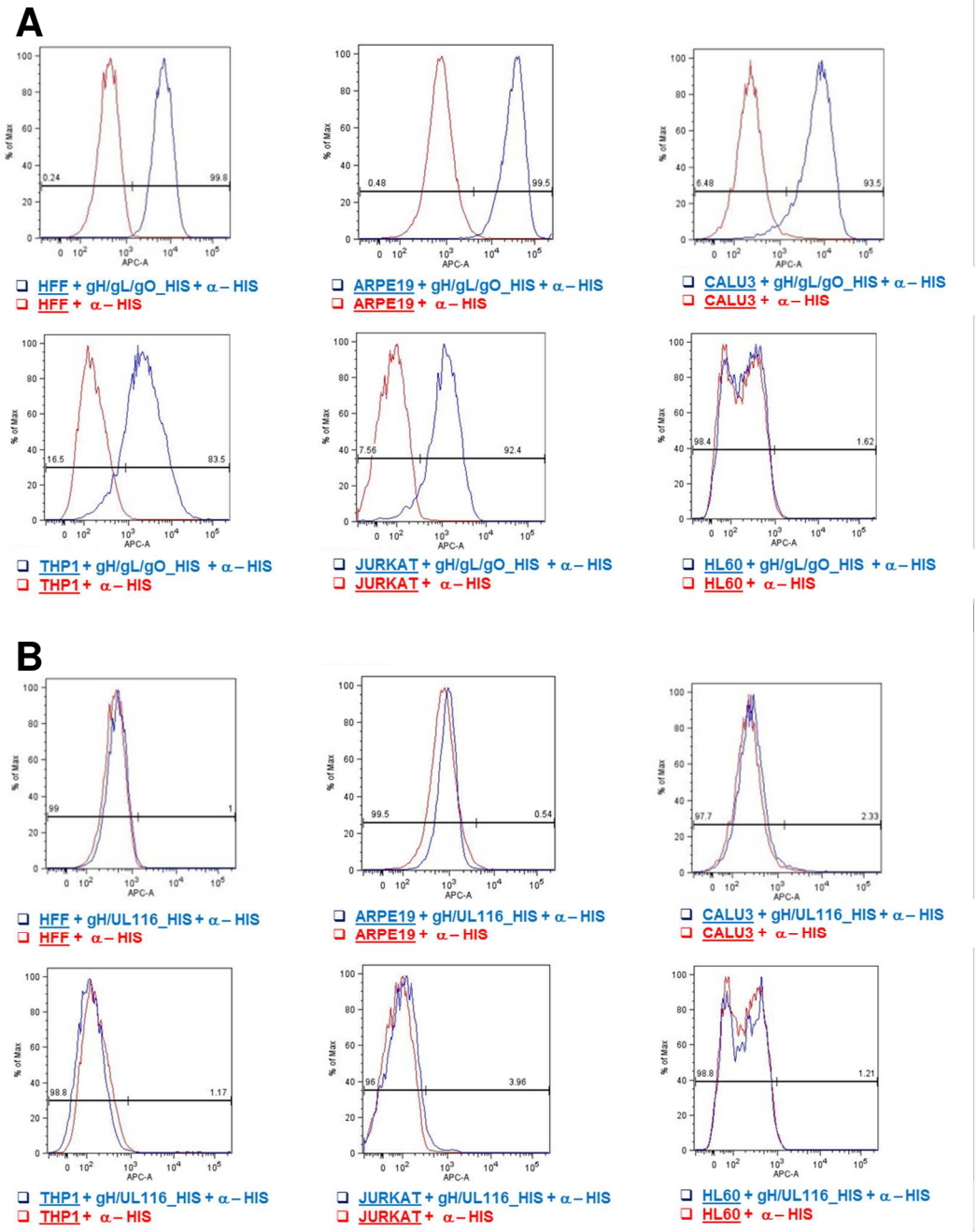

Figure 2. Binding assay reveals that gH/UL116 is not able to interact on cellular surface.
FACS analyses to evaluate the recombinant HCMV complexes ability to bind the surface of different cell lines: gH/gL/gO (A), used as positive control, bound each tested cell lines with the exception of HL-60 cells; gH/UL116 (B) did not bind any of the tested cell lines. The experiment was repeated three times.

Studies on TRG-ΔstopUL115 (gL glycoprotein knock-out) recombinant viruses

The gL glycoprotein (UL115) is essential for the formation of the gH/gL/gO and gH/gL/UL128/UL130/UL131 envelope complexes. Therefore, we decided to generate a recombinant HCMV lacking the expression of the gL glycoprotein and, consequently, the Trimeric and Pentameric complexes on the viral surface. In this way we ensured the presence of only UL116 complexes, to verify if this is enough for the viral replication (Figure 3A).

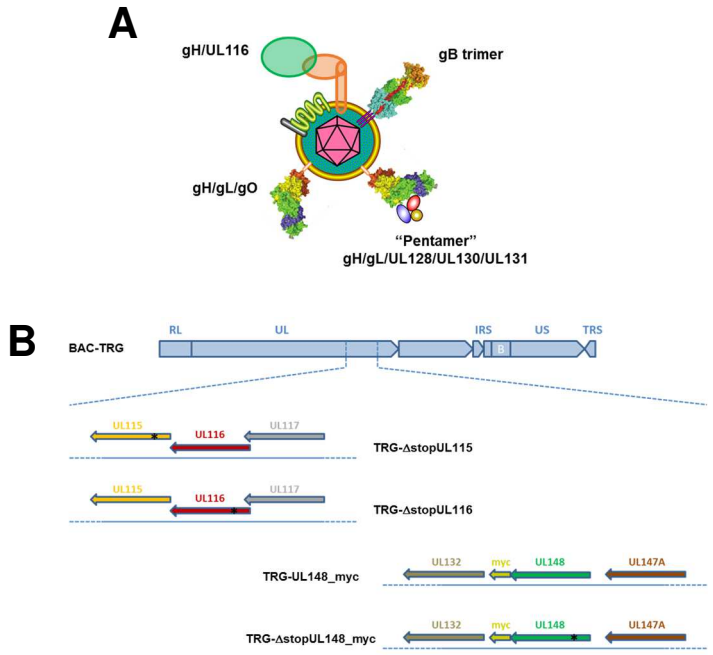

Figure 3. HCMV envelope glycoprotein complexes and generated mutants schematic representation.
(A) Together with glycoprotein B (gB), the gH/gL dimer comprises the "core membrane fusion machinery" conserved among all herpesviruses. gH/gL exists on the viral surface as part of a trimeric complex with gO (gH/gL/gO) or a pentameric complex with UL128, UL130 and UL131A (Pentamer). UL116 exists on the virion surface in association with gH in absence of gL. (B) The TRG-ΔstopUL115 clone was generated changing the cysteine at position 6 (Cys_6) in a STOP codon. The TRG-ΔstopUL116 clone was generated with a single nucleotide insertion in the CDS (between nucleotides in position 4-5) of the UL116 gene causing a frame-shift and a STOP codon formation. The TRG-UL148_myc clone was generated inserting the sequence encoding for a myc-tag in frame at C-TERM. The TRG-ΔstopUL148_myc clone was generated changing the leucine at position 4 (Leu_4) in a STOP codon using the TRG-UL148_myc as template. Each clone was generated through the BAC Mutagenesis technique as described in STAR METHODS.

To this intent, we used the BAC (Bacterial Artificial Chromosome) mutagenesis technique on an infectious BAC clone containing the TR HCMV strain genome to generate the TRG-ΔstopUL115 clone. Introduction of a point mutation in the CDS of the *UL115* gene changed the cysteine at position 6 (Cys_6) in a STOP codon leading to the knock-out of the gene encoding gL (Figure 3B). It is noteworthy that each mutagenized BAC generated in this work carried a GFP CDS insertion, under control of an Immediate Early CMV promoter, in an intergenic region of TR genome. This could allow us to better follow the CMV infection (Viruses, STAR METHODS).

The absence of gL viral glycoprotein impairs the HCMV regeneration and dissemination after BAC electroporation in MRC-5 fibroblasts.

The MRC-5 human fibroblasts are indicated as highly permissive cells for electroporation and viral regeneration. This procedure consists of infectious BAC electroporation in MRC-5 in order to use the cellular machinery to restore infectious viral particles starting from the HCMV genome cloned into a BAC (Paredes and Yu, 2012). Completed this stage, the supernatant of electroporated MRC-5 cells (containing still low levels of viral titers) is used to re-infect HFF-1 human fibroblasts with the aim to obtain high levels of infectious particles. Usually, the dissemination of restoring virus starts from each electroporated cell to adjacent cells through a cell-to-cell spreading, and in a minor extent through the infection of mature virions just generated in the culture supernatant. In these early stages, following the GFP signal, little green outbreaks of infection and some isolate fluorescent cell are visible.

Hence, the TRG-ΔstopUL115 BAC clone was used trying to generate a virus lacking the gL protein after the electroporation in MRC-5 fibroblasts. Unfortunately, the absence of gL, and therefore

of gH/gL/gO and Pentameric complexes, seemed to be crucial for the restoration and dissemination of the virus. In the first three days following electroporation we could see that the cells containing our mutagenized BAC showed a GFP signal, proving that the electroporation was successfully achieved. In the following three days we could still see a GFP signal, however, at day 4-5 post-electroporation the cells that had showed fluorescence died without allowing the spread of the virus to the surrounding cells (data not shown).
This result suggests that gH/UL116 complex alone, in the absence of gH/gL-based complexes, is not sufficient for a productive egress of the virus neither for a cell-to-cell viral infection.

Characterization of TRG-ΔstopUL116 (UL116 knock-out) recombinant virus

The analyses described above suggest that UL116 cannot replace gH/gL-based complexes to primer the early stages of infection, despite its localization on the virus envelope. However, the presence of the gH/UL116 complex on the HCMV virion suggests that UL116 may be involved in mechanisms that influence the infectivity of the virus.
To this aim, we generated a recombinant virus defective in the expression of the UL116 protein. Following the same approach used for the knock-out of gL, we generated the TRG-ΔstopUL116 clone. This clone carries a single nucleotide insertion in the CDS of the UL116 gene causing a frame-shift and a premature STOP codon formation (Figure 3B).

The absence of UL116 expression causes a drastic slowdown of infection in ARPE-19 epithelial cells and a strong reduction of gH-based complexes in virions.

After the regeneration of recombinant viruses in MRC-5 cells electroporated with BACs, supernatants were used to infect HFF-1 fibroblasts to amplify viral particles. Fibroblasts have always been the standard cell type for isolation and propagation of HCMV from patient samples and are still the most efficient producer cell line irrespective of the virus strain. However, epithelial cells are one of the major targets of HCMV infection and are assumed to play an important role during host-to-host transmission since they lay all external body surfaces. Therefore, we investigated infectivity into these two different cell types to verify if the mutations introduced in our recombinant viruses could have an effect on viral tropism.
Infection of both HFF-1 fibroblasts and ARPE-19 epithelial cells was performed at a multiplicity of infection (MOI) 1. The propagation of the infection of three viruses, TRG-wt, TRG-ΔstopUL116 and TRG-ΔstopUL148_myc (recombinant virus, lacking of the UL148 protein, generated as control) was measured by harvesting the cultures at different time points and analyzing their GFP signal by flow cytometry. The propagation of TRG-ΔstopUL116 infection in HFF-1 followed a similar trend to that of the TRG-wt and TRG-ΔstopUL148_myc. Conversely, the propagation of infection in ARPE-19 showed substantial differences: whereas the knock-out of UL148 increased virus dissemination (as known in literature [Li et al., 2015]), the TRG-ΔstopUL116 virus was substantially less infective, roughly half of the cell population compared to the wild-type. These effects were also visualized as cytopathic effect by optical and fluorescence microscopy (Figure 4A and B).

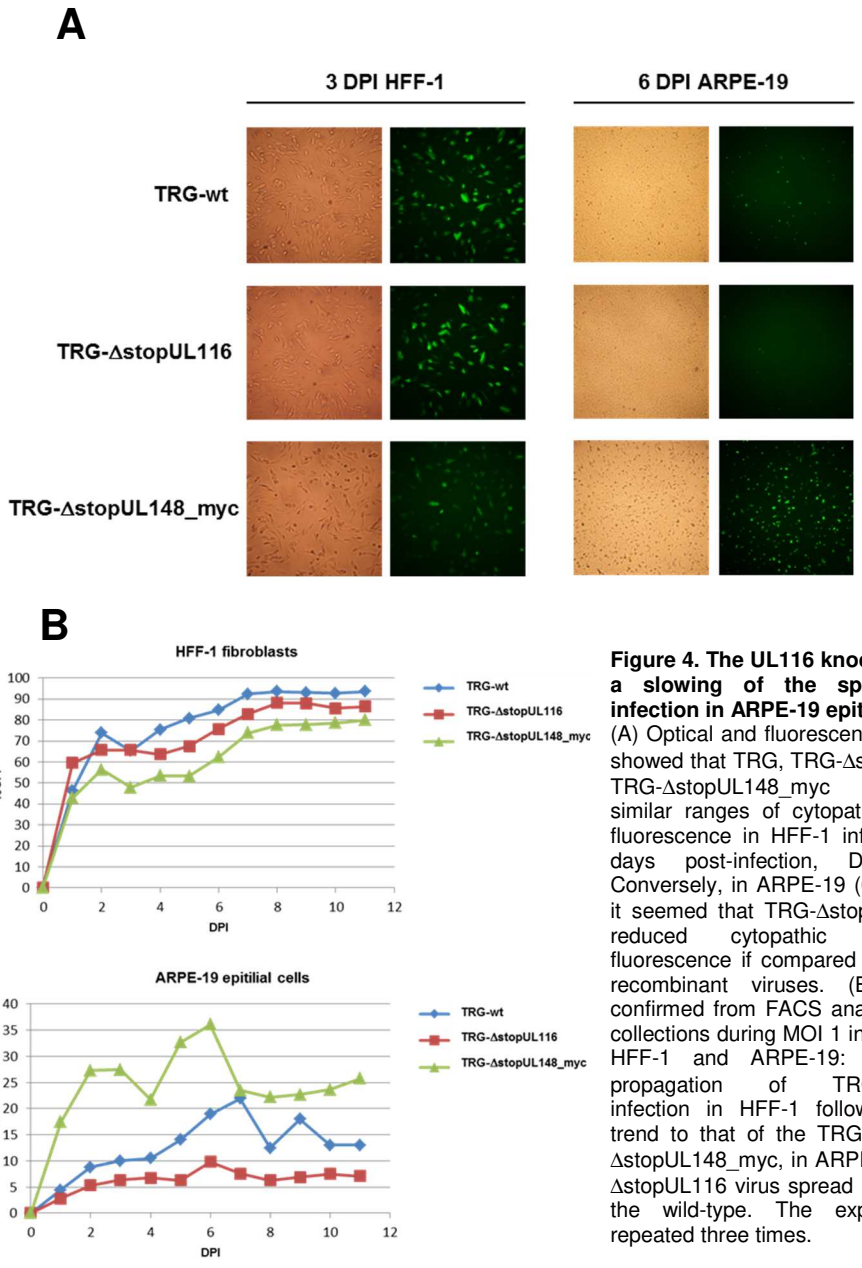

Figure 4. The UL116 knock-out causes a slowing of the spread of the infection in ARPE-19 epithelial cells. (A) Optical and fluorescence microscopy showed that TRG, TRG-ΔstopUL116 and TRG-ΔstopUL148_myc viruses had similar ranges of cytopathic effect and fluorescence in HFF-1 infected cells (3 days post-infection, DPI, images). Conversely, in ARPE-19 (6 DPI images) it seemed that TRG-ΔstopUL116 had a reduced cytopathic effect and fluorescence if compared with the other recombinant viruses. (B) This was confirmed from FACS analyses on daily collections during MOI 1 infection of both HFF-1 and ARPE-19: although the propagation of TRG-ΔstopUL116 infection in HFF-1 followed a similar trend to that of the TRG-wt and TRG-ΔstopUL148_myc, in ARPE-19 the TRG-ΔstopUL116 virus spread at least half of the wild-type. The experiment was repeated three times.

To the aim of verifying whether the different spreading kinetics reflect a different pattern of the main envelope species, we performed the analysis of the gH-based complexes on isolated virions. Using an anti-gH polyclonal antibody to detect gH/gL complexes resolved under non-reducing conditions, we noted that a ~135-kDa band, representing a disulfide-linked complex of gH/gL/UL128, was ~2-fold less abundant in TRG-ΔstopUL116 virions than in TRG-wt virions while, as previously reported (Li et al., 2015), the TRG-ΔstopUL148_myc virion exhibited an increase of the gH/gL/UL128 species (Figure 5A and C). Moreover, the ~300-kDa band representing a disulfide-linked complex of gH/gL/gO, showed a strong reduction in both knock-out mutants. WB analysis in non-reducing conditions is useless in revealing the gH/UL116 complex since these two proteins are non-covalently associated (Caló et al., 2016). However, gH and UL116 can be revealed separately with specific antibodies and the fraction of free-gH detected in virions can be *bona fide* assigned to the species associated to UL116. This assumption is supported by the repeated observation that gH alone does not enter the secretory pathway and remain trapped at early folding stages into the ER. Interestingly, we revealed an intensity increase of both UL116 and gH bands in the TRG-ΔstopUL148_myc virions if compared with the TRG-wt. In addition, despite free-gH was detectable in whole cell lysates of infected HFF-1 (Figure 5B), it was completely undetectable in TRG-ΔstopUL116 virions (Figure 5A). These last observations provide a strong evidence that gH/UL116 is part of the viral envelope complexes.

Altogether, these data demonstrate that the absence of the UL116 gene alters complexes composition on the mature virus without affecting their diffusion in fibroblasts but impairing virus spread in epithelial cells.

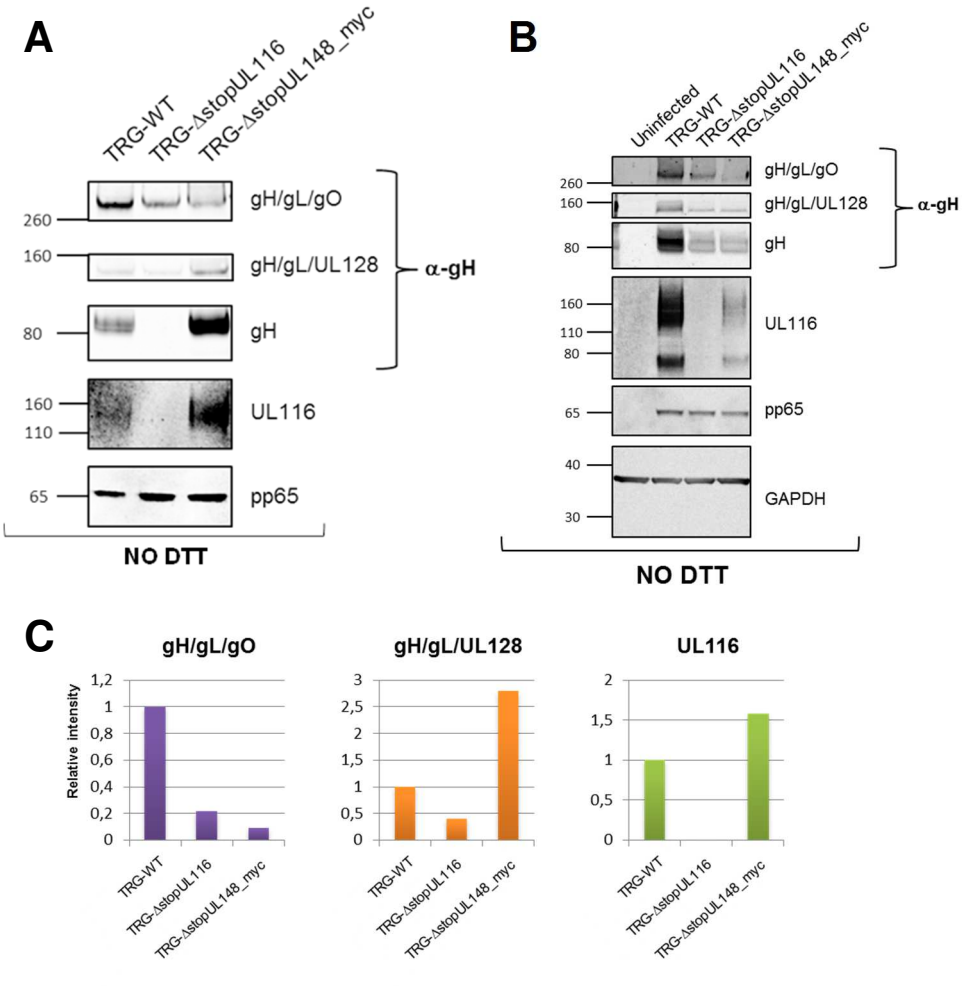

Figure 5. Disruption of UL116 alters the ratio of gH/gL complexes in strain virions. (A) Lysates of sucrose cushion-purified virions (5 DPI) were electrophoresed under nonreducing conditions. gH alone, gH/gL/gO and gH/gL/UL128 complexes were detected using a gH polyclonal antibody. Virion lysates were also assayed for detection of UL116 and pp65 (tegument protein). (B) Whole cell lysates of infected HFF-1 (5 DPI) electrophoresed under nonreducing conditions. (C) Relative intensity of gH/gL/gO, gH/gL/UL128 and UL116 in virion lysates, normalized to pp65 and compared to TRG-wt values. The experiment was repeated two times.

Analyses of the interaction between UL116 and UL148 HCMV proteins

The data shown in previous sections showed that, in the absence of UL116 expression, viral composition of the gH/gL/gO and Pentamer complexes undergo serious modification. This kind of phenotypic variation has been previously observed by *Li et al.* who described the UL148 protein to play crucial role for the complexes switch in the endoplasmic reticulum during the HCMV infection. We first sought to check that interaction between gH and UL116 starts in the ER and then if there is any interaction, either direct or indirect, between UL116 and UL148.

UL148 co-precipitates with UL116 in HFF-1 infected fibroblasts.

To investigate the possibility that UL116 could interact with UL148 during the infection we used the recombinant virus, TRG-UL148_myc, expressing the UL148 protein with a myc-tag in frame at C-TERM. Hence, we performed co-immunoprecipitation (CoIP) experiments on extracts of HFF-1 infected cells. Extracts from cells infected with TRG-wt and TRG-ΔstopUL148_myc viruses were used as controls. CoIP were performed with three different antibodies: anti-UL116, anti-myc (to pull down UL148 associated proteins) and anti-gH mAb MSL-109. Each CoIP elution was separated by SDS-PAGE and revealed by WB. As expected, UL116 was detected in anti-gH CoIP elutions in each infected cell lysate, and UL148_myc was detected only from extracts of cells infected with TRG-UL148_myc (Figure 6). Notably, pulldown of UL116 revealed co-precipitation of UL148_myc. Similarly, immunoprecipitation of UL148_myc resulted in the CoIP of UL116. These results strongly suggest that UL116 is part of the complexes associating with UL148 although they did not distinguish between a direct interaction or an associated mediated by gH as part of the gH/UL116 complex.

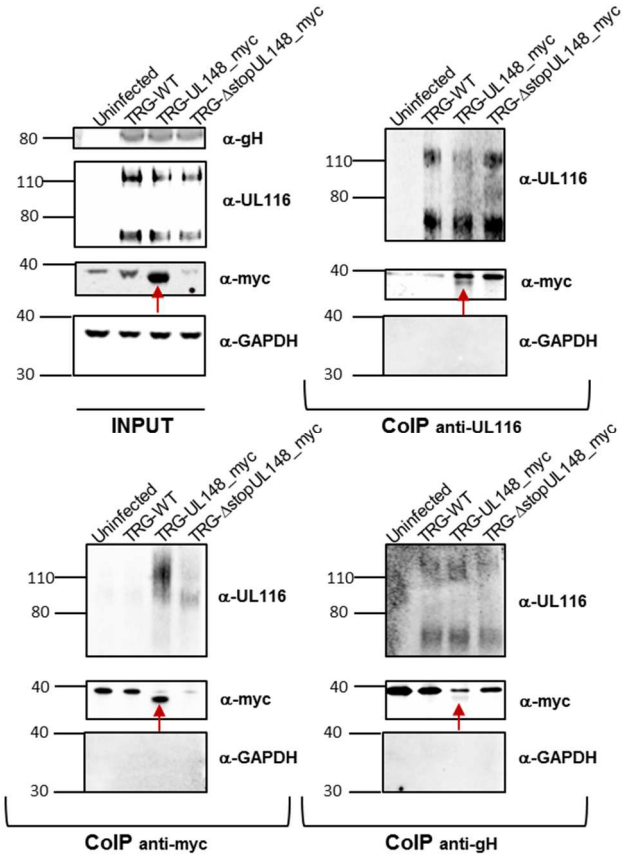

Figure 6. CoIP studies from infected cells.
Anti-UL116 (H4), myc and gH (using MSL-109 antibody) immunoprecipitates from uninfected and infected HFF-1 with TRG-wt, TRG-UL148_myc and TRG-ΔstopUL148_myc were assayed for detection of UL116, UL148_myc and GAPDH. Input lysates were also assayed for detection of gH. Red arrows indicate UL1148_myc band. The experiment was repeated two times.

UL148 co-precipitates directly with UL116 and gH in HEK-293T co-transfected cells

In order to understand whether the interaction between UL116 and UL148 occurs directly or not, we decided to perform CoIP experiments on co-transfected cells. In particular, plasmids expressing tagged either gH, UL116 or UL148 were used to transfect HEK-293T cells including each plasmid alone as control. All the different combinations of these plasmids were considered. Co-immunoprecipitations were performed with an anti-HIS antibody to detect proteins associated with UL148 and by anti-gH mAb MSL-109 to reveal species associated with gH. Each immunoprecipitated sample was treated for WB using anti-UL116, anti-myc (for gH), and anti-HIS (for UL148) as probes. Pulldown of gH resulted in association with both forms of UL116 but not with UL148 (Figure 7, top, lane 1 and 2) excluding a faint signal in correspondence with the co-transfection gH-UL148 (Figure 7, bottom, lane 3). Conversely, immunoprecipitation of UL148 resulted in the co-immunoprecipitation of gH (Figure 7, middle, lane 9 and 11), but only with UL116 lower form (Figure 7, top, lane 9 and 12) in contrast with that we previously have seen in infected cells CoIP.
These results demonstrate that UL148 is able to directly interact both with gH and UL116. Moreover, it seems that UL148 releases UL116 once dimerization with gH is achieved but it still accompanies the gH/UL116 complex through a certain maturation since UL116 acquires complex Golgi-type glycosilation.

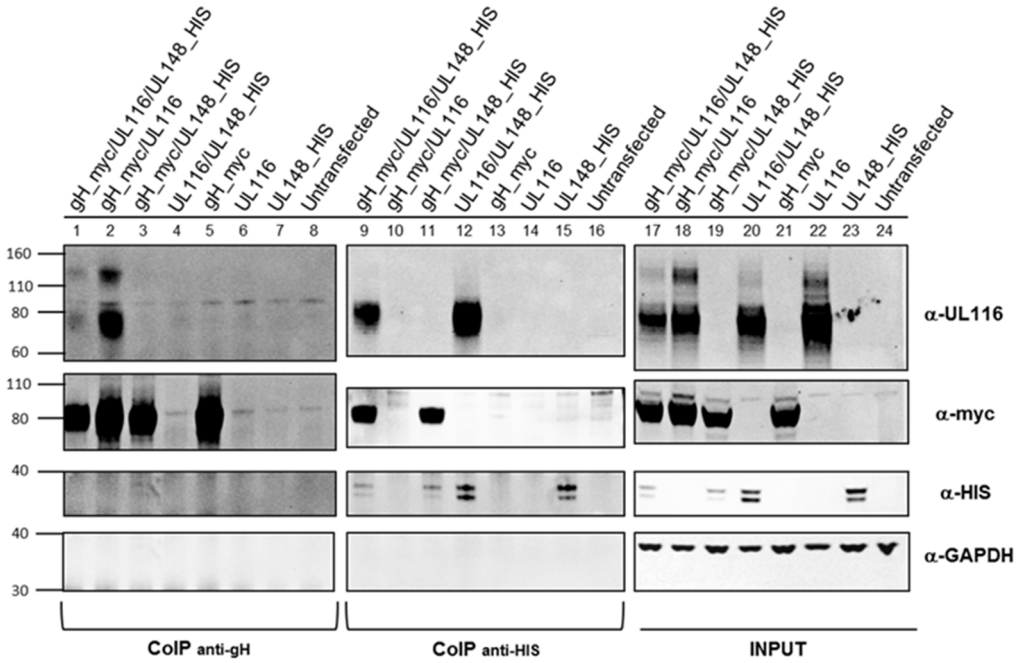

Figure 7. CoIP studies from transfected cells.
Anti-gH (using MSL-109 antibody) and anti-HIS (UL148) immunoprecipitates from untransfected and all the different combinations of transfected HEK-293T cells (plasmid expressing either gH_myc, UL116 or UL148_HIS) were assayed for detection of UL116 (F11), gH_myc, UL148_HIS and GAPDH. Input lysates were also assayed with the same probes. The experiment was repeated two times.

Preliminary investigation on viral proteomic patterns

By analyzing the composition of complexes on virions we observed that UL116 depletion alters the levels of gH/gL/gO and Pentamer as compared to the wild type virus. Conversely, the absence of UL148 causes a decrease in the gH/gL/gO complex and an increase in Pentamer. We have also observed that these differences are mainly reflected during the dissemination in infected ARPE-19 epithelial cells.
In order to investigate if there are other viral factors that are influenced by the UL116 knock-out, and consequently the virus tropism, we decided to perform proteomic analyses on virions.

2D-Electrophoresis and mass spectrometry analyses reveal the loss of pp71 in TRG-ΔstopUL116 virions

Supernatants from infected HFF-1 at MOI 1 with TRG-wt or TRG-ΔstopUL116 were harvested after 5 DPI, the virion were purified and then treated for two-dimensional gel electrophoresis (2-DE). We adopted this approach to comparatively analyze the wild type and mutant HCMV virion proteomes obtaining a refined protein separation on the basis of their isoelectric point and molecular weight. We started with the TRG-wt and TRG-ΔstopUL116 purified virions and the gels obtained, stained in Coomassie blue after the two dimension runs, are shown in *Figure 8*.

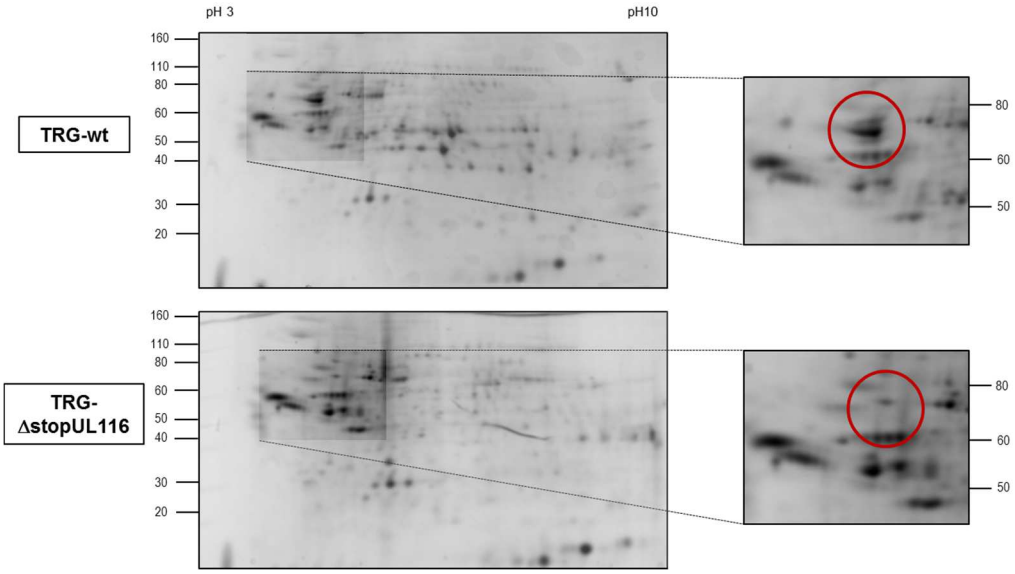

Figure 8. Two-dimensional gel electrophoresis (2-DE) on purified virions.
Purified Virions were harvested from supernatants of HFF-1 infected cells at MOI 1 with TRG-wt or TRG-ΔstopUL116 were treated and separated first by their isoelectric point using gel strips (pH 3-11) and then by their molecular weight through SDS– PAGE. Gels were revealed with colloidal coomassie staining. Each differential point was collected from the gels and treated to be analyzed by mass spectrometry. The HCMV strain Merlin proteome database was used for proteins identification. In red circle is indicated the pp71 protein, detected in TRG-wt and not in TRG-ΔstopUL116.

Comparison between the two gels showed several differential bands that were collected and analyzed by mass spectrometry (MS) to be identified. The most promising results obtained corresponded to a band present in TRG-wt but not in TRG-ΔstopUL116 (Figure 8, red circled). Mass spectrometry analysis allowed us to identify this species as pp71 tegument protein.
Although the MS analysis is still ongoing, this preliminary result seems very interesting given the importance of pp71 in the early phases of viral replication. Indeed, this protein is usually located in the HCMV virion tegument layer, which is defined as the space enclosed by the lipid envelope but outside of the protein capsid, and it is recruited in the nucleus of infected cells immediately after virion entrance to ensure the transcriptional activation of viral Immediate Early genes. The pp71 transactivation role is to facilitate the progression to lytic infection and viral replication of HCMV. Its incorporation mechanism as virion tegument protein is not fully characterized, such as its transport to infected cells nucleus. However it seems that pp71 nuclear localization is regulated by a phosphorylation on its threonine 223 that alters the protein conformation thus impairing its nuclear transport (Baldick et al., 1997; Kalejta, 2008; Shen et al., 2008). If confirmed, this difference could explain the lower propagation observed in UL116-null virus infected cells.

DISCUSSION

HCMV envelope glycoproteins are potential components of a prophylactic HCMV vaccine. Recently, preclinical vaccination studies have focused to the components of the two gH-based complexes: the Trimeric gH/gL/gO and the Pentameric gH/gL/UL128/UL130/UL131A. Both complexes are required to be present on the virions for an efficient infection and their relative amount correlates with specific cell tropism. In spite of the broad range of human cells infected by HCMV, the cell types that have been paradigmatically used to assess infectivity are fibroblasts, for which the gH/gL/gO complex is sufficient, and epithelial/endothelial cells that require Pentamer. Receptors for the two complexes have been recently identified as PDGFR and Nrg2 respectively (Kabanova et al., 2016; Yurochko et al., 2017). Given the clear importance of alternative gH/gL complexes, recent studies have also directed to understand the mechanisms that regulate their relative abundance during infection. The product of the *UL148* gene has been proven to be a viral modulator interacting with gH in the ER and promoting incorporation of the gH/gL/gO complex (Li et al., 2015). More recently, a chaperone-like protein favoring incorporation of Pentamer on the virion envelope has been identified as the product of *US16* gene (Luganini et al., 2017). This protein interacts directly with UL130 and in its absence the Pentamer levels, and consequently the epithelial/endothelial infectivity, is strong reduced.

In our laboratory we achieved a preliminary characterization of the product of the HCMV *UL116* gene from the TR strain. This protein is a virion envelope glycoprotein forming a noncovalent heterodimer with gH, independently from gL. So far, the gH/UL116 complex remains the only gL-independent gH dimer for the entire *herpesviridae* family (Caló et al., 2016). In this contest, it follows that the UL116 functional characterization is particularly relevant both for purely scientific and applicative purposes.

In this work we investigated several hypothetical functional aspects of UL116, trying to outline an overall picture of its role during HCMV infection.
Hence, we first explored the hypothesis that gH/UL116 could participate as viral interactor on cell surface during the early stages of HCMV infection, conducting several analyses on its ability to bind cells in a specific way. Although a preliminary experiment showed that gH/UL116 purified complex interacted *in silico* with TREM-1, IL-6R and TARM-1 cellular membrane receptors, our validation analyses disproved these results. In fact, we showed that gH/UL116 complex was not able to bind the surface of transfected CHO-K1 cells that ectopically expressed each single putative receptor. Moreover, FACS analysis of gH/UL116 complex binding to a panel of human cell lines failed to identify specific binding. Indeed, we concluded that specific binding to TREM-1, IL-6R and TARM-1 were false-positive outputs from *in silico* experiment. However, it could also be possible that the recombinant gH/UL116 complex does not have the same conformation to the one found on the virions. As matter of fact, the dimer used in this study was purified as secreted product from transfected cells with the soluble gH form in combination with UL116, thus in a context devoid of any other viral factor. Anyway, since it is impossible to purify the complex from native virion, the hypothesis of gH/UL116 as cell tropism factor was abandoned.
A second hypothesis we worked on considered UL116 able to substitute gL forming an alternative scaffold equally functional. To this aim, we tried to generate a TRG-ΔstopUL115 (gL knock-out) recombinant virus to ensure the only presence of gH/UL116 complex on the surface of the virus envelope. The gL glycoprotein has been previously described as an essential product for HCMV egress and infection, however it is transcribed in the same transcription unit of UL116 (UL119-UL115 transcript) and we speculate that the previously described knock-outs were made irrespective of the maintenance of the other products. Hence, we constructed a recombinant virus with a stop codon close to gL N-

terminal region, thus maintaining the integrity of the transcription unit. Following transfection of the recombinant BAC into the cells we observed initial HCMV replication but the culture failed to regenerate the virus suggesting that gH/UL116 complex alone is not able to replace absence of gH/gL-based complexes.
Although gH/UL116 did not appear involved in the early stages of HCMV infection, its lack of expression influenced other observed viral aspects. In fact, our analyses on TRG-ΔstopUL116 recombinant viruses showed that the absence of UL116 impaired the HCMV infection progression in ARPE-19 epithelial cells. In addition, we also reveal that TRG-ΔstopUL116 purified virions showed a strong gH/gL/gO and Pentameric complexes reduction. This decrement in gH/gL-based complexes seems to not influence the TRG-ΔstopUL116 spreading in HFF-1 fibroblasts. Our suggestion was that since the HFF-1 cells are very permissive for the replication of the virus, the slowing down of the mutant spreading was more evident and more appreciable in cells, such as ARPE-19, in which the replication of the virus is more problematic. Interestingly, UL116-null virions lack the prominent monomeric gH species readily detected from wild-type HCMV virions following non-reducing SDS-PAGE, suggesting that the gH/UL116 is a significant constituent of HCMV virions.
The evidence of the interaction between gH and UL116 in infected cells and the low levels of the gH/gL/gO and Pentameric complex found in the UL116 knock-out virions led us to investigate a possible involvement of UL116 in the mechanisms that regulate the gH-based complexes formation or stability. Here, we show that UL116 can interact both with gH and UL148. In particular, in infected HFF-1 UL116 co-immunoprecipitates with gH and also with UL148. This interaction was confirmed also in CoIP experiment on transfected HEK-293T cells, which revealed that UL116 interacts with UL148 despite the absence of gH. Interestingly, the UL148-null virions show high levels of UL116 and gH monomeric species. These results seem to suggest that the interaction between UL116 and UL148, which takes place in the

ER, controls the gH/UL116 complex levels. Moreover, it also seems that UL148 remains bound to the immature gH/UL116 complex since, in infected cells, UL148 co-precipitates with the higher mass weight form of UL116, which has identified as the Golgi modified species. Thus, the ER resident UL148 could function as an escort protein for the gH/UL116 complex at least to the early phase of the anterograde transport.

Altogether, our data are consistent with a multifaceted role of UL116 during the entire HCMV life cycle. In the ER, a complex pattern of interactions regulates the amount and the type of gH/gL-based complexes to be exposed on the virions while the absence of either UL116 or UL148 alters these equilibria. Our hypothesis is that UL116, probably together with UL148, is the early gH interactor that stabilizes the gH conformation, being able to either sort the ER in combination with UL116 or covalently link gL to form the known gH/gL complexes (Figure 9, [1]). It would be interesting to define if UL116 establishes additional interactions in the ER, in particular with the US16 protein. The latter appears to be implicated in the complexes switches favoring the Pentamer inclusion, but we do not know any cross-talking with UL116 and/or UL148.

The gH/UL116 complex could have also different roles on the host cell membrane or on the viral envelope. Although we have not been able to demonstrate any direct interaction of the heterodimer with host or other viral proteins, the UL116 absence causes a reduced cell-to-cell spread in epithelial cells that lead us to speculate its involvement in viral dissemination and/or replication in this cell type (Figure 9, [2-3]).

Finally, our preliminary analyses on proteome variation in UL116-null virions showed that the cytoplasmic viral protein pp71, an important transactivator of the early genes, is absent. We do not know what kind of correlation is between gH/UL116 complex and pp71 but, waiting for experimental validation, it is tempting to speculate that gH/UL116 complex is involved in the mechanism of pp71 inclusion within the HCMV tegument.

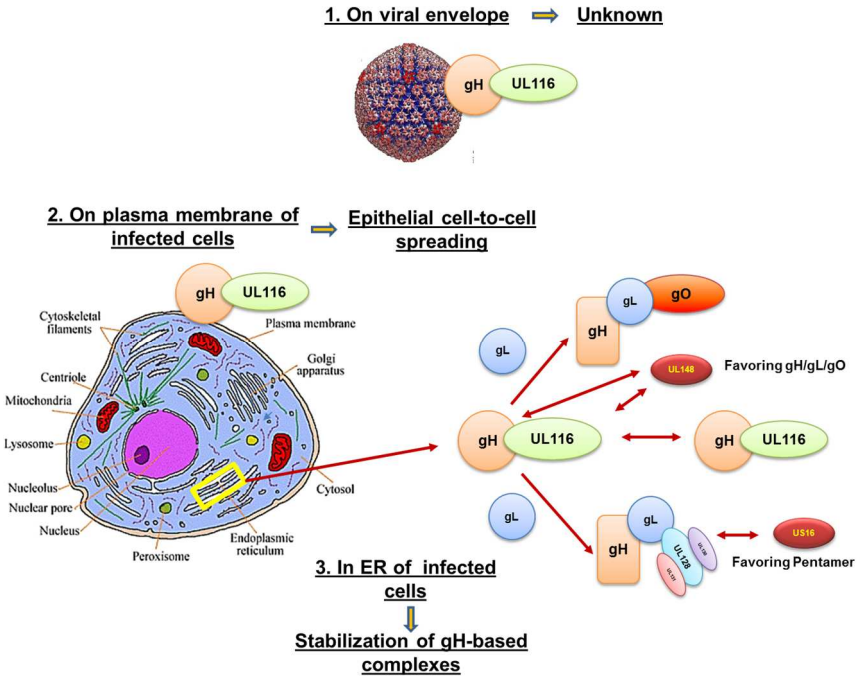

Figure 9. Graphical representation of proposed UL116 functions.

1. The gH/UL116 complex, localized on the viral envelope, still has unknown function.
2. On the plasma membrane of epithelial infected cells, gH/UL116 complex could be involved in the HCMV dissemination through cell-to-cell spreading.
3. In HCMV infected cells, UL116 interacts with both UL148 and gH in the ER. gH protein, stabilized by UL116 interaction, is able to sort the ER either as non-covalent gH/UL116 heterodimer or establish a disulfide bond with gL that replace UL116 on gH. gH/gL dimer could form the gH/gL/gO complex, favored by UL148, or the Pentameric complex, favored by US16.

Transparency Declaration

This study was sponsored by GlaxoSmithKline Biologicals SA.
Diego Amendola is a PhD student and participates in a post graduate studentship program at GSK.

STAR METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

CHO-K1 (Hamster [*Cricetulus griseus*] ovary epithelial-like cells; XX), MRC-5 (Human [*Homo sapiens*] lung normal Fibroblasts; XY), HFF-1 (Human [*Homo sapiens*] skin/foreskin normal Fibroblasts; XY), THP-1 (Human [*Homo sapiens*] peripheral blood acute monocytic leukemia cells; XY), ARPE-19 (Human [*Homo sapiens*] retinal pigmented normal epithelial cells; XY); CALU-3 (Human [*Homo sapiens*] lung adenocarcinoma epithelial cells; XY), Jurkat (Human [*Homo sapiens*] peripheral blood acute leukemia T lymphocytes; XY), HEK293T (Human [*Homo sapiens*] embryonic kidney epithelial cells), HL-60 (Human [*Homo sapiens*] peripheral blood acute leukemia promyeloblast; XX). Cells were all obtained from ATCC. MRC-5, HFF-1, HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine heat inactivated serum, 100 I.U./mL penicillin and 100 mg/mL streptomycin (Penicillin-Streptomycin) and 1 mM sodium pyruvate. CHO-K1, ARPE-19 and CALU-3 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, HEPES) supplemented with 10% fetal bovine heat inactivated serum, 100 I.U./mL penicillin (Penicillin-Streptomycin) and 100 mg/mL streptomycin and 1 mM sodium pyruvate. THP-1 and Jurkat cells were cultured in Roswell Park Memorial Institute (RPMI 1640, GlutaMAX Supplement, HEPES) 1640 with 10% fetal bovine heat inactivated serum, 100 I.U./mL penicillin and 100 mg/mL streptomycin (Penicillin-Streptomycin) and 1 mM sodium pyruvate. HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI 1640, GlutaMAX Supplement, HEPES) 1640 with 20% fetal bovine heat inactivated serum. All cell lines were incubated at 37°C with 5% CO_2.

Viruses

A bacterial artificial chromosome (BAC) containing the genome of the HCMV TR (carrying inside also a GFP immediate early expressing gene cassette in the intergenic region between US32 e US33A genes) strain was obtained from Oregon Health Science University (Murphy et al., 2003b). TR, a clinical HCMV strain derived from an ocular vitreous fluid sample from a patient with HIV disease (Smith et al., 1998), was cloned into a BAC after limited passage in fibroblasts (Murphy et al., 2003a). HCMV strain TR-GFP (TRG) and each recombinant virus were propagated in HFF-1 fibroblasts grown to 70-80% confluency, as previously described (Cell Lines, STAR Methods), using infectious supernatants at an MOI of 1. At 24 hpi (hours post-infection) infected cells show GFP-fluorescence for each of the viruses used here. When cells exhibit 100% CPE (or GFP signal) and the 50% of cells are detached from the plate, medium supernatant was collected and cleared of cell debris by centrifugation for 20 min at 3,200 × g, 4°C before aliquoting and storing at -80°C.

To titrate the viruses it has been performed a Titration Assay previously described (Britt, 2010) with minor modifications. In brief, 8 5-fold serial dilutions of samples were performed in DMEM supplemented with 1% fetal bovine heat inactivated serum and 1 mM sodium pyruvate, and 150 µl of each dilution was applied to duplicate wells of a 96-well flat bottom cluster plate containing 3×10^4 HFF-1 fibroblasts, incubated for over-night (O/N) at 37°C with 5% CO_2 before infection. 24 hpi the infected cells were trypsinized and transferred in a 96-well round bottom cluster plate and treated to evaluate how many cells exhibited GFP-signal through FACS analysis with BD LRSII Special Order System (Becton Dickinson, San Jose, CA) equipped with High Throughput Sampler (HTS) option. Titer was calculated using the following equation: Titer (IU/ml) = $(N \times P)/(V \times D)$ [Note: N = Cell Number in each well used for infection day; P = percentage of GFP positive cells (considering the dilution virus exhibiting GFP

signal < 20%); V = virus volume used for infection in each well (ml); D = dilution fold; IU = infectious unit]

METHOD DETAILS

BAC Mutagenesis

To generate recombinant viruses a Two-step Red-mediated recombination method has been used, as previously described (Karstentischer et al., 2006), with minor modifications, where the BAC TR-GFP was used as starting template. In brief, kanamycin resistance cassette, flanked by I-SceI restriction enzyme cleavage sites, was amplified from pEPkan-S shuttle vector using primers containing homologous regions for the integration in the region of interest. Recombination events were performed with E. coli GS1783 strain containing a BAC clone of the HCMV TR-GFP (TRG) strain, the lambda Red system under the control of a heat-inducible promoter and the I-SceI genes under the control of an arabinose-inducible promoter (Tischer et al., 2010). The first recombination step consists in the electroporation of the purified PCR-amplified cassette in competent, heat-induced GS1783 cells. Positive clones for cassette integration were selected based on kanamycin resistance and screened both by PCR and sequencing. The second recombination was triggered through both heat-shock and arabinose and results in the excision of the kanamycin resistance, leaving the mutation in frame with the gene of interest. Presumptive clones were screened by PCR and sequencing analyzed by Vector NTI. All the primers used are listed in Table S1 (SUPPLEMENTAL TABLES AND FIGURES).

Reconstitution of infectious viruses

To reconstitute the virus MRC-5 fibroblasts were electroporated ("nucleofected") using a Cell Line Nucleofector Kit V according to the manufacturer's protocol. In brief, for each reaction, 1×10^6 freshly trypsinized MRC-5 fibroblasts were pelleted by

centrifugation at 300 × g for 5 min, washed two times with PBS and then resuspended in a solution containing 1,5 μg of BAC and 0,3 μg of pcDNA3.1-pp71 plasmid (cotransfection of HCMV protein pp71-expressing plasmid markedly increases the efficiency of virus reconstitution from transfection of infectious viral DNA because pp71 acts as a viral transactivator to help initiate lytic infection [Baldick et al., 1997] premixed with 100 μL of Nucleofector solution (82 μL of Nucleofector solution and 18 μL of supplement). The cell suspension was then electroportated using a Nucleofector II (program D-023), and then plated and cultured in DMEM supplemented with 1% fetal bovine heat inactivated serum and 1 mM sodium pyruvate. 24h after the electroporation medium was changed and cells were cultured by standard methods. When cells exhibit 100% CPE (or GFP signal) and 50% of cells are detached from the plate, medium supernatant was collected and cleared of cell debris by centrifugation for 20 min at 3,200 × g, 4°C before aliquoting and storing at -80°C. To determine virus titer the "Titration Assay" has been performed as previously described (Viruses, STAR METHODS).

HCMV Virions purification

The supernatant of infected cells was collected 5-7 days after infection and cleared of cell debris by centrifugation for 20 min at 3,200 × g, 4°C. Clear supernatant was transferred to polycarbonate ultracentrifuge tubes under lied with 20% sucrose cushion and centrifuged at 23000 rpm in a Beckman SW32Ti rotor for 50 minutes. The pelleted virus was treated as desired.

Viral Infectivity Studies

These analyses were conducted in 24-well cluster plates which had been seeded 1 day prior to infection in parallel with 5×10^4 HFF or 9×10^4 ARPE cells per well. For each day post-infection (DPI), including the day 0, the infected cells were photographed using a

fluorescence inverted microscope (Zeiss Axiovert 200) and then they were trypsinized and treated to evaluate how many cells exhibited GFP-signal through FACS analysis.

Transfections

Lipofectamine LTX was used to transfect CHO-K1 cells with TREM-1_HA, IL-6R_HIS or TARM-1_HIS expressing plasmids according to the manufacturer's protocol. The CHO-K1 transfected cells were trypsinized 48h post-transfection and used for the "Protein Binding assay on non-permissive cells". Lipofectamine 2000 was used to transfect HEK-293T cells with gH_myc, UL116 or UL148_HIS expressing plasmids according to the manufacturer's protocol. The HEK-293T transfected cells were trypsinized 48h post-transfection and used for immunoprecipitation assays.

Flow Cytometry

For the detection of membrane-exposed TREM-1 or IL-6R CHO-K1 cells transiently transfected were trypsin detached 48h post-transfection, incubated for 20 min at room temperature (RT) with Live/Dead Aqua, diluted 1:400 in PBS, and incubated with 1:200 of anti-TREM-1 Polyclonal Antibody, or anti-IL-6R Monoclonal Antibody [B-R6] for 60 min on ice. After three washes in PBS, the Alexa Fluor 647-conjugated anti-goat or anti-mouse secondary antibody was added at a 1:300 dilution and incubated for 30 min on ice. For Protein Binding assay on non-permissive cells, CHO-K1 transiently transfected cells were incubated for 60 min with blocking buffer (PBS with 1% Bovine Serum Albumin, BSA) + 200 ng/µL of gH/UL116 recombinant complex (HIS-tagged at gH C-TERM). To reveal gH/UL116 protein cells it was used HIS Tag Monoclonal Antibody [HIS.H8] and then Alexa Fluor 647-conjugated anti-mouse secondary antibody. A similar procedure was adopted to reveal gH/UL116 or

gH/gL/gO recombinant complexes for Protein Binding assay on different cell lines (HFF-1, ARPE-19, HL-60, THP-1, JURKAT, CALU-3). A total of 10^5 cells were analyzed for each histogram using FACS BD Canto II (Becton Dickinson, Heidelberg, Germany).

Immunoprecipitations

HFF-1 cells were infected with HCMV TRG-wt, TRG-UL148_myc or TRG-ΔstopUL148_myc. Protein expression was allowed to proceed for 144 hpi, and then cells were washed in 1× PBS and lysed with a lysis buffer (1% TRITON-X100 in PBS) in presence of protease inhibitors (EDTA-free, EASYpack Protease Inhibitor Cocktail). Five hundred micrograms of total protein extract was incubated overnight at 4°C with 5 µg of each type of used antibody (Myc Tag Monoclonal Antibody [Myc.A7], UL116 Monoclonal Antibody [H4], gH Human Monoclonal Antibody [MSL-109]). Complexes were immunoprecipitated using Dynabeads Protein G according to the manufacturer's protocol. The beads were washed in lysis buffer and then boiled for 5 min in SDS-PAGE loading buffer with DTT. Eluated proteins were separated on SDS-PAGE, and immunoblotting was performed as described above.

A similar procedure was adopted for immunoprecipitation from extracts of transfected HEK293T cells. One well of transfected HEK293T cells it has been used for each immunoprecipitation reaction using 5 µg of each antibody (gH Human Monoclonal Antibody [MSL-109], HIS Tag Monoclonal Antibody [HIS.H8])

Immunoblotting

Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% polyacrylamide pre-cast gels (Bolt 4-12% Bis-Tris Plus Gels) under standard conditions. Proteins were transferred to nitrocellulose membranes

(iBlot 7-Minute Blotting System, Invitrogen), and membranes were blocked with PBS containing 0.1% Tween 20 and 10% powdered milk. Antibodies were diluted in PBS containing 0.1% Tween 20 and 1% powdered milk. For detection of primary antibody binding, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody and the West Pico Chemoluminescent Substrate were used according to the manufacturer's instructions. The densitometric analysis of signal intensity in Western blotting was performed with ImageLab software.

Two-Dimensional Electrophoresis (2-DE)

One hundred micrograms of purified virions were resuspended in 100 μL of PBS. Subsequently, it was added to the samples 10ul of a TCA 10X (100%), DOC 0.4% solution and incubated for 4 hours on ice. Samples were then centrifuged at 14000 g for 20 min; formed pellet were first washed with a 10% TCA solution and then twice with cold EtOH. Final pellet was resuspended in a buffer containing 7 M urea, 2 M thiourea, 2% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate, 2% w/v amidosulfobetaine-14, 65 mM DTT, 2 mM tributylphosphine, 20 mM Tris, 2% v/v carrier ampholyte IPG Buffer, 0,01% bromophenol blue. Proteins were adsorbed overnight onto Immobiline DryStrips (11 cm; pH gradient 3–11 non-linear), using the Immobiline Dry-Strip Reswelling Tray (Amersham Biosciences). Proteins were then separated by 2-DE. The first dimension was run using an IPGphor IEF Unit (Amersham Biosciences), applying sequentially 150 V for 60 min, 500 V for 35 min, 1000 V for 30 min, 2600 V for 10 min, 3500 V for 15 min, 4200 V for 15 min, and finally 5000 V to reach 12 kVh. For the second dimension, the strips were equilibrated with a the equilibration buffer containing 6 M urea, 50 mM Tris-HCl, 30% v/v Glycerol, 2% w/v SDS, 0,01% bromophenol blue first adding 2% w/v DTE/DTT and incubating for 5 min, and then with the same equilibration buffer instead adding 2,5% w/v and incubating

for 12 min. Hence, proteins were separated on linear 4–12% polyacrylamide gels pre-cast gels (4–12% Criterion XT Bis-Tris Protein Gel). Gels were stained with Colloidal Coomassie. Protein spots were excised and treated for mass spectrometry analyses as previously described (Ferrari et al., 2006).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Myc Tag Monoclonal Antibody (Myc.A7)	ThermoFisher	MA1-21316
HIS Tag Monoclonal Antibody (HIS.H8)	ThermoFisher	MA1-21315
Human TREM-1 Antibody	R&D Systems	AF1278
IL-6 Receptor Monoclonal Antibody (B-R6)	ThermoFisher	AHR0061
UL116 Monoclonal Antibody (H4)	Takis S.r.l.	N/A
UL116 Monoclonal Antibody (F11)	Takis S.r.l.	N/A
gH Human Monoclonal Antibody (MSL-109)	Ciferri et al., 2015	N/A
gH Rabbit Polyclonal Antibody	Ciferri et al., 2015	N/A
Bacterial and Virus Strains		
E. coli strain GS1783	Tischer et al., 2010	N/A
HCMV TR-GFP; GenBank TR strain sequence: Human herpesvirus 5 strain TR (KF021605.1)	Murphy et al., 2003b	N/A
Chemicals, Peptides, and Recombinant Proteins		
gH/UL116 recombinant complex	Caló et al., 2016	N/A
gH/gL/gO recombinant complex	Ciferri et al., 2015	N/A
Dulbecco's Modified Eagle Medium (DMEM, high glucose)	ThermoFisher	11965092
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, HEPES)	ThermoFisher	11330032
Roswell Park Memorial Institute (RPMI 1640, GlutaMAX Supplement, HEPES)	ThermoFisher	72400021
Penicillin-Streptomycin	ThermoFisher	15140122
Sodium Pyruvate	ThermoFisher	11360070
Phosphate buffered saline (PBS)	GSK LAO	N/A

Triton X-100	SIGMA	T8787
cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail, Roche	SIGMA	05892791001
Trypsin-EDTA (0.25%), phenol red	ThermoFisher	25200072
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	ThermoFisher	L34966
Bovine Serum Albumin	SIGMA	A2058
TWEEN 20	SIGMA	P9416
IPG Buffer, pH 3-11 NL	SIGMA	GE17-6004-40
Immobiline Drystrip pH 3-11 NL, 11 cm	SIGMA	GE17-6003-74
QC Colloidal Coomassie Stain	BIO-RAD	1610803
4–12% Criterion XT Bis-Tris Protein Gel, 11 cm IPG/prep+1 well, 550 µl	BIO-RAD	3450127
Critical Commercial Assays		
Cell Line Nucleofector Kit V	LONZA	VCA-1003
NucleofectorTM 2b Device	LONZA	AAB-1001
Dynabeads Protein G for Immunoprecipitation	ThermoFisher	10003D
Lipofectamine LTX Reagent with PLUS Reagent	ThermoFisher	15338100
Lipofectamine 2000 Transfection Reagent	ThermoFisher	11668027
Experimental Models: Cell Lines		
Hamster: CHO-K1	ATCC	CCL-61
Human: MRC-5	ATCC	CCL-171
Human: HFF-1	ATCC	SCRC-1041
Human: THP-1	ATCC	TIB-202
Human: ARPE-19	ATCC	CRL-2302
Human: CALU-3	ATCC	HTB-55
Human: Jurkat	ATCC	TIB-152
Human: HEK293T	ATCC	CRL-3216

Human: HL-60	ATCC	CCL-240
Recombinant DNA		
pEPkan-S	addgene	Plasmid #41017
pcDNA3.1-pp71; NCBI Reference Sequence: tegument protein pp71 [Human betaherpesvirus 5] (AGL96671.1)	GeneArt	N/A
pcDNA3.1-TREM1_HA; NCBI Reference Sequence: triggering receptor expressed on myeloid cells 1 isoform 1 [Homo sapiens] (NP_061113.1)	GeneArt	N/A
pcDNA3.1-IL6R_HIS; NCBI Reference Sequence: interleukin-6 receptor subunit alpha isoform 1 [Homo sapiens] (NP_000556.1)	GeneArt	N/A
pcDNA3.1-TARM1_HIS; NCBI Reference Sequence: T-cell-interacting, activating receptor on myeloid cells protein 1 isoform 1 [Homo sapiens] (NP_001129158.2)	GeneArt	N/A
pcDNA3.1-gH_myc; NCBI Reference Sequence: envelope glycoprotein H [Human betaherpesvirus 5] (AGL96664.1)	GeneArt	N/A
pcDNA3.1-UL116; NCBI Reference Sequence: protein UL116 [Human betaherpesvirus 5] (AGL96697.1)	GeneArt	N/A
pcDNA3.1-UL148_HIS; NCBI Reference Sequence: membrane protein UL148 [Human betaherpesvirus 5] (AGL96709.1)	GeneArt	N/A

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SUPPLEMENTAL TABLES AND FIGURES

Table S1. Primer sequences.

Primer	Sequence (5′-3′)
UL115-stop-BAC-F	TCGGCGCCAACTGGCTCCTTACCGTCACAC TCTCATCGTGCCGCAGACTTGATGTGCCGC CGCCCGGATTAAGGCTTCTCTTTCTCACCTG GACTAGGGATAACAGGGTAATCGAT
UL115-stop-BAC-R	CTGAGGAAACAATGGACAGCAGAAGGCAAC ACCACAGCAGTATCACCGGTCCAGGTGAGA AAGAGAAGCCTTAATCCGGGCGGCGGCACA TCAAGCCAGTGTTACAACCAATTAAC
UL116insB-stop-BAC-F	ACTTGCCGCTGTACAACGAATTCACCAGCTT TCGCCTGCCCACCTCATGATAGCGGCGGCG TAGGGATAACAGGGTAATCGAT
UL116insB-stop-BAC-R	AAAGCACAGAGCCAGGAAAAGCAACCAGCC CCGCCATCGCCGCCGCCGCTATCATGAGGT GGCCAGTGTTACAACCAATTAAC
UL148b-BAC-myc_F	GGCTCGCGTTTTCATAACCTACCTGGTGTC GCGGCGTCGGGAACAGAAACTGATTAGCGA AGAAGATCTGTAGGGATAACAGGGTAATCG AT
UL148b-BAC-myc_R	GAACGACGTGTGACGAGGACGTGGTTTCCG CAAGCCTCTACAGATCTTCTTCGCTAATCAG TTTCTGTTCGCCAGTGTTACAACCAATTAAC
UL148B-BAC-STOP_F	GGGCGGTGGCCGGCACGCCGCATTTCCTA ACCCGCGCAGCATGTTGCGCTAACTGTTCA CGCTAGGGATAACAGGGTAATCGAT
UL148B-BAC-STOP_R	CTAGCGTTGACAGACGGCCCGTGGAGGGC CAGTAGGACGAGCGTGAACAGTTAGCGCAA CATGCCAGTGTTACAACCAATTAAC
UL116_F	CCACGGTCTGGCCGTCATCGTCG
UL115_R	CAGGCATCGGCGCGTTAGTTCGG
PRE-UL116_F	CTGTTTGACGCCGTAACCCTGTGC
UL116_R	AACCGTGGTGGGAGTGGTGACGG
UL148-CTERM_F	AACGCGCGCCTGACCCGCGG
postUL148_R	GGTTGTAGGTTCGCTACTCG

RINGRAZIAMENTI

Ognuno di noi è il prodotto di ciò che ci accade e di come reagiamo.
Le persone che ci circondano e, soprattutto, la scelta delle persone che vogliamo al nostro fianco e su cui facciamo affidamento, danno un enorme contributo a ciò che noi siamo.
E se oggi sono qui, lo devo alle persone che più amo e che di più hanno condiviso il mio percorso.
Ringrazio la mia Famiglia, vicina più che mai nonostante la distanza che ci separa; ringrazio Giusy, che mi ha supportato e sopportato con amore e gioia, senza mai farmi pesare nulla; infine, ringrazio gli Amici e i Compagni di viaggio, valvole di sfogo indispensabili.
Tutti voi siete, e sarete sempre, il timone che mi guiderà sulla giusta direzione.