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# *Functional and structural characterization of HCMV complexes by dissecting molecular interactions*

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# *GLOSSARY*

**Bacterial Artificial Chromosome (BAC)**: is a DNA construct, based on a functional fertility plasmid (or F-plasmid), used for transforming and cloning in bacteria, usually *E. coli*. The bacterial artificial chromosome's usual insert size is 150–350 kb.

**Cytopathic effect:** refers to structural changes in host cells that are caused by viral invasion. The infecting virus causes lysis of the host cell or when the cell dies without lysis due to an inability to reproduce.

**gH/gL complex**: is required for the fusion of viral and plasma membranes leading to virus entry into the host cell. Following initial binding to host receptor, membrane fusion is mediated by the fusion machinery composed of gB and the heterodimer gH/gL. The complex may also be involved in the fusion between the virion envelope and the outer nuclear membrane during virion morphogenesis.

**gH/gL/gO complex**: is required for the HCMV entry into the fibroblast at the cell surface at neutral pH. gO is a highly glycosylated protein and has been shown to covalently interact with gH/gL. It has been proposed that gO might function as a molecular chaperone to promote gH/gL incorporation.

**Glycoprotein B**: fusion protein responsible for mediating virus and host membrane fusion during viral entry.

**Herpesviridae family**: is a large family of DNA viruses that cause diseases in animals, including humans. The members of this family are also known as herpesviruses. The classification hystorically is based on the architecture of the virion.

**Human Cytomegalovirus (HCMV):** is a species of the Cytomegalovirus genus of viruses, which in turn is a member of the viral family known as Herpesviridae, belongs to the Betaherpesvirinae subfamily.

**Multiplicity of infection (MOI)**: MOI is the ratio of agents to infection targets. For example, MOI is the ratio of the number of virus particles to the number of target cells present in a defined space.

**Pentameric complex**: is composed of 5 proteins, gH/gL/UL128/UL130/UL131A, and is required for virus entry into epithelial, endothelial and monocyte cells. It appears to be involved in fusion at endosomal membranes.

**Recombinant virus:** A recombinant virus is a virus produced by recombining pieces of DNA using recombinant DNA technology. This may be used to produce viral vaccines or gene therapy vectors.
It is also used to refer to naturally occurring recombination between virus genomes in a cell infected by more than one virus strain.

**Virus titration**: involves counting the number of viruses in a specific volume to determine the virus concentration. Examples of specific instances where known viruses need to be quantified include clone screening, multiplicity of infection (MOI) optimization and adaptation of methods to cell culture.

# *SUMMARY*

Human cytomegalovirus (HCMV) is a virus infecting the majority of adults worldwide. In healthy individuals, a strong immune response to HCMV is able to limit and contain the spread of the disease [1]. HCMV can infect a remarkably broad cell range within its host. The broad cell tropism of HCMV may reflect the abundance of distinct glycoprotein complexes in the virion envelope [10]. The core machinery for *Herpesvirus* entry comprises three highly conserved viral glycoproteins, glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL) [21].
In addition to gB and gH/gL, most *Herpesviruses* encode additional glycoproteins that are able to interact with gH/gL. For HCMV, this addition consist of the glycoprotein gO, to form gH/gL/gO complex, or the trimer UL128/UL130/UL131A (referred as "ULs"), to form a pentameric structure often designated as "Pentamer". Viral entry into fibroblast or epithelial/endothelial and lymphoid cells relies on the presence of gH/gL/gO or Pentamer respectively [35, 40].
In an *in vitro* system, specific cysteines have been identified to stabilize these complexes and impairment of disulfide bonds formation abolishes complexes maturation and cellular trafficking [41]. Here we addressed the relevance of these disulfide bonds in the formation of HCMV entry complexes and on the infectivity of point mutated viruses. To this purpose, four recombinant Cys-mutated viruses, generated through mutagenesis of a Bacterial Artificial Chromosome (BAC) containing the entire genome of HCMV TR strain, were analysed for viral tropism on three different cell types. We also checked by Western blot the content of the pentameric proteins expressed by these mutants both in the extracts of infected fibroblasts and monocytic cells (HFF and THP-1, respectively) and in virions produced by infection of human fibroblasts. Surprisingly, results from our analysis showed that

mutation on two specific cysteines involving gL disulfide bonds to gO or UL128 and to gH resulted in the loss of intracellular gL or expression level under the detection power of Wb. Two other Cys mutated viruses showed no differences in the levels of viral structural proteins compared to wt. These results suggest that the impairment of the disulfide bond involving binding of gL to UL128 or gO and gL to gH, cause instability of the gL protein with loss or reduced ability to form higher order complexes and likely cellular degradation.
However, our results show that infectious viruses can achieve a complete life cycle in absence of a crucial protein like as gL but it also raise the question of which pattern of factors, likely interacting with gH, are necessary as "surrogate" gL.

# INTRODUCTION

## 1.1 Human cytomegalovirus general features, pathogenesis and classification

Human cytomegalovirus (HCMV) is a ubiquitous virus infecting the majority of adults worldwide, ranging around 55-60% of the world population, although reaching peaks of more than 90% seroprevalence in given populations grouped according to age-gender-socioeconomic related factors [1]. Horizontal transmission depends on direct contact of infected bodily fluid at mucosal surfaces. In healthy individuals, a strong immune response to HCMV is able to limit and contain the spread of the disease, therefore bland clinical symptoms and spontaneous acute infection resolution are associated to infection of immunocompetent individuals [1]. Nevertheless, the virus is able to establish a lifelong lasting latency with recurrent and spontaneous reactivation, and complete clearance of the virus by the organism is never achieved. Both innate and adaptive immune responses operate to control viral replication and spread. The acute disease associated with HCMV infection occurs only in a small proportion of infected individuals, in particular its onset is observed in patients where the normal immune response is compromised. For example, HCMV infection can lead to severe complications and death in the case of transplant patients when the immune system is shut down to avoid rejection of the organ [2].
Another example of opportunistic superinfection by HCMV is very common in patients with Acquired Immuno-Deficiency Syndrome (AIDS) following HIV infection; the immune-deficient

environment in these patients allows HCMV infection and/or reactivation [3]. In all these particular cases, antiviral therapies are necessary to counteract the severe disease associated to the HCMV primary infection or reactivation. Antiviral agents are currently available for the prevention of HCMV infection or for treatment of overt disease are the nucleoside analogues ganciclovir, valganciclovir and cidofovir. All these substrates inhibit HCMV DNA polymerase and therefore HCMV replication. These agents slow down the replication of HCMV and are able to suppress clinical symptoms, but they cannot eliminate the viruses [4]. The development of vaccines has a high priority, also from the point of view of economic aspects, i.e. costs incurred due to an HCMV disease [5].

HCMV belongs to the *Herpesviridae* family, a classification historically based on the architecture of the virion that includes the *Herpesviruses* of mammals, reptiles and birds. The *Herpesviridae* family is one of the three families, together with the *Alloherpesviridae* and the *Malacoherpesviridae*, encompassed by the *Herpesvirales* order, established later as extensive nucleotide sequence data became available [6]. The *Herpesviridae* family members share several biological properties; the most evident is the ability to establish latent infection in some cell types that usually vary among the different members. Moreover, in addition to similar structural features, they share a large array of enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing. Finally, in all *Herpesviridae* the DNA replication and capsid assembly occur in the nucleus, the final processing of the virion takes place in the cytoplasm and the release of the progeny results in the destruction of the host cell [1]. On the basis of differences in the cellular tropism, genome organization, and gene content, *Herpesviruses* have been classified into three subfamilies: the *Alphaherpesvirinae* (HSV-1, VZV), *Betaherpesvirinae* (HCMV, MCMV), and *Gammaherpesvirinae* (EBV) [7]. All betaherpesviruses exhibit species specificity and only particular

types of differentiated cells are susceptible within a species. All CMV are conveniently propagated in fibroblasts although natural targets include other cell types, such as epithelial, endothelial, macrophage and dendritic cells [8].

## *1.2 HCMV virion structure and genome organization*

As typical of the *Herpesvirus* group, the virion of HCMV is approximately 230 nm in diameter and is composed of an envelope, a tegument, and a capsid containing the double-stranded DNA genome. The virus envelope is a lipid bilayer derived from the host cell endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and contains the viral glycoproteins necessary for the cellular tropism [9]. The HCMV genome is composed of a linear, double-stranded DNA molecule (236 kbp in wild type virus), the largest among the human herpesviruses. The capsid is isosahedral and is composed of four integral protein species (pUL46, pUL80.5, pUL85, pUL104) that are organized into 162 capsomeres (Figure 1). The tegument region is approximately 50 nm thick and includes seven relatively abundant virus-encoded protein species, at least five of which are phosphorylated. Both the tegument and the envelope contain additional less abundant virus-encoded and host-cell proteins, as well as phospholipids, polyamines, and small RNAs [10].

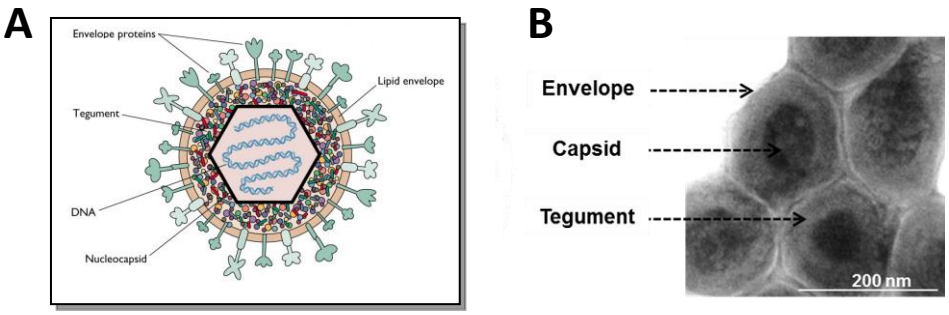


**Figure 1.** (A) Schematic representation of HCMV virion. (B) Negative stained of a purified virion preparation analyzed through electron microscopy.

HCMV is the largest and most complex of all known herpesviruses. It consists of a genome of ~240kb with estimates of 165-252 open reading frames (ORFs). The genome organization reflects the common structure of all herpesviruses, composed of unique long (UL) and unique short (US) genetic regions flanked by two sets of inverted repeats (RL, repeated long and RS, repeated short). Recombination phenomena can occur among identical terminal and internal repeats, leading to genome isomerization. Thus, genetic material isolated from a viral population consists of equal amounts of four different genomic isomers pooled together [1].

While the general genomic arrangement is conserved among HCMV strains, a major difference in the open reading frame (ORF) organization and composition can be observed between "laboratory-adapted strains" and "clinical isolates" [11]. While laboratory strains indicate all the strains extensively passaged and adapted to growth in human fibroblasts, permitting easy

manipulation due to the fast replication and high yields of produced virus, clinical isolates underwent through none or limited passages in cell culture before being cloned as bacterial artificial chromosomes (BACs) and/or sequenced. As a consequence of the fibroblast adaptation, several genomic rearrangements occurred in laboratory adapted strains. The ≈230,000-bp sequence and annotation of the AD169 laboratory strain of HCMV was published in 1990 [12]. This first annotation predicted that AD169 has the potential to encode 208 ORFs, of which 14 are duplicated within the TRL/IRL repeats. AD169 has a restricted cellular tropism as compared with clinical isolates [13], because it is tissue culture-adapted and has undergone duplications, deletions, and numerous more subtle sequence changes in comparison with clinical isolates of HCMV (Figure 2.) The Toledo strain, which has been passaged to a more limited extent in cell culture, contains a block of additional ORFs that are absent in AD169 [14]. Despite this huge coding potential, only a small subset of these proteins constitutes the mature virion: it has been estimated that around 50 [1, 15] proteins are incorporated in the virion.

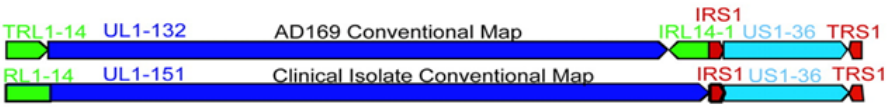


**Figure 2.** HCMV genome organization. ORFs map of conventional laboratory strain AD169 and clinical isolate. The AD169 genome (upper) carries TRL1-14 (green arrow), UL1-132 (dark blue arrow), IRL14-1 (green arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow). In the clinical isolate RL1-14, (green arrow), UL1-151 (dark blue arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow) are present.

## *1.3 Viral replication*

Human cytomegalovirus (HCMV) can infect a remarkably broad cell range within its host, including parenchymal cells and connective tissue cells of virtually any organ and various hematopoietic cell types. Epithelial cells, endothelial cells, fibroblasts and smooth muscle cells are the predominant targets for virus replication. The pathogenesis of acute HCMV infections is greatly influenced by this broad target cell range. Infection of epithelial cells presumably contributes to inter-host transmission. Infection of endothelial cells and hematopoietic cells facilitates systemic spread within the host. Infection of ubiquitous cell types such as fibroblasts and smooth muscle cells provides the platform for efficient proliferation of the virus. In line with the classification of the respective proteins as structural components of the viral envelope, inter-strain differences concerning the infectivity in endothelial cells and macrophages are regulated at the level of viral entry [10] (Figure 3).

The lytic infection of cells can be monitored using protein expression patterns and the replication of nucleic acid. The immediate early (IE) proteins are responsible for the regulation of the early (E) proteins and also for that of the late (L) proteins. After absorption of the virus into the target cell with the aid of viral glycoproteins, the virus envelope fuses with the cell membrane, the capsid is released into the cell and transported to the nucleus where the genome is released. Transcription of the immediate early (IE1/2) proteins then takes place in the cell nucleus with the aid of the RNA polymerase II of the host cell. Tegument proteins of the infecting virus particle act as transactivators for the IE genes. The IE proteins regulate the following stages of viral replication and are also involved in cell regulation including the expression and transport of the HLA antigens (class I MHC proteins) to the proteasome. IE proteins can be used as early markers of the virus infection in cell cultures [15].

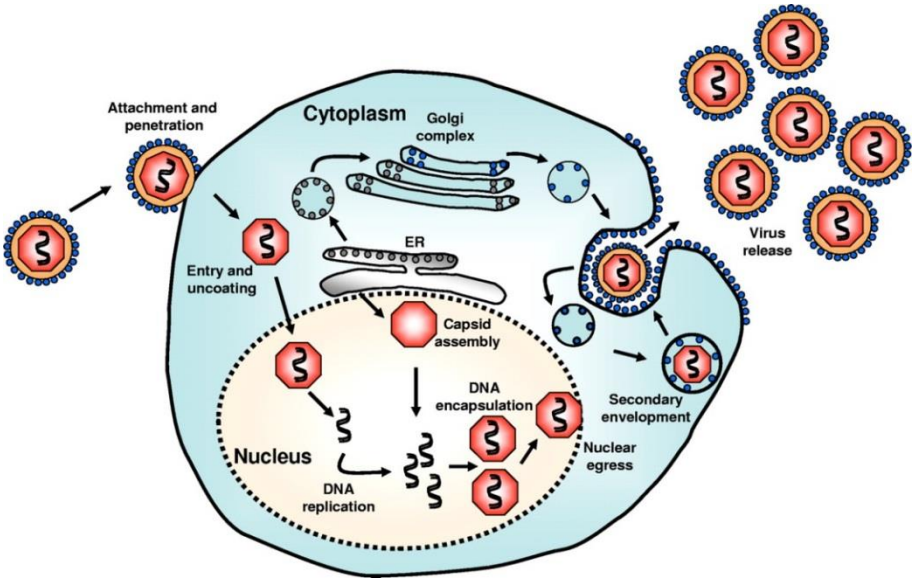


**Figure 3**. HCMV lytic life cycle. The virus attaches to the cell via interactions between viral glycoproteins and a specific surface receptor(s), followed by the fusion of the envelope with the cellular membrane to release nucleocapsids into the cytoplasm. These nucleocapsids are translocated into the nucleus, where viral DNA is released. Viral replication and maturation follow the stimulation and parallel accumulation of viral synthesis function. This process involves the encapsulation of replicated viral DNA as capsids, which are then transported from the nucleus to the cytoplasm. Secondary envelopment occurs in the cytoplasm at the endoplasmic reticulum (ER)-Golgi intermediate compartment. This is followed by a complex two-stage final envelopment and egress process that leads to virion release by exocytosis at the plasma membrane.

The HCMV genome is predicted to encode over 50 glycoproteins and the virion itself has been determined by mass spectrometry to consist of at least 19 different envelope proteins. However, of these, only five glycoproteins are essential for virus replication *in vitro*. These include glycoprotein B (gB or UL55), gM/gN (UL100/UL73), and gH/gL (UL75/UL115). Glycoprotein M, the most abundant glycoprotein, accounting for 10% of the virion mass, is complexed with gN and acts as an attachment receptor. Glycoprotein B, the second most abundant glycoprotein, is both an attachment and fusion receptor [10].

It is known that HCMV entry begins with an initial tethering step to heparin sulfate proteoglycans (HSPGs) on the cell surface, mediated by gB and gM. At least *in vitro*, binding to HSPGs is an essential step in the HCMV entry process, and is thought to help stabilize the virion at the cell surface until other downstream receptors are engaged [17]. HCMV entry is followed by the delivery of both the tegument and DNA containing capsid into the cytoplasm. Tegument proteins with regulatory function dissociate from the capsid and remain in the cytoplasm or migrate independently in the nucleus, where they modulate cellular and viral genes expression. A thick layer of tegument proteins remain tightly associated with the capsid and contributes to the delivery of the DNA to the nucleus. For the efficient delivery of HCMV DNA, an intact microtubular network (MT) is essential. The MT spans from cellular periphery up to the perinuclear MT organizing center (MTOC). HCMV moves along MT branches to reach the nuclear pore complex and to inject the DNA into the nucleus [18]. Various works describing the HCMV entry step suggest that the virus uses distinct cellular receptors, and consequently different entry pathways, depending on the target cell.
HCMV binding and entry induces a global reprogramming of the cellular activity and causes profound changes in infected cell morphology. The most evident is the formation of a large cytoplasmic juxtanuclear region defined virion assembly

complex/compartment (AC), corresponding to the virus final budding site. Before being released, viral particles pass through a series of maturation steps. DNA containing capsids exits the nucleus through succeeding envelopment/de-envelopment process at the nuclear membrane and reach the cytoplasm to complete their morphogenesis. At this stage, partial tegumented nucleocapsids reach the AC to acquire the full spectrum of tegument proteins and envelope glycoproteins [15] (Figure 4).

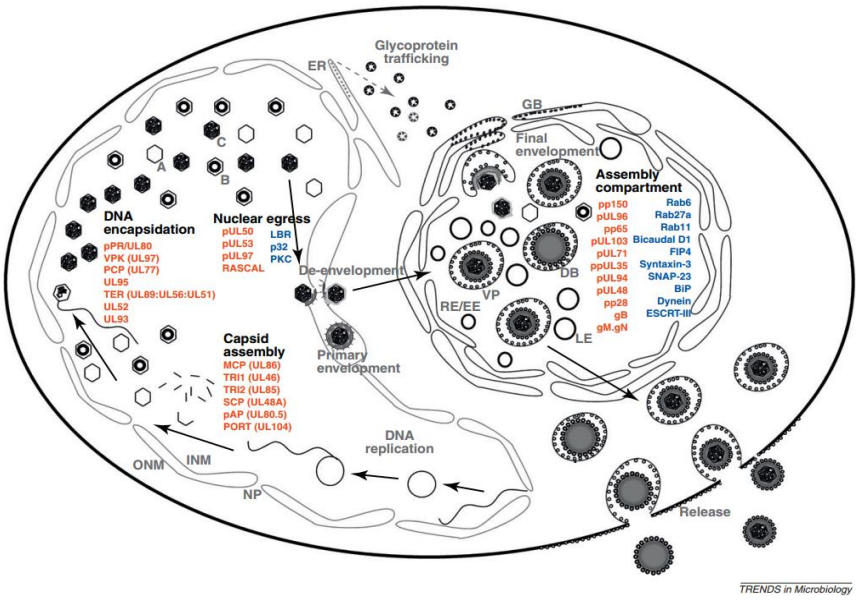


**Figure 4.** HCMV virion particles formation, maturation and budding processes. Major cellular and viral proteins involved in these processes are reported (red and blue, respectively). List of abbreviations: DB, dense body; VP, virus particle; EE/RE, early endosome/recycling endosome; LE, late endosome; GB, Golgi body; ER, endoplasmic reticulum; NP, nuclear pore; INM, inner nuclear membrane; ONM, outer nuclear membrane; A, B and C, types of nuclear capsids.

Moreover, HCMV has developed a huge arsenal of genetic functions committed to modulate both innate and adaptive immune responses. In particular, the virus is able to subvert the immune system mimicking the same strategies and mechanisms used by the host cells to clear the infection. Inhibition of complement cascade and natural killer (NK) cells activation, attenuation of interferon (IFN) response and disruption of antigen presentation are only few examples of the functions hijacked by virus encoded chemokines, cytokine and cellular receptors homologues [19].

The final steps are common among the Herpesviridae family and involve a two-stage envelopment/de-envelopment-envelopment process that drives the nucleocapsid outside the nucleus to the AC to acquire the full spectrum of tegument proteins and envelope glycoproteins, and finally the mature virion to be released by exocytosis at the plasma membrane [9].

## *1.4 Envelope glycoprotein*

The broad cell tropism of HCMV may reflect the abundance of distinct glycoprotein complexes in the virion envelope. Computer predictions and proteomics analyses of purified virions have suggested that HCMV envelope may contain up to 20 different glycoproteins [20]. In enveloped viruses, viral glycoproteins execute a highly regulated fusion event between virion and cellular membranes, thereby delivering the viral genome and other contents of the virion into the host cell. Antibody responses that block entry are considered neutralizing and represent an important host defense against viral pathogens. In many enveloped viruses, one or two viral glycoproteins are sufficient to carry out binding and membrane fusion events that mediate entry. In *Herpesviruses*, however, at least four envelope glycoproteins are typically involved. The core machinery for *Herpesvirus* entry comprises three highly conserved viral glycoproteins, glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL), along with one or more accessory glycoproteins necessary for binding to cell surface receptors [21,22]. In addition there are other two glycoproteins: glycoprotein M, the most abundant glycoprotein, accounting for 10% of the virion mass, is complex with glycoprotein N and acts as an attachment receptor [15].

In a number of *Beta* and *Gamma Herpesviruses*, including the human pathogens *Human Cytomegalovirus* (HCMV), *Human Herpesvirus 6* (HHV-6), and *Epstein–Barr virus* (EBV), two different gH/gL complexes are found on the virion envelope and are necessary for the viruses to enter the full range of cell types that they infect *in vivo*. Of the two gH/gL complexes expressed in HCMV virions, the gH/gL complex with glycoprotein O (gO) (gH/gL/gO), is sufficient for entry into fibroblasts, a cell type in which fusion events at the plasma membrane initiate infection. Infection of several other types of cells, including monocytes, dendritic cells, endothelial cells, and epithelial cells requires the

Pentameric complex made by gH/gL together with three small glycoproteins UL128, UL130, and UL131 (UL128-131) and it appears to involve fusion at endosomal membranes [23, 24] (Figure 5).

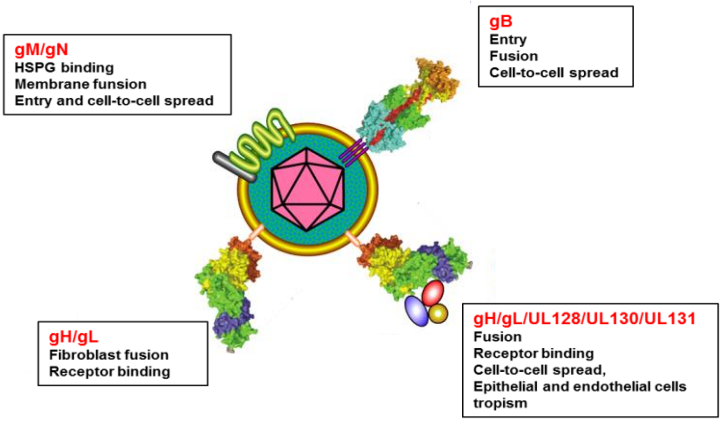


**Figure 5.** HCMV envelope glycoprotein complexes. The proposed role of each complex is indicated in the respective box.

Recent structural and mutagenesis analysis suggested that gB is responsible for mediating virus and host membrane fusion during viral entry [25,26]. Relatively little is known about the specific role of gB in HCMV entry and cell fusion apart from its requirement for both processes and its engagement of cellular signaling receptors. By contrast, more mechanistic details are available for homologous gB proteins from HSV-1, an Alphaherpesvirus, and EBV, a Gammaherpesvirus [27]. Crystal structures of gB from HSV-1 and EBV revealed strong protein fold similarities between the two ectodomains despite low sequence identity (25.9%) and provided important functional insights [28,29,30].

gB proteins are structurally similar to the viral fusion proteins from unrelated viruses Vesicular stomatitis virus G (VSV G) and baculovirus gp64, both of which are necessary and sufficient for viral entry and cell fusion. This structural similarity, in the absence of any sequence similarity, implicated gB as the fusion protein of herpesviruses capable to undergoing large conformational changes to effect fusion [28]. These data suggest that currently available gB structures represent the post-fusion form rather than pre-fusion form of the proteins, although this is not proven. Similarly to VSV-G, for which the structures of post- and pre-fusion forms are available, gB is expected to undergo a conformational transition from its original pre-fusion form as found in the viral envelope to the final post-fusion form occurring after fusion of the viral and host cell membranes [31]. The central role of gB during the fusion event makes gB a prime target of host defense mechanisms.

As well as for other Herpesviruses, the envelope glycoproteins gB form the conserved fusion machinery required for viral entry along with gH/gL. The role of gH/gL in the fusion is less clear because crystal structures of Herpes simplex virus 2 (HSV-2), Pseudo-rabies virus (PrV), and Epstein–Barr virus (EBV), did not reveal any similarity to known viral fusion proteins. It has been proposed that gH/gL heterodimer is involved in the entry process through activation of gB [32, 33].
In addition to gB and gH/gL, most Herpesviruses encode additional glycoproteins that are able to interact with gH/gL and in this way, either mediating binding to specific cellular receptors or regulating the activity of the gH/gL–gB complex [34].

HCMV entry into both epithelial and endothelial cells requires a Pentameric glycoprotein complex (Pentamer) formed between gH/gL and the UL128, UL130, and UL131A proteins [35]. Mutations in the UL131A–UL128 gene locus are sufficient to abolish epithelial/endothelial tropism and they occur spontaneously within only few passages of wild-type (WT) HCMV in fibroblasts.

In addition, Pentamer cell surface overexpression interferes with HCMV entry into epithelial cells, but not into fibroblasts, suggesting the presence of a cell-type specific Pentamer receptor [36, 37]. Strains of HCMV, such as AD169 and Towne, that have undergone extensive serial passage in cultured fibroblasts fail to express the pentameric gH/gL/UL128-131 complex on virions and thus are unable to infect epithelial and endothelial cells; however, repair of a frameshift mutation in the UL131 gene of strain AD169 restores expression of gH/gL/UL128-131 and expands its cell tropism [39].
Although it was proposed that gO might function as a molecular chaperone to promote gH/gL incorporation into the virion [40], it is now clear that for HCMV entry into fibroblasts the gH/gL/gO complex is sufficient when acting neutral pH on the cell surface. gO is a highly glycosylated protein and it covalently bind gL subunit of the gH/gL heterodimeric complex.

As reported by Ciferri *et al.*, specific cysteine residues are involved *in vitro* complexes' formation. They used mass spectrometry (MS) to investigate which cysteines are involved in disulfide formation. Analysis of gH/gL showed that $gLCys_{144}$ in one monomer forms a disulfide bridge with $gLCys_{144}$ in a second monomer, demonstrating that the gL subunit is involved in homodimerization. In addition, MS analysis identified a disulfide bond between $gLCys_{47}$ and $gHCys_{95}$, consistent with the covalent interaction between gH and gL.
Mutation of both cysteines ($gLCys_{47}$ and $gHCys_{95}$) prevented gH/gL secretion, suggesting that this disulfide bonding is essential for gH/gL complex folding. MS analysis of gH/gL/gO showed that $gLCys_{144}$ forms a disulfide bond with $gOCys_{351}$. The $gOCys_{351}$Ser mutation impaired gO expression resulting in secretion of gH/gL only. Finally, MS analysis of the Pentamer showed that $gLCys_{144}$ forms a disulfide bond with $UL128Cys_{162}$. The $UL128Cys_{162}$Ser mutation revealed a lack of covalent interaction between gH/gL and UL128. Therefore, the same cysteine in gL ($gLCys_{144}$), forms

alternative disulfide bonds with gO and UL128 and, in their absence, is available to form disulfide linked gH/gL homodimers. These data are consistent with gH/gL/gO and Pentamer forming mutually exclusive cell entry complexes [41].

# ***AIMS***

In a number of *Beta* and *Gamma Herpesviruses*, including the human pathogens *Human Cytomegalovirus* (HCMV), *Human Herpesvirus 6* (HHV-6), and *Epstein–Barr virus* (EBV), two different gH/gL complexes are found on the virion envelope and both are necessary for the viruses to enter the full range of cell types that they infect *in vivo* [23].

Recent structural and mutagenesis analysis suggested that gB is responsible for mediating virus and host membrane fusion during viral entry [25, 26]. Relatively little is known about the specific role of gB in HCMV entry and cell fusion apart from its requirement for both processes and its engagement of cellular signaling receptors [27]. Similarly, to other Herpesviruses, the envelope glycoproteins gB form the conserved fusion machinery required for viral entry with gH/gL. In addition to gB and gH/gL, most Herpesviruses encode additional glycoproteins that are able to interact with gH/gL and are capable of either mediating binding to specific cellular receptors or regulating the activity of the gH/gL–gB complex [34]. In particular, the glycoprotein complexes gH/gL/gO and Pentamer are key targets of human humoral response against *HCMV* since both are absolutelyrequired for the virus entry into a large number cell lines.

As reported by Ciferri *et al.* specific cysteines are involved in complexes formation *in vitro*. Mass spectrometry and mutagenesis analysis revealed that $gLCys_{144}$ forms disulfide bonds with $gOCys_{351}$ in gH/gL/gO and with $UL128Cys_{162}$ in the Pentamer. In addition, MS analysis identified a disulfide bond between $gLCys_{47}$ and $gHCys_{95}$, consistent with the covalent interaction between gH and gL. The mutations on these cysteines abrogate the covalent interactions of gL with UL128 or gO and of the gH/gL secretion,

suggesting that these disulfides are essential for complex formation [41].

Based on these data, this work aims to a) unravel the molecular mechanisms driving the viral tropism, b) analyze the effects of specific mutations in the protein complexes in the infection model and c) characterize the composition of the complexes with impaired disulfide bonds. Several recombinant viruses expressing mutated proteins were generated using a BAC system containing the complete HCMV genome. In particular, Cys residues involved in complex formation were mutated to serine or arginine and the recombinant viruses used for infection of different cell type. Subsequent analysis of gH-based complexes expressed in infected cells and carried on the viral particles will allow us to understand how the targeted cysteines influence the formation of the complexes and the infectious capacity of the virus.

# RESULTS AND DISCUSSION

## 1.1 Construction of recombinant viruses

In order to investigate if the effects of disulfide bonds impairment observed *in vitro* were reproduced *in vivo*, we generated several recombinant viruses through mutagenesis of a Bacterial Artificial Chromosome (BAC) containing the entire genome of HCMV TR strain. Each mutated virus carried an unique point mutation resulting in an aminoacid changes on specific cysteine residues. We decided to analyze 4 recombinant viruses in which the cysteines involved in complexes' formation are mutated in arginine or serine. In particular, gL cysteine at position 144 ($Cys_{144}$), UL128 cysteine at position 162 ($Cys_{162}$) and gO cysteine at position 351 ($Cys_{351}$), were mutated to arginine. The gH cysteine at position 95 ($Cys_{95}$), was mutated to serine (Figure 1). gL $Cys_{144}$Arg and UL128Cys351Arg should results in absence of disulfide bond formation of gL with UL128 impairing formation of the pentameric complex. Also, this mutation on gL, in addition with the mutation on gO, should reveal in a lack covalent interaction between gH/gL and gO preventing the gH/gL/gO complex. Finally, the gHCys95Ser should avoid the covalent interaction between gH/gL.

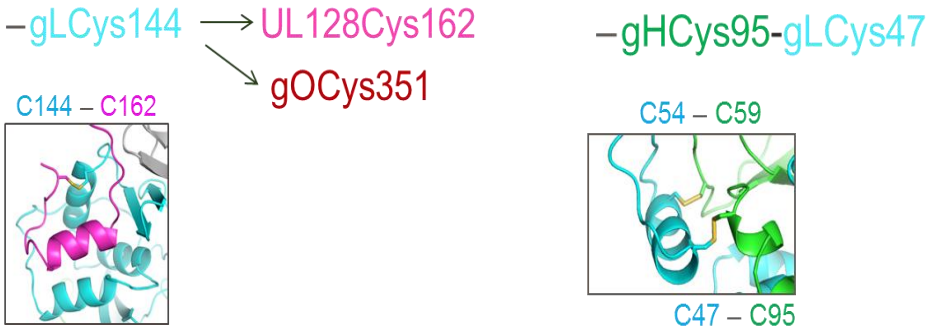


**Figure 1.** Schematic representation of disulfide bonds between the proteins involved in gH/gL, gH/gL/gO and Pentameric complexes formations.

To introduce point mutaton on the TR BAC, the strategy consists of two recombination steps. Briefly, the first allows the integration of the point mutation together with the expression cassette for kanamycin resistance while, in the second step, digestion with I-SceI enzyme permits the alignment of homologous sequences and the excision of the kanamycin resistance cassette leaving the point mutation in-frame with the gene of interest. This is achieved with the implementation of the *E.coli* strain GS1783 containing the TR BAC, the lambda Red system expressed with a heat-inducible promoter and the I-SceI enzyme expression cassette under the control of a promoter inducible by arabinose (see the Material and Methods) [42]. At the end of the recombinant steps, the gene of interest has been sequenced to verify the insertion of point mutation and the analysis of sequencing performed by Vector NTI.

## *1.2 Analysis of infectious capacity*

Due to the broad HCMV tropism, to establish the ability of the mutated viruses to infect different cell type, as attested for the natural infection, a certain number of cell culture models are required *in vitro*. In order to investigate these mutations in an infection system, the recombinant BAC-DNA was transfected by electroporation into Human Fetal Lung Fibroblast Cells (MRC5) to reconstitute the mutant viruses. 7 days post-infection, the supernatants were used to infect Human Foreskin Fibroblast (HFF) to amplify viral particles. Fibroblasts, in fact, 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 [10].

After propagation, we calculated the viral titer to compare the infectious capacity of the mutant viruses compared to wt TR. To this purpose, we performed a cytofluorimetric assay on HFF infected cells. Briefly, HFF were infected with the cell culture supernatants diluted 1:10 in infection media (without FBS) and incubated for 4 hours at 37°C. Then, fresh complete medium was added and infected cells were incubated for additional 24 hrs. Cells were then analyzed by FACS in permeabilized condition and stained with antibody against pp65, the most abundant tegument protein that is generally used as a marker of virus infection in cell culture. All mutant viruses were able to infect HFF cells as shown by the positivity of cells to intracellular pp65 staining (Figure 2).

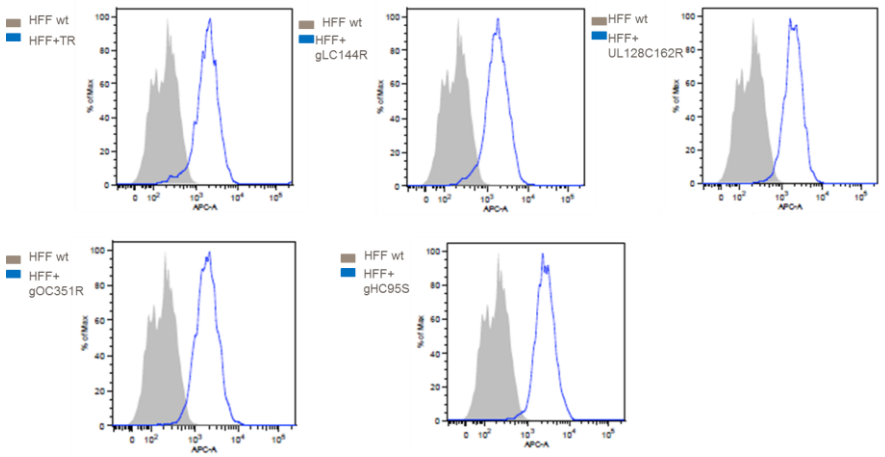


**Figure 2.** Cytofluorimetric analysis comparing the TR virus and infected HFF with different recombinant viruses. In grey the wt HFF used as negative control. Cells were stained with pp65 antibody. Each histogram represent $1x10^5$cells.

The percentage of pp65 positive cells was used to calculate the titer per mL using the following formula:

$$\frac{Number\ of\ cells\ used\ x\ \%\ positive\ cells}{\mu l\ virus\ used}\ x\ 1000$$

As shown in Figure 3, the titers of all recombinant viruses resulted to be almost identical to the one obtained infecting with wt TR ($1x10^8$ titer/mL). This result confirms that the mutations in cysteines do not impair infectious ability of the recombinant viruses.

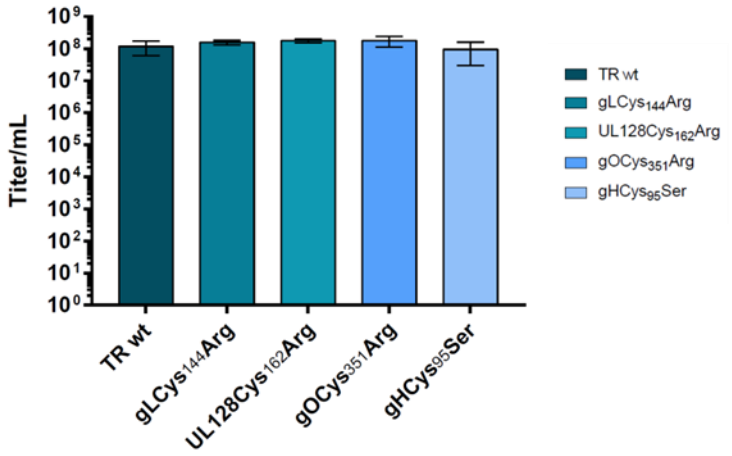


**Figure 3.** Viral titer of recombinant viruses with respect to the TR wild-type virus. The cells were harvested and titrated using FACS with an antibody directed against pp65. The experiment was repeated 3 time.

## *1.3 Analysis of viral tropism*

HCMV can infect a remarkably broad cell range within its host, including parenchymal cells and connective tissue cells of virtually any organ and various hematopoietic cell types. Epithelial cells, endothelial cells, fibroblasts and smooth muscle cells are the predominant targets for virus replication. Infection of endothelial cells and hematopoietic cells facilitates systemic spread within the host. Infection of ubiquitous cell types such as fibroblasts and smooth muscle cells provides the platform for efficient proliferation of the virus [10].
The envelope glycoprotein B cooperates with gH/gL based complexes to form the conserved fusion machinery required for viral entry. In HCMV, the gH/gL/gO and gH/gL/UL128/UL130/UL131A (Pentamer) complexes are required for virus entry into fibroblast and endothelial/monocytic cells, respectively [23, 24].

As HCMV has the capacity to infect different cell types using different entry complexes, we investigated infectivity into different cell types to verify if the mutations introduced in our recombinant viruses could have an effect on viral tropism. Three representative cell lines, exemplifying the major targets of HCMV, have been chosen:

- Human Foreskin Fibroblast (HFF)
- Adult Retinal Pigment Epithelial 19 (ARPE-19)
- Human Leukemic Monocyte (THP-1)

### *1.3.1 Analysis on HFF*

Fibroblasts are not only the standard cell culture system for propagation of HCMV to high titers, but they are also among the major targets of HCMV *in vivo*. Efficient replication in such a ubiquitous cell type opens the possibility for HCMV to replicate in virtually every organ. Consequently, infected connective tissue cells are assumed to contribute to efficient spread of HCMV in organs such as adrenal glands, bone marrow, heart, kidney, liver, lung, pancreas, placenta, small bowel and spleen. If the particular property of cultured fibroblast to generate and release high titers of viral progeny also applies for infected connective tissue cells *in vivo*, then they might contribute greatly to the highly dynamic proliferation of HCMV during acute infections [10].

In order to investigate how the impairment of specific disulfide bonds formation involved in gH/gL/gO and Pentamer complexes have an effect on the infectious ability, we examined the intracellular levels of viral marker in infected cells. Titrated infectious supernatants were used to infect HFF cells at MOI 5. 96 hours post-infection, the cells with 90% cytopathic effects were harvested, lysed and the whole cell extracts subjected to immunoblot. To compare the level of infection of recombinant and wt viruses, levels of the viral marker pp65 was determined. Immunodetection of GAPDH was used as loading control.

As shown in Figure 4, quantitative analysis by Western blot of the pp65 expression level is comparable in all recombinant viruses. This result, confirming the one obtained by FACS, suggests that the impairment of disulfide bonds between the gH/gL ($Cys_{95}$ of gH), gL/UL128 ($Cys_{144}$ of gL and $Cys_{162}$ of UL128) and gL/gO ($Cys_{144}$ of gL and $Cys_{351}$ of gO) does not cause loss of infectious capacity in HFF.

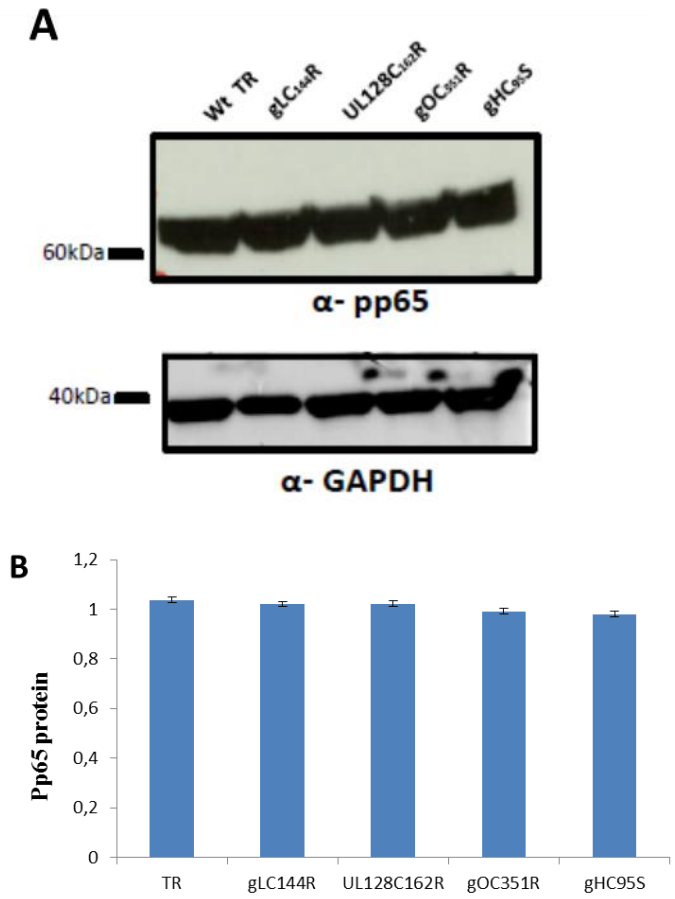


**Figure 4.** Expression of pp65 protein in HCMV-infected fibroblasts. The experiment was repeated three times, and a representative result is shown. **A.** Western blot on lysates of HFF cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, gOCys$_{351}$Arg, gHCys$_{95}$Ser or TR (positive control) at an MOI of 5. Pp65 was used as viral marker protein, while GAPDH detection was used as a protein loading control. **B.** Densitometric analysis of the samples shown in A. The values are expressed as ratio between pp65/GAPDH.

### *1.3.2 Analysis on ARPE-19*

Epithelial cells are one of the major targets of HCMV infection and, therefore, are assumed to play an important role during host-to-host transmission since they lay all external body surfaces [10]. According to the published literature, HCMV entry into both epithelial and endothelial cells requires a Pentameric glycoprotein complex (Pentamer) formed by gH/gL/UL128, UL130, and UL131A proteins [35].

In order to verify the infectious capacity of these recombinant viruses on epithelial cells, the infectious supernatants recovered from virus reconstitution in MRC5 cells were used to infect ARPE-19 (Adult Retinal Pigment Epithelial 19) cells at MOI 5. As described above, the whole cell extracts was subjected to immunoblot using the antibody against viral protein pp65 as marker of infection while the level of GAPDH used as loading control (Figure 5).

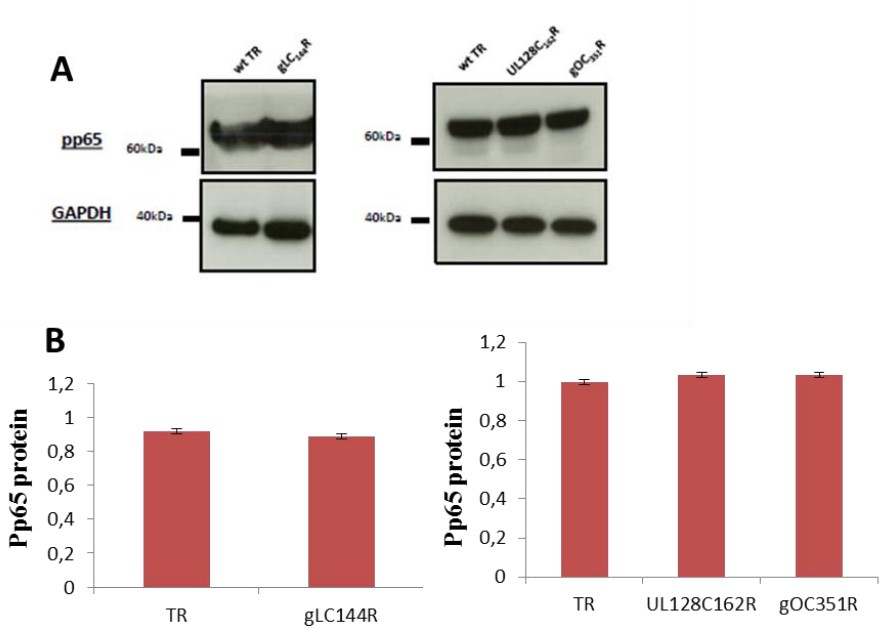


**Figure 5.** Expression of pp65 protein in HCMV-infected ephitelial. The experiment was repeated twice, and a representative result is shown. **A.** Western blot on lysates of ARPE-19 cells infected with $gLCys_{144}Arg$, $UL128Cys_{162}Arg$, $gOCys_{351}Arg$ or TR (positive control) at an MOI of 5. Pp65 was used as viral marker protein, while GAPDH detection was used as a protein loading control. **B.** Densitometric analysis of the samples shown in A. The values are expressed as ratio between pp65/GAPDH.

As shown in Figure 5, impairing disulfide bond formation between $Cys_{144}$ of gL and $Cys_{162}$ of UL128 (cysteines involved in pentameric complex formation), had no effect on the infectious capacity of recombinant viruses, confirming the results obtained on HFF cells. Furthermore, in these cells, disruption of the disulfide bond between gL/gO ($gLCys_{144}$ and $gOC_{351}$) did not lead to loss of infectivity. This result was less surprising since, according to data from literature, the gH/gL/gO complex does not play a major role in viral entry in these cells.

### *1.3.3 Analysis on THP-1*

Monocytes represent a key cell type for HCMV infection generally considered as potential site of latency and efficient vehicles for viral dissemination.These cells represent a good model for our studies since recent studies have demonstrated that the Pentameric complex is required for entry into monocytic cells [43].

We used THP-1 human monocytic cells as model for *in vitro* infection experiments. Following the standard protocol for virus reconstitution, BAC-DNA was transfected into MRC5 cells by electroporation and 7 days post-infection, supernatants were used to infect THP-1. Intriguingly, these supernatants were unable to infect THP-1 cell line although their infectivity on ARPE-19 cells indicated the presence of the pentameric complex (data not show).

This circumstance was already observed in other *herpesviruses* in which the progenies derived from different cell types differ in their cell tropism. For example, for EBV, a gH/gL/gp42 and a gp42-negative gH/gL complex have been described. The latter binds to

integrins αvß6 and αvß8 and promotes entry into epithelial cells by fusion at the plasma membrane [44]. The gH/gL/gp42 complex binds to HLA-DR-ß and promotes entry into B-cells by an endocytotic route. During virus production in B-cells, gp42 is intracellularly targeted to HLA-DR-ß, where it is vulnerable for degradation. Consequently, B-cells release virus particles, with reduced levels of gH/gL/gp42. This virus is directed towards epithelial cells. Epithelial cells on the other hand do not express HLA-DR ß and produce virus highly enriched in gH/gL/gp42 complex, which is directed to B-cells [45]. Thus, the EBV host cell tropism is switched by alternate replication in B- or epithelial cells.

We adopted a different strategy to obtain infection of this cell type. We decided to try to reconstitute the viruses by transfection of the BAC-DNA directly into THP-1 cells. To this aim, we performed electroporation in THP-1 and, 7 days post-infection, used the supernatants to infect fresh THP-1 cells at MOI 5. This approach was successful since we recovered viral particles infectious for THP-1 cells. 96 hours post-infection, the cells were harvested, lysed and the whole cell extracts subjected to immunobloting using pp65 as viral marker. According to what observed in the previous experiments, the disulfide bond impairment on the proteins involved in the Pentameric complex does not affect the virus capacity to entry into the cells (Figure 6). All mutated viruses, indeed, preserved their infectious capacity.

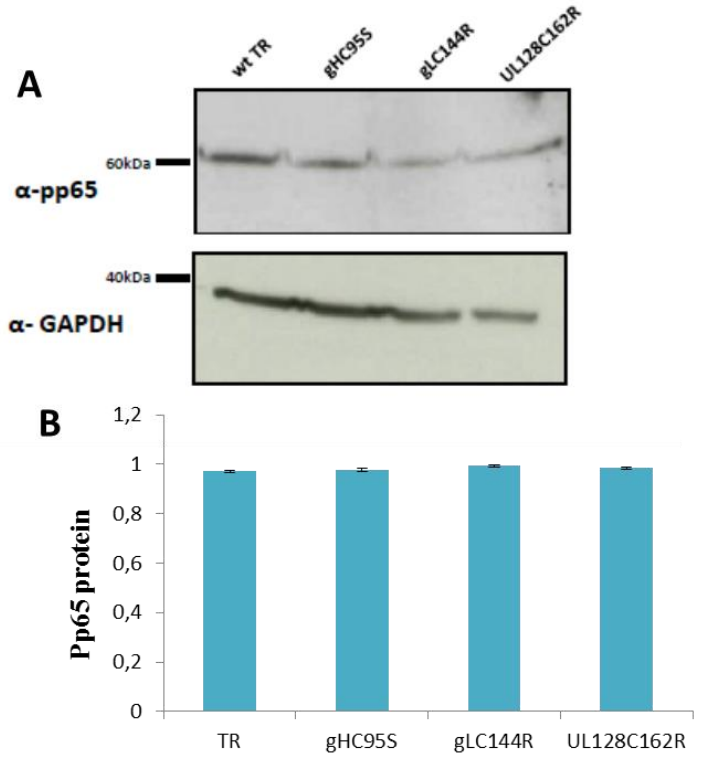


**Figure 6.** Expression of pp65 protein in HCMV-infected monocyte. The experiment was repeated twice, and a representative result is shown. **A.** Western blot on lysates of THP-1 cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, gHCys$_{95}$Ser or TR (positive control) at an MOI of 5. Pp65 was used as viral marker protein, while GAPDH detection was used as a protein loading control. **B.** Densitometric analysis of the samples shown in A. The values are expressed as ratio between pp65/GAPDH.

## *1.4 Cellular expression analysis of HCMV structural proteins*

Having defined that none of the mutations introduced in our recombinant viruses had effect on the infectious capacity of HCMV in all of the three different cell lines, next we investigated the impact of mutations on the expression levels of the viral proteins forming the gH-based complexes. According to the literature, the mutations of these cysteines prevent the covalent interaction that stabilize the complexes and impair their maturation *in vitro*. Consequently, it was expected the impairment of complexes formation when mutations were introduced on the whole viral genome context. In order to shed light into this point, we evaluated the expression of the crucial structural proteins following infection of two representative cell lines, HFF and THP-1. As reported from the literature, in both cell types, the gH/gL/gO and Pentamer complex are responsible for the virus entry respectively.

The first cell line analyzed was HFF. The whole cell extracts from infected HFF at MOI 5 at 96 hours post-infection were subjected to immunoblotting using antibodies against the principal structural proteins gH and gL, which are also essential for complex formation.

As shown in Figure 7, the levels of both proteins in Western blot analysis resulted identical in all recombinant viruses excepted for the recombinant virus containing the mutation in gL $Cys_{144}$. This cysteine is engaged in the disulfide bond between gL and either UL128 or gO, thus crucial for Pentamer or gH/gL/gO formation respectively.

In cells infected with this mutant, the protein gL and gH are not detectable in Western blot. Indeed, this mutant, although has the

same infectivity as wt TR, does not express two of the major structural proteins for the entry complex formation at least at levels comparable with the wt strain.

Although the mutation on $Cys_{162}$ of UL128 (this cysteine forms disulfide bond with $Cys_{144}$ of gL) showed less drastic differences as compared to the wt, however it resulted in a strong reduction of the expression of gL compared to wt TR.

Lastly, the impairment of disulfide bond between gO and gL (mutation on $Cys_{351}$ of gO) and gH and gL (mutation on $Cys_{95}$ of gH) did not show any effect on the relative expression of structural proteins during infection (Figure 7).

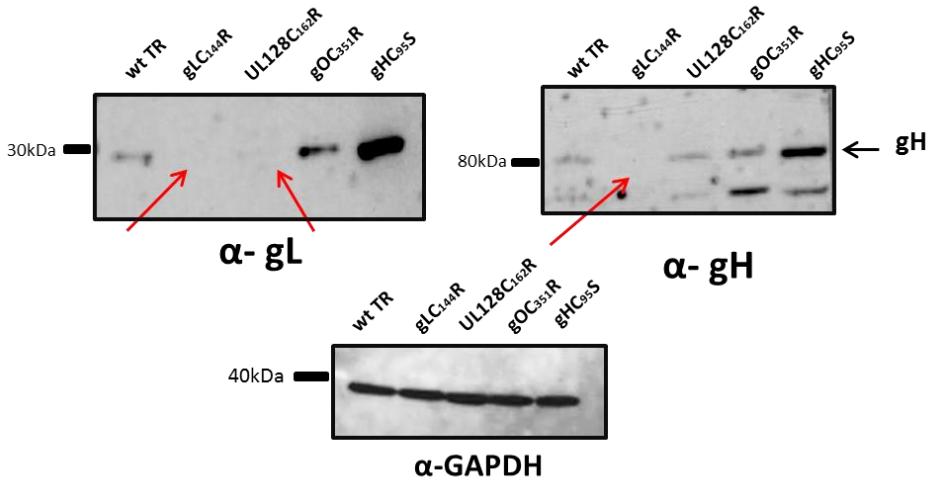


**Figure 7.** Expression of gL and gH protein in HCMV-infected fibroblasts. Western blot on lysates of HFF cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, gOCys$_{351}$Arg, gHCys$_{95}$Ser or TR (positive control) at an MOI of 5. GAPDH detection was used as a protein loading control. The experiment was repeated two times and a representative result is shown.

In order to confirm these results we used an antiserum generated against the Pentameric complex. This antiserum recognizes in Western blot the protein gH, gL and UL128 as shown by figure 8 A on the recombinant pentameric complex. In Figure 8 B, the antiserum was used to detect these protein in extracts from cells infected with wt and mutated viruses. The result confirmed that the protein gL and gH are undetectable in cell extracts of the recombinant virus containing the mutation on $Cys_{144}$ in gL. The level of UL128 protein, instead, is the same compared to wt TR (Figure 8 B). Using the same immunological probe, no differences in the level of viral proteins was revealed in extracts from cells infected with viruses carrying mutation on $Cys_{351}$ of gO or on $Cys_{95}$ of gH (Figure 8B).

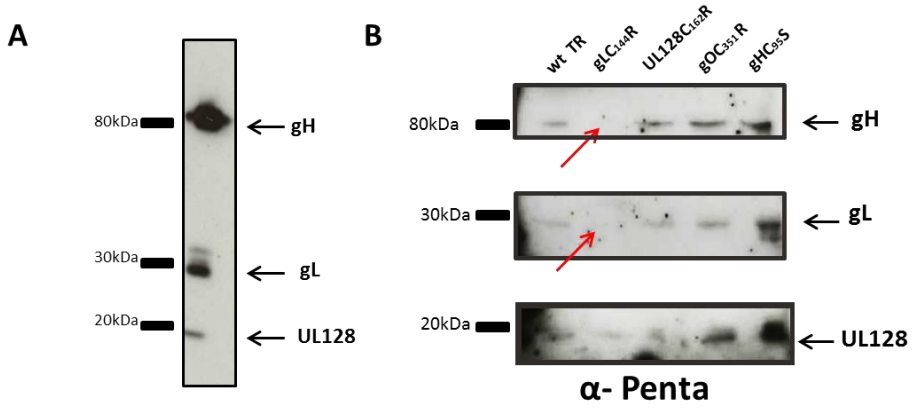


**Figure 8. A.** Expression of gL, gH and UL128 protein in recombinant pentameric complex. **B.** Western blot on lysates of HFF cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, gOCys$_{351}$Arg, gHCys$_{95}$Ser or TR (positive control) at an MOI of 5 using antiserum against Pentamer complex.

The second cell line analyzed in infection with wt and mutated viruses has been THP-1. In this case, infectious viruses from transfected THP-1 cells were used to infect fresh THP-1 cells at MOI 5. Also in this case the whole cell extracts were subjected to immunoblotting using the antibodies against the structural proteins, gH and gL. Results are shown in Figure 9.

The disruption of the disulfide bond between gL and UL128 caused the lack of gL expression in the gL $Cys_{144}$ mutant and a strong reduction in the UL128 $Cys_{162}$ mutant, a result that mirrors the one obtained on HFF infected cells. Opposite to what observed in fibroblasts, in THP-1 the expression levels of gH remains comparable to wt TR for all mutants. The gH $Cys_{95}$ mutant replicating in THP-1 behaves as observed in HFFs. As shown in Figure 9, the expression levels of gH and gL in the infected cell extracts were identical to those in the wt control.

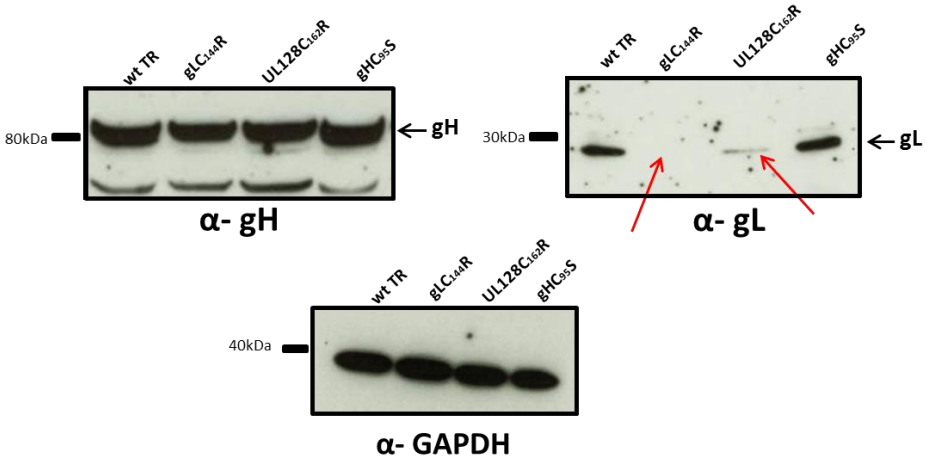


**Figure 9**. Expression of gL and gH protein in HCMV-infected monocyte. Western blot on lysates of THP1 cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, gHCys$_{95}$Ser or TR (positive control) at an MOI of 5 using antibody against gH and gL protein. GAPDH detection was used as a protein loading control. The experiment was repeated two times and a representative result is shown.

## *1.5 Analysis of purified virions*

The analysis of whole cell extracts of infected HFF, ARPE-19 and THP-1 cells showed that impairing disulfide bonds between the proteins involved in complex formation did not modify infectious ability of the mutants but, for some of them, the expression levels of two of the major structural proteins (gH and gL) is strongly impaired. In all *herpesviruses*, the complex gH/gL with the fusion glycoprotein B plays a central role in viral membrane fusion. Since these proteins have been proven to be essential for the cell entry process, we sought to verify the presence of gH and gL on the purified virions.

In the HCMV field, HFFs have always been the standard cell type for isolation and propagation of the virus. We decided to analyze our mutated viruses produced in this cell type. To this aim, HFFs were infected at MOI 5 and the supernatants collected at 4 days post-infection. Purification of viruses was achieved through a sucrose gradient ultracentrifugation. The virions were subjected to SDS-PAGE and immunoblot using antibody against Pentamer that recognize three proteins of the pentameric complexes: gH, gL and UL128. The Western blot analysis of these samples is shown in Figures 10 and 11.

It is remarkable that the protein gL was absent in the gL$Cys_{144}$Arg mutant virion, whereas it carried levels of gH and UL128 comparable to wt TR (Figure 10). The UL128$Cys_{162}$Arg mutant virion had the same level of gH and UL128 as wt TR, but the mutation resulted in a strong reduction of gL expression reflecting the results seen on cell extracts (Figure 10).

The results obtained on the gL$Cys_{144}$Arg and on UL128$Cys_{162}$Arg mutant virion suggest a lower stability of gL protein perhaps because unable to bind its partner. The absence of gL also on the

UL128$Cys_{162}$Arg mutant virion shows the importance of $Cys_{162}$ and its crucial role on the stability of pentameric complex.

Western blot of purified gO$Cys_{351}$Arg mutant virion shows that the levels of gH, gL and UL128 are the same compared to wt TR. This result confirmed what was seen in cell extracts, indicating that impairment of the disulfide bond between gL and gO by mutation on the gO cysteine involved in disulfide bond with gL is irrelevant not only for the infectious capacity but also for the expression levels of both gH and gL. These observations suggest that this cysteine is not essential for the gH/gL/gO complex formation, although we did not check the presence of gO in the complex (Figure 11).

From these results, we concluded that the destruction of disulfide bonds between gL and UL128 is crucial for the formation of the Pentamer. Although the destruction may be obtained by mutating the cysteine on either protein, only the gL protein seems to be affected. The data obtained in this work and the current literature confirms that the ability to form a disulfide bond engaging the cysteine at position 144 is crucial to the stabilization of the gL protein. In its absence, gL appears to be unstable and quickly cleared from cells. Furthermore, $Cys_{144}$ mutated gL seems unable to bind gH, as indicated by the presence of normal levels of gH in the absence of gL.

These conclusions are also supported by the results from the analysis of the gO$Cys_{351}$Arg mutant virion (Figure 11). These mutant viruses carry levels of gH, gL and UL128 comparable to wt virus. Thus, impairment of disufide bond uniquely on the gO side does not impair formation of the Pentamer while the presence of the gH/gL/gO complex remains to be checked.

Finally, the gH$Cys_{95}$Ser mutant virion showed the same levels of gH and UL128 protein in association with a very strong reduction of gL expression (Figure 11). Unexpectedly, this result did not match the one obtained on the cell extract from the infected cells in

which normal levels of all proteins were observed. It can be hypothesized that the impaired formation of a disulfide bond between gH and gL does not prevent the formation of the dimer, but generates a less stable complex which is inefficiently incorporated into mature virions. Among all proteins of these complexes, gH is the only one carrying a transmembrane domain that anchor the protein to the lipidic envelope. gL, which lacks a membrane anchor, is dependent on association with gH for virion incorporation [46, 47].

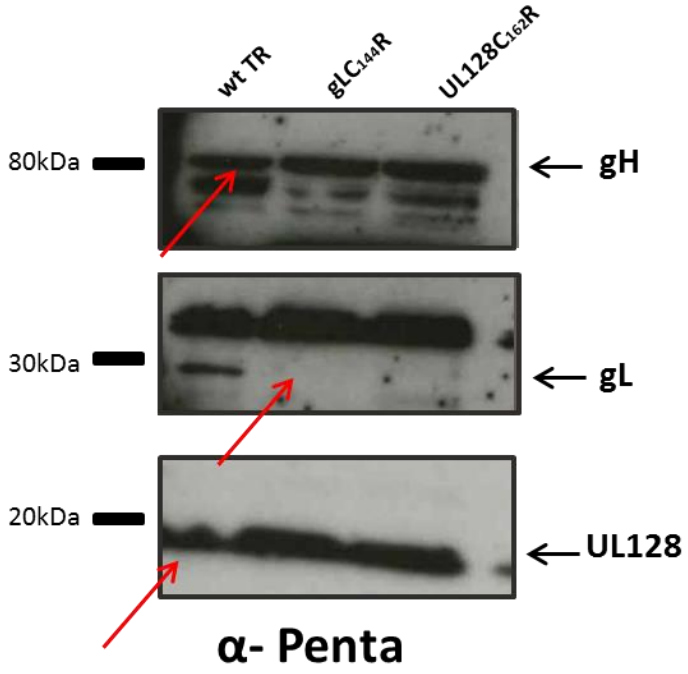


**Figure 10.** Expression of gL, gH and UL128 protein in HCMV-infected fibroblasts. Western blot on purified virions from HFF cells infected with gLCys$_{144}$Arg, UL128Cys$_{162}$Arg, or TR (positive control) at an MOI of 5 using antibody against Pentamer complex. The experiment was repeated two times and a representative result is shown.

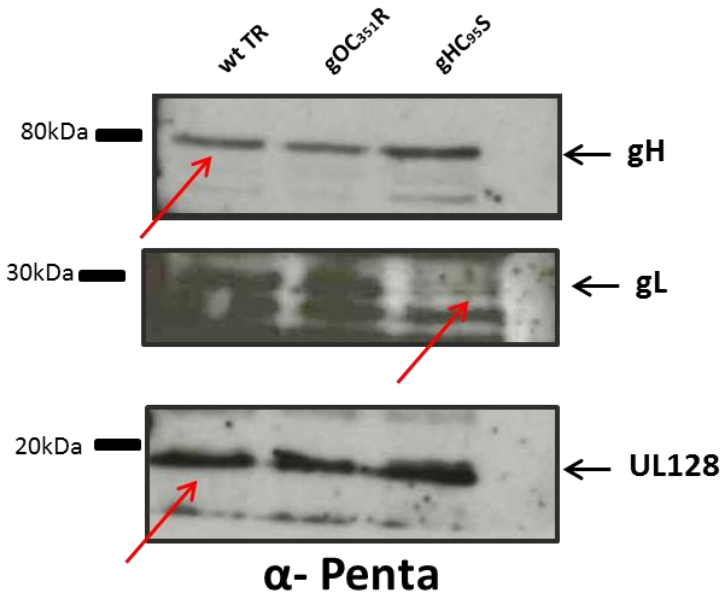


**Figure 11.** Expression of gL, gH and UL128 protein in HCMV-infected fibroblasts. Western blot on purified virions from HFF cells infected with gOCys$_{351}$Arg, gHCys$_{95}$Ser, or TR (positive control) at an MOI of 5 using antibody against Pentamer complex. The experiment was repeated two times and a representative result is shown.

Results obtained in this study are summarized in two tables. Table 1 reports the infectious capacity and the protein level detection in extracts from cell lines infected with wt and mutants. In table 2 the pattern of three "crucial" structural proteins carried on the viral particles is reported.

**Table 1. Summary on the whole cell extracts from infected cells**

| Kind of recombinant virus | Infectious capacity | gH protein expression | gL protein expression | UL128 protein expression |
|---|---|---|---|---|
| TR | √ | √ | √ | √ |
| $gLC_{144}R$ | √ | | | √ |
| $UL128C_{162}R$ | √ | √ | | √ |
| $gOC_{351}R$ | √ | √ | √ | √ |
| $gHC_{95}S$ | √ | √ | √ | √ |

**Table 2. Summary of the proteins carried by purified viruses**

| Kind of recombinant virus | gH protein expression | gL protein expression | UL128 protein expression |
|---|---|---|---|
| TR | √ | √ | √ |
| $gLC_{144}R$ | √ | | √ |
| $UL128C_{162}R$ | √ | | √ |
| $gOC_{351}R$ | √ | √ | √ |
| $gHC_{95}S$ | √ | | √ |

The absolute necessity of the gH/gL dimer to form complexes with other proteins to achieve productive infection is a paradigm of the *herpesviridae* family. Different studies, in fact, demostrated the importance of gH/gL complex for virus entry and for its infectious capacity, in HCMV as well as in other *herpeseviruses*. For example, Roop *et al.* [48] have analyzed a mutant HSV-1 unable to express gL. In the absence of gL, the produced viral particles were also deficient in gH, although they reached the cell surface. Furthermore, gL-negative particles purified from infected cells were able to adsorb on the cells, but were unable to enter cells and initiate an infection [48].

The most striking result shown here is that the infectious ability remains unchanged despite the absence of gL or an imbalanced expression of gL (as compared to gH). gH/gL complexes are clearly important for virion cell tropism of several *beta* and *gamma herpesviruses*, but the mechanisms that regulate their relative abundance during infection have, for the most part, remained elusive. A recent study demostrated that HCMV uses the virally encoded protein UL148 to modulate the relative abundance on virions of the two alternative gH/gL complexes by influencing complex assembly and/or maturation that suggests a novel mechanism for regulation of virion tropism in a *herpesvirus* [49].

The gH/gL complex represents the conserved fusion machinery required for viral entry with envelope glycoprotein B. Recent studies, however, demonstrated the ability of viruses to use alternative complexes in absence of principal proteins that play a major role in the entry process. For example, in Herpes simplex virus (HSV) entry into the cell occurs by fusion, and requires a multipartite apparatus made of a glycoprotein quartet: gD, gB, and the heterodimer gH/gL. gD serves as the receptor-binding glycoprotein and interacts with three alternative receptors, nectin1, herpesvirus entry mediator (HVEM) and modified heparan sulfate [50]. It was hypothesized that gB and gH/gL activation occurs through their recruitment to activated gD and that the C-terminal

profusion domain carries the actual binding sites for gB and gH/gL. An alternative possibility is that the C-terminal profusion domain simply enables the conformational changes in gD, but does not carry the actual binding sites for gB and gH/gL [51]. In a recent work, it was demostrated that gD forms a complex with gH/gL in the absence of gB and forms a complex with gB in the absence of gH/gL [52]. These data suggests that the virus is flexible regard to the complex used for infection and, specifically, that the absence of the gH/gL complex could not modify the infectious capacity of the virus because the virus might use an alternative complex when the principal proteins gH or gL are absent. These results suggest a similar flexibility in complex utilization by other *herpesviruses* because of the high similarity between the entry complexes of divergent *herpesviruses*.

In addition, in this study we have been unable to infect human monocytic cell lines (THP-1) using viruses produced in human fibroblast. Productive infection of THP-1 cells was obtained exclusively when viruses were reconstituted in the same cell line. This is a strong indication that the gH/gL/gO and the Pentamer complexes, whose presence was witnessed by the infectious capacity of the same preparations to infect epithelial cells (ARPE-19), are at least not sufficient to cover all tropism observed for HCMV. Indeed, it is rationale to speculate that still unknown additional complexes are likely able to substitute the canonical one in the entry process. On the viral particles we analyzed, gL was the main structural protein that we found undetectable or produced at very low levels compared to gH.

For HCMV gL-less viruses, the identification of alternative gH-based complexes for entry into cells or mediating a different infectious pathway remains a matter of speculation. To date, only one species has been found to bind gH in alternative to gL: in our laboratory, we demonstrated that the product of the UL116 gene is a HCMV envelope glycoprotein that forms a dimer with gH excluding gL and the gH/UL116 complex remains the only gH-

based, gL-less complex of the entire family [53]. The biological meaning of the envelope gH/UL116 complex still remains unknown, but it suggest that the list of complexes on the *hepesviruses* envelope, and our understanding of their function in infection, is still not complete.

Based on a previous report, we have targeted 4 cysteine residues that were identified to stabilize the proteins of both the gH/gL/gO and Pentamer complex respectively. In a very recent work, the structure on the HCMV Pentamer has been solved and further details have emerged [54]. In particular, the crucial importance of the Cys 144 on gL has been confirmed and corroborates the results obtained in this study. As shown here, its mutation results in the disappearence of the protein, thus creating an impairment of Pentamer formation. However, resolution of the disulfide bridges linking gH and gL has revealed the presence of a double disulfide not detected by the mass spectrometry [54]. In our study we have mutated Cys 95 on gH without imparing the second bond. Nevertheless, we observed a strong reduction of cellular and viral levels of gL. This result strongly indicates that the stabilization that disulfide bonds provide to the whole structure is a crucial event for HCMV complex formation.

The surprisingly data, however, remains that fact that these mutants infect three different cell types with the same efficiency that the wild type. Thus, the infectious capability of recombinant viruses lacking gL, and therefore gH/gL-based complexes, suggests that alternative entry complex(es) may exist whose composition has yet to be elucidated.

Among our future perspectives, we have the intention to search new interactors of gH and investigate whether infection can be carried out. These studies already begun with immunoprecipitation experiments on purified viruses using gH antibody. The mutants described in this work represent an ideal starting biological tool for such experiments since they lack gL and could reveal additional

species interacting with gH. Analysis by Mass Spectrometry of these samples will identify these species.

Also, generation of gL-less mutant viruses will be exploited. Although gL has been found to be essential for viral replication in fibroblasts, the data presented in this work clearly indicate that in absence of the protein, in both cell environment and viral envelope, the entire viral life cycle is correctly achieved.

HCMV is known to contain several escape mechanism to productively infect human and survive in a hostile environment. This is not surprising since the *beta-herpesviruses* are the biggest viral human pathogen and their genome encode an high number of proteins whose function has not been elucidated yet for some of them. Although many proteins have been already discovered to be involved into the immunosurveillance escape mechanism, very few of them have been so far found to be involved in the entry mechanism. All considered the results shown here represent a convincing evidence that the list of structural proteins is not complete and that our mutants represent an ideal starting point for such studies.

# *MATERIALS AND METHODS*

## Cells lines and antibodies

Human Foreskin Fibroblast (HFF), Human Fetal lung Fibroblast (MRC-5) and Adult Retinal Pigment Epithelial 19 (ARPE-19) cell lines were purchased from ATCC (catalogue numbers SCRC-1041, CCL-171, CRL-2302, respectively) and cultured according to supplier's instructions. Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco, Life Technologies) was supplemented with 10% fetal bovine serum (FBS) and penicillin streptomycin glutamine (Gibco, Life Technologies). Human Leukemia Monocyte (THP-1) cell lines were purchased from ATCC (catalogue numbers TIB-202) and cultured according to supplier's instructions. RPMI medium (Gibco, Life Technologies) was supplemented with 10% fetal bovine serum (FBS) and penicillin streptomycin glutamine (Gibco, Life Technologies).

Primary antibodies used were: mouse anti-pp65 (Abcam), rabbit anti-gH, rabbit anti-gL, rabbit anti-Penta (from GSK Protein Biochemistry Function, Rockville), mouse anti-GAPDH (Sigma). Secondary antibodies used in this study were: Alexa Fluor 647-conjugated goat anti-mouse (Invitrogen) and HRP-conjugated secondary antibodies from Perkin Elmer.

## Viruses

Wild-type (WT) HCMV (TR strain) was derived from a bacterial artificial chromosome (BAC) containing the HCMV TR genome. The BAC TR was used to create recombinant viral genomes via a marker-less two-step RED-GAM BAC mutagenesis [42]. Briefly,

kanamycin resistance cassette, flanked by I-SceI restriction enzyme cleavage sites, was amplified from pEP-TAP shuttle vector using primers containing homologous regions for the integration in the region of interest. All the primers used are listed in Table 1. Recombination events were performed with *E. coli* GS1783 strain containing a BAC clone of the HCMV TR 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. 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 point mutation in frame with the gene of interest. Presumptive clones were screened by PCR and sequencing analyzed by Vector NTI, while the integrity of the recombinant HCMV genome was assessed through HindIII and BamHI restriction analysis. To reconstitute the virus, actively growing MRC-5 cells from a nearly-confluent T175-flask were trypsin detached, mixed with about 3 μg fresh prepared BAC DNA and 1 μg pCMVKm2-pp71 plasmid and electroporated in 4 mm cuvettes at 250 V and 950 μF. Supernatant was collected from infected cells when cytopathic effect was >90%. For all following infections, HFF cells were used.

## Virus titration

To assay the virus titration of the viral mutants, one day after seeding $1x10^5$ HFF cells per well in 96-well plate, cells were infected with 50μl of 1:10 dilutions of infectious supernatant in infection media (DMEM with L-Glutamine, sodium pyruvate, and 1% pen/strep) at 37°C for 4 h. After incubation, the infectious

inoculum was removed and fresh medium was added. The next day, the cells were assayed to determine the percentage of infection via FACS using antibody against pp65. The cells were incubated with 0.1-0.3 µg of mouse anti-pp65 diluted in DPBS for 1 h at 4°C. After washing the wells five times with DPBS, 0.1 ml of APC-conjugated goat anti-mouse IgG diluted l/300 in DPBS was added and the plate incubated for 1 h at 4°C. The wells were then washed twice with DPBS and the percentage of postive cells was readed by FACS.

## Flow cytometry

Briefly, cells were incubated 30 min at RT with Live&Dead Agua (Invitrogen) diluted 1:400 in PBS, washed twice and incubated for 30 min with blocking buffer (PBS with 1% Bovine Serum Albumin, BSA). The cells were permeabilized with Cytofix/Cytoperm kit (BD) for 30 min in ice and perm/wash buffer was used in all subsequently steps. The cells were incubated for 1h on 4° with primary antibody against pp65. Cells were washed 3 times in PBS and then incubated with Alexa Fluor 647 fluorophore conjugate secondary antibodies for 30 min on ice. Cells were washed thrice in PBS before being analyzed.
The cells were analyzed using a FACSCanto II (Becton Dickinson).

## SDS-PAGE and immunoblotting

Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% polyacrylamide pre-cast gels (Life Technologies) under standard conditions. Proteins were transferred to nitrocellulose membranes (iBlot® system – Life

Technologies), and membranes were blocked with PBS containing 0.05% Tween 20 and 10% powdered milk. Antibodies were diluted in PBS containing 0.05% 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 (Thermo Scientific) were used according to the manufacturer's instructions.

## HCMV Virions purification

The supernatant of infected cells was collected when 100% of cytopathic effect was observed and subjected to 4000 rpm centrifugation for 20 minutes at 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 60 minutes. The pelleted virus was resuspended in 25ul of RIPA Buffer and incubated for 1h vortexing every 10'. Tubes were centrifuged at 13000 rpm for 20 minutes. Proteins (supernatant) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% polyacrylamide pre-cast gels (Life Technologies) under standard conditions.

## Densitometric analysis

The densitometric analysis of signal intensity in Western blotting was performed with IMAGEJ software.

## Table 1: List of oligos

| Name | Sequence |
|---|---|
| gLCys$_{144}$Arg F | CAGCGCCGCGCTGGATGACGGTAATGCGCGGCTACAGCGAGCGCGGC GATGGCTCGCCGGCCGTTAAGTAGGGATAACAGGGTAATCGAT |
| gLCys$_{144}$Arg R | GGTCGTCCACGCACGTGTACACGGCCGGCGAGCCATCGCCGCGCTCGC TGTAGCCGCGCATTACCAACCAATTAACCAATTCTGATTAG |
| gLCys$_{144}$Arg Screen F | CGTTGGTGAATGTTACCGGGC |
| gLCys$_{144}$Arg Screen R | CGCACGGCGCGGTTGGTACG |
| UL128Cys$_{162}$Arg F | GTACCTGGAGAGCGTTAAGAAACACAAACGGCTGGATGTGCGCCGCGC TAAAATGGGCTATATTAAGTAGGGATAACAGGGTAATCGAT |
| UL128Cys$_{162}$Arg R | CATTTTATTATTCACTGCAGCATATAGCCCATTTTAGCGCGGCGCACAT CCAGCCGTTTGTGTTCAACCAATTAACCAATTCTGATTAG |
| UL128Cys$_{162}$Arg Screen F | CGCTGCGGCAAAGTGAACG |
| UL128Cys$_{162}$Arg Screen R | GCGCTATCGCCAGATATCGCG |
| gOCys$_{351}$Arg F | GTGGGTGTATACCACTCTACGTTACCGGCAAAACCCTTTTAGGGAATC AAGCCGCAACCGAACTAAGTAGGGATAACAGGGTAATCGAT |
| gOCys$_{351}$Arg R | TCATAAATTCTGATACGGCGGTTCGGTTGCGGCTTGATTCCCTAAAAGG GTTTTGCCGGTAACCAACCAATTAACCAATTCTGATTAG |
| gOCys$_{351}$Arg Screen F | CGTCAACCGCTCTGAACG |
| gOCys$_{351}$Arg Screen R | GGTGTAGTTTCGGAAGCCG |
| gHCys$_{95}$Ser F | CCAAAGCTATAATCAATACTATGTATTCCATATGCCTCGAAGTCTTTTT GCGGGTCCTCTGGCTAAGTAGGGATAACAGGGTAATCGAT |
| gHCys$_{95}$Ser R | CCTGGTTCAGAAACTGCTCCGCCAGAGGACCCGCAAAAAGACTTCGAG GCATATGGAATACATCAACCAATTAACCAATTCTGATTAG |
| gHCys$_{95}$Ser Screen F | CGCGTTTCACCTACTACTCAAC |
| gHCys$_{95}$Ser Screen R | CAGCCGTGTGGAGTGGTTTGCG |

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## List of publications

- Caló S., Cortese M., Ciferri C., Bruno L., Gerrein R., Benucci B., Monda G., Gentile M., Kessler T., Uematsu Y., Maione D., Lilja AE., Carfí A., Merola M. *The Human Cytomegalovirus UL116 Gene Encodes an Envelope Glycoprotein Forming a Complex with gH Independently from gL*. J. Virol. 2016; 90(10):4926-38.

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