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Analysis of interactions between HCMV and the host-immune system

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A mia nonna e a Nannina,

per me esempi di forza, punti di riferimento.

Mi avete insegnato che non è importante

quante difficoltà e momenti brutti ci siano,

basta affrontarli, si superano..

e, sempre serenamente, "come Dio vole"

grazie..

INDEX

CHAPTER ONE

INTRODUCTION

1. Human Cytomegalovirus virion structure and genome organization

Human Cytomegalovirus (HCMV) is a member of the *β-herpesviridae* family that includes the herpesviruses of mammals, birds and reptiles [1]. Although more recently validated by genome sequences, historically the members of the family were classified based on the virion structure, composed of an envelope, a tegument and a capsid surrounding an inner core containing the viral genome. The virus envelope is composed of a double host-cell derived lipid layer in which are embedded the viral glycoproteins necessary for the cellular attachment and entry. Compared to the other members of the family, the envelope shape is very irregular and generates viral particles whose diameter reaches up to 300 nm, with an average size of 230 nm diameter. Below the envelope, an amorphous matrix referred as tegument is present. This compartment carries the majority of the HCMV expressed proteins plus a remarkable number of host species including proteins and nucleic acids. Several crucial viral functions are incorporated in the tegument including kinases, trans-activating factors and the most abundant and immunogenic protein pp65. The HCMV capsid is embedded in the tegument. The core of the capsid contains a tightly packed linear double stranded DNA genome. In line with the huge virus dimension, HCMV has the largest genome among its family with approximately 230 kb, 50% larger than HSV, despite the capsids of both viruses being roughly of the same size (110-125 nm) [1].

As for the other herpesviruses, genome organization reflects the common structure 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 phenomenon can occur among identical terminal and internal repeats, leading to genome isomerization.

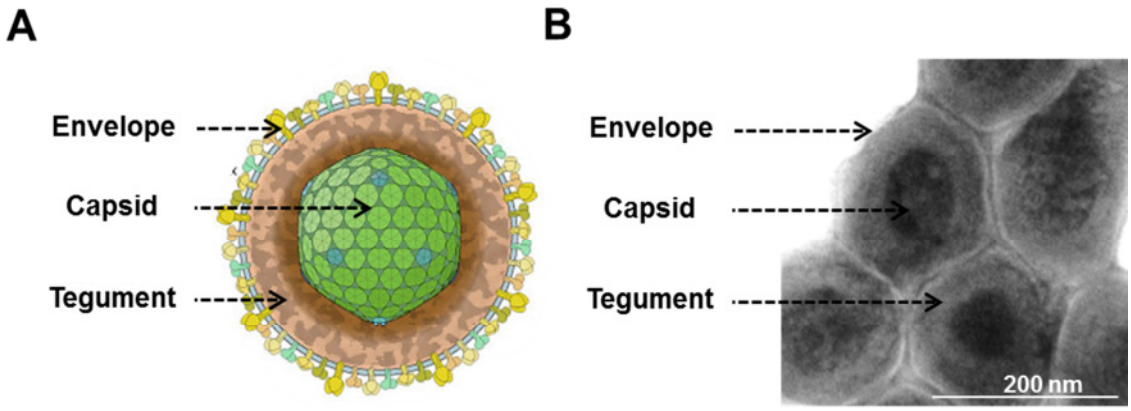

Figure 1: (A) Schematic representation of HCMV virion (modified from http://viralzone.expasy.org/all_by_species/180.html). **(B)** Negative stained purified virion preparation analyzed through electron microscopy.

Thus, genetic material isolated from a viral population consists of equal amounts of four different genomic isomers pooled together [6].

While the general genomic arrangement, consistent of repeated and unique sequences, is conserved among HCMV strains, a major difference in the open reading frame (ORF) composition and organization can be observed among "laboratory strains" and "clinical isolates". The latter are generally defined as viruses that underwent through none or limited passages in fibroblast cells before being sequenced and/or cloned as bacterial artificial chromosomes (BACs) . On the contrary, laboratory strains indicate all the strains extensively passaged and adapted to grow in human fibroblasts. Historically, these ones were the first to be selected and used since they exhibit rapid replication and high yields of produced virus. Consequently to the fibroblast adaptation, severe genomic rearrangements occurred in these viruses. Both AD169 and Towne laboratory strains acquired a large deletion in a multilocus of the UL segment, concomitantly being replaced by duplicated RL region. The abrogation of several envelope glycoproteins encoded in the deleted segment resulted in an impaired tropism, tightly restricted to fibroblast cells only. Due to these rearrangements, a difference in the coding potential can be observed between laboratory strains and clinical isolates. In

particular, while the AD169, a laboratory strain prototype, is predicted to encode for 208 ORFs (including the repeated segments), the coding potential of a clinical isolate is estimated around 252 ORFs [7]. Recently, through an integrated approach combining ribosome footprinting assays, high-resolution mass spectrometry and analysis of mutated viruses, Stern-Ginossar and colleagues were able to identify a total of 751 translated ORFs including short peptides [8]. In spite of this huge coding potential, only a small subset of these proteins constitutes the mature virion: it has been estimated that around 50 [1, 9] proteins are inserted in the virion, divided through the different compartments.

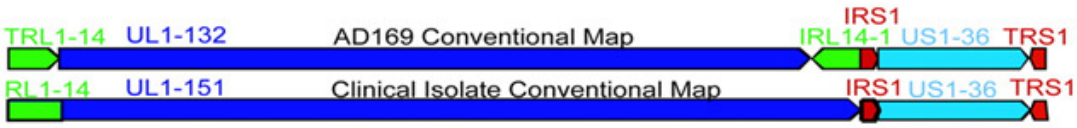

Figure 2: HCMV genome organization. ORFs map of conventional laboratory strain AD169 and clinical isolate. The AD169 genome (upper) carries TRL1-14 (green arrow), UL1-132 (dark blue arrow), IRL14-1 (green arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow). In the clinical isolate RL1-14, (green arrow), UL1-151 (dark blue arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow) are present. Modified from [7].

2. Envelope glycoproteins and viral replication

Envelope glycoproteins play a key role in HCMV pathogenesis, being the modulator of viral entry. Out of the total number of proteins composing the virion, around 20 [1, 9-11] were identified as envelope associated proteins. Notably, only 8 were demonstrated as essential for virus replication *in vitro* in different cell types: glycoproteins B (gB), M (gM), N (gN), L (gL), H (gH), UL128, UL130, UL131 [12]. All these proteins act as complexes on the viral surface. gB is an homotrimeric complex mediating virus attachment and fusion, involved in the first step of heparin sulfate proteoglycans (HSPG) tethering. The most abundant envelope glycoprotein, gM, forms an eterodimeric complex associating with gN that is present on the virion only as 1:10 ratio to gM. Like gB, gM:gN complex is involved in HSPG tethering and stabilization of virus-cell contact. The complex that has been identified as

involved in the fusion event is composed by gH and gL. This complex is sufficient to mediate fibroblast infection while formation of a pentameric complex through additional binding of UL128, UL130 and UL131 gene products is necessary for the infection of epithelial, endothelial, polymorphonuclear leukocyte and dendritic cells (DC). Indeed, the attenuated tropism observed in laboratory strains can be explained by the mutations of the UL128-UL131 locus [7, 13, 14].

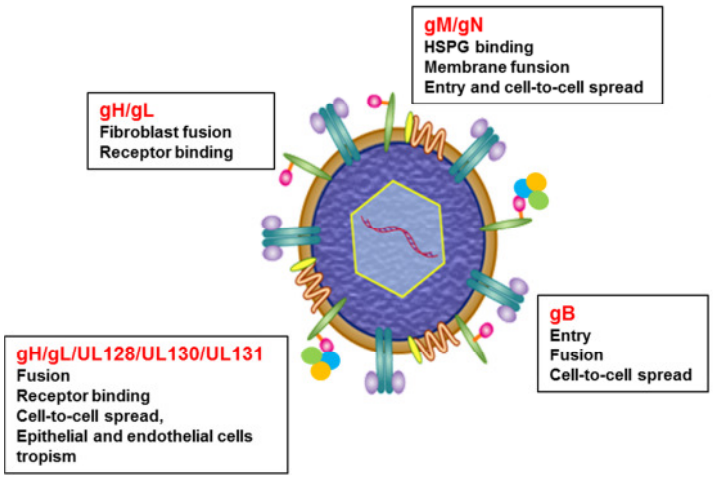

Figure 3: HCMV envelope glycoprotein complexes. The proposed role of each complex is indicated in the respective box. Modified from [2].

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 entry consists of a first low specificity - high avidity tethering step of the cell surface HSPGs mediated by both gB and gM/gN [2, 12]. The interaction with high avidity receptors has been shown to follow the first attachment step. A series of "post-attachment" receptors were proposed over the time, even if none of them completely fulfilled this role. The most accredited cellular interactors are the β1 and, to a lesser extent, the β3 integrins. Engagement of gB by these molecules induce receptors clustering and intracellular signaling. The entry step culminates with gH/gL mediating the fusion of viral and cellular membranes. While direct fusion at neutral pH has been observed *in vitro* in fibroblast cells [15], low pH dependent receptor-mediated endocytosis is required for viral entry in epithelial and endothelial cells [16-18]. Moreover, a recent report suggests that HCMV entry into DCs relies on macropinocytosis-like pathway in a cholesterol-dependent and pH-independent manner

[19]. Interaction between gB and dendritic cell specific C-type lectin DC-SIGN (DC specific ICAM-grabbing non-integrin) has been indicated to have a prominent role in DCs infection [20].

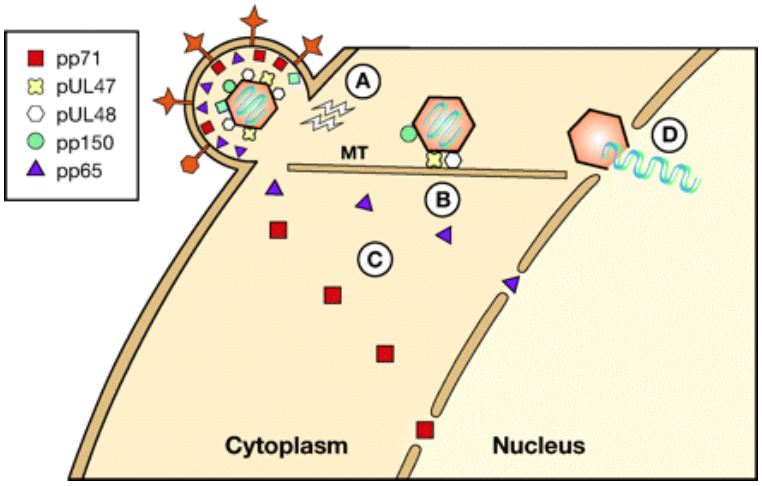

Figure 4: Delivery of HCMV capsid to the nucleus. (A) HCMV capsid and associated tegument proteins are transported along the microtubules (B) toward the nucleus. A set of tegument proteins dissociate from the capsid and migrates independently to the nucleus (C). Viral DNA is released into the nucleus through the nuclear pores (D). Modified from [3].

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 remains tightly associated with the capsid and contributes to the delivery of the DNA to the nucleus [3]. 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 [3]. Viral replicative cycle starts shortly after with the expression of immediate early (IE) genes, that can be detected as short as 4 hour post infection [21-23]. During the replicative cycle, genes expression can be divided in distinct temporal phases, defined as immediate early, early and late, linked together by a tightly regulated temporal cascade. A set of genes expressed with immediate early kinetic, whose transcription do not require *de novo* protein synthesis, act as global regulators of viral genes expression. Early

genes protein products are responsible for DNA replication, while late genes encode for structural proteins and are transcribed following viral DNA replication [3].

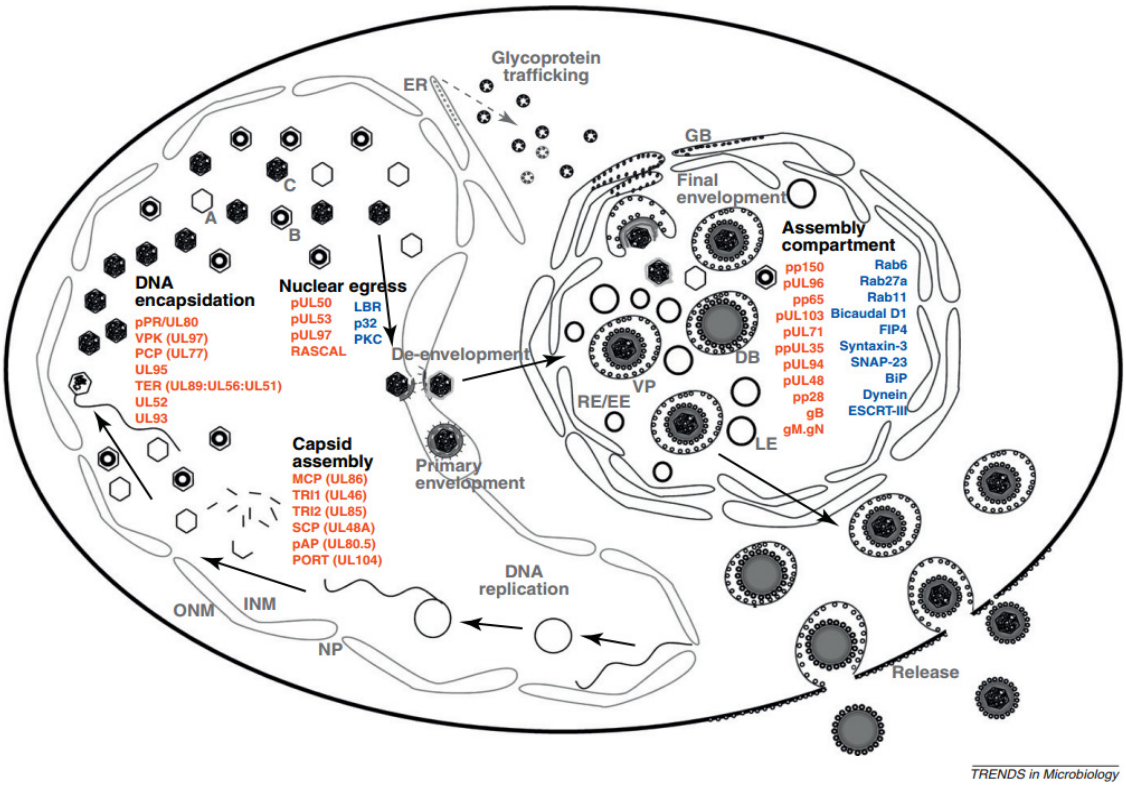

Figure 5: HCMV maturation. The model illustrates the HCMV virion particles formation, maturation and budding processes. Major cellular and viral proteins involved in these processes are reported (red and blue, respectively). List of abbreviations: DB, dense body; VP, virus particle; EE/RE, early endosome/recycling 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. From [5].

HCMV binding and entry induce 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 exit the nucleus through a successive envelopment/de-envelopment process at the nuclear membrane [5, 24] 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 [25]. Viral assembly complex is organized as nested cylinders of secretory apparatus structures, surrounded by the endoplasmic reticulum (ER) network [26, 27]. Golgi and trans-Golgi network membranes constitute the edge of the AC, while the inner part contains vesicles positive for both the early and recycling endosomes markers. Secondary viral envelopment occurs when a viral particle capture a vesicle promoting its bending and fusion around the tegumented nucleocapsid [28]. Vesicles containing mature enveloped HCMV virions fuse with the cellular plasma membrane releasing the viral particles.

3. Pathogenesis

Human cytomegalovirus infection is ubiquitous among adult population, ranging around 55-60% of the world population, although reaching peaks of more than 90% seroprevalence in certain areas grouped according to age-gender-socioeconomic related factors [1]. Horizontal transmission can occur through exchange of bodily fluid at mucosal surfaces. Primary infection is generally asymptomatic in healthy individuals, where a strong immune response to HCMV is able to limit and contain the spread of the disease. Bland clinical symptoms and spontaneous acute infection resolution are associated to infection of immunocompetent individuals; therefore antiviral therapies are usually not indicated in these settings [1]. Nevertheless, complete clearance of the virus by the organism is never achieved and lifelong lasting latency with recurrent and spontaneous viral reactivation is always observed. Viral replication and spread is brought under control by the combined action of both innate and adaptive immune responses: severe diseases, associated with HCMV infection and reactivation, occur in settings where immune response is severely suppressed (transplantations related immunosuppression) or compromised (AIDS patients). Moreover, due to the HCMV ability to cross the transplacental barrier, congenitally acquired infection can pose a severe treat to the fetus that lacks a fully functional immune system able to counteract the viral

infection. Thus in the presence of an impaired or absent immune system, antiviral therapies are necessary to counteract the severe disease associated to the HCMV primary infection or reactivation. However almost all the antiviral agents actually on the market showed a significant number of potential side effects including marrow and organ toxicity and increasing drug resistant viral strains [1].

Passive immunization using hyperimmune globulin preparations with high levels of antibodies against HCMV (CMVIG) are usually implemented as prophylactic tools to reduce the morbidity and mortality associated to HCMV infection in immunocompromised patients. For example, combination of antiviral drug *glancicovir* and CMVIG proved to be effective in preventing HCMV infection in patients undergoing solid organ transplant [29]. Moreover, a study suggests their effectiveness in the treatment and prevention of severe diseases associated to congenital HCMV infection [30].

Nevertheless, rather than a general prophylactic tool, the efficacy and effectiveness of CMVIG in reducing HCMV infection seems to be restricted to specific clinical cases while only modest beneficial effects were shown upon CMVIG treatment in other settings such as hematopoietic cell transplantation (HCT) and blood transfusion in premature newborns [31]. Additionally, CMVIGs exhibit low potency *in vitro* [32] and must be administrated at high doses to reach the amount of neutralizing antibodies required to exert a beneficial effect. To increase the efficacy of a passive immunization therapy, a series of neutralizing monoclonal antibodies against immunodominant HCMV envelope glycoproteins were developed. For example, a highly *in vitro* neutralizing antibody against gH was isolated from the spleen of a CMV seropositive patient. The monoclonal antibody, named MSL-109 or sevirumab, was tested in phase II clinical trials as treatment for CMV-induced retinitis in AIDS patients [33]. Unfortunately the tests were stopped due to lack of efficacy [34].

4. Immune response

Primary infection in immunocompetent individuals is accompanied by a period of viral dissemination (viremia). In this phase, virus excretion in bodily fluids, such as breast milk, blood, urine, saliva and seminal secretions lasts from months to years depending on the host age, and is the primary cause of viral transmission. Drop and clearance of the acute infection phase correlates with the mounting of strong humoral and cellular adaptive immune responses. Despite the setting up of a strong response, a complete clearance of infected cells is never observed. HCMV is able to establishing lifelong latency remaining silent in myeloid lineage cells [35].

4.1 Humoral immunity

Evidence from both the mouse and guinea pig animal models suggest that antibody is important in protection from a lethal infective dose and in reducing fetal [36, 37]. In humans pre-existing anti-cytomegalovirus antibodies generated following primary infection prior to conception play an important role in preventing congenital infection of the foetus during pregnancy [38] and can protect against transfusion borne infection in premature infants [39]. The role of HCMV specific antibodies to control reactivation and viral dissemination in immunosupressed patients is less clear. Evidence for the benefits of administration of HCMV specific antibody (hyperimmune globulin) to immunosuppressed transplant patients either for primary infection or reactivation has been described in renal transplants [40], but the benefit is less clear in allogeneic stem cell transplantation patients, with some evidence that it is beneficial [41] and others suggesting no beneficial outcome [42, 43].

HCMV primary infection elicits antibodies specific for numerous HCMV proteins including structural tegument proteins (e.g. pp65 and pp150), envelope glycoproteins (predominantly gB and gH) as well as non-structural proteins such as the Immediate Early 1 protein (IE1, UL123) [44, 45]. It was initially reported that viral neutralizing activity is

predominantly mediated by antibodies specific for gB and gH in *in vitro* assay systems [46, 47]. More recently it has been recognized that passage of wild-type HCMV strains leads to the mutation and deletion of numerous viral genes [48] and of particular relevance to antibody mediated neutralization is the five member complex of gH/gL/UL128-131A which is required to mediate entry into various cell types including endothelial, epithelia and myeloid cells [13, 14, 49-51]. Antibodies to conformational epitopes formed by two or more members of the complex are generated to HCMV infection and have a superior neutralizing ability when compared to gB and gH antibodies [52]. Further evidence of the role of humoral response in controlling HCMV infection were recently showed in a work reporting correlation between fetal cytomegalovirus transmission and maternal antibodies production and avidity against the pentameric gH/gL/UL128-131A complex [53, 54].

4.2 Cellular immunity: CD8+ T cell responses

There is strong evidence from both the murine MCMV model and from patients undergoing bone marrow (BM) and stem cell transplantations (SCT) that HCMV specific CD8+ T cells are a crucial protective component of the immune response to this virus. Mice are protected from lethal MCMV challenge following adoptive transfer of MCMV immediate early antigen specific CD8+ T cells into animals with an ablated immune system [55], CD8+ T cells were able to prevent lethal infection in the absence of CD4+ T cells [55-57]. In murine models of BMT, removal of reconstituted CD8+ cells leads to lethal disease and reconstituted CD8+ T cells transferred to immunocompromised mice could prevent disease [58]. However, other lymphocyte subsets can provide functional redundancy as following CMV reactivation in B cell deficient mice it has been demonstrated that removal of the CD4+ and NK cells lead to reactivation [58].

Studies on the specificity, function and phenotype of HCMV specific CD8+ T cells have been ongoing for 30 years. The first viral proteins identified as targets of the CD8+ T cell

response to HCMV was the immediate early protein [59] and the tegument protein pp65 (UL83) [60]. Numerous studies have been performed utilizing increasingly sensitive methods of detection (IFNγ secretion either by ELISPOT or intracellular flow cytometry as well as MHC Class I tetramers). They have shown that most HCMV seropositive individuals have a CD8+ T cell responses to pp65 and IE and that the magnitude of these responses is often very high [60-66].

Stimulation of PBMC using US2 to US11 deleted HCMV infected autologous fibroblasts (this virus can no longer cause MHC Class I down regulation, see section 4.4), revealed high frequency responses to pp65 and IE1, pp150 and gB. However, T cells with many other specificities were also generated many of them were immediate early or early viral protein specific, although the ORFs were not defined [67]. The most comprehensive study has determined IFNγ responses from both CD4+ and CD8+ T cells to 213 predicted HCMV encoded open reading frames (ORFs) using 13,687 peptides and a panel of 33 seropositive donors with disparate MHC Class I types. 151 ORF's were shown to elicit a CD4+ or CD8+ T cell response in at least one donor. Three ORF's were recognized by more than half of the cohort, UL48, UL83 (pp65), and UL123 (IE). CD8+ T cell responses from HCMV seropositive donors recognized a median of 8 ORFS, however responses were highly heterogeneous between individuals with some recognizing only a single ORF and as many as 39 [68].

During latency, transcription of viral genes is highly restricted and a number of genes have been shown to be expressed. These include UL111.5 a viral IL-10 homologue [69], UL138 [70] and UL8182AS a viral transcript found antisense to UL81-82 termed latent undefined nuclear antigen (LUNA) [71]. UL138 and a truncated sequence of UL111.5 were included in the T cell proteome screen, UL138 was both a CD4+ and a CD8+ target in just one donor [68]. Despite the extensive number of HCMV proteins that are targets for the

CD8+ T cell response the vast majority of the immunobiology of the CD8+ T cell response in primary infection and long-term memory has been studied using pp65 and IE specific T cells. The total CD8+ T cell response to HCMV is large, an estimation of about 10% of the peripheral CD8+ memory T cells are directed against HCMV determined by screening all the HCMV ORFs in multiple individuals, this group included young to middle aged individuals but not old individuals [68].

4.3 Cellular immunity: CD4+ T cell responses

Human CD4+ T cells include multiple subsets which can be broadly divided into helper T cells (further subdivided into those that provide help to CD8+ T cells (Th1) and those that interact with B cells (Th2), regulatory T cells (Treg) and specialist subsets such as Th17 cells that are involved in inflammation and anti-parasite responses.

Evidence for their protection role in cytomegalovirus immunity is supported by studies in mice infected with MCMV, which show that long term depletion of the whole CD4+ T cell compartment *in vivo*, was associated with persistent virus replication at specific anatomical sites [72].

In humans it has been shown that an extended period of HCMV secretion was seen in young children with an impaired HCMV specific CD4+ T cell response [73]. In renal transplant patients undergoing primary HCMV infection IFNγ-producing CD4+ T cells precede the emergence of the CD8+ T cell response in patients with asymptomatic infection, whereas those patients that had a delay in HCMV specific CD4+ T cell generation developed disease [74]. Following bone marrow transplantation, the maintenance of HCMV specific CD8+ T cell infusions was dependent on the presence of HCMV-specific CD4+ cells [66, 75], suggesting that CD4+ helper T cells are essential for effective CD8+ T cell responses.

Human antigen-specific CD4+ T cells have been identified by intracellular cytokine production, CD4+ T cells often respond to the same ORFs as CD8+ T cells and both pp65 and

IE-specific CD4+ T cells have been detected in a very high proportion of individuals tested [76-79]. Individual peptide epitopes have been identified in pp65, IE, gB and gH [4, 79-81]. An analysis of the CD4+ T cell response to the whole HCMV proteome has shown that the response is very broad, with an individual responding to a median of 12 ORFs, five ORFs (UL55, UL83 (pp65), UL86, UL99, and UL122/123 (IE)) were recognized by more than half the donors tested. It was estimated that the entire HCMV specific CD4+ T cell response in these young to middle aged donors comprised about 10% of the CD4+ T cells present in the peripheral blood [68].

During primary infection (in a kidney transplant model), HCMV specific CD4+ T cells, can be detected 7 days after the detection of HCMV DNA in peripheral blood [82]. These cells produce T helper type 1 (Th1) cytokines IFNγ and TNFα but not the T helper type 2 (Th2) cytokine IL-4 [83]. There is also accumulating evidence suggesting that HCMV specific CD4+ T cells can act as effectors directly upon virally infected cells [82, 84-87]. Subjects with higher levels of HCMV specific CD4+ T cells that secrete IFNγ clear the virus faster and exhibit fewer symptoms [74, 88].

4.4 Manipulation and evasion mechanisms

To escape from the immune system surveillance, HCMV has developed a huge arsenal of genetic functions committed to alter and modulate both innate and adaptive arms of the immune response. In particular, HCMV co-evolution within its host has led to the incorporation of a repertoire of functions with strong homologies to host genes. Thus, the virus is able to subvert the immune system mimicking the same strategies and mechanisms used by hosts to clear the infection. Inhibition of complement cascade and natural killer (NK) cells activation, attenuation of interferon (INF) response and disruption of antigen presentation are only few examples of the functions hijacked by virus encoded chemokines, cytokines and cellular receptors homologues [35].

Table 1: List of selected protein functions encoded by cytomegalovirus that interfere with adaptive and innate immune response. Modified from [35].

Adaptive immune response	Innate immune response
Inhibits MHCI and II expression ***(US3)***	Fcγ Receptor Homologs ***(UL119, RL11, RL13, RL12)***
MHC I and II degradation ***(US2, US11)***	Interferon-Mediated Immunity ***(IE2, UL83)***
Inhibits TCR signallig ***(UL11)***	Natural Killer Cells ***(UL18, UL142, UL141, UL40)***
Binds MHC I molecules ***(US8, US10)***	Cytokine Homologs ***(UL111)***
Inhibits TAP peptide transport ***(US6)***	Viral Chemokine Homologs ***(UL146, UL147, UL128-131)***
Inhibit MHC II expression ***(UL83)***	Apoptosis ***(UL36, UL37x1)***

It has been recognized since early 1990s that MHC Class I expression on the surface of HCMV-infected cells progressively diminishes with increasing time after infection. HCMV employs a number of mechanisms to interfere with the normal cellular MHC Class I processing and presentation pathways in order to prevent CD8+ T cell recognition. HCMV viral genes US2 and US11 degrade newly synthesized MHC Class I heavy chains [89-91]. US3 retains MHC Class I peptide complexes in the endoplasmic reticulum (ER) [92]. US6 blocks peptide translocation into the ER [93]. The number and diversity of these mechanisms is surprising. However, this apparent redundancy may have distinct advantages to the virus. The sequential expression of the US3 and US11 gene products, which would lead to MHC Class I-peptide complex retention in the ER followed by degradation of de novo MHC Class I heavy chains, may be very efficient. The combination of US2 and US11 may allow many different MHC Class I heavy chains to be redirected to the cytosol for degradation. HCMV infects a number of different cell types *in vivo* and it is conceivable that some of these gene products are more efficient in some cell types than others. The genes are also expressed at different times during HCMV replication, US3 is expressed at immediate early times while

the other proteins are expressed from early times, expression then continues through the viral life cycle.

The action of these genes may not completely protect cells from CD8+ T cell recognition dependent presentation of IE antigen to human T cells, pp65 peptides were still presented [94]. There is also some evidence that MHC Class I downregulation genes are not redundant as mutant viruses expressing gpUS2 and gpUS11 alone only incompletely protect HCMV infected fibroblasts from CTL recognition by both IE and pp65 specific T cells [95].

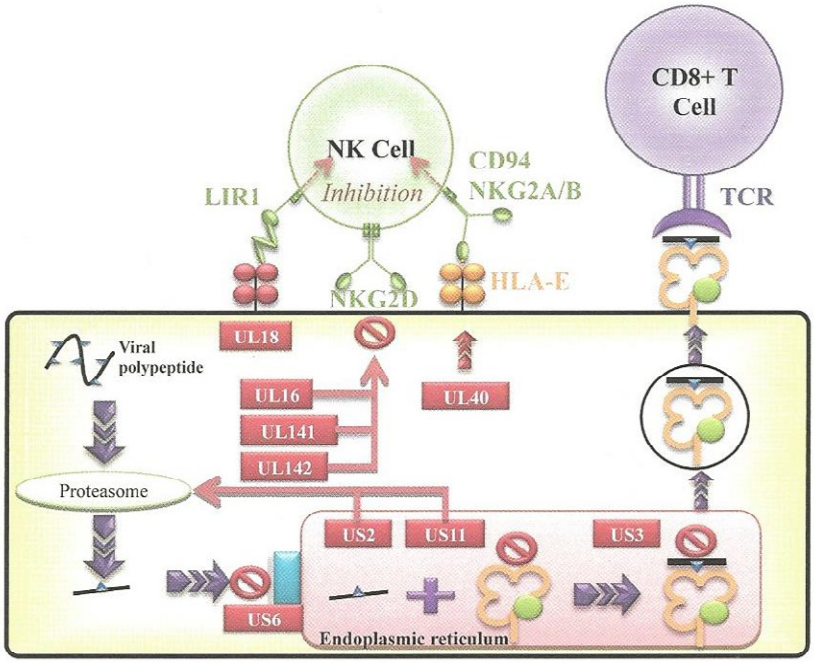

Figure 6: CD8+ T-cell and NK evasion by HCMV-infected cells. Disruption of MHC Class I processing and presentation to CD8+ T-cells by HCMV proteins US2, 3, 6 and 11 causes the inhibition of expression of cell surface MHC Class I loaded with viral peptides and prevents CTL recognition. The lack of cell surface MHC Class I decreases inhibitory signaling to NK cells, which is substituted by UL18 and UL40 expression. Stress-induced ligands of NK cell receptors are prevented from cell surface expression by HCMV expression of UL16, UL141 and UL142 in order to inhibit NK cell activation. From [4].

A large number of HCMV encoded gene products target the MHC class I antigen presentation pathway in an attempt to avoid CD8+ T cell recognition. HCMV also evades the CD4+ T cell response by a number of methods. Disrupting IFNγ induced, upregulation of MHC class II molecules to the cell surface by preventing the expression of Janus kinase 1 and

repression of Class II transactivator mRNA. The virally encoded gene product of US2 also inhibits MHC class II presentation to CD4+ T cells by redirecting the HLA-DRα and HLA-DMα chains to the cytosol where they are degraded [96]. More recently a truncated transcript to UL111A, a viral homologue of the immunomodulatory cytokine IL-10, which is expressed during latency (cmvLA IL-10) has been shown to down regulate expression of MHC class I and II molecules, inhibit proliferation of peripheral blood mononuclear cells and inhibit the production of inflammatory cytokines [97-99]. The same group in a further study showed that the presence of cmvLA IL-10 during latent infection of myeloid progenitor cells, suppressed both allogeneic and autologous recognition by CD4+ T cells and may represent a mechanism by which HCMV latency evades the CD4+ T cell response and aid in the maintenance of the latent state [69]. Taken together these reports show that HCMV dedicates significant resources to target MHC Class I and Class II presentation pathway, and hence the host T-cell response. However, it is nevertheless generated. The production of immune evasion molecules that target MHC pathways may represent only a mechanism, during early time of infection, reactivation or both, to create a window of opportunity for the virus to disseminate and establish latency, before the inevitable induction of the T-cell response and the control of infection. Recent works showed new mechanisms the virus uses to shut down T-cell, interacting directly with their activation pathway, through the TCR [100]. More probably, other mechanisms have still to be uncovered to understand the complex interactions between HCMV and the host immune system.

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PROJECT SUMMARY

Human Cytomegalovirus (HCMV) exerts complex effects on the host immune system through expression of several interfering functions. Although a remarkable number of the genes implicated in immune evasion have been identified, due to the huge coding potential of the HCMV genome and to the numerous ORFs with unassigned functions the list is predicted to grow.

As the humoral response is known to play an important role in the control of HCMV infection and outcomes, we started a characterization of the antibody repertoire in patients infected by the virus. Through a combined approach of Reverse Vaccinology, recombinant mammalian protein expression, immunoblotting and protein microarrays, a total of 25 viral proteins were found to be recognized by Immunoglobulin (human plasma/Cytogam®) of HCMV infected patients. Among them, the majority were known to be involved in the control of host immune response, while others were only predicted ORFs. Based on bioinformatics and literature data we decided to focus our work on the characterization of UL10 and UL139 proteins.

UL10 is a member of the HCMV RL11 family. The characteristics of the UL10 protein, a predicted heavily glycosylated Ig-like membrane protein, that is known to be dispensable for viral replication in cultured cells, suggested a possible role in host-cell interaction. *Ex-vivo* cell based assays (Rosetting assay) and flow cytometry experiments on both lymphoid cell lines and primary blood cells showed that the protein interacts with a ubiquitous receptor on the surface of human lymphocytes. Proliferation assays performed on PBMC purified from blood of healthy donors showed that the protein significantly reduced the proliferation of stimulated T-cells. Further ongoing experiments aim to identify the cellular receptor and characterize downstream signaling functions. Uncovering the role of the UL10 protein during

HCMV infection could allow new insights into the modulation of the immune response triggered by the virus and ultimately the development of new therapies.

UL139 is a predicted ORF coding for a type I membrane glycoprotein. It is located in a polymorphic locus deleted in laboratory adapted strains of HCMV. It has a region of homology with CD24 and bioinformatics analysis predicts a long and quite conserved cytoplasmic tail, suggesting a possible role in as signal transduction/structural protein during the infection cycle.

We characterized UL139 protein during the infection cycle in fibroblasts, using an engineered HCMV TR strain carrying a tagged version of the protein. We observed that the protein is a non-structural viral protein, it is expressed with a late kinetics and it co-localizes with early endosomes marker, enclosing the viral assembly complex. Data obtained by pull-down and MS experiments suggested the presence of cellular interactor(s) involved in the vesicular transport/cytoskeleton reorganization. Preliminary data strongly indicated Fascin, a cellular protein involved in actin rearrangement, as potential UL139 interacting protein. Further ongoing experiments aim to verify this hypothesis and uncover the biological significance of such interaction.

CHAPTER TWO

RECOMBINANT HUMAN CYTOMEGALOVIRUS (HCMV) UL10 INTERACTS WITH A RECEPTOR ON LEUKOCYTES SURFACE AND INHIBITS T CELL PROLIFERATION

1. INTRODUCTION

Human cytomegalovirus (HCMV) is a widespread and highly host-specific member of the *herpesviridae* family that persists indefinitely in immunocompetent hosts despite induction of strong humoral and cell-mediated immune responses [1]. Infection of immunocompetent individuals with HCMV rarely results in symptomatic disease, but it can cause severe morbidity and mortality in individuals who are immunocompromised or immunologically immature, such as AIDS patients, transplant recipients and congenitally infected neonates. Cellular immunity, in particular Natural Killer (NK) cells and $CD8^+$ T cells, has been found to be pivotal in controlling CMV [2, 3]. Yet, despite the induction of strong cellular and humoral immune responses, CMV is able to establish latent infection, reactivation, as well as reinfection with multiple CMV strains, and they seem to be quite frequent events [4-6].

HCMV has the largest genome (235 kb) of any characterized human virus and is believed to encode around 200 proteins and several microRNAs [7-9]. Still, large part of the predicted viral products have to be characterized. Previous work showed that a large percentage of the HCMV genome is dispensable for growth in fibroblasts *in-vitro* [10, 11], as it is likely devoted in modulating host response in order to ensure its efficient replication and survival. A remarkable number of viral gene products are known to be designated to interfere and control the adaptive and innate immune responses through a variety of mechanisms, such as altering

antigen presentation pathways; interfering with natural killer (NK) cellular responses or mimicking the function of cytokines, chemokines and their receptors [12, 13].

As is the case with other large DNA-containing viruses, herpesviruses co-evolution with their hosts allowed them to capture cellular genes in order to augment their replication capacities, as well as their abilities to persist and propagate. During time, these genes have been modelled to create highly specific molecules, which may preserve functions related to the original host protein or, alternatively, they may evolve retaining some key structural properties while performing completely novel functions [14]. CMVs, in particular, are equipped with homologues of major histocompatibility complex (MHC) class-I molecules, Fc receptors, cytokines, chemokines and their receptors [15-18].

It is a long standing observation that T lymphocytes in patients with acute HCMV infection display reduced proliferation capacity [19-23] that may result in transient immunosuppression associated with an increased risk of secondary infection [24, 25]. A number of mechanisms have been proposed by which HCMV may interfere with the priming of T cells as well as with their effector functions. The inhibition of MHC class I antigen presentation pathways by HCMV is well established; limiting the recognition and lysis of infected cells by cytolytic T lymphocytes [12, 26]. Another strategy that acts on the ability of T cells to proliferate is the secretion of host and virally encoded suppressive factors from HCMV infected cells; the virus induces enhanced secretion of transforming growth factor β1 and soluble CD83, and itself encodes an interleukin-10 homologue that suppresses T cell proliferation [26, 27]. Other suppressive functions require direct contact between infected cells and T cells [22]: indeed, upregulation of pro-apoptotic ligands on the surface of HCMV-infected dendritic cells that can induce apoptosis in activated T cells [28]. Another example is the direct interaction of surface exposed UL11 protein (pUL11) on HCMV infected cells with T cells regulatory

receptors CD45 that inhibits proliferation [29]. Additional mechanisms could exist to obtain a complete shutdown of the immune response during HCMV infection

Cellular proteins and also immunomodulatory proteins of various viruses that mediate the interaction with surface proteins of immune cells often contain immunoglobulin-like or MHC-like domains [30-32]. The CMV genome encodes a number of putative transmembrane proteins with such a property [8], the most prominent being the RL11 family, that includes 14 largely uncharacterized proteins. The defining motif of this family is the RL11 domain, which has limited sequence homology to immunoglobulin domains and to the immunomodulatory E3 proteins of adenoviruses [8, 33]. Few members of the RL11 family have been studied more deeply: the TRL11/IRL11 and the TRL13 proteins, that are Fc-receptors that binds human immunoglobulins, presumably mediating escape from recognition by anti-viral immunoglobulins [34-36]; the UL7 protein (pUL7), that is shed from infected cells and impairs cytokine production in DC [37]; and, more recently, the UL11 protein, that binds the CD45 inhibiting T cells proliferation [29]. In this study, we focused on another member of the RL11 family, the UL10 protein (pUL10) that is the neighbor of UL11 on the HCMV genome. pUL10 shares high sequence homology with pUL11 and therefore has the potential to interact with lymphocyte receptor molecules as well. We demonstrate that the UL10 gene codes for an extensively glycosylated transmembrane protein that is shed from the cell surface. Although we didn't identified the receptor of the protein, we show here that it interacts with an ubiquitous molecule on the surface of lymphocyte and interferes with T cell proliferation. These findings strongly suggest that UL10 has a subversive role in host immune control.

2. RESULTS

In silico analysis of pUL10

The *UL10* gene is located at the extremity of the HCMV genome in the unique long region, and belongs to the RL11 family (RL11 domain aa 39-120) [38]. Bioinformatics prediction of the UL10 protein (pUL10) structure provided for a type I transmembrane protein lacking a canonical signal peptide (Figure 1A). This protein consists of, from N- to C- term, an extracellular region predicted to fold as an immunoglobulin superfamily (IgSF) domain (112 amino acids), a hydrophobic transmembrane sequence (17 amino acids) and a cytoplasmic tail (26 amino acids). The pUL10 ectodomain contains four potential N-linked glycosylation sites, three of them located in the Ig-like domain (Figure 1A).

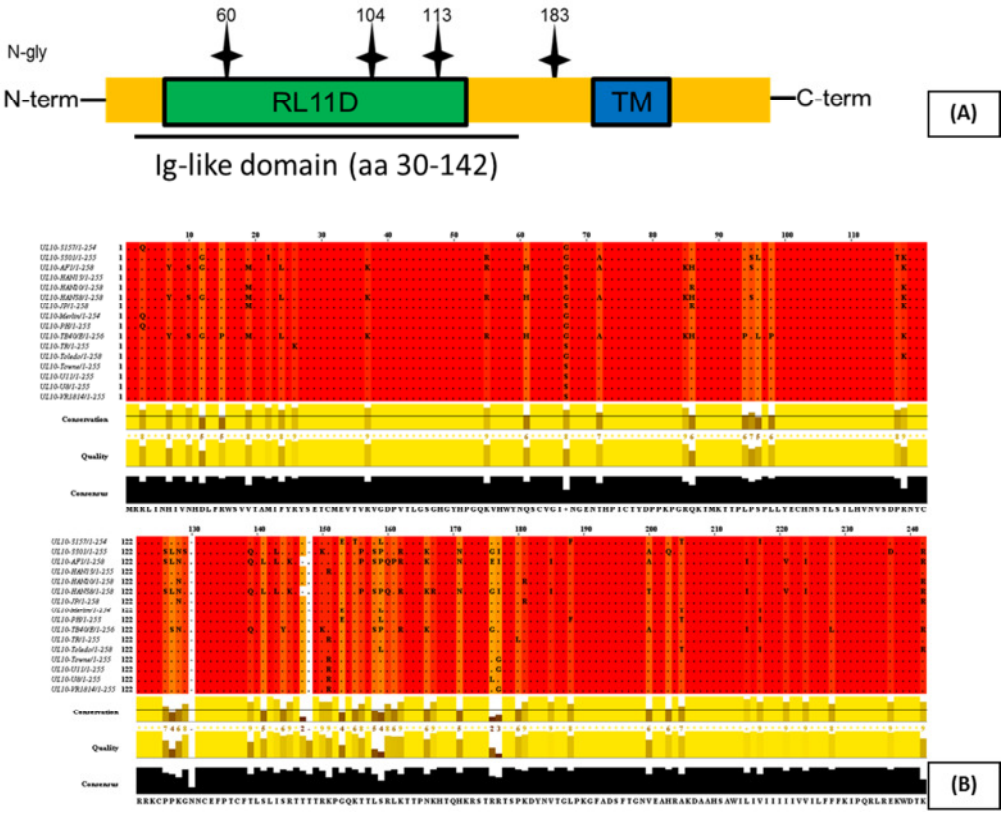


Figure 1. UL10 protein is a predicted type I membrane glycoprotein, highly conserved among HCMV strains. (A) Schematic representation of the UL10 protein predicted features. N-glycosilation sites are indicated. RL11D: RL11 domain; TM: Transmembrane domain **(B)** Multiple alignment of amino acid sequences of the UL10 protein from different HCMV strains. The numbers above the sequences represent amino acid residues. Dots indicate identity while changes in amino acid composition, using TR strain as reference, are shown.

We searched for the presence of pUL10 orthologues within the CMV genomes from the different species sequenced to date. pUL10 is unique to HCMV and orthologues of the protein are absent in those sequenced from primate-, rat- or mouse- specific CMVs. In order to examine the variability of the pUL10, we compared amino acid sequence in a number of HCMV strains deposited in GenBank, including some unpassaged clinical isolates (3301, AF1, JP, U8, and U11), low-passage clinical isolates (Toledo, Merlin, 3157, 6397, HAN13, HAN20, HAN38 and VR1814), two BAC-cloned isolates (TR and PH) and laboratory-adapted strains (Towne and TB40/E). Alignments showed that the pUL10 amino acid sequences, in the 16 HCMV strains analyzed, share a conservation of about 98% (Figure 1B). Thus, among putative membrane proteins, pUL10 shows a high inter-strain-specific conservation level.

Characterization of recombinant pUL10

Expression of the protein from physiologically relevant strains of the virus during the infection cycle has not been analyzed, although relatively low levels of UL10 transcript and protein from the Merlin strain of HCMV in human fibroblast have been reported [39].

To obtain hints on the possible function of pUL10 and to allow us to work with conveniently detectable levels of the protein, we cloned a human codon optimized UL10 sequence from the TR strain of HCMV in a eukaryotic expression vector (pcDNA3.1, Invitrogen), in frame with the coding sequences of the 6His/c-myc tags at the C-terminal (pcDNA-UL10) for monitoring expression of the protein.

To gain some insights into the properties of UL10 protein, we transfected HEK293T cells with pcDNA-UL10 and performed immunoblots of the cell lysates. When an antibody specific for the 6His-tag was used, a band of around 50 kDa and less intense doublets with apparent molecular masses ranging between 55 and 65 kDa were detected (Figure 2A, left

panel). This suggested posttranslational modification of the pUL10 since the predicted molecular mass of the polypeptide backbone is approximately 29 kDa (32 kDa with the addition of the tags at the C-term). A commercial pool of anti-HCMV hyperimmuneglobulin, Cytogam®, was also used as primary antibody on the same cell lysate. A similar band pattern was detected by Western blot (Figure 2A, right panel), thus indicating that antibodies against pUL10 are elicited by immune response during natural HCMV infection.

To investigate the glycosylation status of pUL10, lysates of transfected cells were first treated with Endo H, PNGase F, and then immunoblotted (Figure 2B). Endo H treatment reduced the apparent MW of the 50 kDa band to 32 kDa without changing the migration of the higher MW doublets (Figure 2B, middle lane). The latter species, however, was sensitive to PNGase F digestion and the resulting deglycosylated protein showed a MW of approximately the predicted 32 kDa (Figure 2B, right lane).

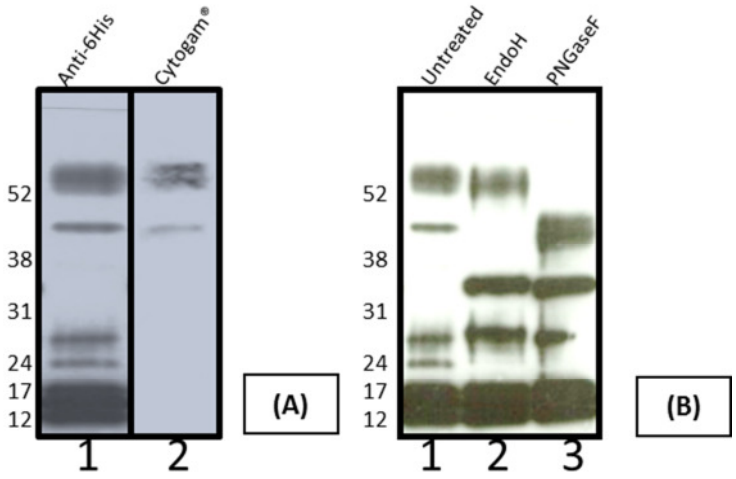


Figure 2. pUL10 is a higly N-glycosilated protein recognized by naturally elicited antibodies. (A) The HEK293T cells were transiently transfected with pCDNA-UL10. After 48h cell lysate was analysed by western blot with an anti-6His mAb (1) followed by anti-mouse-HRP or with Cytogam® followed by anti-human-HRP. **(B)** Deglycosilation analysys of pUL10. The lysates of HEK293T cells transiently transfected with pCDNA-UL10 were treated with or without (lane 1) *N*-Endoglycosidase H (Endo-H, lane 2) or Peptide-N-glycosidase F (PNGaseF, lane 3). Afterwards, the samples were separated under reducing conditions by SDS-PAGE (4-12%) and transferred to a nitrocellulose membrane. The blot was probed with an anti-6His mAb followed by anti-mouse-HRP.

This mass corresponds to the predicted MW of the polipetide backbone. N-linked glycosylation, therefore, appears to be responsible for all the majority post-translational modification of pUL10. The Endo H sensitive species appeared as an immature form of the protein, presumably transiting through the endoplasmic reticulum. However, no O-linked carbohydrate seem to be added since all form of pUL10 remain PNGase sensitive and the protein appears to be completely maturated in our system.

The intracellular localization of the UL10 protein has not been described in literature. *In-silico* analysis predicts pUL10 to be a potential membrane protein due to the presence of a transmembrane region, although it does not possess a predicted canonical signal peptide. We examined the intracellular localization of the protein by confocal microscopy in human epithelial cell line ARPE-19 (ATCC) and in human fibroblast MRC-5 (ATCC) cells. Twenty four hours after transfection of pcDNA-UL10 with nucleofectin®, cells were fixed, permeabilized and stained concurrently with markers of Golgi (GM130), ER (PDI) or plasma membrane (WGA) (Figure 3A). Although pUL10 did not clearly co-localize with the soluble ER marker, it was found to co-localize with the Golgi marker and present on the plasma membrane in both ARPE-19 and MRC-5 (Figure 3A and data not shown respectively). Exposure to the extracellular space of this protein was further confirmed through surface staining and flow cytometry analysis of pUL10 expressing HEK293T cells using a specific polyclonal antiserum (Figure 3B). Thus, pUL10 enters the secretory pathways and reaches the plasma membrane even in the absence of other HCMV proteins.

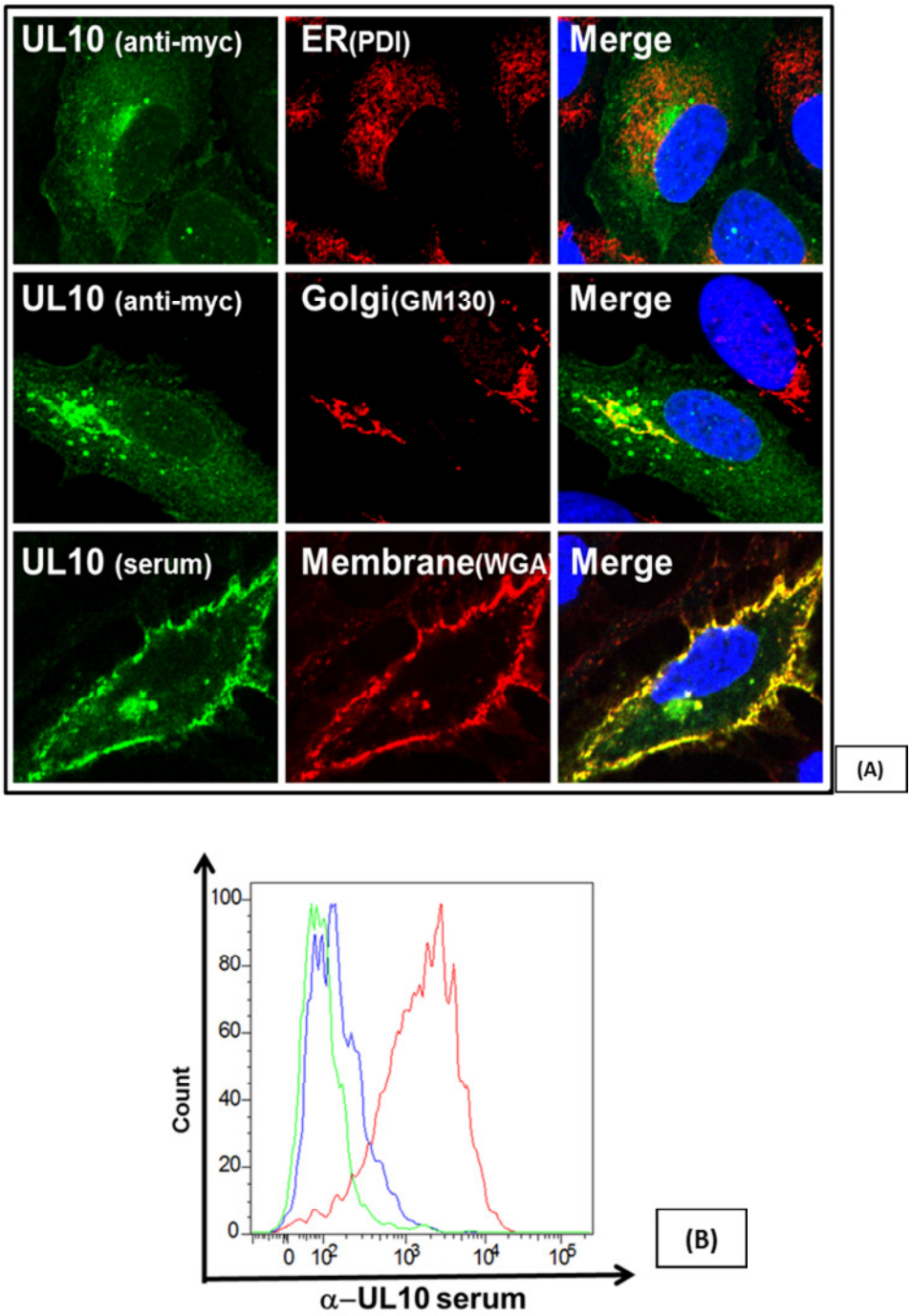


Figure 3. pUL10 is a membrane glycoprotein expressed on the cell surface. (A) Confocal analysis for intracellular localization of pUL10 in epithelial cells. ARPE-19 cells were nucleofected with pCDNA-UL10. After 24h, the cells were fixed and stained with an anti-UL10 mouse serum or a mouse anti-c-myc tag followed by an anti-mouse IgG Alexafluor-488 (Invitrogen). Markers of cell compartment are used to understand the intracellular localization of the protein. Merge panels show colocalization with marker used for the ER compartment (PDI), for the Trans-Golgi network (TG46) and for the membrane (WGA), thus indicating the presence of the protein in the secretarial pathway. **(B)** Flow cytometry analysis of HEK293T unpermeabilized cells transfected with pCDNA-UL10 (red), pCDNA-UL6 (blue) or left untransfected (green), and stained with an anti-UL10 mouse serum followed by anti-mouse Alexafluor-647 (Invitrogen)

pUL10 is cleaved from the plasma membrane

As we have shown above, the intracellular pUL10 can be revealed as one immature species and a set of doublets of mature Golgi-modified glycoprotein in western blot. However, the anti-His antibody also recognizes multiple bands of smaller size. We considered the possibility of a specific proteolysis releasing the N-terminal domain of pUL10. This hypothesis emerged from the fact that pUL7, another HCMV protein, belonging to the RL11 family and sharing the Ig-like domain, was reported to be protelytically cleaved and shed in a soluble form from cells [37]. We tested our hypothesis by analyzing culture supernatants of pUL10 expressing cell. HEK293T were transfected with either pcDNA-UL10 or empty vector and grown for 48h before collecting the culture supernatants. Proteins present in the samples were then separated by SDS-PAGE, blotted and probed with both the anti-His antibody and the anti-UL10 antiserum. As it can be seen in figure 4A, a band of approximately 35 kDa was detected by anti-UL10 antiserum and not by anti-His in the supernatant of HEK293T transfected with pcDNA-UL10. This result shows that UL10, expressed as single gene product, is proteolytically cleaved into the extracellular environment.

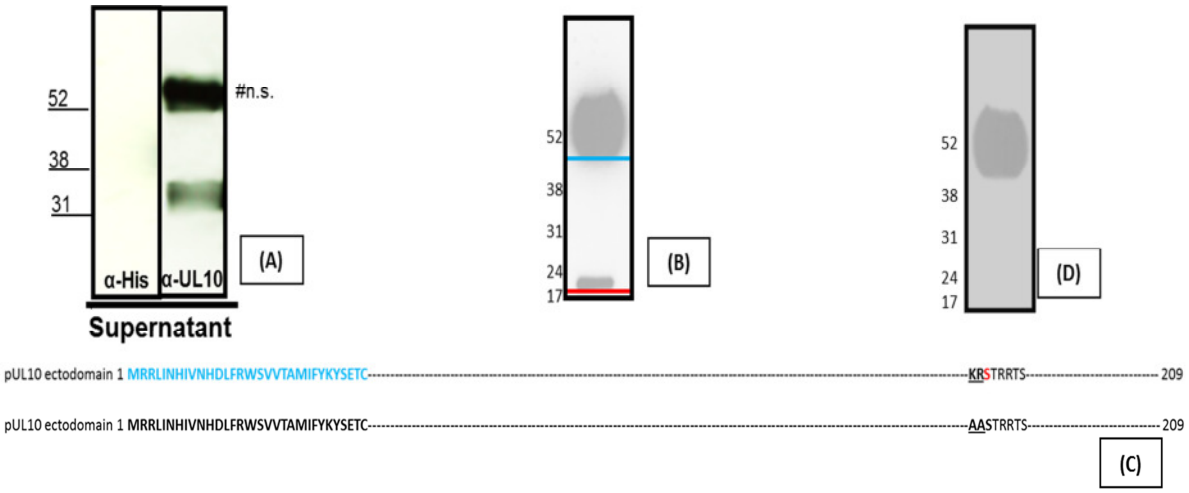


Figure 4. pUL10 is shed from the cell surface. (A) The HEK293T cells were transiently transfected with pCDNA-UL10. After 48h cell culture supernatant was analysed by western blot with anti UL10 mouse serum (α-UL10) or with an anti-His mAb (α-His) followed by anti-mouse-HRP. A band at around 35kDa is visualized only in the supernatant probed with α-UL10, thus indicating that the N-term of the protein is cleaved from the cell surface. **(B)** HEK293T cells were transiently transfected with pCDNA-UL10ectodomain (aa1-209). After 48h cell culture supernatant was analyzed by western blot with an anti-6His mAb (α-His) followed by anti-mouse-HRP. A band at around 45kDa and lower molecular weight one are visualized. The two bands are purified and used for N-term sequencing. The higher band (pale blue) allowed to identify the signal peptide (aa 1-30), the lower band (red) provided indication of the cleavage site (S173). **(C)** Sequences identified in (B) are reported in the upper lane. Underlined the two polar residues mutated into Alanin to avoid the cleavage as showed in the lower line. **(D)** Mutated form of UL10ectodomain. Lower molecular band disappeared, as compared to (B)

To determine the cleavage site of pUL10 we cloned the predicted ectodomain of the protein (aa 1-209 from now on indicated as UL10ecto) into the same pcDNA3.1 vector with the double tag 6His/c-myc in frame at the C-Term, in order to work with a soluble form of the protein. The cleavage of the truncated protein in the extracellular matrix was confirmed, as it can be seen in Figure 4B, where the 52 kDa band is the full length UL10ecto and the 20 kDa species is the post cleavage C-term moiety. After concentration and affinity purification with Cobalt Agarose resin (Pierce) both forms of the protein were separated under reducing condition on SDS-PAGE and transferred to PDVF membrane to perform N-term sequencing. From this analysis we were able to identify the cleavage site (Serine 173) as well as a non-canonical signal peptide of pUL10, since the mature protein starts at aa 30 (Figure 4C upper line).

pUL10 binds leukocytes membrane

The structural features of this viral protein suggested that it could potentially interact with proteins on the surface of neighboring cells, such as infiltrating immune effector cells in

infected tissue, or target host cell receptors for the soluble species. A recent work published by Gabaev and colleagues identified the product of *UL11* gene, a neighbor of *UL10* on the HCMV genome, as interacting partner of the receptor tyrosine phosphatase CD45 leading to functional paralysis of T-cell [29]. Those two proteins share a high degree of conservation. Therefore, we sought to explore the possibility that pUL10 could bind to specific immune cells. To this end, NK, monocytes, T and B cell lines (NK-92, THP1, Jurkat and Ramos respectively) were examined for their capability to bind to pcDNA-UL10 HEK293T transfected cells as compared with cells transfected with others HCMV glycoproteins or the corresponding vector alone in a rosetting assay. Notably, all the cell types showed adherence to pUL10 expressing cells (Figure 5A, data shown only for Ramos cells) but not to the control cells. To rule out the possibility that the binding of the viral protein to lymphocytes was solely mediated through carbohydrate moieties, HEK293T cells were also transfected with a construct expressing the highly glycosylated HCMV molecule pUL6, belonging to the RL11 family, and its ability to interact with lymphocytes was assessed. As shown in Figure 5A, cells did not adhere to pUL6 expressing HEK293T.

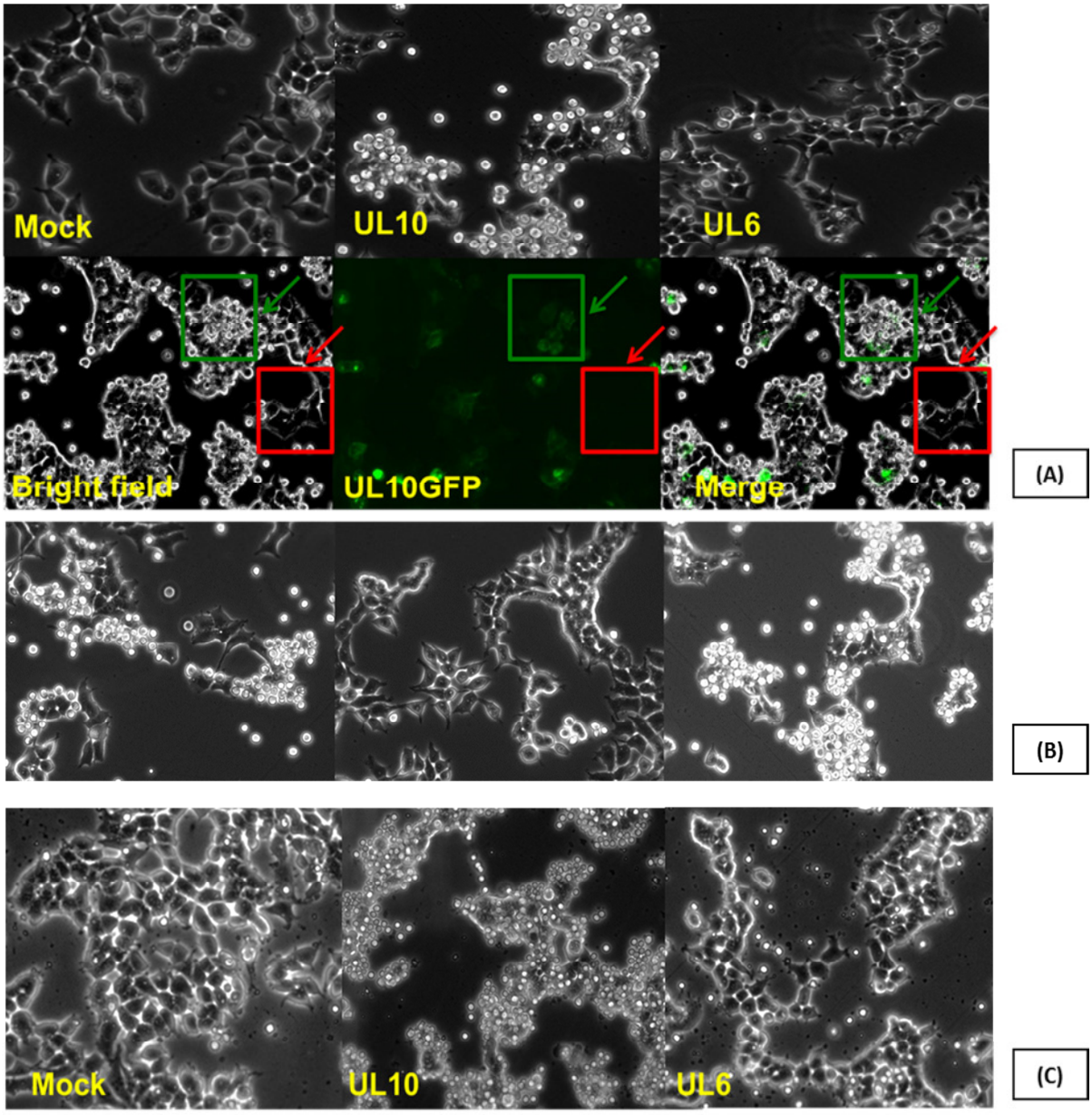


Figure 5. Lymphocites cell lines adhere to pUL10 expressing cells. (A) Rosetting assay. In the upper panel, Ramos cells were incubated with HEK293T transfected 48h earlier with vectors expressing pUL10 (pCDNA-UL10) or UL6 (pCDNA-UL6) or with empty vector (pCDNA). Unbound cells were removed by washing. In the lower panel, HEK293T cells were transfected with pCDNA containing the pUL10 fused with GFP in frame at C-term (pCDNA-UL10GFP) and incubated with Ramos cells. Green and red squares evidence transfected or untrasfected cells. In the merge panel it's evident the adhesion of Ramos only on the transfected cells. **(B)** HEK293T expressing pUL10 were untreated (left), incubated 1 h with anti-UL10 (middle) or pre-immune mouse serum (right) prior to overlay Ramos cells. Unbound cells were removed by extensivley washing. **(C)** HEK293T transfected and depicted as in (A-upper panel) were incubated with PBMCs purified from blood of healthy donors. To avoid variability among donors, the experiment was repeated five times. One representative experiment is shown.

To further demonstrate that the binding was occurring through the ectodomain of pUL10, transfected cells were pretreated with the anti-UL10 mouse serum before addition of cells. As shown in Figure 5B, the anti-UL10 serum completely abrogated the binding, thereby confirming the specificity of the interaction between the ectodomain of pUL10 and lymphocytes.

The same set of experiments was repeated using PBMCs (Peripheral Blood Mononuclear Cell) purified from blood of healthy donors to verify the binding of plasma membrane pUL10 with primary cells as well (Figure 5C). The result indicates that pUL10 is able to interact specifically with a molecule on the surface of lymphocytes.

To analyze the properties of this binding and to search for the cellular receptor, the cleaved N-term portion of pUL10 (aa30-172) was fused in frame with the Fc region of human IgG1, mutated in its binding site to the human Fcγ receptor (pFUSE-IgG1-Fc2e3 – Invivogen), expressed as soluble products in HEK293T cells and purified with a protein-A sepharose resin (Millipore). The recombinant N-term-UL10Fc protein was used to measure interactions of this viral product with six different cell types by flow cytometry. No significant difference in binding, compared to the control used was observed (data not shown), hence, we supposed that the cleaved N-term portion of the protein expressed without downstream amino acid residues was unable to fold correctly. To test this hypothesis, we mutated the two charged amino acid residues of the identified cleavage site of the protein in two non-polar Alanin residues (K171A, R172A). As it can be seen in Figure 4D, in the secreted form of the mutant UL10ecto was not further cleaved. To verify the correct expression and folding of the protein, we repeated the entire set of experiment described above, i.e. localization, deglycosylation and rosetting assay, with the full-length pUL10 carrying the two mutations. The mutant species provided identical results compared to the wild type protein (data not shown).

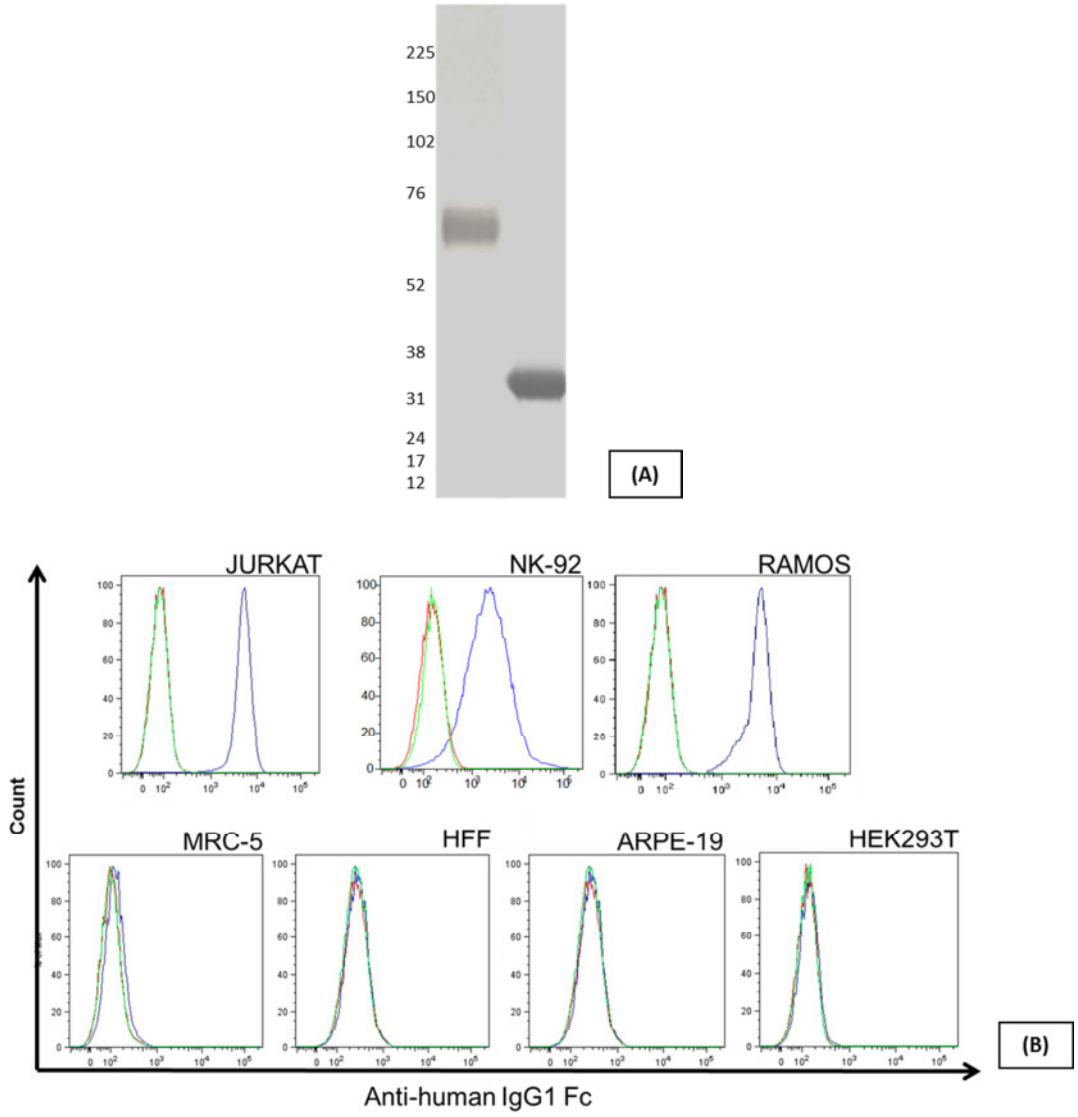
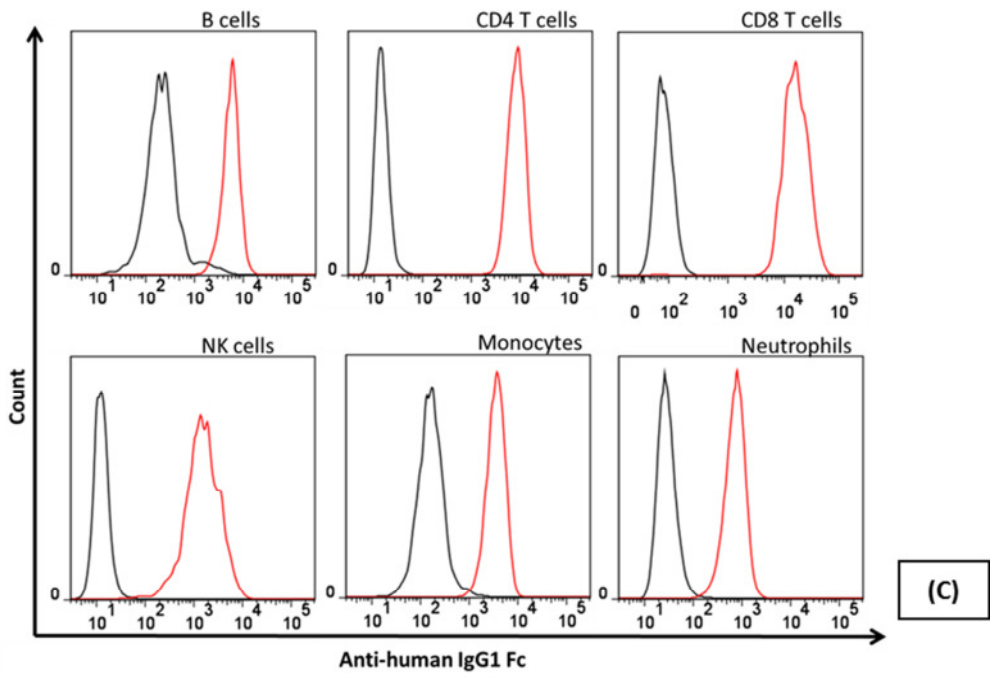


Figure 6. pUL10 interacts with leukocytes. (A) Coomassie staining of purified UL10Fc protein (lane 1) or Fc (lane 2) used for the binding experiments. **(B)** Surface staining of the indicated cell lines with purified UL10Fc (blue lines), the Fc control protein (green lines) or the control isotype antibodies (red lines). **(C)** Surface staining of primary leukocyte with UL10Fc (red) or Fc (black). Surface markers and cell size were used to set gates for different leukocyte subpopulations.



To further analyze the binding properties of the protein, we cloned the mutated ectodomain of pUL10 (aa30-209, K171A-R172A) into the pFUSE-IgG1-Fc2e3, as specified before, and used the corresponding UL10Fc protein (Figure 6A) in all the experiments described before. In the flow cytometry binding assay markedly higher binding of UL10Fc, compared to the Fc domain alone (Fc) and to the isotype control antibody, was detected on the lymphocyte cell lines Ramos, NK-92 and Jurkat but not on the non-hematopoietic cell lines ARPE-19, HEK293T, HFF or MRC-5 (Figure 6B). Extension of the flow cytometry assay to primary PBMCs from a healthy donor demonstrated that UL10Fc was able to bind to the surface of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, monocytes and polymorphonuclear cells (Figure 6C). These data suggest that the rosette formation may result from interaction between pUL10 and protein(s) expressed on the surface of many cell types of hematopoietic origin.

pUL10 inhibits activated T-cell proliferation

T cells are known to play a fundamental role during control of HCMV infection and, recently, it has been demonstrated that the interaction of CD45 co-receptor with the product of the *UL11* gene interferes with the activation and inhibits proliferation of T cells [29]. Since the

two proteins, pUL10 and pUL11, share a high degree of conservation, we sought to determine whether T cell proliferation is also impaired following pUL10 treatment. To this aim, we measured the effects of UL10Fc on the proliferation of primary T cells in response to double stimulation via CD3/CD28. PBMCs were stained with the fluorophore CFSE (Carboxyfluorescein Succinimidyl ester) and then incubated with the anti-CD3 and anti-CD28 antibodies or with the mitogen phytohaemagglutinin (PHA) either in the presence of UL10Fc, the Fc control protein or with isotype control antibody. After 96h, proliferation was measured by flow cytometry and results are shown in Figure 7. A significant inhibitory effect of UL10Fc was observed on proliferation after stimulation of the CD3/CD28 pathway (Figure 7A left panel, Figure 7B) compared to Fc alone or untreated control (Figure 7A right and middle panels respectively). No significant effect was observed after stimulation with PHA (data not shown).

This observation is consistent with an inhibitory effect of the product of the HCMV *UL10* gene on T cell activation following TCR signaling.

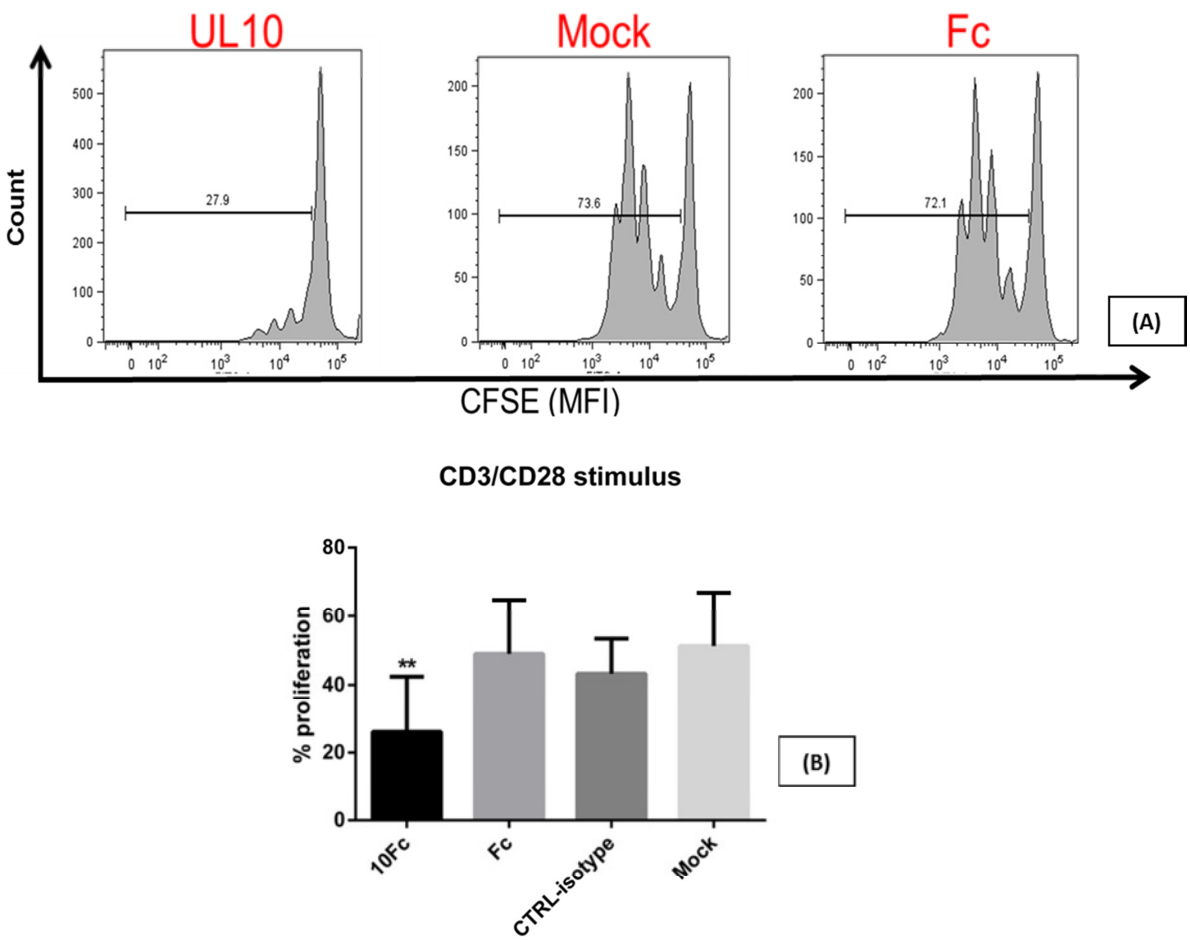


Figure 7. pUL10 inhibits T cell proliferation. (A) Total PBMCs purified from blood of healthy donors were were stained with CFSE and then incubated with anti-CD3 plus anti-CD28 specific antibodies together with 10 µg/ml of UL10Fc (left panel), of the Fc domain (right panel) or left untreated (central) for 4 days. One representative experiment is shown. **(B)** Graph showing the effect of UL10Fc on cell proliferation. Results are expressed as mean±SD of nine experiments performed as described in (A). **P<0.01 (*Anova-Bonferroni test*) UL10Fc group versus control groups. Variations among control groups are not statistically significant.

pUL10 interacts with a 120 kDa receptor on lymphocytes surface

To identify interaction partners of pUL10, proteins were precipitated from cell lysates using UL10Fc as bait. Since pUL10 interacts with a leukocyte surface protein, we wished to determine which of the proteins precipitated from lysates were surface proteins. Jurkat, Ramos or 293T cells were first labeled with membrane impermeable biotin before lysis and precipitation with UL10Fc or the Fc domain alone. UL10Fc bound species were revealed on

SDS-PAGE by blotting and peroxidase-coupled streptavidin detection (Figure 8). The result shows a major band, of about 120 kDa of apparent MW, specifically co-immunoprecipitated by UL10Fc from Jurkat and Ramos cell lysates and few minor species with lower MW. Identification of the species present in the identified bands is ongoing.

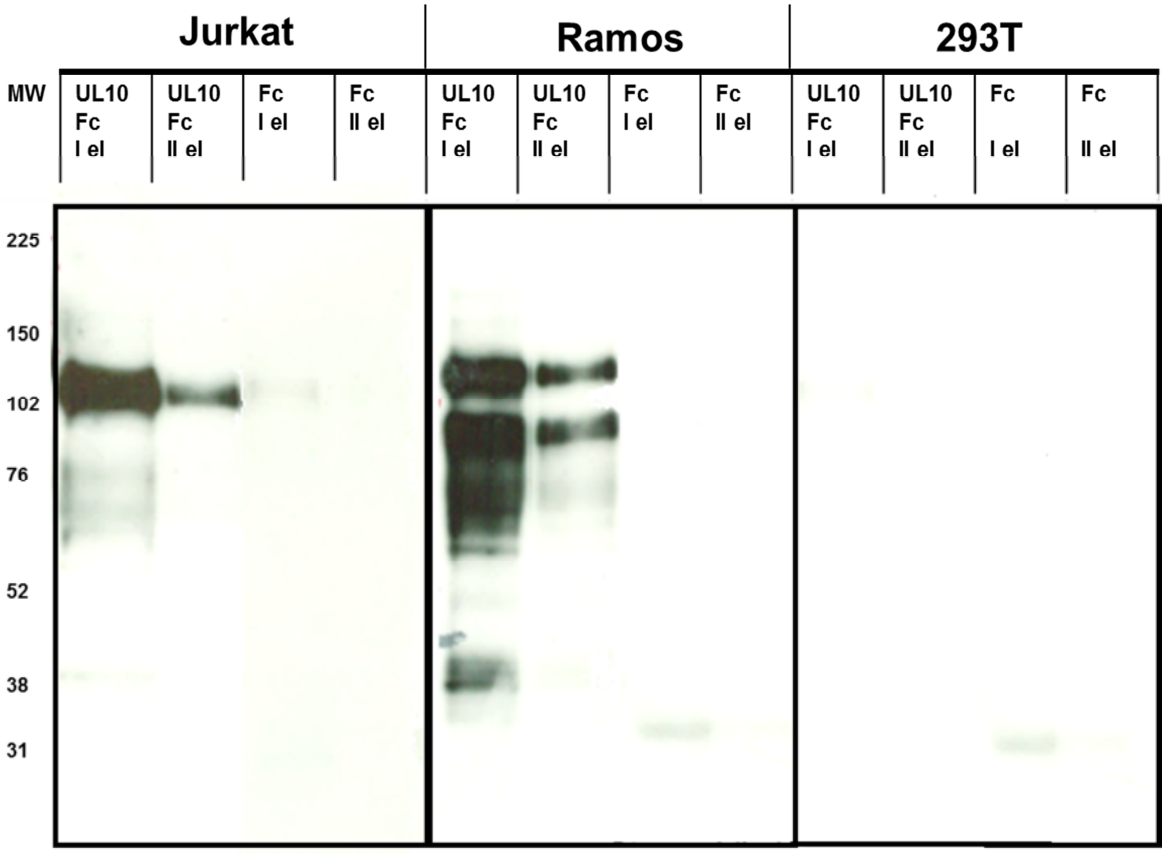


Figure 8. pUL10 interacts with a 120kDa receptor on lymphocytes surface. Jurkat, Ramos or 293T cells were biotinylated prior to lysis. Lysates were incubated with UL10Fc or the Fc control protein (Fc) and protein A sepharose resin. The bound proteins were separated by SDS-PAGE and detected after blotting using HRP-streptavidin.

3. DISCUSSION

HCMV induces robust immune response, involving both innate as well as humoral and cellular immunity. Nevertheless, the virus, as other member of the *herpesviridae* family, is able to establish latent infection inside the host. The asymptomatic lifelong infection with HCMV is thought to be a balance between latency and reactivation. Long-term memory T cell responses are maintained at high frequency and are thought to prevent clinical disease following periodic reactivation of the virus. Thus, a balance is established between the immune response and viral reactivation [2]. T cells are crucial players for control of HCMV infection by their action on revealing and defeating actively infected cells. In this equilibrium, impairment of the immune system lead to serious consequences such as an increased incidence of severe secondary bacterial and fungal infections in solid organ transplant recipients [40]. During the last years, several mechanisms of immune evasion by HCMV and in general by human pathogens belonging to the family of the *herpesviridae* have been described [13], but new functions have to be uncovered to understand the complex process of immune modulation. Here, we report the identification of an HCMV gene that encodes a glycoprotein, pUL10, which is transported to the cell membrane and, at least partially, shed as soluble molecule. The pUL10 ectodomain showed immune modulation properties, interfering with T cell proliferation by interacting with an unknown receptor on human blood cells of lymphoid origin.

As a starting point to identify new HCMV encoded immunosuppressive proteins, we considered the RL11 gene family, composed of 14 members largely uncharacterized (genes RL/TRL11–13, UL1, UL4–11, RL5A and RL6), which are located in close proximity on the 5' extremity of the HCMV genome [33, 41]. All members of this family are dispensable for viral growth in cultured fibroblasts and, most of them, are predicted to encode membrane glycoproteins [10, 11]. The sequence relatedness among RL11 members suggests that they are

the result of a common ancestor, from which they retain the common structural core, that was usurped from the cell, incorporated into the HCMV genome, duplicated and subsequently subjected to functional divergence during co-evolution with the host. The RL11 domain, a variable region of between 65 and 82 residues, own some sequence homology to the adenovirus CR1 domain and to immunoglobulin domains [33]. Adenovirus proteins containing the CR1 domain include immunomodulatory E3 proteins [33][42], and immunoglobulin domains. Both modules are commonly required for cellular and viral protein interactions with cell surface components of the immune system [30, 31].

Acute HCMV infection results in a reduction in the proliferation capacity of lymphocytes, which are not themselves infected by the virus [21]. A similar effect is produced in vitro upon contact between lymphocytes and HCMV infected cells, indicating the potential existence of uncharacterized surface expressed viral proteins with immunomodulatory properties [22, 28]. Recently the *UL11* gene was reported to encode a highly glycosylated protein that binds the CD45 tyrosine phosphatase receptor, causing paralysis of T cell [29]. Differently from the highly polymorphic *UL11*, its neighbor *UL10* resulted highly conserved among laboratory and clinical HCMV strains but functionally and structurally not characterized. The observation that sequences are significantly conserved among HCMV strains, largely retaining critical structural amino acids and N-glycosylation sites, suggests that there is high selective pressure exerted in preserving the structure of this protein. This preservation might be crucial to the appropriate function of UL10 in nature. Therefore, we hypothesized pUL10 to be involved in modulation of the host immune system.

Expression and characterization of the protein from physiologically relevant strains of the virus have not been reported. Consistent with the relatively low levels of UL10 transcript and protein from the Merlin strain of CMV in human fibroblast observed [39], we could detect only UL10 mRNAs in fibroblasts or epithelial cells infected with the TR strain of CMV (data

not shown). Therefore, we cloned the *UL10* gene from TR strain in a eukaryotic expression vector and begun its characterization in several cell lines. Remarkably, we found that the expressed recombinant protein was recognized by human antibodies induced during natural HCMV infection (Cytogam®), suggesting that the protein is expressed during HCMV infection and generate an immune response.

The unescorted pUL10 reached the plasma membrane of fibroblasts and epithelial cells and mediated adhesion to human PBMC. Furthermore, pretreatment with an anti-UL10 mouse serum prevent surface exposed protein to bind leukocytes membrane, both cell lines and freshly purified PBMCs. The binding was further studied using the purified predicted ectodomain in flow cytometry experiments. We observed a general interaction of the protein with all leukocyte sub-classes. These findings clearly indicated that a common UL10 receptor-like factor is present on the surface of immune system cells. As it was also reported previously [29], the binding is not a general property of the RL11D. Due to the high degree of conservation between UL10 and UL11 and to their close proximity on the HCMV genome, we speculated that pUL10 could have a redundant function. pUL11 was previously found to inhibit T cell CD3-dependent proliferation. We tested whether pUL10 could mediate the same effect as pUL11. We found that pretreatment with pUL10 inhibited TCR dependent (CD3/CD28) T cell proliferation while no effect could be seen in presence of a non-physiological stimulus (PHA), differently from what was observed for pUL11. These effects indicate that signal transduction through the TCR is impaired in the presence of pUL10. Furthermore, preliminary experiments showed an interaction with a ubiquitous leukocyte specific receptor, of around 120 kDa, not compatible with the size of CD45, which has been reported to bind pUL11. Although the readout indicates identical functions, pUL10 could probably mediate the inhibitory effect through different signaling pathways from those modulated by pUL11. This implies that the immunomodulatory effects of pUL10, even if

redundant, in vivo may be wide ranging, potentially affecting different function of the immune response.

HCMV is known to encode various soluble factors affecting immune cells function [37, 43]. Recently the RL11 family member pUL7 was shown to be cleaved at cell surface and affect the DCs inflammatory response [37].

Differently from pUL11, but similarly to pUL7, in our study we also observed that pUL10 shed from the cell surface. Ectodomain shedding is believed to constitute an important regulatory mechanism for cellular signaling, either by reduction of the amount of a given receptor or adhesion molecule present on the cell surface and thereby leading to reduced cellular responsiveness to selected stimuli, or by release of soluble receptors that retain their ligand-binding capacity, thus functioning as decoy receptors and natural antagonists [44, 45]. As the released pUL10 product from the cell surface lacks the cytoplasmic tail, it is tempting to speculate that it may function as a decoy receptor consistent with the direct effect on T cell proliferation.

The role of pUL10 in the context of CMV infection is intriguing; a transient general suppression of T cell function during viral infection has been demonstrated [19-23] and the interaction of pUL10 with a receptor on their surface may contribute to this effect. It is also clear that a means for the virus to escape from CMV-specific T cell control could enhance viral replication. Our results, however, do not exclude the possibility that pUL10 might exert other immunomodulatory functions. The consequences of the interaction of pUL10 with a surface receptor may also extend beyond the effects on T cell function, as we observed the interaction with other classes of leukocytes as well. Effects of pUL10 could be cumulative, strengthen the resistance of the virus to the immune system reaction or could be a replacement to answer in different physiological situation. To answer these questions, it will be critical to

identify the cell receptor(s) and to analyze molecular pathways through which pUL10 exerts its effects.

Before these questions can be addressed, however, the expression profile of pUL10 during HCMV infection needs to be understood. We would speculate that the expression of pUL10 may be cell type or state specific, but this remains to be demonstrated.

In conclusion, we have identified HCMV pUL10 as a novel viral immune modulator providing that it reduces T cell proliferation. The interaction of pUL10 with leukocyte receptor appears be an additional way by which HCMV induce immunosuppression. Uncovering all the strategies evolved by the virus to escape the immune response could give a huge boost to the development of therapies or prevention strategies for viral infection. The identification of pUL10 as a novel player in the control of the host immune response is an additional insight in this complex process and could have a potential therapeutic significance.

4. MATERIALS AND METHODS

Cells, plasmids and antibodies

ARPE-19, HFF, MRC-5, HEK293T, RAMOS, JURKAT cell lines were purchased from ATCC (catalogue numbers CRL-2302, SCRC-1041, CCL-171, CRL-11268, CRL-1596 and TIB-152 respectively) and cultured according to the supplier's instructions. DMEM high glucose, DMEM:F12 and RPMI media (Gibco, Invitrogen) were supplemented with 10% fetal calf serum (FCS) and penicillin streptomycin glutamine (Gibco, Invitrogen). Lipofectamine 2000 (Invitrogen) was used to transfect HEK293T cells, while Fugene 6 (Roche) and Nucleofector kit V (Amaxa) were used to transfect ARPE-19 cells as suggested by manufacturer. Human codon-optimized *UL10* gene, from HCMV TR strain, was synthesized

by Geneart and cloned in plasmid pcDNA3.1(-)/c-myc/6His C (Invitrogen) in frame with C-term myc and six histidine tag sequences. Fluorescent fusion protein was obtained by subcloning the genes of interest upstream of the EYFP sequence in pEYFP-N1 vector (Clonetech). Primary antibodies used in this work were: anti-6His (Invitrogen), anti-myc (Invitrogen), anti-PDI (Invitrogen), anti-GM130 (Abcam). Primary antibodies were produced in mouse or rabbit. Mouse anti-sera recognizing UL10 was developed in house. Secondary antibodies used in this study were: Alexa Fluor $F(ab)_2$ fragment of 488-, 568-, and 647-conjugated goat anti-mouse/anti-rabbit (Invitrogen) and HRP-conjugated secondary antibodies from Perkin Elmer. Wheat Germ Agglitinin (WGA) 647-conjugated was purchased from Invitrogen. The mutant of pUL10 cleavage site Quickchange site directed mutagenesis (Stratagene) as suggested by the manufacturer with primers UL10AA Fw 5'-TGGTCCGTCTGGTGCTAGCGGCGTGCTGGGTGTGCTTG – 3' and UL10AA Rev 5'-CAAGCACACCCAGCACGCCGCTAGCACCAGACGGCCA -3'.

Glycosidase treatment

For deglycosylation treatments, 20 µg of protein extract was incubated either with 2.5 µl of Endoglycosidase H (Endo Hf, NEB) or 2.5 µl peptide-N-glycosidase F (PNGaseF, NEB) or buffer only for 3 h at 37°C according to the manufacturer's protocol. Samples were analyzed by Western immunoblotting.

Confocal microscopy analysis

Cells expressing the proteins of interest were grown on glass coverslips or glass chamberslides. For intracellular staining, cells were fixed 48h post transfection with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma). For membrane staining, the permeabilization step was omitted and fixation was performed after staining with

primary and secondary antibodies for 60 min on ice. In both cases, cells were incubated in blocking buffer (PBS + 5% FBS) for 30 min before antibody staining. Antibodies were always diluted in blocking buffer.

The intracellular locations of antibody-tagged or fluorescent fusion proteins were examined under laser illumination in a Zeiss LMS 710 confocal microscope and images were captured using ZEN software (Carl Zeiss). Live microscopy experiments were performed with a Zeiss Axio Observer widefield fluorescence microscope.

Fc fusion protein

The sequence encoding the predicted extracellular domain of pUL10 was amplified from the pCDNA3.1-UL10 using the primers

FW: 5′- CCGGAATTCGTGCATGGAAGTGACCGTGCGC-3′

REV: 5′-GGAAGATCTGTGGGCGGCATCCTTGGCTCT-3′

and cloned into a pFUSE-IgG1-Fc2e3 vector (Invivogen) containing sequences encoding the mouse IL2 signal peptide and the Fc region of human IgG1. UL10Fc and Fc control proteins were purified from serum free supernatants of transfected 293E cells by Protein A affinity chromatography using hiTrap rProtein A Columns (GE Healthcare).

Flow cytometry and rosetting

To define the protein plasma membrane localization, HEK293T cells transiently expressing UL10, UL6 and empty vector were harvested with trypsin 48h post transfection and subjected to staining with mouse anti-serum. Briefly, cells were incubated 30 min at RT with Live&Dead Aqua (Invitrogen) diluted 1:400 in PBS, washed twice and incubated with for 30 min with blocking buffer (PBS with 2% BSA). Mouse anti-sera, diluted 1:100 in blocking buffer, was added for 60 min on ice. Cells were washed 3 times in PBS and then

incubated with Alexa Fluor F(ab)2 fluorophore conjugate secondary antibodies for 30 min on ice. Cells were washed three times in PBS before being analyzed. When intracellular staining was required, cells were permeabilized with Cytofix/Cytoperm kit (BD) and perm/wash buffer was used in all subsequently steps. For cells expressing the c-myc tagged proteins, anti-myc-FITC antibody (Invitrogen) was used at 1:500 dilution. A total of 10^4 cells were analyzed for each histogram using a FACSCanto II (Becton Dickinson).

For flow cytometry based binding assays, 2.5 µg of purified Fc fusion proteins were incubated with 1×10^6 cells in blocking solution (5% goat serum in PBS). Bound Fc proteins were detected using PE-conjugated anti-human IgG (Acris). Sub-populations of PBMCs were identified using antibodies directed to the following surface markers: T cells: anti-AL700 (Immunotools), anti-CD4-APC (Acris), anti-CD8-PerCP (Immunotools). B cells: anti-CD20-Bv421 (Antibodies-online). NK cells: anti-CD56-FITC (Immunotools), anti-CD16-FITC (Immunotools); NK cells were identified as CD56-CD16 positive and CD3 negative cells; monocytes: anti-CD14-PE-Cy7 (Immunotools). Polymorphonuclear cells were identified as CD14 negative cells, morphologically distinguished from lymphocyte. Measurement was performed using a FACS LSRII cytometer and analysis was performed using FlowJo software. To observe leukocyte rosetting, HEK293T cells transfected 48h earlier with plasmid of interest were co-cultured with Jurkat T cells, Ramos B cells or freshly isolated PBMCs at a ratio of 1:10 for 2 h at 37°C, and washed 10 times with PBS. To determine the effects of antisera on leukocyte rosetting, HEK293T cells transfected as described were incubated for 2 h at 37°C with 400 µl of the rabbit anti-UL10 serum or preimmune serum diluted with 600 µl of DMEM. The serum was then removed and the HEK293T co-cultured with Ramos for another 2.5 h, followed by 10 washing steps with PBS, as described before. Images were taken using a Zeiss Axio Observer light/epifluorescence microscope.

T cell assays

To measure proliferation of PBMCs cell were purified from blood by Fycoll gradient and incubated with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE, Invitrogen) in PBS for 10 min. Cells were then pre-incubated with Fc fusion proteins (2 µg) in 200 µl of culture medium. Anti-CD3 and anti-CD28 (1 µg each) were adsorbed onto 96-well plates and 1×10^5 PBMCs were seeded per well. PHA (Oxoid, Basingstoke, UK) was added where indicated at 20 µg/ml. After 96 h, florescence intensity was measured by flow cytometry (FACSCanto II Becton Dickinson). Results are expressed as mean±SD of multiple experiments. ANOVA (one-way probability value) was used with the Bonferroni post hoc test for multiple groups using GraphPad Prism software (San Diego, CA).

Surface protein analysis

Cell surface proteins were biotinylated by incubating 2.5×10^7cells/ml in PBS with 2 mM Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific), for 30 min. The cells were washed three times with 100 mM glycine in PBS and then lysed in NP-40 lysis buffer. Proteins were pulled down or immunoprecipitated from cell lysates prepared from 1×10^8 cells/ml of NP40 lysis buffer. 500 µl of cell lysate precleared by incubation for 20 min with protein A sepharose CL-4B (GE Healthcare) was incubated with 10 µg of Fc proteins and 20 µl protein A sepharose CL-4B for 90 min at 4°C. Revelation of immunoprecipitated proteins was performed by Western Blot with HRP conjiugated Streptavidin (Pierce)

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CHAPTER THREE

pUL139, A NOVEL NON-STRUCTURAL HUMAN CYTOMEGALOVIRUS (HCMV) GLYCOPROTEIN POSSIBLY REGULATING ACTIN DYNAMICS

1. INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the β-herpesvirus subfamily that, like all the other herpesviruses, persists indefinitely in infected individuals through a latent infection. HCMV persistence is associated with an increased risk of age-related pathologies including atherosclerosis [1], immune senescence [2-4] and frailty [5, 6] in otherwise healthy individuals. Reactivation of HCMV from latency in individuals with compromised T cell immunity, including transplant and AIDS patients, is a significant cause of morbidity and mortality [7-9]. Further, HCMV is the leading cause of infectious disease-related birth defects [9-11]. The mechanisms controlling the outcome of infection, in the diverse cell types infected by HCMV in the human host are poorly understood.

HCMV strains vary in virulence and differ for their cell tropism and pathogenic potential. These characters seem to be related to genetic variability exhibited by 'key-genes,' although the definitive associations between gene products and disease have not been established [12, 13]. The HCMV genomic region UL133–UL151 is deleted in the laboratory adapted strain AD169 whereas is present in low passages strains or clinical isolates. The 19 ORFs present in the UL133–UL151 region have been indicated as possible genetic markers for HCMV pathogenesis [14]. This hypothesis is mainly based on the observation that wider infectious properties are exhibited by low passages strains containing an intact version of the UL133-UL151 region. There are very few information on the functions of most of the above-

mentioned ORFs. In this region, UL139 ORF is a polymorphic locus extensively present in HCMV clinical isolates.

Although the effects of the UL139 genomic variability on the biological properties of HCMV infection are still unknown, insertions and non-synonymous substitutions occurring in this ORF and its reported similarity with CD24 [15] might affect viral pathogenic potential with a possible role in immune modulation and/or tissue tropism. The identified variants in the UL139 sequences were clustered clearly into three main groups but, due also to the small sample size, no association between genotypes and disease was found [15].

An interesting observation was that CCMV UL139 is much larger than HCMV UL139 and contains the coding regions of separate homologues in other CMVs. The C-terminal region of CCMV UL139 is homologous to HCMV UL139, whereas the N-terminal region is homologous to rh174, a RhCMV gene that lacks a homologue in HCMV. This suggests that an ancestor of CCMV may have originally contained counterparts of both RhCMV rh174 and HCMV UL139, and that an in- frame deletion resulted in fused coding regions (effectively yielding rh174- UL139).

A region of sequence identity (SETTTGTSSNSSQST) has been noted between the pUL139 and CD24, a cellular glycosyl phosphatidylinositol-linked glycoprotein that is involved in B cell activation [15]. This sequence is present in all the UL139 genotypes identified in the study done by Qi and colleagues, and it is also found in CCMV UL139. It is difficult to assess the significance of this similarity. However, as with pUL139, variation in glycosylation has been observed in CD24, and this has been linked to differences in cell and tissue specificity [16, 17]. Additional roles for CD24 in apoptosis and cell adhesion have been suggested, and more recently also in regulating the responsiveness of a chemokine receptor, CXCR4 [18, 19]. The possibility that UL139 may be a CD24 homologue remains intriguing, but unproven.

The aim of our work was to understand the putative role of pUL139 in the viral pathogenesis. As starting point, we proceeded to the characterization of the protein from HCMV TR strain.

2. RESULTS

UL139 is transcribed with a late kinetics

In the HCMV TR genome, *UL139* extends from nucleotide 186490 to 186924 (435 bp). To analyze potential transcripts arising from the *UL139* ORF, total RNA was isolated from HFF cells at different times after infection with HCMV TR and subjected to RT-PCR. As shown in Figure 1A, *UL139* transcript could be detected with a sharp peak at 48 declining to 72h post infection (p.i.).

HCMV lytic gene expression is conventionally divided into three major kinetic classes of viral genes: immediate-early, early, and late. The transcription of *UL139* RNA in TR-infected cells was completely blocked by the viral DNA polymerase inhibitor phosphonoacetic acid (PAA), consistent with the classification of *UL139* as a late-phase gene. As control we used the product of the *UL99* gene, better known as gB, previously described as a late expressed protein (Figure 1B) [20].

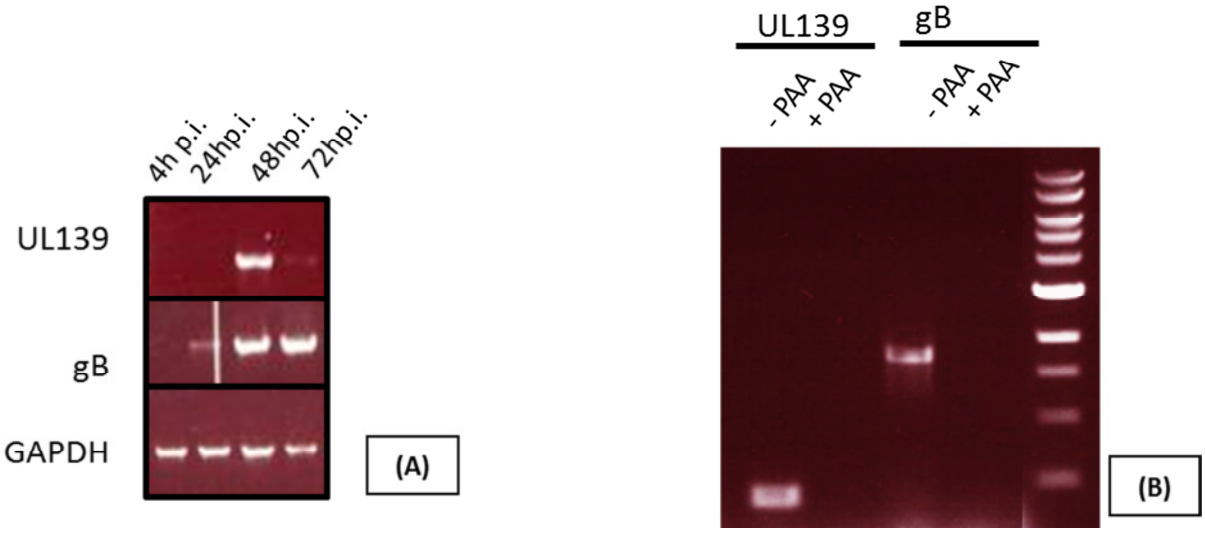


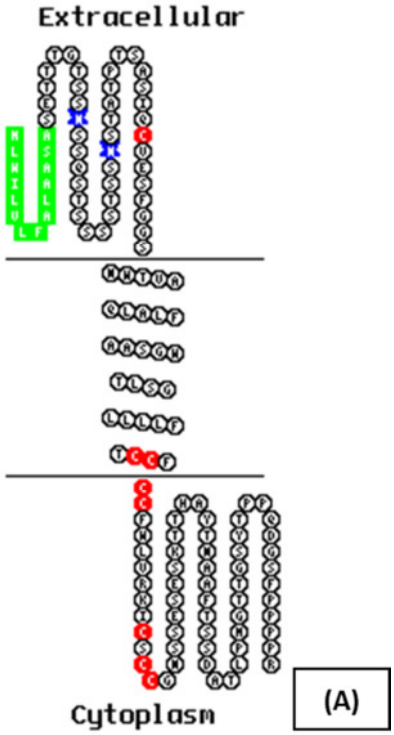
FIG 1: **UL139 RNA is expressed during HCMV infection. (A)** TR infected HFF-1 cells at an MOI of 5 were harvested at indicated time point and the total RNA extracted. Samples were subjected to RT-PCR using primers specific for UL139, gB and GAPDH as cellular control **(B)** Experiments were performed as described above for panel A to assess the effect of PAA treatment (PAA) on late-phase protein expression

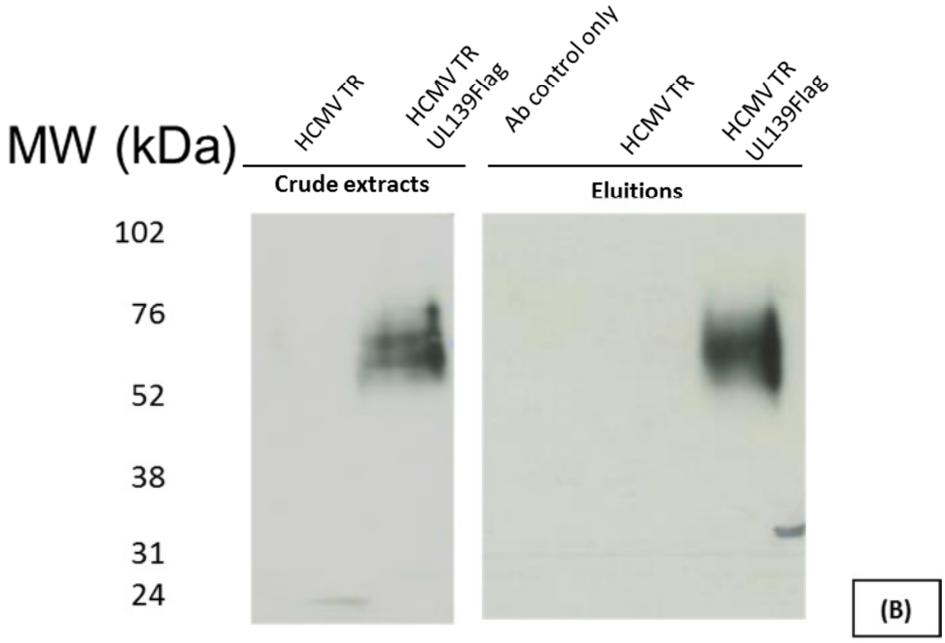
Expression of the UL139 glycoprotein during HCMV replication

The 144 amino acid long UL139 polypeptide (molecular mass about 15 kDa) is predicted to be expressed as a type I glycoprotein with a signal peptide and a membrane anchor (Figure 2A). We investigated the expression kinetics of the protein product of *UL139* gene (pUL139) during productive HCMV infection. For this purpose, we generated an HCMV (TRBAC-derived) recombinant virus with a Flag tag fused to the *UL139* C-terminal. Reconstituted TRUL139Flag was used to infect HFF cells, and the expression of the protein was evaluated. As shown in Figure 2B, the anti-Flag antibody revealed multiple specific bands of apparent molecular weight comprised between 65 kDa and 75 kDa absent in extracts from wild-type TR-infected cells.

The difference between the apparent molecular mass observed for this protein by immunoblotting (around 65-75 kDa) and the predicted molecular mass of the 144 amino acid

polypeptide backbone (15 kDa) suggested that pUL139 undergoes an extensive posttranslational modification process.





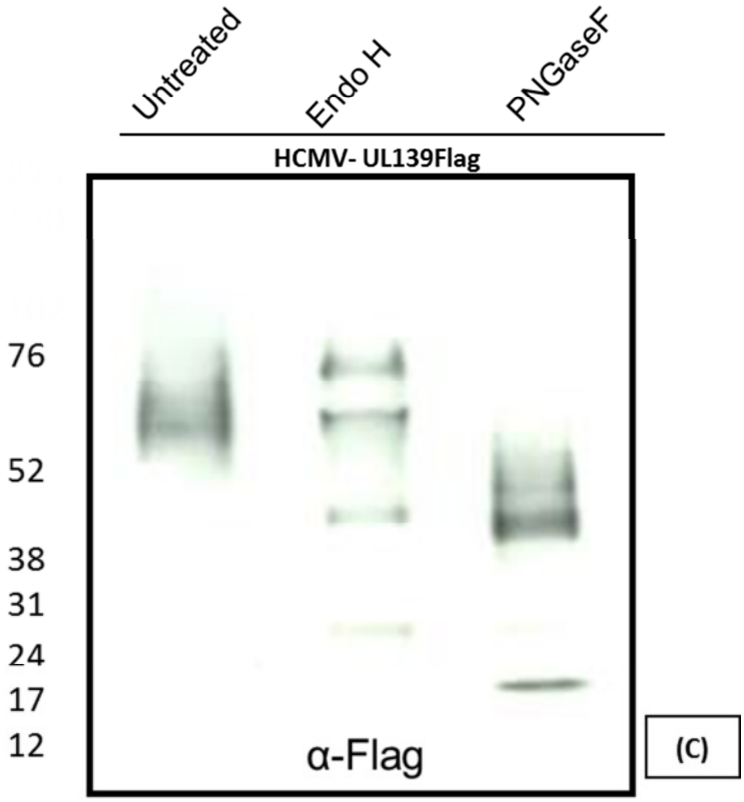
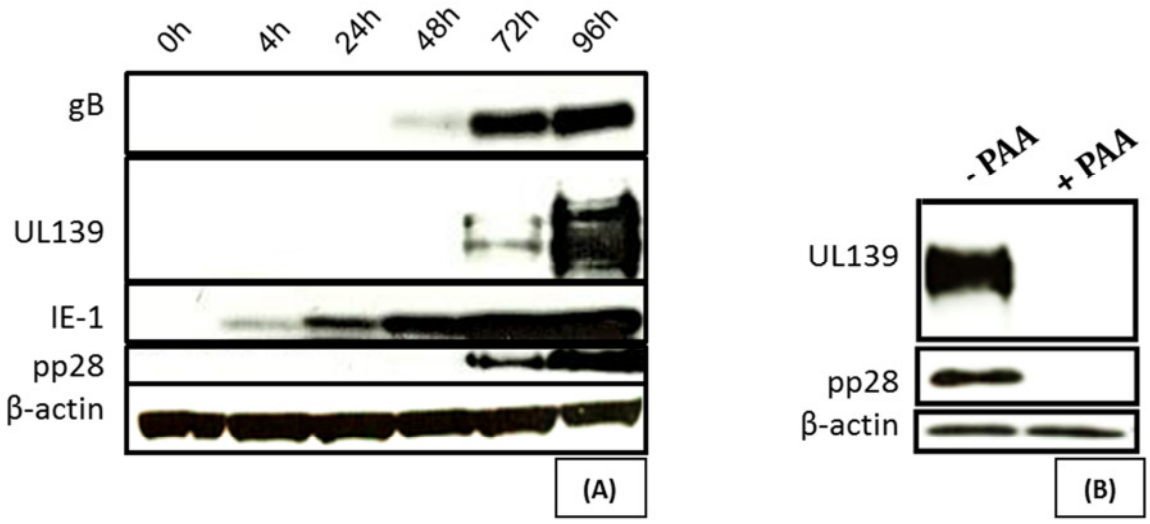


FIG 2: **UL139 is a highly glycosylated viral protein. (A)** Schematic representation of pUL139 predicted features. Green – signal peptide; Blue – N-Glycosylation sites; Red – Cysteine residues **(B)** TR and TRUL139Flag infected HFF-1cells at an MOI of 5 were harvested at 5 days p.i. Equivalent amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with antibodies reactive with Flag tag (left panel). Both lysates are immunoprecipitated with anti-Flag coupled magnetic beads to verify specificity of the binding (right panel). **(C)** Whole-cell lysates of TRUL139Flag infected HFF-1 cells (96h p.i.) were left untreated (lane 1) or treated with N-Endoglycosidase H (Endo-H, lane 2) or Peptide-N-glycosidase F (PNGaseF, lane 3). Afterwards, the samples were separated under reducing conditions by SDS-PAGE (4-12%) and transferred to a nitrocellulose membrane. The blot was probed with an anti-Flag ab followed by anti-mouse-HRP.

The primary amino acid sequence of pUL139 contains two predicted motifs for N-linked glycosylation in the extra-luminal portion of the molecule (Figure 2A). To determine whether the protein was glycosylated, lysates from infected fibroblasts 96h p.i. were treated with endoglycosidases and subsequently analyzed by immunoblotting. Treatment with Endo H, which removes only endoplasmic reticulum-like carbohydrates, resulted in a simplified pattern of species (figure 2B, middle lane). The high molecular weight forms of pUL139 were trimmed to two sharp species migrating at 70 and 80 kDa respectively with an almost

complete loss of smeared proteins. Two faster migrating bands of apparent MW of 33 and 45 kDa appeared as deglycosylated products (figure 2B, middle lane compared to left lane). Treatment with PNGase F, which removes high-mannose N-linked sugars, reduced the molecular weight of the smeared area evidencing a major band at 43 kDa (Figure 2C, right lane) and the appearance of a 17 kDa species, close to the predicted MW of the amino acids backbone. Thus, pUL139 expressed during viral infection receive diversified Golgi-like carbohydrates addition although two major species are generated.

To control for possible proteolytic activity, which may have occurred during the enzymatic digestion, blots were stripped and redeveloped using antibody 27-287, which is specific for the gp58 part of glycoprotein B. In agreement with published data, we observed an 8-kDa reduction with PNGase F and a 3-kDa reduction with Endo H (data not shown).



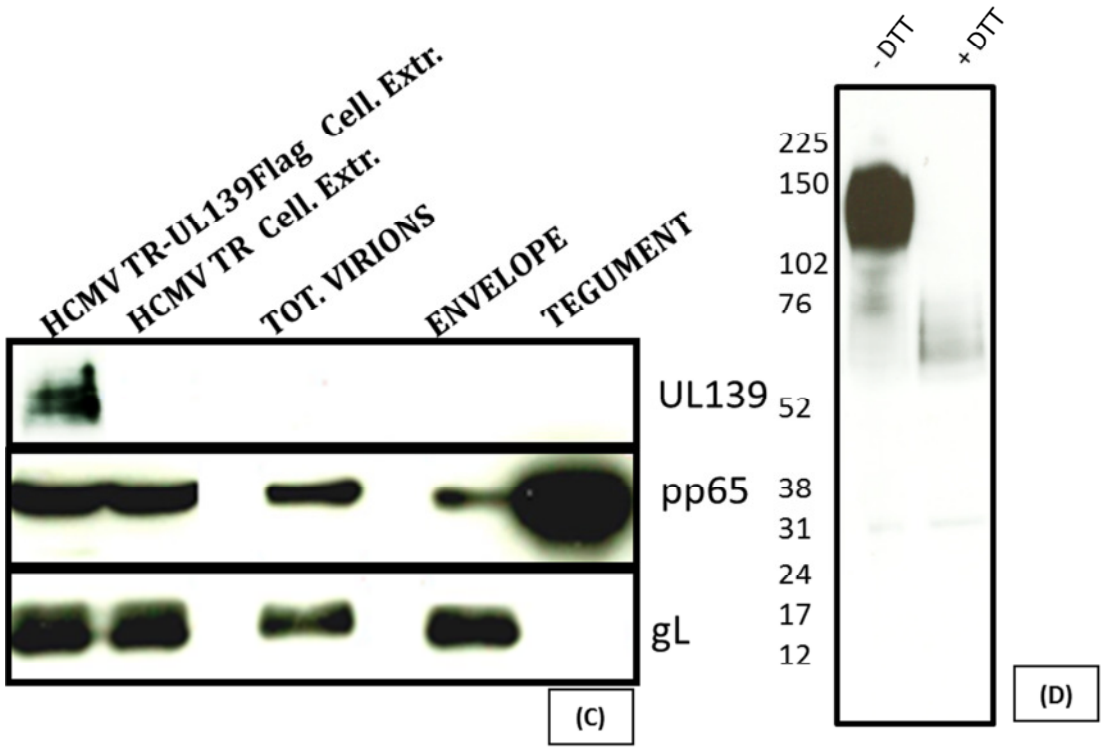


FIG 3*:* UL139 is a true late glycoprotein not present on the virion. (A) Uninfected (0h) and TRUL139Flag infected HFF-1 cells at an MOI of 5 were harvested at the indicated times p.i. Equivalent amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with antibodies reactive with Flag or the HCMV IE1, gB and pp28 proteins. Actin detection was used as a protein loading control. **(B)** Experiments were performed as described above for panel A to assess the effect of PAA treatment (PAA) on late-phase protein expression. **(C)** Western blot performed on purified virions from virus expressing a full-length UL139 with a C-terminal epitope Flag tag. Complete virions (lane 3), envelope fraction (lane 4), and tegument/capsid fraction (5) were probed for the antigens indicated. Whole cell lysate of TRUL139Flag and TR infected HFF-1 cells were used as comparison (lane 1 and 2) **(D)** Fibroblasts were infected with the recombinant virus TR-UL139Flag. 96h p.i. Samples were lysed, separated in absence (lane 1) or presence (lane 2) of Dithiothreitol (DTT) by SDS-PAGE (4-12%) and analyzed by western blot

Kinetic of pUL139 expression was monitored by immunoblot analysis, using an anti-Flag antibody, in cell extracts harvested at different time points ranging from 4 to 96h p.i. HCMV specific mAbs specific for the major immediate early protein IE1 pp72 (UL123), the pp28 (UL99) late phosphoprotein, and the gB (UL55) late glycoprotein were used as controls (Figure 3A). The band profile corresponding to pUL139 were weakly detectable starting from

72h p.i. but its expression remarkably raised at 96h p.i., coincident with the expression kinetics of pp28 [20]. Concerning the control proteins, IE1 pp72 was detectable as soon as 4h p.i. and its expression level sustained for the entire HCMV replication cycle. Consistent with the observed kinetic pattern and the results obtained at the transcriptional level, a metabolic blockade with PAA resulted in the disappearance of the pUL139 specific bands (Figure 3B), further supporting the observation of pUL139 as late expressed protein during HCMV replication in fibroblasts.

Since pUL139 is expressed with a true late kinetics and it is highly glycosylated, we sought to assess whether pUL139 is a virion structural glycoprotein. To this aim, we purified TRUL139Flag particles by negative-viscosity–positive-glycerol-tartrate gradient centrifugation. This purification process facilitates the isolation of intact virions, separated from non-infectious enveloped particles (NIEPs), dense bodies (DBs), and the removal of contaminating host cell debris. Purified virions were subjected to an extraction procedure that separate the tegument/capsid fraction from the viral envelope allowing a more precise evaluation of protein localization. Immunoblotting of the two fractions with the anti-Flag antibody was performed on the TRUL139Flag. Purified wild-type TR virion particles were used as a negative control due to the absence of the Flag epitope on the UL139 protein. Furthermore, the Flag-tagged pUL139 specific band observed in lysates of TRUL139Flag infected cells was used as a positive control. As it can be observed in Figure 3C, the control proteins were present in their expected location, i.e. tegument and envelope for pp65 or the glycoprotein L (gL) respectively, while pUL139 was absent in viral derived fractions and present only in the extract of infected cells. These results indicated that pUL139 is not an HCMV structural protein.

Many of the HCMV glycoproteins form high molecular weight disulfide-linked oligomers, such as the gCI complex, composed of multidimers of gB [21, 22]. Therefore, we analyzed

pUL139 migration patterns under both non-reducing and reducing conditions. As shown in Figure 3D, the reduced sample showed specific bands of apparent molecular weight comprised between 65 kDa and 75 kDa (figure 5A, right lane). In absence of reducing agent, however, the apparent molecular weight of pUL139 ranged between 120 kDa and 150 kDa (Figure 3D), consistent with the presence of disulfide-bonded dimeric proteins with composite glycosylation. Alternatively, pUL139 might form a disulfide-bonded complex with different cellular or viral protein.

Subcellular localization of pUL139 in infected cells

Consistent with the previous result, that excluded the presence of pUL139 on the viral particles, we tried to acquire information about its role in the physiopathology of HCMV by analyzing the cellular localization of pUL139 during the infection cycle in human fibroblasts. The Flag epitope of the TRUL139Flag virus was employed to trace its subcellular distribution by confocal microscopy in comparison with a panel of molecular markers of cellular compartment. Results from this analysis are shown in Figure 4. In HFF cells, following fixation at 96h p.i., pUL139Flag appeared to be included mainly in vesicles diffused in the cytoplasm, with minor colocalization with the endoplasmic reticulum (ER) marker (PDI) and a more pronounced association with the cellular membrane marker (WGA). Markers for the endocytic pathway, such as the early endosomal marker EEA-1, showed almost complete colocalization with the pUL139 around a juxtanuclear cytoplasmic site that could possibly represent the viral assembly complex (Figure 4A). This pattern was not detectable in uninfected and wild-type TR-infected HFF cells (data not shown), thus confirming the specificity of the observed staining pattern.

During its final envelopment, HCMV acquires membranes containing endosomal and trans-Golgi network markers in the so-called assembly complex (AC) where the budding is thought

to occur. Lysosomes and cis- and medial-Golgi markers are excluded from this structure but surround the virus envelopment and assembly site [23]. The envelopment of the infectious HCMV particles was proposed previously to take place within a juxtanuclear cytoplasmic site [24, 25]. To localize the viral assembly complex in our system and understand the pUL139 localization in relation with other HCMV structural proteins, we employed antibodies specific for the tegument phosphoprotein pp28 and for one of the major virion envelope glycoprotein gH (UL75). HCMV pp28 was reported previously to be acquired from the cytoplasm to the AC and to colocalize with other tegument and viral envelope proteins. gH follows a different pathway being targeted to plasmamembrane first and then transported to the AC by an endocytic process at late phase of the infectious cycle [25, 26]. In infected fibroblast, pUL139 do not colocalize with pp28 and gH (Figure 4B), supporting our previous observation that it is not carried by the viral particles released from the cells. Altogether, these results suggest that pUL139 in infected cells is localized on the cell membrane and, through an endocytotic mechanism, accumulates in vesicles surrounding the virus assembly compartment.

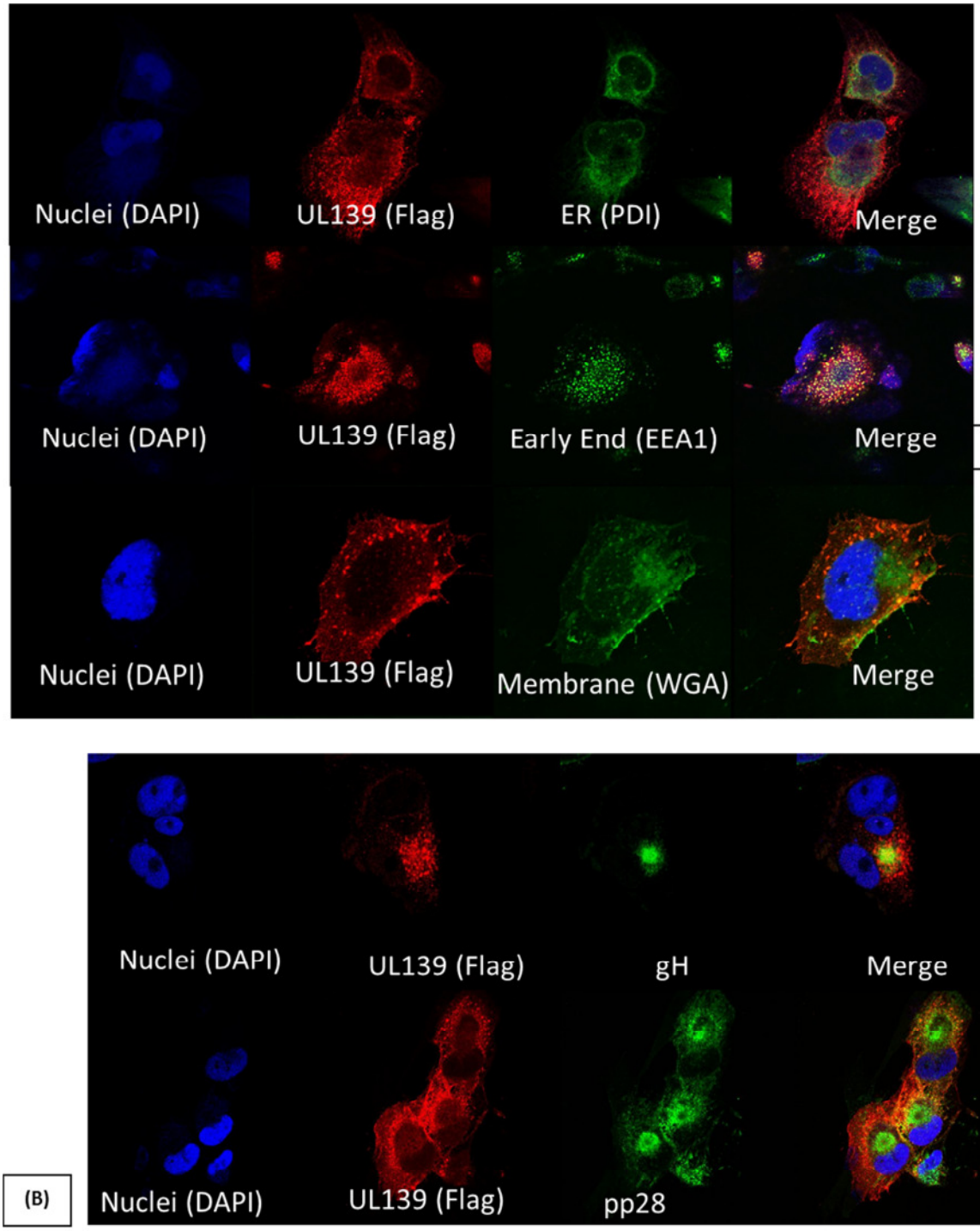
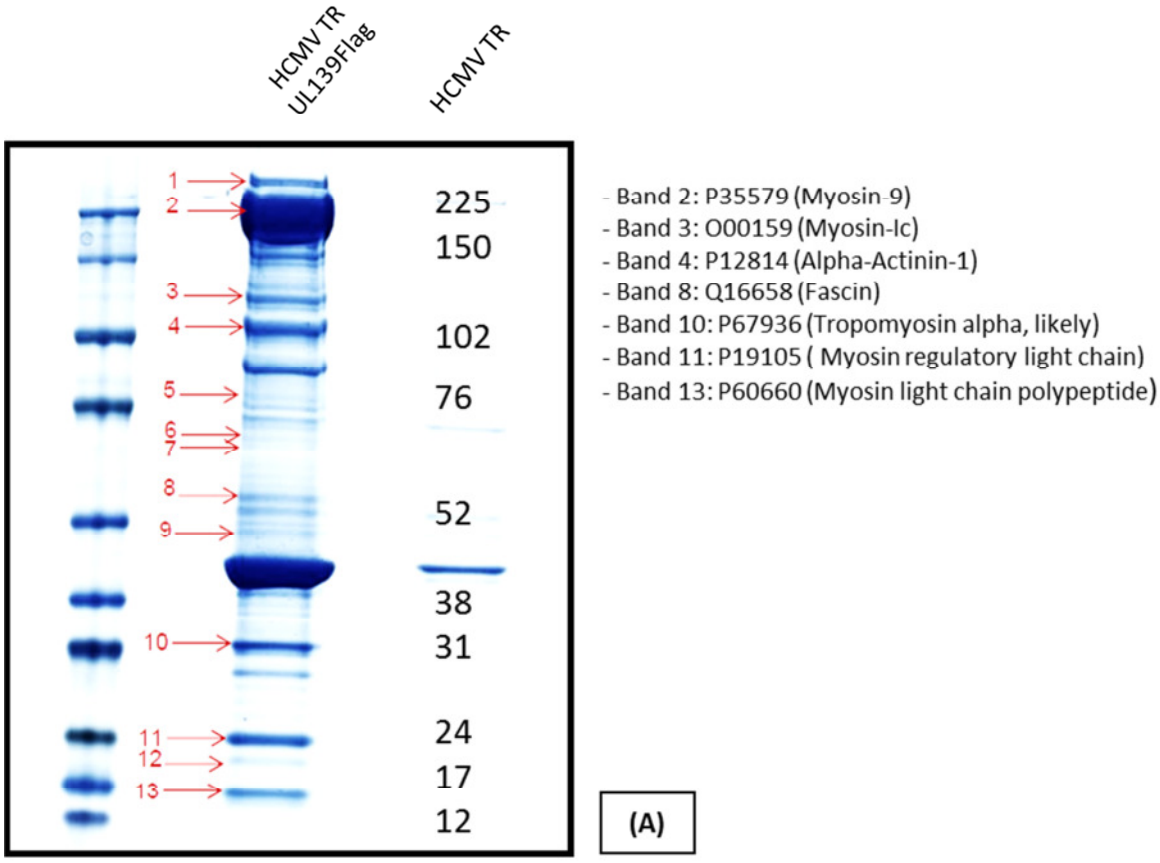


FIG 4: Intracellular localization of pUL139 in TR-infected HFF-1 cells. **(A)**Fibroblasts were infected with the recombinant virus TRUL139Flag for 96h. The intracellular localization of the individual proteins was determined by comparing the signal from Flag specific antibody (pUL139) with those of antibodies specific for a component of the secretory pathway Endoplasmatic Reticulum (PDI), Early endosome (EEA1) and cellular membrane (WGA). The merge panel shows colocalization of the signals, cell nuclei are also stained blue.(B) Cells were treated as above and signal of pUL139 was compared with marker of viral infection as HCMV envelope glycoprotein gH, tegument protein pp28.

pUL139 complex formation

To investigate the existence of cellular and/or viral interactors of pUL139 we performed a pull-down from lysates of TRUL139Flag infected HFF. Wild type TR-infected fibroblasts were used as control. 96h p.i. cells were lysed and the extract incubated with magnetic beads coupled anti-Flag antibody. After extensive washing, eluted proteins were separated on SDS-PAGE and stained with Coomassie reagent (Figure 5A). A different band pattern was observed as compared to the control. To determine the identity of those proteins, bands were excised and subjected to mass spectrometric analysis. Results are reported in Figure 5A. All the proteins identified are known as actin-binding species.



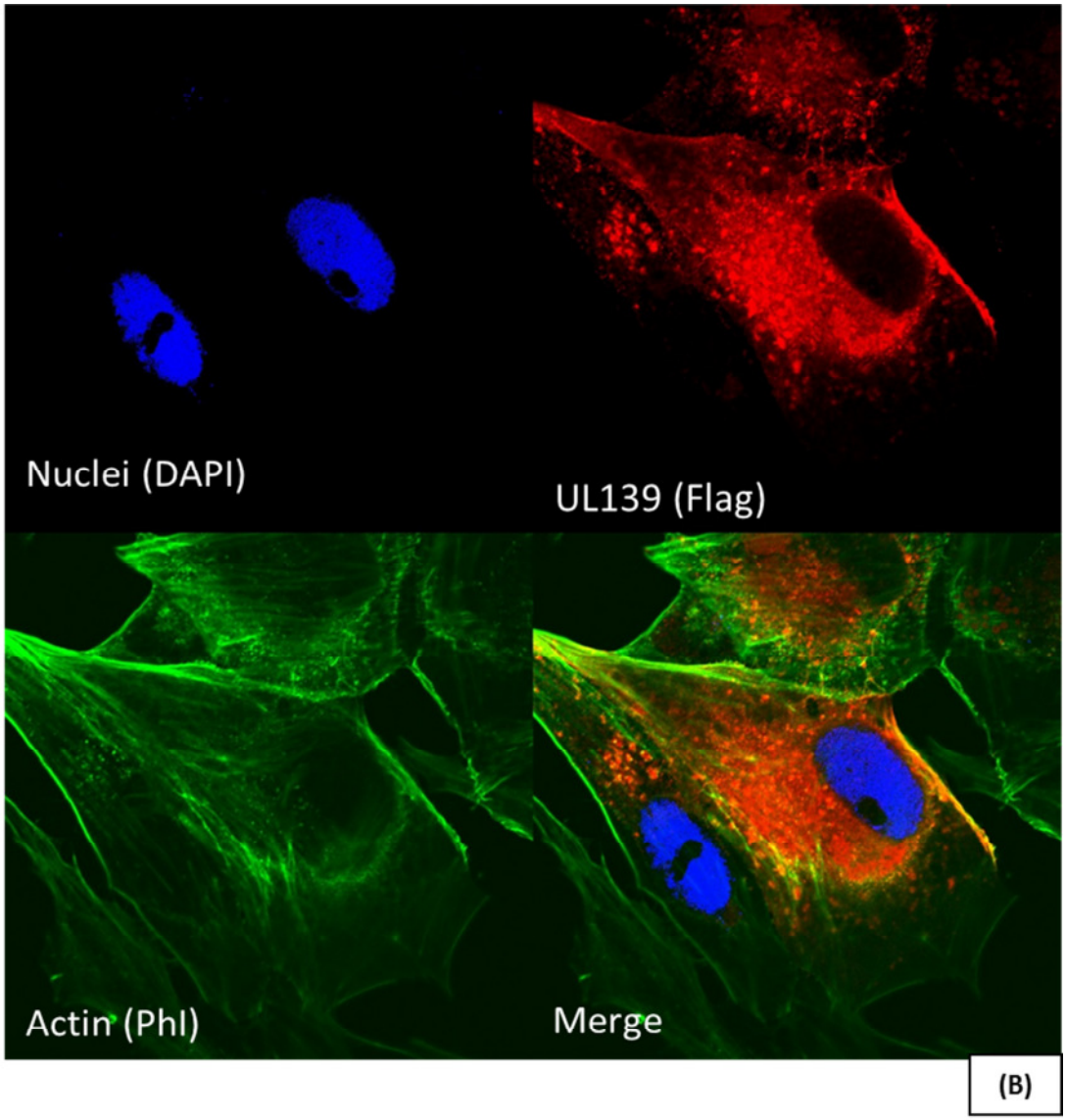


FIG 5: Possible interactors of UL139 in infected HFF-1 cells. (A) Pull-down using anti-Flag magnetic beads from whole cell lysate of fibroblasts infected with the recombinant virus TR-UL139Flag 96h p.i. Samples were separated under reducing conditions by SDS-PAGE (4-12%) and stained with Coomassie reagent. Band indicated were analyzed by mass-spectrometry and identified cellular protein reported (B) Fibroblasts were infected with the recombinant virus TRUL139Flag for 96h and stained with Flag-specific antibody (pUL139) and Phalloidin (Actin). The merge panel shows colocalization of the signals, cell nuclei are also stained blue.

To date, there are no reports about HCMV proteins interacting with cellular motors, but inter-strains differences in trafficking and actin dynamics were observed in THP-1 cells [27]. We investigated the possible association of pUL139 with actin by confocal microscopy, staining the HFF TRUL139Flag infected cell with Phalloidin (high-affinity filamentous actin probe).

As it can be observed in Figure 5B there is a partial colocalization of pUL139 with filamentous actin on the cell membrane and in some vesicular structures localized around a juxtanuclear space, previously identified as the assembly complex.

Among the list of proteins identified as possible pUL139 interactors, we focused our attention on Fascin. It has a role in regulating cytoskeletal structures for the maintenance of cell adhesion, coordinating motility and invasion through interactions with signalling pathways [28]. It localizes to actin-rich protrusions at the cell surface called filopdia. Fascin has also been found to bind beta-catenin [29] and forms a complex present at the leading edges and borders of cells. In our confocal microscopy staining (Figure 4A lower panel and Figure 5B) we observed pUL139 localized in protrusions at cell surface as well, thus, we decided to investigate whether pUL139 could interact with Fascin or a Fascin-binding protein. To this aim, HFF infected with TRUL139Flag were lysed 96h p.i. and incubated with anti-Flag or anti-Fascin coupled magnetic beads. After extensive washing and elution, samples were separated on SDS-PAGE and analyzed by Western blot. Results are shown in Figure 6. The anti-Flag immunoprecipitation clearly revealed that Fascin, although present in high cellular amount and massively eluted with the flow-through, remained associated with pUL139 (middle panel). The other way around, i.e. using anti-Fascin for immunoprecipitation and anti-Flag as probe, showed the presence of pUL139 (lower panel). The high molar excess of Fascin, not associated with the HCMV protein, could explain the low amount of pUL139 detected in this last experiment. TR wild-type infected cells and beads uncoupled with antibodies were used as control and no bands were present (data not shown).

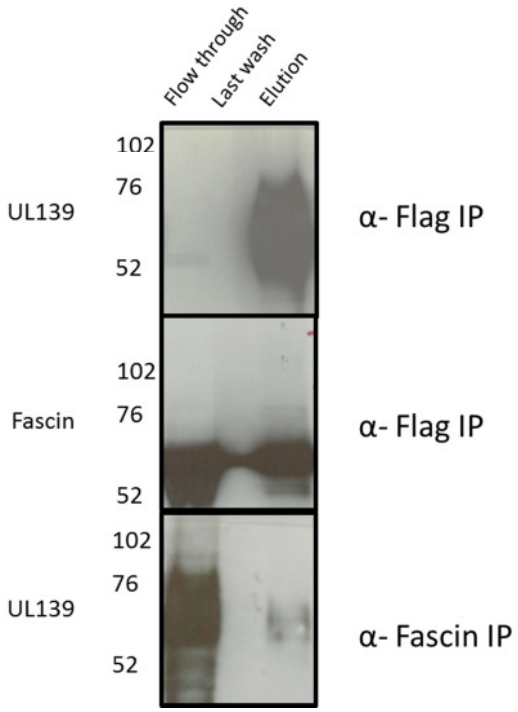


FIG 6: **pUL139-Fascin interacion.** Pull-down using anti-Flag magnetic beads (first and second panel) or anti-Fascin magnetic beads (third panel) from whole cell lysate of fibroblasts infected with the recombinant virus TRUL139Flag 96h p.i. Samples were separated under reducing conditions by SDS-PAGE (4-12%) and western blot with indicated antibodies was performed

3. DISCUSSION

From bioinformatics analysis, the primary translation product of *UL139,* from N- to C-terminal, consists of a signal peptide sequence, the CD24-related region, a hyper variable region, a transmembrane anchor, and a highly conserved cytoplasmic tail. Variations in the N-terminal region are due to substitutions or deletions of variable size in a portion rich in Ser and Thr residues that contains O-linked glycosylation consensus sites. The pUL139 C-terminal region is relatively conserved as well as the 8 cysteine residues. In addition, it was reported that the post-translational modification consensus sites, such as glycosylation/phosphorylation/myristoylation sites, differ in numbers but were conserved within the strains of all the identified clusters [15]. Thus, sequence variability seemed to affect only partially the features of the pUL139.

In this report, we have shown that pUL139 is expressed during viral infection with a late kinetics and that the monomer exhibits variable glycosylations that generate species ranging from 65 to 75 kDa. Thus, pUL139 is a highly glycosylated protein since the predicted MW of the amino acid bulk is about 15 kDa. The presence of cysteines allows the formation of disulfide linked complex, most likely pUL139 dimers. pUL139 contains two consensus sequences for the addition of N-linked carbohydrates and their usage, confirmed by the partial sensitivity to Endo H, provided a proof that it is a type I glycoprotein with its amino-terminal domain being inserted in the lumen of the endoplasmic reticulum and consequently exposed on the surface of infected cells. The sensitivity of the protein to PNGase F indicated that the protein in infected cells carries complex N-linked sugars. . In addition to complex N-linked carbohydrates, pUL139 most probably carries O-linked sugars, as suggested by the partial diffuse migration of the protein after removal of the N-linked carbohydrates. According to bioinformatics predictions, pUL139 carries 35 sites for addition of O-linked glycosylation. Additional modifications such as phosphorylation, which has also been detected on other

HCMV glycoproteins such as gB, could further add to the diffuse migration pattern seen in SDS-PAGE [30].

The current model of herpesvirus morphogenesis postulates that capsids obtain a primary envelope as they pass through the inner nuclear membrane. On exit from the outer nuclear membrane, they undergo de-envelopment. Capsids that are released in the cytoplasm are subsequently coated with tegument proteins and finally wrapped by membranes of a post-Golgi compartment that has been termed the assembly compartment [25]. This compartment has been shown to contain proteins found in the trans-Golgi network and late endosomes [31, 32]. Thus, the structural glycoproteins must be targeted to this compartment and, late in infection, become concentrated within this organelle. This proposed assembly pathway appears to be a default assembly pathway for all herpesviruses [31].

Both confocal analysis on infected cells and characterization of the secreted viral particles indicated that pUL139 is not included in the virion and does not localize in the assembly complex. However, pUL139 appeared to be included mainly in vesicles present around the assembly complex but diffused in the cytoplasm with minor colocalization with the endoplasmic reticulum (ER) marker (PDI) and a more pronounced association with the cellular membrane (WGA) and early endosome (EEA-1) markers. The presence of this protein also on the plasmamembrane of infected cells suggests that pUL139 follows the secretory pathway and is then internalized via endocytosis. Analysis of the Tyr residues and their surrounding sequences revealed similarities to sequences that are capable of binding the SH2 domains of signal transducer protein. Two tyrosine-based motifs are found at positions YTNA114, YTPP134. YXXA, YXXP, YXXT, and YXXV motifs have been shown to bind SH2 domains within proteins such as the Src family of kinases, Shc, Abl, Nck, and phosphatidylinositol 3-kinase [33, 34]. These observations, together with the presence of

CD24 region of identity let us to hypothesize that pUL139 could have a possible cellular or viral interaction partner and being involved in signaling or transport of viral proteins.

Pull-down experiments from HCMV infected cells provided us a panel of possible pUL139 interactors. Surprisingly, all proteins that were found associated to pUL139 in infected cells are involved in cellular transport. Our preliminary results showed a possible interaction of pUL139 and Fascin, or Fascin-binding protein, although further experiments are required to confirm this result and to dissect its biological significance. The localization of *UL139* ORF in a locus non-essential for viral replication in fibroblast, would rather exclude a structural role of its protein product in the context of lytic infection in fibroblasts [35]. Other genes in the genomic region UL133-UL151 are known to be involved in the control of immune response [36-39] and latency [40-43]. Moreover, the observation that inter-strains differences in trafficking and actin dynamics were observed in THP-1 cells [27] and our results about a possible interaction of pUL139 and actin-binding protein, raised the intriguing hypothesis that pUL139 could be involved in the regulation of infectious cycle in non-fibroblast cell types. Also, pUL139 could also be one of the factors involved in the still unclear latency process. While the productive HCMV infection has been roughly characterized, mechanisms contributing to HCMV persistence and latency remain poorly understood. Latent viral coexistence with the host requires the maintenance of viral genomes, suppression of viral replication, inhibition of antiviral responses (i.e., apoptosis and type I interferon response), and evasion the host immune response. Having established a latent infection, the virus retains the ability to reactivate from the latent state given an appropriate stimulus and, ultimately, to replicate productively. HCMV proteins localized throughout the secretory pathway play important roles in viral egress and in modulating intracellular protein trafficking to suppress cell surface presentation of MHC-I [9, 44, 45]. It could be possible that with its location in the secretory pathway, pUL139 contributes to latency by blocking late viral glycoprotein

trafficking or viral egress [44]. Indeed, pUL139 might act as a "molecular crossing guard" in the secretorial apparatus, regulating the movement and activity of viral and/or cellular proteins to orchestrate the switch from productive to latent infection.

It would be beneficial to characterize the role of the protein in an infection model different from fibroblasts, such as monocyte cells thought to be the viral reservoir in the human body.

pUL139 in our experimental setting, showed a late kinetics of expression. This observation could not fit well with a possible role in the control of the outcome of the infection. However, recent works have shown that kinetics of expression could be different outside the context of cultured fibroblasts [46-49]. Thus, this challenged the dogma of strictly temporally regulated cascade of viral gene expression. pUL139 could be subjected to a different regulation as well, depending by the strain or the particular cellular environment.

This work represents the first characterization of the product of the polymorphic gene *UL139.* The protein from TR strain belongs to the group that includes about 25% of the existing isolates of HCMV. For the first time a Cytomegalovirus protein potentially able to regulate actin kinetics or transport is reported, even if the biological significance of this data is not clear. Although we are aware that further studies will be required to have a clear picture of the pUL139 role in the context of viral infection, our results provide intriguing hints on the putative function of pUL139. Variability of this gene could be an additional way the virus uses to adapt to host differences and polymorphisms. However, this hypothesis remains to be substantiated in the future. Future study in different experimental settings will decipher the role of pUL139, unraveling additional mechanisms the virus uses to control cell and survive inside the host.

4. MATERIALS AND METHODS

Cells and antibodies

MRC-5 and HFF cell lines were purchased from ATCC (catalogue numbers CCL-171 and CRL-2429, respectively) and cultured according to the supplier's instructions. DMEM high-glucose (Gibco, Invitrogen) were supplemented with 10% fetal calf serum (FCS) and penicillin streptomycin glutamine (Gibco, Invitrogen). Primary antibodies used in this work were: anti-PDI (Invitrogen), anti-Flag Clone-M2 (Sigma), rabbit-anti-Flag (Sigma), anti-TGN46 (AbD Serotec), anti-actin (Abcam), anti-EEA1 (Abcam), anti-gB (Abcam), anti-pp28 (AbD Serotech), anti-Fascin (Abcam), anti-gL (Abcam), anti-gH (AbD Serotech). All but rabbit-anti-Flag primary antibodies were produced in mouse. Secondary antibodies used in this study were: Alexa Fluor $F(ab)_2$ fragment of 488-, 568-, and 647-conjugated goat anti-mouse/rabbit (Invitrogen) and HRP-conjugated secondary antibodies from Perkin Elmer. 647-conjugated Phalloidin and Wheat Germ Agglutinin (WGA) were purchased from Invitrogen.

RNA analysis

HFF cells seeded in 10cm dishes were infected at MOI 5 at 90% confluence. Cells were lysed with 1ml of Trizol Reagent (Invitrogen) and RNA extracted following manufacturer instructions. cDNA was prepared using QuantiTect Reverse Transcription Kit (QIAGEN). Oligonucleotides used for cDNA detection are reported in Table 1. Late-phase gene expression was inhibited by the use of phosphonoacetic acid (PAA; Sigma-Aldrich). A total of 250 μg/ml of PAA was added to the medium at the time of infection and maintained throughout infection.

Glycosidase

For deglycosylation treatments, 20 µg of protein extract was incubated either with 2.5 µl of Endoglycosidase H (Endo Hf, NEB) or 2.5 µl peptide-N-glycosidase F (PNGaseF, NEB) or buffer only for 3 h at 37°C according to the manufacturer's protocol. Samples were analyzed by Western immunoblotting.

Confocal microscopy analysis

HFF cells seeded on glass coverslips and infected at MOI 5. For intracellular staining, cells were fixed 96 h post infection with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma). For membrane staining, the permeabilization step was omitted and fixation was performed after staining with primary and secondary antibodies for 60 min on ice. In both cases, cells were incubated in blocking buffer (PBS + 5% non-immune human serum in order to reduce non-specific signal) for 30 min before antibody staining. Antibodies were always diluted in blocking buffer.

Markers were examined under laser illumination in a Zeiss LMS 710 confocal microscope and images were captured using ZEN software (Carl Zeiss). Live microscopy experiments were performed with a Zeiss Axio Observer widefield fluorescence microscope.

HCMV virions purification and virion proteins fractionation

Mature HCMV virions were separated from dense bodies (DB) and noninfectious enveloped particles (NIEPs) through a positive density/negative viscosity step-gradient centrifugation as described previously [50]. Briefly, medium from infected cells was collected when 100% cytopathic effect was observed and subjected to 3500 rpm centrifugation for 20 min at 16°C. Supernatant was transferred to polycarbonate tubes under lied with 20% sucrose and centrifuged for 60 min at 23000 rpm in a Beckman SW32Ti rotor. Pelleted virus was

resuspended in 1 ml PBS and 2 ml of 4 different solutions containing decreasing concentration of sucrose and increasing concentration of glycerol tartarate were layered underneath. Tubes were centrifuged at 42000 rpm for 60 min at 10°C in a Beckman SW28Ti rotor. Band containing mature virions was collected through a syringe, resuspended in PBS, centrifuged for 60 min at 23000 rpm in a Beckman SW32Ti rotor and the pellet containing virus was resuspended in PBS. Quality of the purification was assessed through negative staining electron microscopy (EM) analysis. To separate envelope from capsid and tegument proteins, purified virions were mixed 1:1 with envelope extraction buffer (1% NP-40 and 4% Sodium Deoxycholate) and incubated on ice for 30 min with occasional vortexing. Soluble fraction was collected through max speed centrifugation in a benchtop centrifuge for 30 min at 4°C. The insoluble pellet was washed twice in PBS before being solubilized in SDS-PAGE sample buffer. For each extraction, a total of 4 confluent T175 cm^2 flasks of HFF cells were infected. Virus was purified as described above and 10% was mixed with SDS-PAGE loading buffer while the rest was subjected to proteins extraction.

Construction and generation of TRUL139Flag

Marker-less two-step RED-GAM BAC mutagenesis was used to generate recombinant virus harboring tagged version of pUL139 gene in its genome [48]. Briefly, kanamycin resistance cassette, flanked by I-SceI restriction enzyme cleavage site, was PCR amplified from pEP-KanS shuttle vector. The primers used contained homologous regions to allow the integration of the amplicon in the region of interest of the BAC DNA through lambda Red recombinase induction. Combination of I-SceI cleavage with a second Red recombination event removed the resistance gene leaving only the new sequences of interest. The primers used to generate the mutated HCMV TRUL139Flag strain were (Table 1, supplemental material): UL139FlagFw and UL139FlagRv on pEP-KanS plasmid as template. Recombination events

were performed with *E. coli* GS1783 strain containing a BAC clone of the HCMV TR strain. The *E. coli* strain contains also the lambda Red system and the I-SceI genes under the control of heat shock- inducible and arabinose-inducible promoters, respectively. The desired mutations were confirmed by sequencing and integrity of the whole recombinant HCMV genomes was checked through restriction analysis.

To reconstitute the virus, MRC-5 cells from a confluent T175 cm^2 were trypsin detached, mixed with 10 µl fresh prepared BAC DNA (around 3 µg) and 1 µg of pCMVKm2-pp71 plasmid and electroporated in 4 mm cuvette at 250 V and 950 µF. Supernatant containing virus was collected from infected cells when cytopathic effects were >90%. For all following infection experiments, human foreskin fibroblast (HFF) cells were used.

Protein analysis

HFF cells seeded in T-175 flasks were infected at MOI 5 at 90% confluence. Cells were trypsin detached 6 days p.i. and lysed in NP-40 lysis buffer (300 mM NaCl, 1% NP40, 50 mM Tris-HCl pH 7.2, 1% glycerol, protease inhibitor cocktail [Roche]) after washes in PBS . Proteins were pulled down or immunoprecipitated from cell lysates prepared from 15×10^7 cells/ml of NP40 lysis buffer. 300 µl of cell lysate were incubated with 50 µl of anti-Flag coupled magnetic beads (Invitrogen) or Protein G coupled magnetic beads (Invitrogen) plus rabbit anti-Fascin (Abcam) for 90 min at 4°C. The preparative pull-down for mass spectrometric analysis used 15×10^7 cells lysed in 1 ml of NP40 lysis buffer, incubated with 30 µl of anti-Flag coupled magnetic beads. For Coomassie staining of proteins, SDS-PAGE gels were washed twice in Milliq water then fixed in ethanol 50%/phosphoric acid 2%. Gels were then washed again with MilliQ water and incubated in methanol 34%/ammonium sulfate 17%/phosphoric acid 2% for 45 min, followed by addition of Coomassie G-250 (Sigma) 0,065% and stained for 72h. For detection of pUL139 and Fascin a mouse anti-Flag antibody

(Sigma) and the mouse anti-Fascin (Abcam) were used, respectively, followed by incubation with HRP-conjugated anti-mouse (Invitrogen).

Table 1: Oligonucleotides used in this study.

Name	Sequence
UL139Flag Fw	cagggtcgtacactcccccacaggacggctcatttccacctccgcctcgggattacaagg atgacgacgataagtagggataacagggtaat
UL139Flag Rv	ggctttccgaaaccgcgtcagattcaacgtgggtttcggtttagcctgcgtcacttatcg tcgtcatccttgtaatcccgaggcggaggtggaaatgcaaccaattaaccaattctgatt ag
UL139TR Fw	atgctgtgcgatattagttttattt
UL139TR Rev	tcaccgaggcggaggtggaaa
gB TR Fw	cgctgctctgcgtccagac
gB TR Rev	tcgttggtgtaaggcggag
GAPDH Fw	atcccatcaccatcttccag
GAPDH Rev	ccatcacgccacagtttcc

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