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Characterization of the Influences of Human Cytomegalovirus Glycoprotein O (gO) Expression on gH/gL Complexes Assembly and Its Polymorphisms on Cell-free and Cell-to-cell Spread, and Antibody Neutralization.

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Abstract Title: Characterization of the influences of Human Cytomegalovirus glycoprotein O (gO) expression on gH/gL complexes assembly and its polymorphisms on cell-free and cell-to-cell spread, and antibody neutralization.

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Human cytomegalovirus (HCMV) is widely spread throughout the world and immunocompromised individuals can suffer severe diseases from HCMV infection. Once the infection is established, HCMV can spread through the body and infect many major somatic cell types. The glycoproteins H and L (gH/gL) on HCMV envelope can be bound by either gO or the UL128-131 proteins to form complexes gH/gL/gO and gH/gL/UL128-131 that are critical for viral entry and spread, and these two complexes are important targets of neutralizing antibodies. Strains of HCMV vary considerably in the levels of gH/gL/gO and gH/gL/UL128-131. gO is one of the most diverse loci among strains with 10-30% of amino acid sequence differences. In this thesis I explored the mechanisms behind the complex assembly differences between strains and the impacts of interstrain gO diversity on the biology of HCMV. My results uncovered that the strain variations in the assembly of gH/gL complexes is due to the differences in the expression level of gO and UL128-131, while gO amino acid sequence differences have no influence on the complexes assembly. Interestingly, the diversity of gO has dramatic impacts on HCMV cell-free and cell-to-cell spread as well as on antibody neutralization and these effects of gO polymorphisms are epistatically dependent on other variable loci in the virus genome. My study could help to understand the complexity of genotypes observed in clinical samples and decode the challenge for intervention approaches against HCMV.

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Chapter I. Introduction

Overview of Human Herpesviruses

a. Herpesviridae

Herpesviridae is a large family of DNA viruses and the members of this family are also known as herpesviruses. There are more than 130 herpesviruses in total and they are found in mammals, fish, reptiles, birds, and mollusks. Based on biological and genetic properties, the herpesviruses are divided into three subfamilies: alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae [1]. In hosts, the life cycle of herpesviruses can be divided into lytic and latent infections. The lytic infection leads to production of progenies, while the viral replication is suppressed during latent infection. The establishment of latency is a unique characteristic of herpesviruses.

b. Human Herpesviruses

There are eight herpesviruses that are known to utilize human as primary host: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus-6 (HHV-6), human herpesvirus-7 (HHV-7), and Kaposi's sarcoma herpes virus (KSHV).

HSV-1, HSV-2, and VZV belong to the *alpha* subfamily and they have the characteristics of short replication cycle in the host, rapid growth, and the establishment of latency in sensory ganglia [1]. The *beta* human herpesviruses, including HCMV, HHV-6, and HHV-7, have relative long replication cycle in the host

compared to *alpha* human herpesviruses. The latency of *beta* herpesviruses are established in lymphocytes, secretory glands, and other cell types [4]. EBV and KSHV, the *gamma* herpesviruses, have limited host cell tropism compared to alphaherpesvirinae and betaherpesvirinae. They develop long-term latency in B-cells/ memory B-cells and replicate in epithelial cells [Table 1.1].

Table 1.1. Major properties of human herpesviruses

Name	Formal name	Type	Primary target cells	Main sites of latency
Herpes simplex virus-1 (HSV-1)	Human herpesvirus 1	Alpha	Mucoepithelia	Sensory and cranial nerve ganglia
Herpes simplex virus-2 (HSV-2)	Human herpesvirus 2	Alpha	Mucoepithelia	Sensory and cranial nerve ganglia
Varicella zoster virus (VZV)	Human herpesvirus 3	Alpha	Mucoepithelia	Sensory and cranial nerve ganglia
Epstein-Barr virus (EBV)	Human herpesvirus 4	Gamma	Epithelial and B-cell	Memory B-cells
Human Cytomegalovirus (HCMV)	Human herpesvirus 5	Beta	Monocytes, lymphocytes, epithelial, fibroblasts…	Monocytes, lymphocytes
Roseola virus (HHV-6)	Human herpesvirus 6	Beta	T-cells	Various leukocytes
Rosela virus (HHV-7)	Human herpesvirus 7	Beta	T-cells	T-cells, epithelia
Kaposi's sarcoma-associated virus (KSHV)	Human herpesvirus 8	Gamma	lymphocytes and epithelia	B-cells

Overview of Human Cytomegalovirus

a. HCMV structure

The virion of HCMV is approximately 230nm in diameter and consists four major components: the core, capsid, tegument, and envelope [2](Fig 1.1). The core contains a linear double-stranded viral DNA genome, which is located inside an icosahedral-shaped capsid. Between the capsid and lipid envelope, there is a layer of tegument proteins, which are responsible for connecting the capsid to the envelope and storing viral proteins that are essential for initiation of infection. The outermost layer is viral lipid bilayer envelope and it contains viral glycoproteins that facilitate virus attachment, signaling transduction, and fusion into the host cell.

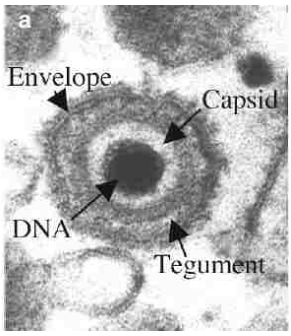

Fig 1.1. Virion particle in cytoplasm of HCMV-infected cell. (Modified from [2])

1. Genome

HCMV has the largest genome among human herpesviruses. The whole genome size is about 235kb, which is over 50% larger than that of herpes simplex virus type 1(HSV-1). According to the herpesviruses genome structure classification [5], HCMV genome has class E repeated region organization and it is the most complex genome

structure among human herpesviruses. The two major domains are known as the long and short genome segments (L and S) and each includes a unique region (U_L and U_S) at the center. The unique regions are flanked by repeated sequences at both the two ends of the genome (TR_L and TR_S) and the intersection between the long and short segments (IR_L and IR_S). Besides, there are few hundred base pair repeated at the two ends of the viral genome and the IR_L-IR_S junction, which is termed as *a* sequence [6]. Consequently, HCMV genome has the structure organization of: *a*-TR_L-U_L-IR_L-*a'*-IR_S-U_S-TR_S-*a* (Fig 1.2).

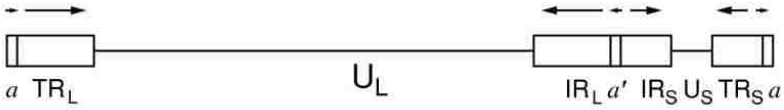

Fig 1.2. HCMV genome structure. (Modified from [6])

2. Capsid

HCMV has an icosahedral-shaped capsid, which is important for both protecting the large DNA viral genome and releasing the viral genome into host nucleus. There are at least five proteins involved in capsid formation: UL86 (the major capsid protein), UL48-49 (the smallest capsid protein, SCP), UL85 (the minor capsid protein), UL46 (minor capsid binding protein), and fragments of UL80 (assembly protein). These protein components assemble into the capsid through extensive intermolecular networks, such as formation of disulfide bonds and non-covalent interactions.

3. Tegument

The tegument layer is formed through sequential adding of proteins, which starts in the nucleus and completes in the cytoplasm. Previous studies have suggested that the formation of tegument requires an intact capsid [8]. Tegument proteins play important roles in regulating viral gene expression and modifying host immune responses to HCMV infection. For example, tegument protein pp71 activates the immediate early gene expression to initiate HCMV replication and ppUL83 inhibits expression of the host proteins associated with induction of interferon response [9-13]. In addition, tegument proteins are involved in capsids egress by modifying nuclear cytoskeleton and nuclear membranes [14].

4. Envelope

The envelope of HCMV is a lipid bilayer and viral glycoproteins are embedded in this lipid bilayer. Mass spectrometry studies have revealed that HCMV virion contains at least 19 different glycoproteins and some of them are indispensible for viral replication in vitro, including glycoprotein B (gB), gM/gN and gH/gL [15]. The relative abundance of these essential glycoproteins follows the order of: gM/gN > gB > gH/gL.

gM from different strains of HCMV have very few amino acid sequence changes, while gN from clinical isolates have up to 20% amino acid sequence variability in the ectodomain whereas the short cytoplasmic tail is conserved. Nearly 67% of gN's mass is from carbohydrate, which are almost exclusively O-linked sugars attached to

the ectodomain. Despite the variation in amino acid sequence, the total O-linked glycan modification sites are relatively conserved among gN isoforms [16].

The fusion protein gB is a type-I membrane fusion protein with 110kDa ectodomain disulfide linked to a 55kDa transmembrane domain. gB exists in virion envelope in the form of homotrimer, which is linked together by inter-chain disulfide bonds [17]. gB is also heavily glycosylated and it has 50-60kDa of N-linked glycan and at least one O-linked glycan modification [18].

On the HCMV envelope, gH/gL is decorated by accessory proteins and exists as two complex forms: gH/gL/gO and gH/gL/UL128/UL130/UL131 (gH/gL/UL128-131). Compared to gH/gL and UL128-131, gO amino acid sequence is highly diverse among HCMV strains and the phylogenetic analysis showed that there are 8 genotypes of gO [19]. Within each genotype, the gO sequences are 98-100% identical, while between groups there are up to 50% differences [19]. The diverse regions are scattered across the gO sequence but mostly locate at the N-terminus [64]. Nearly half of the mass of gO is contributed by glycan, the amino acid sequence divergence may contribute to variation of glycan sites and glycan compositions among different gO isoforms.

b. HCMV lifecycle

1. Entry and cell tropism

The very first step of viral replication cycle is entry into host cell. HCMV's entry process has high complexity and one unique property of HCMV is that this virus can infect a broad spectrum of cell types, such as dendritic cells, endothelial cells, epithelial cells, fibroblast cells, and monocyte/macrophage cells.

The entry event is mediated by envelope glycoproteins: gB, gM/gN, gH/gL/gO and gH/gL/UL128-131. The gM/gN complex interacts with heparin sulfate proteoglycans on the host cell surface to facilitate viral particle attachement to the host cell [28]. As with other human herpesviruses, gB together with gH/gL serve as the "core" membrane fusion machinery for HCMV. gH/gL complexes trigger gB to dramatically rearrange its structure and mediate fusion between viral and cellular membranes [29,30]. However, the mechanisms for how gH/gL complexes interact or regulate the gB fusogen remain unclear.

The gH/gL complexes, including gH/gL/gO and gH/gL/UL128-131, have big impacts on the tropism of HCMV. For virus floating outside of cells, gH/gL/gO on the virion envelope is required for infecting all cell types. It has been shown that gH/gL/gO can bind to the platelet-derived growth factor receptor-alpha (PDGFR-α) on fibroblasts through gO [31, 32]. Particularly for infection on certain cell types, such as leukocytes, epithelial cells, and endothelial cells, gH/gL/UL128-131 is additionally required. Neuropilin-2 (Nrp2) has been identified as a receptor for gH/gL/UL128-131 [33]. gH/gL/gO-mediated entry into fibroblast cell occurs through a rapid macropinocytosis in a pH-independent manner [34]. However, the

entry into epithelial and endothelial cells, which depend on both gH/gL/gO and gH/gL/UL128-131, requires low pH and involves endocytosis [35]. Over the years, many host cell surface proteins have been suggested to function as potential receptors, co-receptors, or accessory proteins for gH/gL complexes, which contribute to viral entry. However, the mechanisms of how they affect the entry event are not well understood [36-39].

2. Delivery of viral genome into the nucleus and initiation of viral gene expression

After fusion between virion envelope and host cell plasma membrane or endosome membrane, not only the capsid containing viral genome but also the proteins within tegument layer are released into the cell. The tegument proteins are mainly in charge of delivering the viral genome to the nucleus and initiating viral gene expression. For example, tegument proteins pUL47 and pUL48, which are tightly associated with the nucleocapsid, can interact with microtubules in the cytosol to accomplish delivery of viral nucleocapsid to the nucleus. Another tegument protein pp71 can bind and degrade host proteins inside of the nucleus that inhibit viral genome transcription, thus facilitating the initiation of viral replication.

3. Progeny assembly

The assembly of progeny starts with capsid formation in the nucleus and is followed by incorporating the viral DNA genome into the capsid through DNA packaging enzyme (terminase) [23]. After nucleocapsid egress from nucleus to cytoplasm, the tegument proteins are added to the particle [26]. Once the particle is completely

tegumented, the immature particle buds into the virion assembly compartment, which is a complex derived from the endoplasmic reticulum, Golgi apparatus, and endosomal machinery. The immature particles acquire their envelope along with all the virion envelope glycoproteins inside of the virion assembly compartment [27]. Afterwards, the fully assembled progeny virus is either released out of the cell or transported towards the cell-cell interface.

4. Latency and reactivation

Like all herpes viruses, HCMV's ability of establishing latency is critical for its lifelong persistence in the host. Compared to acute HCMV infection, which has very broad cell tropism, latent virus resides in restricted cell types. Based on clinical study using a highly sensitive PCR approach, it has been found that myeloid lineage in the peripheral blood is an important site for HCMV latency. More specifically, $CD14^+$ monocyte population is the dominant carrier of the HCMV latent genome. The $CD34^+$ cells that reside in bone marrow are also shown to be HCMV genome positive [40-43].

The reactivation of HCMV gene expression and productive infection is associated with differentiation of $CD34^+$ cells, which is stimulated by inflammatory cytokines and/or growth factors [48-49]. The differentiation of $CD34^+$ cells to mature macrophages and dendritic cells change the level of cellular transcription factors and these changes lead to viral gene expression.

c. HCMV genetic variability

HCMV has the largest genome among all known viruses that infect humans. With the development of Next Generation Sequencing (NGS), researchers in the field started to look into HCMV sequence in clinical specimens.

Based on clinical samples collected worldwide, NGS analyses showed that HCMV has significantly higher diversity compared to other human herpesviruses [54]. There are 21 out of 165 loci scattered in the genome that are hyper-variable across clinical samples, while the majority are conserved [54-55]. Since most regions are conserved, the linkage disequilibrium is low among loci, which enables pervasive recombination between viral genomes. Among the 21 high diversity loci, some of them encode for glycoproteins that are critical for viral tropism and escape from host immunity. For example, UL74 encodes for gO, which is involved in viral entry and spread. The product of the UL11 gene is a membrane glycoprotein, which can modulate T-cell signaling. For each of the high diversity loci, 2-14 different alleles exist [153]. It is possible that HCMV utilizes extensive recombination to constantly diversify the loci that are critical for cell tropism and escaping from immune responses, while maintaining the rest of the genome that is optimally adapted to the asymptomatic lifecycle.

High HCMV intra-host diversity has also been described by genomic sequencing research. This diversity was observed among a range of human hosts, including healthy adults, children, congenitally infected infants, organ transplant recipients,

and AIDS patients [56-60]. The analyses also uncovered higher viral genetic diversity in plasma compared to certain body compartments, which suggests that the various genomes in the peripheral blood might undergo selection pressure for fitness in different host compartments [66]. Some potential links have been drawn between the diversity of viral glycoproteins and dissemination into distinct body compartments. For example, UL74 (gO) locus diversity may affect dissemination to certain body compartments by influencing the ratio between gH/gL/gO and gH/gL/UL128-131 complexes, thus impacting cell tropism [66]. It has also been found that UL55(gB) genotypes are associated with particular host compartments [65].

Taken together, these studies highlight the complexity of HCMV genome and brought a new direction for understanding HCMV's various pathological outcomes.

d. HCMV immune evasion

The replication and spread of HCMV can induce host immune responses, including recruitment of natural killer cells, production of neutralizing antibodies, and activation of $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T cells [67].

HCMV as one of the most ancient human viruses has sophisticated mechanisms for escaping both innate and acquired immune responses. It has been shown that viral protein IE2-86 could inhibit transcription of interferon-beta [70]. There is also a list of viral proteins involved in interfering with MHC-I antigen presentation. For

example, US2 and US11 translocate MHC heavy chain from ER to cytosol for proteasome degradation [72]. US3 interacts with MHC-I and leads to ER retention [73-74]. US6 inhibits peptide transport and prevents viral peptide being loaded to MHC-I [75]. In addition, UL141 interferes with NK cell activation by retaining CD155, which is a ligand for the activation receptor on NK cells, inside the ER [78].

For the acquired immune response, HCMV can interfere with T cell stimulation mainly through viral protein UL144. UL144 interacts with T cell attenuator, which inhibits T cell proliferation [81]. Interestingly, the UL144 gene has significant strain-specific variability and the amino acid sequences vary up to 20% among HCMV strains [82-84].

e. HCMV Pathology

HCMV is an opportunistic pathogen and it does not cause disease in healthy people. However, the infection in hosts with immature or compromised immune system can cause severe pathology.

1. Congenital and neonatal infection

HCMV is the most frequent among all the congenital viral infections, which account for more than 40,000 cases in the United States every year. 13% of infected infants are born with symptoms and 0.5% of the infections are fatal [85]. For the symptomatic infants, 20% of them suffer from sensorineural hearing loss and the rest show various symptoms including physical impairment, vision loss, behavioral

and cognition delays [86]. Clinical studies have shown that the seronegative mothers who became infected during pregnancy had very high risk of transmitting the virus to the developing child [87]. Women infected by HCMV before pregnancy also had the risk of bearing an infected infant, which resulted from infection with a new strain during conception [88].

2. Hematopoietic stem cell transplantation (HCT) patients

Following HCT, HCMV infection-caused pneumonia is one of the most feared cases at clinics, and mortality remains high even with treatment [89]. The HCMV infection-associated gastrointestinal disease is the most common one observed in the clinic among HCT recipients, which can affect both upper and lower tracts [89]. Since HCMV has broad cell tropism, the infection also frequently causes retinitis, hepatitis, and encephalitis. The most important pre-transplant risk factor for HCMV disease is the serological status of the donor and recipient. The seropositive recipients are considered as the highest risk, which is mostly caused by HCMV reactivation diseases [90]. For the cases where the donors are seropositive, recipients have the risk of getting re-infected by different HCMV strains [90].

3. Solid organ transplantation (SOT) patients

Among SOT patients, HCMV can cause a febrile syndrome with leukopenia and/or transaminitis. The indirect effects related to HCMV infection include allograft rejection, decreased graft, and patient death [92]. Compared to HCT patients, HCMV reactivation diseases in seropositive recipients are less common in SOT patients.

The highest risk occurs when the organ donor is HCMV seropositive and the recipient is seronegative.

4. HIV/AIDS patients

HCMV diseases are observed in HIV-1-infected patients whose immune systems are heavily under attack. Retinitis is the most common clinical manifestation, followed by gastrointestinal disease and encephalitis [93].

5. Immuno-competent hosts

HCMV infection may occur at any time during lifespan, such as childhood acquisition in a day care setting, adulthood latent infection reactivation, and transmission through blood transfusions. Primary infection is typically asymptomatic in immune-competent hosts. Occasionally, HCMV causes pneumonia or gastrointestinal diseases [94].

f. Treatment and prevention

Ganciclovir, foscarnet, and cidofovir are the drugs frequently applied for treating HCMV infection in the clinic. Ganciclovir is a Guanosine analog and after phosphorylation by HCMV UL97 kinase, it acts as a chain terminator during viral DNA replication. Cidofovir is a nucleoside monophosphate analog and foscarnet is a pyrophosphate analog that inhibits viral DNA polymerase activity. Unlike ganciclovir, neither cidofovir nor foscarnet requires activation by other viral proteins [94]. Ganciclovir has been tested in both SOT and HIV patients, which could

be given through intravenous therapy, oral formulations, and locally to the eye for sight-threatening retinitis [95]. However, the clinical trials for cidofovir have not been performed in SOT patients.

These drugs have various side effects. For example, ganciclovir's principal toxicity is causing neutropenia and the main side effects of foscarnet are renal toxicity and electrolyte imbalance [96]. It has been observed that HCMV in patients developed drug resistance to all these three drugs during treatment [97]. The resistance to ganciclovir has been observed when viral UL97 kinase and viral DNA polymerase mutations occurred [98]. The resistance mutations against foscarnet and cidofovir also arise at the DNA polymerase gene [99]. Some of these mutations at the viral DNA polymerase gene locus could lead to resistance to more than one drug [99].

Considering the tremendous amount of healthcare costs associated with HCMV infection, much effort has been put into developing vaccines against this virus as a prevention strategy. The history of vaccine development for HCMV can be traced back 30 years. However, no HCMV vaccine appears to be approaching imminent licensure. A variety of strategies have been employed, including live attenuated vaccines and subunit vaccines, and a number of them have been evaluated in clinical trials [100].

The first live attenuated vaccine tested in human was developed based on the laboratory-adapted strain AD169. Later on another live attenuated vaccine based on

strain Towne was confirmed to elicit neutralizing antibodies as well as $CD4^+$ and $CD8^+$ T lymphocyte responses. The efficacy of the Towne vaccine was tested in studies with renal transplant recipients. This vaccine was also tested in a placebo-controlled study in seronegative mothers who had children attending daycare. The study found that immunization with Towne based vaccine failed to protect these women from acquiring HCMV infection from their children [103]. However, this vaccine did protect women with pre-existing immunity against HCMV from getting re-infected by different strains [103]. Recent evidence indicated that the defect in Towne live attenuated vaccine might be due to insufficient $CD4^+$ and $CD8^+$ T cell responses. In order to overcome this defect, adjuvant interleukin-12 (rhIL-12) was co-administered with live attenuated Towne vaccine and it is currently under clinical trial [104]. In addition, chimeric viruses between strain Towne and Toledo were generated as live attenuated vaccines. Four of these Towne/Toledo chimeric vaccines were tested in a clinical trial and all of them were well tolerated with no sign of virus shedding in the blood and body fluids [105]. However, the major concern or risk for live attenuated vaccines is that they may establish latent HCMV infections.

Subunit vaccines are designed against specific immunogenic viral proteins, which are expressed by various techniques and tested either alone or in combination. Based on clinical observations among HCMV-seropositive individuals, up to 70% of neutralizing antibodies respond to gB [106], which makes gB a promising candidate for subunit vaccine development. The vaccines based on gB demonstrated

protection against HCMV infection disease in murine and guinea pig models [107]. In current clinical trials, soluble gB ectodomain expressed in Chinese hamster ovary (CHO) cells are purified and combined with adjuvants MF59 or alum as vaccines [108]. These vaccines are currently under test among seronegative adults, a limited number of toddlers, young HCMV-seronegative women, and renal transplant patients who are waiting for transplantation. Based on the results reported to date, the level of gB-specific antibodies and virus neutralizing activity after 3 doses exceeded those observed in HCMV-seropositive controls [109-111].

pp65 has been identified as a target for subunit vaccine because it is the dominant trigger for CD8+ T cell responses [113-114]. During clinical trial, pp65 vaccine recipients were observed with a similar level of HCMV-specific CD8+ cytotoxic T cell responses as the seropositive controls [115]. The pp65 vaccine is also currently under clinical trial in a trivalent formulation together with gB and IE1 vaccines [115].

Focus of the dissertation

Human cytomegalovirus (HCMV) glycoproteins H and L (gH/gL) can be bound by either gO or UL128, UL130, and UL131 proteins to form complexes: gH/gL/gO and gH/gL/UL128-131, which facilitate viral entry and spread [122-127, 129-132]. The epitopes on gH/gL in two complexes are important targets of neutralizing antibodies [166-170]. Strains of HCMV vary dramatically in their levels of gH/gL/gO and gH/gL/UL128-131. The UL74 locus that encodes for gO is one of the most

diverse loci among HCMV strains. Most phylogenetic groupings indicate gO has 8 genotypes, which differ in 10% to 30% of amino acid sequences [64, 135]. The diverse regions of gO predominately locate at the N-terminus and these amino acid polymorphisms can potentially affect the N-linked glycan sites on gO [64].

The two major questions addressed in this dissertation include:

- What are the mechanisms behind gH/gL complex assembly differences between strains?
- How do natural inter-strain variations in the amino acid sequence of gO influence the biology of HCMV?

The studies in Chapter 2 address the first question by utilizing recombinant viruses in which the UL74 (gO) ORF was swapped. I picked strain TR to represent strains with gH/gL/gO as the dominant gH/gL complex on the envelope, and strain Merlin (ME) represented for gH/gL/UL128-131-rich viruses. I observed that swapping UL74 (gO) had no effect on gH/gL complexes assembly for both strains. To explore whether the abundance of viral proteins could influence gH/gL complex formation, I applied a quantitative immunoprecipitation approach and revealed that gO expression level was 20-fold lower in ME compared to TR. Overall, strain variations in the assembly of gH/gL complexes are mostly due to the viral protein expression level difference and gO amino acid sequence does not affect the gH/gL complexes assembly process.

The Chapter 3 summarizes my results for investigating the second question. I constructed heterologous gO recombinants with 6 out of 8 genotypes (GT) and analyzed them in both strain TR and strain ME backgrounds. I found that gO isoforms could impact viral entry, spread, and sensitivity to anti-gH neutralizing antibodies and these effects were subjected to epistatic global strain genetic differences. Characterization of these gO recombinants has also revealed that gH/gL/gO utilizes different mechanisms for facilitating cell-free and cell-to-cell spread, and gH/gL/gO performs its functions in viral attachment and fusion in a separate manner.

In summary, this dissertation explored the significance of gO diversity in critical aspects of HCMV biology and highlighted the epistatic effects in HCMV phenotype determination. These findings may provide information for better understanding of HCMV pathology and bring novel insights on vaccine design strategy.

Chapter II. Expression levels of glycoprotein O (gO) vary between strains of Human Cytomegalovirus, influencing the assembly of gH/gL complexes and virion infectivity.

This chapter is modified version of the manuscript published in Journal of Virology in May 2018; 92: e00606-18.

Introduction

Human Cytomegalovirus (HCMV) is widely spread through the world and it is found in approximately 60% of adults in developed countries and in 100% of adults in developing countries [116-119]. HCMV is an opportunistic pathogen and immunocompromised individuals, such as HIV patients, transplant recipients under immunosuppression treatments can suffer severe HCMV infection related pathologies, including gastroenteritis, encephalitis, retinitis, and vasculopathies. The congenital infection of HCMV is a significant cause of congenital neurological impairments and sensorineural hearing loss. The transmission of HCMV is mainly through body fluid, such as urine and saliva. Once infection is established, the virus spreads throughout the body, infecting many of the major somatic cell types, neurons, and leukocytes.

Much focus has been on the gH/gL complexes, which likely engage cell receptors and promote infection by contributing to the gB-mediated membrane fusion event or through activating cell signaling pathways [120-122]. During virus assembly, the HCMV UL128-131 proteins and gO compete for binding to gH/gL to form the pentameric complex gH/gL/UL128-131 or the trimeric complex gH/gL/gO. Structural studies involving purified soluble complexes showed that gO and UL128 can each make a disulfide bond with cysteine 144 of gL, and this was suggested to be the basis of the competitive assembly of the complexes [123]. However, Stegmann et al. demonstrated that mutant gO lacking the cysteine implicated in the disulfide

bond with gL formed intact and functional gH/gL/gO [124]. This suggests that gO can engage in extensive non-covalent interactions with gH/gL. The gH/gL/UL128-131 complex is dispensable for infection of cultured fibroblasts and neuronal cells but is required for infection of epithelial and endothelial cells and monocytes-macrophages [125-129]. In contrast, gH/gL/gO is critical for infection of all cell types [130-133]. Both complexes likely interact with cell receptors. gH/gL/gO can bind platelet-derived growth factor receptor alpha (PDGFRα) through the gO subunit, and this interaction is critical for infection of fibroblasts [31-32, 134]. Epithelial and endothelial cells do not express PDGFRα, but blocking of gH/gL/gO with either neutralizing antibodies or soluble PDGFRα can inhibit infection of these cells, suggesting the existence of other gH/gL/gO receptors [31-32]. Receptors for gH/gL/UL128-131 might include epidermal growth factor receptor (EGFR) (also known as ErbB1) and β1 or β3 integrins, and these interactions may induce signaling cascades critical for infection of selected cell types, such as epithelial and endothelial cells and monocytes-macrophages [31, 35].

Zhou et al. reported that the amounts of gH/gL/gO and gH/gL/UL128-131 in the virion envelope differ dramatically among strains of HCMV and this difference affects the infectivity of the virions [64,133]. The major results of those studies were that 1) Merlin (ME) virions contained gH/gL mostly in the form of gH/gL/UL128-131, whereas TR and TB 40/E (TB) virions had mostly gH/gL/gO; 2) in terms of "total gH/gL," the amount of gH/gL/gO in TR and TB virions was larger than the amount of gH/gL/UL128-131 in ME virions; 3) the infectivity of all three strains on

both fibroblasts and epithelial cells correlated with the amount of gH/gL/gO; and 4) when the expression of UL128-131 was suppressed in ME, virions contained dramatically less gH/gL/UL128-131 but only slightly more gH/gL/gO. The latter point was especially curious since the model that gO and UL128-131 proteins compete for binding to gH/gL would predict that the fraction of gH/gL normally bound by UL128-131 would, in their absence, be bound by gO instead. This discrepancy could be explained by differences in the stoichiometric expression of gH/gL, gO, and UL128-131 between strains. An alternative hypothesis was suggested by the fact that there are at least eight alleles of the UL74 gene that encodes gO [135]. The amino acid sequence of gO among these eight genotypes can vary between 10 and 30%, and this could affect competition with UL128-131 for binding to gH/gL. Both of these non-mutually-exclusive hypotheses were addressed in this chapter.

Results

Strains of HCMV display different patterns of glycoprotein expression and trafficking to virion assembly compartments.

The dramatic differences in the compositions of gH/gL complexes in TR and ME virions described previously by Zhou et al. [64, 133] suggested corresponding differences in glycoprotein expression and/or the trafficking of glycoproteins to virion assembly compartments (ACs). To address these possibilities, cells were infected for 2 days (Fig 2.1A) or 5 days (Fig 2.1B) with TR or ME, and steady-state amounts of viral proteins were compared by immunoblotting. At 2 days

postinfection (dpi), immediate early protein 1/2 (IE1/2) levels were similar for both TR and ME, consistent with an equal multiplicity of infection. At 5 dpi, the levels of the virion structural proteins major capsid protein (MCP), gB, gH, and gL were also very similar between the two strains. In contrast, ME-infected cells contained dramatically more UL128-131 protein than did TR-infected cells. The UL148 protein was also included in these analyses because it was recently described as an endoplasmic reticulum (ER) chaperone protein that influences the ratio of gH/gL complexes [136]. In TR-infected cells, an anti-UL148 antibody detected a prominent 35-kDa protein species, consistent with the previous description of the UL148 protein [136]. This 35-kDa species was not detected in ME-infected cells. Instead, ME-infected cells contained two species that were less abundant and of higher and lower electrophoretic motilities than the single UL148 species detected in TR-infected cells. The basis of the apparent size difference was not characterized but could reflect differences in translational start/stop codon usage, splicing of the UL148 mRNA, or posttranslational modifications of the UL148 protein between strains. Overall, the pattern of expression of the UL128-131 and UL148 proteins correlated well with the previously described pentamer-rich nature of ME virions and the trimer-rich nature of TR virions [64, 133]. Note that the expression of gO was not addressed in these analyses because the gO amino acid sequence differences between strains affect antibody recognition and preclude direct comparison [64].

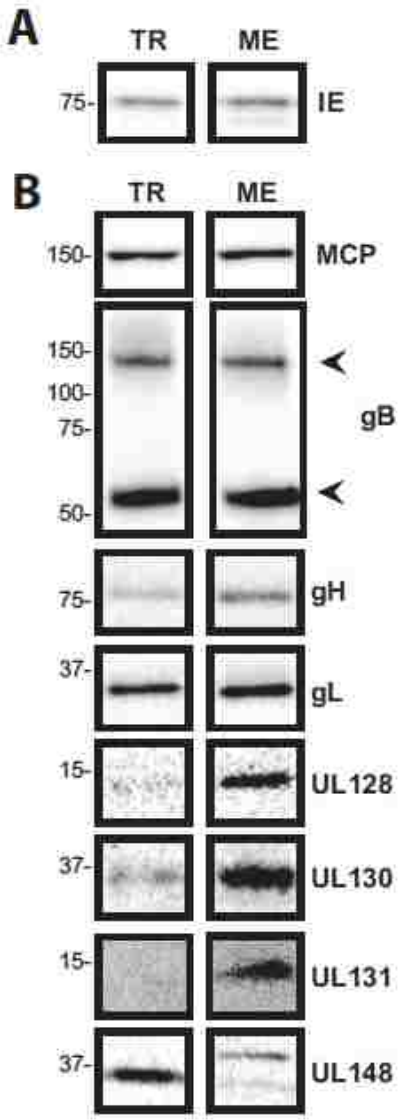

Fig 2.1 Comparison of protein expression between TR and ME. nHDF were infected with 1 PFU/cell of TR or ME. At day 2 (A) or day 5 (B), total cell extracts were separated by reducing SDS-PAGE and analyzed by immunoblot analysis probing for immediate early protein 1/2 (IE1/2), major capsid protein (MCP), gB, gH, gL, UL128, UL130, UL131, or UL148. Arrowheads indicate the positions of the cleaved 100-kDa and 55-kDa fragments of gB.

Trafficking of gH/gL from the ER to trans-Golgi network (TGN)-derived assembly compartments was assessed by treating the infected-cell extracts at 5 dpi with either endoglycosidase H (endo H) or peptide N-glycosidase F (PNGase F) and then analyzing gH and gL by immunoblotting (Fig 2.2). The majority of gH and gL in TR-

infected cells was endo H resistant, consistent with efficient transport from the ER to trans-Golgi network-derived ACs. In contrast, most of the gH and gL in ME-infected cells was sensitive to endo H digestion. In HFFFtet cells, which repress transcription from the UL128-131 locus [64, 137], there was even less endo H-resistant gH and gL. This suggested that the bulk of gH/gL trafficked to ACs in ME-infected neonatal human dermal fibroblasts (nHDF), which allow UL128-131 expression, represented gH/gL/UL128-131 and is consistent with previous observations that (i) the bulk of gH/gL in the ME virion is pentamers and (ii) the loss of gH/gL in the form of pentamers in ME-T virions due to the repression of the UL128-131 proteins is apparently not fully compensated for by the formation of complexes with gO [64, 133].

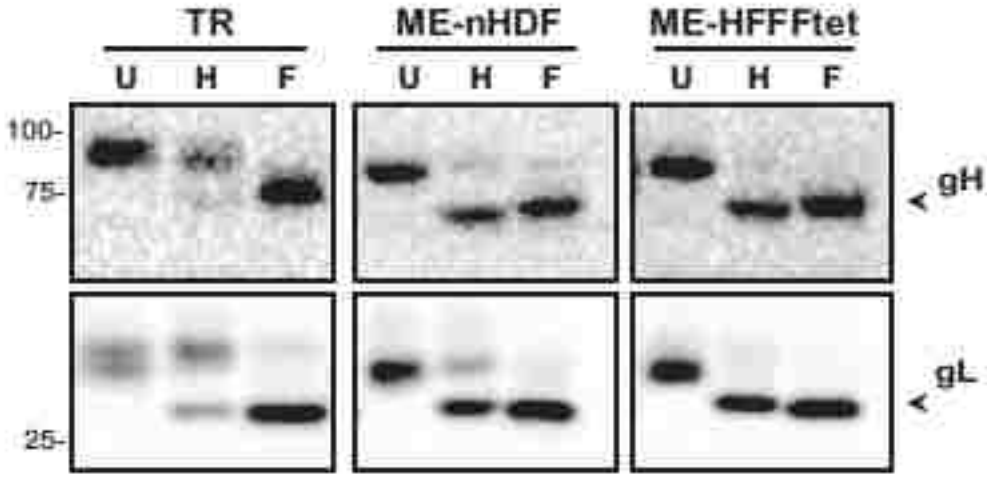

Fig 2.2 Analysis of ER-to-trans-Golgi compartment trafficking of glycoproteins in TR- or ME-infected cells. Extracts of nHDF infected with TR or ME or HFFFtet cells infected with ME were treated with endoglycosidase H (H) or PNGase F (F) or left untreated (U) and then separated by reducing SDS-PAGE and analyzed by immunoblot analysis probing for gH or gL. Arrowheads indicate the positions of the faster-migrating, deglycosylated species.

Differences in amino acid sequence of gO between TR and ME do not affect the infectivity of cell-free virus.

The predicted amino acid sequence of gO differs by 25% between TR and ME. This sequence divergence precluded direct comparison of gO expression levels because antibodies do not cross-react [64]. Furthermore, these sequence differences could potentially affect the ability of the distinct gO isoforms to compete with the UL128-131 proteins for binding to gH/gL (thus influencing the amounts of gH/gL complexes in the mature virion envelope) or the function(s) of gO during entry, such as binding PDGFRα or other receptors. To address these possibilities, bacterial artificial chromosome (BAC) recombineering methods were used to replace the gO open reading frame (ORF) (UL74) of TR with the analogous sequences from ME, and visa versa, to generate recombinant viruses denoted TR_MEgO and ME_TRgO. Zhou et al. demonstrated a positive correlation between the infectivity of HCMV virions and the amounts of gH/gL/gO in the virion envelope [133]. To assess the effects of gO sequences on infectivity, cell-free virus stocks of the parental wild type and heterologous gO recombinants were analyzed by quantitative PCR (qPCR) to determine the number of virions, and infectivity was determined by a plaque assay. No difference in particles/PFU was observed between TR and the corresponding recombinant TR_MEgO (Fig 2.3) or between ME and the corresponding recombinant ME_TRgO (Fig 2.3). When ME-based HCMV was grown in HFFFtet cells, which repress UL128-131 expression, the resultant virions, ME-T and ME-T_TRgO, were dramatically more infectious, as shown previously [133, 137], but consistently, there were no differences due to the isoform of gO expressed (Fig 2.3). In parallel

analyses, the amounts of gH/gL complexes were analyzed by nonreducing immunoblot probing for gL to detect intact, disulfide-linked gH/gL/gO and disulfide-linked gH/gL/UL128 (note that UL130 and UL131 are not disulfide linked to the intact pentamer complex and are thus separated by SDS-PAGE) (Fig 2.4). Consistent with our previous reports [64, 133], TR virions contained much larger amounts of total gH/gL, mostly in the form of gH/gL/gO, whereas ME virions contained less gH/gL, mostly as gH/gL/UL128-131. Repression of the UL128-131 proteins (ME-T) drastically reduced the amount of gH/gL/UL128-131 and increased the amount of gH/gL/gO. However, note that the amount of gH/gL/gO in ME-T virions was still smaller than the amount of gH/gL/UL128-131 in ME virions, indicating that the repression of UL128-131 was not fully compensated for by gO. In no case did the expression of the heterologous gO isoform detectably influence the amounts of gH/gL complexes in HCMV virions. Together, these results suggest that the amino acid sequence differences between TR and ME gO do not influence gH/gL complex assembly or the function of gO in entry into fibroblasts.

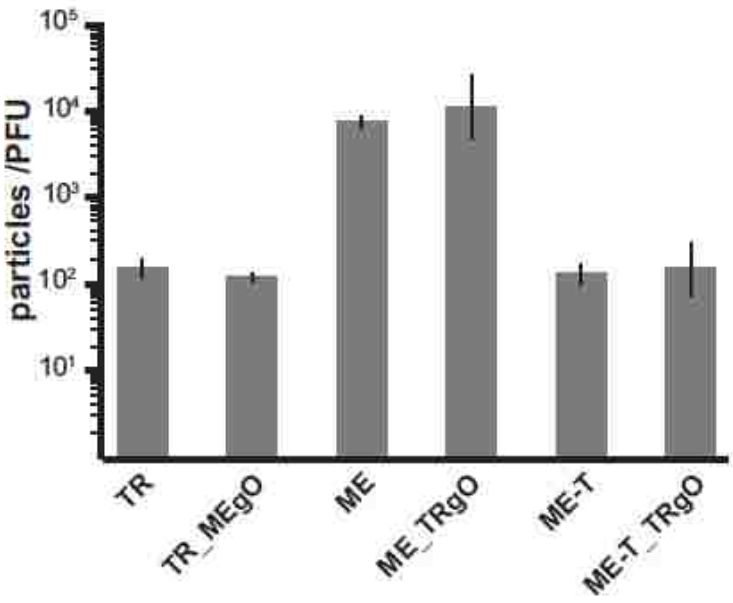

Fig 2.3 Specific infectivity of parental and TR-ME heterologous gO recombinants. Extracellular virions of TR, TR_MEgO, ME, ME_TRgO, ME-T, or ME-T_TRgO were analyzed by quantitative PCR for viral genomes, and PFU were determined by a plaque assay on nHDF. Average particle/PFU ratios from at least 4 independent experiments are plotted. Error bars represent standard deviations.

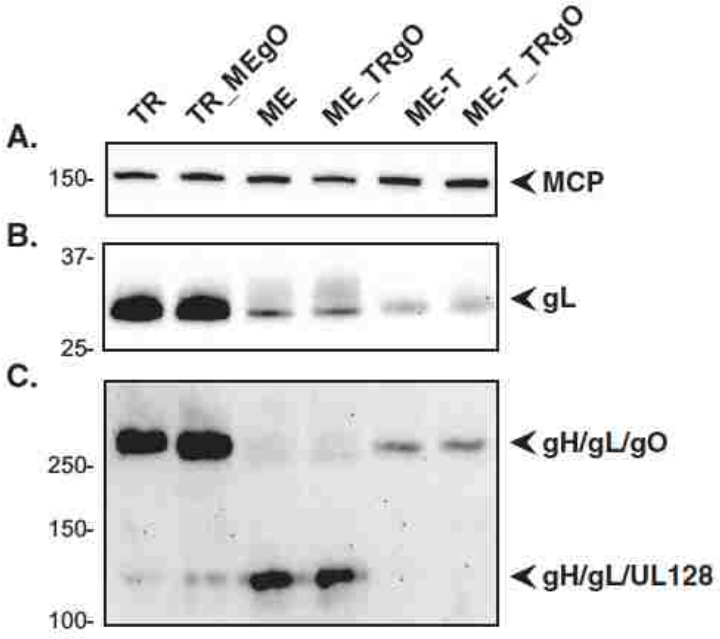

Fig 2.4 Immunoblot analysis of gH/gL complexes in parental viruses and TR-ME heterologous gO recombinants. Extracellular virion extracts of TR, TR_MEgO, ME, ME_TRgO, ME-T, or ME-T_TRgO were separated by reducing (A and B) or nonreducing (C) SDS-PAGE and analyzed by immunoblot probing for major capsid protein (A) or gL (B and C).

ME expresses less gO during replication than does TR.

The heterologous gO recombinants allowed comparison of gO expression levels between TR and ME. In the first analyses, cells infected with the parental virus or the heterologous gO recombinants were analyzed by reducing immunoblot analysis using TR- and ME-specific anti-gO antibodies [64] (Fig 2.5). TR-specific gO antibodies detected two bands in TR-infected cells, a prominent species migrating just above the 100-kDa marker and a minor, more diffuse species migrating at approximately 130 to 140 kDa. The ME-specific antibodies detected similarly migrating bands in TR_MEgO-infected cells; however, their relative abundances appeared more equal. No similar bands were detected in cells infected with ME or ME_TRgO analyzed with either gO antiserum. The failure to detect either isoform of gO in cells infected with ME-based HCMV suggested that the protein expression level from the UL74 locus of ME was lower than that in TR.

To directly compare differences in glycoprotein expression between TR and ME, infected cells were labeled with [^{35}S]methionine-cysteine for 15 min and then analyzed by immunoprecipitation with antipeptide antibodies specific for gH, gL, or gO, followed by SDS-PAGE and band density analysis (Fig 2.6 and Tables 1 and 2). Two approaches were taken to allow direct quantitative comparisons of labeled proteins between extracts. First, cell extracts were denatured and reduced with

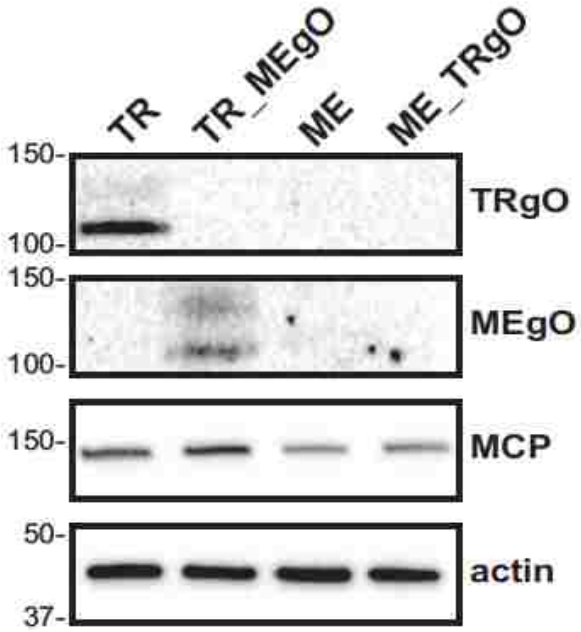

Fig 2.5 Immunoblot analysis of gO expression in cells infected with parental viruses and TR-ME heterologous gO recombinants. nHDF were infected with 1 PFU/cell of TR, TR_MEgO, ME, or ME_TRgO. At day 5, total cell extracts of infected cells were separated by reducing SDS-PAGE and analyzed by immunoblot probing for TRgO, MEgO, MCP, or actin.

SDS-dichlorodiphenyltrichloroethane (DDT) prior to immunoprecipitation to allow maximum epitope access by the antipeptide antibodies. Second, for each analysis, multiple immunoprecipitation reactions were performed in parallel with increasing amounts of protein extract input to ensure that antibodies were not limiting. In these experiments, expression levels of gH were nearly identical between TR and ME, and the gL expression level was approximately 4-fold higher for TR than for ME, but the gO expression level was strikingly 27-fold higher for TR than for ME (Fig 2.6A and Table 2.1). To address the possibility that the MEgO-specific antibodies were simply less efficient at capturing MEgO from ME extracts, similar experiments were performed with the TR-ME heterologous gO recombinants (Fig 2.6B and Table

2.2). Again, gH and gL levels were similar between TR_MEgO and ME_TRgO, but gO levels were approximately 20-fold higher for the TR-based virus. To address the hypothesis that differences in gO expression between TR and ME reflect differences in protein turnover, the $^{[35S]}$methionine-cysteine label was chased for up to 6 h (Fig 2.7). The patterns of gH detection over the chase time were very similar for both TR and ME samples. In both cases, the amounts of labeled gH dropped to 60% after 3 h and to 30 to 40% after 6 h. The pattern of gO detection for both TR and ME was comparable to that of gH detection. Together, these results confirmed that ME-infected cells express less gO than do TR-infected cells and suggested differences in early steps of expression, such as mRNA transcription, translation, or rapid ER-associated degradation, which can degrade proteins in the time scale of minutes [138].

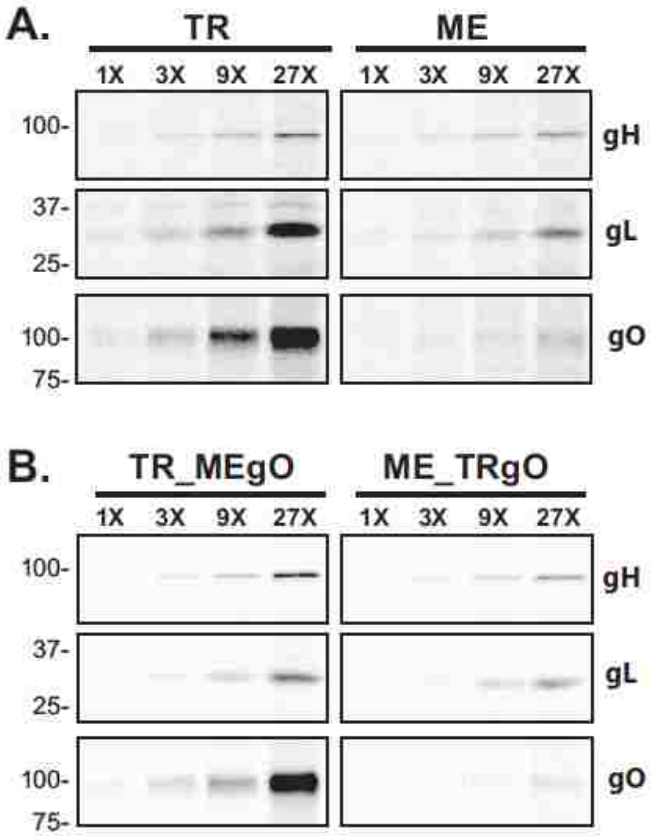

Fig 2.6 Quantitative comparison of glycoprotein expression in TR- and ME-infected cells. nHDF were infected with 1 PFU/cell of TR or ME (A) or TR_MEgO or ME_TRgO (B). At 5 dpi, infected cells were metabolically labeled with [^{35}S]cysteine-methionine for 15 min, and membrane proteins were extracted in 1% Triton X-100. All samples were adjusted to 2% SDS–30 mM DTT, heated to 75°C for 10 min, cooled to room temperature, and then diluted 35-fold. Parallel immunoprecipitations were performed, in which equal amounts of anti-gH, gL, or gO (TR- or ME-specific) antibodies were reacted with 3-fold-increasing amounts of protein extract as the input, and precipitated proteins were analyzed by SDS-PAGE.

Table 2.1 Quantitative comparison of glycoprotein expression in TR- and ME-infected cells[d]

mmunoprecipitation ntibody[a]	Extract input[b] (ml)	Strain				Fold difference[f]	Mean fold differen (±SD)[g]
		TR		ME			
		Density[c]	Adjusted density[e]	Density	Adjusted density		
nti-gH	0.04	ND	ND	ND	ND	ND	1.4 (±0.1)
	0.13	136.7	4.6	106.6	3.4	1.3	
	0.40	476.9	15.9	337.8	10.9	1.5	
	1.20	1,200.7	40.0	872.9	28.2	1.4	
nti-gL	0.04	143.8	11.1	ND	ND	ND	4.0 (±1.2)
	0.13	679.1	52.2	127.2	9.8	5.3	
	0.40	1,627.3	125.2	509.3	39.2	3.2	
	1.20	6,071.3	467.0	1,809.9	139.2	3.4	
nti-gO	0.04	267.0	12.1	ND	ND	ND	27.2 (±9.4)
	0.13	1,008.6	45.8	ND	ND	ND	
	0.40	3,805.4	173.0	120.8	5.0	34.4	
	1.20	9,251.8	420.5	478.9	20.0	21.1	

[a]Seven microliters of rabbit antipeptide serum per immunoprecipitation reaction mixture.

[b]Preparation of radiolabeled cell extracts is described in the legend to Fig 2.6 and in Materials and Methods.

[c]Pixel density of bands shown in Fig 2.6A as determined using ImageJ version 1.48.

[d]ND, band density not detected.

[e]Density divided by the predicted number of methionine (met) and cysteine (cys) residues: TRgH (17 met, 13 cys), MEgH (17 met, 14 cys), TRgL (3 met, 10 cys), MEgL (3 met, 10 cys), TRgO (16 met, 6 cys), MEgO (18 met, 6 cys).

[f]Adjusted density of TR divided by adjusted density of ME.

[g]Average fold difference between TR and ME ± standard deviation.

Table 2.2 Quantitative comparison of glycoprotein expression in TR_MEgO- and ME_TRgO-infected cells[d].

Immunoprecipitation antibody[a]	Extract input[b] (ml)	Strain				Fold difference[f]	Mean fold difference (±SD)[g]
		TR_MEgO		ME_TRgO			
		Density[c]	Adjusted density[e]	Density	Adjusted density		
Anti-gH	0.04	68.4	2.3	29.5	1.0	2.4	1.6 (±0.5)
	0.13	181.6	6.1	163.1	5.3	1.1	
	0.40	539.5	18.0	410.6	13.2	1.4	
	1.20	1,697.7	56.6	1,064.0	34.3	1.6	
Anti-gL	0.04	ND	ND	ND	ND	ND	1.5 (±0.4)
	0.13	196.6	15.1	153.8	11.8	1.3	
	0.40	645.0	49.6	508.3	39.1	1.3	
	1.20	2,547.5	196.0	1,269.6	97.7	2.0	
Anti-gO	0.04	187.8	7.8	ND	ND	ND	19.7 (±1.7)
	0.13	945.8	39.4	ND	ND	ND	
	0.40	2,580.3	107.5	127.8	5.8	18.5	
	1.20	10,502.7	437.6	460.1	20.9	20.9	

[a]Seven microliters of rabbit antipeptide serum per immunoprecipitation reaction mixture.

[b]Preparation of radiolabeled cell extracts is described in the legend to Fig 2.6 and in Materials and Methods.

[c]Pixel density of bands shown in Fig. 6B as determined using ImageJ version 1.48.

[d]ND, band density not detected.

[e]Density divided by the predicted number of methionine (met) and cysteine (cys) residues: TRgH (17 met, 13 cys), MEgH (17 met, 14 cys), TRgL (3 met, 10 cys), MEgL (3 met, 10 cys), TRgO (16 met, 6 cys), MEgO (18 met, 6 cys).

[f]Adjusted density of TR_MEgO divided by adjusted density of ME_TRgO.

[g]Average fold difference between TR_MEgO and ME_TRgO ± standard deviation.

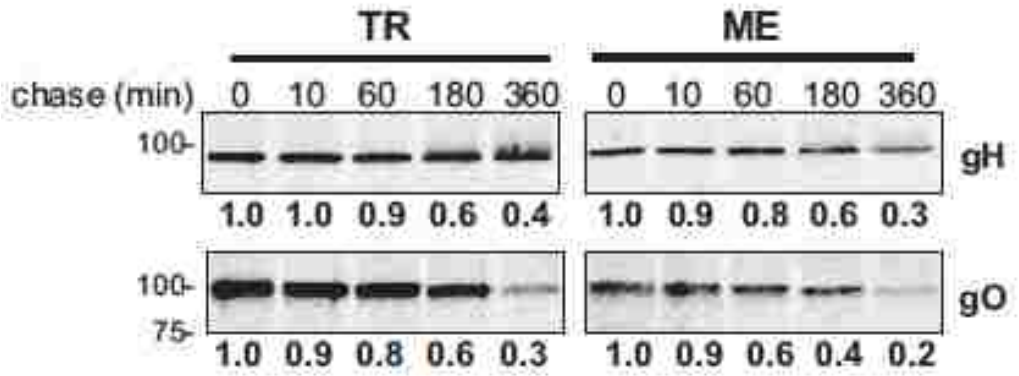

Fig 2.7 Analysis of glycoprotein turnover in TR- and ME-infected cells. nHDF were infected with 1 PFU/cell of TR or ME. At 5 dpi, infected cells were metabolically labeled with [^{35}S]cysteine-methionine for 15 min, and the label was then chased for 0, 10, 60, 180, or 360 min. Membrane proteins were extracted in 1%Triton X-100, adjusted to 2% SDS–30mM DTT, heated to 75°C for 10 min, cooled to room temperature, and then diluted 35-fold. Immunoprecipitation was performed with anti-gH and -gO (TR- or ME-specific) antibodies, and precipitated proteins were analyzed by SDS-PAGE. Band densities were determined relative to the 0-min chase time. Results shown are representative of data from 4 independent experiments.

Overexpression of gO during ME replication increases gH/gL/gO assembly and virus infectivity.

To directly test the hypothesis that the low abundance of gH/gL/gO in ME virions was due not simply to competition from the UL128-131 proteins but also to low gO expression, adenovirus (Ad) vectors were used to increase gO levels during ME replication. Ad vectors expressing green fluorescent protein (GFP) were used to control for potential effects of the Ad vectors themselves. Consistent with data from the above-described analyses, gO levels were below the limits of immunoblot detection in ME-infected nHDF or HFFFtet cells, but gO was readily detected in cells superinfected with AdMEgO (Fig 2.8A). The overall expression of gL in ME-infected cells was reduced by the presence of either Ad vector (Fig 2.8A). In the case of the control AdGFP, the lower intracellular gL level correlated with reduced levels of gH/gL/gO complexes in virions from HFFFtet cells (ME-T) (Fig 2.8B), and this in

turn correlated with reduced infectivity (i.e., increased particle/PFU ratio) (Fig 2.9). The "Ad effect" on virion gH/gL levels and infectivity was less apparent in human foreskin fibroblast (HFF) cells (ME), perhaps masked by the overall larger amounts of gH/gL and the much lower infectivity of these virions (Fig 2.8B and 2.9). Controlling for the Ad effect, AdMEgO expression in HFFFtet cells increased the amounts of gH/gL/gO in ME-T virions compared to AdGFP, and this resulted in a 6-fold enhancement of infectivity, beyond the 40-fold enhanced infectivity resulting from the repression of UL128-131 alone (Fig 2.8B and 2.9). In contrast, AdMEgO expression had little effect on the virions from HFF cells.

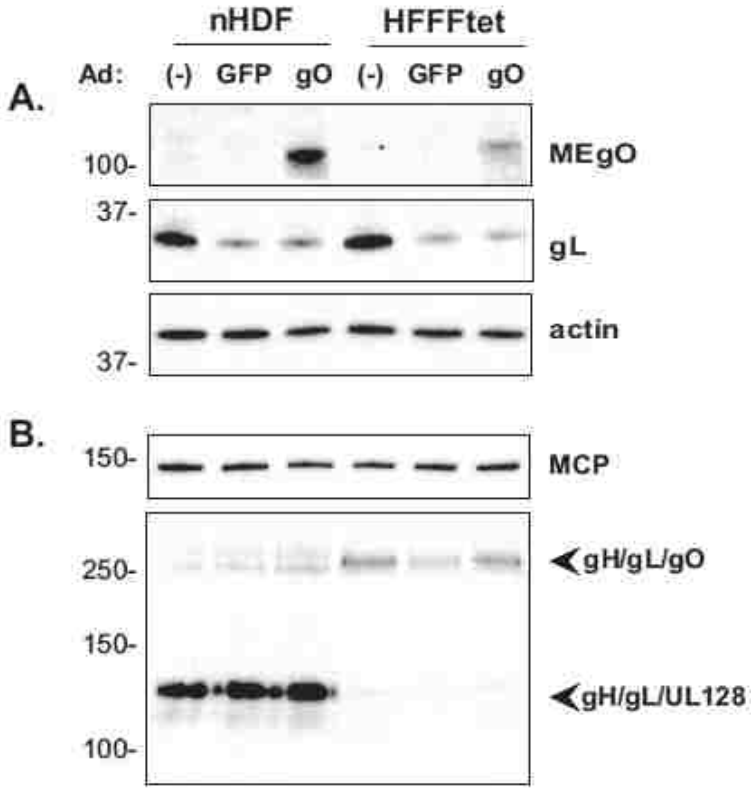

FIG 2.8 Ad vector overexpression of gO during ME replication. nHDF or HFFFtet cells were infected with ME for 2 days and then superinfected with Ad vectors expressing either GFP or MEgO for an additional 4 days. Extracts of infected cells (A) or extracellular virions (B) were separated by reducing (A and B, top) or nonreducing (B, bottom) SDS-PAGE and analyzed by immunoblot probing for MEgO, actin, MCP, or gL, as indicated to the right.

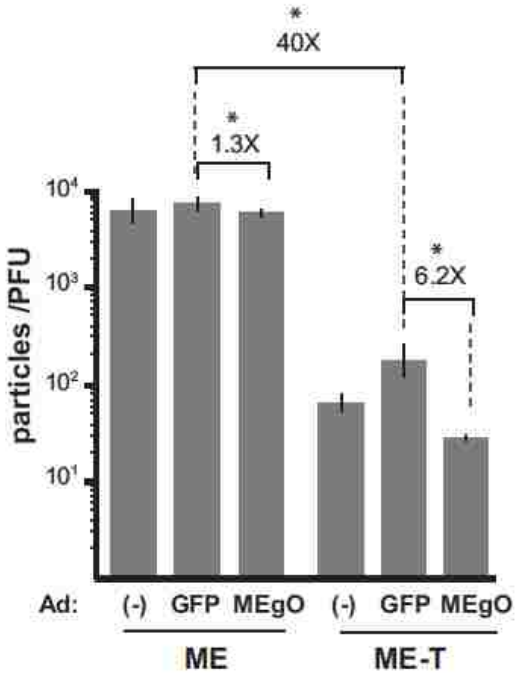

FIG 2.9 Specific infectivity of ME virions produced under conditions of gO overexpression. nHDF or HFFFtet cells were infected with ME for 2 days and then superinfected with Ad vectors expressing either GFP or MEgO for an additional 4 days. Extracellular virions from nHDF (ME) or HFFFtet (ME-T) cells were analyzed by quantitative PCR for viral genomes, and PFU were determined by a plaque assay on nHDF. Shown are average particle/PFU ratios of virions produced in 2 independent experiments, each analyzed in triplicate. Error bars represent the standard deviations. Asterisks above fold differences indicate a P value of <0.03 (determined by Student's unpaired t test [2 tailed]).

Discussion

Recent population genetic studies have demonstrated a greater degree of genetic diversity of HCMV in clinical specimens than had been previously appreciated [54, 61, 139]. The cell type and propagation methods likely narrow the resultant genotypes by purifying selection [140-141]. During propagation in cultured fibroblasts, inactivating mutations in the UL128-131 ORFs are rapidly selected in a BAC clone of ME, and this selective pressure can be relieved by transcriptional repression of the UL131 promoter, which reduces the expression of pentameric gH/gL/UL128-131 [137]. In contrast, the UL128-131 ORFs are more stable in BAC clones of strains TR and TB [141-142]. The UL128-131 ORF of TB contains a single

nucleotide polymorphism (SNP) relative to ME that reduces the splicing of the mRNA encoding the UL128 protein, which may help stabilize the UL128-131 ORFs through reduced expression of gH/gL/UL128-131 [142]. However, TR is identical to ME at this nucleotide position, and recombinant ME in which the UL128-131 locus was replaced with the UL128-131 sequences from TR was as sensitive to the selective inactivation of the locus as wild-type ME [142]. Together, these observations suggest that factors beyond the expression levels of the UL128-131 proteins can influence the selective pressures on the UL128-131 ORFs.

The results reported here demonstrated that TR and ME differ in the stoichiometry of expression of gO and UL128-131, and this seems to be a major factor determining the abundances of gH/gL/gO and gH/gL/UL128-131 in the virion envelope and the infectivity of cell-free virions. The steady-state levels of gH/gL in fibroblasts infected with TR and ME were found to be comparable, but ME-infected cells contained more UL128-131 than did TR-infected cells. In ME-infected cells, most of the gH/gL was in an ER-associated form, whereas TR-infected cells contained a large amount of Golgi compartment-associated gH/gL. This correlated well with previous observations that TR contained more total gH/gL than did ME virions [64, 133]. The amount of Golgi compartment-associated gH/gL in ME-infected cells was reduced when the expression of the UL128-131 proteins was repressed, consistent with the observation that most of the gH/gL in ME virions was in the form of gH/gL/UL128-131 [64,133]. Comparison of gO expression levels between strains was complicated because the amino acid sequence differences between genotypes affected antibody

recognition [64]. To circumvent this caveat, HCMV recombinants were engineered, in which the UL74(gO) ORFs of TR were replaced with the homologous sequences of ME, and vice versa. This approach allowed the analysis of the expression of both gO isoforms in both genetic backgrounds, eliminating the possibility that the results were due to differences in antibody-antigen affinities. Immunoblot and radiolabeling experiments clearly demonstrated that ME infected cells contained less gO than did TR-infected cells. The overexpression of gO during ME replication had no effect on the levels of gH/gL/gO or the infectivity of the virions unless UL128-131 proteins were also transcriptionally repressed, and even then, gH/gL/gO levels and infectivity were only modestly enhanced. Together, these results underscore the competition between gO and UL128-131 for binding to gH/gL and suggest that other factors may influence the efficiency of gH/gL/gO assembly.

The molecular mechanisms underpinning the discrepancy between TR and ME in the expression UL128-131 and gO remain unclear. As mentioned above, Murrell et al. described a SNP in the TB UL128-131 locus that affected mRNA splicing, in part explaining the lower expression levels of these proteins in TB [142]. However, this splicing effect does not explain the difference in UL128-131 expression levels between TR and ME, since this nucleotide position is conserved between these strains. For gO, the radiolabeling analyses reported in Fig 2.6 and 2.7 suggest that the differences are due to early events in UL74(gO) expression, such as transcription, mRNA processing/stability, translation, or rapid ER-associated

degradation, occurring in the time scale of minutes [138]. Attempts to analyze UL74(gO) mRNA levels between TR and ME by quantitative reverse transcription-PCR (RT-PCR) were complicated by the fact that HCMV genomes contain many overlapping RNA polymerase II (RNAPII) transcription units that vary between strains [143, 144]. It is interesting that ME-infected cells contained less UL148 than did TR-infected cells. UL148 was first described as an ER-resident chaperone protein that promotes the assembly of gH/gL/gO [136]. The mechanism may well involve interactions between UL148 and the cellular ER-associated degradation pathway (C. Nguyen, M. Siddiquey, H. Zhang, and J. Kamil, presented at the 42nd International Herpesvirus Workshop, Ghent, Belgium, 2017).

The TR-ME heterologous gO recombinant viruses also allowed analysis of the effects of gO amino acid sequence differences on the assembly of gH/gL complexes and the function of gO in entry. No differences were observed between TR and TR_MEgO or between ME and ME_TRgO in either the amounts of gH/gL complexes in virions or cell-free infectivity. These results argue against the notion that the amino acid sequence differences between gO genotypes affect interactions with gH/gL or the binding of the fibroblast entry receptor PDGFRα. Interestingly, Kalser et al. showed that replacing the endogenous gO protein of TB with the gO protein from Towne did not alter replication in cultured fibroblasts but enhanced replication in epithelial cell cultures [145]. Thus, it may be that gO sequence variation affects interactions with receptors other than PDGFRα that mediate infection of epithelial cells.

Laib-Sampaio et al. reported that mutational disruption of UL74(gO) expression in ME had little effect on replication unless the UL128-131 locus was also disrupted [132]. Those authors suggested that the spread of ME was mediated principally by gH/gL/UL128-131 in a cell-associated manner, but when UL128-131 was inactivated, spread could also occur in a cell-free manner, mediated by gH/gL/gO. This is in stark contrast to the dramatic phenotype reported for a gO-null TR mutant [130]. Our finding that the level of expression of gO by ME is low compared to that by TR may provide a partial explanation for these different gO-null phenotypes.

It remains unclear whether the described difference in gO expression between TR and ME represents a bona fide variation that naturally exists between HCMV genotypes in vivo or reflects differential selection on de novo mutations that occurred during the independent isolation of these strains from clinical specimens. It seems clear that serial propagation of ME in cultured fibroblasts selects for de novo mutations that reduce or abolish the robust expression of the UL128-131 proteins [137, 141]. The selective pressure that fixes these mutations in the culture population may be explained by data from the specific infectivity analyses reported here (Fig 2.3 and 2.9) and by Zhou et al. [133]. In both analyses, the specific infectivity of TR was measured at approximately 100 to 200 particles/PFU, whereas ME was 30- to 50-fold less infectious. Repression of the UL128-131 proteins enhanced the infectivity of ME ("ME-T") to levels comparable to those of TR (approximately 100 particles/PFU). While the infectivities of ME-T and TR virions were comparable, ME-T virions still contained far less gH/gL/gO than did TR virions

(Fig 2.4)[133]. Ad vector overexpression of gO enhanced the infectivity of ME only 6-fold beyond the enhancement due to UL128-131 repression alone (Fig 2.8 and 2.9). Together, these observations seem to suggest that in vitro selective pressures for reduced UL128-131 expression are much more pronounced than any for enhanced gO expression. Thus, it is possible that the difference in gO expression between HCMV TR and ME is derived not from the selection of de novo mutations occurring during propagation in culture but from nonselective, random sampling of the multitude of different genotypes that likely preexist in clinical specimens [54, 61, 139]. Distinguishing between these possibilities will require clear identification of the genomic sequences that determine gO expression levels.

Materials and methods

Cell lines. Primary neonatal human dermal fibroblasts (nHDF; Thermo Fisher Scientific), MRC-5 fibroblasts (ATCC CCL-171; American Type Culture Collection), and HFFFtet cells (which express the tetracycline [Tet] repressor protein; provided by Richard Stanton) [137] were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 6% heat-inactivated fetal bovine serum (FBS; Rocky Mountain Biologicals, Inc., Missoula, MT, USA) and 6% bovine growth serum (BGS; Rocky Mountain Biologicals, Inc., Missoula, MT, USA). Human cytomegaloviruses. All HCMV strains were derived from bacterial artificial chromosome (BAC) clones. The BAC clone of TR was provided by Jay Nelson (Oregon Health and Sciences University, Portland, OR, USA) [146]. The BAC clone of Merlin (ME) (pAL1393), which carries tetracycline operator sequences in the

transcriptional promoter of UL130 and UL131, was provided by Richard Stanton. [137]. Infectious HCMV was recovered by electroporation of BAC DNA into MRC-5 fibroblasts, as described previously by Wille et al. [130]. Cell-free HCMV stocks were produced by infecting HFF or HFFFtet cells at 2 PFU per cell. At 8 to 10 days postinfection (when cells were still visually intact), culture supernatants were harvested, and cellular contaminants were removed by centrifugation at 1,000 X g for 10 min and again at 6,000 X g for 10 min. Stocks were judged to be cell free by the lack of calnexin and actin by Western blot analyses and then stored at -80°C. Freeze-thaw cycles were avoided. PFU were determined by plating a series of 10-fold dilutions of each stock onto replicate cultures of HFF for 2 h at 37°C and replacing the inoculum with DMEM supplemented with 5% FBS and 0.6% SeaPlaque agarose (to limit cell-free spread). Plaques were counted by light microscopy 3 weeks after infection.

Heterologous UL74(gO) recombinant HCMV. A two-step BAC recombineering process was performed as previously described [137]. In the first step, the endogenous UL74 ORF from the start codon to the stop codon of both TR and ME was replaced by a selectable marker. Briefly, cultures of Escherichia coli SW102 containing either the BAC clone of TR or ME were grown at 32°C until an optical density at 600 nm (OD600) of 0.55 was reached. Recombination genes were induced by incubation at 42°C for 15min. The purified PCR product containing the KanR/LacZ/RpsL selectable marker cassette flanked by sequences homologous to 80 bp upstream and downstream of the TR or ME UL74 ORF was electroporated

into the bacteria, and cultures were incubated for 1 h at 32°C and then selected on medium containing kanamycin (15 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μM), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (20 μg/ml), and chloramphenicol (12.5 μg/ml). First-step primer sequences were 5'-CTTGGTGGACTATGCTTAACGCTCTCATTCTCATGGGAGCTTTTTGTATCGTATTAC GACATTGCTGTTTCCAGAACTCCTGTGACGGAAGATCACTTCG-3' and 5'-CGACCAGAATCAGCAGTGAGTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGT GCGGGGTCTCCTCCTCTGTCCTGAGGTTCTTATGGCTCTTG-3' for TR and 5'-CCTGGTGGACTATGCTTAACGCTCTCATTCTGATGGGAGCTTTTTGTATCGTATTACGAC ATTGCTGCTTCCAGAACTCCTGTGACGGAAGATCACTTCG-3' and 5'-CGACCAGAATCAGCAGTGAGTACACGCAGGCAAACCAAACCACAAGGCAGACGGACGGT GCGGGGTCTCCTCCTCTGTACTGAGGTTCTTATGGCTCTTG-3' for ME.

In the second step, the selectable marker cassette in the TR and ME first-step intermediate BACs was replaced with the UL74(gO) sequence from the heterologous strain. Briefly, E. coli cultures were prepared for recombination as described above for step 1 and electroporated with purified PCR products containing the UL74 ORF from the TR or ME strain flanked by sequence homologous to 80 bp upstream and downstream of the opposite strain. Transformed E. coli cells were selected for the removal of the KanR/LacZ/RpsL cassette by growth on medium containing streptomycin (1.5 mg/ml), IPTG (50 μM), X-gal (20 μg/ml), and chloramphenicol (12.5 μg/ml). Primers used to generate the second-step PCR product were 5'-GCCTGGTGGACTATGCTTAACGCTCTCATTCTGATGGGAGCTTTTTGTATCGTATTACGA

CATTGCTGCTTCCAGAACTTTACTGCAACCACCACCAAAG-3' and 5'-CGACCAGAATCAGCAGTGAGTACACGCAGGCAAACCAAACCACAAGGCAGACGGACGGTGCGGGGTCTCCTCCTCTGTAATGGGGAGAAAAGGAGAGATG-3'for the transformation of TR UL74 into ME and 5'-GGCTTGGTGGACTATGCTTAACGCTCTCATTCTCATGGGAGCTTTTTGTATCGTATTACGACATTGCTGTTTCCAGAACTTTACTGCGACCACCACCAAA-3' and 5'-CAGAATCAGCAGTGAGTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGTGCGGGGTCTCCTCCTCTGTCATGGGGAAAAAAGAGATGATAATGG for the transformation of ME UL74 into TR.

The final heterologous UL74(gO) recombinants were verified by Sanger sequencing of PCR products using the following primers: 5'-GATGATTTTTACAAGGCACATTGTACATC-3' and 5'-AACTAGGTCGTCTTGGAAGC-3' for TRΔMEgO and 5'-CTCACAATGATTTTTACAATGCG-3' and 5'-AACTAGGTCGTCTTGGAAGC-3' for MEΔTRgO.

Antibodies. Rabbit polyclonal antipeptide antibodies specific for TBgO and MEgO were described previously [64]. Rabbit polyclonal antibodies specific for UL148 were described previously [136]. Rabbit polyclonal anti-peptide antibodies against gH, gL, UL130, and UL131 were provided by David Johnson (Oregon Health and Sciences University, Portland, OR, USA) [147]. Anti-UL128 monoclonal antibody (MAb) 4B10 was provided by Tom Shenk (Princeton University, Princeton, NJ, USA) [148]. MAb 28-4 directed against major capsid protein (MCP) and MAb 27-156

directed against gB were provided by Bill Britt [149-150]. MAb CH160 against cytomegalovirus (CMV) immediate early protein 1 (IE1) and IE2 was purchased from Abcam (Cambridge, MA, USA).

Immunoblotting. HCMV-infected cells or cell-free virions were solubilized in 2% SDS–20 mM Tris-buffered saline (TBS) (pH 6.8). Insoluble material was cleared by centrifugation at 16,000 X g for 15min, and extracts were then boiled for 10 min. For endoglycosidase H (endo H) or peptide N-glycosidaseF (PNGase F) treatment assays, proteins were extracted in 1% Triton X-100 (TX100) plus 0.5% sodium deoxycholate (DOC) in 20 mM Tris (pH 6.8) plus 100 mM NaCl (TBS-TX-DOC). Extracts were clarified by centrifugation at 16,000 X g for 15 min and treated with endo H or PNGase F according to the manufacturer's instructions (New England BioLabs). For reducing blots, dithiothreitol (DTT) was added to extracts to a final concentration of 25 mM. After separation by SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Whatman) in a buffer containing 10 mM $NaHCO_3$ and 3mM Na_2CO_3 (pH 9.9) plus 10% methanol. Transferred proteins were probed with MAbs or rabbit polyclonal antibodies, anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich), and Pierce ECL-Western blotting substrate (Thermo Fisher Scientific). Chemiluminescence was detected using a Bio-Rad ChemiDoc MP imaging system.

Radiolabeling proteins. Cell cultures were incubated in labeling medium (Met-Cys-free DMEM plus 2% dialyzed FBS lacking methionine and cysteine) for 2 h at 37°C, and [^{35}S]methionine-cysteine was then added to 1 mCi/ml (EasyTag Express ^{35}S protein labeling mix; PerkinElmer). For chase experiments, label medium was removed, and cultures were washed twice in DMEM plus 2% FBS supplemented with a 10-fold excess of nonradioactive methionine and cysteine and then incubated in this medium for the indicated times.

Immunoprecipitation. Cell extracts were harvested in TBS-TX-DOC supplemented with 0.5% bovine serum albumin (BSA) and 1 mM phenylmethylsulfonyl fluoride (PMSF), clarified by centrifugation at 16,000 X g for 15 min, adjusted to 2% SDS–30 mM DTT, and heated at 75°C for 15 min. The extracts were then diluted 35-fold with TBS-TX-DOC supplemented with 0.5% BSA and 10 mM iodoacetamide, incubated on ice for 15 min, and precleared with protein A-agarose beads (Invitrogen/Thermo Fisher Scientific) for at 4°C for 2 h. Immunoprecipitation reactions were set up with specific antibodies and protein A-agarose beads, and the mixtures were incubated overnight at 4°C. Protein A-agarose beads were washed 3 times with TBS-TX-DOC, and proteins were eluted with 2% SDS and 30 mM DTT in TBS at room temperature (RT) for 15 min, followed by 75°C for 10 min. Eluted proteins were separated by SDS-PAGE and analyzed with a Typhoon FLA-9500 imager (GE Healthcare Life Sciences). Band densities were determined using ImageJ version 1.48 software.

Quantitative PCR. Viral genomes were determined as described previously [133]. Briefly, cell-free HCMV stocks were treated with DNase I before extraction of viral genomic DNA (PureLink viral RNA/DNA minikit; Life Technologies/Thermo Fisher Scientific). Primers specific for sequences within UL83 were used with the MyiQ real-time PCR detection system (Bio-Rad).

Superinfection of HCMV-infected cells with replication-defective adenovirus vectors. The construction of Ad vectors expressing MEgO or GFP was described previously [64]. Two days after HCMV infection, cells were superinfected with 20 PFU/cell of AdMEgO or AdGFP. Six days later, cell-free HCMV was collected from the supernatant culture by centrifugation, and cells were harvested for immunoblotting.

Chapter III. Polymorphisms in Human Cytomeglaovirus gO exert epistatic influences on cell-free and cell-to-cell spread, and antibody neutralization on gH epitopes.

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Introduction

Recent application of state-of-the-art genomics approaches have begun to uncover a greater and more complex genetic diversity of human cytomegalovirus (HCMV) than had been appreciated [54, 55, 61, 139, 151-154]. Of the 165 canonical open reading frames (ORFs) in the 235 kbp HCMV genome, 21 show particularly high nucleotide diversity and are distributed throughout the otherwise highly conserved genome. Links between specific genotypes and observed phenotypes are not well understood and as a corollary outcome, the factors driving HCMV genetic diversity and evolution remain speculative. This is further complicated by recombination between genotypes that can shuffle the diverse loci into various combinations, and this may result in epistasis where the phenotypic manifestation of a specific genotype of one locus may be influenced by the specific genotypes of other loci. Thus, realizing the full potential of modern genomics approaches towards the design of new interventions, clinical assessments and predictions will require better mechanistic understanding of the links between genotypes and phenotypes.

The UL74 ORF codes for glycoprotein (g) O and is one of the aforementioned highly diverse loci of HCMV [19, 64, 155,156]. Most phylogenetic groupings indicate 8 genotypes or alleles of gO that differ in 10-30% of amino acids, predominately near the N-terminus and in a short central region. These amino acid polymorphisms also affect predicted N-linked glycan sites. The evolutionary origins of gO genotype diversity are not understood. Studies that followed infected humans through latency-reactivation cycles over several years demonstrated remarkable stability in

UL74(gO) sequences, arguing against the idea of selective pressure from a dynamically adapting host immune system as a driving force for gO diversity [59, 155]. The functional significance of gO diversity has only recently been addressed and centers around its role as a subunit of the envelope glycoprotein complex gH/gL/gO, which is involved in the initiation of infection into different cell types.

The general model for herpesvirus entry involves fusion between the virion envelope and cell membranes mediated by the fusion protein gB and the regulatory protein gH/gL [120, 122, 157]. The HCMV gH/gL can be unbound, or bound by gO or the set of UL128-131 proteins [148, 158-160]. How these gH/gL complexes participate to mediate infection is complicated and seems to depend on both the cell type and whether the infection is by cell-free virus or direct cell-to-cell spread. Efficient infection of all cultured cell types by cell-free HCMV is dependent on gH/gL/gO, whereas infection of select cell types including epithelial and endothelial cells additionally requires gH/gL/UL128-131 [125, 126, 130, 131, 133, 161]. Experiments involving HCMV mutants lacking either gO or UL128-131 suggested that cell-to-cell spread in fibroblast cultures can be mediated by either gH/gL/gO or gH/gL/UL128-131, whereas in endothelial and epithelial cells gH/gL/UL128-131 is required, and it has remained unclear whether gH/gL/gO plays any role [126, 130, 132, 162]. While it is clear that gH/gL/gO can bind to the cell surface protein PDGFRα via gO, and that gH/gL/UL128-131 can bind NRP2 and OR14I1 via UL128-131, the specific function(s) of these receptor engagements is unclear, but may include virion attachment, regulation of gB fusion activity, or activation of signal

transduction pathways [31, 33, 163]. In the case of gH/gL/gO, binding to PDGFRα activates signaling pathways, but these are not required for entry [31, 162, 134]. Stegmann et al. showed that binding of a gO null HCMV to fibroblasts and endothelial cells was impaired, yet it is unclear whether this was due to lack of PDGFRα engagement. [32]. Finally, Wu et al. reported coimmunoprecipitation of gB with gH/gL/gO and PDGFRα, consistent with a role for the gH/gL/gO-PDGFRα interaction in promoting gB fusion activity [134]. However, unbound gH/gL has been shown to mediate cell-cell fusion and has also been found in stable complex with gB in extracts of infected cells and extracellular virions [160, 164]. Thus, although many of the key factors in HCMV entry and cell-to-cell spread have been identified, their interplay in the various entry pathways is unclear. Moreover, the influence of gO diversity remains a mystery.

The gH/gL complexes have been extensively studied as potential vaccine candidates and neutralizing antibodies have been described that react with epitopes on gH/gL, on UL128-131 and on gO [165–173]. Anti-UL128-131 antibodies neutralize with high potency, but only on cell types for which gH/gL/UL128-131 is required for entry; e.g., epithelial cells. In contrast, antibodies that react with epitopes on gH/gL tend to neutralize virus on both fibroblasts and epithelial cells, but are far less potent on fibroblasts, where only gH/gL/gO is needed for entry. One explanation for these observations is that gO, with its extensive N-linked glycan decorations presents more steric hindrance to antibodies accessing the underlying gH/gL epitopes than do the UL128-131 proteins. Similar effects of glycans in shielding

neutralizing epitopes have been described for HIV env, and for HCMV gN [174-175]. In support of this hypothesis for gO, Jiang et al. showed that focal spread of a gO null HCMV in fibroblasts was more sensitive to anti-gH antibodies [176]. Recently, Cui et al. described antibodies that reacted to a linear epitope on gH that exhibited strain-selective neutralization that could not be explained by polymorphisms within the gH epitope [177]. One possible explanation was that gO polymorphisms between the strains imposed differential steric hindrances on these antibodies.

In this study we utilized a set of HCMV BAC-clones that represent the range of phenotypic diversity in terms of gH/gL complexes. HCMV TB40/e (TB), TR and Merlin (ME) differ dramatically in the amounts of gH/gL complexes in the virion envelope and their infectivity on fibroblasts and epithelial cells. Extracellular virions of TB and TR contain gH/gL predominately in the form of gH/gL/gO and are far more infectious on both fibroblasts and epithelial cells than ME, which contains overall lower amounts of gH/gL, predominately as gH/gL/UL128-131 [64, 133]. Each of these strains encodes a different representative of the 8 gO genotypes. In a previous report, we demonstrated that variation in the UL74(gO) ORF was not responsible for the observed differences between TR and ME. [178]. Rather, it was shown that the amounts of gH/gL/gO in ME and TR virions were influenced by different steady-state levels of gO present during progeny assembly. Kalser et al. showed that replacing the gO of TB with that of Towne (TN) also did not affect the levels of gH/gL complexes but may have enhanced the ability of TB to spread in epithelial cell cultures [145]. Here, we have generated a set of heterologous gO

recombinants to include 6 of the 8 genotypes in the genetic backgrounds of the gH/gL/gO-rich strain TR and the gH/gL/UL128-131-rich ME to analyze how the differences in gO sequence influence HCMV biology. The results demonstrate that gO variation can have dramatic effects on cell-free entry, cell-to-cell spread and the neutralization by anti-gH antibodies. In some cases opposite influences were observed for a given gO genotype in the different backgrounds of TR and ME, indicating epistasis with other genetic differences between these strains.

Results

Influences of gO polymorphisms on cell-free infectivity and tropism can be dependent on the background strain. To examine the effects of gO polymorphism, a set of recombinant viruses was constructed in which the endogenous UL74(gO) ORFs of strain TR and ME were replaced with the UL74(gO) ORFs from 5 other strains. BAC-cloned strains TR and ME were chosen as the backgrounds for these studies since they represent gH/gL/gO-rich and gH/gL/UL128-131-rich strains respectively [64, 133, 145]. Additionally, ME is restricted to a cell-to-cell mode of spread in culture, whereas TR is capable of both cell-free and cell-to143 cell modes of spread [126, 137, 179]. The intended changes to UL74(gO) in each recombinant BAC were verified by sequencing the UL74 ORF and the flanking regions used for BAC recombineering. However, it was recently reported that HCMV BAC-clones can sustain various genetic deletions, and rearrangements, and mutations during rescue in fibroblasts or epithelial cells, resulting in mixed genotype populations [141]. To ensure that phenotypes characterized were the associated with the intended

changes to UL74(gO) and not to other genetic changes sustained during BAC rescue in fibroblasts, all analyses were performed on at least three independently BAC-rescued viral stocks.

As a basis for interpretation of the later biological comparisons among recombinants, the levels of gH/gL complexes incorporated into the virion envelope were analyzed by immunoblot as previously described [64, 133]. As in the previous reports, TR contained predominantly gH/gL/gO, whereas ME contained mostly gH/gL/UL128-131 (Fig 3.1, compare lane 1 in panels A and B). Propagation of ME under conditions of UL131 transcriptional repression (denoted "Merlin-T" (MT) as described [133, 137]), resulted in more gH/gL/gO and less gH/gL/UL128-131 (Fig 3.1C, lane 1). Some minor differences in the amounts of total gL, gH/gL/gO, and gH/gL/UL128-131 were observed for some of the heterologous gO recombinants relative to their parental strains. However, band density analyses showed that all apparent differences were less than 3-fold and few reached statistical significance when compared across multiple experiments, likely reflecting the limitations of immunoblot as a precise quantitative method, as well as stock-to-stock variability in glycoprotein composition (Table 3.1). Thus, consistent with our previous report, differences between strains TR and ME in the abundance of gH/gL complexes are predominately influenced by genetic background differences outside the UL74(gO) ORF [178].

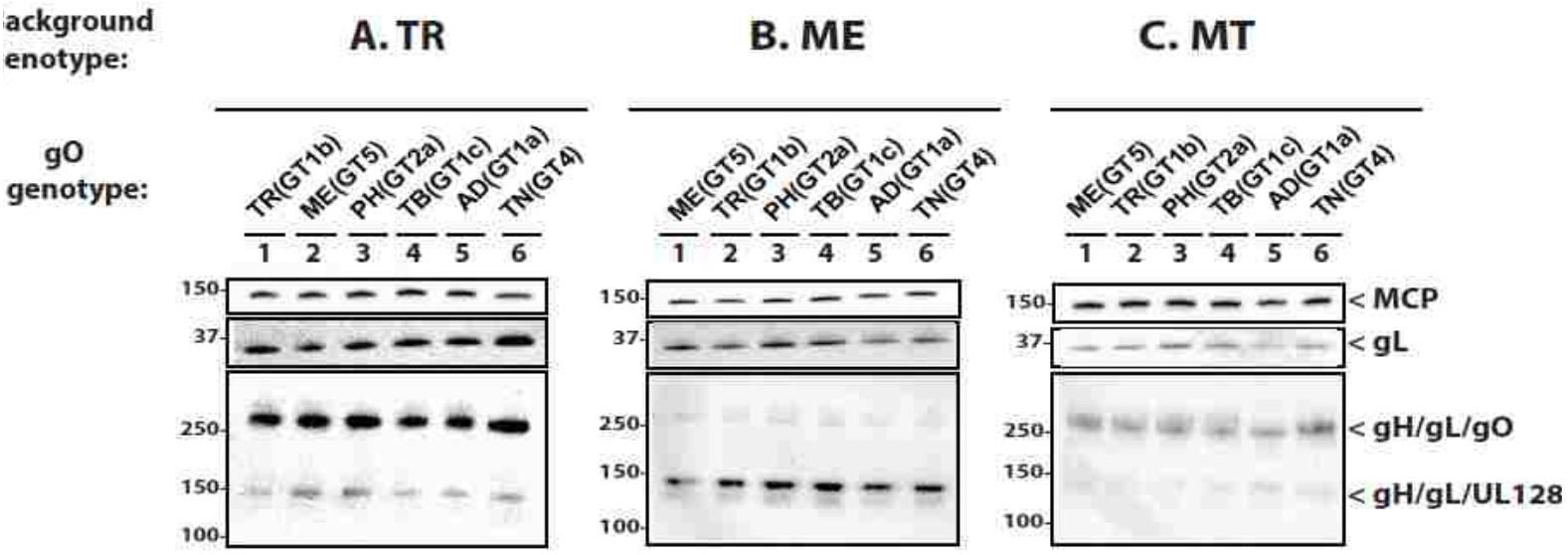

Fig 3.1 Immunoblot analysis of gH/gL complexes in parental and heterologous gO recombinant HCMV. Equal number of cell-free virions (as determined by qPCR) of HCMV TR (A), ME (B), or MT (C) or the corresponding heterologous gO recombinants were separated by reducing (upper two panels) or non-reducing (bottom panel) SDS-PAGE, and analyzed by immunoblot with antibodies specific for major capsid protein (MCP) or gL. Blots shown are representative of three independent experiments. Molecular mass markers(kDa) indicated on each panel.

Table 3.1 Immunoblot band density analyses of parental and heterologous gO recombinants.

Genotype Background	Virion Protein(s) Analyzed							
TR	MCP		gL		gH/gL/gO		gH/gL/UL128	
gO genotype	Fold[b]	ANOVA[c]	Fold	p-value	Fold	ANOVA	Fold	ANOVA
TR(GT1b)	-	-	-	-	-	-	-	-
MEgO(GT5)	1.1	ns	0.6	ns	1.4	ns	2.0	ns
PHgO(GT2a)	1.1	ns	0.9	ns	1.8	ns	2.3	*
TBgO (GT1c)	1.2	ns	0.8	ns	0.9	ns	0.9	ns
ADgO (GT1a)	1.1	ns	0.9	ns	0.9	ns	1.0	ns
TNgO (GT4)	1.1	ns	2.0	ns	2.7	ns	2.1	ns
ME	MCP		gL		gH/gL/gO		gH/gL/UL128	
gO genotype	Fold	ANOVA	Fold	ANOVA	Fold	ANOVA	Fold	ANOVA
MEgO(GT5)	-	-	-	-	-	-	-	-
TR(GT1b)	0.9	ns	0.8	ns	0.9	ns	1.1	ns
PHgO(GT2a)	1.1	ns	1.1	ns	1.4	ns	1.4	ns
TBgO (GT1c)	1.3	ns	1.2	ns	1.0	ns	1.4	ns
ADgO (GT1a)	1.0	ns	0.7	ns	0.9	ns	1.1	ns
TNgO (GT4)	1.1	ns	0.8	ns	0.9	ns	1.4	ns
MT	MCP		gL		gH/gL/gO		gH/gL/UL128	
gO genotype	Fold	ANOVA	Fold	ANOVA	Fold	ANOVA	Fold	ANOVA
MEgO(GT5)	-	-	-	-	-	-	-	-
TR(GT1b)	1.1	ns	1.2	ns	0.7	ns	0.9	ns
PHgO(GT2a)	1.1	ns	1.6	ns	1.4	ns	1.1	ns
TBgO (GT1c)	1.1	ns	1.3	ns	1.1	ns	1.6	ns
ADgO (GT1a)	0.8	ns	0.5	ns	0.6	ns	1.7	ns
TNgO (GT4)	0.9	ns	0.7	ns	1.4	ns	1.8	ns

a. Three independent stocks of cell-free virions collected from infected nHDF (for TR and ME) or HFFF-tet (for MT) culture supernatants and analyzed by immunoblot as described for Figure 1.
b. Mean fold difference of chemiluminescent band densities obtained for each recombinant compared to the parental TR in three independent experiments.
c. One-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental in three independent experiments. (*) $p \leq 0.05$, (ns) not significant.

While gH/gL/gO is clearly important for entry into both fibroblasts and epithelial cells, the mechanisms are likely different since 1) fibroblasts clearly express the gH/gL/gO receptor PDGFRα on their surface, whereas ARPE19 epithelial cells express little or none of this protein [31, 162, 134, 180], and 2) entry into epithelial cells requires gH/gL/UL128-131 in addition to gH/gL/gO [126, 161, 133]. Thus, it was possible that gO polymorphisms would differentially affect replication in these two cell types. To address this, fibroblast-to-epithelial tropism ratios were determined for each parental strain and gO recombinant by inoculating cultures of

fibroblasts and epithelial cells in parallel with equivalent amounts of cell-free virus stocks. The number of infected cells in each culture was then determined by flow cytometry using GFP expressed from the virus genome. Figure 3.2 shows the results of these experiments as the fold preference for either cell type as a ratio, where "1" indicates equal infection of both cell types. Stocks of the parental TR were approximately 20-fold more infectious on fibroblasts than on epithelial cells (Fig 3.2A). Preference towards fibroblasts was greater for TR-recombinants expressing MEgO(GT5), PHgO(GT2a), and TBgO(GT1c). In contrast, tropism ratios of TR recombinants expressing ADgO(GT1a) and TNgO(GT4) were closer to 1, indicating more equal infection of both cell types. Parental ME and all of the ME-based gO recombinants had tropism ratios within the range of 6 in favor of fibroblasts to 3 in favor of epithelial cells. Several of these viruses had variability between replicate stocks where some had slight fibroblasts preference and others slight epithelial preference (Fig 3.2B). Propagation of the ME-based viruses as MT greatly increased the preference towards fibroblasts infection for all recombinants to a range of 30-300 fold (Fig 3.2B). These results suggested that for the more gH/gL/gO-rich TR and MT, gO polymorphisms may differentially influence the infection of fibroblasts and epithelial cells, shifting the apparent relative tropism. However, such influences were less pronounced for ME, consistent with the low abundance of gH/gL/gO expressed by this virus.

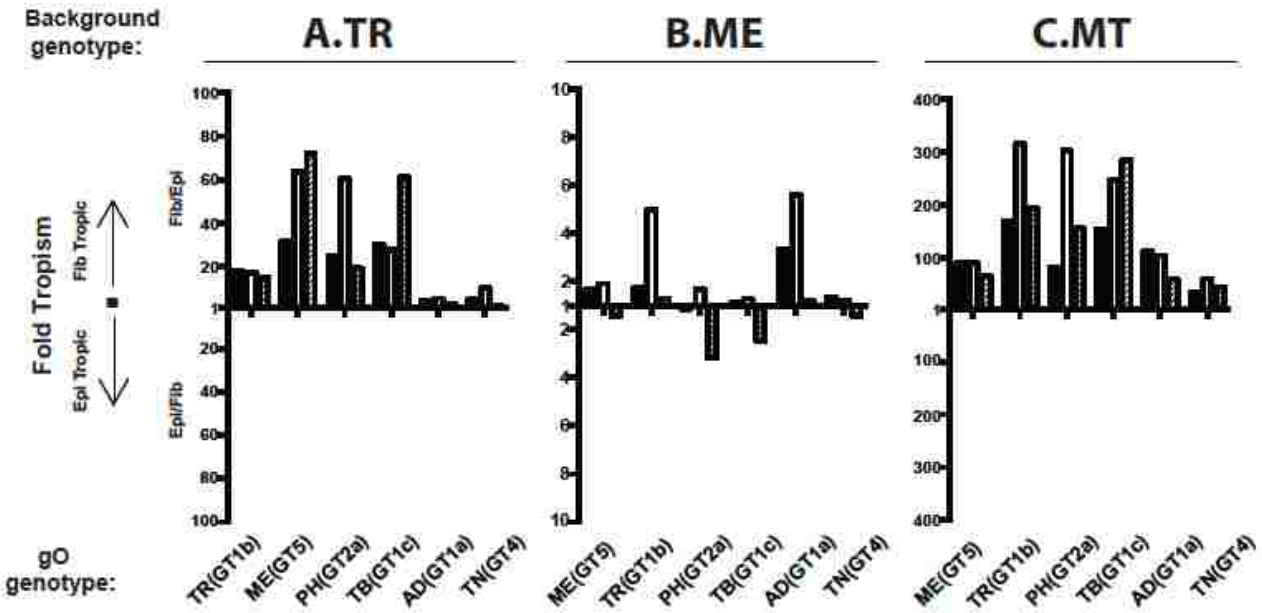

Fig 3.2 Relative fibroblast and epithelial cell tropism of parental and heterologous gO recombinant HCMV. Cell-free stocks of HCMV TR (A), ME (B), or MT (C) or the corresponding heterologous gO recombinants were serially diluted, and side-by-side cultures of nHDF fibroblasts and ARPE19 epithelial cells were inoculated with equal volumes of the dilutions. The number of infected cells was determined by flow cytometry for GFP at 2 days post infection. Ratios greater than or equal to 1 of the number of each cell type infected (fib/epi or epi/fib) are plotted for each of three independent sets of virus stocks (black, open and striped bars).

It was not clear if the observed differences in tropism ratios were due to enhanced infection of one cell type, reduced infection of the other cell type or a mixture of both. To address this, specific infectivity (ratio of the number of virions to the number of infectious units) was determined for each parental and recombinant on both fibroblasts and epithelial cells. Multiple independent supernatant stocks of each recombinant were analyzed by qPCR for encapsidated viral genomes and infectious titers on both cell types were determined by flow cytometry quantification of GFP-positive cells (Fig 3.3). For the TR-based viruses on fibroblasts, MEgO(GT5), TBgO(GT1c), and TNgO(GT4) each resulted in moderately enhanced infectivity (2 to 10-fold fewer genomes/IU) compared to the parental TR,

and PHgO(GT2a) enhanced infectivity 30-fold. In contrast, ADgO(GT1a) dropped TR infectivity below the detection limit of the flow cytometry-based assay (Fig 3.3A, top panel). In our previous report, expression of MEgO in the TR background did not appear to affect infectivity on fibroblasts [178]. This discrepancy was likely due to the more sensitive flow cytometry readout used in the current studies as compared to the plaque assay readout used previously. The infectivity of parental TR on epithelial cells was about 20-fold lower than on fibroblasts (i.e., 20-fold higher genomes/IU), but the relative effect of each heterologous gO was similar to that observed on fibroblasts (Fig 3.3A, bottom panel). Thus, some of the gO changes had dramatic effects on the infectivity of TR. Although these effects were manifest on both cell types, they were more pronounced on fibroblasts and this explains the observed differences in fibroblast preferences reported in Figure 3.2A.

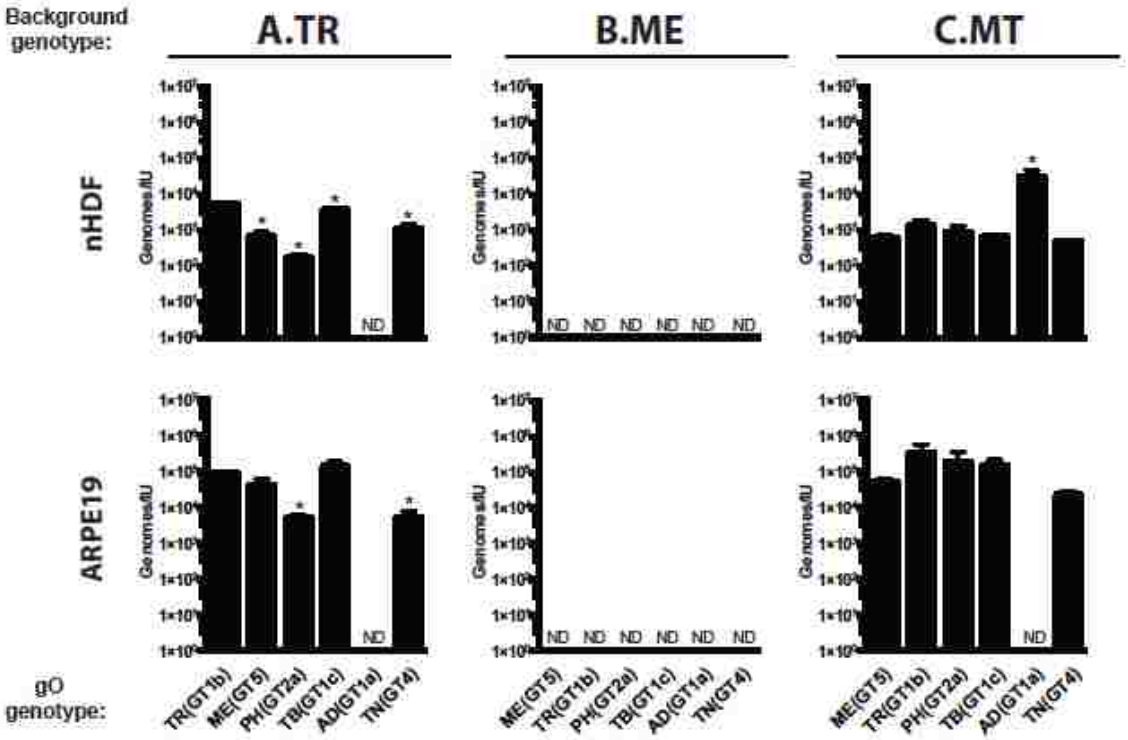

Fig 3.3 Specific infectivity of parental and heterologous gO recombinant HCMV. Extracellular HCMV stocks of HCMV TR (A), ME (B), or MT (C) or the corresponding heterologous gO recombinants were quantified by qPCR for viral genomes, and infectious units (IU) were determined by flow cytometry quantification of GFP-expressing nHDF fibroblasts or ARPE-19 epithelial cells, 2 days post infection. Average genomes/IU of 3 independent set of virus stock are plotted, with error bars representing standard deviations. Undetectable levels of infectivity indicated by ND (not determined). Asterisks (*) denote p-values ≤ 0.05; one way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental in three independent experiments.

The infectivity of cell-free ME virions on both cell types was below the detection limit of the flow cytometry-based assay and none of the changes to gO rescued infectivity (Fig 3.3B). These results indicated that the cell-free virions of all of the ME-based viruses were virtually non-infectious. When propagated as MT, infectivity on both cell types was improved to levels comparable to TR and this was consistent with our previous results (Fig 3.2C) [133, 178]. The only significant effect of gO changes on MT was ADgO(GT1a), which reduced infectivity on both cell types,. Thus,

as in the TR background, some changes to gO influenced infectivity of MT and this was disproportionally manifest on fibroblasts compared to epithelial cells, but the overall preference of all of the MT-based viruses was strongly in favor of fibroblasts.

It has been reported that gO-null HCMV are impaired for attachment to cells and that soluble gH/gL/gO can block HCMV attachment [32, 181]. Thus, it was possible that the observed changes to cell-free infectivity due to gO polymorphisms were related to a role for gO in attachment. To test this hypothesis, each heterologous gO recombinant was compared to the corresponding parental strain by applying cell-free virus stocks to fibroblast or epithelial cell cultures for approximately 20 min, washing away the unbound virus and then counting the numbers of cell-associated virions by immunofluorescence staining of the capsid-associated tegument protein pp150 [32] (Fig 3.4 and Tables 3.2 and 3.3). Given the short incubation time, high concentrations of input viruses were used to, and these inputs were equal for each set of parental and heterologous gO recombinants within the constraints of the stock concentrations. Higher inputs were required for ME to obtain detectable numbers of bound virus, consistent with the low amounts of gH/gL/gO in these virions. The average number of cell-associated virions per cell varied considerable between experiments, likely reflecting the complex parameters expected to influence virus attachment including stock concentration, cell state and variability in the incubation time between experiments. In some cases, a given recombinant was significantly different from parental in only one or two of the three experiments. It was concluded that these specific gO isoforms did not affect binding or attachment

of HCMV to cells. However, binding of TR_TNgO(GT4) and MT_ADgO(GT1a) were each significantly lower than their respective parental viruses in all three experiments on both fibroblasts and epithelial cells. While it was possible that the reduced binding of MT_ADgO(GT1a) was due in part to the slightly lower amounts of gH/gL/gO (Fig 3.1C and Table 3.1), the reduced binding of TR_TNgO(GT4) could not be similarly explained since this virus had slightly more gH/gL/gO than the parental TR (Fig 3.1A, Table 3.1). Moreover, reduced binding may help explain the lower infectivity of MT_ADgO(GT1a)(Fig 3.3C), but the poor infectivity of TR_ADgO(GT1a) could not be explained by poor binding, and the reduced binding of TR_TNgO(GT4) did not result in reduction of infectivity (Fig 3.3A).

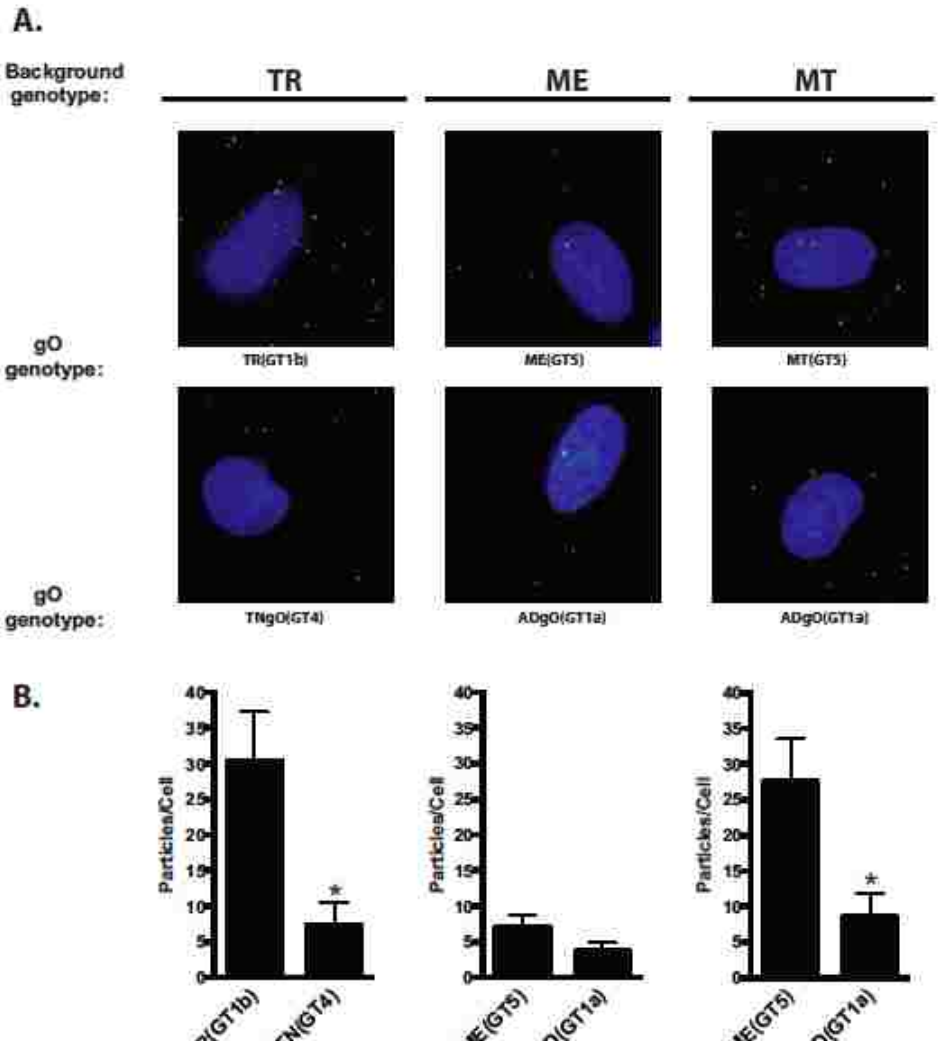

Fig 3.4 Binding of parental and heterologous gO recombinant HCMV to fibroblasts. Extracellular HCMV TR, ME, MT or the corresponding heterologous gO recombinants were applied to nHDF for 20 min. Multiplicities (genomes/cell) were: TR-background viruses (1 x 10^4), ME-background viruses (5 x 10^4), MT- background viruses (1 x 10^4). After washing away unbound virus, cultures were fixed and permeabilized with acetone and cell-associated virus particles were detected by immunofluorescence using antibodies specific for the capsid-associated tegument protein, pp150. Cells were visualized by staining nuclei with DAPI. (A)Representative fields of parental TR, ME, MT and heterologous gO recombinants that consistently reduced binding in 3 independent experiments (Table 3.2). (B) Mean particles per cell for representative experiments. Error bars represent the standard deviation. Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

Table 3.2 Binding of parental and heterologous gO recombinant HCMV to fibroblasts.

Genotype Background	Experiment 1 (input[a])			Experiment 2 (input)			Experiment 3 (input)		
TR	(6.2×10^7)			(7.5×10^7)			(1.0×10^8)		
gO genotype	Mean[b]	Fold[c]	ANOVA[d]	Mean	Fold	ANOVA	Mean	Fold	ANOVA
TR(GT1b)	17.8	-	-	31.2	-	-	30.4	-	-
MEgO(GT5)	21.2	-	ns	44.7	1.4	*	37.9	-	ns
PHgO(GT2a)	24.3	-	ns	12.7	0.41	*	35.3	-	ns
TBgO (GT1c)	18.8	-	ns	30.5	-	ns	33.7	-	ns
ADgO (GT1a)	25.7	-	ns	24.7	-	ns	23.3	-	ns
TNgO (GT4)[e]	**4.9**	**0.27**	*	**6.9**	**0.22**	*	**7.3**	**0.24**	*
ME	(2.0×10^6)			(5.0×10^6)			(5.0×10^8)		
gO genotype	Mean	Fold	ANOVA	Mean	Fold	ANOVA	Mean	Fold	ANOVA
MEgO(GT5)	21.6	-	-	5.8	-	-	7	-	-
TR(GT1b)	5.3	0.25	*	7.1	-	ns	3.9	0.56	*
PHgO(GT2a)	8.0	0.37	*	7.5	-	ns	2.3	0.33	*
TBgO (GT1c)	15.9	0.74	*	9.0	-	ns	7	-	ns
ADgO (GT1a)	2.4	0.11	*	2.4	-	ns	3.7	0.53	*
TNgO (GT4)	5.8	0.27	*	6.5	-	ns	7.4	-	ns
MT	(1.0×10^6)			(2.0×10^6)			(5.0×10^6)		
gO genotype	Mean	Fold	ANOVA	Mean	Fold	ANOVA	Mean	Fold	ANOVA
MEgO(GT5)	27.5	-	-	63.9	-	-	120.9	-	-
TR(GT1b)	28.5	-	ns	40.2	0.63	*	159.4	-	ns
PHgO(GT2a)	33.4	-	ns	50.4	-	ns	222	1.84	*
TBgO (GT1c)	44.6	1.6	*	66.2	-	ns	220.8	1.83	*
ADgO (GT1a)	**8.5**	**0.31**	*	**13.4**	**0.21**	*	**23.6**	**0.2**	*
TNgO (GT4)	32.5	-	ns	61.8	-	ns	133.2	-	ns

a. Concentration of cell-free virus stock (genomes/mL) applied to cells.

b. Average pp150 puncta detected by immunofluorescence per cell in 10 microscopy fields; approximately 4 to 6 cells per field.

c. Fold difference in mean pp150 puncta per cell as compared to parental virus. Determined for recombinant viruses that were significantly different ($p \leq 0.05$) from parental within an experiment. (-) indicates value not calculated.

d. One-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental. (*);$p \leq 0.05$, , (ns); not significant.

e. Bold font indicates recombinant viruses that were significantly different from the parental in the same direction (> or <) in all 3 experiments.

Table 3.3 Binding of parental and heterologous gO recombinant HCMV to epithelial cells.

Genotype Background	Experiment 1 (input[a])			Experiment 2 (input)			Experiment 3 (input)		
TR	(6.2×10^7)			(7.5×10^7)			(1.0×10^8)		
gO genotype	Mean[b]	Fold[c]	ANOVA[d]	Mean	Fold	ANOVA	Mean	Fold	ANOVA
TR(GT1b)	26.2	-	-	41.7	-	-	43.7	-	-
MEgO(GT5)	35.5	1.35	*	38.3	-	ns	56.8	-	ns
PHgO(GT2a)	33.4	-	ns	19.3	0.46	*	61	1.4	*
TBgO (GT1c)	24.1	-	ns	35.4	-	ns	58.7	1.34	*
ADgO (GT1a)	36.4	1.39	*	22.2	0.53	*	36	-	ns
TNgO (GT4)[e]	**16.2**	**0.62**	*	**18.62**	**0.45**	*	**23.4**	**0.54**	*
ME	(2.0×10^8)			(5.0×10^8)			(5.0×10^8)		
gO genotype	Mean	Fold	ANOVA	Mean	Fold	ANOVA	Mean	Fold	ANOVA
MEgO(GT5)	37.3	-	-	18	-	-	15	-	-
TR(GT1b)	17.7	0.47	*	24.9	-	ns	10.4	0.69	*
PHgO(GT2a)	22.3	0.6	*	23	-	ns	9.4	0.62	*
TBgO (GT1c)	34.1	-	ns	32.3	1.79	*	18.6	-	ns
ADgO (GT1a)	14.4	0.39	*	11.4	-	ns	10.8	0.72	*
TNgO (GT4)	24.4	0.65	*	25.9	1.44	*	14.3	-	ns
MT	(1.0×10^8)			(2.0×10^8)			(5.0×10^8)		
gO genotype	Mean	Fold	ANOVA	Mean	Fold	ANOVA	Mean	Fold	ANOVA
MEgO(GT5)	33.2	-	-	68	-	-	236.8	-	-
TR(GT1b)	35.3	-	ns	46.1	0.68	*	210.1	-	ns
PHgO(GT2a)	46.5	-	ns	78	-	ns	383.2	1.62	*
TBgO (GT1c)	63.4	1.91	*	69.6	-	ns	238.3	-	ns
ADgO (GT1a)	**16.7**	**0.5**	*	**26.1**	**0.38**	*	**26.6**	**0.11**	*
TNgO (GT4)	44.1	-	ns	48.1	0.71	*	150.9	0.64	*

a. Concentration of cell-free virus stock (genomes/mL) applied to cells.
b. Average pp150 puncta detected by immunofluorescence per cell in 10 microscopy fields; approximately 4 to 6 cells per field.
c. Fold difference in mean pp150 puncta per cell as compared to parental virus. Determined for recombinant viruses that were significantly different ($p \leq 0.05$) from parental within an experiment. (-) indicates value not calculated.
d. One-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental. (*) $p \leq 0.05$, (ns) not significant
e. Bold font indicates recombinant viruses that were significantly different from the parental in the same direction (> or <) in all 3 experiments.

In sum, these analyses indicated that; 1) gO polymorphisms can influence the cell-free infectivity of HCMV. In some cases this was independent of any effects on abundance of gH/gL/gO in the virion envelope or binding to cells (e.g. parental TR and TR recombinants harboring MEgO(GT5), TBgO(GT1c), and ADgO(GT1a), had dramatically different infectivity but comparable levels of gH/gL/gO and cell binding). 2) The influence of some gO isoforms was dependent on the background strain (e.g., PHgO(GT2a) enhanced TR infectivity but did not affect ME or MT and TNgO(GT4) reduced binding of TR but had no effect on binding of ME or MT). 3) While some heterologous gO recombinants had quantitatively different effects on infectivity on fibroblast compared to epithelial cells, these did not change the fundamental fibroblast preferences for either TR or MT. 4) Some of the heterologous gOs did appear to change relative tropism of ME. However, the relevance of tropism ratios for these viruses is questionable since the specific infectivity (genomes/IU) analyses suggested that all ME-based recombinants were noninfectious on either cell type. This was consistent with the highly cell-associated nature of ME [137, 179].

Polymorphisms in gO can differentially influence the mechanisms of cell-free and cell-to-cell spread. The analyses described above focused on the cell-free infectivity of HCMV, as indicative of a cell-free mode of spread. Cell-to-cell spread mechanisms are likely important for HCMV, and while gH/gL complexes are clearly important for cell-to-cell spread, the mechanisms in these processes are poorly

characterized in comparison to cell-free infection. Strains TR and ME are well-suited to compare the effects of gO polymorphisms on cell-free and cell-to-cell spread since ME is mostly restricted to cell-to-cell due to the poor infectivity of cell-free virions but can be allowed to also spread cell-free by propagation as MT, whereas TR can spread by both cell-free and cell-to-cell mechanisms [126, 133, 137, 179].

To compare spread among heterologous gO recombinants, replicate cultures were infected at low multiplicity, and at 12 dpi, foci morphology was documented by fluorescence microscopy and the increased number of infected cells was determined by flow cytometry. In fibroblasts cultures, parental TR and MT showed more diffuse foci compared to the tight, localized focal pattern of parental ME, consistent with the notion that TR and MT spread by both cell-free and cell-to-cell mechanisms whereas ME was restricted to cell-to-cell spread (Fig 3.5A). Quantitatively, spread by parental TR increased the numbers of infected cells 55-fold over 12 days, whereas spread of TR_MEgO(GT5) and TR_PHgO(GT2a) were significantly reduced (Fig 3.5B). Spread of ME was slightly reduced by ADgO(GT1a), but was increased by TNgO(GT4) (Fig 3.5C). Surprisingly, different effects on spread were observed for MT where TBgO(GT1c) and TNgO(GT4) reduced spread, and ADgO(GT1a) increased spread.

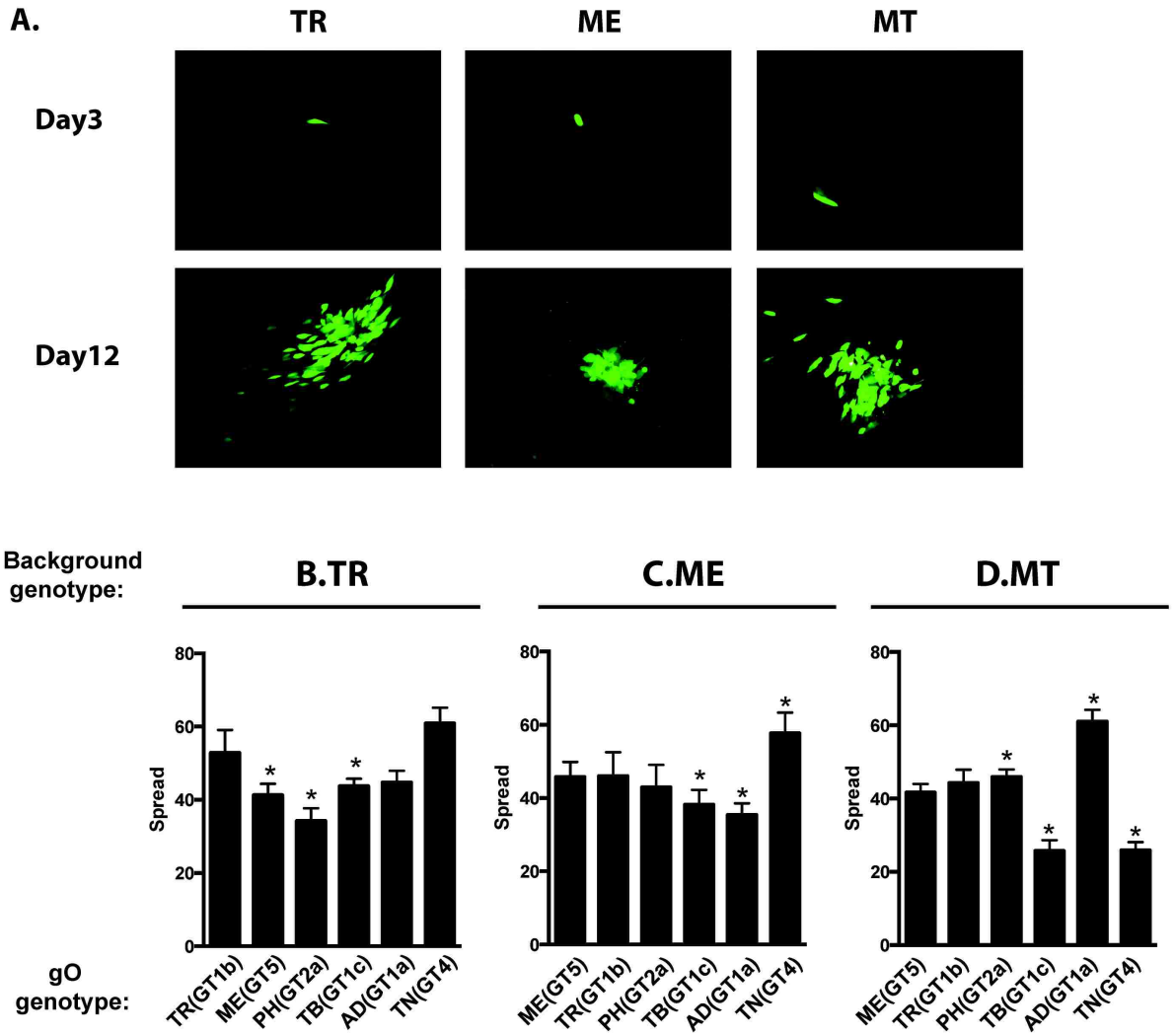

Fig 3.5 Spread of parental and heterologous gO recombinant HCMV in fibroblast cultures. Confluent monolayers of nHDF or HFFFTet (for "MT") were infected with 0.003/cell of HCMV TR (A, B), ME (A, C), MT (A, D) or the corresponding heterologous gO recombinants. At 3 and 12 days post infection cultures were analyzed by fluorescence microscopy (A) or by flow cytometry to quantitate the total number of infected (GFP+) cells (B-D). Plotted are the average number of infected cells at day 12 per infected cell at day 3 in 3 independent experiments. Error bars represent standard deviations. Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

A number of interesting incongruities were observed when comparing the cell-free infectivity of some gO recombinants on fibroblasts to their respective spread characteristics in fibroblasts; 1) Spread of TR_PHgO in fibroblasts was reduced compared to the parental TR (Fig 3.5B), but the cell-free infectivity of this recombinant was actually better (Fig 3.3A). Similarly, spread of both MT_TBgO(GT1c) and MT_TNgO(GT4) were reduced in fibroblasts (Fig 3.5D), but cell-free infectivity of both viruses was comparable to parental MT. 2) Conversely, MT_ADgO(GT1a) spread better in fibroblasts (Fig 3.5D), but the cell-free infectivity was substantially worse (Fig 3.3C). Since the efficiency of cell-free spread should depend on both the specific infectivity and the quantities of progeny virus released to the culture supernatants, it was possible that some of these incongruities reflected offsetting differences in the quantity of cell-free virus released as compared to their infectivity. To test this, progeny released from infected fibroblasts into culture supernatants were quantified by qPCR. There were no significant differences in the quantity of progeny released per cell for any of the TR or ME-based recombinants (Fig 3.6A, and B). Likewise, all of MT-based recombinants released similar numbers of cell-free progeny exceptcfor MT_ADgO(GT1a), which was reduced by approximately 4-fold (Fig 3.6C). Thus, the discrepancies between efficiency of spread and cell-free infectivity could not be explained by offsetting differences in the release of cell-free progeny. Rather, these results suggested that gO polymorphisms can differentially influence the mechanisms of cell-free and cell-to-cell spread in fibroblasts. The interpretation that gH/gL/gO can provide a specific function for cell-to-cell spread was supported by the results that expression of

ADgO(GT1a) and TNgO(GT4), respectively reduced and increased spread of the strain ME, for which spread is almost exclusively cell-to-cell (Fig 3.5C).

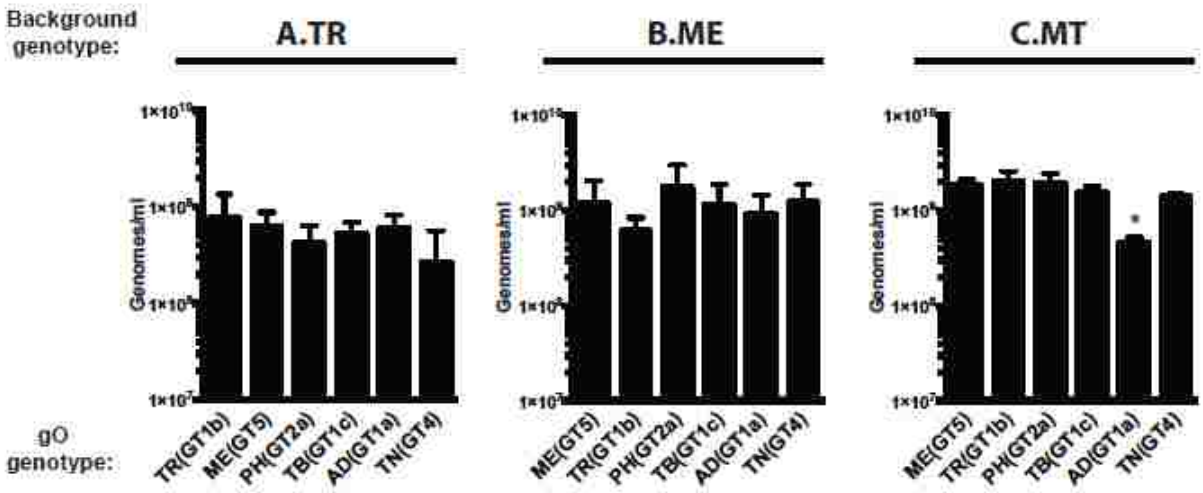

Fig 3.6 Release of extracellular progeny by parental and heterologous gO recombinant HCMV in fibroblast cultures. Cultures of nHDF or HFFFTet (for "MT") were infected with 1 IU/cell of HCMV TR (A), ME (B), MT (C) or the corresponding heterologous gO recombinants for 8 days. The number of infected cells was determined by flow cytometry and progeny virus in culture supernatants was quantified by qPCR for viral genomes. The average number of extracellular virions per mL in each of 3 independent experiments is plotted. Error bars represent standard deviations. Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

Spread was also analyzed in epithelial cell cultures. Here, foci of both TR and ME remained tightly localized, suggesting predominantly cell-to-cell modes of spread for both strains in this cell type (Fig 3.7A). The number of TR-infected cells increased by only 5-6 fold over 12 days compared to approximately 25-fold for ME (Fig 3.7B and C). The low efficiency of spread for TR in epithelial cells compared to ME was documented previously and may relate to the low expression of gH/gL/UL128-131 by TR compared to ME [126, 133, 142]. Expression of TNgO(GT4) further reduced TR spread in epithelial cells (Fig 3.7B). In contrast, ME spread was slightly reduced by TBgO(GT1c) and ADgO(GT1a), but nearly doubled by

TNgO(GT4). The observed increase in ME spread due to TNgO(GT4) was not attributed to increased release of progeny to the culture supernatants in epithelial cells (Fig 3.8). Note that spread of MT could not be addressed in epithelial cells, since gH/gL/UL128-131 is clearly required for spread in these cells and its repression would complicate analysis of the contribution of gO polymorphisms [126]. Nevertheless, it is clear from these experiments that gO polymorphisms can affect spread in epithelial cells and that this can depend on the background strain. Specifically, TNgO(GT4) reduced TR spread but increased ME spread. This suggested that although gH/gL/UL128-131 is required for efficient cell-to-cell spread in epithelial cells, and may even be sufficient in the case of gO-null HCMV [130, 132], gH/gL/gO may also contribute to the mechanism when present.

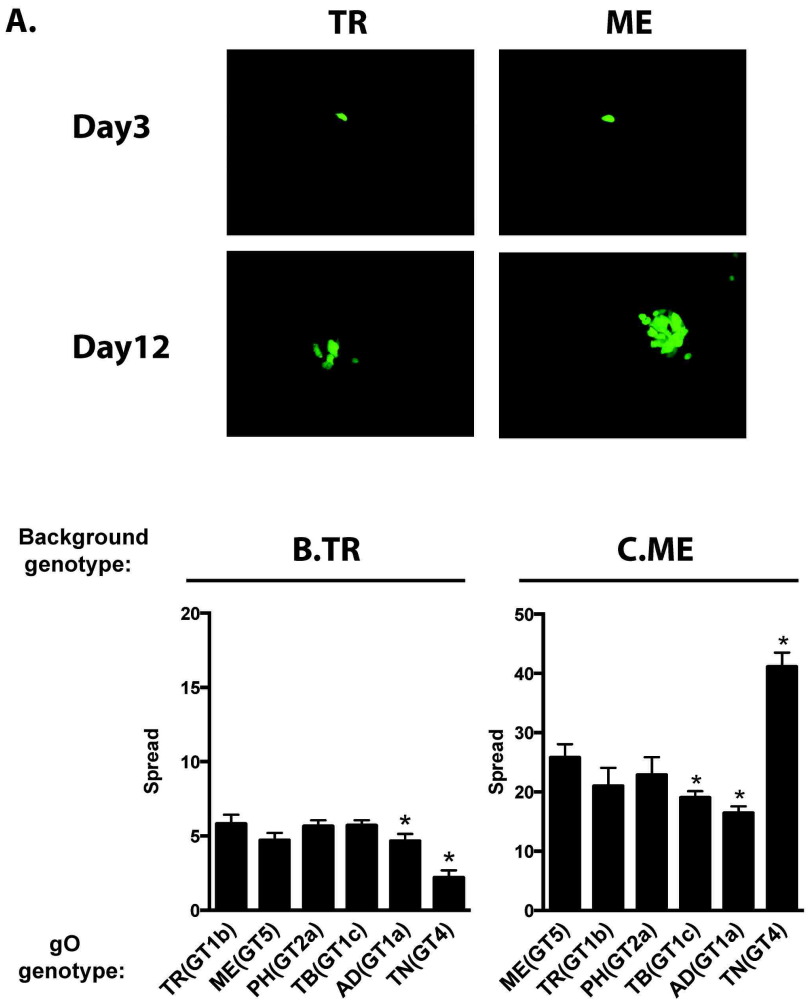

Fig 3.7 Spread of parental and heterologous gO recombinant HCMV in epithelial cell cultures. Confluent monolayers of ARPE19 cells were infected with 0.003 IU/cell of HCMV TR (A, B), ME (A, C), or the corresponding heterologous gO recombinants. At 3 and 12 days post infection cultures were analyzed by fluorescence microscopy (A) or by flow cytometry to quantitate the total number of infected (GFP+) cells (B-D). Plotted are the average number of infected cells at day 12 per 829 infected cell at day 3 in 3 independent experiments. Error bars represent standard deviations Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

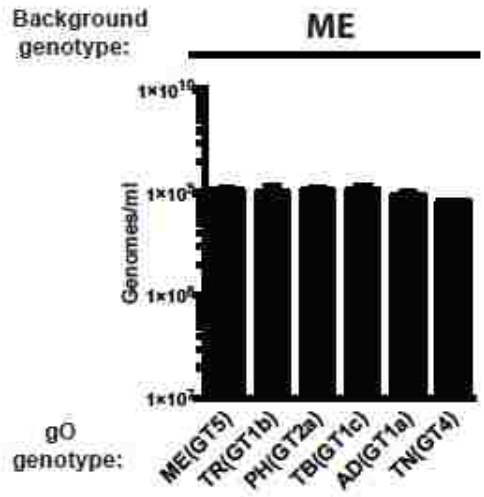

Fig 3.8 Release of extracellular progeny by parental and heterologous gO recombinant HCMV ME in epithelial cell cultures. Cultures of ARPE19 epithelial cells were infected with HFFF-tet-derived MT or corresponding heterologous gO recombinants at the highest multiplicities possible given the specific infectivity of stocks reported in Fig 3.3 (approximately 0.0005 IU/cell). (Note: since APRE19 cells do not express TetR, after the initial infection, MT replicates as ME). Cultures were then propagated by trypsinization and reseeding of intact cells until the number of infected cells approached 90-100% by microscopy inspection for GFP+ cells. After 8 more days, culture supernatants were then analyzed by quantified by qPCR for viral genomes. The average number of extracellular virions per mL in each of 3 independent experiments is plotted. Error bars represent standard deviations. Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

Polymorphisms in gO can affect antibody neutralization on gH epitopes.

The extensive N-linked glycosylation of gO raised the possibility that gO could present steric hindrance to the binding of antibodies to epitopes on gH/gL, as was shown for HCMV gN and also HIV env [174, 175]. A corollary hypothesis was that such effects might vary with the polymorphisms among gO isoforms. To address this, neutralization experiments were conducted using two monoclonal anti-gH antibodies; 14-4b, which recognizes a discontinuous epitope likely located near the membrane proximal ectodomain of gH [165, 166] and AP86, which binds to a continuous epitope near the N-terminus of gH [182]. Note that these experiments

could only be performed with TR- and MT-based recombinants since the cell-free progeny of ME-based viruses were found to be only marginally infectious (Fig 3.3B).

Parental TR and recombinants encoding MEgO(GT5), PHgO(GT2a) and TBgO(GT1c) were completely neutralized on fibroblasts by mAb 14-4b, whereas TR_ADgO(GT1a) and TR_TNgO(GT4) were significantly resistant (Fig 3.9A). There was more variability among TR-based recombinants with mAb AP86 (Fig 3.9B). Here, parental TR could only be neutralized to approximately 40% residual infection. TNgO(GT4) rendered TR totally resistant to mAb AP86, and MEgO(GT5) also significantly protected TR. In contrast, TR_TBgO(GT1c) and TR_ADgO(GT1a) were more sensitive to mAb AP86. On epithelial cells neutralization by both antibodies was more potent and complete than on fibroblasts, and there was less variability among gO recombinants (Fig 3.9C, and D). This was consistent with the interpretation that both 14-4b and AP86 could bind their epitopes on gH/gL/UL128-131 and that this represented the majority of the observed neutralization on epithelial cells. However, TR_TNgO(GT4) still displayed some reduced sensitivity to both antibodies, suggesting that gH/gL/gO epitopes also contributed to neutralization on epithelial cells.

MT-based recombinants were generally more sensitive to neutralization by 14-4b than were TR-based viruses (compare 14-4b concentrations in Fig 3.9A and 3.10A). Strikingly, whereas TNgO(GT4) conferred 14-4b resistance to TR, it did not in MT, and instead ADgO(GT1a) provided resistance to 14-4b (Fig 3.10A). As was observed

for TR-based recombinants, 14-4b neutralization on epithelial cells was less affected by gO polymorphisms (Fig 3.10B). Note that neutralization of MT-based recombinants by AP86 could not be tested since MEgH harbors a polymorphism in the linear AP86 epitope that precludes reactivity [182]. Together, these results indicated that differences among gO genotypes can differentially affect antibody neutralization on gH epitopes. Moreover, which gO genotype could protect against which antibody depended on the background strain, suggesting the combined effects of gO polymorphisms and gH/gL polymorphisms.

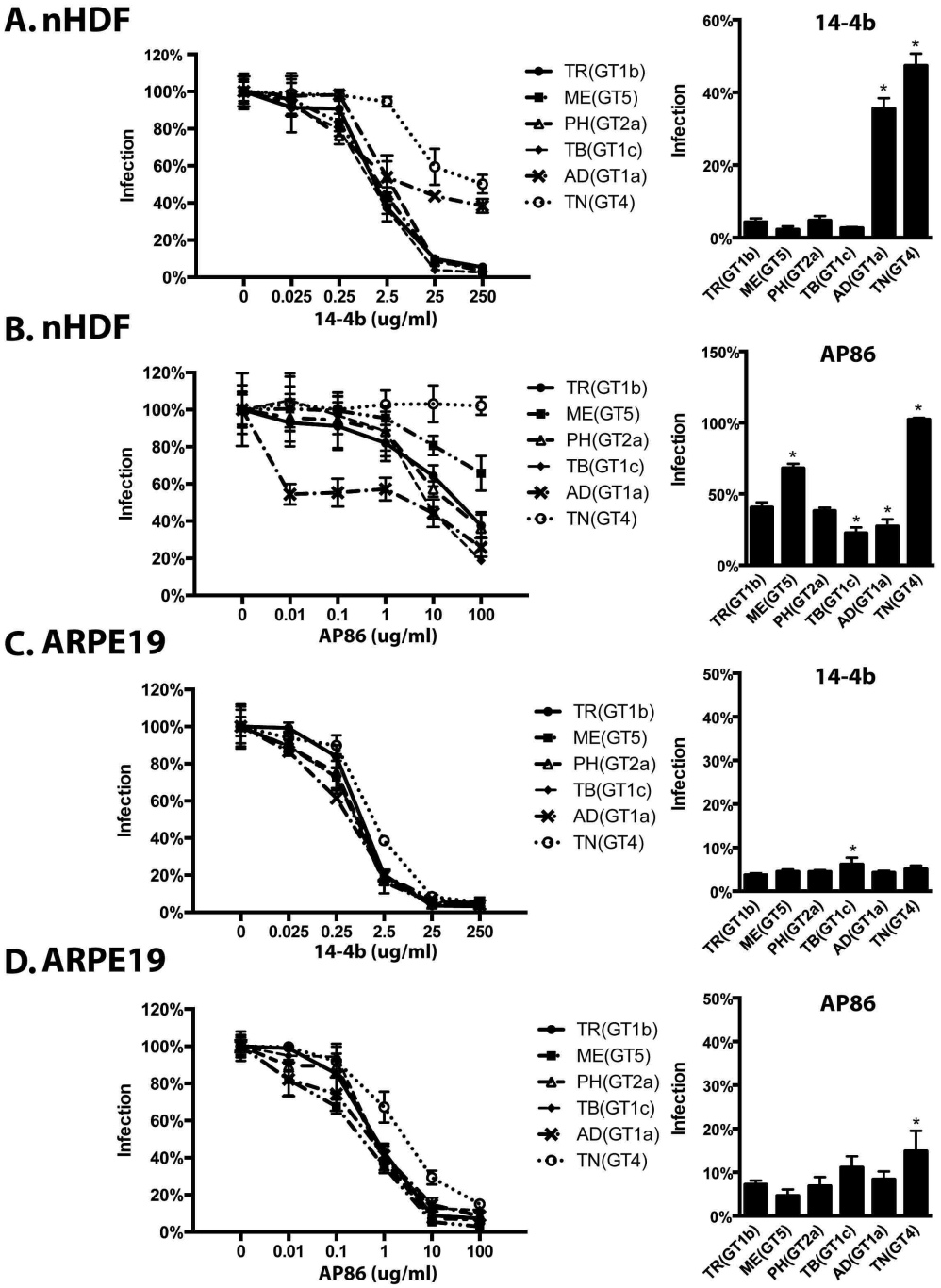

Fig 3.9 Neutralization of parental HCMV TR and heterologous gO recombinant by anti-gH antibodies. Genome equivalents of extracellular HCMV TR or the corresponding heterologous gO recombinants were incubated with 0.025-250 mg/mL of anti-gH mAb 14-4b, or 0.01-100 mg/mL of anti-gH mAb AP86 and then plated on cultures of nHDF fibroblasts (A and B) or ARPE19 epithelial cells (C and D). At 2 days post infection the number of infected (GFP+) cells was determined by flow cytometry and plotted as the percent of the no antibody control. (Left panels) Full titration curves shown are representative of three independent experiments, each performed in triplicate. (Right panels) Average percent of cells infected at the highest antibody concentrations in 3 independent experiments. Error bars represent standard deviations. Asterisks (*) denote p values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

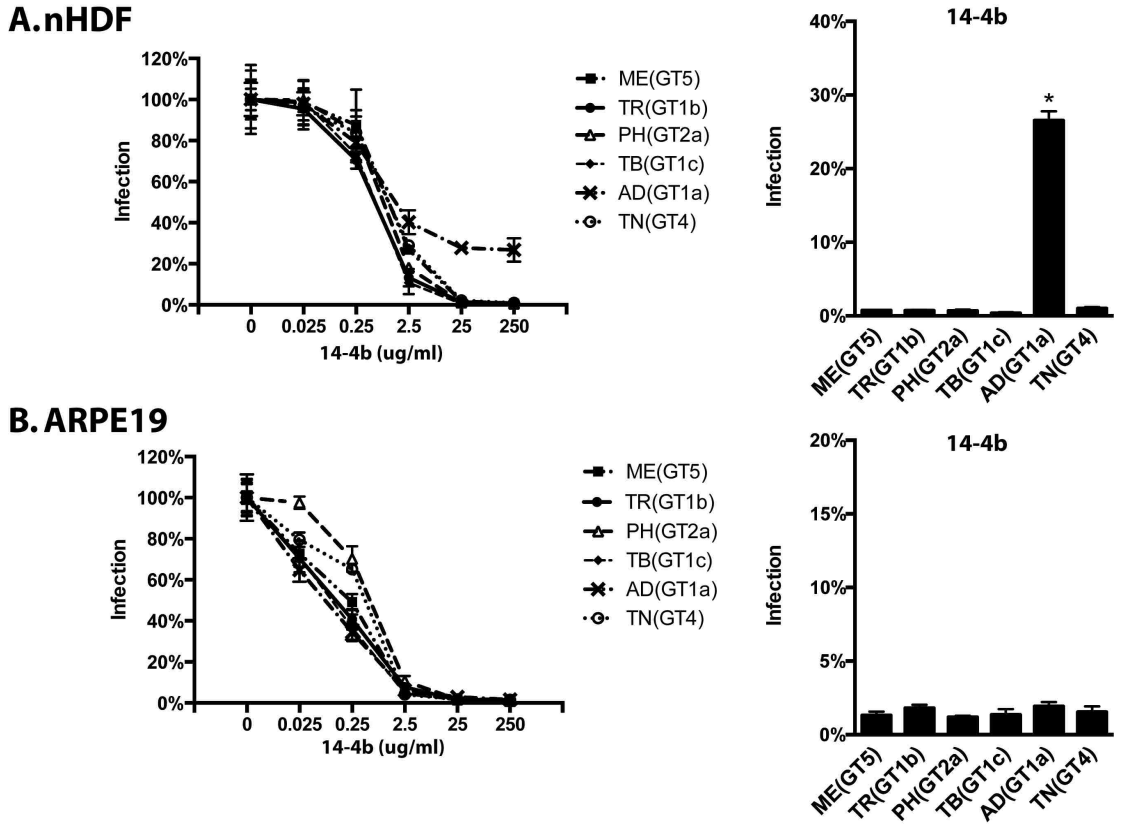

Fig 3.10 Neutralization of parental HCMV MT and heterologous gO recombinant by anti-gH antibodies. Genome equivalents of extracellular extracellular HCMV MT or the corresponding heterologous gO recombinants were incubated with 0.025-250 mg/mL of anti-gH mAb 14-4b and then plated on cultures of nHDF fibroblasts (A) or ARPE19 epithelial cells (B). At 2 days post infection the number of infected (GFP+) cells was determined by flow cytometry and plotted as the percent of the no antibody control. (Left panels) Full titration curves shown are representative of three independent experiments, each performed in triplicate. (Right panels) Average percent of cells infected at the highest antibody concentrations in 3 independent experiments. Error bars represent standard deviations. Asterisks (*) denote p-values ≤ 0.05; one-way ANOVA with Dunnett's multiple comparisons test comparing each recombinant to the parental.

Discussion

Efficient cell-free infection of most, if not all cell types requires gH/gL/gO [130, 131, 133]. However, the details of the mechanisms, and the distinctions between the roles of gH/gL/gO in cell-free and cell-to-cell spread remain to be clarified. While there are naturally occurring amino acid polymorphisms in each subunit of

gH/gL/gO, gO has the most dramatic variation, with 8 known genotypes (or alleles) that differ between 10-30% of amino acids [64, 135, 155, 156]. All isoforms of gO are predicted to have extensive N-linked glycan modifications and some of the amino acid differences alter the predicted sites. In a previous report, we sought to determine if gO polymorphisms were a factor influencing the different levels of gH/gL/gO and gH/gL/UL128-131 in strains TR and ME. On the contrary, results suggested that genetic differences outside the UL74(gO) ORF result in more rapid degradation of gO in the ME-infected cells compared to TR, and this influences the pool of gO available during progeny assembly [178]. Kalser et al. reported that gO polymorphisms could differentially affect multi step replication kinetics in fibroblasts and epithelial cells [145]. However, only TB was analyzed as the background and distinctions between effects on cell-free and cell-to-cell spread were unclear. In this report we constructed a matched set of heterologous gO recombinants in the well-characterized, BAC-cloned strains TR and ME. Studies included address aspects of cell-free and cell-to-cell spread, cell-type tropism and neutralization by anti-gH antibodies. The results demonstrate that gO polymorphisms can influence each of these parameters and the effects in some cases were dependent on the genetic background, suggesting a number of possible epistatic phenomena at play.

A commonly used measure to assess the tropism of HCMV strains, isolates and recombinants is the ratio of infection between fibroblasts and other cell types, including epithelial and endothelial cells [142, 145, 183, 184]. Expressions of this

ratio have varied, but have generally involved a normalization of the epithelial or endothelial infection to that of fibroblasts. Here we similarly determined the infectious titer of each of the parental strains and heterologous gO recombinants on both fibroblasts and epithelial cells and expressed ratios ≥1 (either fibroblasts/epithelial or epithelial/fibroblasts) to indicate the fold cell type preference or tropism of each virus (Fig 3.2). Both gH/gL/gO-rich viruses, TR and MT, were strongly fibroblast-tropic and some heterologous gO isoforms enhanced this preference, while others reduced it. In contrast, the gH/gL/UL128-131-rich virus ME infected both cell type more equally (ratios closer to 1), and gO polymorphisms had little effect. The limitation of any such measure of relative tropism is that it does not determine whether the virus in question can efficiently infect one cell type in particular, both or neither. Thus, any 2 viruses compared may have the same fibroblast-to-epithelial cell infectivity ratio for completely different reasons. To address this we also compared infectivity on both cell types using a common comparison for all viruses, i.e., the number of virions in the stock as determined by qPCR for DNAse-protected viral genomes in the cell-free virus stocks (Fig 3.3). This analysis provided a measure of specific infectivity as the number of genomes/IU, where the lower ratio indicates more efficient infection. Whether higher genomes/IU values reflect the presence of greater numbers of bona fide "defective" virions, or a lower probability or efficiency of each viable virion in the stock to accomplish a detectable infection, and whether or how these two possibilities are different is difficult to know for any type of virus. Nevertheless, these analyses provided important insights to the tropism ratios reported. In

general, the specific infectivity ratios of the gH/gL/gO-rich viruses TR and MT in these experiments were in the range of 500-5000 genomes/IU on fibroblasts, but these viruses were approximately 20-100 fold less infectious on epithelial cells, explaining the strong fibroblast preference exhibited by these strains. The effect of most heterologous gO isoforms was similar on both cell types, but often of larger magnitude on fibroblasts. Thus, while all of the TR and MT-based gO recombinants remained fibroblast tropic, the quantitatively different effects on the two cell types influenced the magnitude of fibroblasts preference. Importantly, in no case did the change of gO affect the fundamental fibroblast preference of either TR or MT. The infectivity of the gH/gL/UL128-131-rich, ME-based viruses on both cell types was undetectable in these assays. Thus, the near neutral fibroblast-to-epithelial tropism ratios of the ME-based viruses seem to reflect an equal inability to infect either cell type and any assertion of a "preference" for either cell type for extracellular ME virions seems spurious.

Binding to PDGFRα through gO is clearly critical for infection of fibroblasts [31]. However, while gH/gL/gO is also important for infection of epithelial cells, the literature is conflicted on the expression of PDGFRα and its importance for HCMV infection in epithelial and endothelial cells [32, 133, 134,162, 163]. On either cell type, possible mechanisms of gH/gL/gO include facilitating initial attachment to cells, promoting gB mediated membrane fusion, and signaling though PDGFRα or other receptors. While Wu et al. were able to coimmunoprecipitate gB with gH/gL/gO and PDGFRα, Vanarsdall et al. showed that gH/gL without gO or UL128-

131 can directly interact with gB and promote gB-fusion activity [134, 160, 164]. It has also been shown that gH/gL/gO engagement of PDFGRα can elicit signaling cascades, but that this is not required for infection [31, 134, 162]. In contrast, there is evidence that gH/gL/gO can help facilitate initial virion attachment [32, 181]. In our studies, TNgO(GT4) reduced binding of TR to both fibroblasts and epithelial cells (Fig 3.4, Tables 3.2 and 3.3). However, the reduced binding of TR_TNgO(GT4) did not result in reduced infection of either cell type, and there were other isoforms of gO that either resulted in increased or decreased infectivity but were not associated with any detectable alteration in binding. Thus, while gH/gL/gO may contribute to initial binding, it is likely involved in other important mechanisms that facilitate infection and these can be influenced by gO polymorphisms. For example, it is possible that polymorphisms in gO can affect the nature and outcome of PDGFRα engagement. In support of this hypothesis, Stegmann et al. showed that mutation of conserved residues within the N-terminal variable domain of gO were critical for PDGFRα binding [185]. Thus it is conceivable that the variable residues of gO can alter the architecture of the interaction with PDGFRa. Alternatively, it may be that there are other receptors on both cell types for gH/gL/gO and that gO polymorphisms can affect those interactions. Also, the effects of several specific gO isoforms observed in the TR-background were not observed in the ME or MT-backgrounds. Possible explanations for the apparent epistasis include not only the differential contributions of polymorphisms in gH/gL, but also potential differences between strains in other envelope glycoproteins, such as gB, or gM/gN may influence the relative importance of gH/gL/gO for binding and infection.

The mechanistic distinctions between cell-free and cell-to-cell spread of HCMV are unclear. Spread of ME in both fibroblast, epithelial and endothelial cells is almost exclusively cell-to-cell and this can be at least partially explained by the non-infectious nature of cell-free ME virions (Fig 3.3) [132, 137, 142, 179]. Laib Sampaio et al. showed that inactivation of the UL74(gO)ORF in ME did not impair spread but that a dual inactivation of both gO and UL128 completely abrogated spread [132]. This indicates that gH/gL/UL128-131 is sufficient for cell-to-cell spread in fibroblasts or endothelial cells in the absence of gH/gL/gO, and it seems likely that spread in epithelial cells might be similar in this respect. Our finding that various heterologous gO isoforms can enhance or reduce spread of ME without affecting the cell-free infectivity strongly suggest that while gH/gL/UL128-131 may be sufficient for cell-to-cell spread, gH/gL/gO can modulate or mediate the process, if present in sufficient amounts. In the context of MT, where expression of gH/gL/UL128-131 is reduced to sub detectable levels [133, 137] the virus gained cell-free spread capability, and yet some of the heterologous gO isoforms had opposite effects on cell-free infectivity and spread (compare Fig 3.3C to 3.5D). Similar discorrelations between cell-free infectivity and spread were observed for the naturally gH/gL/gO-rich strain TR, albeit with different heterologous gO isoforms involved. That gO polymorphisms can have opposite effects on cell-free and cell-to-cell spread supports a hypothesis of mechanistic differences in how gH/gL/gO mediates the two processes, and again these effects seem dependent on epistatic influences of the different genetic backgrounds.

Beyond the roles of gH/gL/gO in replication, the complex is likely a significant target of neutralizing antibodies, and therefore a valid candidate for vaccine design. Several groups have reported neutralizing antibodies that react with epitopes contained on the gH/gL base of both gH/gL/UL128-131 and gH/gL/gO and others that react to gO [165-173]. We found that changing the gO isoform can have dramatic effects on the sensitivity to two anti-gH mAbs (Figs 3.9 and 3.10). In the TR background on fibroblasts, both ADgO(GT1a) and TNgO(GT4) conferred significant resistance to neutralization by 14-4b, which likely reacts to a discontinuous epitope near the membrane proximal ectodomain of gH [165-166]. TNgO(GT4) also conferred resistance to AP86, which reacts to a linear epitope near the N-terminus of gH [182], whereas ADgO(GT1a) actually increased sensitivity of TR to AP86. Neutralization by either antibody on epithelial cells was not significantly affected, consistent with the notion that these antibodies can also neutralize by reacting to gH/gL/UL128-131. Again, the strain background exerted considerable influence over the effects of gO polymorphisms. For MT, it was ADgO(GT1a) that conferred resistance to 14-4b, and the other isoforms had little or no effect. The observed effects on neutralization on gH epitopes likely involve differences in how gO variable regions or associated glycans fold onto gH/gL to exert differential steric effects. Relatedly, the differential influence of gO isoforms in the two genetic backgrounds suggests epistasis involving the additive effects of gO polymorphisms with the more subtle gH polymorphisms, which together can differentially affect the global conformation of the gH/gL/gO trimer.

Previous analyses have suggested two groups of gH sequences defined by polymorphisms at the N terminus, including the AP86 epitope [182, 186]. Of the strains represented in this study, TB, TR and AD belong to the gH1 genotype and are sensitive to AP86, whereas ME, TN and PH belong to gH2 genotype and are resistant to AP86. The differential effects of gO recombinants reported here raise questions about the combinations of gH and gO genotypes in HCMV circulating in human populations. The recently published genome sequence datasets from clinical specimens have been collected with short-read sequencing approaches, which allow sensitive detection of the various gH and gO genotypes within samples, but not the combinations of the two ORFs on individual genomes [54, 55, 139, 153]. To address, this we analyzed 236 complete HCMV genome sequences of isolated strains and BAC clones in the NCBI database (Fig 3.11). Approximately half the sequences were gH1 and the other half gH2. ADgO(GT1a) and TBgO(GT1c) genotypes were exclusively linked to gH1, whereas MEgO(GT5) was exclusively linked to gH2. Other gO genotypes were found mixed with both gH genotypes, but in most cases, disproportionally with one of the gH genotypes. These analyses agreed with Rasmussen et al who suggested a strong linkage between gH1 and gO1 genotypes (note that their study predated the GT1a, 1, b, and 1c subdivisions) [135]. Thus, it appears that gH and gO genotypes are non-randomly linked. This may be due in part to the adjacent position of UL74(gO) and UL75(gH) on the HCMV genome and the sequence diversity, together limiting the frequency of recombination, as suggested by the high linkage-disequilibrium of this region reported by Lassalle et al [55]. In

addition, our results may suggest linkage pressures based on functional compatibility of gH and gO. However, it was worth noting that among the more striking effects reported were the loss of cell-free infectivity and differential sensitivity to neutralization by gH antibodies of TR_ADgO(GT1a). Together, with the fact that TR and AD are of the same gH genotype, these results suggest epistatic interplay of genetic variation of other loci with that of gH and gO.

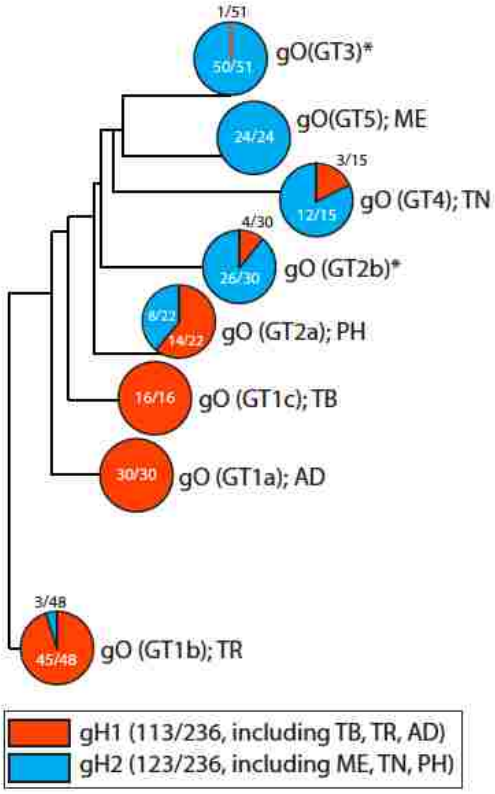

Fig 3.11 Association of gH and gO genotypes in 236 complete HCMV genome sequences in the NCBI database. Complete HCMV genome sequences were retrieved from the NCBI nucleotide database using the keywords filter <human herpesvirus type 5 complete genome>. The resulting set of 350 sequences was curated to remove duplicates or genomes missing any of the UL74(gO) and UL75(gH) open reading frames, generating a working set of 236 complete HCMV genomes, which were analyzed using MAFFT FFT-NS-I (v7.429) phylogeny software. UL74(gO) and UL75(gH) sequences were assigned to their respective genotype groups as defined previously; UL75(gH) genotypes 1 and 2 [182, 186]; UL74(gO) genotypes 1a, 1b, 1c, 2a, 2b, 3, 4 and 5 [19, 156]. Shown is a phylogenetic tree of the 8 gO genotypes with the frequency of pairing with either gH1 or gH2 . Asterisks (*) indicate gO genotypes that were not analyzed in the experiments described herein.

In conclusion, we have shown that naturally occurring polymorphisms in the HCMV gO can have a dramatic influence on significant aspects of HCMV biology including, cell-free and cell-to-cell spread, and neutralization by anti-gH antibodies. These effects could not be explained by changes to the levels of gH/gL complexes in the virion envelope, but rather point to changes in the mechanism(s) of gH/gL/gO in the processes of cell-free and cell-to-cell spread. The associated epistasis with the global genetic background highlights a particular challenge for intervention approaches since humans can be superinfected with several combinations of HCMV genotypes and recombination may occur frequently [54, 55, 61, 139, 151–154]. Moreover, these observations could help explain the incomplete protection observed for the natural antibody response against HCMV.

Materials and methods

Cell lines. Primary neonatal human dermal fibroblasts (nHDF; Thermo Fisher Scientific), MRC-5 fibroblasts (ATCC CCL-171; American Type Culture Collection), and HFFFtet cells (which express the tetracycline [Tet] repressor protein; provided by Richard Stanton) [137] were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 6% heat-inactivated fetal bovine serum (FBS; Rocky Mountain Biologicals, Inc., Missoula, MT, USA) and 6% bovine growth serum (BGS; Rocky Mountain Biologicals, Inc., Missoula, MT, USA) and and with penicillin streptomycin, gentamycin and amphotericin B. Retinal pigment epithelial cells (ARPE19) (American Type Culture Collection, Manassas, VA,

USA) were grown in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM:F-12)(Gibco) and supplemented with 10% FBS and with penicillin streptomycin, gentamycin and amphotericin B.

Human Cytomegalovirus (HCMV). All HCMV were derived from bacterial artificial chromosome (BAC) clones. The BAC clone of TR was provided by Jay Nelson (Oregon Health and Sciences University, Portland, OR, USA) [146]. The BAC clone of Merlin (ME) (pAL1393), which carries tetracycline operator sequences in the transcriptional promoter of UL130 and UL131, was provided by Richard Stanton [137]. All BAC clones were modified to express green fluorescent protein (GFP) by replacing the US11 ORF with the eGFP gene under the control of the murine CMV major immediate early promoter. The constitutive expression of eGFP allows the monitoring of HCMV infection early and was strain-independent. Infectious HCMV was recovered by electroporation of BAC DNA into MRC-5 fibroblasts, as described previously by Wille et al. [130] and then coculturing with nHDF or HFFFtet cells. Cell-free HCMV stocks were produced by infecting HFF or HFFFtet cells at 2 PFU per cell and harvesting culture supernatants at 8 to 10 days postinfection (when cells were still visually intact). Harvested culture supernatants were clarified by centrifugation at 1,000 X g for 15 min. Stock aliquots were stored at -80°C. Freeze-thaw cycles were avoided. Infectious unit (IU) were determined by infecting replicate cultures of nHDF or ARPE19 with serial 10-fold dilutions and using flow cytometry to count GFP positive cells at 48 hours post infection.

Heterologous UL74(gO) recombinant HCMV. A modified, three step BAC En Passant recombineering technique was performed [187-188]. In the first step, the endogenous UL74 ORF from the start codon to the stop codon of both TR and ME was replaced by a selectable marker. This necessary step was added to prevent formation of chimeric UL74 gene by internal recombination of the UL74 BAC sequence and the incoming heterologous UL74 ORF. A purified PCR product containing the ampicillin resistance selectable marker (AmpR) cassette from the pUC18 plasmid flanked by sequences homologous to 50 bp upstream and downstream of the TR or ME UL74 ORF was electroporated into the bacteria, recombination was induced and the recombinant-positive bacteria were selected on medium containing ampicillin (50 μg/ml) and chloramphenicol (12.5 μg/ml). The primers used to produce the TR- and ME-specific AmpR PCR bands are For74TRamp, 5'-CATGGGAGCTTTTTGTATCGTATTACGACATTGCTGTTTCCAGAACTTTAcgcggaaccccta tttgtttatttttctaaatac, For74MEamp, 5'-GATGGGAGCTTTTTGTATCGTATTACGACATTGCTGCTTCCAGAACTTTAcgcggaaccccta tttgtttatttttctaaatac, and Rev74amp (used for both TR and ME PCR reactions), 5'-CCAAACCACAAGGCAGACGGACGGTGCGGGGTCTCCTCCTCTGTCATGGGGttaccaatgctta atcagtgaggcacc. The lower case nucleotides correspond to the AmpR gene from the pUC18 plasmid, the upper case nucleotides to the TR and ME BAC sequences immediately upstream and downstream of the UL74 ORF.

In the second step, the AmpR cassette in the TR and ME first-step intermediate BACs was replaced with the UL74(gO) sequence from the heterologous strain containing the En Passant cassette [187-188]. Briefly, E. coli cultures were prepared for recombination as described above for step 1 and electroporated with purified PCR products containing the UL74 ORF from the TR or ME strain flanked by sequence homologous to 50 bp upstream and downstream of the opposite strain. The UL74 ORF also contained an inserted En Passant cassette (an I-SceI site followed by a kanamycin resistance gene surrounded by a 50-bp duplication of the UL74 nucleotides of the insertion site). Transformed E. coli cells were induced for recombination and then selected for the swap of the UL74 En Passant sequence into the BAC by growth on medium containing kanamycin (50 μg/ml) and chloramphenicol (12.5 μg/ml). A PCR reaction analysis with primers located upstream and downstream of UL74 was used to confirm the swap of the AmpR cassette by the En Passant cassette/UL74 gene.

In the third step, several sequencing validated colonies of the second step were subjected to the last step of the En Passant recombineering, that is, an induction of both the I-SceI endonuclease and the recombinase [187-188]. The activity of these enzymes lead to an intramolecular recombination in the UL74 sequence around the En Passant cassette and thus the restoration of an uninterrupted, full length UL74 ORF. The final heterologous UL74(gO) recombinants were verified by Sanger sequencing of PCR products using primers located upstream and downstream of the UL74 gene.

Antibodies. Monoclonal antibodies (MAbs) specific to HCMV major capsid protein (MCP), pp150, and gH (14-4b and AP86) were provided by Bill Britt (University of Alabama, Birmingham, AL) [165, 182, 189-190]. 14-4b and AP86 were purified by FPLC and quantified by the University of Montana Integrated Structural Biology Core Facility. Rabbit polyclonal sera against HCMV gL was described previously [64, 133].

Immunoblotting. HCMV cell-free virions were solubilized in 2% SDS–20 mM Tris-buffered saline (TBS) (pH 6.8). Insoluble material was cleared by centrifugation at 16,000 X g for 15min, and extracts were then boiled for 10 min. For reducing blots, dithiothreitol (DTT) was added to extracts to a final concentration of 25 mM. After separation by SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Whatman) in a buffer containing 10 mM NaHCO3 and 3mM Na2CO3 (pH 9.9) plus 10% methanol. Transferred proteins were probed with MAbs or rabbit polyclonal antibodies, anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich), and Pierce ECL-Western blotting substrate (Thermo Fisher Scientific). Chemiluminescence was detected using a Bio-Rad ChemiDoc MP imaging system. Band densities were quantified using BioRad Image Lab v 5.1.

Quantitative PCR. Viral genomes were determined as described previously [133]. Briefly, cell-free HCMV stocks were treated with DNase I before extraction of viral

genomic DNA (PureLink viral RNA/DNA minikit; Life Technologies/Thermo Fisher Scientific). Primers specific for sequences within UL83 were used with the MyiQ real-time PCR detection system (Bio-Rad).

Flow cytometry. Recombinant GFP-expressing HCMV-infected cells were washed twice with PBS and lifted with trypsin. Trypsin was quenched with DMEM containing 10% FBS and cells were collected at 500Xg for 5 min at RT. Cells were fixed in PBS containing 2% paraformaldehyde for 10 min at RT, then washed and resuspended in PBS. Samples were analyzed using an AttuneNxT flow cytometer. Cells were identified using FSC-A and SSC-A, and single cells were gated using FSC-W and FSC-H. BL-1 laser (488nm) was used to identify GFP+ cells, and only cells with median GFP intensities 10-fold above background were considered positive.

Virus particle binding. nHDF or ARPE19 cells were seeded at density of 35,000 cells per cm^2 on chamber slides (Nunc Lab Tek II). 2 days later, virus stocks were diluted with media to equal numbers of virus particles based on genome quantification by qPCR. Binding of virus particles to the cells was allowed for 20min at 37°C. Then the inoculum was removed, and the cells were washed once with medium to remove unbound virus before fixation and permeabilization with 80% acetone for 5min. Bound virus particles were stained with an antibody against the capsid-associated tegument protein pp150 [26] which allowed to detect enveloped particles attached to the plasma membrane as well as internalized particles. For visualization, a goat anti-mouse Alexa Fluor 488 (Invitrogen) secondary antibody

was used. Unbound secondary antibody was washed off before the chambers were removed and the cells were mounted with medium containing DAPI (Fluoroshield) and sealed with a cover slide for later immunofluorescence analysis. Images were taken with a Leica DM5500 at 630-fold magnification. For each sample 10 images with 4 to 6 cells per image were taken and the number of cell nuclei as well as the number of virus particles was determined using Image J Fiji software (v 1.0). Three independent virus stocks were tested in 3 independent experiments.

Antibody neutralization assays. Equal numbers of nHDF-derived cell-free parental viruses and heterologous gO recombinants were incubated with multiple concentrations of anti-gH mAb 14-4b or AP86 for 1hr at RT then plated on nHDF or ARPE19 for 4hrs at 37°C. Cells were then cultured in the appropriate growth medium supplemented with 2% FBS. After 2 days, cells were detected from the dish and fixed for flow cytometry analyses. Each antibody concentration was performed in triplicate and 3 independent experiments were conducted.

Chapter IV. Conclusion, discussion, and future directions

HCMV epidemiology, clinical pathology and genetic diversity

Human cytomegalovirus (HCMV) has high seroprevalence within human population. Based on clinical observations, 50% of people acquire HCMV during childhood and the rest contract this virus later on in their life [191]. Most human viruses attack certain cell types, tissues, or organs, for example HIV specifically infects T-cells and SARS-Cov-2 targets lungs [192-193]. In contrast, one unique characteristic of HCMV is that it can spread throughout the body and cause clinical pathologies, including gastroenteritis, encephalitis, retinitis, vasculopathies and so on [194].

With the development of high-throughput sequencing techniques in the past few years, some geneticists and bioinformaticians have revealed a great degree of genetic diversity of HCMV in clinical specimens [53-55,61,66,139,151,153,154]. Based on these studies, 21 out of 165 canonical genes have high diversity and they are distributed in clusters across the viral genome. For each diverse gene, there are 2 to 14 different alleles detected among the clinical specimen samples. Since the majority of the viral genome is conserved, recombination sites are widely spread through the genome. The low linkage disequilibrium among the diverse loci suggests the variable alleles may get shuffled among strains by recombination [55]. Theoretically, more than 10^{15} unique strains can be generated by possible recombination events [Jean-Marc Lanchy, personnel communication]. Of the approximately 240 complete genome sequences in the databases, each are unique in their combination of these variable alleles. The inter-strain recombination suggests that more than one individual HCMV virion may infect the same cell and actively

replicate at the same time. Another important finding from the clinical specimen sequencing study is that while there are many HCMV strains, which contain distinct viral genomes, circulating in the peripheral blood, each body compartment may have a limited number of strains.

UL74, which codes for glycoprotein O (gO), is one of the most diverse loci among the variable genes described above. Based on the sequencing results using isolates collected from congenitally infected newborns, transplant recipients, and HIV/AIDS patients, 8 genotypes of gO are identified [135,156,195]. There are five major genotypes (gO1, gO2, gO3, gO4 and gO5) and some of them are further divided into minor subtypes (gO1a, gO1b, gO1c, gO2a, gO2b). The divergence of nucleotide sequence between genotypes ranges between 3% to up to 55% [156]. The amino acid sequence alignment of representative gO isoform from each genotype has revealed regions of high diversity and regions of conservation (Fig 4.1A). The majority of the diverse regions locate within the N-terminal 100 residues. A 20-22-amino acid hydrophobic domain serving as the signal peptide that begins at the 14th amino acid from the N-terminus [196]. After cleavage of signal peptide, the mature gO has approximately 70 amino acids that are diverse at the N-terminus and the residues from 270 to 340 also harbor notable diversity. There are 6 conserved cysteine residues among gO isoforms, which locate at positions 31, 152, 160, 178, 229, and 354. Apart from cysteine 31 that is within the signal peptide, cysteine 354 is involved in forming disulfide bond with gH/gL and cysteine 178, 229 are important for viral infectivity [64,124]. The amino acid sequence differences may

lead to distinct characteristics and a good example is that the predicted N-link glycosylation sites vary among the gO isoforms (Fig 4.1B).

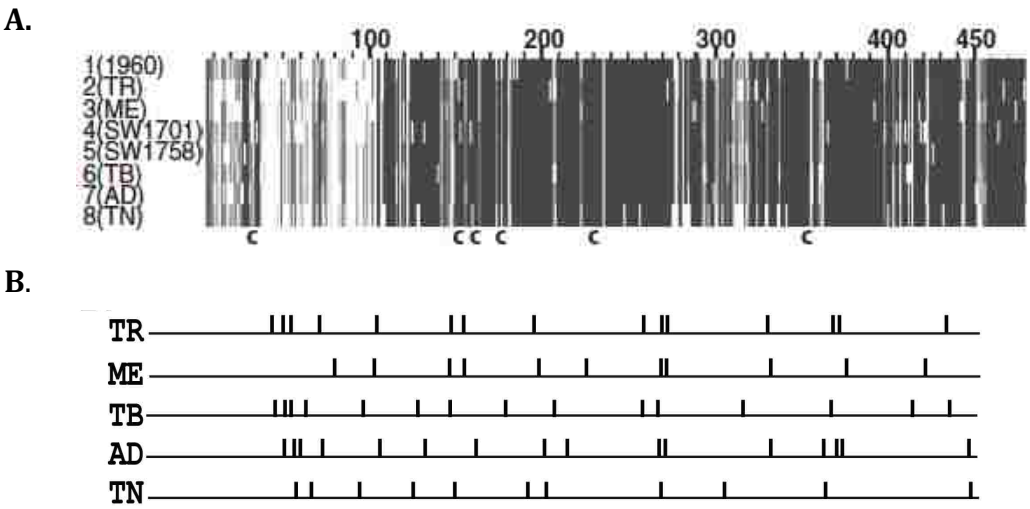

Fig 4.1. (A) gO sequence alignment. Dark shading indicates conserved sequences. The approximate positions of 6 conserved cysteine (C) are indicated. (Modified from [64]) (B) Predicted N-link glycosylation sites on gO.

The role of gO in HCMV biology

gO does not have a transmembrane domain and it locates on the virion envelope through forming a complex with glycoprotein H (gH) and glycoprotein L (gL). gH and gL are found in all herpesviruses and they are part of the core membrane fusion machinery for the virus family [120,122,197]. Within the gH/gL/gO complex, gH is the one containing the transmembrane domain and anchoring the complex on the lipid envelope, while gL forms disulfide bonds with both gH and gO. The gH/gL heterodimer in the virion envelope can alternatively be found being bound by UL128, UL130, and UL131, forming complex gH/gL/UL128-131 [159,161]. The same gL cysteine 144 mediates binding to both gO and UL128, which suggests the two gH/gL complexes are formed through competition [123].

gH/gL/gO is critical for HCMV cell-free infection on all cell types, while gH/gL/UL128-131 is additionally required for infecting certain cell types, such as epithelial, endothelial, and leukocytes [125,126,130,131,132,133,161]. It has been shown that platelet-derived growth factor receptor-α (PDGFR-α) on fibroblast cell is a receptor for gH/gL/gO, and gO directly interacts with PDGFR-α [31,32]. The interaction between gO and the receptor may contribute to viral infection by enhancing viral attachment to the host cell and/or facilitating post-attachment events, such as initiating signal transduction or triggering membrane fusion.

Compared to cell-free spread, the mechanisms of gH/gL complexes in cell-to-cell spread are less understood. Previous study has shown that gO-null virus could still spread cell-to-cell on fibroblasts and epithelial cells, which was most likely facilitated by gH/gL/UL128-131. In contrast, the UL128-131-null virus could spread cell-to-cell on fibroblasts but not on epithelial cells [179]. gO and UL128-131 double knock out mutant completely abolished cell-to-cell spread, which suggests a requirement for the participation of at least one gH/gL complex [132].

Based on studies using clinical human blood samples and an animal immunization models, the epitopes on gH/gL are prime targets for neutralizing antibodies and there is evidence suggesting that gO protects the virus from antibody neutralization [116,166,168,176,198].

Despite the clear diversity at gO locus, there has been no evidence showing the differences in gO sequence could cause functional variability. Since previous work studied each role of gO within only one strain context, it was not clear whether the observed gO functions could be affected by gO sequence diversity and whether strain genetic background was involved in determining the phenotype outcome. In this dissertation, I explored the significance of gO diversity in the following critical aspects of HCMV biology in different strain genetic backgrounds:

- The assembly of gH/gL/gO and gH/gL/UL128-131
- The infectivity and tropism
- The cell-free vs. cell-to-cell spread
- The sensitivity to neutralizing antibody
- The epistatic effect of gO polymorphism

1.The assembly of gH/gL/gO and gH/gL/UL128-131

Previous studies have shown that strains containing distinct gO genotypes have various amounts of gH/gL/gO and gH/gL/UL128-131 in their virion envelope [64]. Strain TB and TR have more gH/gL in total than strain ME. The gH/gL in strain TB and TR are mostly in the gH/gL/gO form while ME is abundant in gH/gL/UL128-131.

In this topic, I tested two factors that could contribute to the differential level of gH/gL complexes among strains:

- The expression level of gH/gL, gO and UL128, UL130, UL131

- The competition ability of gO sequences in gH/gL complexes formation

Strain TR was chosen as a representative for gH/gL/gO-rich virus and strain ME represented for gH/gL/UL128-131 abundant virus. The results in Chapter 2 showed that TR expressed similar amount of gH/gL as strain ME. However, the gH/gL in ME-infected cells were mostly associated with the ER, while TR's gH/gL were predominantly residing in Golgi, indicating a higher chance of being assembled into virion. This result was consistent with the observation that TR had more total amount of gH/gL in the virion than ME. Much more UL128, 130, 131 were detected in ME-infected cells than TR-infected cells, which directly correlated with ME's high gH/gL/UL128-131 phenotype. To study gO expression level, TR-ME gO open reading frame swap mutants were generated to eliminate the caveat of using strain-specific antibodies to make a comparison. Both the immunoblot and quantitative radiolabeling experiment results showed that ME produced significantly less amounts of gO than TR. These results clearly demonstrated that the stoichiometry of gO and UL128-131 expression plays an important role in gH/gL/gO and gH/gL/UL128-131 assembly.

There was one previous study related to addressing the expression level of UL128-131. It was found that strain TB, a gH/gL/gO-rich strain, possessed a G>T single nucleotide polymorphism (SNP) compared to ME, which reduced the splicing of mRNA encoding UL128, thus limited the assembly of gH/gL/UL128-131 [142]. However, this G>T SNP cannot fully explain strain TB's low amount of

gH/gL/UL128-131, since strain TR, which also contains small amounts of gH/gL/UL128-131, has nucleotide G at the SNP position. The research on gO expression was rather incomplete before this dissertation study. Based on the quantitative radiolabeling experiment conducted in Chapter 2, ME infected cells produced 27-fold less of gO than TR within 15min, which suggested the differences in gO expression were due to some early stage events, such as UL74 (gO) mRNA transcription, mRNA degradation, translation efficiency, or ER-associated rapid degradation. Nguyen et al. provided evidence supporting the possibility of ER-associated degradation by showing that viral protein UL148 interacts with SEL-1, a factor that plays a key role in ER-associated degradation. This study also showed that gO was a substrate for ER-associated degradation and that UL148 could protect gO from degradation [199]. UL148 has also been considered as a chaperon protein in the ER facilitating gH/gL/gO formation [136], which correlates well with the result in Chapter 2 that ME-infected cells contained less UL148 than TR.

The results in Chapter 2 further tested whether the low abundance of gO in ME-infected cells was the determinant factor for its low gH/gL/gO phenotype. The overexpression of gO during ME replication did not increase the level of gH/gL/gO in the virion unless the expression of UL128-131 was suppressed. Even then, the change of gH/gL/gO level was marginal. These data taken together suggest that there might be some other factors that affect the assembly of gH/gL complexes. One potential candidate was discovered by Calo et al., who found that HCMV viral glycoprotein UL116 competes with gL and forms a heterodimer with gH, and this

gH/UL116 complex is located on infectious virion envelope [200]. It's unclear whether gL-gO or gL-UL128 competes with UL116 for binding to gH. If these competitions do exist, which one is more competitive? This interesting question could be followed up.

Swapping 6 gO isoforms into strain TR and ME had no impact on the level of gH/gL complexes in the virion envelope. These results suggest that the strain genetic background, loci outside of UL74 (gO), determines the level of gH/gL complexes in the virion, and the diversity within the gO open reading frame does not affect gH/gL complexes assembly.

<u>New ideas for future work:</u>

The mechanisms of differential expression levels of gO and UL128-131 between TR and ME haven't been fully characterized in this study and some potential future work can be followed up on this topic.

1) Comprehensive RNA sequencing analysis on infected cells with multiple time points may help to clear up whether UL74 of ME is transcribed with low efficiency and/or whether ME's UL74 mRNA has poor stability. Also, RNA sequencing data would also provide information on understanding the mechanisms of UL128-131 expression differences between TR and ME.

2) Several connections around UL148: i) it favors gH/gL/gO formation, ii) it is involved in ER-associated rapid degradation, and iii) ME expresses significantly less UL148 than TR. Since polymorphisms exist in the UL148 locus between TR and ME,

one specific direction for going forward is to generate UL148 swap mutants between TR and ME, which may help to narrow down UL148's function in determining the gH/gL complexes assembly.

3) UL116 is a potential factor that influences gH/gL complex assembly. It is unclear whether gL-gO or gL-UL128 can compete with UL116 for binding to gH inside of ER. Adenovirus can be used as vector to deliver genes of interest into cells and afterwards analyze the gH-associated complex by immunoprecipitation. Based on the result from Chapter 3 that gO isoforms do not affect gH/gL composition in the virion, it is hard to believe that the gO sequence has an impact on competition with UL116. However, there are genetic polymorphisms at the UL116 locus. Between strain TR and strain ME, there are 6 amino acid variations in UL116 sequence, including TR>ME: Gly34 > Ser, Thr82 > Ala, Pro86 > Leu, Gly99 > Arg, Pro115 > Ser, and Ala123 > Val. It is worth to look into whether these polymorphisms could potentially change its structure or interaction with gH.

2. Infectivity and tropism

Strains TB, TR, and ME containing different gO genotypes have distinct cell-free infectivity on different cell types [133]. The gH/gL/gO-rich strains TB and TR are much more infectious than strain ME, which is abundant in gH/gL/UL128-131. However, the level of gH/gL/gO in the virion cannot fully explain the infectivity differences. In the case of MT, where the level of gH/gL/UL128-131 is reduced during strain ME replication, the virion has slightly increased amounts of gH/gL/gO and the infectivity is greatly improved. Despite much less of gH/gL/gO in the MT

virion than strain TR, MT is more infectious than TR. From these previous analyses, it seems that both the quantity and the quality of gH/gL/gO can impact the cell-free infectivity. Since gO can directly interact with gH/gL/gO receptor PDGFR-α, it is possible that gO isoforms have different characteristics in interacting with receptor [31], which lead to various efficiencies in attachment, signal transduction, and membrane fusion.

This dissertation tested the hypothesis that the amino acid sequences of gO directly affect viral infectivity and tropism.

Since all the gO isoforms have no influence on the assembly of gH/gL complexes in both strain TR and strain ME background. The gO swap mutant library was a suitable reagent set for testing this hypothesis.

The cell-free infectivity analysis in Chapter 3 showed that several gO isoforms significantly affected viral infectivity on both fibroblast and epithelial cells. For example, MEgO, PHgO, and TNgO enhanced TR's viral infectivity, while ADgO dramatically dropped the infectivity down to below the detection limit of this flow cytometry-based assay.

A common parameter used in the field for measuring the tropism is the ratio of infection between fibroblast cells and other cell types, such as epithelial and endothelial cells [142,145,184]. In this dissertation, I generated ratio of infectious

titer between fibroblast cells and epithelial cells to look into the cell type preference or tropism for each gO swap mutant virus. TR and MT, which contain much more gH/gL/gO than gH/gL/UL128-131 in the virion envelope, were strongly fibroblast tropic, and some gO isoforms enhanced this preference, while others reduced it. However, in strain ME with abundant gH/gL/UL128-131, the tropism ratio was close to equal. This result revealed the limitation of using the ratio of infection between cell types as the parameter for evaluating viral tropism. The ratio parameter overlooked the situation where viruses could not efficiently infect one cell type in particular or both. Since none of the gO swap mutant in ME background had detectable cell-free infectivity, it is a moot point to generate the tropism ratio. The near neutral tropism ratio reflected these viruses' equal incapability for infecting fibroblast and epithelial cells. For MT gO swap mutants and majority of gO swap mutants in TR background, they were capable of establishing cell-free infection with reproducible viral infectivity. These viruses were approximately 20- to 100-fold more infectious on fibroblast cells than on epithelial cells, thus showing a preference for fibroblast cells. The impacts of gO isoforms in TR and MT were similar on both cell types but exhibited a larger range on fibroblast cells. Thus, although all of the TR- and MT-based gO swap mutants remained fibroblast cell tropic, they were quantitatively different due to the various magnitude of fibroblast cell preference.

Possible functions or mechanisms of gH/gL/gO in cell-free infection include engaging receptor binding/attachment to cell, promoting gB-mediated fusion, and

signaling through its receptor. Within the past few years, studies in the field have investigated PDGFR-α as a receptor for gH/gL/gO on fibroblast cells. The cryo-EM structure analysis indicated the interaction between gH/gL/gO and PDGFR- α was through component gO [31]. However, it is unclear whether gH/gL/gO engages binding to receptors on epithelial or endothelial cells. That soluble PDGFR-α pre-incubation with virus significantly prevented the infection on endothelial cells suggested it is highly likely gH/gL/gO also has the function of receptor binding on epithelial and endothelial cells [32]. Wu et al. showed that fusion protein gB could be co-immunoprecipitated with gH/gL/gO and PDGFR-α, which supported the hypothetical model in the field that gH/gL/gO interacts with gB to facilitate fusion and this function may rely on receptor binding [134]. There was another study that revealed that the gH/gL heterodimer could form a stable complex with the fusion protein gB in the virion to perform fusion [160], however, distinguishing gB-gH/gL-complex-mediated fusion compared to gH/gL/gO- or gH/gL/UL128-131-facilitated fusion needs to be further investigated. The binding of gH/gL/gO does trigger cell signaling through PDGFR-α. However, Wu et al. reported that only the extracellular domain of PDGFR-α was critical for virus entry while the cell line with a PDGFR-α intracellular tyrosine kinase domain knocked out had no influence on cell-free infection [162].

For binding between gH/gL/gO and PDGFR-α, Stegmann et al. went one step further and explored that the N-terminus of gO was important for binding to PDGFR-α. Mutagenesis analysis uncovered the binding sites on gO were aa56-61 and aa117-

121. These two binding sites are conserved within 3 out of 8 gO genotypes, which are GT1a, GT1c and GT2 [185]. It remains to be explored whether other gO genotypes bind to PDGFR-α through different binding sites or whether they utilize other proteins on host cell surface as receptors. Since ADgO and PHgO contain the conserved sites for binding to PDGFR-α, the previous findings cannot explain the dramatic infectivity differences between TR_ADgO and TR_PHgO, where TR_PHgO was 30-fold more infectious than TR and TR_ADgO's infectivity was too poor to be detected by the flow cytometry-based infectivity assay. It is possible that although ADgO and PHgO contain the conserved binding sites, the co-folding between gH/gL and gO are differentially altered by the sequence outside of the binding sites, which leads to their distinct accessibility for binding to PDGFR-α and cell-free infectivity. However, this possibility is framed under the assumption that PDGFR-α is the only receptor for gH/gL/gO and the binding event between them is conserved. It is possible that gH/gL/gO utilize multiple receptors on the host cell surface. A similar phenomenon has been observed for herpes simplex virus-1 envelope glycoprotein gD, which could bind to nectin-1, HVEM, or 3-O-sulfacted HS [201].

Considering that gH/gL/gO is involved in receptor binding, the study in Chapter 3 investigated whether the cell-free infectivity differences among gO recombinant mutants were due to their various abilities for attachment. The results from the attachment assay could not explain the distinct cell-free infectivity. For example, ADgO in TR background dramatically dropped the infectivity down to below the detection limit of the flow cytometry-based assay. However, TR_ADgO had no

deficiency in binding to cells. In contrast, TR_TNgO was more infectious than TR, while it had significantly less attachment than others on both fibroblast and epithelial cells. This incongruity between cell-free infectivity and attachment indicated that although gH/gL/gO-mediated attachment and fusion closely coordinate, the efficiency of these two events are not completely linked together. It is possible that gH/gL/gO with certain gO isoforms can bind to PDGFR-α with higher affinity, however, the binding will not necessarily lead to fusion and vice versa. Fewer virus particles attached to cells does not preclude the possibility that the gO isoform could contribute to a hyper-fusogenic gH/gL/gO.

New ideas for future work:

The results of gO's disproportional impacts on gH/gL/gO's attachment and fusion highlighted a big unknown in the field about how gH/gL/gO interacts with gB and leads to fusion, and whether this interaction requires gH/gL/gO binding to its receptor. It is also unclear whether gO is involved in this interaction interface between gB and gH/gL/gO. It remains to be understood whether gH/gL/gO carrying different gO genotypes have different binding affinity to PDGFR-α. In addition, the viral attachment could be aided not only by the interaction between gH/gL/gO and PDGFR-α, but also by other potential receptors for gH/gL/gO or other viral proteins on the virion envelope. In order to explore these unknowns, some structure and proteomics approaches would be fruitful.

1) In order to obtain information on the interaction between gB and gH/gL/gO, the most direct approach is a structural study. Compared to crystallography, cryo-EM is

a better approach for keeping all the glycan on the protein of interest. However, since cryo-EM requires proteins being purified, it is going to be challenging to co-purify gB and gH/gL/gO as a complex.

2) In regard to finding novel receptors for gH/gL/gO, the future work can start with using soluble expressed and purified gH/gL/gO to bind to fibroblast or epithelial cells, using immunoprecipitation and mass spectrometry to investigate gH/gL/gO-associated host cell proteins existed in the cytoplasmic membrane fraction. With the development of high-resolution single-cell mass spectrometry technology, it will be even possible to study the preference of gH/gL/gO for utilizing its receptors [202].

3) To investigate whether gH/gL/gO containing different gO isoforms have various affinity for binding to PDGFR-α, surface plasmon resonance (SPR) can be utilized to obtain the binding kinetics information.

3. The cell-free vs. cell-to-cell spread

HCMV cell-to-cell spread requires gH/gL complexes and either gH/gL/gO or gH/gL/UL128-131 is sufficient for facilitating cell-to-cell spread on fibroblast cells, while gH/gL/UL128-131 is indispensable for cell-to-cell spread on epithelial and endothelial cells [132,179]. However, the roles of gH/gL complexes in cell-to-cell spread have only been studied in cases of gO-null or UL128-131-null mutant. The sufficiency of gH/gL/UL128-131 in cell-to-cell spread observed with gO-null mutant could not rule out gH/gL/gO's potential contribution in cell-to-cell spread. Schultz et al. reported that strains were inherently different in their spread [205]. For example, strain TB is highly dependent on cell-free mode of spread, while strain ME

spreads in a cell-to-cell manner. Since previous studies were based on different single strain, it remains unclear whether gH/gL/gO and gH/gL/UL128-131 have differential participations in spread for distinct strains.

To understand the mechanism of gH/gL/gO in facilitating cell-to-cell spread, the following questions remained to be answered:

- Does gH/gL/gO-mediated cell-to-cell spread also require receptor binding?
- How does gO polymorphism affect the cell-to-cell mode of spread?
- How does gH/gL/gO contribute to cell-to-cell spread with the presence of gH/gL/UL128-131?

The results from spread analysis in Chapter 3 uncovered the influence of gO polymorphism in cell-to-cell spread. On fibroblast cell, TR_PHgO had the best cell-free infectivity across all the mutants, however, the total spread rate of this mutant (combination of cell-free and cell-to-cell spread) was the lowest, which indicated gH/gL/PHgO was poorly efficient in cell-to-cell spread. Quite opposite, ADgO in TR background was non-infectious for cell-free infection. However, TR_ADgO's total spread rate was similar to TR on fibroblast cells, which revealed that TR_ADgO had potent ability in cell-to-cell spread and it could compensate the poor contribution from cell-free spread. On epithelial cell, however, TR_ADgO had relatively slower spread rate than TR, while TR_TNgO, which spread similar to TR on fibroblast cells, had significantly reduced spread on epithelial cells, so the effects of gO isoforms on viral spread were dependent on the cell type. Since cell-free viruses of ME gO

recombinants were non-infectious, the spread observed in Chapter 3 were dominantly contributed by cell-to-cell mode. TBgO and ADgO significantly reduced the spread of ME, while TNgO dramatically increased the spread on both fibroblast and epithelial cells.

The indications from results mentioned above cleared up some confusion about gH/gL/gO's function in viral spread in the HCMV field. The differential impacts of gO isoforms on two modes of spread enlightened that gH/gL/gO utilizes different mechanisms in cell-free and cell-to-cell spread. It is possible that gH/gL/gO binds to different receptors for facilitating the two modes of spread or the interaction between gH/gL/gO and fusion protein gB is different at the cell-cell junction compared to cell surface. The differential effects of gO polymorphisms on ME spread in both fibroblast and epithelial cells uncovered that although gH/gL/UL128-131 was sufficient for facilitating cell-to-cell spread, gH/gL/gO could also contribute to this process. In addition, the phenomenon of gO isoform's various impacts between cell types brought to light that gH/gL/gO participates in spread with different fashion based on the cell type. One possibility that remains to be further studied is the differential coordination/preference between gH/gL/gO and gH/gL/UL128-131 in facilitating membrane fusion in distinct cell types.

4. The sensitivity to neutralizing antibody

Previous studies reported that in clinical human samples and animal immunization model, HCMV infection elicited antibodies mostly against gB, gH/gL, and UL128-131

[166-170,198]. The common observation from these studies was that the antibodies against gH/gL and UL128-131 in either human or animal serum neutralized HCMV infection on epithelial or endothelial cells much more potently than on fibroblast cells. There are many possible explanations for this phenomenon, such as antibodies against UL128-131 can efficiently block the gH/gL/UL128-131 thus inhibiting the entry on epithelial and endothelial cells. Another possibility is that the anti-gH/gL antibodies elicited can't bind to gH/gL/gO as easily as gH/gL/UL128-131, which suggests a new potential function for gO as part of HCMV's strategy for escaping the host immune response. Several studies supported this possibility. Jiang et al. showed that the focal growth of gO-null virus was much more sensitive to anti-gH antibody than WT [176]. Cui et al. discovered that two HCMV strains, which contained identical gH epitopes but distinct gO genotypes had different sensitivities to anti-gH antibody [177]. All these observations taken together, generated one hypothesis:

- gO provides steric hindrance against anti-gH antibody binding, and this protective effect varies among gO isoforms.

The gO recombinant library is a great tool set for addressing this hypothesis. The neutralization assay in Chapter 3 tested the sensitivity of gO swap mutants to two anti-gH monoclonal antibodies. Both TR_ADgO and TR_TNgO conferred significant resistance to antibody 14-4b, which recognizes a conformational epitope located near the transmembrane domain of gH. Anti-gH antibody AP86, which targets a linear epitope near the N-terminus of gH. TR_TBgO and TR_ADgO, turned out to be

more sensitive to AP86 than TR, while MEgO provided some resistance and TR_TNgO showed 100% resistance to AP86 neutralization. In the MT context, ADgO showed resistance to 14-4b, while others had similar sensitivity.

Taken together, these results indicated that gO could protect HCMV against anti-gH antibody neutralization and that different gO isoforms might cover distinct epitopes on gH. These findings may help to explain the great challenges in HCMV vaccine development for HCMV where immunization with one or two attenuated strains, or viral proteins failed to prevent natural acquisition of this virus during clinical trials [203].

New ideas for future work:

In order to explore the mechanisms behind gO polymorphism's differential impacts on viral sensitivity to neutralizing antibody, three future studies are proposed here.

1) Characterization of binding between antibody and gO recombinant viruses.

For the recombinant virus that conferred resistance to antibody neutralization, if the antibody does not bind or has weaker binding to the virus, it can simply explain the resistance to neutralization is due to poor antibody binding. However, if the antibody can bind to these recombinant viruses as well as others, it suggests that gO may affect the folding of gH/gL during gH/gL/gO assembly. It is possible that gH that is bound by the antibody yet still can interact with gB to trigger fusion.

2) Structural study of gH/gL/gO formed with various gO isoforms.

Since gH, gL and gO are heavily glycosylated and the glycans may play important roles in preventing antibody binding, solving structure of gH/gL/gO with glycan information is critical. Compared to crystallography, which requires the glycans being trimmed off from the proteins, cryo-EM is a better method for this study.

3) Characterization and comparison of the glycans on different gO isoforms by mass spectrometry.

Wei et al. demonstrated that HIV utilized glycan on Env protein as an escaping strategy for antibody neutralization [174]. Considering that gO is heavily glycosylated and the predicted N-link glycan sites vary among gO isoforms, it is possible that the glycan on gO can provide steric hindrance against the binding of neutralizing antibody and gO isoforms containing different amino acid sequences may carry distinct glycan compositions. However, the knowledge about gO-associated glycans are very limited currently and further study on this topic, especially including different gO isoforms, will be very helpful for the comprehensive understanding of gO.

5. The epistatic effect of gO polymorphism

Another set of interesting observations from my study was that some impacts of gO isoforms were dependent on global strain genetic background.

The infectivity analyses in Chapter 3 showed that swapping ADgO into TR and MT reduced the viral infectivity, however, PHgO and TNgO significantly enhanced the viral infectivity in TR background on both fibroblast and epithelial cells but not in

MT situation. Interestingly, the effects of certain gO isoforms on viral attachment were also not identical in TR, ME and MT backgrounds, such as that TR_ADgO attached to cell as well as TR, while MT_ADgO had significant reduced binding compared to MT.

The characterization of viral spread on gO recombinants in TR and ME background proved that the impacts of gO polymorphism in spread phenotype were also dependent on strain genetic background. The isoform TNgO had no influence for spread in TR background, but significantly enhanced the spread in ME, while PHgO reduced the spread in TR background but not in ME.

Based on the neutralization analysis in Chapter 3, TNgO provided protection for TR against antibody 14-4b neutralization, while TNgO did not protect MT. This observation is more evidence indicating that the impacts of gO polymorphism are subject to the global genetic background. One difference between TR and ME background that may directly contribute to the epistatic effect in this neutralization study is that the amino acid sequence 34-43 at the N-terminus of gH is not identical between two strains. Based on this polymorphism at N-terminus, gH across different strains of HCMV is assigned as gH1 genotype and gH2 genotype. Compared to TR-gH (gH1), ME-gH (gH2) has a Proline missing and a substitution from Histidine to Lysine [182]. Since Proline is often considered as a “helix breaker” and Histidine has higher chance of being found around protein’s active or binding sites, it is possible that the polymorphisms in gH can affect the structure of gH/gL/gO.

To investigate the frequency of linkage between gH and gO genotypes, 236 complete HCMV genome sequences were analyzed. The result in Chapter 3 showed that ADgO and TBgO genotypes were exclusively linked to gH1, whereas MEgO was exclusively linked to gH2. Other gO genotypes were found together with gH1 and gH2, however, each gO genotype seemed to have a preference type between the two gH genotypes. The preference of genotype combination between gO and gH may be due to the fact that they locate right next to each other in the HCMV genome, with gO locating at UL74 and gH at UL75, so the recombination chance is rare. It is also possible that there are genes coded on the opposite strand of DNA, which restricts the recombination between gH and gO locus. The sequencing analysis also confirmed the high linkage disequilibrium between gH and gO locus [55]. The caveat for this part of the study is that the genome sequences subjected to this analysis were isolated strains and BAC clones, majority of which were extensively passaged on fibroblast cells and went through selection pressure for adapting to propagation in tissue culture. More advanced analyses based on the clinical specimen sequencing data are required for better understanding of the linkage between gH genotypes and gO genotypes.

In summary, the effects of gO isoforms were tested in different virus strain genetic backgrounds in multiple aspects, including the infectivity, attachment, spread, and sensitivity to neutralizing antibody. These findings stressed the important roles of other variable loci apart from gO in determining the phenotypic features of HCMV

and the observed influences of gO polymorphism were epistatic outcomes between gO and other variable loci across the viral genome.

Perspectives

With more Next Generation Sequencing and bioinformatics data analyzed directly on clinical samples, the connections and disconnections between laboratory adapted strains and HCMV in clinical specimens started drawing more and more attention.

The studies characterizing HCMV genomic sequence directly from clinical samples revealed great complexity and diversity of HCMV [153-154]. There is substantial strain diversity among individuals and this high diversity is likely due to high frequency of mutations at nonfunctional genes and pervasive recombination between strains. Within the host, multiple-strain infections are observed and up to 5 strains can be detected inside of one individual. The strains inside body compartments of hosts are rather conserved and stable, suggesting the compartments may place selection pressure on strain genotypes.

The major factor that causes disconnection between lab strains and clinical samples is the method of isolating virus from clinical specimens. One piece of evidence from studies in the field was that propagation of clinical isolates on fibroblasts could rapidly select out mutations that heavily reduced or even abolished the expression of UL128-131 proteins by inserting a stop codon within UL128 open reading frame

[137,141]. The cell type for clinical sample propagation also plays critical role in the process of isolation. The disrupting mutations in UL128 observed during fibroblast propagation were not seen in epithelial cell passaging, which could be interpreted as that gH/gL/UL128-131 has indispensible function on epithelial cell infection [140]. It has also been observed that 29 out of 30 clinical HCMV isolates that had the potential to spread in endothelial cells lost that ability after long-term adaptation in fibroblast cultures. In contrast, virus subjected to long-term adaptation on endothelial cells retained both fibroblast tropism and endothelial tropism [204]. However, since it was unclear whether the isolates contained single or multiple strains, the phenomenon described above might be due to mutation and/or strain selection. Besides, there are mutations in genes occurred in regardless of culture cell types, such as RL13, which encodes a highly glycosylated virion envelope protein and has the potential to modulate tropism. Stanton et al. reported that a rapid and reproducible frame-shift mutation occurred at RL13 locus when strain ME was passaged on both fibroblast and epithelial cells and the mutation led to dramatic repression on RL13 expression [137]. For other strains, such as TR, although the same phenomenon was not observed, it is still under investigation whether RL13 in these strains can be translated into protein and perform its function. In addition, other sporadic mutations can be selected across HCMV genome during the passaging of laboratory virus clones [141].

The result in Chapter 2 showed that overexpression of gO during ME replication enhanced the infectivity by only 6-fold, while repression of UL128-131 enhanced

ME's infectivity by 30- to 50-fold. These infectivity analyses suggested that the selective pressure for losing UL128-131 was much stronger than gaining gO, thus it is possible that the difference in gO expression between TR and ME reflects sampling of the different genotypes exist in clinical specimens rather than mutations raised during tissue culture propagation.

Although the question about how well the laboratory-adapted strains could represent HCMV exist in nature remains unclear, multiple studies that conducted Next Generation Sequencing analysis on clinical specimen samples concurred the diversity of gO sequence in nature [53-55,66,153-154]. This dissertation focused on studying the effects of gO sequence diversity as the connection between laboratory research and HCMV exist in nature, and characterized the influences of gO polymorphism on critical aspects of HCMV biology.

In conclusion, this dissertation research is fundamentally based on questions raised from clinical sequencing studies and aimed for connecting the barrier between laboratory study and clinical observations. The new findings include the mechanism of gH/gL complex assembly and the dramatic influence of gO polymorphism on cell-free, cell-to-cell spread and neutralization by anti-gH antibodies. This study also firstly elaborated that the impacts of gO polymorphism are subjected to epistatic influences of the global genetic background. Although the study has inevitable limitations in reflecting the features of virus found in clinic, hopefully these findings

can provide more information for better understanding of HCMV pathology and bring novel insights on vaccine design strategy.

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