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Article

# Human Cytomegalovirus Escapes a Naturally Occurring Neutralizing Antibody by Incorporating It into Assembling Virions

Kate Manley,[1] John Anderson,[1] Fan Yang,[1] Joseph Szustakowski,[1] Edward J. Oakeley,[1] Teresa Compton,[1,2] and Adam L. Feire[1,*]
[1]Novartis Institutes for Biomedical Research, 500 Technology Square, Cambridge, MA 02139, USA
[2]Present address: Biogen Idec, 14 Cambridge Center, Cambridge, MA 02142, USA
*Correspondence: adam.feire@novartis.com
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## SUMMARY

**Human cytomegalovirus (CMV) is a common but difficult to treat infection of immunocompromised patients. MSL-109 is a human monoclonal IgG isolated from a CMV seropositive individual that recognizes the viral glycoprotein H (gH) surface antigen complexes that mediate entry. Although MSL-109 blocks CMV infection in vitro, it lacked sufficient efficacy in human trials, and CMV isolated from treated patients suggested the evolution of MSL-109 resistance. To understand how CMV escapes MSL-109, we characterized a MSL-109-resistant CMV strain. Our results elucidate a nongenetic escape mechanism in which the antibody is selectively taken up by infected cells and incorporated into assembling virions in a dose-dependent manner. The resistant virus then utilizes the Fc domain of the incorporated antibody to infect naive nonimmune cells. This resistance mechanism may explain the clinical failure of MSL-109, illustrate a general mechanism of viral antibody escape, and inform antiviral vaccine and therapeutic development.**

## INTRODUCTION

Human cytomegalovirus (CMV) infects between 60% and 80% of the adult population worldwide (Ho, 1990). Infection is usually asymptomatic but results in a lifelong latent infection. Immunocompromised populations such as chemically immunosuppressed transplant patients, immune-naive neonates, and AIDS patients commonly exhibit significant morbidity and mortality as a result of CMV infection. The current therapy for CMV disease is treatment with either ganciclovir or valganciclovir, which are associated with significant toxicity and not approved for use in pregnant women. As an alternative, CMV antibodies have been used in the successful treatment of CMV-induced disease. CMV hyperimmunoglobulin has demonstrated efficacy in certain solid organ transplant recipients (Snydman, 1990), and a more recent study found that pooled human IgG products from CMV-positive individuals were effective at protecting infants from CMV disease when born to women with a primary infection (Nigro et al., 2005). These findings suggest that antibody therapy may prove to be an effective and safe alternative/addition to the current forms of CMV therapy.

CMV entry is reliant upon the stepwise coordination of multiple virus-encoded envelope proteins. Following the initial attachment of the virus to the cell through interactions between the virus gM/gN complex and heparan sulfate proteoglycans (HSPGs) (Kari et al., 1994), the virus induces envelope fusion with cellular membranes through a combination of glycoprotein B (gB) and gH/gL complexes. The latter of these exists in two forms; gH/gL/gO is required for replication in fibroblast cell types, while gH/gL/UL128/UL130/UL131a is required for virus entry into epithelial, endothelial, and dendritic cells but is typically mutated to eliminate function when the virus is passaged in fibroblast cells (Hahn et al., 2004). In fibroblasts, CMV entry occurs by fusion at the plasma membrane and is not dependent on the lowering of pH in endocytic compartments (Compton et al., 1992). In addition, there are also data supporting alternative routes of entry depending on the virus strain and cell type tested (Patrone et al., 2007; Ryckman et al., 2006; Wang et al., 2007), demonstrating that CMV entry is a complicated process which may involve multiple entry routes depending on the circumstances of infection.

MSL-109 is a human monoclonal IgG isolated from the spleen of a CMV seropositive individual. It recognizes gH surface antigen complexes and in vitro blocks the infection of fibroblasts by laboratory and clinical strains of CMV (Nokta et al., 1994). MSL-109 was tested as an adjuvant treatment for AIDS patients with CMV-induced retinitis, but development was halted during phase 2/3 clinical trials when data showed that MSL-109 lacked sufficient efficacy (Jabs et al., 2002). There are a number of possible reasons for its failure to work in vivo. For example, in silico analysis of the amino acid sequence of MSL-109 revealed potential deamidation sites within complementarity determining region (CDR) regions that may have affected its physiochemical properties in vivo. Also, the patient population in which it was tested was possibly suboptimal for measuring its efficacy, as the eye is an immune privileged site. Although both of these explanations are possible, a previous study of CMV isolated from MSL-109-treated patients suggested the virus had generated resistance to the antibody (Li et al., 1995). The mechanism of resistance was found not to require the mutation of the target protein, suggesting that CMV has alternative nongenetic mechanisms to escape naturally occurring neutralizing antibodies. To further understand this mechanism, we generated a MSL-109-resistant strain of CMV. Our findings confirm that antibody resistance can occur without any genetic changes and is generated rapidly by the selective incorporation of the antibody into the virion in a dose-dependent manner. This unusual mechanism of resistance provides an important insight into how CMV may escape the immune systems antibody response, and offers valuable information for the successful design of future monoclonal antibody therapies for CMV.

## RESULTS

### Characterization of MSL-109 Binding

MSL-109 blocks entry of several CMV clinical isolates into multiple cell types with $IC_{50}$ values ranging between 0.03 and 1.02 μg/ml (see Table S1 available with this article online). The reported target of MSL-109 is the virus envelope glycoprotein, gH (Lakeman et al., 1991). To confirm that MSL-109 binds to gH-containing complexes, we tested antibody binding to cells expressing gH in combination with glycoprotein L (gL). The gH/gL or gB complexes were displayed on the surface of adult retinal pigment epithelial-19 (ARPE-19) cells by adenovirus transduction, and FACS analysis was used to detect antibody binding. We also tested two other naturally occurring human antibodies, 13H11 and ITC88, which target gH and gB, respectively. Both MSL-109 and 13H11 bound to gH/gL-expressing cells, but not to mock-transduced cells or gB-expressing cells (Figure 1A). ITC88 bound to gB-expressing cells, but not to mock-transduced cells or gH/gL-expressing cells. In addition, we also tested the ability of MSL-109 to immunoprecipitate various forms of gH/gL-containing complexes from neonatal-normal human dermal fibroblasts (NN-NHDFs). MSL-109 was found to immunoprecipitate gH, but not an irrelevant cellular transmembrane receptor, DDR1, indicating that it specifically binds to the known gH-containing complexes (Figure 1B). UL131a was also immunoprecipitated from cells expressing the gH/gL/UL128/UL130/UL131a complex. These data confirm that MSL-109 recognizes gH/gL complexes on cells.

To test the ability of MSL-109 to immunoprecipitate gH from virions, purified VR1814 was complexed with either MSL-109, 13H11, or ITC88. As expected, ITC88 immunoprecipitated gB from virion lysates (Figure 1C). 13H11 immunoprecipitated gH but not gB, but in contrast MSL-109 was unable to immunoprecipitate either gB or gH from virions. Therefore, MSL-109 is capable of recognizing gH when it is expressed in cells but is not capable of recognizing gH when it is mixed with cell-free virions. To confirm this, we performed ELISA assays using either soluble gH/gL/UL128/UL130/UL131a complex, soluble gB, or purified VR1814 virions. ITC88 was bound to soluble gB and VR1814 virions, while 13H11 bound to soluble gH/gL/UL128/UL130/UL131a complexes and VR1814 virions (Figures 1D–1G). The pooled CMV hyperimmunoglobulin Cytotect bound to soluble gB and gH/gL/UL128/UL130/UL131a in addition to intact virions. MSL-109 bound soluble gH/gL/UL128/UL130/UL131a, confirming that it is capable of recognizing gH in this complex, but was unable to bind intact VR1814 virions. Additionally, experiments mixing free VR1814 virions with antibody in solution also demonstrated a lack of binding (Figure S1). These data show that MSL-109 binds to gH/gL complexes via an epitope that is only exposed following interaction with the cell and blocks a step subsequent to the initial attachment of the virus to the cell.

### Generation of MSL-109-Resistant Virus

To generate MSL-109-resistant virus, we subjected VR1814 (moi 0.5) to multiple rounds of infection in the presence of antibody. Initially multiple concentrations of antibody were tested to generate resistance and 4.4 μg/ml was found to be the lowest dose that generated a shift in the $IC_{50}$ value of over 100-fold (data not shown). This was the concentration of antibody used to generate the initial preparation of resistant virus, and subsequent rounds were generated in 6.6 μg/ml. NN-NHDF cells were infected with VR1814 in the presence of the antibody, and the flask was monitored for cytopathic effects (CPEs). When all the cells demonstrated CPE, the virus was harvested and used for the next round of infection. A total of three rounds were carried out before the virus was assessed for its susceptibility to MSL-109, 13H11, ITC88, or an additional anti-gH antibody, 3G16. MSL-109 completely neutralized parental VR1814 with $IC_{50}$ values in the 1 μg/ml range. Virus grown in the presence of MSL-109 approached an $IC_{50}$ value ~100-fold higher and was not able to neutralize more than 50% of the input virus (Figure 2A). These data indicate that the virus had escaped antibody neutralization, and we subsequently called this virus VR1814-MSL109R. The escape virus was still susceptible to the anti-gB antibody ITC88, with an $IC_{50}$ value of 0.24 μg/ml, and to two antibodies that recognize different epitopes on gH (13H11 and 3G16) with $IC_{50}$ values of 1.24 μg/ml and 6.11 μg/ml, respectively (Figure 2A). The ability of both gH-targeting antibodies and ITC88 to block VR1814-MSL109R infection indicates that the virus still expresses and uses gH and gB to enter cells.

Plaque-purified MSL-109-resistant clones were generated and tested for antibody susceptibility. All of the clones were resistant to MSL-109 with $IC_{50}$ values over 100 μg/ml (Figure 2B). To examine if there was a loss of fitness associated with resistance, plaque-purified VR1814-MSL109R was used to generate growth curves and compared to parental VR1814. VR1814-MSL109R grew at a similar rate to wild-type VR1814, although at day 16 there was a slight reduction in VR1814-MSL109R as compared to VR1814 (Figure 2C). These data indicate that there is little to no fitness cost to the generation of resistance.

### CMV Resistance to MSL-109 Is Not Dependent on a Genetic Change and Is Reversible

To identify the mechanism of resistance, all of the CMV glycoproteins that are known to be required for virus entry (gH, gL, gO, UL128, UL130, UL131a, gB, gM, and gN) were sequenced. VR1814-MSL109R sequences were aligned with those of both the parental VR1814 virus and the published VR1814 sequence. There were no coding mutations identified in any of the genes with known roles in virus entry, including both gH and gL. To establish if there were genetic mutations anywhere in the 234 kbp genome that tracked with resistance, we sequenced the full genome of eight plaque-purified viruses in addition to the parental VR1814 virus. We obtained complete coverage for all nine genomes (Table S2), and the sequencing reads were mapped onto the published VR1814 sequence. We analyzed all of the known and predicted HCMV open reading frames (ORFs)

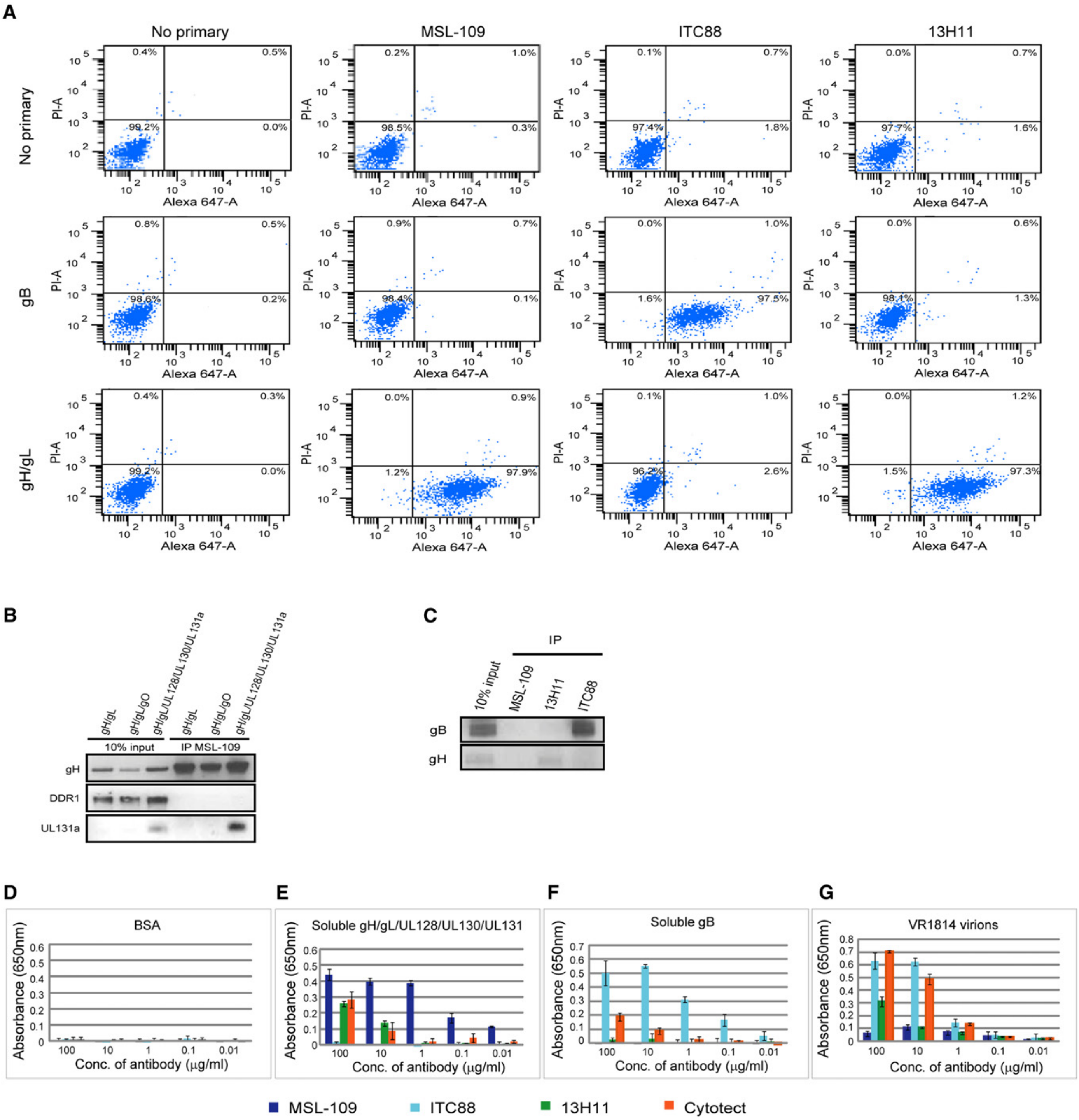


**Figure 1. MSL-109 Binds to HCMV gH/gL in a Cell-Associated Form**

(A) Adenoviruses were used to express either gH/gL or gB in ARPE-19 cells. Cell surface glycoprotein expression was detected using MSL-109, ITC88, or 13H11 antibodies.

(B) NN-NHDF cells were transduced with adenoviruses to express different gH containing complexes. Forty-eight hours p.i., lysates were harvested and immunoprecipitated using MSL-109. Immunoblotting was used to detect the presence of gH, UL131a, or DDR1.

(C) gH and gB were immunoprecipitated from an equivalent number of purified infectious virions using MSL-109, 13H11, or ITC88. The presence of gH or gB was detected by immunoblotting.

(D–G) MSL-109, 13H11, and ITC88 were bound to immobilized BSA (D), soluble gH/gL/128/130/131 complex (E), soluble gB (F), or VR1814 virions (G). Binding was detected using an anti-human HRP conjugated antibody (also see Figure S1 and Table S1). Error bars represent ±SD.

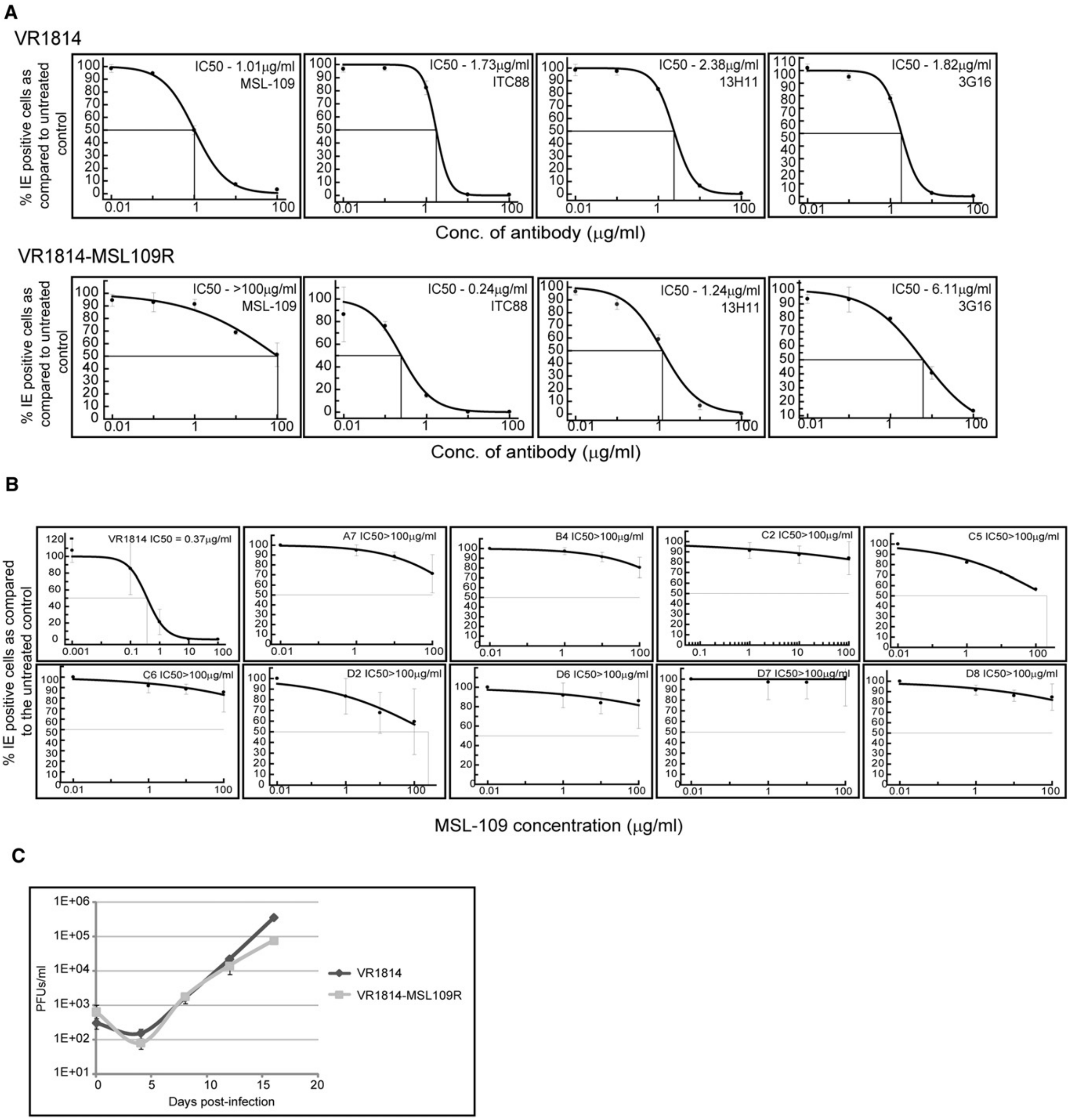


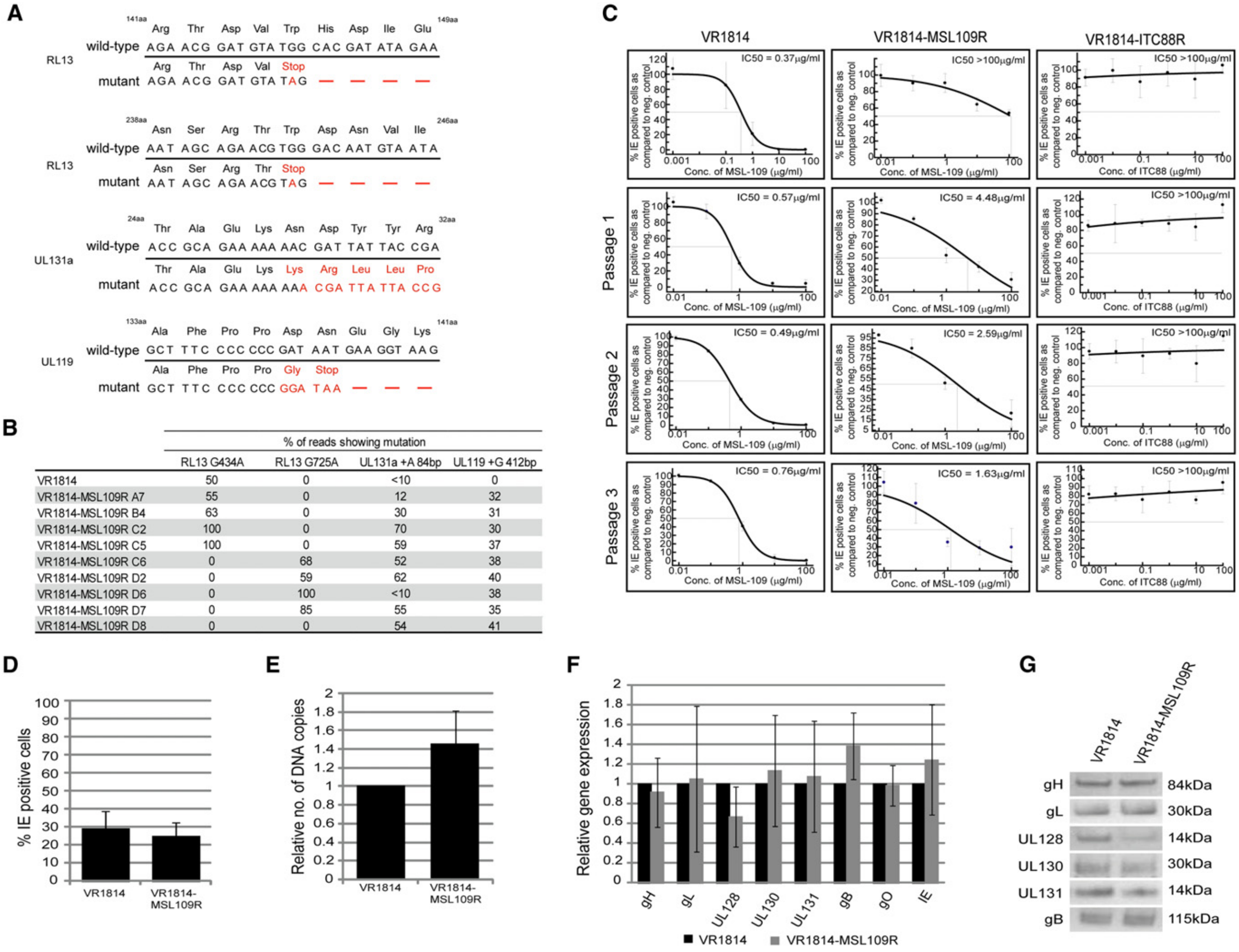
**Figure 2. VR1814-MSL109R Has Escaped MSL-109**

(A) VR1814 or VR1814-MSL109R was incubated with 10-fold dilutions of antibody for 1 hr prior to the infection of NN-NHDF cells. Twenty-four hours p.i., cells were stained for IE1/2 protein. The $IC_{50}$ value was calculated for each antibody using the percentage of IE1/2-positive cells compared to the untreated control; see also Table S1.

(B) Plaque-purified VR1814-MSL109R clones were tested for their susceptibility to MSL-109 as described in (A).

(C) NN-NHDF cells were washed three times in serum-free media and infected with either VR1814 or VR1814-MSL109R at an moi of 0.05. VR1814-MSL109R was infected and maintained in the presence of 6.6 μg/ml of MSL-109. At 0, 4, 8, 12, and 16 days p.i. virus was harvested. Plaque assay was used to establish the number of infectious particles. Error bars represent ±SD.

to identify ORFs in which a high frequency of mutations occurred. A high frequency of mutations occurred in three ORFs, RL13, UL119, and UL131a (Figure 3A). Both RL13 and UL131a frequently undergo mutations when the virus is continually passaged in fibroblasts (Dargan et al., 2010), so it is unsurprising that these genes show an accumulation of mutations.



| | % of reads showing mutation | | | |
|---|---|---|---|---|
| | RL13 G434A | RL13 G725A | UL131a +A 84bp | UL119 +G 412bp |
| VR1814 | 50 | 0 | <10 | 0 |
| VR1814-MSL109R A7 | 55 | 0 | 12 | 32 |
| VR1814-MSL109R B4 | 63 | 0 | 30 | 31 |
| VR1814-MSL109R C2 | 100 | 0 | 70 | 30 |
| VR1814-MSL109R C5 | 100 | 0 | 59 | 37 |
| VR1814-MSL109R C6 | 0 | 68 | 52 | 38 |
| VR1814-MSL109R D2 | 0 | 59 | 62 | 40 |
| VR1814-MSL109R D6 | 0 | 100 | <10 | 38 |
| VR1814-MSL109R D7 | 0 | 85 | 55 | 35 |
| VR1814-MSL109R D8 | 0 | 0 | 54 | 41 |

**Figure 3. MSL-109 Escape Is Not Due to a Genetic Change**

(A and B) The genomes of VR1814 and eight plaque-purified VR1814R-MSL109R clones were sequenced; ORFs RL13, UL131a, and UL119 accumulated mutations in the resistance clones (A); see also Table S2. The percentage of reads containing each of these mutations for each clone is given (B).

(C) VR1814-, VR1814-MSL109R-, and VR1814-ITC88R-resistant viruses were grown in NN-NHDF cells in the absence of antibody. Susceptibility to MSL-109 or ITC88 was tested by incubating virus with antibody for 1 hr before infection. The percent of IE1/2-expressing cells was determined and the $IC_{50}$ was calculated.

(D–F) NN-NHDF cells were infected with VR1814 or VR1814-MSL109R for 3 days. Cells were then either stained for IE1/2 (D) or harvested for DNA (E) or RNA (F). Quantitative real-time PCR was used to determine the DNA copy number or relative gene expression of virus glycoproteins.

(G) Equivalent numbers of infectious particles were separated using SDS-PAGE electrophoresis and then immunoblotted for the presence of virus glycoproteins. Band intensity was determined using the ChemiDoc XRS and Quantity One software (Bio-Rad) (see also Table S2). Error bars represent ±SD.

UL119 has not previously been identified as an ORF that undergoes mutation during passage in fibroblasts. It is a single-pass transmembrane receptor present in virions which binds to human IgG antibodies by the Fc domain, and it has been speculated that this is may be a mechanism by which the virus evades detection by immune cells (Sprague et al., 2008). Despite a high frequency of mutations occurring in these three ORFs, the percentage of individual reads containing the mutations varied considerably in the plaque-purified clones (Figure 3B), indicating that each clone was a heterogeneous population for these mutations. As these clones were plaque purified and maintained in the presence of the antibody, they should all be homogenous for the mutation if it is responsible for resistance. In addition, the parental VR1814 reads also demonstrated that there was a small population of virus that contained mutations in RL13 and UL131a. Together the sequencing data indicate that a simple genetic change is not responsible for antibody resistance in VR1814-MSL109R.

To support this conclusion, we examined the reversibility of the resistance phenotype by passaging VR1814-MSL109R in the absence of the antibody and testing each passage of virus for susceptibility to MSL-109. VR1814-ITC88R, which is resistant to neutralization by the anti-gB antibody ITC88, was tested in parallel. This virus contains a single nucleotide polymorphism (SNP) in the binding site of the antibody. The $IC_{50}$ value for MSL-109 and VR1814-MSL109R was 4.48 μg/ml after just one passage, and the virus remained susceptible to the antibody for the second and third passages with $IC_{50}$ values of 2.59 μg/ml and 1.63 μg/ml, respectively (Figure 3C). In contrast, VR1814-ITC88R remained resistant to ITC88 even when grown in the absence of the antibody. To confirm that RL13, UL131a, and UL119 are not responsible for MSL-109 resistance, we sequenced the reverted VR1814-MSL109R virus and found that the mutations in RL13, UL131a, and UL119 (Figure 3A) remained in the reverted virus (data not shown). The rapid reversion of the resistance phenotype argues against a permanent genetic change being responsible for the resistance phenotype.

## VR1814-MSL109R Expresses and Contains Wild-Type gH

To address alternative mechanisms of resistance, we tested VR1814-MSL109R for gH expression using qRT-PCR on RNA harvested from VR1814-MSL109R- or VR1814-infected NN-NHDF cells at 3 days postinfection (p.i.). The cells were infected at an moi of 0.5, and to ensure that the equivalent number of cells were infected, we fixed and stained some of the cells for IE1/2 (Figure 3D). We also used qRT-PCR to confirm an equivalent number of DNA genomes in the infected cells (Figure 3E). The qRT-PCR data demonstrated that there was little difference in the gene expression levels of IE1/2, gH, gL, UL128, UL130, UL131a, gB, or gO (Figure 3F). These data indicate that resistance was not due to an alteration in the expression of gH or any of the other proteins currently known to interact with gH.

To test if reduced gH abundance in virions could account for MSL-109 resistance, we compared protein levels of purified resistant virus. To ensure that an equal number of virus particles were compared, the number of infectious particles was quantified using immunofloresence staining for infected cells, and data were coupled with equivalent protein concentrations. Equivalent amounts of virus were pelleted through a sorbitol cushion, and the pellet was resuspended in lysis buffer and SDS-PAGE electrophoresis used to separate the proteins. Immunoblots confirmed that gH, gL, UL130, UL131a, and gB were present in the VR1814-MSL109R virions to similar levels as compared to wild-type VR1814 (Figure 3G). There was 62% less UL128 present in VR1814-MSL109R as compared to wild-type VR1814, but as MSL-109 can still block the entry of laboratory strains of CMV that do not have UL128 at all (Nokta et al., 1994), it is unlikely that this is the cause of resistance. Together with the data showing that other gH-targeting antibodies still neutralize VR1814-MSL109R infection (Figure 2), these data demonstrate that there is no loss of wild-type gH in VR1814-MSL109R.

## MSL-109 Resistance Is Dose Dependent and Involves the Incorporation of the Antibody into Assembling Virions

Since our data indicate that a genetic change is not responsible for resistance, studies were performed to establish the relationship between antibody concentration and resistance. We infected NN-NHDF cells with VR1814 and p.i. added various concentrations of MSL-109. The cells were left for 9 days to allow for virus production. The supernatant and intracellular virus was harvested from each sample and tested for susceptibility to MSL-109. Surprisingly, virus harvested from within the cell was susceptible to MSL-109 regardless of the antibody concentration present in the culture media (Figure 4A), while virus obtained from the supernatant had become resistant to MSL-109 in a dose-dependent manner. These data indicate a close relationship between the concentration of antibody and the generation of resistance.

As there is a close relationship between antibody concentration and the generation of resistance, we tested wild-type or resistant virions for the direct presence of the antibody. VR1814-MSL109R was grown in the presence of increasing doses of MSL-109 and compared to VR1814 grown in the absence of any antibody, and the ITC88-resistant virus (VR1814-ITC88R) was grown in the presence of 24 μg/ml of ITC88. Purified virions were normalized to equivalent number of infectious particles using both immunoflorescence of infected cells and protein concentration determination as described previously. Equivalent amounts of infectious virions were then pelleted and resuspended in lysis buffer. The heavy chain of human IgG was detected in VR1814-MSL109R virion lysates in a dose-dependent manner, but not in VR1814 or VR1814-ITC88R virions (Figure 4B). In addition, protein A/G immunoprecipitation of the human IgG from VR1814-MSL109R revealed that it was bound to gH (Figure 4C). These data suggest that the mechanism of antibody resistance is reliant upon the incorporation of the antibody by the virion in a dose-dependent manner.

## MSL-109 Is Selectively Taken Up into Infected Cells and Colocalizes with gH in an Intracellular Compartment

Our data demonstrate that MSL-109 only binds gH when it is associated with the cell. Therefore, in order for MSL-109 to become incorporated into the virion it must bind to gH while it is in its cell-associated form. Previous data have shown that human IgG is taken up into CMV-infected cells (Keller et al., 1976), likely due to virally encoded Fc receptors. To test this with MSL-109, NN-NHDF cells were infected with VR1814 followed by the addition of cell culture media containing 6.6 μg/ml of MSL-109. The cells were then washed and fixed 3 days p.i. To detect the presence of MSL-109, cells were stained with an anti-human AF488-conjugated antibody and colocalized with an anti-gH mouse antibody. There was clear uptake of MSL-109 into cells staining positive for gH, but there was no detectable uptake into cells that showed no staining (Figure 4D). There was also a strong colocalization between MSL-109 and gH, indicating that the two proteins reach the same intracellular compartment. This process, however, was not limited to MSL-109 uptake, as ITC88 was also taken up into infected cells and colocalized in the same compartment as gH. These data confirm that there is a virus-induced mechanism for the uptake of human IgG into cells that allows for the interaction of MSL-109 with gH during the assembly and maturation of the virion.

## Bound MSL-109 Disrupts gH/gL/UL128/UL130/UL131a Function

To investigate the fitness cost of virion-bound MSL-109, we tested the function of the gH/gL/UL128/UL130/UL131a complex. This complex is required for entry into epithelial, endothelial, macrophage, and monocyte cell lines but is not required for entry into fibroblasts (Ryckman et al., 2006). To test its function, we first tried to infect NN-NHDF cells, ARPE-19 cells,

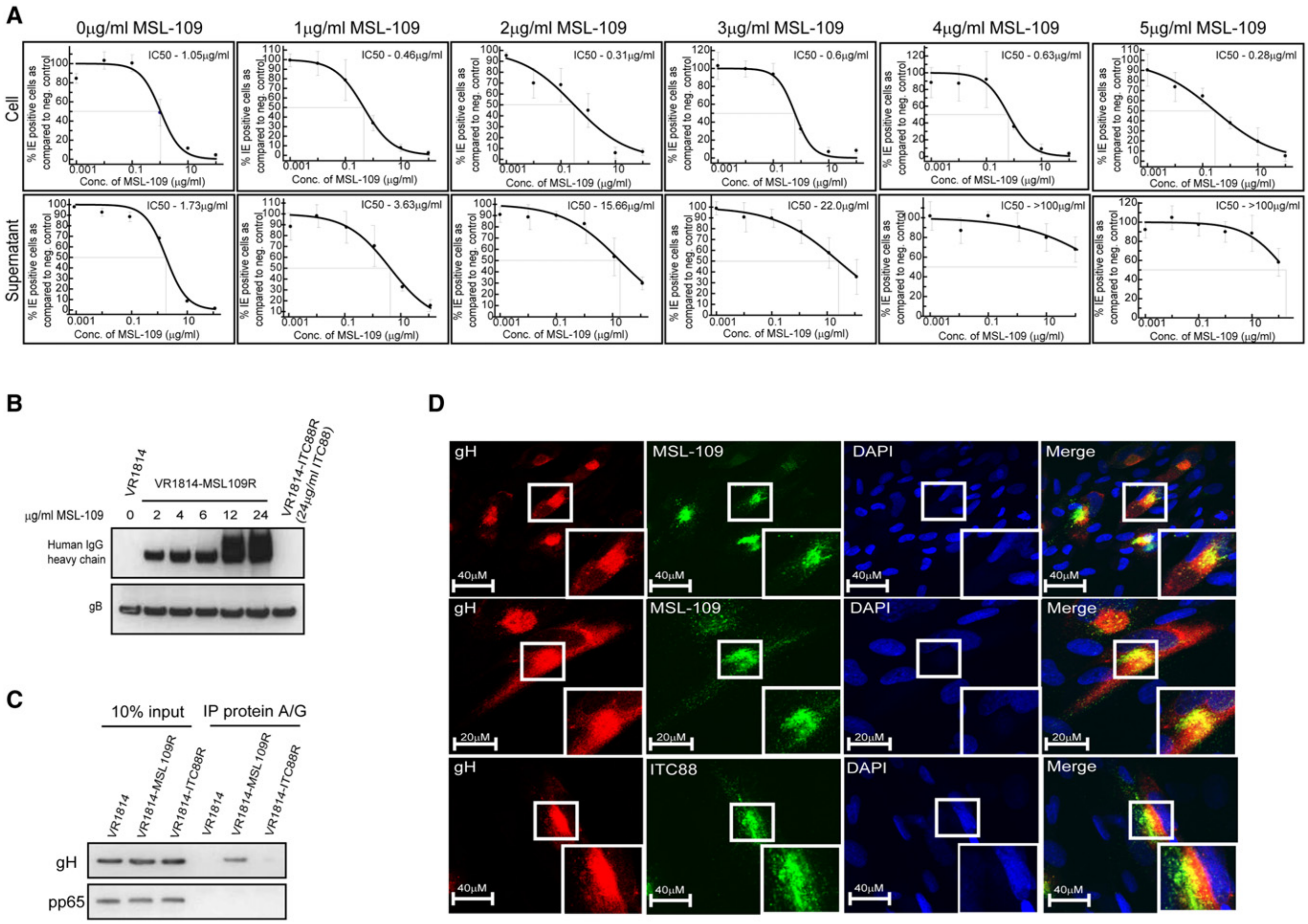


**Figure 4. MSL-109 Is Incorporated into Resistant Virions in a Dose-Dependent Manner**

(A) NN-NHDF cells were infected with VR1814, and 24 hr p.i. cells were treated with increasing concentrations of MSL-109. Nine days p.i. virus was harvested and tested for MSL-109 susceptibility as described above. Error bars represent ±SD.

(B) Equivalent numbers of infectious purified particles were subjected to SDS-PAGE electrophoresis, and the presence of human IgG or gB was detected using an anti-human HRP-conjugated secondary.

(C) Human IgG was immunoprecipitated from virus lysates and blotted for the presence of gH or pp65.

(D) NN-NHDF cells were infected with VR1814 and incubated with 6.6 μg/ml of either MSL-109 or ITC88 for 3 days before being fixed and stained. gH (red) was detected using an anti-gH mouse antibody. MSL-109 or ITC88 were detected using an anti-human AF488-conjugated secondary antibody.

human umbilical vein endothelial cells (HUVECs), or human foreskin fibroblasts (HFFs) with wild-type VR1814, VR1814-MSL109R, or VR1814-ITC88R. VR1814 and VR1814-ITC88R infected all four cell types (Figure 5A). VR1814-MSL109R was only able to infect NN-NHDF and HFF cells, indicating that the virus is defective for entry into nonfibroblast cell types. This is seen in CMV strains that are deficient for gH/gL/UL128/UL130/UL131a function.

Laboratory-adapted strains of CMV, such as AD169, lack the gH/gL/UL128/UL130/UL131a complex and are unable to infect nonfibroblast cell types. The block to infection in these cells is thought to be at the stage of virus fusion and can be overcome by the addition of PEG, which induces fusion of the virus membrane with the plasma membrane. To test if the block to VR1814-MSL109R infection of ARPE-19 cells is at the stage of virus fusion, increasing amounts of VR1814, AD169, or VR1814-MSL109R were added to ARPE-19 cells, and the cells were then treated with PEG as previously described (Ryckman et al., 2006). Twenty-four hours after p.i., the number of IE1/2-positive cells was determined as an indication that a productive virus entry event had occurred. Treatment with PEG resulted in an increase of IE1/2-positive cells for both VR1814-MSL109R and AD169 (Figure 5B), suggesting that like AD169, VR1814-MSL109R lacks a functional gH/gL/UL128/UL130/UL131a complex.

Since the resistance phenotype is reversible, we tested whether the defect in tropism was reversible. ARPE-19 cells infected with VR1814-MSL109R, which had been grown in the absence of antibody and was now susceptible to MSL-109, were monitored for CPE for 21 days. The reverted virus was capable of growing in ARPE-19 cells, and CPE rapidly spread throughout the cell culture at a similar rate to the parental VR1814 (Figure 5C), suggesting that although the incorporation of MSL-109 causes a defect in gH/gL/UL128/UL130/UL131a function, it can be reversed by removal of the antibody.

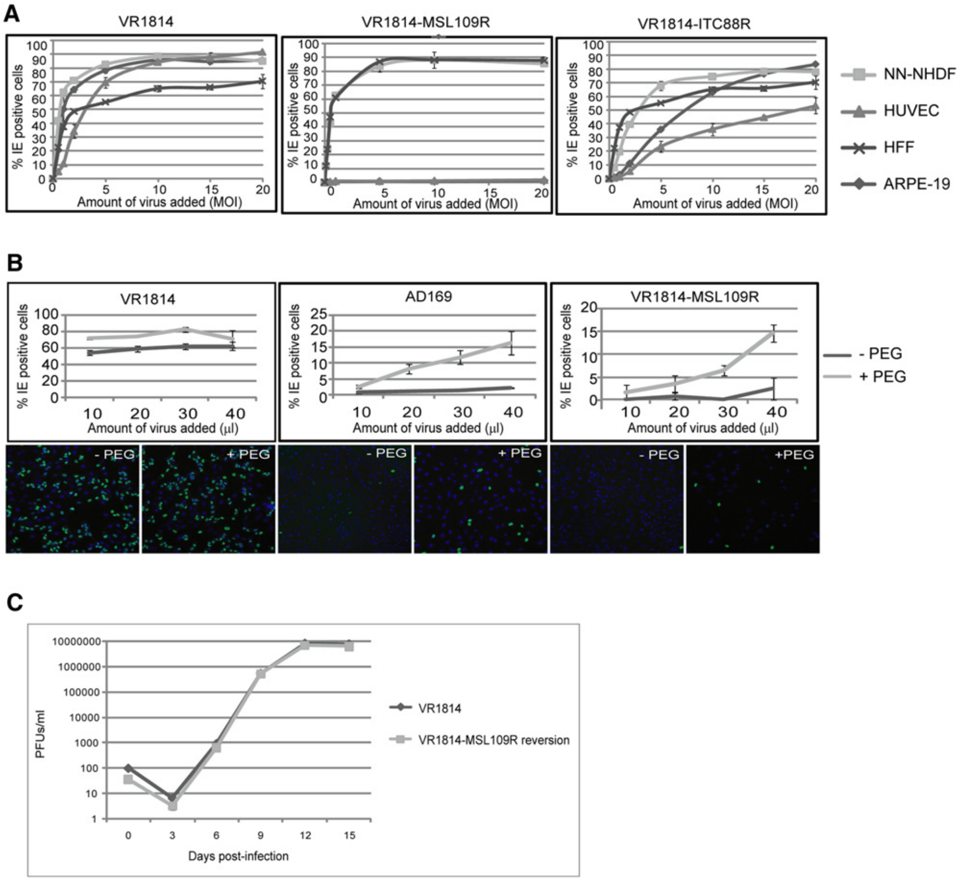


**Figure 5. VR1814-MSL109R Has an Altered Cellular Tropism**

(A) NN-NHDF, HUVEC, HFF, and ARPE-19 cells were infected with increasing amounts of VR1814, VR1814-MSL109R, or VR1814-ITC88R. Twenty-four hours p.i. the cells were stained for IE.

(B) VR1814, AD169, and VR1814-MSL109R were allowed to bind to ARPE-19 cells for 1 hr before the addition of PEG. Twenty-four hours p.i. the cells were stained for IE1/2 protein.

(C) The reverted virus was used to infect ARPE-19 cells, and infection was monitored for 15 days p.i. Error bars represent ±SD.

### VR1814-MSL109R Enters Naive Cells by pH-Dependant Endocytosis and Requires the Fc Domain of MSL-109

Since MSL-109 is attached to the virion but not able to block entry of VR1814-MSL109R, we tested for any differences in the mechanism of entry. We first compared the entry kinetics of VR1814-MSL109R with those of parental VR1814. The resistant virus entered cells at a slower rate than parental VR1814 (Figure 6A). At 3 hr p.i., over 50% of parental VR1814 was resistant to low pH neutralization, while only about 30% of VR1814-MSL109R was resistant. These data indicate a slower rate of entry for VR1814-MSL109R.

CMV enters fibroblasts by direct fusion of the virus envelope with the plasma membrane at neutral pH (Compton et al., 1992). To test if VR1814-MSL109R entry still occurs at a neutral pH, the acidification of endosomal and lysosomal compartments was prevented by treating NN-NHDF cells with nontoxic concentrations of either ammonium chloride or chloroquine. The cells were then infected with either parental VR1814 or VR1814-MSL109R. VR1814 was resistant to both ammonium chloride (Figure 6B) and chloroquine (Figure 6C) treatment. In contrast, VR1814-MSL109R was susceptible to treatment with both ammonium chloride and chloroquine (Figures 6B and 6C). These data confirm that VR1814-MSL109R enters cells using an alternative mechanism that requires low pH conditions.

To test for the involvement of clathrin-dependent endocytosis or lipid raft-mediated endocytosis, including caveolae-mediated endocytosis, cells were treated with either chlorpromazine or nystatin, respectively. Cells were pretreated with nontoxic concentrations of compound for 1 hr prior to infection with either VR1814 or VR1814-MSL109R. As expected, VR1814 infection was unaffected by the presence of either chlorpromazine or nystatin (Figures 6D and 6E). In contrast, VR1814-MSL109R exhibited a dose-dependent decrease in infection in the presence of chlorpromazine (Figure 6D). VR1814-MSL109R was unaffected by treatment with nystatin (Figure 6E). These data suggest that the early stages of VR1814-MSL109R require clathrin-dependent endocytosis.

To determine if the antibody itself was playing a role in VR1814-MSL109R entry, we first tested the ability of an anti-human IgG antibody to block VR1814-MSL109R entry. Fibroblasts were infected with either VR1814 or VR1814-MSL109R that had been incubated with either MSL-109 or an anti-human

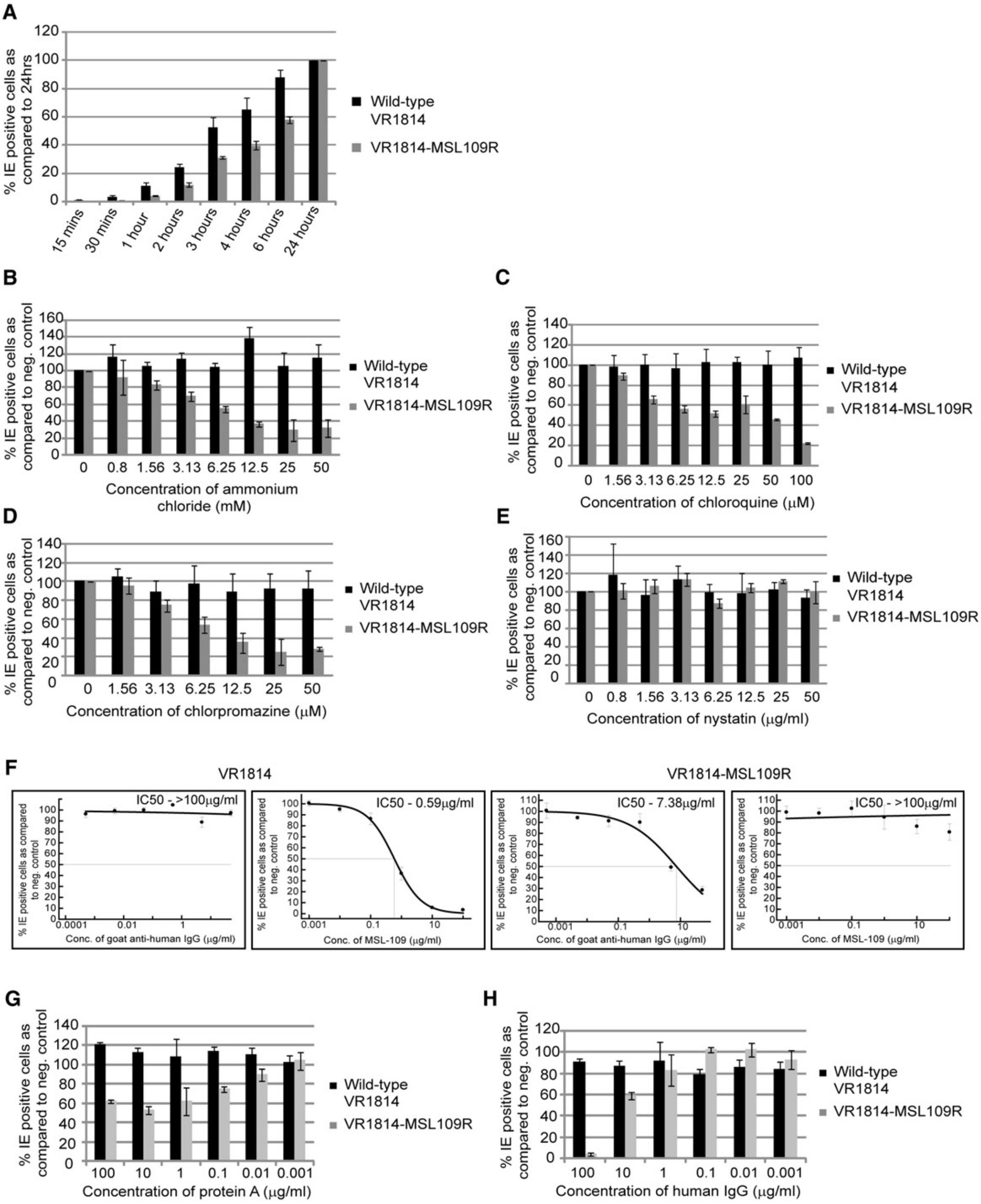


**Figure 6. VR1814-MSL109R Entry Is Ph Dependent and Reliant upon MSL-109 Fc Domain Availability**

(A) VR1814 and VR1814-MSL-109R were allowed to bind to NN-NHDF cells for 45 min at 4°C before being shifted to 37°C. At various time points cells were treated with low pH citrate buffer. Twenty-four hours p.i. cells were stained for IE1/2.

(B–E) Cells were treated with compound for 1 hr prior to infection. Infected cells were detected by IE1/2 staining.

(F) VR1814 or VR1814-MSL109R was treated for 1 hr with either a goat anti-human IgG antibody or MSL-109 before infecting NN-NHDF cells.

(G) NN-NHDF cells were treated with human IgG for 1 hr at 4°C. Infection was allowed to occur at 37°C for 2 hr before cells were treated with low pH citrate buffer.

(H) VR1814 and VR1814-MSL109R were incubated with protein A for 1 hr prior to the infection (see also Figure S2). Error bars represent ±SD.

IgG antibody. VR1814 was blocked by MSL-109 with an $IC_{50}$ value of 0.59 μg/ml but was completely unaffected by pretreatment with the anti-human IgG antibody (Figure 6F). The converse was true for VR1814-MSL109R, which was resistant to MSL-109 but was blocked by the anti-human IgG antibody with an $IC_{50}$ value of 7.38 μg/ml. These data suggest that the Fc domain of

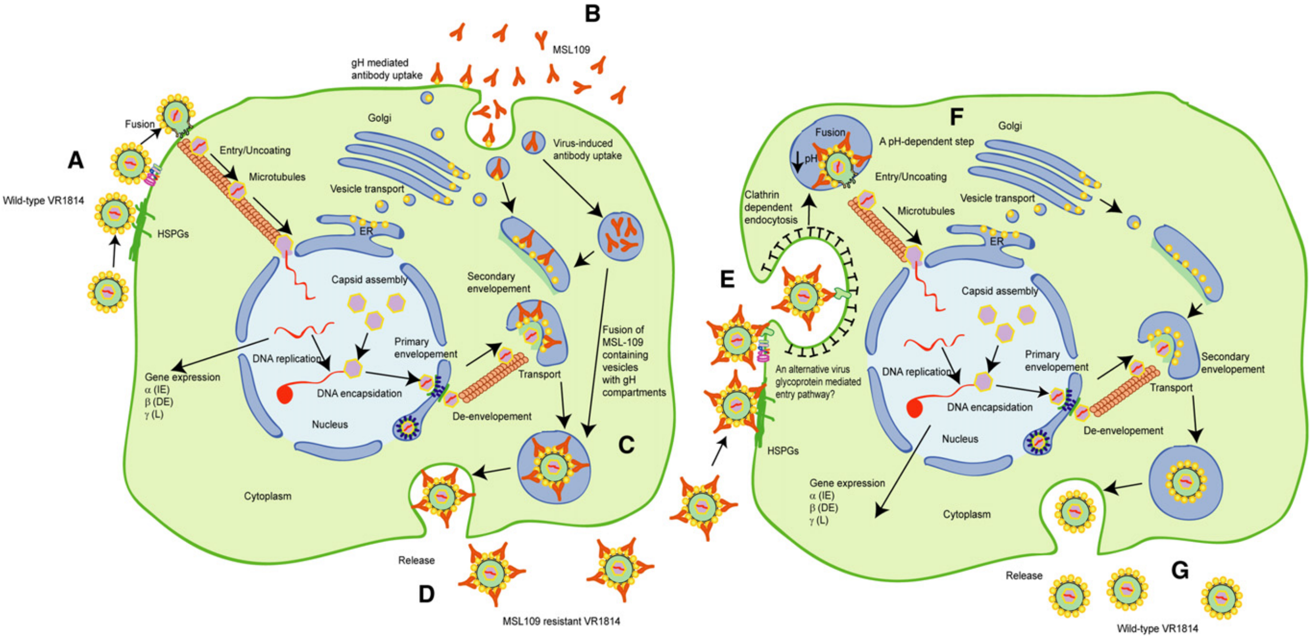

B
MSL109
gH mediated antibody uptake
Fusion
A
Entry/Uncoating
Golgi
Microtubules
Virus-induced antibody uptake
Wild-type VR1814
Vesicle transport
HSPGs
ER
Capsid assembly
Secondary envelopement
Fusion of MSL-109 containing vesicles with gH compartments
Primary envelopement
Gene expression
α (IE)
β (DE)
γ (L)
DNA replication
Transport
DNA encapsidation
Nucleus
De-envelopement
C
Cytoplasm
Release
D
MSL109 resistant VR1814
F
Fusion
A pH-dependent step
Golgi
pH
Entry/Uncoating
Clathrin dependent endocytosis
Vesicle transport
Microtubules
ER
E
Capsid assembly
An alternative virus glycoprotein mediated entry pathway?
DNA replication
Primary envelopement
Secondary envelopement
Transport
DNA encapsidation
HSPGs
Nucleus
De-envelopement
Gene expression
α (IE)
β (DE)
γ (L)
Cytoplasm
Release
G
Wild-type VR1814

**Figure 7. Model of MSL-109 Resistance**

CMV infection (A) of cells triggers the uptake of MSL-109 (B). MSL-109 colocalizes with gH in an intracellular compartment, which allows for the attachment and incorporation of MSL-109 into the virion (C). New virions are released with MSL-109 attached (D) and infect naive cells using the Fc domain of MSL-109 (E). The infection process requires a pH-dependent step (F). New virions generated from cells in the absence of antibody do not contain the antibody and are now susceptible to MSL-109 (G) (see also Figure S3 and Table S3).

the MSL-109 antibody is important for VR1814-MSL109R entry. To confirm this, we tested whether protein A was capable of blocking VR1814-MSL109R infection. NN-NHDF cells were infected with either VR1814 or VR1814-MSL109R which had been preincubated with protein A. Protein A treatment inhibited VR1814-MSL109, but not VR1814, in a dose-dependent manner (Figure 6G). Since both anti-human IgG and protein A were capable of inhibiting resistant virus by binding to MSL109 in the virion, we tested whether saturating Fc binding sites on cells would also inhibit VR1814-MSL109R infection. Cells were incubated with irrelevant human IgG at 4°C before adding virus at 37°C. Irrelevant human IgG inhibited the entry of VR1814-MSL109R but had no effect on parental VR1814 (Figure 6H). These data confirm a role for the Fc domain of MSL-109 in the infection of NN-NHDF cells by VR1814-MSL109R.

## DISCUSSION

MSL-109 is a naturally occurring human antibody which in vitro is capable of blocking CMV infection of multiple cell types by targeting gH glycoprotein complexes, but was unsuccessful as a therapy for CMV disease. In this study MSL-109-resistant CMV was generated to establish whether virus resistance could account for its inefficacy in vivo. Here we demonstrate a mechanism of CMV escape from a neutralizing antibody. The virus generated rapid resistance by the dose-dependent attachment of the antibody to gH complexes within CMV-infected cells, and the resulting resistant virus demonstrated an alteration in the mechanism of virus entry (Figure 7). The resistance mechanism is not reliant upon the generation of a genetic mutation and is reversible upon removal of the antibody. The virus was able to replicate in fibroblast cells to similar levels as wild-type CMV, but there was a fitness cost to the generation of resistance, as the virus was unable to infect nonfibroblast cell types. This defect is not permanent, as the removal of the antibody produces virions capable of infecting epithelial cells. The reversal of this defect, and the lack of a requirement for a genetic change, suggests that in vivo the virus would be able to replicate in a reservoir of cells that do not require the gH/gL/UL128/UL130/UL131a complex for infection even in the presence of the antibody. This mechanism of antibody escape offers perhaps the best explanation for the inability of MSL-109 to block CMV disease progression, and when considering the dose dependence of the escape mechanism in the context of MSL-109-treated patients, it is tempting to speculate that the high doses administered in these studies may have further enhanced the resistance phenotype.

The binding data confirming that gH/gL-containing complexes are the target for MSL-109 demonstrate that it binds to these complexes when they are associated with the cell but not the "resting" virion. The lack of binding to cell-free virions indicates that MSL-109 acts at a postattachment step, requiring the virus to interact with the cell before the antibody can bind to its epitope. This implies that the epitope for MSL-109 is either protected in the virion and is only revealed upon binding to the cell, or that gH/gL complexes undergo conformational changes upon binding to the cell which generate the MSL-109 epitope. This is not uncommon, as other neutralizing antibodies have been found to bind epitopes only exposed during virus entry (Copeland et al., 1986). Further study is required to truly establish the

MSL-109 epitope and the mechanism of neutralization, which will provide useful information about the role of gH/gL complexes in CMV entry.

Our data show that while there is little cost to growth of the resistant virus in fibroblasts, there is a restriction of virus tropism. The loss of tropism likely stems from a defect in the gH/gL/UL128/UL130/UL131a complex, which is known to be required for entry into nonfibroblast cell types. The sequencing data indicate that mutations begin to accumulate in UL131a during MSL-109 selection in fibroblasts, which may explain the loss of tropism, but this is not the case for all of the resistant clones, as some were homogenously wild-type and still showed a restriction in tropism. The reduction in UL128 protein in VR1814-MSL109R virions (Figure 3G) indicates that the binding of MSL-109 to gH/gL complexes may disrupt the proper formation of the gH/gL/UL128/UL130/UL131a complex. This may offer a better explanation for the loss of tropism and explain the ability of the virus to regain its epithelial cell tropism following reversion.

Our data show that the mechanism of resistance to MSL-109 is not a genetic one. This stems from whole genome sequencing in which we were unable to identify any genetic changes which tracked with the resistance phenotype. In addition, the rapid generation and reversion of resistance within a single passage support the conclusion that resistance is not due to a permanent genetic change. Sequencing data did identify accumulating mutations in UL119, but there are multiple reasons to eliminate this as the cause of MSL-109 resistance: first, plaque purification of resistant virus clones should eliminate any heterogeneity in the population with regards to a mutation that confers resistance, but this was not the case for the resistant clones; second, there were no corrective mutations in UL119 following reversion of the resistance phenotype; and third, the ability of F(ab′)2 fragments to neutralize infection (Figure S2) confirms that the gH/gL-targeting CDR region of MSL-109 is responsible for its neutralizing capabilities and that UL119 has no role in the mechanism of neutralization.

While UL119 is not the cause of resistance, the mutation of UL119 may be the result of the resistance phenotype. UL119 and UL118 are virally encoded Fc receptors that are expressed on both the surface of infected cells and in the virion, but both are dispensable for growth in vitro (Atalay et al., 2002; Sprague et al., 2008). It is thought that their function in the virion is to protect it from immune cell recognition by binding to the Fc domain of antibodies bound to the virion. Our data implicate the Fc domain of MSL-109 in facilitating MSL-109-resistant virus infection of naive cells (Figure 6), so the presence of Fc binding receptors in the virion would be detrimental to the fitness of the resistant virus. The accumulation of mutations in UL119 that eliminate it from the virion supports this hypothesis and lends weight to the argument that the Fc domain of MSL-109 is important for the generation of MSL-109 resistance. Although it should be noted that UL118 did not show any mutations in the resistant virus, so further examination of the relationship between UL119, UL118, and MSL-109 resistance is required to establish why UL119 is mutated but UL118 is not.

Our data support a model in which the Fc domain of virus-attached MSL-109 is aiding in the entry of the virus into naive cells. The precise mechanism of how resistant virus enters cells and the role the Fc domain plays in mediating this process remain unclear. Currently our lab is attempting to generate F(ab′)2-resistant virus. Following three passages of virus growth in the presence of 20 times the $IC_{50}$ value for MSL-109 F(ab′)2, no resistance has been observed (data not shown). The difficulties in generating resistance to F(ab′)2 also argue that the Fc domain of MSL-109 has a role to play in the generation of resistance. However, it should be noted that the role of the Fc domain may be 2-fold—first, in the uptake of the antibody into infected cells to generate resistance, and second, the subsequent infection of naive cells by resistant virus. Further experimentation is needed to fully understand how the antibody is being taken up into cells to generate resistance, how the resistant virus is being taken up into naive cells, and what role the Fc domain of the antibody is playing in either of these processes.

Although there is a role for the Fc domain of the antibody in mediating new rounds of infection into naive nonimmune cells, the cellular factors engaged by this IgG-containing resistant virus remain unknown. Other viruses, but not CMV, have previously been shown to utilize the Fc domain of bound antibodies to aid uptake into cells (Halstead et al., 1984). However, there are key differences between antibody-dependent enhancement (ADE) and what occurs with MSL-109-resistant CMV. First, ADE-based mechanisms described to date are defined by the ability of nonneutralizing antibodies to mediate entry. This is not the case for MSL-109-resistant virus, as MSL-109 is capable of neutralizing parental VR1814. Second, in classic ADE-based entry it is the antibody itself that is responsible for the uptake of the virus into immune cells by binding to Fc-$\gamma$ receptors. VR1814-MSL109R does not infect immune cells, such as differentiated THP-1 cells, which express high levels of Fc-$\gamma$ receptors (Figure S3). Third, of the known Fc receptors, NN-NHDF cells only express the neonatal Fc receptor (FcRn). The FcRn is not found on the surface of human cells and is therefore unlikely to be involved in the uptake of the virus into the cell. Previously published data have suggested that CMV may utilize nonneutralizing and low avidity neutralizing antibodies to bind the FcRn and direct the virus across cell monolayers (Maidji et al., 2006). Our data describing MSL-109 uptake into cells and binding to virions suggest that this mechanism may also occur for potently blocking antibodies. Thus, antibody-mediated resistance and transcytosis may be an epitope-driven effect rather than an affinity-driven process. Further study is required to determine the exact role of Fc-mediated uptake by the resistant virus and to determine how common this nongenetic resistance mechanism is among naturally elicited CMV-neutralizing antibodies.

Antibody-based therapies represent a safe addition to the current therapy for CMV disease and provide a potentially promising avenue for treating CMV disease in the congenital setting. However, the failure of MSL-109 in clinical trials called this into question. The implications of our findings for the generation of an antibody therapy for CMV are significant. These data offer a promising explanation as to why MSL-109 did not work in the clinic by showing that CMV can utilize a nonconventional mechanism of antibody escape. However, while this offers the best explanation for MSL-109's ineffectiveness, other explanations, such as antibody exposure in immune privileged sites like the eye, cannot be excluded. If CMV antibodies are to be developed as a therapy for CMV disease, the question remains about how common this mechanism of resistance is and whether it occurs preferentially against certain glycoprotein targets. Our lab has tried to address this question by generating escape viruses to other potently neutralizing antibodies, including to other gH antibodies, and found that the generation of resistance is significantly slower in comparison to the generation of resistance to MSL-109, indicating that this mechanism of escape is not a common one (Table S3). These data would suggest that antibodies targeting other epitopes may prove more successful at offering the high barrier of resistance that is seen with current therapy. What is apparent is the importance of establishing the exact mechanism of antibody neutralization and escape before further development of a neutralizing antibody as a therapy for CMV disease.

## EXPERIMENTAL PROCEDURES

### Cells, Antibodies, Viruses, and Other Reagents

NN-NHDF, HFFs, and ARPE-19 cells were grown according to ATCC guidelines. HUVECs were grown in endothelial basal media (EBM) supplemented with the EGM-2 bullet kit (LONZA). MSL-109, ITC88, 13H11, and 3G16 are human monoclonal IgGs and have all been previously described (Macagno et al., 2009; Nokta et al., 1994; Ohlin et al., 1993). Primary antibodies used include IE1/2 (6F8.2) (Chemicon), gB (1.B.223) (US Biologicals), gB (2F12) (Abcam), pp65 (Chemicon), gH (1.B.224) (Abcam), and DDR1 (C-20) (Santa Cruz Biotechnology). gH, gL, UL130, and UL131a polyclonal rabbit antibodies were used for immunoblotting (Kinzler et al., 2002; Ryckman et al., 2008). A polyclonal rabbit antibody to UL128 was generated by New England Peptides against a KLH-conjugated peptide (CNYNPLYLEADGR). Secondary antibodies included horseradish peroxidase (HRP)-conjugated antibodies against mouse IgG (Millipore), rabbit IgG (Santa Cruz Biotechnology), and human IgG (Jackson ImmunoResearch Laboratories Inc.), and an anti-mouse AP-conjugated secondary (SouthernBiotech). Alexa Fluor-conjugated secondary antibodies were purchased from Invitrogen. VR1814 was propagated as previously described (Nowlin et al., 1991). VR1814-MSL109R and VR1814-ITC88R were propagated in the presence of 6.6 μg/ml and 10 μg/ml of their respective antibodies. Polyethylene glycol (PEG), ammonium chloride ($NH_4Cl$), chloroquine, chlorpromazine, and nystatin were purchased from Fisher Scientific. Protein A and normal human IgG were obtained from Invitrogen. Soluble gH/gL/UL128/UL130/UL131a complex was generated by Redbiotec.

### FACS Analysis

Cells were detached using cell dissociation buffer (Invitrogen) and washed in 1×PBS (0.137M NaCl, 0.0027M KCl, 0.1M, Na2HPO4, 0.002M KH2PO4 [pH7.4]). The cells were resuspended in 1×PBS containing 2% FBS and incubated with primary antibodies (1:500 in 1×PBS containing 2% FBS). The cells were washed three times in 1×PBS and incubated in anti-human Alexa Fluor 647-conjugated secondary antibody (1:1000). The cells were washed three times in 1×PBS and resuspended in 150 μl of 1×PBS with propidium iodide (Invitrogen). Florescence was measured using a BD Biosciences LSRII Cell Analyzer.

### Immunoprecipitation

NN-NHDF cells were infected with adenovirus particles expressing VR1814 glycoproteins (20 PFUs/cell). Protein expression was allowed to proceed for 72 hr before the cells were washed in 1×PBS and lysed in 1% NP-40. Protein (500 μg) was incubated with 5 μg of antibody for 16 hr at 4°C. Alternatively, equivalent numbers of virions were semipurified through a 20% sorbitol cushion before being lysed as described above. Complexes were immunoprecipitated using the Classic IP Kit (Pierce Biotechnology) according to the manufacturer's protocol. The complexes were washed four times in lysis buffer. Samples were boiled for 5 min at 100°C before immunoblotting.

### ELISA Assays

Soluble gB or gH/gL/UL128/UL130/UL131a complexes were diluted in 1×PBS to a concentration of 5 μg/ml. VR1814 was partially purified through a 20% sorbitol cushion and resuspended in 1×PBS. Fifty microliters of either soluble protein or diluted virus was added to each well of a MaxiSorp 96-well flat-bottomed plate (Nunc) and allowed to absorb overnight at 4°C. Unbound protein was removed and the plate washed three times with 1×PBST. Plates were blocked in 1×PBS with 5% milk (Bio-Rad) for 2 hr. Serial dilutions of the antibodies were made in 1×PBS containing 1% BSA (Fisher Scientific). Primary antibody (50 μl/well) was incubated at room temperature for 1 hr. The plate was washed three times with 1×PBST, and primary antibody was detected using a 1:5000 dilution of anti-human HRP-conjugated antibody for 1 hr at room temperature. The plate was washed three times with 1×PBST, and signal was detected with SureBlue TMB substrate (KPL). Plates were read at 650 nm by a Spectramax 340PC plate reader.

### Immunofluorescense

For confocal microscopy, cells were grown on tissue culture-treated chamber slides (BD Falcon) and fixed in 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X and incubated with mouse anti-gH antibody (1:500) for 2 hr. Cells were washed three times in 1×PBS and incubated with anti-mouse AF546 (1:2000), anti-human AF488 (1:1000), and DAPI (1:5000) for 1 hr. The slides were washed three times in 1×PBS and mounted with coverslips (Fisher Scientific) using fluorsave (Calbiochem). A Zeiss LSM 510 Confocal Microscope was used for imaging. For antibody neutralization assays, 12,000 cells per well were plated in 96-well plates and infected with virus that had been preincubated with antibody. Twenty-four hours p.i., cells were stained as above using a mouse anti-IE antibody (1:2000) and an anti-mouse Alexa Fluor 594 secondary antibody (1:3000). The number of IE-positive cells was counted using Cellomics Arrayscan. The $IC_{50}$ values of the antibodies were generated using XLfit.

### Plaque Assay

$2 \times 10^5$ NN-NHDF cells were plated per well of a 24-well dish. Virus was diluted to $10^{-2}$ in serum-free media, and 10-fold dilutions were made out to $10^{-7}$. Cells were washed three times in serum-free media before virus was added. Three hours p.i. the media was removed and cells were overlaid with 1.5 ml of a 1:1 mix of 2×DMEM and 1.2% agarose. An additional 1.5 ml of 1×DMEM was added to each well. The VR1814-MSL109R virus was maintained in 6.6 μg/ml of MSL-109. The cells were left for 14 days at 37°C in 5% $CO_2$. Cells were washed and fixed for 10 min in 100% methanol at room temperature. Cells were permeabilized for 10 min in 1×PBS containing 0.5% Triton X and stained for gB using an anti-CMV gB antibody (US Biologicals) at 1:500 for 1 hr. Signal was detected using a goat anti-mouse AP-conjugated secondary (SouthernBiotech) and BCIP/NBT solution (KPL Research).

### Genome Sequencing

Genomic DNA was fragmented using a Covaris S2 sonication device (K-Biosciences). DNA was subjected to end repair and linker ligation as per the SOLiD 3+ recommended procedure (Applied Biosystems). The library was size selected, and molecular barcodes were added to each sample (Life Technologies, Foster City). The libraries were quantitatively pooled and 6 pmoles attached to magnetic beads (Life Technologies, Foster City). Emulsion PCR, bead enrichment, purification, and slide deposition were performed as recommended (Life Technologies). The beads were checked for polyclonality using a standard WFA run on the SOLiD3+ system. The production flow cell was deposited and samples subjected to 50 bases of ligation-mediated sequencing on the SOLiD3+ system.

Sequences were aligned to the reference (GU179289.1) using the BFAST algorithm as follows: SOLiD CSFASTA files and QUAL files were converted to FASTQ format using the BFAST utility program "solid2fastq." Ten BFAST reference genome indices were built using the recommended masks. The BFAST match, localalign, and postprocess routines were run. Reads with a unique best-scoring alignment were output in SAM format. The GATK (The Genome Analysis Toolkit) package was used to call SNPs and INDELs (Homer et al., 2009a, 2009b). The SAM files from BFAST were recalibrated as recommended. Local realignment was run on candidate sites detected by the software. SNPs and INDELs were called separately by "UnifiedGenotyper" and "IndelGenotyperV2."

### Quantitative Real-Time PCR

Cells were infected for 3 days before total RNA or DNA were harvested using the QIAGEN RNeasy mini kit or the QIAGEN Blood DNA extraction kit. RNA was reverse transcribed by Taqman Reverse Transcription Reagents (Applied Biosystems). DNA copy number was detected using Taqman Gene Expression Master Mix and a 7900HT Fast Real-Time PCR system (Applied Biosystems). Primers and probes for each target gene were designed using Primer Express software (Applied Biosystems) against conserved regions (Supplemental Experimental Procedures). The probes were labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) (Integrated DNA Technologies).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chom.2011.07.010.

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