Specialization to cell-free or cell-associated spread by BAC-cloned HCMV strains not determined by the UL128-131 and RL13 loci.

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A widely held view is that clinical isolates of human cytomegalovirus (HCMV) are cell-associated and that mutations affecting the UL128-131 and RL13 loci arise during subsequent passage in culture and lead to the appearance of a cell-free spread phenotype. To distinguish the factors influencing cell-associated and cell-free spread, we analyzed the spread characteristics of three HCMV BAC-clones; Merlin (ME), which expresses high levels of UL128-131 and harbors a frameshift mutation within RL13, and TB40/e (TB) and TR, which are both low in UL128-131 and intact at RL13. Quantitation of the number of newly infected cells over 12 days by flow cytometry revealed remarkably similar spread efficiencies in fibroblasts among strains. However, comparing the inhibition of spread by neutralizing antibodies and the quantities and infectivity of progeny virus indicated that TB and TR spread was predominately cell-free, whereas spread of ME was predominantly cell-associated. While transcriptional repression of UL128-131 greatly enhanced cell-free spread by ME, the efficiency of the cell-associated mode was not affected. Spread in epithelial cultures was highly cell-associated for all strains, and ME was the most efficient. Repression of UL128-131 reduced the efficiency of ME spread in epithelial cells, but did not affect the predominate mode of spread. Spread in RL13-expressing cells was comparably reduced for all strains, and more pronounced in fibroblasts than in epithelial cells. RL13 effects could not be clearly explained by changes in production, release, or infectivity of progeny virus, and there were no changes to the proclivity of strains for cell-free or cell-associated spread. In sum, the specialization of HCMV strains to cell-free spread is linked to the quantity and infectivity of cell-free progeny, which can be influenced by UL128-131 levels, but the cell-associated specialist phenotype is likely determined by factors beyond the UL128-131 or RL13 loci.

Experimental distinctions between cell-free and cell-associated modes of spread for HCMV have been largely relativistic. When cell-free spread is inhibited by neutralizing antibodies, or if particular strain of virus is poor at cell-free spread, then the observed spread may be simply defined as “cell-associated”. However, such a view does not easily lend towards analysis of the efficiency of cell-associated spread or the factors involved. In our study, we measured the kinetics of HCMV cell-free and cell-associated spread as independent processes and show evidence that HCMV strains can be highly specialized to one or the other mode of
spread. The genetic factors that determine specialization for mode of spread are unclear, but given the genetic diversity of HCMV circulating in human populations, it seems likely that both modes of are represented. The efficacy of intervention approaches is likely affected by the mode of spread. For example, neutralizing antibodies are less effective to limit cell-associated spread. Our results provide a conceptual approach to evaluating intervention approaches such as neutralizing antibodies raised by vaccine candidates and drug compounds that target viral replication processes for their ability to limit cell-free or cell-associated modes of spread as independent processes.
INTRODUCTION

Intervention approaches to control human cytomegalovirus (HCMV) infection, including DNA replication inhibitors such as ganciclovir, and vaccines designed to elicit neutralizing antibodies have had limited success (1, 2), and this may be due in part to the complex genetic diversity of HCMV circulating within human populations (3–9). Basic annotations of the 235kbp HCMV genome identify 165 canonical open reading frames (ORFs), although there is evidence of extensive transcription and translation beyond these loci (10, 11). While nucleotide polymorphisms are found throughout the genome, most sequences are well conserved. 21 of the 165 canonical ORFs show high nucleotide diversity and are distributed as islands throughout the genome. Several groups have reported evidence of frequent recombination within the more conserved regions, which may effectively mix and match the more diverse loci into many different genotype combinations (3, 4, 7, 8). Adding to this complexity, several studies show evidence of gene inactivating mutations (pseudogenes), and gene deletions (7, 8). While some infected individuals may harbor relatively pure populations of HCMV genotypes, complex, multiple genotype infections, and sequential infections by genetically distinct populations also occur. Cloning of HCMV from clinical samples on bacterial artificial chromosomes (BAC) has resulted in numerous, genetically distinct strains for use in laboratory studies. It is not clear how the genetic and phenotypic differences among BAC-cloned HCMV might reflect the natural diversity of HCMV.

The glycoprotein (g) H/L complex is functionally conserved among the herpesvirus family and is involved in the initial receptor engagement and the regulation of the membrane fusion protein gB (reviewed in (12)). The HCMV gH/gL is present in the virion envelope as a gH/gL/gO complex (13, 14), a gH/gL/UL128-131 complex (15–18), and in complex with gB (19). Transient expression of HCMV gH/gL and gB is sufficient to drive cell-cell fusion, but is unclear which forms of gH/gL contribute to membrane fusion during HCMV infection (20). The gH/gL/gO and gH/gL/UL128-131 complexes bind to various cell surface receptors through the gO and UL128-131 domains (21–23) and these interactions are important for entry into a broad range of cell types. gH/gL/gO is likely critical for infection of most, or all cell types, fibroblasts, epithelial and endothelial cells being the most extensively studied (24–27). Mutants of HCMV lacking gO are deficient at adsorption, and soluble gH/gL/gO blocks virus adsorption (25, 28). In contrast, gH/gL/UL128-131 is dispensable for infection of fibroblasts and neuronal cells but critical for epithelial and endothelial cells,
monocyte-macrophages, and dendritic cells (16, 18, 29–31). Soluble gH/gL/UL128-131 did not block virus adsorption, but there is evidence that engagement of receptors can elicit signal transduction pathways that influence the nature of the resulting infection pathway (21, 28, 30). Murine and guinea pig CMVs contain homologous gH/gL complexes that play similar roles in cell tropism, although there are some differences in the reported requirements for the complexes for infection of different cell types (32–37).

We and others have noted striking phenotypic variation among three HCMV strains, TB40/e (TB), TR, and Merlin (ME) in terms of content of the gH/gL complexes in the virion envelope and the corollary effects on entry and cell type tropism. TB and TR contain gH/gL mostly in the form of gH/gL/gO, and very little gH/gL/UL128-131, whereas ME virions contain overall lower amounts of gH/gL and this is mostly in the form of gH/gL/UL128-131 (27). The genetic polymorphism(s) responsible for these differences are unclear. Murrell et al. described a G>T mutation in the UL128 locus of TB that when engineered into ME, reduced the assembly of gH/gL/UL128-131 through effects on mRNA splicing (38). However, this did not fully explain the observed strain differences since TR is also low in gH/gL/UL128-131 but is congenic to ME at this locus (27). Zhang et al. showed that expression of gO during replication is lower in ME-infected cells as compared to TR, but again the genetic correlates of this difference were not clearly identified (39). The cell-free infectivity of HCMV strains on both fibroblasts and epithelial cells correlates with the amounts of gH/gL/gO in the virion envelope; TB is by far the most infectious, followed by TR, while ME virions are poorly infectious (27). Repression of the UL131 promoter in ME resulted in virions with dramatically reduced amounts of gH/gL/UL128-131, somewhat higher levels of gH/gL/gO, and dramatically improved cell-free infectivity (27, 40). It seems likely that the low infectivity of cell-free ME at least partially reflects a role for gH/gL/gO in virion adsorption (25, 28).

HCMV can spread through monolayer cell cultures by diffusion of cell-free virus in the culture supernatant or by a more cell-associated mode, but the mechanistic distinctions between these modes of spread are not well characterized. Strains vary with respect to their apparent cell-free and cell-associated nature. ME spreads with a characteristic cell-associated pattern whereas TB shows more cell-free spread (40, 41). While this basic observation can be explained by the respective low and high infectivity of ME and TB virions described above, it is not clear how strains compare in their ability to spread by cell-associated modes, nor are the genetic correlates of cell-associated spread well defined. Multiple reports have monitored
foci size in the presence of neutralizing antibodies and attributed the cell-associated nature of ME to the expression of the UL128-131 and RL13 loci (24, 42). It seems clear that either gH/gL/gO or gH/gL/UL128-131 is required for cell-associated spread on fibroblasts, whereas gH/gL/UL128-131 seems to be required for cell-associated spread in epithelial or endothelial cells (24, 26, 43, 44). Moreover, the pathway of cell-associated spread for HCMV has not been extensively studied. There have been suggestions of limited fusion between infected and uninfected cells allowing the transfer of subviral components, but the efficiency of these processes to facilitate the spread of HCMV infection is not clear (45–47). Syncytium formation has been observed in HCMV infected cultures, but it is not clear whether these cell-cell fusions contribute to viral spread, or merely represent the coalescence of late-stage infected cells long after progeny virus has exited and spread to adjacent cells (48, 49). Here, we report the use of flow cytometry to compare the spread of HCMV TB, TR, and ME in fibroblasts and epithelial cells. The results showed that these strains are specialists for either the cell-free or the cell-associated modes, and that this specialization is not determined by the UL128-131 and RL13 loci.

RESULTS

HCMV strains exhibit differences in focal pattern and spread efficiency in fibroblasts and epithelial cell cultures. We previously described dramatic differences among BAC-cloned HCMV strains TB, TR, and ME in the levels of gH/gL complexes displayed on the virion envelope, and the corollary effects on infectivity of extracellular virions, which impact the cell-free mode of spread (27, 39, 50). To extend comparative analyses to include both cell-free and cell-associated modes of spread, visual patterns of focal growth were documented under conditions where either mode of spread was accessible. Cultures of fibroblasts or epithelial cells were infected at low multiplicities with GFP-expressing TB, TR, or ME, and spread through the cultures was monitored by fluorescence microscopy over 18 days (Fig 1). In fibroblasts, the spread of all three strains was localized to small, tight foci for up to 12 dpi. By day 18, TB and TR foci were larger and more diffuse, whereas ME foci remained smaller and more localized (Fig 1A). In contrast, foci of all three strains in epithelial cells were more similar to one another throughout the experiment, smaller and more tightly localized than in fibroblasts (Fig. 1B). The diffuse appearance of TB and TR foci on fibroblasts suggested efficient cell-free spread whereas the localized ME foci suggested a more cell-
associated mode of spread on these cells. In contrast, all three strains appeared more cell-associated in epithelial cultures.

For quantitative comparisons of spread, experiments similar to those described for Figure 1 were performed, but instead of microscopy, flow cytometry was used to measure the number of infected (GFP-expressing) cells over time (Fig 2). In fibroblasts, the number of infected cells for both TB and TR increased exponentially over 12 days, and fit well to a log-linear regression, indicating a constant rate of spread over the course of the experiment (Fig 2A, B). In contrast, ME spread somewhat faster between days 3 and 6, and slower between days 9 and 12. Despite, average spread rates (LN GFP+ cells/day), as indicated by the regression slopes (m), were within 1.2-fold among strains (Fig. 2C). Spread rates for all three stains were lower in epithelial cells than in fibroblasts, and the strains were more different form one another. ME was approximately 2-fold faster than TR and 1.5 fold faster than TB (Fig 2D-F). While differences in the average spread rate among strains on either cell type were small, they were statistically significant (one-way ANOVA: fibroblast, p=0.0176; epithelial, p <0.0001).

HCMV strains have strong preferences towards either cell-free or cell-associated modes of spread. To assess the contribution of cell-free and cell-associated virus to the observed spread characteristics, the quantity and infectivity of progeny virus in fibroblast and epithelial cell cultures was determined (Fig 3 and 4). Note that since these experiments were intended to correlate with the spread characterizations described above, infectivity of fibroblasts-derived virus was determined on fibroblasts cultures, and infectivity of epithelial-derived virus was determined on epithelial cell cultures.

In fibroblasts, TB, TR, and ME generated similar numbers of progeny genomes both within the cells and in culture supernatants (Fig 3A and C). This suggested that DNA replication, nucelocapsid assembly, and egress pathways in these cells were comparable for all three strains. In contrast, the specific infectivity of the progeny was dramatically different among strains. For the cell-free progeny, TB was the most infectious with specific infectivity of approx. 40 genomes per infectious unit (IU), TR was over 100-fold less infectious (6000 genomes/IU), and ME was by far the least infectious (>10^6 genomes/IU) (Fig. 3B). These comparisons of infectivity were consistent with previous analyses, and were likely due at least in part to the described differences in the levels of gH/gL/gO and gH/gL/UL128-131 (27). Sonication of infected cells was performed to determine the infectivity of cell-associated virus. However, specific infectivity analysis of cell-associated
virus was complicated by the fact that a large number of intracellular viral genomes likely remain in the nucleus and are unlikely to contribute to infectivity. Thus, nuclei were removed and the quantity of viral genomes remaining in cytoplasmic fractions was determined. When analyzed in this manner, intracellular virus infectivity resembled that of the cell-free virus with TB the most infectious, followed by TR, and ME was by far the least infectious (Fig 3D).

In epithelial cells, the release of cell-free virus was notably different among strains (Figs 4A and C). ME released 80 virions per cell compared to 12 for TB and only 2 for TR (Fig 4A). The specific infectivity of cell-free, epithelial-derived TB (epi-TB) was approximately 600 genomes/IU whereas cell-free epi-TR and epi-ME were 3 and 5-fold less infectious, respectively (TR=1600 genomes/IU; ME=3000 genomes/IU) (Fig 4B). Note that while TB released 6-fold fewer cell-free progeny than ME, the cell-free TB progeny were 5-fold more infectious than the ME progeny (compare Figs 4A and B). This indicates that TB and ME generate comparable amounts of total infectivity into epithelial cell culture supernatants. In contrast, TR produced far less total cell-free infectivity in these cultures since the low quantity of progeny released was not compensated by better infectivity. The accumulation of epithelial cell-associated progeny was comparable among strains and the cell-associated infectivity was within the range of 15,000-45,000 genomes/IU for all strains (Fig 4C and D.).

To further evaluate the contribution of cell-free and cell-associated spread mechanisms, neutralizing antibodies were used with the rationale that they inhibit cell-free spread more potently than cell-associated spread. Neutralizing antibodies chosen for these experiments were mAb 14-4b, which likely targets a discontinuous epitope at the membrane proximal region of gH (51, 52), as well as a mixture of rabbit anti-peptide sera that target UL130 and UL131 (17). The relative potency of these antibodies to neutralize cell-free TB, TR, and ME was verified in neutralization experiments (Fig. S1). Spread rates of each strain in either fibroblast or epithelial cell cultures were then determined in the presence or absence of anti-gH mAb 14-4b or anti-UL130/131 sera at concentrations sufficient for complete neutralization of cell-free virions. To demonstrate that the observed spread required production of progeny viral genomes and was not the result of transfer of cytoplasmic contents from infected to uninfected cells, spread in the presence of ganciclovir was also determined (Fig 6).
In fibroblasts, anti-gH 14-4b reduced the spread rate of TB and TR to 30% and 45% of control, respectively (Fig 5A-B). ME was notably more resistant to mAb 14-4b, with a spread rate of 75% of control (Fig 5C). This was especially noteworthy since cell-free ME was more sensitive to neutralization than either TB or TR (Fig. S1). Consistent with the dispensability of gH/gL/UL128-131 in fibroblasts, anti-UL130/131 sera had no effect on the spread rates of any of the three strains in fibroblasts (Fig 5, A-C). The residual spread of all strains in the presence of neutralizing antibodies was greater than with ganciclovir treatment, even for TR, which is known to harbor resistance mutations in the UL97 kinase (53). These results support the hypothesis that spread of TB in fibroblasts predominately involved cell-free virions that were sensitive to neutralizing antibodies, whereas ME spread predominately in a manner not sensitive to antibody neutralization (i.e., not by cell-free virions). This interpretation is consistent with the earlier observations that while all strains release similar numbers of cell-free progeny, ME virions are far less infectious, and thus less likely to contribute to efficient cell-free spread (Fig 3). The intermediate antibody sensitivity of TR spread might indicate an intermediate contribution of cell-free and cell-associated spread mechanisms. However, it may also reflect the lack of complete neutralization of cell-free TR (Fig S1).

In epithelial cell cultures, the presence of anti-gH 14-4b had no effect on the spread rates for TB, TR, or ME, and anti-UL130/131 had a marginal effect, with a 30% reduction for TR being the largest (Fig 5D-F). If cell-free virus significantly contributed to spread in epithelial cells, inhibition by both anti-gH and anti-UL130/131 would have been expected since both antibodies potently neutralized cell-free virus of all three strains on these cells (Fig S1). This suggests that the observed spread of all strains in epithelial cells predominately involved a cell-associated mode that was resistant to neutralization of cell-free virus. The modest inhibition by anti-UL130/131 may suggest differences in the specific mechanisms blocked by these antibodies compared to anti-gH 14-4b. While both types of antibodies can evidently block cell-free infection, the anti-UL130/131 appears to be able to inhibit the cell-associated mode, albeit less efficiently than they block cell-free infection. This interpretation is consistent with conclusions of Murrell et al. that cell-associated spread of ME may be blocked by antibodies, but less potently (42).

In sum, the results of this set of experiments indicate; 1) HCMV can spread in fibroblast cultures with similar efficiencies through either a cell-free mode, exemplified by TB, or a cell-associated mode, exemplified by ME. 2) In APRE19 epithelial cells, spread by all three strains was predominantly cell-associated, but
efficiencies varied, ME was by far the most efficient. 3) Efficient cell-free spread (as exemplified by TB in fibroblasts) correlated with the detection of higher quantity and infectivity of cell-free progeny in culture supernatants. Conversely, efficient cell-associated spread (exemplified by ME in either fibroblasts or epithelial cells) did not correlate with the quantity or infectivity of cell-associated progeny in cell sonicates.

The specialization to cell-associated spread of HCMV ME is not determined by high levels of gH/gL/UL128-131. The cell-associated nature of ME has been linked to the high expression levels of UL128-131 and the corollary poor infectivity of cell-free ME virions (24, 27, 40, 42). To address this in our quantitative system, we made use of the previously described tetracycline (Tet)-repression system developed by Stanton et al. (40). Briefly, the BAC clone of ME used in these studies contained tetracycline-operator (TetO) sequences in the promoter of UL131. We previously showed that replication of this recombinant ME in fibroblasts expressing the tetracycline-repressor (TetR) protein produced extracellular virus with amounts of gH/gL/UL128-131 at or below that of TB and TR, marginally higher amounts of gH/gL/gO, and these changes in glycoproteins greatly improved cell-free infectivity (27). In order to test the role of high UL128-131 expression for ME spread, experiments were conducted using these TetR-expressing fibroblasts (HFFFtet), as well as a newly generated TetR-expressing ARPE19 epithelial cell line (ARPEtet). Both HFFFtet and ARPEtet showed efficient repression of a TetO-regulated luciferase reporter (Fig. 6A). Consistent with our previous characterizations, ME virions produced in HFFFtet cells contained drastically reduced amounts of gH/gL/UL128-131, increased gH/gL/gO, and infectivity was dramatically improved (Fig 6B and C). Similarly, ME virions produced in ARPEtet cells contained dramatically reduced amounts of gH/gL/UL128-131 compared to virions from control ARPE cells, but did not contain increased gH/gL/gO, or display enhanced infectivity as was observed for fibroblasts-derived virus (Fig 6B). Note that even without repression of gH/gL/UL128-131, the specific infectivity of epi-ME on epithelial cells was dramatically better than fib-ME on fibroblasts (Fig 6C).

In fibroblasts, gH/gL/UL128-131 repression did not influence spread rates or the sensitivity of spread to neutralizing antibodies (Fig 7A). Thus, the efficient cell-associated spread of ME in fibroblasts does not require high levels of gH/gL/UL128-131. In epithelial cells, repression of gH/gL/UL128-131 resulted in a modest decrease in average spread rate (from 0.38 to 0.29), and small increase in the sensitivity of spread to anti-UL130/131, (from 10% to 30% inhibition) (Fig 7B). The reduction in spread was consistent with the
important role of gH/gL/UL128-131 for HCMV in these cells. On the other hand, the increase in antibody sensitivity was more complicated to interpret. One possibility is that this reflected a shift towards increased cell-free spread. This seems unlikely since repression of gH/gL/UL128-131 did not enhance cell-free infectivity (Fig 6). Alternatively, it may be that reduced gH/gL/UL128-131 expression allowed for more efficient blocking of the cell-associated spread by these antibodies. In any case, ME spread in ARPEtet cells was still highly resistant to antibodies as compared to the spread of TB in fibroblasts (compare 30% antibody resistant spread for TB in fibroblasts; Fig 5A, to 70% resistant for ME in ARPEtet; Fig 7B). Overall, these results indicated that while reduction of gH/gL/UL128-131 may enhance cell-free modes of spread, at least on fibroblasts, high expression levels of the gH/gL/UL128-131 could not explain the highly specialized cell-associated spread of ME.

Spread inhibition due to RL13 expression is more pronounced in fibroblasts than in epithelial cells, and influences both cell-free and cell-associated modes of spread. The RL13 ORF encodes an envelope glycoprotein that has been described as an inhibitor of HCMV replication in culture and mutations in RL13 have been documented during cell-free passage in fibroblasts and epithelial cells (40, 54, 55). While the ME recombinant used in the present studies harbors such a RL13 mutation, the RL13 expression status of the TB and TR BAC-clones is unclear. Both TB and TR have intact RL13 ORFs confirmed by DNA sequencing, but lack of quality antibodies makes protein detection difficult. To compare the effect of RL13 on the spread of all three strains, and to avoid potential selection of RL13 mutants during spread experiments, fibroblast and epithelial cell lines that express RL13 were engineered. Immunoblot analysis demonstrated that these cell lines expressed both mature and immature proteoforms, and flow cytometry confirmed similar expression levels (Fig S2). The effects of RL13 expression on spread rates, and the quantity and infectivity of cell-free and cell-associated progeny virus were then analyzed (Fig 8).

In fibroblasts, RL13 expression resulted in a comparable 40-50% reduction in spread rate for all three strains (Fig 8A). Inhibition of spread by neutralizing antibodies was not affected for TB or TR, but spread of ME in RL13 expressing cells was approximately 50% more sensitive than in control cells (Fig 8B). These observations could not be universally explained by effects on progeny virus production since effects of RL13 expression on these parameters were generally small and varied among strains (Fig 8C). Moreover, the only
statistically significant change in progeny infectivity due to RL13 expression was observed for cell-free TB, which was 6-fold less infectious than virus produced from control fibroblasts (Fig. 8D).

In epithelial cells, RL13 expression reduced spread by approximately 20% for all three strains, but this only reached statistical significance in the case of ME (Fig 8E). Spread of TB was notably more sensitive to antibody inhibition, whereas TR and ME were less affected (Fig 8F). As in fibroblasts, these effects were not readily attributable to the quantity or infectivity of progeny since 1) there were no significant changes to the quantity of cell-associated or cell-free progeny virus for any of the three strains (Fig 8G), and 2) the only notable effect on progeny infectivity was in the case of cell-free ME, which was 120-fold less infectious when produced in RL13-expressing epithelial cells as compared to control cells (Fig. 8H).

To determine if the observed reduction in infectivity of cell-free ME produced in RL13 expressing epithelial cells was related changes in the virion content of gH/gL complexes or gB, equal quantities of fibroblast- or epithelial-derived extracellular virions of ME were subjected to non-reducing SDS-PAGE and immunoblot analysis with antibodies specific for gL to detect gH/gL complexes, or with anti-gB monoclonal antibodies that react with both the full length 165-170 kDa form and the 55 kDa portion of the furin-cleaved form of gB (56–58) (Fig 9). Fib-ME virions contained gH/gL primarily in the form of gH/gL/UL128-131, and most of the gB was in the furin-cleaved form. RL13 expression in fibroblasts seemed to have little effect on either gH/gL complexes or gB. However, there were notable differences in epithelial cells. First, epi-ME virions contained greater amounts of gH/gL/UL128-131 than fib-ME, and this was somewhat reduced by RL13 expression. Secondly, epi-ME had more uncleaved gB, whereas ME virions produced by RL13 expressing epithelial cells had less overall gB, and it was mostly furin-cleaved. Together, these results indicated that the processing and incorporation of gH/gL complexes and gB into cell-free progeny can be influenced by the cell type, and in epithelial cells, this may be further affected by RL13 status. While the effects on gB seem to correlate with the observed reduction in ME infectivity in RL13 epithelial cells, a strong causal connection would be a premature conclusion.

In sum, these results indicate that 1) RL13 expression tempers the spread of HCMV in fibroblasts by either the cell-free mode exemplified by TB, or the cell-associated mode exemplified by ME, 2) The effect of RL13 on spread in epithelial cells is less dramatic than in fibroblasts, 3) effects of RL13 on progeny production and release from either cell type are generally small, and 4) RL13-expression in epithelial cells dramatically
reduces the infectivity of cell-free ME progeny, and this is coincident with changes in gH/gL complexes and gB.

**DISCUSSION**

Distinctions between cell-free and cell-associated phenotypes of HCMV strains have been noted, but the mechanistic differences, and the genetic loci involved in these modes of spread remain to be understood. Sinzger et al. showed that during the initial rounds of cell-associated subculturing in fibroblasts, clinical HCMV isolates formed small and tightly packed foci, but tended towards larger and more diffuse foci during later rounds of passage (59). The change in foci morphology coincided with the appearance of infectious virus in culture supernatants and the basic interpretation was that the adaptation of clinical HCMV to the cell culture environment involved a shift from cell-associated to cell-free modes of spread. A more recent report by Galitska et al. noted variation in clinical HCMV samples, with some displaying the characteristic small foci, but others showing larger diffuse foci even in early rounds of subculturing (60). This variation fits well with the notion of genotypic diversity of HCMV populations within clinical specimens (3, 4, 6, 8, 9) and the earlier suggestion by Sinzger et al. that the observed culture adaptation of HCMV clinical isolates might represent random sampling or purifying selection of preexisting genotypic variants, rather than the acquisition of *de novo* mutations (59). In line with this view, Subramanian et al. used a flow cytometry method to determine the increase in HCMV infected cells over time to better quantitate spread kinetics and found that some low passage clinical isolates spread more rapidly than others (61). They interpreted slow kinetics to represent a cell-associated-only phenotype and fast kinetics to represent a phenotype of both cell-associated and cell-free spread. Indeed, the faster spreading isolates were more sensitive to the presence of neutralizing antibodies. Together, these and other studies suggest considerable variation in the modes of spread exhibited by genetically distinct HCMV, but the exact genetic variations that determine these phenotypes remain unclear.

Here we applied a flow cytometry-based approach to compare the spread characteristics of three commonly studied and genetically distinct BAC clones of HCMV, TB, TR and ME in both fibroblast and epithelial cell cultures. In addition to measuring the rates of spread in the presence or absence of neutralizing antibodies, we also characterized the quantity and infectivity of cell-free and cell-associated progeny produced. TB and ME markedly preferred cell-free and cell-associated modes of spread, respectively, whereas TR seemed to be overall less efficient. While either mode could suffice for efficient spread in
fibroblast cultures, spread in epithelial cell cultures seemed generally skewed towards a cell-associated mode. Consistent with this, ME demonstrated more efficient spread in epithelial cells than either TB or TR. Finally, while the efficiency of cell-free spread correlated with the abundance and infectivity of progeny virus released to culture supernatants, the efficiency of cell-associated spread did not correlate with cell-associated infectivity. In both cell types, ME was far more efficient at cell-associated spread than TB or TR, but did not produce more cell-associated infectivity as measured by titration of sonicated infected cells. This suggests that the efficient cell-associated spread mechanism of ME depends on the intact cell monolayer. Moreover, it seems logical that the poor infectivity of ME virions would be highly detrimental to efficient cell-free spread, whereas cell-associated mechanisms might be less sensitive to poor infectivity. On the other hand, it seems unlikely that the log-folds better infectivity of TB and TR virions would be detrimental to the efficiency of cell-associated spread by these strains (Fig 10). Thus, it seems that ME has a specific mechanism(s) to enhance cell-associated spread that does not depend on production of highly infectious virions. The mechanisms driving the highly efficient cell-associated spread of ME remain unclear, but have been suggested to involve the functions of gH/gL/UL128-131 and RL13 (24, 40, 42, 62).

Our finding that the level of gH/gL/UL128-131 expression did not affect the spread rate of ME in fibroblasts conflicts with Stanton et al., which showed UL128-null ME plaques significantly larger than UL128-intact ME plaques (40). The apparent contradiction may be explained by the fact that our flow cytometry assays measured spread rates averaged over 12 days as compared to the measurements of 21 day plaque sizes by Stanton et al. Linear regression analyses of the data collected by flow cytometry indicated that ME spread at a faster rate between days 3 and 6, and then began to slow after day 9 (Figure 2). This slowdown likely reflects the limiting number of potential uninfected target cells available via cell-associated spread during focal expansion. Contrast this with the constant rates of TB and TR spread, likely indicative of unlimited potential uninfected target cells available via diffusion of progeny virus throughout the culture supernatant. Thus, the increase in 21 day plaque size of a UL128-null ME documented by Stanton is consistent with enhanced cell-free infectivity, but our analysis of spread rate over 12 days indicates this does not come at the expense of a distinct and comparably efficient mode of cell-associated spread. This interpretation is supported by the observation that the 12 day spread rate was highly resistant to neutralizing antibodies regardless of gH/gL/UL128-131 expression level (Fig 8) and is consistent with Laib Sampaio et al. who
showed much greater cell-free dispersion of UL128-null ME foci, but more similar antibody resistant, cell-associated spread with or without UL128 (24). In epithelial cells, repression of gHgLUL128-131 did reduce the 12 day spread rate for ME (Fig 8). This was consistent with reduced plaques sizes of UL128-null ME documented by Murrell et al., and likely reflects the important role of gHgLUL128-UL131 in epithelial cells (38). However, as observed in fibroblasts, spread in epithelial cells was still highly resistant to neutralizing antibodies when gHgLUL128-131 was repressed. This was different than Murrell et al. who concluded that spread of ME in epithelial cells was more sensitive to antibodies when gHgLUL128-131 was reduced (42). Again the discrepancy likely reflects that analysis of 21-day plaque size likely accentuates the effects of cell-free infectivity and masks the contribution of cell-associated spread. Thus, by measuring average spread rates over 12 days, we find that gHgLUL128-131 levels do not determine the cell-free versus cell-associated spread phenotype for ME on either fibroblasts or epithelial cells.

The RL13 glycoprotein was another candidate for a factor influencing the cell-free and cell-associated phenotypes. Previous reports, using a number of different approaches, concluded that expression of RL13 limits HCMV replication in both fibroblast, epithelial, and endothelial cell cultures (24, 40, 54, 55). Our results are in general agreement with these reports, and also demonstrate some variability in the effects of RL13 with respect to the HCMV strain and the cell type. For each of the strains TB, TR, and ME, RL13 expression reduced spread in fibroblasts more substantially than in epithelial cells. In the case of TB on fibroblasts, RL13 expression reduced the release of cell-free progeny, but this effect was less pronounced for TR or ME on fibroblasts, and there was no effect on cell-free progeny release in epithelial cells for any strain. Interestingly, RL13 expression dramatically reduced the infectivity of cell-free virions released by ME from epithelial cells, and this was consistent with results of Stanton et al. showing low cell-free titers for RL13-intact ME in epithelial cell cultures compared to RL13 mutant ME (40). The physiological basis of this effect on ME infectivity remains unclear, but it seemed to correlate with differences in gHgL complex and gB incorporation. Epi-ME virions contained more gB than fib-derived ME virions and much of the elevated gB was in the uncleaved form. Strive et al. showed that furin cleavage was not required for the function of HCMV gB (63). Therefore, the increased levels of gB in epi-ME may partly explain the increased infectivity relative to fib-ME. RL13 expression in epithelial cells reversed both the increase in uncleaved gB and reduced virion infectivity. Finally, RL13 expression did not appear to promote cell-associated spread since the sensitivity of spread to
neutralizing antibodies in RL13-expressing cells was either unaffected or marginally increased for all three strains. Thus, RL13 seems to dampen one or more aspects of HCMV replication that can manifest through either mode of spread. The specific effects of RL13 may vary depending on other physiologic differences between strains and cell types.

Mutational inactivation of the UL128-131 and RL13 loci during serial propagation of HCMV in culture is a phenomenon that has been universally ascribed to HCMV, despite clear strain variability. While ME is highly sensitive to the selection of de novo UL128-131 in fibroblast cultures, TB and TR are remarkably stable in this respect (40, 55, 59). This disparity might simply indicate that many subculturing methods, including supernatant transfer, cell sonication, and high split-ratio transfer of intact infected cells, would be expected to heavily favor cell-free spread. Thus, given the highly efficient cell-associated spread of ME described in the analyses presented here, propagation by low split-ratio passage of intact, infected cells might reduce the selective bottleneck on UL128-131 function for efficient cell-associated spreaders like ME. RL13 inactivating mutations have also been reported for many strains and clinical isolates in studies involving fibroblasts, epithelial, and endothelial cells (10, 54). The BAC clones of TB and TR have intact RL13 ORFs, but since expression of RL13 from transduced cells reduced replication by these strains, it may be that both strains contain mutations or polymorphisms in the signal peptide, or other regulatory sequences, that impact RL13 expression. Moreover, our results suggest that the selection against RL13 may be more extensive in fibroblasts but less pronounced in epithelial cells. Notably, a recent report by Ourahmane et al. showed that the presence of neutralizing antibodies during subculturing of clinical urine-derived HCMV in fibroblasts stabilized the UL128-131 and RL13 loci in the resultant virus populations (62). If these clinical isolates were similar to ME in their proclivity for cell-associated spread, (note that ME was also isolated from a urine sample (10)), it would seem logical that neutralizing antibodies would reduce the selective advantage of cell-free infectivity conferred by UL128-131 mutation. The preservation of RL13 is less intuitive since RL13 seems to impact both cell-free and cell-associated modes of spread. However, given the variable effects we observed of RL13 on different strains, these observations may point to epistasis phenomena, where the relative effect of any given perturbation at one variable locus is influenced by variable physiology related to other polymorphic loci.
Our data also shed new light on the idea that the cell type might influence phenotypic properties in a
non-genetic manner through changes to the protein composition of the progeny virus. Among the
herpesviruses, these so-called “producer cell effects” have been particularly well characterized for EBV, where
the mechanism alternates the tropism of progeny virus between epithelial and B-cells (reviewed in (64)).

Scrivano et al. suggested a similar phenomenon for HCMV based in part on data showing that progeny virus
produced from endothelial cells had less UL128 protein in the envelope compared to fibroblast-derived
progeny (35). This is in stark contrast to our results that epithelial-derived HCMV contained greater amounts
of gH/gL/UL128-131 than fibroblast-derived virus (Fig 9). There are several possible, non-mutually exclusive
explanations for this discrepancy. This could reflect differences between strains as the Scrivano et al.
experiments used TB, whereas our analyses used ME only since the production of TB and TR from epithelial
cells was too low to allow immunoblot analyses for these strains. There could also be differences between
endothelial and epithelial cells. Finally, the Scrivano et al. analysis normalized virion immunoblots to gB,
whereas we used major capsid protein (MCP). It is possible that incorporation of gB into progeny virus is
affected by glycoprotein processing/secretory pathway differences between cell type, whereas plasticity in the
number of capsid subunits per virion seems less likely. Indeed, we observed differences in the proteolytic
processing and virion incorporation of gB when ME was produced in fibroblast and epithelial cells.

Nevertheless, Scrivano et al. also noted an apparent change in the relative fibroblast versus endothelial
tropism depending on nature of the producer cell. While we observed differences in infectivity between
fibroblast and epithelial cell-derived virus, it is important to note that our analyses involved congruent cell
types to correspond with the observed kinetics of viral spread in monolayer cultures. Questions regarding the
spread of HCMV between cell types were outside the scope of the present studies.

In conclusion, we have characterized very different cell-free and cell-associated spread phenotypes
among three commonly studied BAC clones of HCMV. It seems likely that these phenotypic variances are
manifest through the combined influences of diversity at multiple genetic loci other than UL128-131 and RL13.
How this phenotypic variation is represented in vivo remains unclear. There is little clear evidence to support the
notion that HCMV is predominately cell-associated in vivo. The endemic nature, and the pleomorphic
disseminated disease presentations suggest that HCMV is able to thrive in many different bodily environments
as it spreads within and among individual human hosts. Copious amounts of cell-free virus are shed in urine,
saliva, and breast milk, and this is likely a major route of transmission between individuals, whereas HCMV in
the blood is likely highly associated with leukocytes (65–67). Thus, it seems likely that both cell-free and cell-
associated modes of spread are important to the natural history of HCMV.

MATERIALS AND METHODS

Cell lines. Primary neonatal human dermal fibroblasts (nHDF; Thermo Fisher Scientific), RL13-nHDFs
(nHDFs transduced with lentiviral vectors encoding RL13 of HCMV strain Merlin, selected with puromycin
resistance), MRC-5 fibroblasts (American Type Culture Collection; CCL-171), and HFFF-tet (40) (provided by
Richard Stanton, Cardiff University, United Kingdom) were grown in Dulbecco’s modified Eagle’s medium
(DMEM)(Sigma) supplemented with 6% heat-inactivated fetal bovine serum (FBS) (Rocky Mountain
Biologicals, Missoula, MT, USA) and 6% Fetalgro® (Rocky Mountain Biologicals, Missoula, MT, USA).
Retinal pigment epithelial cells (ARPE19)(American Type Culture Collection, Manassas, VA, USA), ARPE-tet
(ARPE19 cells transduced with retroviral vectors encoding the tetracycline repressor protein, and selected by
puromycin resistance), and RL13-ARPE (transduced with lentiviral vectors encoding RL13 of HCMV strain
Merlin, selected with puromycin resistance) were grown in a 1:1 mixture of DMEM and Ham’s F-12 medium
(DMEM:F-12)(Gibco) and supplemented with 10% FBS.

Retro- and lentiviral vectors. The Tet repressor protein bearing a nuclear localization signal was extracted
by PCR from the integrated sequence in the HFFF-tet chromosomal DNA and cloned into the same pMXs
retrovirus vector used to construct HFFF-tet (40). The pMXs retrovirus vector plasmid was a gift from Dr.
Toshio Kitamura at the Institute of Medical Science, University of Tokyo (68). The tet-containing vector
plasmid was transformed in 293T cells together with the pUMVC and pMD2.G helper plasmids. The pUMVC
helper plasmid was a gift from Bob Weinberg (Addgene plasmid # 8449) (69). Two days after transformation,
the retroviral particles in the supernatant were purified from cell debris thru syringe filtration and centrifugation.
After titration, the particles were used to transduce low passage ARPE-19 cells. After a week of puromycin
selection, cells aliquots were tested for tetR expression after transfection of a firefly luciferase tetR-reporter
system and aliquots were stored in liquid nitrogen until further usage. The codon-optimized RL13 with an
intact ORF from Merlin HCMV strain (NCBI ref YP_081461) was constructed by Gibson Assembly and used to
replace the eGFP ORF in the pLJM1-EGFP lentiviral transfer vector plasmid. The pLJM1-EGFP plasmid was
a gift from David Sabatini (Addgene plasmid # 19319) (70). The RL13-containing vector plasmid was
transformed in 293T cells together with three lentiviral helper plasmids. The pMDLg/pRRE, pRSV-Rev, and pMD2.G helper plasmids were a gift from Didier Trono (Addgene plasmids # 12251, # 12253, # 12259, respectively) (71). Two days after transformation, the lentiviral particles in the supernatant were purified from cell debris thru syringe filtration and centrifugation. After titration, the particles were used to transduce either low passage nHDF or ARPE-19 cells. After a week of puromycin selection, cells were tested for RL13 expression and aliquots were stored in liquid nitrogen until further usage.

**HCMV.** All human cytomegalovirus (HCMV) strains were derived from bacterial artificial chromosome (BAC) clones. BAC clone TB40/e (BAC4) was provided by Christian Sinzger (University of Ulm, Germany) (41). BAC clone TR was provided by Jay Nelson (Oregon Health and Sciences University, Portland, OR, USA) (72). BAC clone Merlin (ME)(pAL1393), which contains tetracycline operator sequences within the transcriptional promoter of UL130 and UL131, was provided by Richard Stanton (Cardiff University, Cardiff, United Kingdom) (40). All BAC clones were modified to express green fluorescent protein (GFP) with En passant recombineering (73) by replacing US11 with the eGFP gene. The constitutive expression of eGFP allows the monitoring of HCMV infection early and is strain-independent. Infectious HCMV was recovered by electroporation of BAC-DNA into HFFFs, as previously described (26). For infectious unit (IU) determination, viruses were serial diluted and infectivity was determined on fibroblasts or epithelial cells using flow cytometry 48 hours post infection.

**Antibodies.** Monoclonal antibodies specific to HCMV major capsid protein (MCP), gH (14-4b), and gB (27-156) were provided by Bill Britt (University of Alabama, Birmingham, AL) (51, 57, 74). 14-4b was purified by FPLC prior to use. Rabbit polyclonal sera against HCMV gL, UL130, and UL131 were provided by David Johnson (Oregon Health and Sciences University, Portland, OR) (17).

**Vrial spread assays.** Approx. 1x10^5 (3x10^5) nHDFs (ARPEs) were seeded into 6-well culture plates and allowed to grow to confluence. Confluent monolayers of nHDFs or ARPE cells were inoculated with 100-1000 IUs of strains TB, TR, or ME at for 4hrs at 37°C. Cells were then washed extensively with PBS and cultured in the appropriate growth medium supplemented with 2% FBS. Viral spread in the presence or absence of neutralizing antibodies or ganciclovir was monitored over 12 days by flow cytometry. All experiments were performed in triplicate and a minimum of 3 experiments was conducted for each condition. Spread rates were
determined by plotting LN GFP+ cells over time and fitting the data to the log-linear rate expression \( \ln(I_t) = m(t) + \ln(I_0) \) where \( I_t \) is the number of GFP+ cells, \( t \) is the time in days, and \( m \) is the spread rate.

**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 spun 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. RL13 expression was measured using an intracellular staining kit (Thermo) and an anti-6His antibody conjugated to AlexaFluor-647 using the RL-2 laser (647nm).

**qPCR.** The real time quantitative PCR (qPCR) assay used to quantify viral or cellular DNA molecules was performed as previously described (27). Briefly, HCMV DNA inside cell-free particles was purified using the PureLink Viral RNA/DNA mini kit (Thermo Scientific). A region within UL83 conserved among ME, TR, and TB was chosen as the HCMV-specific amplicon, and viral genomes were quantified by SYBR green qPCR as previously described. Standard curves were performed using serial dilutions of a single PCR DNA band containing the sequences of the viral UL83 and cellular beta2-microglobulin (see below) amplicons and the corresponding set of primers. Finally, the concentration of HCMV DNA genomes in the supernatant was extrapolated from the UL83 standard curve and expressed as genome molecules per ml of media (genome/ml). Total intracellular or cytoplasmic HCMV DNA was quantified with the UL83-based qPCR after extracting viral and cellular DNA from infected cells with the PureLink Genomic DNA mini kit (Thermo Scientific). We also measured the chromosomal DNA molecules present in these samples with an amplicon located in the human beta-2 microglobulin gene (75). These chromosomes numbers were used to correct for the number of cells when measuring the specific infectivity of cell-associated HCMV particles from different samples. To that purpose, another aliquot of infected cells was first subjected to a Subcellular Fractionation Kit for cultured cells (Thermo Scientific) to purify the cytoplasmic HCMV particles from the excess of nuclear HCMV DNA. Lastly, genomes present in the cytoplasmic fraction were purified using the Genomic DNA mini kit and their number was measured with the UL83-based qPCR assay.
Particle production. Production of intracellular and extracellular HCMV genomes was determined over a time course of 4 days. Briefly, nHDFs and ARPEs were infected with HCMV strains TB, TR, or ME at an MOI of 1, and the cells were extensively washed 3 days post infection. At 3, 5, and 7 dpi culture supernatants were collected and spun at 500xg for 10 minutes at RT, while cells were lifted with trypsin and then quenched with culture media containing 10% FBS. Cells were spun at 500xg and resuspended into aliquots. Cells were either resuspended in PBS for whole cell analysis, fractionated for specific infectivity analysis, sonicated to release cell-associated virus, or processed for flow cytometry to determine the number of infected cells. The number of HMCV genomes in the whole cell and supernatant was measured by qPCR, normalized to load, and divided by the number of GFP+ cells. For specific infectivities, the number of HCMV genomes per mL in the supernatant or cytoplasmic fractions was divided by the IUs per mL of the supernatants and cell sonicates. The accumulation at day 7 from nHDFs and ARPEs is shown in Fig 3 and 4, respectfully.

Immunoblot analyses. Cell-free virions were solubilized in a buffer containing 20mM Tris-buffered saline (TBS) (pH 6.8) and 2% SDS. Protein samples were separated by SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes in a buffer containing 10mM NaHCO₃ and 3mM Na₂CO₃ (pH 9.9) and 10% methanol. Transferred proteins were first probed with MAbs or rabbit sera, then anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma), and Pierce ECL-Western Blotting substrate (Sigma). Chemiluminescence was detected using a Bio-Rad ChemiDoc MP imaging system.

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FIGURE LEGENDS
Fig 1. Comparison of focal spread patterns of distinct HCMV stains. Confluent monolayers of fibroblasts (A) or epithelial cells (B) were infected at an MOI of 0.001 with GFP-expressing HCMV strains TB, TR, or ME. Foci were documented by fluorescence microscopy at 6, 12, and 18 days post infection. Four representative fields (10X) are shown for each.

Fig 2. Quantitation of HCMV spread by in fibroblasts and epithelial cells. Confluent 6cm monolayers of fibroblasts (A) or epithelial cells (D) were infected with 1000 IUs of GFP-expressing HCMV strains TB, TR, or ME. The number of infected cells at 3, 6, 9, and 12 days post infection was determined by flow cytometry. To determine exponential growth rates, the LN GFP+ cells is plotted at each time point for fibroblasts (B) and epithelial cells (E). The average spread rates (LN GFP+ cells per day) of the three strains were determined for fibroblasts (C) and epithelial cells (F). All experiments were performed at least three times and error bars represent standard error between all experiments. Error bars are omitted if smaller than the data marker, and p-values were generated using one-way ANOVA.

Fig 3. Production of cell-free and cell-associated HCMV progeny virus in fibroblasts. Replicate cultures of fibroblasts were infected at MOI 1 with TB, TR, or ME. At 7 days post infection, the number of infected cells in each culture was determined by flow cytometry for GFP expression, and the cumulative number of HCMV genomes in each culture was determined by qPCR; (A) culture supernatants, (C) cell-associated genomes. Specific infectivity was determined by titration on fibroblasts cultures and expressed as the ratio of genomes per infectious unit (IU); (B) cell-free, (D) cell-associated. Error bars represent the standard error of three separate experiments, and p-values were generated using one-way ANOVA.

Fig 4. Production of cell-free and cell-associated HCMV progeny virus in epithelial cells. Replicate cultures of epithelial cells were infected with TB, TR or ME. At 7 days post infection, the number of infected cells in each culture was determined by flow cytometry for GFP expression, and the cumulative number of HCMV genomes in each culture was determined by qPCR; (A) culture supernatants, (C) cell-associated genomes. Specific infectivity was determined by titration on epithelial cells and expressed as the ratio of genomes per infectious unit (IU); (B) cell-free, (D) cell-associated. Error bars represent the standard error of three separate experiments, and p-values were generated using one-way ANOVA.

Fig 5. Effects of neutralizing antibodies on spread of HCMV strains in fibroblasts and epithelial cells. Confluent 6cm monolayers of fibroblasts or epithelial cells were infected with 1000 IUs GFP-expressing
HCMV strains TB, TR, or ME and allowed to spread in the presence of either anti-gH mAb 14-4b, anti-UL130/131 rabbit sera, or ganciclovir. The average spread rates over 12 days (LN GFP+ cells per day; as in Fig 2) for TB, TR, and ME on fibroblasts (A-C) and epithelial cells (D-F) are shown. Conditions where no spread was detected are designated “n.d.”. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test vs. mock (*<0.05, **<0.01, ***<0.001, ****<0.0001).

**Fig 6. Repression of gH/gL/UL128-131 expression by HCMV ME in fibroblasts and epithelial cells.** (A) Tetracycline-repressor protein (tetR) expressing fibroblasts (HFFFtet) or epithelial cells (ARPEtet) or control fibroblasts and epithelial cells were transfected with TetO-firefly luciferase reporter and the relative luciferase activity was measured. (B) Non-reducing SDS-PAGE analysis of ME virions derived from HFFFtet or ARPEtet or control cells. (C) Specific infectivity of virions released from HFFFtet or ARPEtet or control cells.

**Fig 7. Effect of gH/gL/UL128-131 repression on spread of HCMV strain ME.** Spread rates (LN GFP+ cells per day; as in Fig 2) for HCMV strain ME on tetR-expressing or control fibroblasts (A) or epithelial cells (B) in the presence or absence of neutralizing antibodies. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test comparing to control cells (*<0.05, **<0.01).

**Fig 8. Replication and spread of HCMV strains TB, TR, and ME in RL13-expressing fibroblasts and epithelial cells.** (A,E) Spread rates in RL13+ fibroblasts or epithelial cells were determined as in Fig 2 and expressed as relative to the same analyses performed in control cells. (B,F) Spread rates in the presence of neutralizing antibodies were determined as in Fig 5 and expressed as percent inhibition in RL13 fibroblasts or epithelial cells, relative to the same analyses performed in control cells. (C,G, and D,H) Quantity and infectivity (genomes/IU) of cell-associated (CA) and cell-free (CF) progeny virus produced by RL13 expressing fibroblasts or epithelial cells were determined as in Figs 3-4 and expressed as relative to the same analyses performed in control cells. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test to RL13(-) cells (*<0.05, **<0.01, ***<0.001, ****<0.0001).

**Fig 9. Effect of RL13 expression on levels of gH/gL complexes and gB in ME virions.** Equal amounts of ME virions derived from RL13-expressing or control fibroblasts or epithelial cells were separated by SDS-
PAGE under non-reducing (A) or reducing (B and C) conditions and analyzed by immunoblot with antibodies directed against gL (A), gB, or major capsid protein (C).

**Figure 10. Model of cell-free and cell-associated modes of spread by HCMV.** 1. Progeny virions assemble into compartments derived from the trans-Golgi network. 2. Trafficking and release of progeny directed towards apical cell surfaces (2a) or cell junctions (2b). 3. Apically released, cell-free virus diffuses in the extracellular fluid and attaches to an adjacent or a distant cell (3a), whereas virus released to the cell junctions is immediately in contact with the adjacent cell (3b). 4. Entry into the next cell by either the cell-free (4a) or the cell-associated (4b) route may be mediated by either direct fusion between the virion envelope and plasma membrane, or by fusion from within an endosome. 5. Alternatively, some studies have suggested that subviral components may be transferred directly to adjacent cells through transient pores or other intercellular connection mechanisms, such as tunneling nanotubes (45, 76, 77). Overall, specific infectivity of virions might have a dramatic effect on the efficiency of the cell-free route (2a-4a) where the virus is subject to physical limitations related to diffusion and to inactivation by neutralizing antibodies, complement components, and other host defenses. In contrast, the efficiency of cell-associated routes (2b-4b, and 5) might be more influenced by mechanisms of directed trafficking and intercellular connectivity.

**Fig S1. Antibody neutralization of cell-free HCMV.** Equal numbers of fibroblast-derived (A and B) or epithelial-derived (C and D) HCMV TB, TR, or ME were incubated with multiple concentrations of anti-gH mAb 14-4b (A and C) or anti-UL130/131 rabbit sera (B and D) for 1hr at RT. Remaining infectivity was determined by titration on either fibroblast (A and B) or epithelial cell (C and D) cultures, and plotted as percent of no antibody control. All experiments were performed in triplicate and error bars represent standard error.

**Fig S2. RL13-expressing fibroblasts and epithelial cell lines.** RL13-expressing or control fibroblasts or epithelial cells were analyzed by immunoblot analysis (A), and the relative expression levels were determined by flow cytometry (B). In both analyses, anti-His antibodies were used to detect the His tag on the transduced RL13.
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Fig 1. Comparison of focal spread patterns of distinct HCMV stains. Confluent monolayers of fibroblasts (A) or epithelial cells (B) were infected at an MOI of 0.001 with GFP-expressing HCMV strains TB, TR, or ME. Foci were documented by fluorescence microscopy at 6, 12, and 18 days post infection. Four representative fields (10X) are shown for each.
Fig 2. Quantitation of HCMV spread by in fibroblasts and epithelial cells. Confluent 6cm monolayers of fibroblasts (A) or epithelial cells (D) were infected with 1000 IUs of GFP-expressing HCMV strains TB, TR, or ME. The number of infected cells at 3, 6, 9, and 12 days post infection was determined by flow cytometry. To determine exponential growth rates, the LN GFP+ cells is plotted at each time point for fibroblasts (B) and epithelial cells (E). The average spread rates (LN GFP+ cells per day) of the three strains were determined for fibroblasts (C) and epithelial cells (F). All experiments were performed at least three times and error bars represent standard error between all experiments. Error bars are omitted if smaller than the data marker, and p-values were generated using one-way ANOVA.
Fig 3. Production of cell-free and cell-associated HCMV progeny virus in fibroblasts. Replicate cultures of fibroblasts were infected at MOI 1 with TB, TR, or ME. At 7 days post infection, the number of infected cells in each culture was determined by flow cytometry for GFP expression, and the cumulative number of HCMV genomes in each culture was determined by qPCR; (A) culture supernatants, (C) cell-associated genomes. Specific infectivity was determined by titration on fibroblasts cultures and expressed as the ratio of genomes per infectious unit (IU); (B) cell-free, (D) cell-associated. Error bars represent the standard error of three separate experiments, and p-values were generated using one-way ANOVA.
Fig 4. Production of cell-free and cell-associated HCMV progeny virus in epithelial cells. Replicate cultures of epithelial cells were infected with TB, TR or ME. At 7 days post infection, the number of infected cells in each culture was determined by flow cytometry for GFP expression, and the cumulative number of HCMV genomes in each culture was determined by qPCR; (A) culture supernatants, (C) cell-associated genomes. Specific infectivity was determined by titration on epithelial cells and expressed as the ratio of genomes per infectious unit (IU); (B) cell-free, (D) cell-associated. Error bars represent the standard error of three separate experiments, and p-values were generated using one-way ANOVA.
Fig S1. Antibody neutralization of cell-free HCMV. Equal numbers of fibroblast-derived (A and B) or epithelial-derived (C and D) HCMV TB, TR, or ME were incubated with multiple concentrations of anti-gH mAb 14-4b (A and C) or anti-UL130/131 rabbit sera (B and D) for 1hr at RT. Remaining infectivity was determined by titration on either fibroblast (A and B) or epithelial cell (C and D) cultures, and plotted as percent of no antibody control. All experiments were performed in triplicate and error bars represent standard error.
Fig 5. Effects of neutralizing antibodies on spread of HCMV strains in fibroblasts and epithelial cells.
Confluent 6cm monolayers of fibroblasts or epithelial cells were infected with 1000 IUs GFP-expressing HCMV strains TB, TR, or ME and allowed to spread in the presence of either anti-gH mAb 14-4b, anti-UL130/131 rabbit sera, or ganciclovir. The average spread rates over 12 days (LN GFP+ cells per day; as in Fig 2) for TB, TR, and ME on fibroblasts (A-C) and epithelial cells (D-F) are shown. Conditions where no spread was detected are designated “n.d.”. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test vs. mock (*<0.05, **<0.01, ***<0.001, ****<0.0001).
Fig 6. Repression of gH/gL/UL128-131 expression by HCMV ME in fibroblasts and epithelial cells. (A) Tetracycline-repressor protein (tetR) expressing fibroblasts (HFFFtet) or epithelial cells (ARPEtet) or control fibroblasts and epithelial cells were transfected with TetO-firefly luciferase reporter and the relative luciferase activity was measured. (B) Non-reducing SDS-PAGE analysis of ME virions derived from HFFFtet or ARPEtet or control cells. (C) Specific infectivity of virions released from HFFFtet or ARPEtet or control cells. P-values were generated by unpaired t-test to tetR(-) cells (*<0.05, **<0.01, ***<0.001, ****<0.0001).
Fig 7. Effect of gH/gL/UL128-131 repression on spread of HCMV strain ME. Spread rates (LN GFP+ cells per day; as in Fig 2) for HCMV strain ME on tetR-expressing or control fibroblasts (A) or epithelial cells (B) in the presence of absence of neutralizing antibodies. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test comparing to control cells (*<0.05, **<0.01).
**Figure S2**

**A**

| nHDF | ARPE |
|------|------|
| -    | -    | RL13-transduced |
| 100  | 100  | +                |
| 75   | 75   | mature           |
| 50   | 50   | immature         |

**B**

**Fig S2. RL13-expressing fibroblasts and epithelial cell lines.** RL13-expressing or control fibroblasts or epithelial cells were analyzed by immunoblot analysis (A), and the relative expression levels were determined by flow cytometry (B). In both analyses, anti-His antibodies were used to detect the His tag on the transduced RL13.
Fig 8. Replication and spread of HCMV strains TB, TR, and ME in RL13-expressing fibroblasts and epithelial cells. (A,E) Spread rates in RL13+ fibroblasts or epithelial cells were determined as in Fig 2 and expressed as relative to the same analyses performed in control cells. (B,F) Spread rates in the presence of neutralizing antibodies were determined as in Fig 5 and expressed as percent inhibition in RL13 fibroblasts or epithelial cells, relative to the same analyses performed in control cells. (C,G) Quantity and infectivity (genomes/IU) of cell-associated (CA) and cell-free (CF) progeny virus produced by RL13 expressing fibroblasts or epithelial cells were determined as in Figs 3-4 and expressed as relative to the same analyses performed in control cells. All experiments were performed three times and error bars represent standard error. P-values were generated by unpaired t-test to RL13(-) cells (*<0.05, **<0.01, ***<0.001, ****<0.0001).
Fig 9. Effect of RL13 expression on levels of gH/gL complexes and gB in ME virions. Equal amounts of ME virions derived from RL13-expressing or control fibroblasts or epithelial cells were separated by SDS-PAGE under non-reducing (A) or reducing (B and C) conditions and analyzed by immunoblot with antibodies directed against gL (A), gB, or major capsid protein (C).
Figure 10. Model of cell-free and cell-associated modes of spread by HCMV. 1. Progeny virions assemble into compartments derived from the trans-Golgi network. 2. Trafficking and release of progeny directed towards apical cell surfaces (2a) or cell junctions (2b). 3. Apically released, cell-free virus diffuses in the extracellular fluid and attaches to an adjacent or a distant cell (3a), whereas virus released to the cell junctions is immediately in contact with the adjacent cell. 4. Entry into the next cell by either the cell-free (4a) or the cell-associated (4b) route may be mediated by either direct fusion between the virion envelope and plasma membrane, or by fusion from within an endosome. 5. Alternatively, some studies have suggested that subviral components may be transferred directly to adjacent cells through transient pores or other intercellular connection mechanisms, such as tunneling nanotubes (45, 76, 77). Overall, specific infectivity of virions might have a dramatic effect on the efficiency of the cell-free route (2a-4a) where the virus is subject to physical limitations related to diffusion and to inactivation by neutralizing antibodies, complement components, and other host defenses. In contrast, the efficiency of cell-associated routes (2b-4b, and 5) might be more influenced by mechanisms of directed trafficking and intercellular connectivity.
Figure 2
Figure 8
A. Fibroblasts

|       | 6 d.p.i. | 12 d.p.i. | 18 d.p.i. |
|-------|----------|-----------|-----------|
| TB    |          |           |           |
| TR    |          |           |           |
| ME    |          |           |           |

B. Epithelial Cells

|       | 6 d.p.i. | 12 d.p.i. | 18 d.p.i. |
|-------|----------|-----------|-----------|
| TB    |          |           |           |
| TR    |          |           |           |
| ME    |          |           |           |

Figure 1
Figure 3

A Cell-free Production

B Cell-free Infectivity

C Cell-associated Production

D Cell-associated Infectivity

$p = 0.1043$

$p = <0.0001$

$p = 0.2205$

$p = <0.0001$
Figure 4

A Cell-free Production

B Cell-free Infectivity

C Cell-associated Production

D Cell-associated Infectivity

$p = 0.3818$

$p = 0.0028$

$p = <0.0001$

$p = 0.0008$
Figure 5
Figure 6
Figure 7

(A) Spread Rate (m) for nHDF and HFFFtet.

(B) Spread Rate (m) for ARPE and ARPEtet.
Figure 9
