The pentameric complex drives immunologically covert cell-cell transmission of wild-type human cytomegalovirus

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Human cytomegalovirus (HCMV) strains that have been passaged *in vitro* rapidly acquire mutations that impact viral growth. These laboratory-adapted strains of HCMV generally exhibit restricted tropism, produce high levels of cell-free virus and develop susceptibility to natural killer cells. To permit experimentation with a virus that retained the phenotype of clinical virus, we reconstructed a wild-type (wt) HCMV genome using bacterial artificial chromosome technology. Like clinical virus, this genome proved to be unstable in cell culture, however propagation of intact virus was achieved by placing the RL13 and UL128 genes under conditional expression. In this study, we show that wt-HCMV produces extremely low titers of cell-free virus but can efficiently infect fibroblasts, epithelial, monocyte-derived dendritic and Langerhans cells via direct cell-cell transmission. This process of cell-cell transfer required the UL128 locus, but not the RL13 gene, and was significantly less vulnerable to the disruptive effects of interferon, cellular restriction factors and neutralizing antibodies compared with cell-free entry. Resistance to neutralizing antibodies was dependent on high-level expression of the pentameric gH/gL/gpUL128–131A complex, a feature of wt but not passaged strains of HCMV.

Virology | Immune evasion | herpesvirus | HCMV | cell-cell spread

Human cytomegalovirus is a major cause of morbidity and mortality in the immunocompromised, and the leading infectious cause of congenital malformation. As a result, a vaccine has been designated of the highest priority. However, basic studies of clinically relevant isolates that inform our understanding of the disease process are limited, due to the rapid accumulation of genetic mutations during *in vitro* passage of HCMV. The same three genetic sites are reproducibly affected: the RL13 gene, the UL128 locus (UL128L), which comprises UL128, UL30 and UL131A, and the ~15kb U_L/*b*' gene region (1). Deletions in the U_L/*b*' region can affect tropism (UL148 (2)), latency (UL136 and UL138 (3-7)) and resistance to NK cells (UL141 (8-10), UL142 (11, 12) and UL135 (13)). Disabling mutations in RL13 and UL128L independently contribute to the release of high-titer cell-free virus, while loss of UL128L restricts virus entry to fibroblasts alone by preventing assembly of a pentameric glycoprotein complex (gH/gL/pUL128/pUL130/pUL131A) in the virion envelope (1, 14, 15).

The rapid selection of mutations is a major obstacle to the propagation of genetically intact 'clinical' strains of HCMV, which in turn has led to significant gaps in our understanding of the affiliated disease processes (16). In particular, HCMV is largely cell-associated *in vivo* (17), and clinical isolates exhibit a similar phenotype *in vitro* (17, 18), yet most studies in the field are based on laboratory strains that produce high titers of cell-free virus (19). As a consequence, little is known about the fundamental processes involved in the infectious spread of cell-associated HCMV.

In this study, we used a system that permits *in vitro* experimentation with a genetically complete virus that retains the cell-associated phenotype displayed by clinical isolates of HCMV. Our data show that cell-cell transfer differs both qualitatively and quantitatively from cell-free infection, enabling the virus to overcome intrinsic cellular restriction factors, interferon-induced antiviral factors and antibody-mediated neutralization. Moreover, the ability of wild-type (wt)-HCMV to spread via the cell-cell route below the radar of humoral immune defences can be attributed to high-level expression of the pentameric complex. Collectively, these findings provide new insights into the mechanisms that facilitate the *in vivo* persistence of HCMV.

RESULTS

Cell-cell spread is more efficient than cell-free infection

To recapitulate cell-cell spread of wt-HCMV *in vitro*, the complete strain Merlin genome was captured in a bacterial artificial chromosome (BAC) (14). The virus was then propagated *in vitro* with RL13 and UL128-131A under the control of a tetracycline repressor (tetR). This strategy prevented the acquisition of mutations in culture (20). The pentameric complex and gpRL13 were subsequently restored to virions during a single infectious cycle in fibroblasts lacking tetR (Fig. 1A). Extremely low quantities of cell-free virus were produced under these conditions, akin to clinical isolates, with peak titers below 70 PFU/ml, which equates to 1 PFU per 1,750 infected cells (Fig. 1B).

As this extremely low yield of cell-free virions would not be sufficient to sustain the infection, we reasoned that wt-Merlin must be transmitted via direct cell-cell spread. Initial experiments

Significance

Strains of human cytomegalovirus (HCMV) that have been passaged in the laboratory fail to recapitulate the phenotypic characteristics of ex vivo isolates due to the rapid acquisition of genetic mutations. In particular, clinical viruses spread via direct cell-cell contact, while passaged strains spread via diffusion of cell-free virions. We developed a method that enables in vitro propagation of genetically intact HCMV. Using this system, we now report that cell-cell transmission of wild-type HCMV is not only highly efficient, but also enables the virus to overcome key innate and adaptive immune defences. These findings inform our understanding of viral persistence and validate a new approach to experimentation that may facilitate the design of improved prophylactic and therapeutic interventions against HCMV.

Reserved for Publication Footnotes

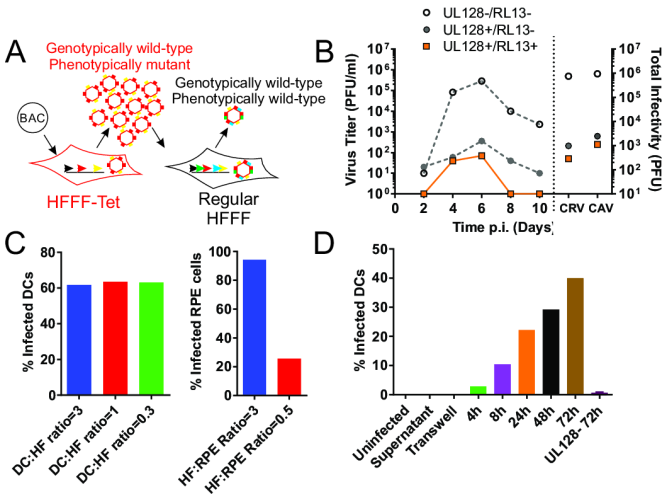

Fig. 1. Characterization of cell-cell infection. (**A**) Diagrammatic representation of the method used to grow wild-type Merlin *in vitro* without risk of mutation. (**B**) HFFFs were infected with variants of the Merlin BAC containing mutations in both UL128 and RL13, wt (tet-regulated) UL128, or wt (tet-regulated) UL128 and wt (tet-regulated) RL13 (MOI = 5). Virus was titrated from supernatants or sonicated cell preparations. Cell-released virus (CRV) and cell-associated virus (CAV) represent the total infectivity obtained from cells or supernatant, respectively, over the course of the experiment. (**C**) HFFFs infected with Merlin expressing IE2-GFP (MOI = 5; 72 h post-infection) were stained with DDAO, then co-cultured with either DCs or RPE-1 cells at the indicated ratios. Samples were analyzed by flow cytometry after a further 72 h to determine the percentage of infected DCs or RPE-1 cells ($DDAO^-/GFP^+$). Representative data are shown. (**D**) DCs were co-cultured with Merlin-infected HFFFs (MOI = 5; 72 h post-infection) for the indicated time periods. Non-adherent cells were then removed and incubated alone prior to flow cytometric analysis 72 h after the start of co-culture. In some experiments, a transwell was placed between the DCs and HFFFs, and the cultures were incubated for 72 h. Alternatively, DCs were infected via the cell-free route using virus harvested from Merlin-infected HFFF supernatants and incubated in isolation for 72 h. Virus lacking UL128–131A expression ($UL128^-$) was used in one well. Representative data are shown.

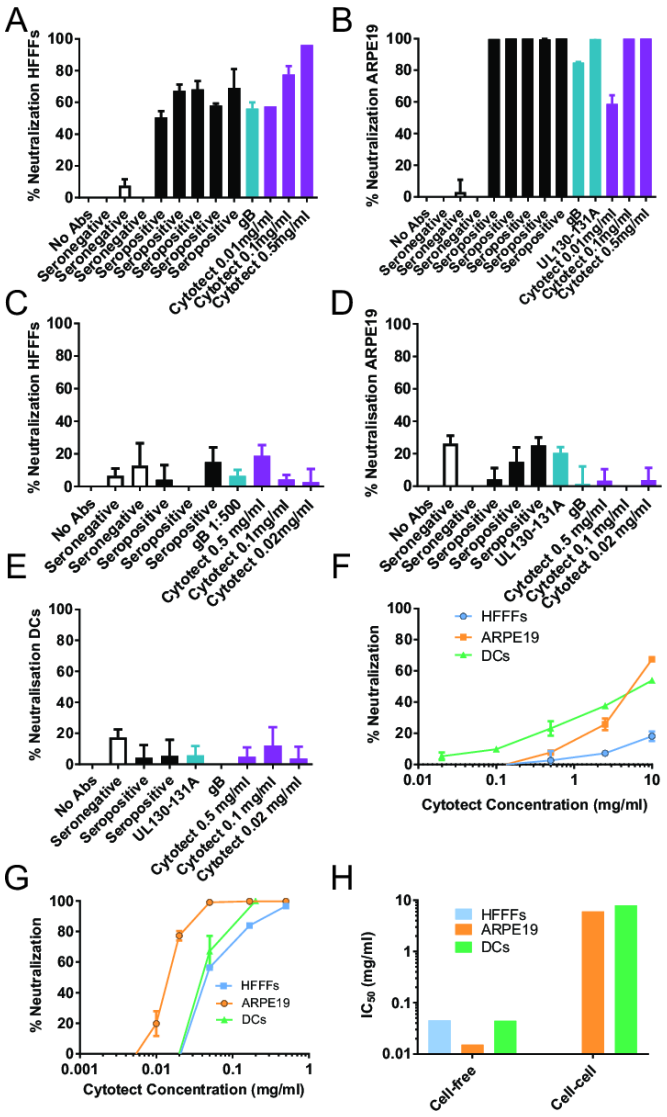

Fig. 2. Cell-cell spread is resistant to neutralizing antibodies. (**A**,**B**) Cell-free Merlin-GFP was incubated for 1 h with specific antibodies (light blue) against gB (C23; 3.6 μg/ml) or UL130 and UL131A (a 1:50 dilution of a 50:50 mixture of polyclonal rabbit sera raised against peptides from UL130 and UL131A), Cytotect (purple) or serum from donors testing seronegative (white) or seropositive (black) for HCMV (1:50). HFFFs (A) or ARPE-19 cells (B) were then infected for 2 h, overlaid and incubated for 2 weeks. Infected cells were quantified by plaque assay. Percent neutralization was calculated relative to the negative control (no antibody). (**C–E**) HFFFs were infected with Merlin-GFP (MOI = 5), then stained with DDAO after 72 h and incubated with HFFFs (C), ARPE-19 cells (D) or DCs (E) in the presence of antibodies or sera as described in (A,B). Infected cells were quantified by flow cytometry 48 h after the start of co-culture. Percent neutralization was calculated relative to the negative control (no antibody). (**F**) HFFFs were infected with Merlin-GFP (MOI = 5), then stained with DDAO after 72 h and incubated with HFFFs, ARPE-19 cells or DCs in the presence of the indicated concentrations of Cytotect. Infected cells were quantified by flow cytometry 48 h after the start of co-culture. (**G**) Cell-free Merlin was incubated with the indicated concentrations of Cytotect for 30 min. HFFFs, ARPE-19 cells or DCs were then infected for 2 h. After a further 24 h, cells were fixed and stained for IE-1. Infected cells were quantified by flow cytometry. Percent neutralization was calculated relative to the negative control (no Cytotect). (**H**) The concentration of Cytotect that inhibited infection by 50% was calculated from panels F and G.

were therefore performed with a virus (hereon termed simply 'Merlin') lacking RL13, which complicates the production of cell-free virus (14), to compare the different modes of infection. Infected human fetal foreskin fibroblasts (HFFFs; Fig 1A) were pre-stained with DDAO and mixed with uninfected cells. Subsequent infection was then identified via GFP expression linked to the viral IE-2 gene (Supplemental Fig. 1A). Both adherent epithelial (RPE-1) and non-adherent dendritic cells (DCs) were readily infected in these assays (Fig. 1C). Notably, the donor-recipient cell ratio strongly influenced the efficiency of infection in co-cultures with RPE-1 cells, while little effect was observed in co-cultures with DCs.

To determine the kinetics of cell-cell transfer, DCs were removed from co-culture at different time points and incubated in isolation for 72 h (Fig. 1D). For comparison with cell-free infection, DCs were incubated for the same length of time with supernatant collected from infected HFFFs over a period of 72 h. Alternatively, transwells were used to separate DCs from infected HFFFs. In both cases, the DCs remained uninfected, indicating that cell-cell contact is required for infection with Merlin. In contrast, infected DCs were readily detected within 4 h of co-culture and accumulated progressively with time. After 24 h, one infected DC was detected for every infected HFFF, making cell-cell transfer of Merlin > 1,750-fold more efficient than cell-free infection. Importantly, virus lacking the pentameric complex was unable to spread into DCs, and provided an additional control to verify that only newly infected DCs were counted in these analyses. To verify that GFP expression was linked to productive infection, GFP^+ cells were first sorted to purity using flow cytometry (Supplemental Fig. 1B). Subsequent imaging revealed that the GFP signal was localized to the nucleus, consistent with *de*

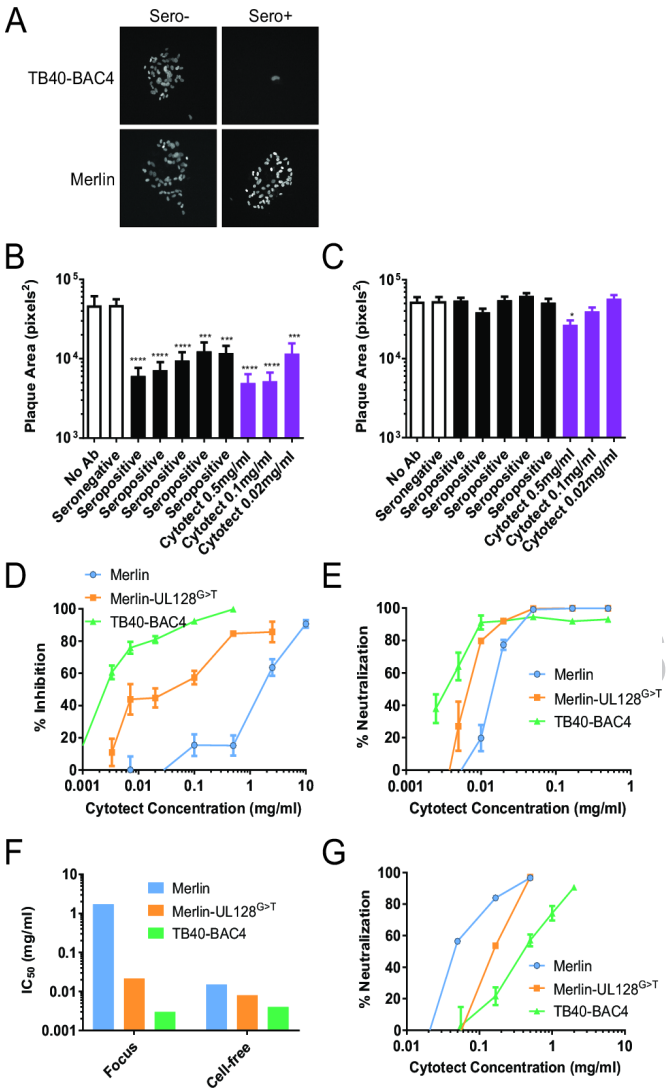

Fig. 3. Levels of the pentameric complex correlate with resistance to neutralizing antibodies. (**A**) ARPE-19 cells were infected at 60 PFU/well with either TB40-BAC4 or Merlin and incubated for 21 days in the presence of seronegative or seropositive sera (1:50). Cells were imaged after fixing and staining for IE-1. (**B, C**) ARPE-19 cells were infected with either TB40-BAC4 (B) or Merlin (C) and incubated for 21 days in the presence of Cytotect (purple) or seronegative (white) or seropositive (black) sera (1:50). Plaque size was measured after fixing and staining for IE-1. (**D**) ARPE-19 cells were infected with 60 PFU/well of either TB40-BAC4, Merlin or Merlin-UL128$^{G>T}$ and incubated for 21 days in the presence of the indicated concentrations of Cytotect. Plaque size was measured after fixing and staining for IE-1. Percent inhibition was calculated relative to the negative control (no Cytotect). (**E**) Cell-free Merlin, Merlin-UL128$^{G>T}$ or TB40-BAC4 were incubated with the indicated concentrations of Cytotect for 30 min. ARPE-19 cells were then infected for 2 h and incubated for a further 24 h. Infected cells were counted after fixing and staining for IE-1. Percent neutralization was calculated relative to the negative control (no Cytotect). (**F**) The concentration of Cytotect that inhibited plaque formation or cell-free infection by 50% was calculated from panels D and E. (**G**)Cell-free Merlin, Merlin-UL128$^{G>T}$ or TB40-BAC4 were incubated with the indicated concentrations of Cytotect for 30 min. HFFFs were then infected for 2 h and incubated for a further 24 h. Infected cells were counted after fixing and staining for IE-1. Percent neutralization was calculated relative to the negative control (no Cytotect). Error bars represent SEM.

novo expression of the IE-2-GFP fusion protein (Supplemental Fig. 1C). In addition, virus was readily transferred back to HFFFs in co-cultures with flow-purified DCs (Supplemental Fig. 1D).

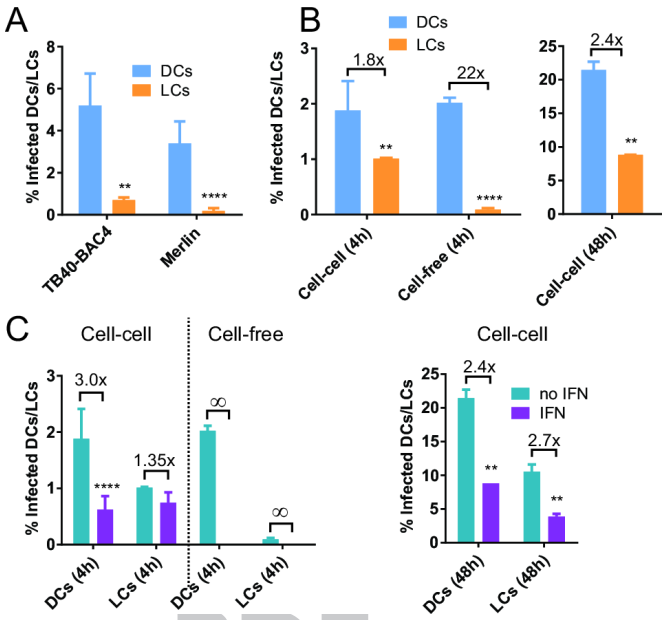

Fig. 4. Cell-cell spread in Langerhans cells is highly efficient and resistant to IFNα. (**A**) Immature DCs or LCs were incubated for 24 h with cell-free preparations of TB40-BAC4 or Merlin. The percentage of infected cells was calculated by microscopy after staining with a mouse anti-IE-1 antibody and DAPI. (**B**) DCs or LCs were incubated for 4 h or 48 h at a 1:1 ratio with Merlin-GFP-infected HFFFs (MOI = 5; 72 h post-infection) or cell-free Merlin-GFP. Non-adherent cells were then removed and incubated alone prior to flow cytometric analysis 72 h after the start of co-culture or cell-free infection. (**C**) DCs or LCs were cultured for 24 h in the presence or absence of IFNα, then incubated for 4 h or 48 h with Merlin-GFP-infected HFFFs (MOI = 5; 72 h post-infection) or cell-free Merlin-GFP. Non-adherent cells were then removed and incubated alone prior to flow cytometric analysis 72 h after the start of co-culture or cell-free infection. Assays were performed in quadruplicate. ** $P < 0.01$, **** $P < 0.0001$ (ANOVA). Error bars represent SEM.

Collectively, these data show that UL128L expression correlates with cell association during the replicative cycle of a HCMV isolate containing a clinical genome, and facilitates efficient cell-cell transmission into epithelial and myeloid cells.

Cell-cell spread is resistant to neutralizing antibodies

Many current vaccine strategies are designed to induce a neutralizing antibody response (21-27). However, it is unclear whether the transmission of HCMV is sensitive or resistant to neutralization, potentially because many studies have used viruses that spread efficiently via the cell-free route (19, 28-36). To determine the neutralization sensitivity of Merlin, we tested a panel of antibody preparations. Cell-free infection of HFFFs and ARPE-19 epithelial cells was inhibited in all cases (Fig.2A,B), while direct cell-cell transmission into HFFFs, ARPE-19 epithelial cells and DCs was largely unaffected (Fig.2C–E).

Cytotect is a clinical grade product containing high neutralizing antibody titers against HCMV. In a recent clinical trial, this formulation was used at a concentration of 100 mg/kg (37). Based on data from other intravenous immunoglobulin preparations, this could increase serum IgG levels by up to 2 mg/ml (38). To test the efficiency of cell-cell transfer under clinically relevant conditions, HFFFs, ARPE-19 epithelial cells or DCs were infected via the cell-cell route in the presence of Cytotect concentrations as high as 10mg/ml (Fig. 2F). Cell-cell transfer into ARPE-19 epithelial cells and DCs was inhibited by 50% at concentrations of 6–8mg/ml, while cell-cell transfer into HFFFs was inhibited by < 50% at a concentration of 10 mg/ml. Similarly, only weak inhibition of cell-cell transfer of virus from flow-purified DCs back into HFFFs or ARPE-19 epithelial cells was observed at maximal concentrations of Cytotect (Supplemental Fig. 2); at

these high concentrations, it is even possible that the effects observed are non-specific, rather than being due to anti-CMV antibodies. In contrast, cell-free infection with the same virus was inhibited by 50% at concentrations < 50 μg/ml (HFFFs and DCs) or < 15 μg/ml (ARPE-19 epithelial cells) (Fig. 2G,H).

To provide comparisons with cell-free infection, experiments thus far had been performed with virus lacking RL13. Identical assays were therefore performed using a virus expressing the complete wt-HCMV proteome (Merlin-RL13^{+}). No consistent differences in the efficiency of cell-cell transfer were observed between Merlin and Merlin-RL13^{+}, and both viruses were equally resistant to the inhibitory effects of Cytotect (Supplement Fig.3).

Thus, cell-cell spread of Merlin is only inhibited at extremely high antibody concentrations, while cell-free infection is > 400-fold more susceptible to neutralization.

Resistance to neutralizing antibodies is dependent on high levels of the pentameric complex

Our findings that cell-cell spread into epithelial cells is resistant to neutralising antibodies is in direct contrast with a large number of studies in which focus formation in epithelial cells was found to be highly sensitive to neutralizing antibodies (28, 30, 32-34, 39). To resolve this discrepancy, we performed focus expansion assays using the TB40-BAC4 strain of HCMV. TB40-BAC4 is exceptional in retaining the capacity to infect endothelial, epithelial and myeloid cells following *in vitro* culture, and has been widely used for these types of study. Focal spread of TB40-BAC4 was highly sensitive to neutralization (Fig. 3A,B), while minimal inhibition was observed in parallel assays with Merlin (Fig. 3A,C). These findings were confirmed in similar experiments with higher concentrations of Cytotect, which revealed that Merlin was almost 600-fold more resistant to inhibition compared with TB40-BAC (Fig. 3D).

In common with most passaged strains of HCMV, TB40-BAC4 produces high titers of cell-free virus and incorporates reduced levels of gpUL128–131A in the virion, a characteristic partially endowed by a G>T nucleotide substitution in one of the UL128 introns (19). Mutation of the same nucleotide in the Merlin strain (Merlin-UL128$^{G>T}$) reduces both cell-associated and virion-associated expression of the UL128 protein, such that it is intermediate between TB40-BAC4 and Merlin (Supplemental Fig. 4)(19). In line with UL128 expression levels, focal spread of Merlin-UL128$^{G>T}$ displayed an intermediate susceptibility to inhibition compared with Merlin and TB40-BAC4 (Fig. 3D). The same phenomenon was observed in assays with cell-free viruses (Fig. 3E), although the differences between strains were less pronounced (Fig. 3F). In contrast, this pattern was reversed in cell-free neutralization studies with HFFFs (Fig. 3G). These differences presumably reflect the fact that entry into fibroblasts is dependent on the gH/gL/gO complex. Viruses with reduced levels of gpUL128–131A (19) typically express greater amounts of gH/gL/gO (40) because gO and gpUL128–131A compete for binding to gL (41).

Thus, efficient expression of gpUL128–131A, a feature unique to strains containing a clinical genome (19), renders cell-cell spread highly resistant to humoral immunity, and cell-free entry partially resistant to antibodies that neutralise epithelial cell entry.

Cell-cell spread is resistant to intrinsic LC restriction factors

In further experiments, we investigated whether cell-cell transfer might differ in other ways from cell-free transmission. Langerhans cells (LCs) are specialized tissue-resident DCs thought to play a key role in the early stages of natural infection with HCMV (42). *In vitro*, immature DCs are readily infected via the cell-free route, whereas immature LCs are not (43, 44). This dichotomy was recapitulated with Merlin (Fig. 4A). In contrast, much greater numbers of immature LCs were infected via the cell-cell route over an equivalent period of time (Fig. 4B). Similar patterns were observed, but with even higher infection frequencies, in longer term assays. Thus, not only were LCs more readily infected when using the cell-cell route, but the absolute number of infected cells that it was possible to achieve using cell-cell spread was much higher than that achieved by cell-free spread.

To ensure these differences were not dependent on the infected-cell secretome, we repeated the co-culture experiment with a transwell between TB40-BAC4-infected HFFFs and uninfected DCs or LCs (Supplemental Fig. 5A). Alternatively, DCs or LCs were removed from the transwell after incubation for 72 h over Merlin-infected HFFFs and exposed to cell-free Merlin (Supplemental Fig. 5B). In both cases, LCs remained significantly more resistant to infection compared with DCs.

Immature LCs are therefore significantly more susceptible to cell-cell infection relative to cell-free infection, consistent with greater intrinsic restriction of the free virion life cycle.

Cell-cell spread is resistant to interferon-induced antiviral factors

To extend this line of inquiry, we investigated the antiviral effect of interferon (IFN)α. In these experiments, DCs or LCs were treated with IFNα, then infected either via co-culture or by exposure to cell-free virus (Fig. 4C). Cell-free infection of both DCs and LCs was abrogated in the presence of IFNα. Comparable results were obtained with virus infections performed with transwell membranes placed between infected HFFFs and uninfected DCs or LCs, indicating that these differences were not due to exposure to the infected cell secretome (Supplemental Fig. 5C). In contrast, cell-cell transmission was inhibited only moderately by IFNα, even after 48 h. Similarly, cell-free infection of HFFFs was suppressed by IFNα, while cell-cell transfer between HFFFs (Supplemental Fig 5D), or from Merlin-infected DCs or LCs into uninfected HFFFs or ARPE-19 cells (Supplemental Fig. 5E) was only minimally affected by IFNα.

Accordingly, cell-cell transfer is more resistant to innate immunity compared with cell-free entry across a range of cell types. It is also notable that RL13 expression did not influence the susceptibility of this process to the antiviral effects of IFNα or the intrinsic restrictive properties of LCs (Supplemental Fig. 5F).

In sum, the findings reported here demonstrate that virus expressing the complete HCMV proteome spreads efficiently via the cell-cell route, a mode of propagation that confers resistance to multiple arms of the immune system.

DISCUSSION

The data presented here show that a genetically defined and clinically relevant strain of HCMV can infect a wide range of cell types via a process of direct transfer that differs qualitatively from cell-free entry and likely predominates *in vivo*. Previous studies have been limited to strains capable of cell-free transmission, such as FIX and TB40-BAC4, which incorporate mutations that reduce expression of the pentameric glycoprotein complex (19). However, clinical isolates are almost entirely cell-associated *in vitro* (18, 45), and the majority of virus is cell-associated *in vivo* (17). Moreover, cell-cell spread is essential for viral replication in animal models of CMV infection (46). Despite these fundamental observations, remarkably little is known about the physiological mechanism of cell-cell transfer. It is established that soluble proteins can be transmitted between infected cells (47), and that small fusion events can occur between infected endothelial cells and polymorphonuclear leukocytes (48). In addition, virus lacking UL99, an essential tegument protein required for free virion formation, can still spread via the cell-cell route in fibroblasts (49). Nonetheless, polymorphonuclear leukocytes are not productively infected, and virus lacking UL99 spreads very inefficiently compared with wt-HCMV. These studies also relied on virus strains that do not express the complete wt-HCMV proteome (1, 14, 16, 19). In contrast, our experiments with fully

reconstituted strain Merlin allowed us to demonstrate that high levels of gpUL128–131A drive efficient cell-cell transmission and confer resistance to innate and adaptive immune defences.

These results will need to be validated using other strains of HCMV, since it is possible that there is strain-strain variation (32). This will require the construction of additional BACs containing genomes that match original clinical isolates, and contain tet operators upstream of UL128L and RL13 (14). Nonetheless, it seems likely that the pentameric complex is normally expressed at high levels *in vivo* given that clinical isolates grow in a cell-associated manner akin to Merlin and rapidly acquire similar ablative mutations in UL128L *in vitro* (1), and that passaged strains with intact UL128L express lower levels of the protein due to acquired mutations (19). It remains unclear whether the observed effects on cell-cell transfer arise from virion- or cell-associated membrane expression of the pentameric complex, both of which are reduced by the G>T mutation used in this work. Previous studies have shown that HCMV forms syncytia in ARPE-19 and fibroblast cells, and that this can be inhibited by neutralizing antibodies (50, 51). These observations suggest a functional role for membrane-associated glycoproteins. However, relatively few syncytia were observed with TB40-BAC4, and very few syncytia were observed with Merlin or the Merlin-UL128$^{G>T}$ mutant.

Cell-cell spread has been particularly well studied in the context of retroviruses. Akin to HCMV, these viruses propagate significantly more efficiently via direct transfer compared with the cell-free route (52-54), and better overcome specific restriction factors and innate immune responses in the process (55-57). However, these similarities do not necessarily reflect common mechanisms of viral spread (58, 59). For example, cell-cell transfer of HIV can be inhibited by many different neutralizing antibodies (60-62), and although it is more resistant than cell-free infection with some antibodies (60, 63-67), the differences are significantly lower than then the 400-fold observed for Merlin; the resistance of Merlin to all but the highest concentrations of neutralizing antibodies potentially indicates a qualitatively distinct mode of transmission. The data presented here also have substantial implications for the control of HCMV *in vivo*. During natural infection, LCs in the oral epithelium are most likely the first antigen-presenting cells to encounter HCMV (42). Immature LCs are resistant to infection via the cell-free route (44, 68), and it has been suggested that this potential bottleneck could feasibly be exploited to interrupt transmission (42). The propensity of wt-HCMV to spread via the cell-cell route would undermine any such efforts. In addition, cell-cell spread enhances the kinetics of viral infection (52, 69, 70) and enables drug resistance to develop more quickly in the setting of HIV (71, 72). These phenotypes can be attributed to the fact that multiple virions are delivered per cell during a cell-cell transmission event (71, 73). It remains to be determined whether the same is true for HCMV.

It will be interesting to determine whether antibodies capable of suppressing cell-cell spread are also effective at neutralising cell-free virus. Nevertheless, the concentrations of IgG required to inhibit cell-cell transmission of Merlin may be difficult to achieve *in vivo* (38). This observation has profound ramifications for the development of therapeutic interventions, especially as current vaccine studies are focused on neutralizing antibodies (21-27). Several of these antibodies are considerably more potent than Cytotect when measured using cell-free passaged HCMV (21, 22, 33), raising the possibility that they could have efficacy against cell-cell spread of clinical virus. However, until they are tested against virus expressing the wildtype HCMV proteome, it is not possible to determine whether potent inhibition of cell-free infection correlates with inhibition of cell-cell spread, or whether inhibition of cell-cell spread is simply dependent upon antibody concentration. These antibodies may of course have efficacy *in vivo* by inhibiting initial cell-free infection from body fluids, yet our data suggests that once an initial infection has occurred, they may be less effective at preventing intra-host spread. Similarly, in both congenital infection and transplantation, where virus spread to the fetus/transplant recipient may conceivably occur by cell-cell spread, such antibodies may be less effective. It is notable in this context that studies of the role of neutralising antibodies *in vivo* are somewhat mixed; studies have documented an inverse correlation between neutralization titers and intrauterine transmission (74-77), and virus-specific antibodies have been shown to confer a degree of protection in animal models (78, 79) and in a trial in transplant patients (80). Yet the administration of polyclonal human IgG containing high titers of neutralizing antibodies failed to prevent congenital infection in a randomized clinical trial (37), and although anti-gB antibody titres correlated with protection in a vaccine study of transplant recipients, neutralising titres did not (81).

Collectively, our data highlight the importance of testing genetically defined viruses that accurately represent the causative agent of disease and provide evidence to suggest that neutralizing antibodies may only limit intra-host spread if present at very high concentrations. A successful vaccine may therefore need to also stimulate cytotoxic responses to eliminate cells that are already infected with HCMV (82).

MATERIALS AND METHODS

Co-culture

Infected HFFFs were washed prior to co-culture, incubated for 15 min with 222 ng/ml CellTrace Far Red (DDAO-SE; Invitrogen) in PBS, washed again and resuspended in regular growth medium. For co-culture with adherent cells (HFFFs, ARPE-19), infected HFFFs were trypsinized and overlaid on targets 3–7 days post-infection. For co-culture with non-adherent cells (DCs or LCs), targets were added directly to infected HFFFs 3–4 days post-infection. Non-adherent targets were washed off at various time points during co-culture. In all cases, cells were maintained for 72 h to allow expression of IE2-GFP. For analysis, cells were harvested (if non-adherent) or trypsinized (if adherent), stained with Fixable Viability 570 (BD), then either fixed in 2% paraformaldehyde and acquired using an Accuri C6 cytometer (BD) or acquired directly without fixation using a FACSAria II cytometer (BD). Informed consent was obtained from all volunteer blood donors, and experiments were approved by Cardiff University Research Ethics Committee

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