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REVIEW

Principles for studying in vivo attenuation of virus mutants: defining the role of the cytomegalovirus gH/gL/gO complex as a paradigm

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Abstract Initial virus entry into cells of host organs and subsequent spread of viral progeny between tissue cells are events fundamental to viral pathogenesis. Glycoprotein complexes inserted in the virion envelope are critically involved in the cell entry process. Here we review and discuss recent work that has shed light on the in vivo role of the trimeric glycoprotein complex gH/gL/gO of murine cytomegalovirus (mCMV) as a model to propose the role of the corresponding complex of human CMV, for which experimental studies in vivo are not feasible due to the host species specificity of CMVs and evident ethical constraints. A novel approach combining gO transcomplementation of a genetically gO-deficient virus and a mathematical log-linear regression analysis of the viral multiplication kinetics in host tissues revealed a critical role of mCMV gH/gL/gO only in first target cell entry of virions arriving with the circulation, whereas intra-tissue spread proceeded unaffected also in the absence of gH/gL/gO. These findings predict that targeting gO for an antiviral intervention may be of prophylactic value in preventing the seeding of virus to organs, but will likely fail to interfere with an established primary organ infection or with recurrent infection after virus reactivation from latency within tissue cells. The demonstration in the murine model of alternative gH/gL complexes gH/gL/gO and gH/gL/MCK-2, substituting one another in a redundant fashion for securing viral spread in tissues, has the medically interesting bearing that targeting the gH/gL core complex directly may be a promising approach to preventing primary, established, and recurrent CMV infections.

Keywords Antiviral intervention · Envelope glycoproteins · Mathematical modeling of virus multiplication · Transcomplementation · Virus entry · Virus spread

Introduction

Glycoprotein complexes inserted in the virion envelope of enveloped viruses are supposed to be critically involved in the process of virus entry into host cells (reviewed in [1]), a process basic to virus multiplication and pathogenesis. In the case of cytopathogenic viruses, such as the cytomegaloviruses (CMVs), virus spread from initially infected cells to neighboring cells in tissues directly accounts for tissue destruction that can end up in functional organ failure associated with morbidity and often mortality. Based primarily on cell culture studies with human CMV (hCMV), four virion envelope glycoprotein complexes, largely conserved among CMV species, are thought to be more directly involved in host cell attachment and entry, and thus in viral host cell tropism: the gB homotrimer [2–7], the gM/gN heterodimer [8, 9], the trimeric complex gH/gL/gO [10–12], and an alternative gH/gL complex identified for hCMV as a pentameric complex composed of the gH/gL core complex [13, 14] and

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the gene products of open reading frames (ORFs) UL128, UL130, and UL131A [15–17] (reviewed in [18–20]).

The structure of hCMV complexes gH/gL/gO and gH/gL/UL128-131A has been resolved very recently and located the neutralizing antibody binding sites [21]. Although the pentameric complex is not required for infection of fibroblasts but appears to be involved in the entry into other cell types, with reported examples being endothelial cells (EC), epithelial cells, and myeloid lineage cells including monocytes/macrophages and dendritic cells (DC), this may not necessarily apply to all subtypes of the named cell-type categories and there may also exist differences between long-term cell lines, ex vivo matured and propagated cells, and cells in their natural microenvironment in situ. Specifically, virion entry into $CD34^+$ stem/progenitor cell-derived immature and mature Langerhans cells (iLCs and mLCs), in vitro models for subtypes of DCs that reside in the skin and oronasal mucosae, proved not to strictly depend on the pentameric complex, although the rate of infection of mLC was higher when the pentamer mode of entry was available [22, 23]. Similarly, virion entry into human trophoblast progenitor cells (TBPCs), the progenitors of epithelial cytotrophoblasts and syncytiotrophoblasts, occurred with attenuated strains lacking the pentameric complex, and antibodies directed against the pentamer proteins pUL130/pUL131A failed to block infection [24].

In the case of murine CMV (mCMV), an alternative gH/gL complex is, instead, formed with MCK-2, the gene product of ORF m131–129 [25]. Notably, besides being part of an alternative gH/gL complex, pUL128 of hCMV and MCK-2 of mCMV share the property to act as C–C chemokines recruiting cells of the myeloid lineage [26–29].

Genetic deletion of glycoprotein-coding genes in respective knockout viruses is an experimental strategy to study the roles of virion envelope glycoprotein complexes. Whereas cell culture studies with various cell types have greatly contributed to our mechanistic understanding of the implication of glycoprotein complexes in the entry process and cell tropism (reviewed in [20]), studies with deletion mutants to reveal the in vivo role of glycoprotein complexes in host organ infection and pathogenesis are limited to animal models. Here we focus on discussing recent work in the murine model on the in vivo role of mCMV gH/gL/gO [30], for which preceding cell culture studies had indicated functional homology to gH/gL/gO of hCMV [31], so that results from the model are likely to be of predictive value.

Principles for defining the critical step underlying viral growth attenuation

The effect of gene deletions or mutations on virus multiplication (virus growth) in vivo is usually studied by comparing viral load in host tissues at certain times or in the time course (measured as infectivity, viral genome numbers, or numbers of infected cells) after host infection with the mutated virus and the corresponding wild-type (WT) virus or, preferably, the corresponding revertant virus. When viral loads in organs differ at selected time points and, in particular, when differences increase over time, it is often concluded that the mutation under study has attenuated the virus in its capacity to replicate within the respective host tissue. Although such an interpretation is widespread in the scientific community, it is not always mechanistically accurate. The inherent problem is illustrated by mathematical modeling of virus growth curves under the following boundary conditions (Fig. 1): *Scenario A* proposes attenuation of mutant virus in its ability to reach an organ and/or enter the tissue cells for initiating organ infection, as indicated by a lower starting number of infected tissue cells, followed by exponential growth of WT and mutant virus with identical doubling times (vDT). vDT represents the growth constant that defines the capacity of a virus to replicate within the respective tissue. *Scenario B* proposes identical capacity of WT and mutant virus in initiating organ infection, as indicated by an equal starting number of infected tissue cells, followed by exponential growth of the mutant virus with a slower (higher numerical value) vDT. This—and only this—indicates virus attenuation for growth within the respective tissue. Finally, *Scenario C*, combining scenarios A and B, proposes mutant virus attenuation in both properties, as indicated by a reduced starting number of infected tissue cells and subsequent exponential growth with slower (higher numerical value) vDT.

The resulting exponential growth curves (Fig. 1a) described by the function $y(t) = y(0) \times 2^{t/\mathrm{vDT}}$ (with t representing the independent variable as time in days after the start of exponential growth, $y(t)$ the measured values of viral burden, and $y(0)$ the starting number of, for instance, infected tissue cells) look at first glance very similar in that they all reveal divergence of the numbers of infected cells, $y(t)$, over time. If one compares replication data $y(t)$ of both viruses at a certain time by bar diagrams and P values for significance, as it is usually done, one is tempted to conclude that in all three cases the mutant virus is attenuated for growth within the tissue under study. Importantly, the simulation of Scenario A, however, tells us that absolute numbers of infected cells diverge over time even though in that case the mutant virus is not attenuated for growth within tissue but grows like WT virus once it has successfully entered tissue cells for a first round of infection.

The fundamental differences between scenarios A, B, and C become graphically evident only after log-transformation of the ordinate (y-axis, dependent variable) data followed by linear regression analysis (Fig. 1b), leading to the log-linear

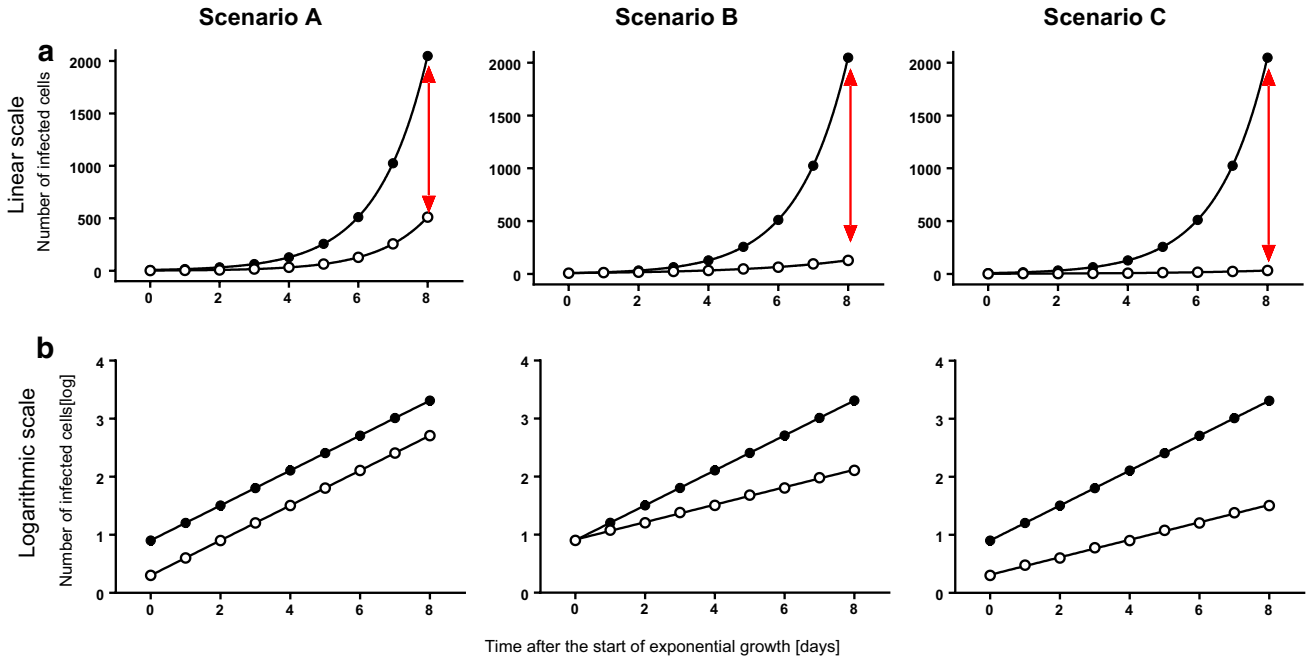


Fig. 1 Mathematical simulation of viral growth kinetics patterns. **a** Exponential *growth curves* showing increasing numbers of infected cells (ordinate: dependent variable values) over time (abscissa: independent variable values). **b** Log-linear *growth curves* (*linear regression lines*) derived from exponential *growth curves* by log-transformation of the ordinate values. See the text for the corresponding mathematical equations. *Closed* and *open circle* symbols represent WT and attenuated mutant virus, respectively. Simulations are performed for different starting numbers of infected cells in tissues, $y(0)$, and different viral doubling times in days, vDT. *Scenario A* WT, $y(0) = 8$; vDT = 1. Mutant, $y(0) = 2$; vDT = 1. *Scenario B* WT, $y(0) = 8$; vDT = 1. Mutant, $y(0) = 8$; vDT = 2. *Scenario C* WT, $y(0) = 8$; vDT = 1. Mutant, $y(0) = 2$; vDT = 2

growth function $\log y(t) = \log y(0) + \log 2/\text{vDT} \times t$ (with log2/vDT representing the slope of the regression line). In *Scenario A*, the regression lines are parallel with a *y*-axis distance from each other that reflects the different starting numbers. In *Scenario B*, the regression lines share the starting number but diverge over time based on different slopes log2/vDT. Finally, *Scenario C* combines different starting number and divergence of the regression lines over time. As the quintessence, congruent regression lines prove identical growth properties and thus the absence of attenuation of the mutant virus. Parallel regression lines for WT and mutant virus growth within a tissue of interest indicate mutant virus replication unaffected by the mutation, whereas divergent log-linear regression lines indicate different capacities of the two viruses to replicate within that particular tissue.

Importantly, the constant that defines the growth characteristics, namely vDT, was found not to depend on the type of the measured variable $y(t)$ [32], be it the number of viral genomes determined by quantitative PCR (qPCR), infectivity determined by virus plaque (plaque-forming units, PFU) assay in fibroblast cell culture, or the number of infected cells determined by in situ techniques, such as immunohistochemistry (IHC) or in situ hybridization (ISH) specific for viral genome or transcripts. However, to avoid a systematic bias, it must be emphasized that the assay for the measured variable $y(t)$ must, of course, be independent of the mutation. For instance, in vivo attenuation of a virus by a mutation that also affects the in vitro infection of fibroblasts cannot be studied by quantitating virus in an in vitro infectivity (e.g., PFU) assay that is based on fibroblasts. For this reason, assays quantitating viral replication directly, such as qPCR, IHC, or ISH, are to be preferred.

This type of quantitative analysis of in vivo virus growth has been successfully employed by us previously in the murine model to reveal that a ΔIE1 virus is attenuated for growth within host tissues [33], following the here modeled scenario B, and is routinely used by us to screen mutated viruses for their in vivo replicative efficiency (fitness) in host tissues (reviewed in [34]). Attenuation of ΔIE1 virus according to scenario B made good sense in view of the known function of IE1 as a transactivator of viral and cellular promoters. In contrast, a role for virion envelope glycoproteins in host cell entry should rather lead to attenuation of knockout mutants with growth characteristics that follow scenario A, in case only first entry is affected, or scenario C, in case first entry and cell-to-cell spread are both affected.

Quantitation of in vivo virus entry events reveals synchronicity of viral gene expression in different hepatic cell types

Recent work has applied these principles to study the role of gO, and thus of the gH/gL/gO complex, in the murine model of CMV infection of host organs, with a focus on liver infection of experimentally immunoablated mice after intravenous virus application [30]. Under such conditions, mCMV virions reach the liver sinusoids with the circulation within seconds and can, in principle, initiate a synchronized infection of various permissive liver cell types, including liver macrophages (MΦ, also known as Kupffer cells), sinusoidal and vascular endothelial cells (EC), and the epithelial cell type of liver parenchyma, the hepatocyte (Hc). As sketched in Fig. 2a, virions arriving in the sinusoidal lumen have direct access to sinusoidal EC, which line the sinusoids, as well as to MΦ that attach to EC from within the sinusoids. In contrast, the sinusoidal endothelium physically separates the sinusoidal lumen from the transendothelial space of Disse and the apical side of Hc so that virus arriving in the sinusoids has to migrate through the endothelium to infect the Hc, the main virus-producing cell type in the liver [35].

One may thus have expected a delay in Hc infection by one round of productive virus replication in EC. Previous work [35] has shown that a floxed reporter virus recombines to express enhanced green fluorescent protein (EGFP) in EC of Tie2-Cre mice within 24 h and that rec-egfp progeny virions spread to Hc detectable by day 2. Reciprocally, however, the floxed reporter virus also recombined within 24 h in Hc of Alb-Cre mice, spreading to EC by day 2. Taken together, these cell-type-specific reporter virus studies indicated that the sinusoidal endothelium is no barrier to mCMV and that, accordingly, infection of Hc does not require a preceding productive viral replication cycle in EC.

These findings are best explained by the cytomorphological fact that liver sinusoidal EC (LSEC) are "fenestrated," like a sieve, by multiple pores, the so-called fenestrae (for a very useful review, see [36]). In the mouse, LSEC form 14 ± 5 of such pores per μm^2 of sinusoidal lining surface, and the diameter of the pores has been determined to be 99 ± 18 nm, sufficient to be passed at least by the rigid virion capsid. To our knowledge, it is not known to what extent the more flexible virion tegument can be compressed by the pressure gradient between sinusoidal lumen and the space of Disse; rather, it is important to note that LSEC-fenestrae are dynamic structures shaped primarily by a calcium-calmodulin-actomyosin system that responds to a number of stimuli by contracting or dilating the fenestrae. Though we are not aware of studies specifically on LSEC-fenestrae dynamics in CMV infections, one can envisage the possibility that early cytokine responses to CMV infection dilate the LSEC-fenestrae so that virions can pass through.

Based on the theoretical considerations on virus growth kinetics in tissues, an "in vivo virus entry assay" has aimed at determining the starting number of infected cells in the liver [30]. As shown by three-color IHC imaging in Fig. 2b, $F4/80^+$ turquoise-stained liver MΦ, $CD31^+$ black-stained EC, and Hc, distinctive by cytomorphology, express *red*-stained intra-nuclear viral immediate-early (IE)1 protein at 24 h after intravenous infection with WT virus. This is in accordance with the reporter virus studies by Sacher and colleagues [35], discussed above, indicating that the sinusoidal endothelium does not pose notable hindrance to an infection of Hc. Normalized to the different quantitative presence of cell types in the liver (Hc > EC > MΦ) [30], EC were preferentially infected, followed by Hc and MΦ (Fig. 2c) [30]. If this indicates different inherent susceptibilities of the different cell types to virus entry and viral gene expression is not clear, however, in the case of EC, the huge surface of the endothelial lining of the sinusoids may account for a high probability for successful hits.

At 24 h, and regardless of the cell type, ~10 % of the infected cells were still in the IE phase of viral gene expression ($IE1^+E1^-$ in two-color IHC), whereas ~90 % had proceeded to the early (E) phase ($IE1^+E1^+$ in two-color IHC). Notably, viral gene expression had not yet proceeded to the late (L) phase, as indicated by the absence of the essential major capsid protein, MCP (Fig. 2c), thus excluding completion of the first viral replication cycle [30]. This finding is important, as it confirms that the "in vivo virus entry assay" really quantitated successful entry events, not obscured by virus spread to neighboring cells for a second round of infection. Also remarkable is the observed synchronicity of viral gene expression in the three cell types under study, again demonstrating that Hc become infected without delay.

Initiation of the infection in different hepatic cell types depends on virion envelope glycoprotein gO

The "in vivo virus entry assay," characterized above, was then used to investigate whether gO plays any role in virus colonization of the liver, differentiated by liver cell type [30]. To this end, mice were infected under otherwise identical conditions with a gO-knockout virus, mCMV-ΔgO, which lacks 532 bp at the 5′ end of the gO-encoding ORF m74 [25, 31]. The deletion was introduced on the genetic background of bacterial artificial chromosome (BAC) pSM3fr-MCK-2fl, in which a preexisting frameshift mutation in m129/MCK-2 had been repaired [37] to make sure that the alternative gH/gL complex, gH/gL/MCK-2, was available for virus entry into the liver cells. As shown in

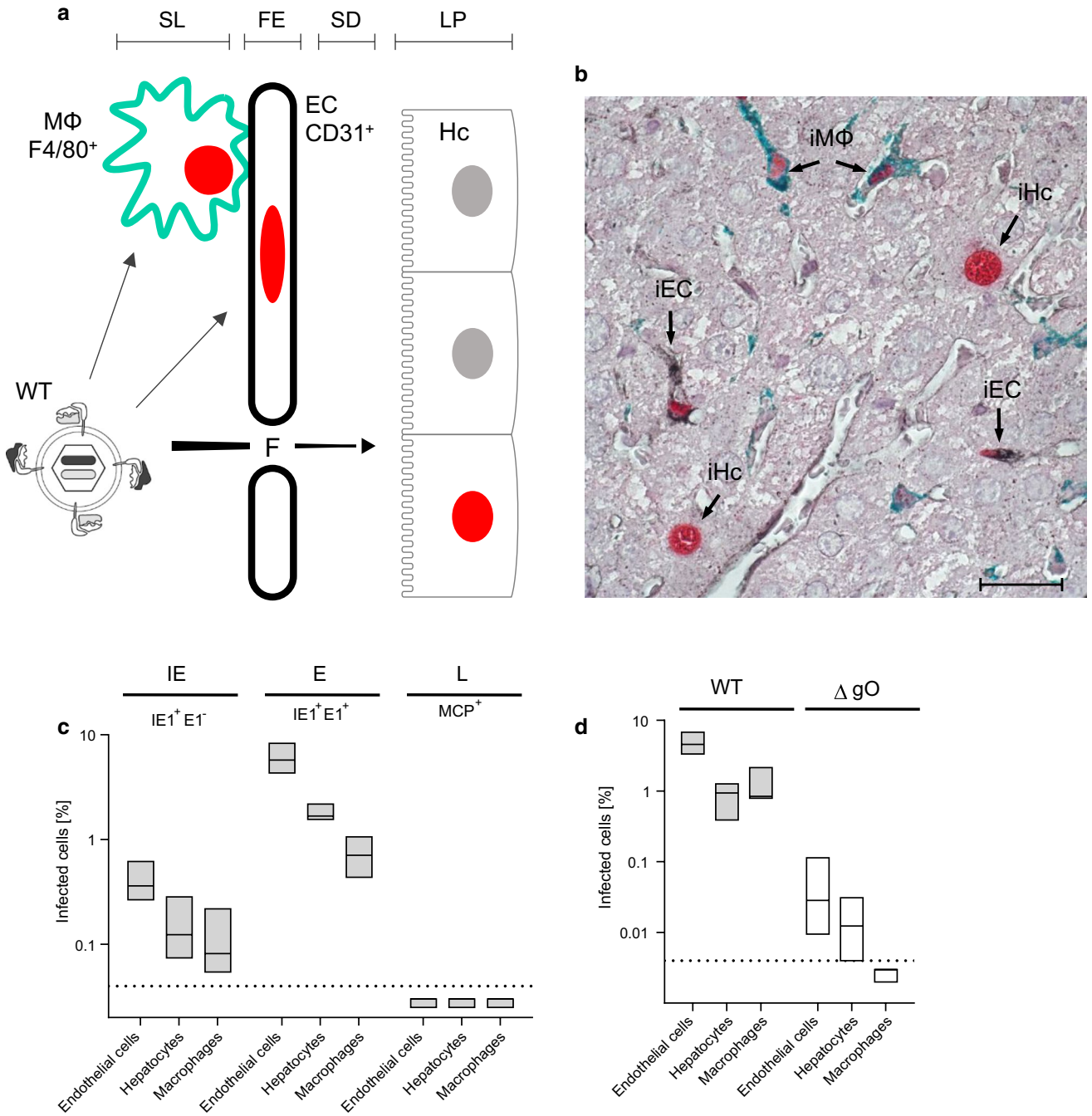


Fig. 2 Quantitation of viral entry events in hepatic cell types. **a** Sketch of the liver sinusoid–parenchyma interface. *SL* sinusoidal lumen. *FE* fenestrated endothelium. *SD* space of Disse. *LP* liver parenchyma. *F* fenestrum within a CD31$^+$ (*black*) liver sinusoidal endothelial cell, (LS)EC. MΦ, F4/80$^+$ (turquoise) macrophage (Kupffer cell). Hc, hepatocyte. Arrows point to the cell types targeted by the virus. The tapered arrow symbolizes flow direction and pressure gradient. *Red color* symbolizes the presence of viral IE1 protein in nuclei of infected cells. **b** Three-color IHC image of a liver tissue section taken at 24 h after intravenous infection with WT virus and stained for intra-nuclear IE1 protein (*red*; infected cells), CD31 (*black*, EC), and F4/80 (turquoise, MΦ). The image is analogous to the one published in [30], but reproduced with new tissue sections from livers of mice of the same experiment. Arrows point to infected hepatocytes (iHc), infected macrophages (iMΦ), and infected endothelial cells (iEC). *Bar marker* represents 25 µm. **c** Percentages of infected cells, differentiated by hepatic cell types and by the stage of viral gene expression, IE (IE1$^+$E1$^-$), E (IE1$^+$E1$^+$), and L (MCP$^+$). **d** Percentages of infected cells (IE1$^+$), differentiated by cell type, after infection with WT virus (*gray-shaded bars*) or mutant virus mCMV-ΔgO (*open bars*). *Bar diagrams* represent median values and ranges based on data published in Ref. [30] with permission by *PloS Pathogens*

Fig. 2d, absence of gO, and thus of the gH/gL/gO complex, drastically reduced (≳100-fold) the number of infected IE1$^+$ cells, regardless of the cell type. In a very formal view, one could argue that residual infection in the absence of gO means that gO is not essential for virus entry; however, gO is certainly critical for an efficient entry with pathogenetic consequences.

In summary, these findings revealed that—in vivo—gO is critical for efficient virus entry into EC, Hc, and MΦ. The findings also imply that gO cannot be equivalently substituted in this function with the gH/gL core complex, the alternative complex gH/gL/MCK-2, or any other virion envelope glycoproteins. At least for MΦ, this result was not anticipated, because own previous work [25], supported by more recent work of others [38], has indicated that MCK-2, by being part of a gH/gL/MCK-2 complex, promotes the infection of MΦ. These earlier studies, however, were based on viruses expressing or lacking MCK-2 in the presence of gO. In a refined view, we therefore now propose that gH/gL/gO is critical for host cell entry, including entry into MΦ, at least the F4/80$^+$ subtype thereof, but that gH/gL/MCK-2 further supports entry into F4/80$^+$ MΦ provided that gH/gL/gO is also present.

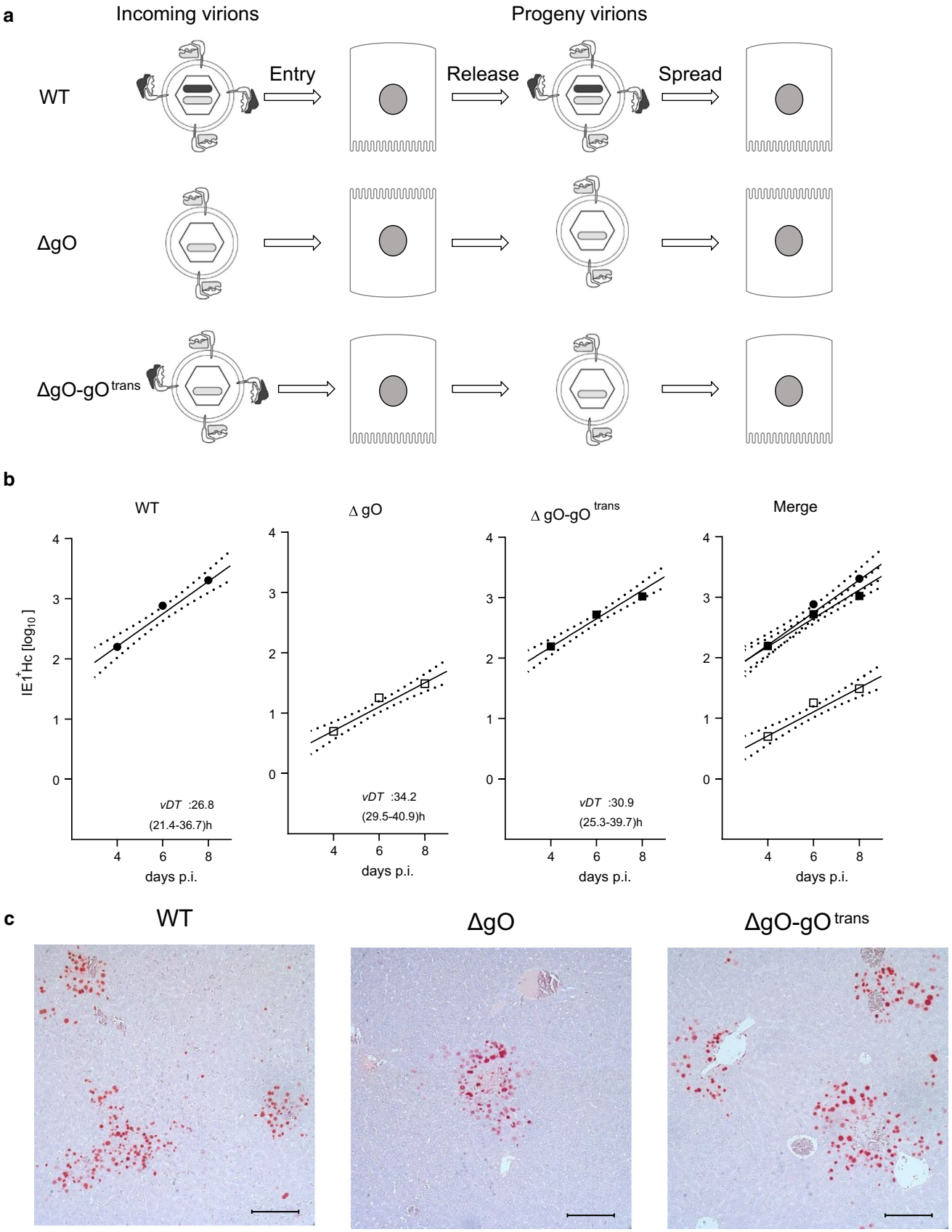
Intra-tissue spread of virus takes place unaltered also in the absence of gO

Intuitively, one might think that virus entry into host cells, irrespective of whether it occurs by envelope-cell membrane fusion or by receptor-mediated endocytosis, requires a certain machinery involving viral entry complexes interacting with host cell receptors, regardless of whether incoming virions arrive from the circulation at first-line target cells or whether progeny virions released from the first-line target cells enter neighboring cells for intra-tissue spread. The findings for the requirement of mCMV gO contradict such a simplified view [30].

gO requirement for first target cell entry and subsequent spread in liver tissue was studied for the example of hepatocytes by determining the log-linear virus growth kinetics [30] (Fig. 3, for the concept recall Fig. 1) of WT mCMV, mutant virus mCMV-ΔgO, and a genetic ΔgO mutant transcomplemented for gO by propagation in the gO-expressing cell line NIH-gO, mCMV-ΔgO-gO^{trans} [30, 31]. As illustrated in Fig. 3a by virion pictograms sketching the genetic composition and gH/gL complex equipment of the three viruses, the gH/gL/gO complex is available upon infection with mCMV-ΔgO-gO^{trans} only for the first target cell entry of incoming virions, whereas progeny virions lack gO in all further rounds of virus replication. This allows experimental dissection of the two steps: entry and spread. As already predicted by the "in vivo virus entry assay" conducted 24 h after infection (see above), gO requirement for first target cell entry resulted in log-linear growth of mCMV-ΔgO shifted on the *y*-axis (representing numbers of infected cells) to below the regression line of WT mCMV (Fig. 3b). Intriguingly, consistent with scenario A of the mathematical modeling (recall Fig. 1), the two experimentally determined regression lines are parallel to each other within the 95 % confidence regions, indicating that WT and ΔgO viruses spread in liver parenchyma with virtually identical vDT, independent of gO. In accordance with this, gO transcomplementation in virus mCMV-ΔgO-gO^{trans} repaired the entry defect of ΔgO virus with no influence on vDT, resulting in a regression line congruent with the regression line of WT virus growth and parallel to the regression line of ΔgO virus growth (Fig. 3b, outer right panel). Identical spread of all three viruses within liver tissue has a histopathological correlate in comparable sizes of plaque-like lesions in liver parenchyma, though the number of such infection foci is reduced for ΔgO virus (Fig. 3c), which results from the reduced number of initially infected cells (Fig. 2d).

The principles reviewed here paradigmatically for hepatocytes applied also to liver EC and F4/80$^+$ liver MΦ [30], as well as to virus spread in other organs, such as lungs and spleen [30]. It should also be noted that virus spreads not only among cells of the same cell type, but also between different cell types of a tissue. Specifically, previous work has shown that cell-type-specific recombination of floxed reporter virus in EC of Tie2-Cre mice and in Hc of Alb-Cre mice led to spread of recombined EGFP-expressing progeny virus to Hc and EC, respectively [35]. Whether this applies likewise to spread directly from Hc to MΦ, which would require retrograde transport through LSEC-fenestrae against a pressure gradient (recall Fig. 2a), is awaiting further investigation, but appears less likely. One could

Fig. 3 Growth characteristics of viruses revealing gO independence of viral spread in liver tissue. **a** Sketch of the concept with WT, ΔgO, and ΔgO-gO^{trans} virion pictograms explaining the gH/gL complex equipment of viruses upon first cell entry (incoming virions) and of their progeny participating in subsequent intra-tissue spread. *Gray-shaded* symbols: MCK-2 gene and protein. *Black* symbols: gO gene and protein. **b** Log-linear *growth curves* (*linear regression lines*), showing the increase in numbers of infected IE1$^+$ hepatocytes (per representative 10-mm^2 liver tissue sections) over time post-infection (p.i) with viruses WT (*closed circles*), ΔgO (*open squares*), and ΔgO-gO^{trans} (*closed squares*). *Symbols* represent median values. *Dotted curves* represent the 95 % confidence areas for the regression lines, determined by linear regression analysis including data from all individual mice of all time points. Data are reproduced, in new compilation, from [30] with permission by *PloS Pathogens.* **c** Immunohistological images (*red* staining of IE1 protein, primarily in Hc cell nuclei) of representative day-8 liver tissue section areas, illustrating different numbers but comparable sizes of infection foci caused by viruses WT (*left panel*), ΔgO (*center panel*), and ΔgO-gO^{trans} (*right panel*). *Bar markers* represent 25 µm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Incoming virions
Progeny virions
WT
Entry
Release
Spread
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IE1+Hc [log10]
vDT :26.8
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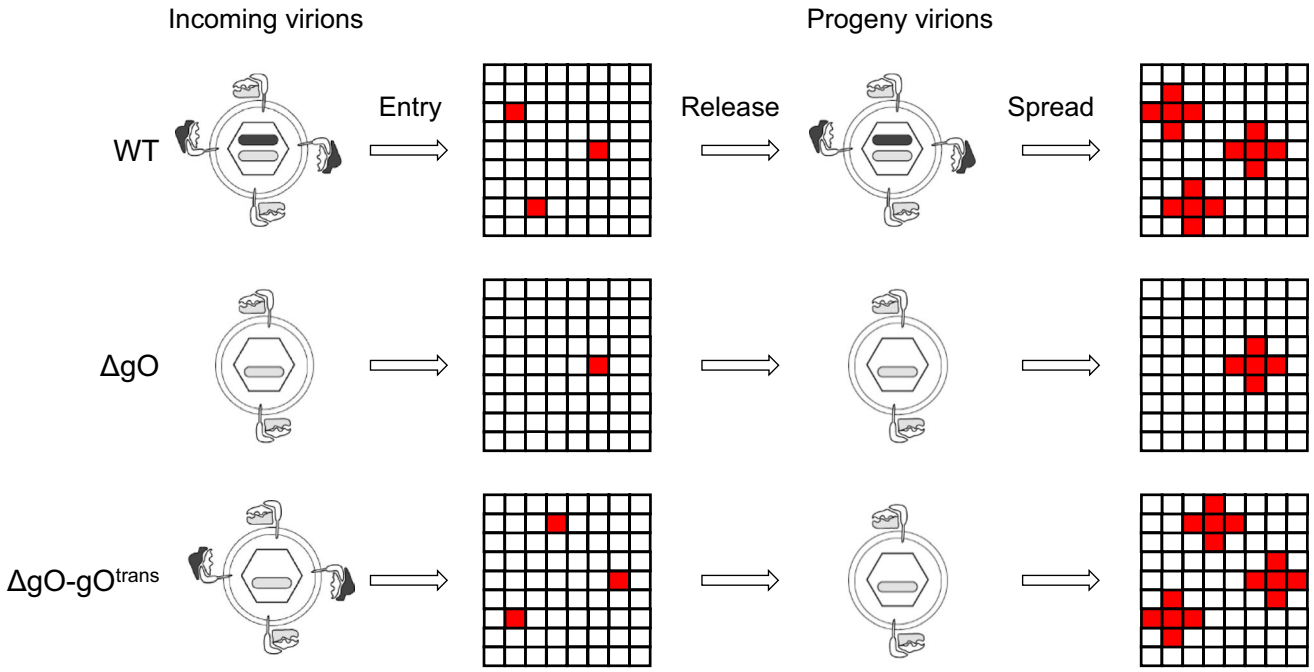

Fig. 4 Synopsis illustrating the differential requirement of the gH/gL/gO complex in viral entry and spread

envisage, however, that virus spreads first from Hc to EC and from there further to MΦ.

Synopsis, discussion, and open questions

The here reviewed data (mainly from Ref. [30]), sketched in their quintessence in Fig. 4, have identified a critical role for gO, and thus for the gH/gL/gO complex, for the efficacy of initial target cell entry of incoming virions, but not for subsequent cell-to-cell spread within tissues, regardless of differences in cell-type composition and overall tissue architecture of different organs. This is a medically relevant finding, as it predicts that therapeutic interventions targeting only gO are unlikely to interfere with established organ infection or to prevent recurrent organ infection after local reactivation of latent virus within tissues, such as in the liver from latently infected LSEC ([39], reviewed in [40, 41]). Targeting gO, however, can attenuate pathogenicity and organ manifestations of primary infection/disease, since—according to Scenario 1 (recall Fig. 1)—any reduction in the initial numbers of infected tissue cells develops into high differences in tissue destruction. Indeed, as we have shown in the highly susceptible model of immunologically immature neonatal mice, ΔgO virus is strongly attenuated in clinical terms, resulting in survival, whereas neonatal mice succumbed to CMV disease after infection with gO-transcomplemented virus [30].

An obvious question has been whether cell-to-cell spread is independent of gH/gL complexes or even independent of virion envelope glycoproteins in general. The idea that spread might occur through cell junctions, not at all involving virion release from infected cells and new entry into neighboring cells, is difficult to reconcile with accessibility of spreading virus to antiviral antibodies, as it was indicated by the previous finding that polyclonal antiviral antibodies in immune serum prevented virus spread in the liver when administered intravenously at a time when infection was already established in liver tissue, so that prevention of entry was not an issue in these experiments [42]. Also, and in line with the findings by Wirtz and colleagues [42], the documented virus spread from infected Hc to LSEC and vice versa (discussed above, [35]) definitively requires virus transfer through the space of Disse, as these two cell types do not establish physical contact (see Fig. 2a). This question has been addressed and answered [30] by showing that dual deletion of the two alternative gH/gL complexes in virus mCMV-ΔgOΔMCK-2-gO^{trans} abolished organ infection, although initial virus entry was ensured by the transcomplementation of gO. This finding implies that the gH/gL complex alone or in association with other virion envelope (glyco)proteins cannot mediate virus spread. On the other hand, virus mCMV-ΔMCK-2, lacking the gH/gL/MCK-2 but expressing the gH/gL/gO complex, was found to spread in the liver like WT virus [30]. Taken together, we see no other interpretation than redundance

of the two alternative gH/gL complexes in ensuring intra-tissue virus spread.

From redundance in the outcome, one cannot necessarily infer that the mode of spread mediated by the two complexes is identical. Specifically, the question remained whether both types of spread are accessible to blocking antibodies. The above-mentioned inhibition of spread in the liver by polyclonal antibodies [42] was observed with the ATCC VR-194 Smith strain of mCMV, meanwhile known to represent a 1:3 mixture of virions with intact MCK-2, capable of forming a functional gH/gL/MCK-2 complex, and MCK-2 truncated as a result of a frameshift point mutation, respectively [37, 43]. Since infection foci in the liver are clonal, as we have frequently observed in coinfection models with genetically different mCMV recombinants [30, 44–48], antibody-resistant spread mediated by either of the two complexes should have led to infection foci in numbers reflecting the ratio of virions in the mixture. However, in the original publication [42] and in a reanalysis of the stored tissue section slides (author J.P.), no infection foci were seen to have developed after antibody immunotherapy, which implies that spread through gH/gL/gO and spread through gH/gL/MCK-2 are both accessible to blocking antibodies.

Why MCK-2 can substitute for gO for intra-tissue spread but not for initial virus entry from the circulation is an open question. We can currently only speculate about an issue of binding avidity and multiplicity of infection. One could envisage that virions arrive from the circulation at any individual first-line target cell at very low numbers and under conditions of shearing stress by flow velocity [49], requiring high-avidity interactions for efficient entry proposed to be duly appropriated only by the gH/gL/gO complex. In contrast, a high density of virions released from infected cells under conditions of low-to-absent flow velocity within tissue can be created at polarized contact sites, known from other virus infections as "virological synapses" (reviewed in [50]), securing spread also by low-avidity interactions proposed to be discharged also by the gO/gL/MCK-2 complex. It is proposed that the gH/gL core complex provides the lowest interaction avidity, and this may explain why viral evolution has recruited additional (glyco)proteins into the complex. Future investigation will have to address the issue of interaction avidity of alternative gH/gL complexes.

Regardless of the precise molecular mechanism, our data have the medically interesting bearing that interventional strategies that directly target gH/gL, and thus all gH/gL complexes, should be more promising to prevent CMV organ infection than are strategies targeting only either complex. Clinical trials [51, 52], which had enrolled patients with an acute AIDS-associated CMV retinitis, gave disappointing results of an adjuvant therapy with the neutralizing anti-gH monoclonal antibody MSL-109 [53], which may relate to established infection of an immune privileged site. A beneficial effect of MSL-109, however, is reported for prevention of primary CMV infection after allogeneic hematopoietic cell transplantation (allo-HCT) in the high-risk constellation of a CMV-negative recipient and a CMV-positive donor [54]. It must be recalled in this context that any other anti-CMV therapy, for instance therapy with antivirals as well as CD8 T cell-based immunotherapy, is most effective as a so-called "preemptive therapy" initiated upon first detection of CMV infection by highly sensitive methods, whereas "therapy" of established organ infection is generally more demanding. This is clinical experience (reviewed in [55]) and the result of "proof of concept" studies in the mouse model ([56, 57], reviewed in [58]). Regarding the specific failures of MSL-109, a new mode of resistance by incorporating neutralizing antibody into assembling virions [59] as well as resistance mutations within gH [53] might have been involved. However, these more general limitations of neutralizing antibody therapies do, in our opinion, not argue against the concept that gH/gL, rather than gO or the UL128-131A proteins, is the preferable target for an antiviral intervention.

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