Combined knockdown of RL13 and UL128 for release of cell-free infectivity from recent HCMV isolates

Nina Weiler[1], Kerstin Laib Sampaio[1], Richard James Stanton[2], and Christian Sinzger[1]

1 Institute for Virology, Ulm University Medical Center, Germany

2 Division of Infection and Immunity, Cardiff University School of Medicine, United Kingdom

Abstract:

Due to strictly cell-associated growth, experiments requiring cell-free virus are not applicable to recent clinical HCMV isolates to date. On the other hand, adaptation to cell-free growth is associated with undesirable changes in the viral gene regions RL13 and UL128. We had previously found that siRNA-mediated reduction of UL128 allowed transient release of cell-free virus by clinical isolates, and now hypothesized that virus yield could be further increased by additional knockdown of RL13. Despite the extensive polymorphism of RL13, effective RL13-specific siRNAs could be designed for three recent isolates and the Merlin strain. Knockdown efficiency was demonstrated at the protein level with a Merlin variant expressing V5-tagged pRL13. Knockdown of RL13 alone did not result in measurable release of cell-free virus, but combined knockdown of RL13 and UL128 increased infectivity in cell-free supernatants by a factor of 10-2000 compared to knockdown of UL128 alone. These supernatants could be used in dose-response assays to compare the effect of a neutralizing antibody on the various HCMV isolates. In summary, combined knockdown of RL13 and UL128 by specific siRNAs allows reliable release of cell-free infectivity from otherwise strictly cell-associated HCMV isolates without the need to modify the viral genome.

Introduction:

Human Cytomegalovirus (HCMV) belongs to the betaherpesvirus family and is spread worldwide (Cannon et al., 2010; Zuhair et al., 2019) with lifelong persistence in its host after infection (Collins-McMillen et al., 2018; Landolfo et al., 2003; Mocarski, 2007). Both initial infection and reactivation can cause severe health problems for immunocompromised patients (Boeckh and Ljungman, 2009; Griffiths and Reeves, 2021; Ljungman et al., 2019). Several available antiviral drugs, particularly ganciclovir, foscarnet or letermovir, have provided great clinical improvement in the prevention or treatment of HCMV-induced diseases, but all of them are still limited in their application as they have toxic side effects or may induce resistance (Chou, 2020; De Clercq and Li, 2016; Douglas et al., 2020; El Helou and Razonable, 2019; Jung et al., 2019; Marty et al., 2017). Hence, the development of additional antiviral therapeutic approaches is desirable, and in this context it is important to include

recent HCMV isolates that, unlike the available laboratory strains, have not yet mutated by adapting to growth in cell culture (Dargan et al., 2010; Frascaroli and Sinzger, 2014; Sinzger et al., 1999).

Mutations in the UL128 gene locus and the RL13 gene of the viral genome repeatedly occur in freshly isolated HCMV after only a few passages in cell culture (Dargan et al., 2010), apparently driven by strong selection pressure against these gene regions. The selection advantage that arises from disruption of the RL13 and the UL128 locus is most likely the switch from pure cell-associated focal spread of the virus to a combination of cell-associated and cell-free virus transmission (Stanton et al., 2010). This results in increased focal size and a diffuse, homogeneous pattern of infection mediated by the appearance of infectivity in the supernatant (Sinzger et al., 1999). The UL128 protein is part of the pentameric complex, which consists of the glycoproteins H and L and the three proteins of the UL128 locus as accessory proteins (pUL128, pUL130, and pUL131A) (Adler et al., 2006; Ryckman et al., 2008; Wang and Shenk, 2005). Disruptive mutations that entail complete loss of the pentamer complex or smaller mutations that reduce its expression level not only affect the tropism of the virus for endothelial and epithelial cells but also increase the capacity of the virus to spread in fibroblasts via the cell-free infection route (Hahn et al., 2004; Laib Sampaio et al., 2016; Murrell et al., 2013; Wang and Shenk, 2005). The RL13 gene belongs to the RL11 gene family and is highly polymorphic (Dolan, 2004; Li et al., 2011; Sekulin et al., 2007). It encodes a glycoprotein that is also present in the viral envelope, and its restrictive effect on virus spread has been shown in the context of a genetically restored version of the HCMV laboratory strain Merlin (Stanton et al., 2010; Laib Sampaio et al., 2016). Mechanistically, it is conceivable that the envelope protein pRL13 regulates the release of cell free virions or modifies their infectivity. While the phenotypic effect of mutations in RL13 and the UL128 locus greatly facilitates work with cell culture-adapted HCMV laboratory strains by increasing the virus titers that can be achieved, one drawback is that their glycoprotein composition differs from genetically unaltered isolates (Weiler et al., 2021). This affects any conclusions regarding virus transmission and entry drawn solely from results obtained with such strains.

On the other hand, working with recent isolates is complicated by several obstacles. Since they spread in fibroblast cultures in a strictly cell-associated manner with almost no infectivity detectable in the supernatant (Sinzger et al., 1999; Yamane et al., 1983), they must be passaged by co-culture of infected and uninfected cells (Frascaroli and Sinzger, 2014). This precludes synchronized infections with defined multiplicities of infection, which in turn is an important prerequisite for precise quantitative analysis of individual replication steps. This is particularly evident for the step of virus entry into the cell and its inhibition, e.g., by neutralizing antibodies or soluble receptors. In addition, approaches that rely on cell-associated transmission are also constrained by the limited timespan until mutations occur. Remarkably, a bacterial artificial chromosome (BAC)-cloned strain Merlin, which was genetically restored, mutated in RL13 or the UL128 locus within the first 3 passages of the reconstitution

procedure in fibroblasts (Stanton et al., 2010). This emphasizes how strong the selection against these gene regions is. In one strain, Tet operators were introduced upstream of RL13 and the UL128 locus to temporarily turn off expression and alleviate the selective pressure and thereby provide a solution to the problem. However, this approach requires genetic engineering, precluding its application to a recent isolate that has not yet been cloned as a BAC. Transient knockdown of UL128 is an alternative approach to release cell-free infectivity from genetically intact HCMV, and this procedure can in principle be applied to any recent isolate (Weiler et al., 2021). Yet, the amount of virus in the supernatant greatly differed with the two isolates tested and may be insufficient for certain downstream applications.

In this situation, additional knockdown of RL13 may help to increase virus yield in the supernatant, as disruption of RL13 has been associated with release of cell-free virus in the context of recent isolates, and the causative role of RL13 has been demonstrated in the Merlin strain (Dargan et al., 2010; Stanton et al., 2010). A similar increase of virus yield was observed when RL13 expression was repressed in the tet-regulated Merlin mutant (Laib Sampaio et al., 2016). It is tempting to suppose that siRNA-mediated knockdown of RL13 could have the same effect in the context of clinical isolates. In contrast to the largely conserved UL128, RL13 is highly polymorphic, and it is questionable whether a suitable siRNA can be found for each variant of RL13. Furthermore, it is unclear whether partial knockdown also leads to the release of cell-free virus, as has been described for complete disruption. The aim of this project was to investigate these aspects and evaluate whether combined siRNA-mediated knockdown of both RL13 and UL128 results in higher virus titers that can further facilitate work with recent HCMV isolates.

Material and Methods:

Cells:

Human foreskin fibroblasts (HFF) were cultivated in Gibco Dulbecco's Modified Eagle Medium with GlutaMAX (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) with 10 % fetal bovine serum (FCS, PAN Biotech, Aidenbach, Germany) and 100 µg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA). Human fetal foreskin fibroblasts (HFFF-tet) are immortalized by constitutive expression of hTERT and, in addition, constantly express the Tet repressor (Stanton et al., 2010). These cells were cultured in the same medium as the HFF for propagation.

Viruses:

Merlin with V5-tagged RL13 (RCMV1511) is a bacterial artificial chromosome (BAC) cloned HCMV strain which has a reconstructed wildtype genome, with tet-operator sequences in front of the RL13 and UL131A genes (Stanton et al., 2010). RCMV1511 was cultivated in HFFF-tet cells until cell-free virus

could be harvested, which was then added to uninfected HFF to achieve cell-associated growth for later use in siRNA knockdown experiments.

Recent clinical HCMV isolates were provided by the diagnostic laboratory of the Institute of Virology in Ulm. They were initially isolated on HFFs from throat washings from patients of the Ulm University Medical Center and passaged for several weeks until the desired cytopathic effect of about 40-60 % was observed. To test whether the isolates were still cell-associated at this stage, medium from infected cultures was harvested and then centrifuged for 10 min at 4000 rpm to remove cells and debris. The cell-free supernatants were then transferred onto adherent HFFs and one day after incubation an immunofluorescence staining for viral immediate early (IE) antigens was performed. The number of infected cells never exceeded 1 per 15.000 cells. After this confirmation of the cell-associated phenotype, aliquots of infected cells were frozen at -80 °C until used in subsequent experiments.

Isolates were selected for this study according to the effect of UL128-knockdown on the release of infectivity into the supernatant. We chose two isolates that released different levels of cell-free infectivity after transfection with UL128-siRNA, as previously reported (Weiler et al., 2021), and one isolate that did not release cell-free infectivity upon the same treatment.

Design of specific RL13-siRNAs:

After the DNA of the different isolates was purified by using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), a PCR was performed using the RL13-specific primer sets (Tab. 1). The RL13 amplification products were then purified with NucleoSpin PCR Clean-up Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and checked by subsequent Sanger sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany). For each isolate and strain Merlin, three RL13- specific siRNAs were designed using the prediction tool by Sigma-Aldrich with the following settings: high score with G/C-content close to 50% (40-60%), low seed seq. frequency: YES, requires sense modification: NO and min. no. mismatches (sense and antisense): 3 or more. All three candidate siRNAs were purchased from Sigma-Aldrich and tested in combination with a UL128-specific siRNA regarding virus release into the supernatant as previously described (Weiler et al., 2021). With each isolate, at least two of the three designed RL13-siRNAs resulted in the release of infectious virus, and the siRNAs with the highest yield was selected for further use (Tab. 2; Fig. 1)

Knockdown of gene expression with siRNA:

HFFs infected with a clinical HCMV isolate or with RCMV1511 were transfected with 200 nmol/l siRNA using the transfection reagent Lipofectamine RNAiMAX (Life Technologies). For knockdown of UL128, the previously described siRNA 5’ -CUGCUACAGUCCCGAGAAA(dT)(dT)-3’was used (Weiler et al., 2021).

For knockdown of RL13, specific RL13-siRNAs were designed as described above (Tab. 2). A pool of non-targeting (NT) siRNAs was included as a control for unspecific effects of transfection with siRNA (D-001206-14; Thermo Fisher Scientific).

Detection of viral immediate early proteins by indirect immunofluorescence:

Infected cells were fixed with 80 % acetone for 5 min at room temperature, washed with PBS, and then incubated for 2 h with the antibody E13 (Argene/Biomerieux, Marcy-l'Étoile, France) or CH160 (Virusys, Taneytown, Maryland, USA), both directed against viral immediate early (IE) antigen (UL122/123). This incubation was followed by another washing step with PBS and an incubation for 45 – 60 min with Cy3-goat-anti-mouse Ig F(ab')2 fragments (Jackson ImmunoResearch, West Grove, PA, USA). Next, the cells were washed again with PBS and counterstained with 4',6-Diamidin-2-phenylindol (DAPI, Sigma-Aldrich). With appropriate fluorescence excitation, infected cells showed red nuclear fluorescence and all cells showed blue nuclear fluorescence. Fluorescence microscope images were taken with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany) and, if necessary, quantified using Zen software (Zeiss).

Immunoblotting:

Samples were lysed in 2x Laemmli lysis buffer (Laemmli, 1970) three and six days after transfection, and boiled for 5 min at 95 °C. Precipitates were removed by centrifugation at 14,800 rpm for 5 min, and clarified lysates were stored at -80 °C. For the subsequent immunoblotting under reducing conditions, the lysates were thawed and treated with 5 % β-mercaptoethanol for 5 min at 95 °C. The lysates were loaded onto 15 % polyacrylamide gels, and electrophoresis was performed in tris glycine SDS buffer. The proteins were then transferred to PVDF membranes in Tris-glycine buffer with 15 % methanol for 3 h at 18 V. The membranes were blocked and cooled overnight with PBS plus 0.1 % Tween and 5 % milk powder and then incubated with the mouse monoclonal antibody R960-25 directed against V5-tag (Thermo Fisher Scientific) or MAb 63-27 directed against pUL123 (kindly provided by W. Britt) for 2 h. After repeated washing in PBS-Tween, the membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse-Ig (Agilent DAKO, Santa Clara, CA, USA) as a secondary antibody. To visualize bound antibodies and then quantify the results, Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific) was used with FusionCapt Advance Solo (v.7 Vilber Lourmat, Eberhardzell, Germany).

Viral neutralization assay:

The monoclonal antibody MCMV5322A, which binds to the CMV envelope protein glycoprotein H (gH) and neutralizes the entry of viral laboratory strains into fibroblasts (Ishida et al., 2015), was used to test its neutralization capability on different clinical HCMV isolates. HFF cultures infected with clinical

HCMV isolates were transfected with UL128- and RL13-siRNA, and cell-free supernatants were harvested at 6 days post transfection (dpt). These supernatants were incubated with the antibody at varying concentrations for 2 h at 37 °C, and then added on a confluent monolayer of HFF. After overnight incubation, detection of viral immediate early proteins by indirect immunofluorescence followed as described above.

Statistical analyses:

To test whether there are significant differences between different conditions, a one-way ANOVA using the built-in data analyses function of OriginLab was performed on data sets with more than two data groups (Fig. 2 and 3). If ANOVA indicated significant differences between the groups in the data set tested, appropriate post-hoc analyzes were performed to identify the groups that differ from the untreated control or from the UL128 knockdown alone (Fisher's exact tests). Differences between the individual groups of data sets were then rated as marginally significant if p-values were <0.05, significant if p-values were <0.01 and highly significant if p-values were <0.001.

Results:

RL13 is highly polymorphic and specific siRNAs need to be designed for different isolates

Previously, we have shown that siRNA-mediated knockdown of UL128 allows transient release of cell-free infectivity (Weiler et al., 2021). However, the achieved titers differed between isolates and may not always be sufficient for the desired downstream applications. Therefore, we aimed to increase the amount of cell-free infectivity with combined siRNA-mediated knockdown of UL128 and RL13.

The UL128-specific siRNA that we previously evaluated for knockdown of the pentamer in HCMV isolates (Weiler et al., 2021) is expected to match with any given HCMV isolate due to the high conservation of this reading frame at the DNA level. In contrast, RL13 is one of the most divergent genes of HCMV, which complicates the design of siRNA candidates.

As a starting point, an extensive dataset of RL13 sequences from clinical samples (Suárez et al., 2019) was used to generate a consensus sequence and identify PCR primer pairs that matched relatively conserved regions of RL13 (Tab. 1). PCR amplification and sequencing allowed us to determine the RL13 sequence of the three isolates selected for our study, except for a few nucleotides at both ends of the reading frame (Fig. 1). Not unexpectedly, the RL13 sequences of the three isolates diverged greatly from each other and from the RL13 sequence of strain Merlin. Due to this polymorphism, attempts to predict RL13-specific siRNAs did not yield a candidate that would match all sequences. Instead, RL13-specific siRNAs were predicted for each isolate and strain Merlin individually. For each virus, the three highest scoring candidates were tested in combination with the UL128-siRNA for release of infectivity in an initial experiment, and the siRNA that yielded the highest titer was chosen for further experiments (supplementary figure 1).

Combined knockdown of UL128 and RL13 leads to substantial release of cell-free infectivity by strain Merlin

First, we wanted to address the question of whether treatment with RL13-specific siRNAs can downregulate the respective protein (pRL13). As antibodies against the RL13 protein itself are not available, we used a BAC-cloned variant of strain Merlin that encodes a V5 tagged pRL13 and resembles recent HCMV isolates regarding cell associated growth in HFF cultures (RCMV1511). This reporter virus was used to simulate the situation of cultures infected with recent isolates.

Since HCMV freshly isolated in HFF cultures from clinical material does not release cell-free infectivity it must be passaged and grown by coculture with uninfected cells, resulting in an asynchronous mixture of infected cells in different stages of infection along with cells not yet infected. The highest infection rate that we could achieve before the risk of cell culture adaptation and associated genetic alterations increased was about 60 %. To mimic this situation with RCMV1511, we first used cell-free virus stocks generated on HFFFtet cells to infect normal HFFs for three days at a high multiplicity of infection (MOI = 2), and then cocultured the infected culture with a threefold excess of uninfected cells for another three days. This procedure resulted in an infection rate of approximately 60 % with infected cells being in different stages of the viral replication cycle, closely resembling the condition of our isolate cultures at the time of storage.

These cultures were then transfected with the respective si-RNAs to knockdown either RL13 and UL128 individually or in combination. As a mock-treated control, we used a non-targeting (NT) siRNA, which was also included in the individual knockdowns to compensate for the missing amount of siRNA and thus ensure equal amounts of siRNA in all experiments. Cell lysates were then produced at 6 d post transfection (dpt) and used for western blot analysis, with equivalent cell numbers loaded in each lane. pRL13 was detected by chemiluminescence using antibodies against the V5 tag and the signal was quantitatively analyzed. The total level of pRL13 in lysates is influenced not only by the expression level in individual infected cells but also by the number of infected cells. While in all cell cultures about 60 % of the cells were infected at the time of knockdown, the efficiency of virus spread during the following days may depend on the siRNAs applied, resulting in different infection levels when the cells are lysed after 6 d for western blot analysis. To compensate for this factor, we also detected the viral immediate early protein pUL123 as a reference value reflecting the fraction of infected cells within the culture. The pRL13/pUL123 ratio was then calculated to estimate the efficiency of RL13 knockdown in infected cells (Fig. 2A).

As expected from our previous experience with knockdown of UL128 in recent isolates (Weiler et al., 2021), pUL123-levels were increased upon treatment with UL128-siRNA as compared to cultures treated with NT-siRNA only, indicating enhanced virus spread in those cultures (Fig. 2A). Comparison

of pRL13/pUL123 ratios as an indicator of RL13 expression per infected cell showed that knockdown of RL13 significantly reduced this value to about 60 % of the control (2xNT), indicating that the RL13-siRNA was effective. Remarkably, treatment with UL128-siRNA alone also reduced the pRL13/pUL123 ratio (to about 50 %) suggesting an interaction of the two proteins. The combination with RL13-specific siRNA further decreased this value (to about 40 %), but this effect was far from being significant ($p = 0.67$, Fig. 2A).

Since knockdown of RL13 was obviously effective on the protein level, the next question to be addressed was whether this knockdown would promote release of cell-free infectivity. Therefore, we harvested supernatants at 3 dpt and 6 dpt, removed cells and debris, and analyzed their cell-free infectivity by overnight inoculation of HFF cultures, immunofluorescence detection of viral IE antigens and calculation of the respective virus titers (Fig. 2B). The mock-treated control cultures with transfection of non-targeting siRNA released only a small amount of cell-free infectivity, as expected, and knockdown of RL13 alone did not change this. In contrast, individual knockdown of UL128 increased the cell-free infectivity up to 4-fold as compared to the 2xNT control, which was expected from our previous experience with UL128 knockdown in HFFs infected with recent HCMV isolates (Weiler et al., 2021). The main question in this study, however, was whether a combination with siRNA against RL13 would further enhance this effect. Indeed, the combined knockdown of UL128 and RL13 augmented the detectable infectivity in the supernatant by a factor of 10-50 and yielded virus titers around 1,800 IU/ml at 6 dpt, which was a highly significant increase as compared to the nontargeting control or the knockdown of UL128 alone.

In summary, these experiments showed that RL13-siRNA effectively represses the expression of pRL13 and strongly enhances the effect of UL128-siRNAs on the release of cell-free infectivity by the Merlin strain.

UL128-siRNA-mediated release of cell-free infectivity from recent HCMV isolates is significantly increased by additional knockdown of RL13

The finding that knockdown of RL13 was effective in strain Merlin suggested that it might also enhance the effect of the UL128 knockdown on release of infectious virus by clinical isolates. Therefore, we tested a set of three different clinical isolates, including one that did not release cell-free virus into the supernatant upon UL128 knockdown alone and two that already showed release of cell-free infectivity with knockdown of UL128 (Weiler et al., 2021).

We analyzed these three different isolates using the same experimental design described in the previous section for the Merlin BAC, i.e., at the time of treatment, cultures of the isolates consisted of 40-60% infected cells at various stages of the viral replication cycle. Again, a non-targeting siRNA was included as a mock-treated control, siRNAs specific for UL128 and RL13 were used to knock down these

genes individually or in combination, and non-targeting siRNA was added in the individual treatments to adjust the total level of siRNA. Supernatants were then harvested at 3 dpt and 6 dpt and analyzed for cell-free infectivity by overnight inoculation of HFF cultures and detection of viral IE antigens. The number of infected cells was counted, virus titers were calculated, and to facilitate comparison of the three isolates, all titers were normalized to the respective mock-treated controls.

As with strain Merlin in the previous set of experiments, knockdown of RL13 alone did not significantly increase cell-free virus titers above baseline levels with any of the isolates (Fig. 3). The effect of the UL128-siRNA was variable: the first isolate showed almost no cell-free infectivity after knockdown of UL128 alone, whereas virus titers of isolates 2 and 3 increased significantly to around 300 IU/ml and 60 IU/ml, respectively. With all three isolates, the combination of both siRNAs resulted in a significant increase in cell-free infectivity above the titers measured with the nontargeting control or the single knockdowns of RL13 and UL128. When compared with UL128 alone, the combination of both siRNAs was 1-3 log fold more effective, resulting in titers of around 1,500 IU/ml with isolate 1, 4,000 IU/ml with isolate 2 and 42,000 IU/ml with isolate 3. Consistently, the increase of cell-free infectivity was higher at 6 dpt when compared to 3 dpt.

In conclusion, siRNA-mediated knockdown of RL13 in addition to that of UL128 increased the cell-free infectivity significantly in all tested isolates to titers that allow for downstream applications requiring cell-free infectivity.

Application of cell-free virus from recent HCMV isolates in a neutralization assay

As an example of such a downstream application, we used the cell-free virus generated by combined knockdown of UL128 and RL13 in neutralization assays to analyze the efficacy of the monoclonal antibody MCMV5322A, which had previously been in phase I and II trials (Ishida et al., 2017, 2015). This antibody is directed against glycoprotein H of HCMV and inhibited infection by strain VR1814 at a half-maximal concentration of 0.2 - 0.5 µg/ml. Our novel approach to release cell-free infectious virus by siRNA-mediated knockdown of UL128 and RL13 allowed the antibody to be tested not only on cell culture adapted laboratory strains, but also on recent isolates, for the first time.

HFF cultures infected with the respective isolate were transfected with siRNA against UL128 and RL13, and cell-free supernatants were harvested from the transfected cultures at 6 dpt. We expected similar virus titers as in the knockdowns before (1,000 - 50,000 IU/ml) resulting in MOIs of about 0.005 – 0.25, depending on the isolate used. These supernatants were incubated for 2 h with various concentrations of the antibody MCMV5322A in a 4-fold dilution series at final concentrations ranging from 2 to 9,600 ng/ml. The virus-antibody mixture was then added to uninfected HFFs and incubated overnight. It is important to note that the MOI was always markedly below 1 infectious unit per cell, which ensures

that neutralization of virus particles directly translates into a reduced number of infected cells. The following day, the cultures were fixed, the viral IE antigen was visualized by immunofluorescence, and the respective infection rates were determined. Infection rates for each of the three isolates were plotted against antibody concentrations to generate dose-response curves.

The antibody showed a complete neutralizing effect on all three isolates, with EC50 values varying between 14 and 41 ng/ml (Fig. 4). When compared to the reported effect on strain VR1814 (Ishida et al., 2015), the three isolates appeared to be approximately 10-fold more sensitive, which indicates that the inclusion of such isolates can provide additional information as compared to laboratory strains.

Discussion:

Our finding that siRNA-mediated knockdown of RL13 alone does not increase the cell-free infectivity of recent HCMV isolates but substantially amplifies virus titers achieved with UL128-specific siRNA has the potential to facilitate research on genetically intact HCMV isolates, as it enables many experimental approaches that were previously impossible due to the strict cell-association of such isolates.

The results of this study are mainly in line with previous reports on the contribution of RL13 and the UL128 locus in restricting HCMV to the cell-associated transmission route (Dargan et al., 2010; Laib Sampaio et al., 2016; Stanton et al., 2010). The three proteins encoded by the UL128 locus form the pentameric envelope complex together with gH/gL and compete for these two proteins with gO which is the accessory protein of the trimeric complex (Adler et al., 2006; Chandramouli et al., 2017; Kschonsak et al., 2021; Li et al., 2015; Ryckman et al., 2008; Wang and Shenk, 2005). In the absence of gO, HCMV spreads in a strictly cell-associated manner, whereas reduced expression of the pentamer is associated with increased cell-free infectivity (Jiang et al., 2008; Murrell et al., 2013; Wille et al., 2010). The glycoprotein encoded by RL13 was also detected in the viral envelope, and disruption of this gene also increased the level of cell-free infectivity (Stanton et al., 2010).

However, RL13 and the pentamer are apparently not completely equivalent in their contribution to the cell-associated phenotype of HCMV. While partial knockdown of UL128 alone was sufficient to release cell-free infectivity from two isolates, RL13-siRNA had no such effect in any of the three isolates included in this study. This result differs slightly from a previous report that both RL13 and UL128 are necessary for maintaining the cell-associated phenotype of HCMV (Stanton et al., 2010). Deletion of RL13 alone increased infectivity in the supernatant compared with genetically intact virus, albeit the effect was smaller than for deletion of UL128. While RL13 expression was completely absent in that deletion virus, our siRNA treatment caused only a partial knockdown of RL13 expression. It is conceivable that the remaining expression of RL13 is sufficient to keep the virus cell-associated unless UL128 is also knocked down. Our finding indicates that the two gene regions may act by different mechanisms and that their interaction is more complex than being simply additive, which is further

supported by the unexpected finding that knockdown of UL128 also affected the level of RL13 expression in the infected culture (Fig. 2A).

While it is tempting to speculate about possible interactions between pRL13 and pentamer either during virus maturation or in the envelope of the mature particle, this was beyond the scope of this project. Questions to be resolved in future studies include, (i) whether these proteins rather regulate the release of virions into the extracellular space or rather modulate the infectivity of particles that have been released, (ii) whether UL128 promotes RL13 expression at the transcriptional level or stabilizes the translated protein, (iii) whether there are threshold effects and (iv) whether further viral factors are involved. The latter is conceivable as the effect strength of the knockdowns differed notably between the three isolates. While UL128 knockdown was most effective in isolate 2, the additional knockdown of RL13 had the strongest effect in isolate 3. As UL128 is highly conserved among these isolates, these differences are more likely explained by differences in RL13 or in a yet unknown third factor.

While previous findings on the function of RL13 and UL128 had suggested both genes as potential targets for knockdown to increase cell-free infectivity, the two gene regions differ in terms of practical considerations. UL128 is highly conserved (Baldanti et al., 2006; Paradowska et al., 2019; Sun et al., 2009), which means that the same siRNA will work in most, if not all, isolates. In contrast, RL13 is highly polymorphic (Dolan, 2004; Suárez et al., 2019) and therefore different RL13-siRNAs had to be designed for each of the three isolates and the Merlin strain. Starting with three predicted siRNAs per virus, two of them usually proved effective in initial trials, and the most effective one was then used for further experiments. Since the siRNA that failed in the experiment was usually the one with the lowest score in the prediction, the effort might be reduced to only testing the two siRNAs with the highest score. Ten different genotypes were reported for RL13 (Suárez et al., 2019), and five of them are covered with the four siRNAs that we have successfully tested. It would be interesting to see whether a set of RL13-siRNAs covering all ten genotypes can be effective in releasing virus from a new unknown isolate, or whether sequencing and selection of genotype-specific siRNA is required.

Unfortunately, no antibodies against the RL13 protein itself are available. Therefore, we could only investigate knockdown efficiency at the protein level in a Merlin variant expressing a V5-tagged RL13 protein (Stanton et al., 2010), where we observed an average reduction of about 40%. However, we can indirectly infer knockdown efficiency in the isolates, as the combined application of siRNAs against RL13 and UL128 significantly increased the release of infectivity in all of them compared to the combination of UL128-siRNA and the nontargeted control RNA. This can only be explained by a specific effect of the RL13-siRNA.

Regarding virus release, it is important that even in an isolate where knockdown of UL128 alone was ineffective, a titer of about 1000-2000 IU/ml could be achieved with the combination of siRNAs against RL13 and UL128. This will allow for many downstream applications that require cell-free infection, including transfer of the virus to other cell types, neutralization assays, the analysis of replication kinetics, or the determination of particle/infectivity ratios. Applications that may require higher virus titers like the immunoblot analysis of the glycoprotein composition of virion particles can still be applied to a subset of isolates represented by isolates 2 and 3 in our study, where titers up to 40,000 IU/ml were reached. A limitation of this knockdown approach is that reduction of pUL128 will certainly lead to a reduction of pentamer levels in infected cells, which may not only affect the cell-associated phenotype but also to some extent alter the cell tropism. The reduction in pRL13 may also have yet unknown effects beyond increased release of infectivity. This should be considered when interpreting the results in terms of the behavior of the natural virus in its host. However, taking this caveat into account, the method allows for the first time the inclusion of any HCMV isolate in procedures requiring cell-free virus.

As an example of how this procedure can be applied, we analyzed our three isolates regarding the neutralizing activity of a monoclonal antibody, MCMV5322A, previously tested in clinical trials for prophylactic or therapeutic use (Ishida et al., 2017, 2015). MCMV5322A is directed against the HCMV glycoprotein H, which is part of both the trimeric and the pentameric complex, and it blocked infection of various cell types by the laboratory strain VR1814 at half-maximal concentrations of 0.2 to 0.5 µg/ml. In combination with another monoclonal antibody against the pentameric complex it was effective at limiting CMV viremia within 24 weeks posttransplant, median time to CMV viremia, and incidence of CMV disease, however it did not significantly reduce the primary endpoint of CMV viremia within 12 weeks posttransplant (Ishida et al., 2017). In addition to possible explanations mentioned by the authors, inefficacy against cell-associated spread or yet unidentified target cells, we speculated that MCMV5322A might be ineffective against many of the HCMV strains in the treated patients and tested this hypothesis using our combination of siRNAs against RL13 and UL128. The procedure reliably yielded cell-free infectious virus from all three isolates at titers sufficient to perform neutralization assays. Surprisingly, all isolates were actually 10-fold more sensitive to neutralization by MCMV5322A than reported for the laboratory strain, which argues strongly against the assumption that this antibody failed against the majority of patient strains, but also demonstrates that isolates can differ from laboratory strains and should be included in such analyses.

In conclusion, the combined siRNA-mediated knockdown of UL128 and RL13 is superior to knockdown of UL128 alone in terms of achievable viral titers and will therefore further facilitate synchronized, precisely dosed cell-free infection with otherwise strictly cell-associated HCMV isolates. This may be particularly important in the development of new anti-HCMV treatment options.

References:

Adler, B., Scrivano, L., Ruzcics, Z., Rupp, B., Sinzger, C., Koszinowski, U., 2006. Role of human cytomegalovirus UL131A in cell type-specific virus entry and release. J. Gen. Virol. 87, 2451–2460. https://doi.org/10.1099/vir.0.81921-0

Baldanti, F., Paolucci, S., Campanini, G., Sarasini, A., Percivalle, E., Revello, M.G., Gerna, G., 2006. Human cytomegalovirus UL131A, UL130 and UL128 genes are highly conserved among field isolates. Arch. Virol. 151, 1225–1233. https://doi.org/10.1007/s00705-005-0696-5

Boeckh, M., Ljungman, P., 2009. How I treat cytomegalovirus in hematopoietic cell transplant recipients 113, 10.

Cannon, M.J., Schmid, D.S., Hyde, T.B., 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev. Med. Virol. 20, 202–213. https://doi.org/10.1002/rmv.655

Chandramouli, S., Malito, E., Nguyen, T., Luisi, K., Donnarumma, D., Xing, Y., Norais, N., Yu, D., Carfi, A., 2017. Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. Sci. Immunol. 2, eaan1457. https://doi.org/10.1126/sciimmunol.aan1457

Chou, S., 2020. Advances in the genotypic diagnosis of cytomegalovirus antiviral drug resistance. Antiviral Res. 176, 104711. https://doi.org/10.1016/j.antiviral.2020.104711

Collins-McMillen, D., Buehler, J., Peppenelli, M., Goodrum, F., 2018. Molecular Determinants and the Regulation of Human Cytomegalovirus Latency and Reactivation. Viruses 10, 444. https://doi.org/10.3390/v10080444

Dargan, D.J., Douglas, E., Cunningham, C., Jamieson, F., Stanton, R.J., Baluchova, K., McSharry, B.P., Tomasec, P., Emery, V.C., Percivalle, E., Sarasini, A., Gerna, G., Wilkinson, G.W.G., Davison, A.J., 2010. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. J. Gen. Virol. 91, 1535–1546. https://doi.org/10.1099/vir.0.018994-0

De Clercq, E., Li, G., 2016. Approved Antiviral Drugs over the Past 50 Years. Clin. Microbiol. Rev. 29, 695–747. https://doi.org/10.1128/CMR.00102-15

Dolan, A., 2004. Genetic content of wild-type human cytomegalovirus. J. Gen. Virol. 85, 1301–1312. https://doi.org/10.1099/vir.0.79888-0

Douglas, C.M., Barnard, R., Holder, D., Leavitt, R., Levitan, D., Maguire, M., Nickle, D., Teal, V., Wan, H., van Alewijk, D.C.J.G., van Doorn, L.-J., Chou, S., Strizki, J., 2020. Letermovir Resistance Analysis in a Clinical Trial of Cytomegalovirus Prophylaxis for Hematopoietic Stem Cell Transplant Recipients. J. Infect. Dis. 221, 1117–1126. https://doi.org/10.1093/infdis/jiz577

El Helou, G., Razonable, R.R., 2019. Safety considerations with current and emerging antiviral therapies for cytomegalovirus infection in transplantation. Expert Opin. Drug Saf. 18, 1017–1030. https://doi.org/10.1080/14740338.2019.1662787

Frascaroli, G., Sinzger, C., 2014. Distinct Properties of Human Cytomegalovirus Strains and the Appropriate Choice of Strains for Particular Studies, in: Yurochko, A.D., Miller, W.E. (Eds.), Human Cytomegaloviruses. Humana Press, Totowa, NJ, pp. 29–46. https://doi.org/10.1007/978-1-62703-788-4_3

Griffiths, P., Reeves, M., 2021. Pathogenesis of human cytomegalovirus in the immunocompromised host. Nat. Rev. Microbiol. 19, 759–773. https://doi.org/10.1038/s41579-021-00582-z

Hahn, G., Revello, M.G., Patrone, M., Percivalle, E., Campanini, G., Sarasini, A., Wagner, M., Gallina, A., Milanesi, G., Koszinowski, U., Baldanti, F., Gerna, G., 2004. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. J. Virol. 78, 10023–10033. https://doi.org/10.1128/JVI.78.18.10023-10033.2004

Ishida, J.H., Burgess, T., Derby, M.A., Brown, P.A., Maia, M., Deng, R., Emu, B., Feierbach, B., Fouts, A.E., Liao, X.C., Tavel, J.A., 2015. Phase 1 Randomized, Double-Blind, Placebo-Controlled Study of RG7667, an Anticytomegalovirus Combination Monoclonal Antibody Therapy, in Healthy Adults. Antimicrob. Agents Chemother. 59, 4919–4929. https://doi.org/10.1128/AAC.00523-15

Ishida, J.H., Patel, A., Mehta, A.K., Gatault, P., McBride, J.M., Burgess, T., Derby, M.A., Snydman, D.R., Emu, B., Feierbach, B., Fouts, A.E., Maia, M., Deng, R., Rosenberger, C.M., Gennaro, L.A.,

Striano, N.S., Liao, X.C., Tavel, J.A., 2017. Phase 2 Randomized, Double-Blind, Placebo-Controlled Trial of RG7667, a Combination Monoclonal Antibody, for Prevention of Cytomegalovirus Infection in High-Risk Kidney Transplant Recipients. Antimicrob Agents Chemother 61. https://doi.org/10.1128/aac.01794-16

Jiang, X.J., Adler, B., Sampaio, K.L., Digel, M., Jahn, G., Ettischer, N., Stierhof, Y.-D., Scrivano, L., Koszinowski, U., Mach, M., Sinzger, C., 2008. UL74 of human cytomegalovirus contributes to virus release by promoting secondary envelopment of virions. J. Virol. 82, 2802–2812. https://doi.org/10.1128/JVI.01550-07

Jung, S., Michel, M., Stamminger, T., Michel, D., 2019. Fast breakthrough of resistant cytomegalovirus during secondary letermovir prophylaxis in a hematopoietic stem cell transplant recipient. BMC Infect. Dis. 19, 388. https://doi.org/10.1186/s12879-019-4016-1

Kschonsak, M., Rougé, L., Arthur, C.P., Hoangdung, H., Patel, N., Kim, I., Johnson, M.C., Kraft, E., Rohou, A.L., Gill, A., Martinez-Martin, N., Payandeh, J., Ciferri, C., 2021. Structures of HCMV Trimer reveal the basis for receptor recognition and cell entry. Cell 184, 1232-1244.e16. https://doi.org/10.1016/j.cell.2021.01.036

Laemmli, U.K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0

Laib Sampaio, K., Stegmann, C., Brizic, I., Adler, B., Stanton, R.J., Sinzger, C., 2016. The contribution of pUL74 to growth of human cytomegalovirus is masked in the presence of RL13 and UL128 expression. J. Gen. Virol. 97, 1917–1927. https://doi.org/10.1099/jgv.0.000475

Landolfo, S., Gariglio, M., Gribaudo, G., Lembo, D., 2003. The human cytomegalovirus. Pharmacol. Ther. 98, 269–297. https://doi.org/10.1016/S0163-7258(03)00034-2

Li, G., Nguyen, C.C., Ryckman, B.J., Britt, W.J., Kamil, J.P., 2015. A viral regulator of glycoprotein complexes contributes to human cytomegalovirus cell tropism. Proc. Natl. Acad. Sci. U. S. A. 112, 4471–4476. https://doi.org/10.1073/pnas.1419875112

Li, M., Ma, Y., Ji, Y., He, R., Qi, Y., Sun, Z., Wang, N., Gao, S., Ruan, Q., 2011. Human cytomegalovirus RL13 gene transcripts in a clinical strain. Virus Genes 43, 327–334. https://doi.org/10.1007/s11262-011-0641-5

Ljungman, P., de la Camara, R., Robin, C., Crocchiolo, R., Einsele, H., Hill, J.A., Hubacek, P., Navarro, D., Cordonnier, C., Ward, K.N., 2019. Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). Lancet Infect. Dis. 19, e260–e272. https://doi.org/10.1016/S1473-3099(19)30107-0

Marty, F.M., Ljungman, P., Chemaly, R.F., Maertens, J., Dadwal, S.S., Duarte, R.F., Haider, S., Ullmann, A.J., Katayama, Y., Brown, J., Mullane, K.M., Boeckh, M., Blumberg, E.A., Einsele, H., Snydman, D.R., Kanda, Y., DiNubile, M.J., Teal, V.L., Wan, H., Murata, Y., Kartsonis, N.A., Leavitt, R.Y., Badshah, C., 2017. Letermovir Prophylaxis for Cytomegalovirus in Hematopoietic-Cell Transplantation. N. Engl. J. Med. 377, 2433–2444. https://doi.org/10.1056/NEJMoa1706640

Mocarski, E.S.Jr., 2007. Betaherpes viral genes and their functions, in: Arvin, A., Campadelli-Fiume, G., Mocarski, E., Moore, P.S., Roizman, B., Whitley, R., Yamanishi, K. (Eds.), Human Herpesviruses. Cambridge University Press, Cambridge, pp. 204–230. https://doi.org/10.1017/CBO9780511545313.016

Murrell, I., Tomasec, P., Wilkie, G.S., Dargan, D.J., Davison, A.J., Stanton, R.J., 2013. Impact of sequence variation in the UL128 locus on production of human cytomegalovirus in fibroblast and epithelial cells. J. Virol. 87, 10489–10500. https://doi.org/10.1128/JVI.01546-13

Paradowska, E., Jabłońska, A., Studzińska, M., Kasztelewicz, B., Wiśniewska-Ligier, M., Dzierżanowska-Fangrat, K., Woźniakowska-Gęsicka, T., Czech-Kowalska, J., 2019. Distribution of the CMV glycoprotein gH/gL/gO and gH/gL/pUL128/pUL130/pUL131A complex variants and associated clinical manifestations in infants infected congenitally or postnatally. Sci. Rep. 9, 16352. https://doi.org/10.1038/s41598-019-52906-y

Ryckman, B.J., Rainish, B.L., Chase, M.C., Borton, J.A., Nelson, J.A., Jarvis, M.A., Johnson, D.C., 2008. Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates

entry into epithelial and endothelial cells. J. Virol. 82, 60–70. https://doi.org/10.1128/JVI.01910-07
Sekulin, K., Görzer, I., Heiss-Czedik, D., Puchhammer-Stöckl, E., 2007. Analysis of the variability of CMV strains in the RL11D domain of the RL11 multigene family. Virus Genes 35, 577–583. https://doi.org/10.1007/s11262-007-0158-0
Sinzger, C., Schmidt, K., Knapp, J., Kahl, M., Beck, R., Waldman, J., Hebart, H., Einsele, H., Jahn, G., 1999. Modification of human cytomegalovirus tropism through propagation in vitro is associated with changes in the viral genome. J. Gen. Virol. 80 (Pt 11), 2867–2877. https://doi.org/10.1099/0022-1317-80-11-2867
Stanton, R.J., Baluchova, K., Dargan, D.J., Cunningham, C., Sheehy, O., Seirafian, S., McSharry, B.P., Neale, M.L., Davies, J.A., Tomasec, P., Davison, A.J., Wilkinson, G.W.G., 2010. Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication. J. Clin. Invest. 120, 3191–3208. https://doi.org/10.1172/JCI42955
Suárez, N.M., Wilkie, G.S., Hage, E., Camiolo, S., Holton, M., Hughes, J., Maabar, M., Vattipally, S.B., Dhingra, A., Gompels, U.A., Wilkinson, G.W.G., Baldanti, F., Furione, M., Lilleri, D., Arossa, A., Ganzenmueller, T., Gerna, G., Hubáček, P., Schulz, T.F., Wolf, D., Zavattoni, M., Davison, A.J., 2019. Human Cytomegalovirus Genomes Sequenced Directly From Clinical Material: Variation, Multiple-Strain Infection, Recombination, and Gene Loss. J. Infect. Dis. 220, 781–791. https://doi.org/10.1093/infdis/jiz208
Sun, Z.-R., Ji, Y.-H., Ruan, Q., He, R., Ma, Y.-P., Qi, Y., Mao, Z.-Q., Huang, Y.-J., 2009. Structure characterization of human cytomegalovirus UL131A, UL130 and UL128 genes in clinical strains in China. Genet. Mol. Res. GMR 8, 1191–1201. https://doi.org/10.4238/vol8-3gmr654
Wang, D., Shenk, T., 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc. Natl. Acad. Sci. U. S. A. 102, 18153–18158. https://doi.org/10.1073/pnas.0509201102
Weiler, N., Paal, C., Adams, K., Calcaterra, C., Fischer, D., Stanton, R.J., Stöhr, D., Laib Sampaio, K., Sinzger, C., 2021. Role of Envelope Glycoprotein Complexes in Cell-Associated Spread of Human Cytomegalovirus. Viruses 13. https://doi.org/10.3390/v13040614
Wille, P.T., Knoche, A.J., Nelson, J.A., Jarvis, M.A., Johnson, D.C., 2010. A human cytomegalovirus gO-null mutant fails to incorporate gH/gL into the virion envelope and is unable to enter fibroblasts and epithelial and endothelial cells. J. Virol. 84, 2585–2596. https://doi.org/10.1128/JVI.02249-09
Yamane, Y., Furukawa, T., Plotkin, S.A., 1983. Supernatant virus release as a differentiating marker between low passage and vaccine strains of human cytomegalovirus. Vaccine 1, 23–25. https://doi.org/10.1016/0264-410x(83)90008-7
Zuhair, M., Smit, G.S.A., Wallis, G., Jabbar, F., Smith, C., Devleesschauwer, B., Griffiths, P., 2019. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. Rev. Med. Virol. 29, e2034. https://doi.org/10.1002/rmv.2034

Figures and Tables:

Table 1: Sequencing primers

Primer	**Sequence (5'-3')**
RL13-1 for	TTACGGTTACGTGGACG
RL13-1 rev	TTAGTCCAAAACTCATCTGG
RL13-2 for	TTGCGATTACATGGACG
RL13-2 rev	TTAGTCCAAGACTCATCTGG

Table 2: Specific RL13-siRNAs for different isolates and strain Merlin

RL13-siRNA	**Sequence (5'-3')**
Merlin	GAACCAAUAUGCACCGUUA(dT)(dT)
Isolate 1	GCUACAAUCUCACGGUAAU(dT)(dT)
Isolate 2	GCAUAAAAUGUGCGAAUUA(dT)(dT)
Isolate 3	UGAACAAUCUGUACGAGUA(dT)(dT)

```
Consensus  ATGCACTGGCATCTTGCGATTACATGGACGGTAATCATATTAACGTTTTCRRAAKSNTGCAACCAAACTTGTCCG
Merlin     ..............................................GG..TGT.A..............
isolate 1  ------------------------------------------------------------.............
isolate 2  -------------------------------------------------AA..GC-.....T.....C...T.C
isolate 3  ----------------------------------------------------------.T..A.C...G.....T

Consensus  TGTCMMTGN-TYTSTGTNAATTCTACAACMGTTAACWWWTCCACAARNNNTGAAACA-N-ACRTCTAAAAACATY
Merlin     ..C.CC..CA.T.G...C...........A....G.ATA.....C.G---.......---..G..........C
isolate 1  ...TCC..CG.T.G...C...........A..C...ATA.......A---.......---..G......GC...C
isolate 2  ....AA..--.CCC...-.G.---..T..C......TAT.....T.GTAC...G...GCC..A..A.C.T...GT
isolate 3  ....AA..--.C.C...-...GAC..C..CACA..TTAT....T..ATAC.ACT.GCACA..AGT..TT....AT

Consensus  ACWMCAACTACCA-CGACAAATTNACCACAAAAACWA--CGTMAAGNNTTGYTACTACTACAMCCKCAYCAGYAA
Merlin     ..TC.........-.........--...A......A.--...C...TA...C...C......C..T.GTT..T..
isolate 1  ..TC.....G.T.-........--...G.......A.--...C...CC...T..T.......C.GT..T...T..
isolate 2  ..AA..GT..T..G.A.T...AGC..TT..G..T.T.-----.A..---...C.-.....G..A.TG..C...C..
isolate 3  .TAAGC.......--..G.TCC.CT.G...CC.-.T.CT...AC..ATCA.T.CAG.-....A..G..CA.AC..

Consensus  CNN-N-NCANNNCCGTTTCTACWGMASCGTCG--AWCAACCAYAC-NATATCTCAAAC--CAANCRCANN-GTCN
Merlin     .GACTGG..AAGT.........T.C.G.....---T......T.---..........--...C.G..---...-
isolate 1  .GATTAG..AAG....G.....T.C.G.T..---.T.....T..---..........--...T.G..---...-
isolate 2  .------..---.........AAA.C.....AA.A......C..AG.....CAC...GA...ATA..AAC..TG
isolate 3  .------..---.........A.A.C.A...GTTA......CG.GG.....---...ATT..G.A..AAAA..G

Consensus  ATACTASYANATGTNCCAWCACAACCMCRA-NNNNAMACGYGANNGTTTGAATNATANNNN--ATAWMACRKGMK
Merlin     ....G.GT.-...--.T.T.......C.G.-----.A...C..---.......-...------...TC..GG.AT
isolate 1  ....A.GT.-....--..T.......C.A.-----.A...C.---.......-...------...TC..GG.AT
isolate 2  .G....CC.C....A...A...C...A.G.CCGTT.CTT.T..TG....C...T...CAGTCC...AA.GAT.CG
isolate 3  .A....CC.CG..CA.G.AT.....TA.A.ACGTT.C...T..TG...A....T...CAGTTT...AA..AT.CG

Consensus  ATSKTRGCYAMGAAGTRANN-TCAACKTMACTGGATMMKTTGGKAGYWACATTNNNNN-NNNAAATG-RWTCMGG
Merlin     ..GT.G..C.A.....G.CTC.A...T.C.......CAT---.G.ATT.....---------C....-GT..C..
isolate 1  ..GT.G..C.A.....G.CTT.....T.C.G.....CAT..T.G..CT.....---------G....-GT..C..
isolate 2  .CCGCA.TT.C..G..A.---.....G.A..A....ACG....TG.CA....AACTCTAAAA.....CAA..A.A
isolate 3  G.CG.A..T.C.....A.---.....G.A..G...CACG....T..TA..G..ACTCTGAAA.....TAA..AC-

Consensus  -----NTAYGGYWCNNTSYAGRCTGGMTTTATTMYKMGGAACC--------N--N-AATATGYRCNMRTTASNNG
Merlin     ------..C..TT.--.CC..G....C......CCTC.....-------------.....CA.-CG...CCA.
isolate 1  ------..CA.TT.--.CC..G....C......CCTC.....-------------.....CA.-CG...CCAA
isolate 2  CTGAGAA.T..CA.AA.GT..A....A..C...ATGA.T.C..------CACGCAT..A...TG.GAA...G--.
isolate 3  --ACAA..T..CAAAA.GTG.AT...A..A.A.ATGAA.....TTATTCTTCATAC..G...TG.GAA...G--.

Consensus  CAAYTATCATCATACTWYMCCWCGTGGYRMCATATGTTTTGATTGYAACRWSACAWMACTTAYTATYTACRATCT
Merlin     ...T.........TTC..T.....TGCTC.T..C........TG..ATG...AA....CTA..T...G....
isolate 1  ..GT.........TTC..T.....TAC.T.......C.....T...ATG...AA.T..GT...T...G....
isolate 2  ...C.....C..A..AACA..A..GCACGA...........C..C...GAC..CTCC..A.C...C...A.CT.
isolate 3  ...C..C.....G...ACA..A..CA.CAA...........G..C...GAC..TTCT..G.CC..C...A....

Consensus  AACGTYAAAMRAYKCTGGAAARTANNNTRYCANASAGAAC-CGTCATRACRRTCAATACGAAGAANMATNNTACW
Merlin     G....T...CG.TT.......G..CGT.GT..-.G.....G..C...G..AA.............GC..GC...A
isolate 1  .....T...CG.TT.......G..CGT.GT..-.G.....A......G..AA.............GC..GC...A
isolate 2  ...CAC..GAA.CG.......A..---.AC..GGC.TC..-...G..A..GG....---......-A..--...T
isolate 3  ...TGC.G.AA.CG.C..G..A..---.AC..G.CG.G..-...T..A..GG.---.........-A..--...T

Consensus  ACSTMACGGTRWTWTTTGCMRACACRACN-NNNNNGTYAYCAACWN-NNGCACRTGYSMTRKAARAYAWACAG-A
Merlin     G.C.C.....GA.T.....CA....G..ATCCATA..C.C....A----.A..G..TGA.AG..A.CGA....-.
isolate 1  .TC.C.....AA.T.A...CA....G..AGCCATA..T.C....A----.G..G..TGA.AG..G.C.A...A-.
isolate 2  ..G.A.....GT.AA...GAG....A..-------..T.T.C..TCTTG....A..CCC.GT..G.T.T.A..--
isolate 3  ..G.A.....AT.A.C..AAGGTT.A..-------..CCT....TCCTG....A..CCCCGT..A.T.T.G..T.

Consensus  AAATACAG--ACACTACKAACMMTGAA---NNNATCGGRARWMAYATTATWGAAACYATTAAGAAAGCYAACATT
Merlin     ........--.......T...CA....------.....G.AAC.T.....T.....T...........C......
isolate 1  ........--.......T...CA..G.------.....G.AAC.T.....T.....T.....A.....C......
isolate 2  -...---------...GG...AC....AACACC..T..A.GTA.C..C..AA....C...G.......T......
isolate 3  ....C...TG.AT....G...AC....GGTACCG....A.GTA.CC....A.....C...CG...TA.T.....C

Consensus  CCCCTGGGAATTCATGCTGTRTGGGCGGGYATAGTGG-TATCAGTGGCACTYATAGCGCTRTAYATGG-GTARCC
Merlin     ..T..........C.....A........T.....A.-....G........C........A..T....-.C.A..
isolate 1  ........G...........G........C.......-............C.....A..A..T....-...A..
isolate 2  ....................A.....A..CG......-..........G..T......T.G..C....-...G..
isolate 3  ....................G...CG...T.......G............T........G..C....G...G..

Consensus  GTCGCAKNCCCAGRAAACCGCGTTATACCAGACTTCCYAAATAYGATCCAGATGAGTTTTGGA-CTAAAACCTAA
Merlin     ......GA.....G.......................T.....C.............C.....-...........
isolate 1  ....T.GG.....G.......................T.....C..C..............-....-------
isolate 2  A.....TT....AA.......A...C....A......C.....T...........T.......A.....------
isolate 3  ......TC..T..A..G....................C-..C.T..-...A------------------------
```

Figure 1: Comparison of RL13 sequences from Merlin and three recent HCMV isolates with location of strain-specific siRNAs. DNA samples of the three HCMV isolates were amplified and sequenced using conserved RL13-specific primers, and the obtained isolate-specific sequences were compared with the known RL13 sequence of Merlin. The top line represents the consensus of all four sequences to which the four virus-specific sequences were aligned. Start and stop codons are highlighted by bold letters. Dots indicate agreement with the consensus sequence, base substitutions are indicated by the corresponding letters, and missing amino acids are indicated by a dash. The gray highlights indicate the positions of the RL13 siRNAs specifically designed for each virus.

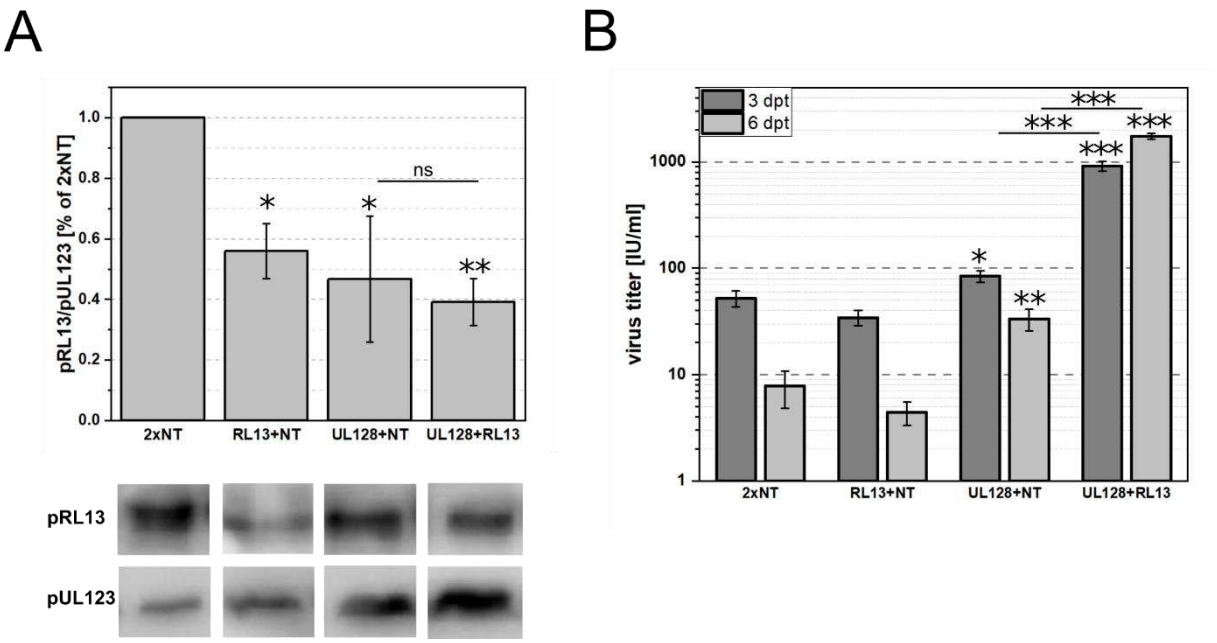

Figure 2: Effect of siRNA-mediated knockdown of UL128 and RL13 on release of cell free infectivity from RCMV1511. Human fibroblast cultures (HFFs) in which approximately 60 % of cells were infected with RCMV1511 at various stages of viral replication were transfected with siRNAs targeting transcripts of the viral genes UL128 and/or RL13. Non-targeted siRNAs (NT) were used as controls and were also added in single UL128 and RL13 knockdown experiments to keep the total amount of RNA constant in all samples. (A) Cell lysates were prepared and analyzed six days post transfection (dpt) by immunoblotting for expression of pRL13 and for levels of pUL123 as a reference representing the number of infected cells. Chemiluminescence signals were analyzed by densitometry, and pRL13/pUL123 ratios were calculated as a readout for the level of RL13 per infected cell. For each experiment, pRL13/pUL123 ratios were normalized to the 2xNT control. Bars indicate mean values of 3 replicates and error bars represent the standard error of the mean (SEM). The asterisks indicate significant differences as compared with the 2xNT control (**, $p < 0.01$; *, $p < 0.05$), and ns indicates that the difference between UL128+NT and UL128+RL13 was not significant. (B) Supernatants of the transfected cultures were harvested at 3 and 6 dpt, clarified from cells and debris, and titrated by

immunostaining for viral immediate early (IE) antigen at 24h. Bars indicate mean values of 3-6 replicates and error bars represent the standard error of the mean (SEM). The asterisks indicate significant differences as compared with the 2xNT control (***, $p < 0.001$; **, $p < 0.01$, *, $p < 0.05$).

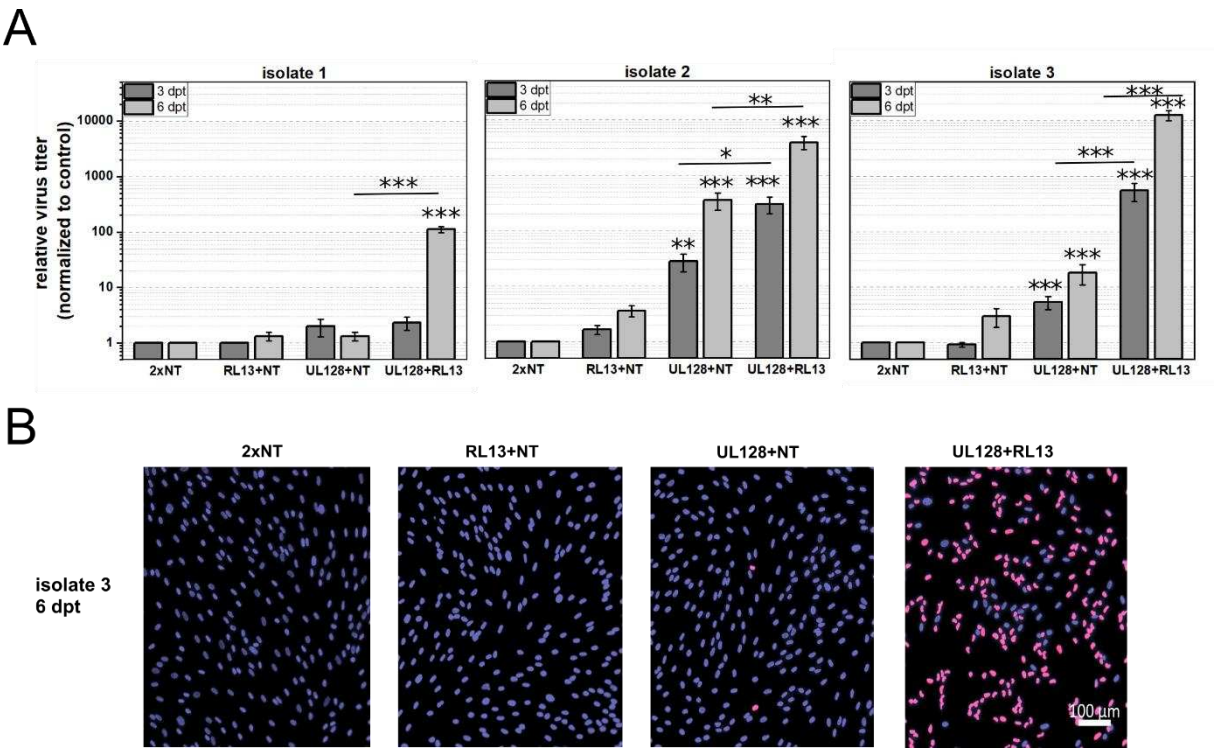

Figure 3: Effect of siRNA-mediated knockdown of UL128 and RL13 on release of cell free infectivity from recent clinical HCMV isolates. Human fibroblast cultures (HFFs) in which approximately 40-60 % of cells were infected with recent HCMV isolates at various stages of viral replication were transfected with siRNAs targeting transcripts of the viral genes UL128 and/or RL13. As a control, non-targeting siRNAs (NT) were used, and were also added in single UL128 and RL13 knockdown experiments to keep the total amount of RNA constant in all samples. Supernatants of the transfected cultures were harvested three and six days after transfection (dpt), clarified from cells and debris and tested for their infectivity. For that, uninfected HFFs were incubated with the cell-free supernatants overnight, fixed and immunostained for viral immediate early (IE) antigen. (A) The number of infected cells was counted, the virus titer calculated and normalized to the NT control. Bars indicate mean values of 4-6 replicates and error bars represent the standard error of the mean (SEM). The asterisks above the bar indicate significant differences as compared with the 2xNT control and the asterisks above the line indicate significant differences as compared with UL128+NT (**, $p < 0.01$; *, $p < 0.05$). (B) Representative examples of the immunostainings are shown for 6 dpt for isolate 3.

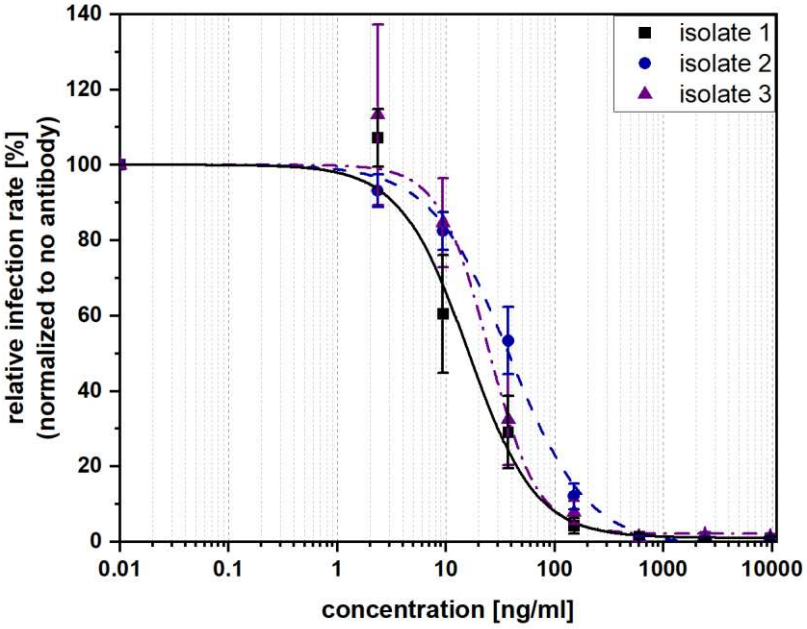

Figure 4: Neutralization of recent clinical HCMV isolates by MCMV5322A. The human monoclonal antibody MCMV5322A was diluted in a 4-fold dilution series and mixed with supernatants of recent HCMV isolates, harvested after an siRNA-mediated knockdown of UL128 and RL13. The virus was incubated with the antibody for 2h and the mixture was then added to HFF overnight. The infection rates of all cell cultures were determined using indirect immunofluorescence, and mean values from three experiments were normalized to untreated controls without antibody. Symbols represent mean values and bars represent the standard error of the mean (SEM).