Recombinant viruses as tools to study human cytomegalovirus immune modulation

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Abstract Infections with cytomegaloviruses are characterized by an intricate balance between the expression of immunomodulatory viral proteins and antiviral immune defence. For human cytomegalovirus (HCMV), several proteins have been described that interfere with the recognition of infected cells by CD8 T lymphocytes. Although the modes of action of these proteins have been elucidated on the molecular level, thus rendering them useful models to understand MHC class I peptide loading and transport, their role during viral infection has remained enigmatic. We exemplify here, how HCMV mutants can help to understand the importance of individual immunomodulatory proteins in the context of viral infection.

Keywords Cytomegalovirus · Immune evasion · Immune modulation · pp65 · IE1

Introduction

Cytomegaloviruses induce a vigorous immune response in their respective hosts. This most likely would have led to the eradication of these viruses if they would not express a whole set of immunomodulatory viral proteins that impede immune defence and thus allow viral replication and shedding. In human cytomegalovirus (HCMV) infected cells, one family of glycoproteins (gpUS2, gpUS3, gpUS6 and gpUS11), termed immunoevasins has been identified that

target MHC class I mediated peptide presentation [1–3], thereby impairing recognition by cytolytic CD8 T lymphocytes (CTL). These antiviral CTL, on the other hand, have been identified as key players in the HCMV-specific immune response, as they are able to terminate acute infection and prevent viral reactivation from latency [4–6].

A wealth of information has been gathered about the molecular activities of gpUS2-11, using heterologous expression systems and this topic has been covered by recent reviews [2, 7–9]. For murine cytomegalovirus, viral mutants have been employed to study the activities of the orthologous proteins, also termed *viral regulators of antigen presentation* (vRAPs) in vivo [10–13]. However, HCMV and MCMV MHC class I modulator proteins are distinct in their mechanisms of targeting the presentation pathway [2]. Consequently, knowledge obtained from MCMV cannot be directly applied to understand immune evasion in HCMV infected cells.

For HCMV, surprisingly little is known about the role of each particular HCMV evasion protein with respect to the protection of infected cells against CD8 T cell recognition and killing. Knowledge about cooperative or, as exemplified in MCMV [12], converse effects of these immunoevasins on MHC I mediated presentation of infected cells may be of pivotal importance in the design of immunotherapeutic strategies against HCMV infection. This has to be considered in the light of recent data using HCMV mutants devoid of the whole genomic region US2-US11 for ex vivo stimulation of T lymphocytes from infected donors [14, 15]. In these studies, a considerable fraction of the virus-specific CD8 T cells that grew out were directed against antigens presented by fibroblasts infected with the US2-US11 negative virus, but not wild-type (wt) HCMV. These results showed that despite immune evasion, a broad repertoire of antiviral CD8 T cells is primed during HCMV

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infection, indicating that peptides from a number of viral proteins are presented by professional APC *in vivo*. We have recently shown that also in fibroblasts, immune evasion is “leaky”, at least with respect to the presentation of one immunodominant peptide from the tegument protein pp65 [16]. In this article we will discuss how HCMV mutants can help to understand the interplay between MHC class I immune evasion and antigen presentation of infected cells. This knowledge may subsequently be used to define better the requirements of antiviral immune intervention strategies.

RV-ΔUS2-11 infected cells still suppress IE1-peptide presentation early after infection

Cloning of the complete viral DNA genome in bacterial artificial chromosome vectors (BACs) has significantly promoted HCMV research [17]. Comparing wt-viruses with mutants that carry targeted deletions or insertions provides valuable information on the functionality of particular genes in the context of viral infection (reviewed in [18]). With respect to MHC class I immune evasion, viruses deficient in the complete US2-US11 gene region have been used to show that downregulation of MHC class I on the surface of HCMV infected cells is critically dependent on the presence of these genes [1]. Using such a mutant, termed RV-ΔUS2-11 [19] (Fig. 1), we could recently show that MHC class I presentation of the immunodominant peptide NLVPMVATV (pp65_{NLV}) from the tegument protein pp65 (ppUL83), which is partially affected by immune eva-

sion in wt-HCMV infected cells, is completely restored on cells infected with the mutant virus [16]. In addition, as previously shown by others [14, 20], presentation of peptides derived from the IE1-protein is also sensitive to US2-US11; in our hands, infection of cells with RV-ΔUS2-11 resulted in efficient IE1-peptide presentation from 24 to 96 h p.i., as evaluated by interferon- γ (IFN- γ) ELISpot assay, in contrast to complete suppression of IE1-peptide presentation on wt-HCMV infected cells during the same period of time [16].

Manley and colleagues [14] reported that cells infected with a US2-11 negative virus were not lysed by IE1-specific CTL after a 2- or 4-h infection period, using a 4-h chromium release assay as read-out. However, because of the extension of the infection period by the 4-h incubation period of the assay, it remained unclear, if there was presentation immediately after infection. To test this, we infected fibroblasts with RV-ΔUS2-11 at an m.o.i. of 5 for 1, 3, 6, 12, and 24 h and subsequently fixed the cells using paraformaldehyde (Fig. 2a, b). The cells were then used as stimulators in an IFN- γ -ELISpot assay with CTL clones directed against pp65_{NLV} (pp65_{NLV}-CTL) or against the HLA-A2 presented IE1-peptide TMYGGISLL (IE1_{TMY}-CTL) [16, 21]. Surprisingly, only limited IE1 presentation was detectable at all time points p.i. tested, despite the lack of US2-11 expression. No significant presentation was observed at 1, 3, or 6 h p.i. Only few spots became visible at 12 and 24 h p.i. In the parallel control assay, using pp65_{NLV}-CTL, significant spot numbers became apparent already at 3 h p.i. and the presentation significantly increased in the periods thereafter. Note that low IE1 presentation was seen even with a responder number of 500 CTL, as opposed of 200 CTL in the pp65-specific assay (Fig. 2a, b).

One argument that may be put forward to explain the lack of IE1 presentation at very early phases of infection, even in the absence of the immunoevasins US2-11, was that IE1 expression was limiting. To investigate this, fibroblasts were infected the same way as for ELISpot analysis and were analyzed at the corresponding time points p.i., using indirect immunofluorescence (Fig. 2c, d). As expected, pp65 was detectable in the cells already 1 h p.i. This tegument protein is known to be introduced into cells by viral particles. The IE1 was not detectable at 1 h p.i. There was, however, some expression seen at 3 h p.i. No difference between the number of pp65- and IE1-expressing cells was detectable at 6, 12 or 24 h after infection. As there was a significant discrepancy in MHC class I presentation of pp65- and IE1-derived peptides at these time points after infection (Fig. 2a, b), these results indicate that there is an impairment of IE1 presentation already very early after infection and that this phenomenon is independent of US2-11 expression.

Fig. 1 Schematic representation of viral US2-11- or pp65-deletion mutants. The HCMV genome of strain Ad169 with restriction sites for *HindIII* and *EcoRI* is shown on top. The structure of the different viruses used is given below in blow-ups of the genomic regions of interest. The HCMV strain Ad169-BAC is a pp65 and US2-11 competent revertant virus of RV-HB5 [17, 23]. The virus RV-ΔUS2-11 lacks the genomic region encoding US2-11 [19]. The recombinant RV-Hd65 is an Ad169-BAC-derived pp65-deletion mutant [16]. Broken lines indicate the genomic regions deleted in the respective viral mutant. Light grey boxes indicate genes flanking UL83 or the US2/3, US6 or US11 genes, respectively

Fig. 2 Interferon- γ (IFN- γ) ELISpot and indirect immunofluorescence analyses of RV- Δ US2-11 infected cells. Fibroblasts were infected at an m.o.i. of 5. For ELISpot analyses (**a**, **b**), cells were fixed at the indicated time points, using 0.5% paraformaldehyde to prevent further peptide processing, MHC class I loading and surface transport. Paraformaldehyde fixation and ELISpot assays were performed as described before [16]. CTL lines specific for the HLA-A0201 (A2) restricted HCMV-derived peptides pp65₄₉₅₋₅₀₃ [34, 35] (pp65_{NLV}-CTL) and IE1₂₉₇₋₃₀₅ [36] (IE1_{TMY}-CTL) were used in these analyses. The CTL lines have been generated by immunizing HLA-A2/huCD8

double-transgenic (tg) mice [16]. Mean values and standard deviations of triplicate samples from one out of at least three independent experiments are shown. For indirect immunofluorescence analyses (**c**, **d**), infected cells were fixed in 90% acetone for at least 20 min at RT and incubated for 1 h with primary murine monoclonal antibodies 65–33 (pp65) and p63–27 [37] at 37°C. After four washing steps in PBS-Triton (0.1%), cells were incubated with a FITC-conjugated anti-mouse secondary antibody for 1 h at 37°C. After additional four washing steps in PBS-Triton (0.1%), micrographs were taken at magnifications of $\times 200$ (**c**) and $\times 1,000$ (**d**), using a Canon Powershot digital camera

RV-Hd65 infected cells do not stimulate IFN- γ secretion by IE1_{TMY}-CTL even very early after infection

The results presented above confirmed an early US2-11 independent interference with IE1-peptide presentation on HCMV infected cells, as previously suggested by Gilbert and colleagues [22]. Using recombinant vaccinia viruses

expressing HCMV IE1 or pp65, these authors demonstrated that co-infection with vacIE1 and vacpp65 leads to a selective impairment of IE1-specific cytolysis with IE1-specific CTL lines being used as effectors. They went on to show that suppression of IE1-specific lysis in HCMV infected cells was abrogated when a pp65 deletion mutant of the virus was used for infection. Hyperphosphorylation of IE1

by a pp65-associated kinase was suggested by these authors to be important, yet the molecular basis for the pp65-mediated suppression of IE1-specific cytotoxicity remained unexplained [22]. In our experiments, IE1_{TMY}-CTL were well stimulated for IFN- γ release by HFF infected with a US2-11 negative virus from 24 to 96 h p.i. (Fig. 3a). Note that, in contrast to the experiment shown in Fig. 2b, paraformaldehyde fixation was not used, thus extending the incubation period by 20-h ELISpot incubation. In contrast, no spots were detectable in cells infected with RV-Hd65, a pp65-negative derivative of the US2-11 competent strain Ad169-BAC [16, 23] (Figs. 1, 3a).

To address the question, whether lack of pp65-expression in HCMV infected cells would enable stimulation of IFN- γ secretion from IE1-specific CTL at a very early time point after infection, HFF were infected with RV-Hd65 at an m.o.i. of 5 for 1, 3, 6, 12, and 24 h. After that, cells were fixed with paraformaldehyde to prevent further protein processing and MHC peptide loading as well as surface transport during the subsequent incubation period of the ELISpot assay (Fig. 3b). Surprisingly, under the conditions used here, IE1_{TMY}-CTL were not significantly stimulated to secrete IFN- γ at any of the time points investigated. Thus deletion of the pp65-gene alone appeared to be insufficient to restore IE1_{TMY} presentation at 1–24 h p.i. and it also had no effect on presentation of the IE1-peptide at later time points (Fig. 3a).

These results seem to conflict with those obtained by Gilbert and colleagues [22]. There are, however, several differences in the experimental setup, which make it difficult to compare the results. One major difference is that an m.o.i. of 25 in combination with cycloheximide/actinomycin D enhancement of IE-gene expression was used by Gilbert and colleagues, whereas an m.o.i. of 5 and no enhancement was employed here. The former protocol

may result in a steady-state level of IE-proteins, including IE1, that may considerably exceed the level obtained in our setup. Consequently, as a matter of quantity, early expression of US2-11 may have been insufficient to completely block IE1 presentation in the absence of pp65. In contrast, in the experiments presented here, US2-11 expression, particularly the expression of the IE-protein gpUS3 may have been sufficient to prevent stimulation of IFN- γ secretion by IE1-specific CTL. There are a number of other differences, particularly with regard to the read-out assays and the origin of the CTL used for analysis, which may also add to discrepancies in the results. As discussed by Manley and colleagues [14], pp65-mediated suppression of IE1 presentation may be transient. In addition, expression of US2-11 may contribute to the suppression, making it difficult to analyze the effect using viruses with a US2-11 positive background. Analyses of the pp65-mediated effect on antigen presentation thus have to be performed using viruses in which both the pp65 and the US2-11 gene loci are deleted.

IE1-derived peptides are well presented in latently infected U138-MG cells

Ever since the first report about their detection in healthy individuals by Borysiewicz and colleagues [24], there has been considerable debate about the role of IE1-specific cytotoxic T cells in the control of HCMV infection in vivo. The knowledge of their failure to lyse HCMV infected cells in vitro [20] contrasted with reports, providing circumstantial evidence for their protective role in vivo [25]. In the murine model of CMV infection, IE1-specific CTL are highly effective in the control of viral infection and in preventing viral reactivation from latency [26, 27]. The latter

Fig. 3 IE1_{TMY}-specific ELISpot analysis of early and late infected fibroblasts. Fibroblasts were infected at an m.o.i. of 5 for the indicated times and were subjected to ELISpot analysis using IE1_{TMY}-CTL. Viruses used for infection are indicated. **a** Cells were infected with the respective viruses and were subsequently used as stimulators in

ELISpot analyses; **b** Cells were infected with RV-Hd65 for the indicated times, fixed using 0.5% paraformaldehyde and used as stimulators in ELISpot analyses. Mean values and standard deviations of triplicate samples are shown from one out of at least two independent experiments (**a**, **b**)

function may define a crucial role for IE1-specific CTL also during the human infection. Viral reactivation is frequent in T cell deficient patients, e.g., following T cell depletion in the course of tumour therapy in combination with hematopoietic stem cell transplantation [28, 29]. As IE-genes are the first to be expressed during viral reactivation, IE1 would provide a perfect target for antiviral CTL to recognize cells in order to prevent viral reactivation. We have, some time ago, established a cell culture model of HCMV latency and reactivation in the glioblastoma cell line U138-MG [30]. For this, the cells were originally infected with recombinants of the Ad169 strain or the Towne strain of HCMV, both of which expressed the Neomycin-phosphotransferase (Neo^R) gene. Using G418 selection of Neo^R expression, these cells maintained the complete HCMV genome and expressed considerable levels of IE1 and moderate levels of the IE2 protein. The cell lines were named according to the strain used for infection as U138-RVA (Ad169) or U138-RVT (Towne). Without induction, most of these cells failed to express early or late proteins of the virus. Upon induction, e.g., with phorbol esters, the cells expressed early genes without releasing viral progeny. However, superinfection with heterologous strains of HCMV reactivated the endogenous virus to produce progeny [30]. Thus these cells may be an interesting model to study immune recognition of HCMV during reactivation.

To be able to perform initial studies using IE1_{TMV}-CTL, the U138-RVA and U138-RVT cell lines were converted to a HLA-A2+ phenotype by transfecting an HLA-A0201-encoding cDNA, expressed from a pcDNA6 vector. Cells were obtained that stably expressed HLA-A2 on the cell surface (data not shown). These cells were then used in chromium release and in ELISpot assays to test the presentation of the IE1-peptide (Fig. 4). Both cell lines U138-RVA and U138-RVT were lysed by IE1_{TMV}-CTL, as tested

in chromium release assays, although U138-RVA appeared to be better targets than U138-RVT. U138-RVA and -RVT were also good stimulators of IFN- γ secretion from IE1_{TMV}-CTL in ELISpot tests, although, in this instance, U138-RVT were better stimulators compared to U138-RVA. The reason for this dichotomy is unclear, but may be related to the variations in IE1 expression in these cells [30]. Further evaluation of these cells is warranted to clarify this point.

At first sight, these results were surprising, considering the complete suppression of IE1 presentation on permissively infected cells (see above). However, as compared to the permissive situation, no significant amounts of pp65 could be detected in uninduced U138-RVA and U138-RVT and thus no interference with IE1 presentation mediated by the tegument protein may be expected. There is no information on the expression of US2-11 in these cells at the current stage. Considering the temporal pattern of US2-11 expression in permissive cells, it may be speculated that there is only limited gpUS2-11 synthesis in uninduced U138-RVA and U138-RVT. This has to be further analyzed. However, from these data it may be hypothesized that, as in the murine model, IE1-specific CTL can readily detect and kill reactivating cells and thus may contribute to the control of HCMV recurrence. The U138-RVA and U138-RVT cells provide a versatile cell culture model to address the role of IE1-specific CTL in the control of HCMV latency in greater detail.

Impairment of pp65-peptide presentation is a slow, m.o.i.-dependent process

In contrast to IE1-derived peptides, pp65-derived peptides are readily presented in the context of MHC class I by

Fig. 4 Presentation of IE1_{TMV} on U138-RVA or U138-RVT cells. **a** Chromium release assay of U138-RVA- or U138-RVT- target cells, using IE1_{TMV}-CTL as effectors. The assay was performed as described before [38]. Mean values are shown from duplicate samples. **b** IE1_{TMV}

specific ELISpot analysis of U138-RVA or U138-RVT cells. Mean values and standard deviations of triplicate samples are shown from one out of three independent experiments. U138-MG cells were used as negative control (**a**, **b**)

HCMV infected fibroblasts, and this has been attributed mainly to the exogenous introduction of the tegument protein by incoming viral particles [31, 32]. We could recently show that pp65-peptide presentation is subject to immune evasion between 24 and 96 h p.i. [16]. From these studies, however, it remained unclear, at which time point after infection pp65-peptide presentation was starting to be affected by US2-11. An experimental strategy was thus designed to address this question (Fig. 5a). Fibroblasts were infected with the US2-11 competent strain AD169-BAC [23] and, as control, with the RV-ΔUS2-11 strain. MHC I peptide complexes were removed from the surface of infected cells at various times p.i. by acid wash. The removal of MHC class I peptide complexes from the cell surface by acid wash was performed as published recently [16]. As shown there, the procedure is highly efficient, as spot numbers were reduced by over 90% when RV-ΔUS2-11 infected cells were tested with pp65_{NLV}-CTL subsequent to acid treatment.

After acid wash, cells were further incubated to allow restoration of MHC I peptide complexes on the cell surface until a total infection period of 24 h was reached. Cells were subjected to ELISpot analysis, using pp65_{NLV}-CTL as

responder cells (Fig. 5b, c). As expected, cells infected with RV-ΔUS2-11 were fully capable of restoring pp65_{NLV} presentation, irrespective of the time of acid wash p.i.. Cells infected with AD169-BAC showed fewer spots compared to cells infected with RV-ΔUS2-11 at any of the time points tested. However, when MHC class I peptide complexes were removed at 1, 6 or 14 h p.i., the cells were still able to restore peptide presentation to the level of the untreated control. Only when the acid wash was performed at 16 h p.i. or later, some reduction in peptide presentation became apparent, as evidenced by a fewer number of spots. These results indicated that, with respect to pp65-derived peptide presentation, immune evasion implemented by US2-11 was a slow process.

To analyze whether the effectiveness of immune evasion was dependent on the m.o.i. used, cells were infected with the US2-11 competent strain AD169-BAC at various infectious doses (Fig. 6). As control, cells were either mock-infected or infected with RV-ΔUS2-11 at an m.o.i. of 5. Cells were infected for 24 h and were then subjected to ELISpot analysis. Spot numbers increased with m.o.i., thus indicating, that immune evasive effects could be overcome with respect to pp65-peptide presentation by infectivity.

Fig. 5 Kinetics of the restoration of pp65_{NLV} presentation on infected fibroblasts depending on the expression of immune evasion proteins US2-11. **a** Scheme of the experimental setup used. Cells were infected with the parental strain Ad169-BAC or the recombinant virus RV-ΔUS2-11 for the time points indicated on top of the bars and were subsequently stripped of MHC-peptide complexes on the cell surface by acid treatment. Cells were further incubated until a total of 24-h infec-

tion time was reached and were subsequently used as stimulators for IFN-γ-ELISpot analysis with pp65_{NLV}-CTL as responder cells. **b** IFN-γ-ELISpot of cells infected with RV-ΔUS2-11 (grey bars). **c** IFN-γ-ELISpot of cells infected with AD169-BAC (black bars). Mean values and standard deviations of triplicate samples are shown from one out of two independent experiments

Fig. 6 Impact of m.o.i. on immune evasion mediated impairment of pp65_{NLV} presentation. Fibroblasts were infected with the recombinant virus RV-ΔUS2-11 at an m.o.i. of 5 or with Ad169-BAC at the indicated m.o.s for 24 h. Cells were then subjected to ELISpot analysis using pp65_{NLV}-CTL as responder cells. Mean values and standard deviations of triplicate samples are shown from one out of two independent experiments

Outlook

Cytomegaloviruses have established an intricate balance with their hosts to ensure survival and persistence. Modulation of the immune response is one level, upon which multiple viral players act to avoid detection of viral infection of the host cell. This co-existence with the host is disturbed, once immune surveillance is compromised for other reasons, e.g., in the case of iatrogenic intervention during tumor therapy. Understanding the role of different immunomodulatory proteins during infection is essential to design novel approaches of immunointervention and therapy of HCMV infection. The cloning of the large HCMV genome into BACs has opened a gateway to precisely look at individual immunomodulatory genes using site-directed deletion [17, 18]. Refinements in BAC technologies allow seamless modification down to single amino acids within proteins of recombinant viruses [33], thus enabling their detailed analysis on the molecular level. With respect to the MHC class I evasion proteins gpUS2-11, mutants that express single genes or combinations of these genes will enforce assessment of their role during permissive and latent infection. HCMV mutants will also allow to investigate the controversial role of pp65 in mediating IE1 immune evasion in more detail. Results obtained from these studies will not only help to understand HCMV immune modulation, they will, even more importantly, also

provide insight into the mechanisms the host has developed to cope with the viral infection. This information will be essential for the design of targeted intervention strategies against HCMV.

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