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ORIGINAL INVESTIGATION

Function of the cargo sorting dileucine motif in a cytomegalovirus immune evasion protein

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Abstract

As an immune evasion mechanism, cytomegaloviruses (CMVs) have evolved proteins that interfere with cell surface trafficking of MHC class-I (MHC-I) molecules to tone down recognition by antiviral CD8 T cells. This interference can affect the trafficking of recently peptide-loaded MHC-I from the endoplasmic reticulum to the cell surface, thus modulating the presentation of viral peptides, as well as the recycling of pre-existing cell surface MHC-I, resulting in reduction of the level of overall MHC-I cell surface expression. Murine cytomegalovirus (mCMV) was paradigmatic in that it led to the discovery of this immune evasion strategy of CMVs. Members of its *m02-m16* gene family code for type-I transmembrane glycoproteins, proven or predicted, most of which carry cargo sorting motifs in their cytoplasmic, C-terminal tail. For the *m06* gene product m06 (gp48), the cargo has been identified as being MHC-I, which is linked by m06 to cellular adapter proteins AP-1A and AP-3A through the dileucine motif EPLARLL. Both APs are involved in trans-Golgi network (TGN) cargo sorting and, based on transfection studies, their engagement by the dileucine motif was proposed to be absolutely required to prevent MHC-I exposure at the cell surface. Here, we have tested this prediction in an infection system with the herein newly described recombinant virus mCMV-m06AA, in which the dileucine motif is destroyed by replacing EPLARLL with EPLARAA. This mutation has a phenotype in that the transition of m06-MHC-I complexes from early endosomes (EE) to late endosomes (LE)/lysosomes for degradation is blocked. Consistent with the binding of the MHC-I α-chain to the luminal domain of m06, the m06-mediated disposal of MHC-I did not require the β2m chain of mature MHC-I. Unexpectedly, however, disconnecting MHC-I cargo from AP-1A/3A by the motif mutation in m06 had no notable rescuing impact on overall cell surface MHC-I, though it resulted in some improvement of the presentation of viral antigenic peptides by recently peptide-loaded MHC-I. Thus, the current view on the mechanism by which m06 mediates immune evasion needs to be revised. While the cargo sorting motif is critically involved in the disposal of m06-bound MHC-I in the endosomal/lysosomal pathway at the stage of EE to LE transition, this motif-mediated disposal is not the critical step by which m06 causes immune evasion. We rather propose that engagement of AP-1A/3A by the cargo sorting motif in m06 routes the m06-MHC-I complexes into the endosomal pathway and thereby detracts them from the constitutive cell surface transport.

Keywords Adapter proteins (AP-1, AP-3) · Antigen presentation · Cargo sorting · Cytomegalovirus · Dileucine motif · Early endosomes (EE) · Endosomal pathway · Endosomal recycling compartment (ERC) · Late endosomes (LE) · Immune evasion · MHC class-I · MHC class-I trafficking · m06 protein · Trans-Golgi network (TGN)

Introduction

Murine cytomegalovirus (mCMV) genes *m04* and *m06*, members of the *m02-m16* gene family ([1], see also map and list in [2]), code for type-I transmembrane glycoproteins m04 (gp34) and m06 (gp48) that stably bind to MHC class-I molecules (MHC-I), specifically to the respective α-chains, via their luminal domains (m04: [3–6] and m06: [7]). They thereby alter the trafficking of MHC-I from the

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endoplasmic reticulum (ER) to the cell surface by disconnecting them from the constitutive vesicular flow for mediating immune evasion (for reviews, see [8–12]). It is instructive to recall that correct folding of MHC-I complexes in the ER requires peptide-loading onto the MHC-I α-chain as well as complex formation with the β2 microglobulin (β2m) chain. As only folded complexes are efficiently transported to the cell surface via the constitutive vesicular flow, all MHC-I complexes that localize to compartments beyond the ER carry peptide, self or foreign. For ease, we here use pMHC-I to indicate binding of a viral peptide, whereas we use MHC-I when the peptide is not specified and derived from intrinsic self proteins. While m04 does not reduce levels of cell surface MHC-I but escorts newly generated pMHC-I to the cell surface in the form of m04-pMHC-I complexes [3–5], m06 strongly reduces levels of cell surface MHC-I [12, 13], thought to operate by routing m06-MHC-I complexes to lysosomes for degradation [7]. In functional terms, m04-pMHC-I cell surface complexes mediate innate immune evasion by silencing natural killer (NK) cells through ligation of certain inhibitory Ly49-family NK cell receptors ([14], reviewed in [15]), whereas m06-mediated disposal of MHC-I is associated with adaptive immune evasion of CD8 T cells by reducing the cell surface transport of recently folded pMHC-I complexes in an interplay with the third immune evasion protein, m152 (p36/gp40) [13, 16–18].

A typical feature common to members of the m02-m16 glycoprotein family is the presence of cargo sorting motifs in their cytoplasmic, C-terminals tails (listed in [2]). Most members carry a tyrosine-based sorting motif, YXXΦ, where Φ denotes a hydrophobic amino acid residue. The m04 glycoprotein belongs to this group. Mediated by its highly conserved YXXΦ motif YRRF [2] it connects the m04-pMHC-I complex primarily with the cellular adapter protein AP-2 [19], which then links the trimeric complex to the clathrin triskelion for endocytosis. Thus, m04 can be viewed as a viral adapter protein that links MHC-I cargo to a cellular cargo sorting adapter protein, thereby functioning as a "connector" or an "adapter–adapter". As we have shown in previous work, disconnecting m04-pMHC-I from AP-2 by mutation of the motif to ARRF in recombinant virus mCMV-m04.Y248A stabilizes m04-pMHC-I at the cell surface, resulting in enhanced NK cell silencing in BALB/c (MHC-I $K^dD^dL^d$) mice [2] that have NK cell subsets expressing inhibitory Ly49-family receptors Ly49A, Ly49C, or Ly49G2, of which the $Ly49G2^+$ NK cell subset is involved most [14]. This result, showing a role of the motif in dampening NK cell silencing by enhancing internalization of inhibitory NK cell ligand m04-pMHC-I was surprising, as it rather appears to contradict the interest of the virus to evade immune recognition. One idea is that in virus-host co-evolution on the polymorphic host population level, this function of the motif may be part of a delicate balance by reducing ligation of activatory Ly49 family NK cell receptors [20], thereby dampening innate immune control.

In this report, we focus on the m04 antagonist m06 (gp48) that also stably binds MHC-I and thus competes with m04 for the cargo. In this context, it is of interest to note that structural analyses based on m04 as a paradigm revealed a shared β-sandwich immunoglobulin variable (Ig-V)-like fold (briefly β-fold) in the luminal domains of m04 and m06 [6, 21] to where MHC-I binds. A functionally relevant distinction is the absence of an endocytic, AP-2/4-binding YXXΦ motif, and instead the presence of a highly conserved transmembrane-proximal dileucine motif EXXXXLL, specifically EPLARLL ([22] and Fig. 1) in the cytoplasmic tail of m06. This motif links the m06-MHC-I complex to cellular adapter proteins AP-1A/3A [22] involved in trans-Golgi network (TGN) cargo sorting into the endosomal-lysosomal pathway. Thus, like its sister molecule m04, m06 serves as a "connector" or "adapter–adapter" molecule, with the fundamental difference that it mediates sorting of the MHC-I cargo for disposal.

As proposed by Reusch and colleagues based on transfection studies in AP mutant cells [22], motif-mediated recruitment of AP-1A/3A for routing MHC-I to lysosomal degradation is a required step in the immune evasion mechanism by which m06 prevents cell surface exposure and re-exposure of newly generated pMHC-I and recycled MHC-I, respectively. We have here tested this hypothesis in cells infected with viruses mCMV-m06LL and mCMV-m06AA expressing functional motif EPLARLL and mutated motif EPLARAA, respectively. Our data call for a revision of the current view that assumes the cargo sorting motif is required for m06-mediated immune evasion.

Materials and methods

Mice

Female BALB/c and C57BL/6 mice were purchased from Janvier Labs, and $\beta2m^{-/-}$ mice from Charles River. They were housed in the translational animal research center (TARC) of the University Medical Center of the Johannes Gutenberg-University Mainz for at least 1 week under specified-pathogen-free (SPF) conditions. Mice were used at the age of 8 weeks. Mouse embryonal/fetal fibroblasts (MEF) were prepared from BALB/c or C57BL/6 mice or from $\beta2m^{-/-}$ mice by a standard protocol [26].

Viruses and infection of cells

Bacterial artificial chromosome (BAC)-cloned virus MW97.01 derived from BAC plasmid pSM3fr [23, 24] is herein referred to as mCMV-m06LL, expressing functional

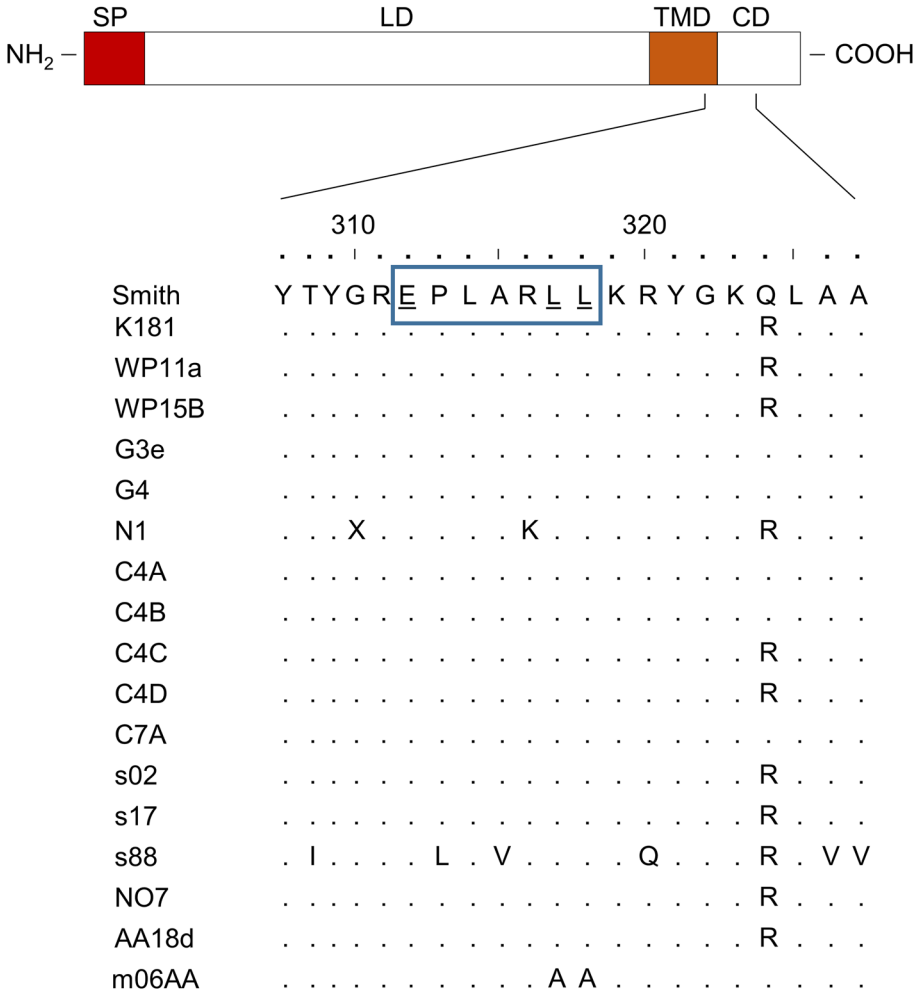

Fig. 1 Conservation of the dileucine motif EPLARLL in m06/gp48. Top: schematic representation (not drawn to scale) of the 336-amino acid *m06* gene product, which is a type-1 transmembrane glycoprotein. *SP* signal peptide, *LD* luminal domain, *TMD* transmembrane domain, *CD* cytoplasmic domain. Bottom: alignment of predicted amino acid sequences between positions 307–327 for mCMV laboratory strains and wild-derived isolates, as well as for mutant virus mCMV-m06AA. Amino acid replacements relative to the Smith reference strain are indicated. The dileucine motif is highlighted by a blue box. Amino acids essential for the motif are underlined

cargo sorting motif EPLARLL in the cytoplasmic tail of m06 (gp48). Recombinant virus mCMV-Δm06 has been described previously [25], replacing discredited virus mCMV-$\Delta m06^{W}$ [13] with mCMV-$\Delta m06^{L}$ [12]. Cell culture derived high titer virus stocks were generated by a standard protocol [26].

MEF in the third passage were pre-treated for 48 h with 20 ng of IFN-γ per ml of culture medium or were left untreated [25]. After this period, MEF were infected with the indicated viruses at a multiplicity of infection (MOI) of 4, using the method of centrifugal infection ([26, 27] and references therein). Based on the Poisson distribution function, almost all cells are hit by an infectious dose of virus at an MOI of 4.

Generation of mCMV-m06AA

For inactivating cargo sorting motif EPLARLL in m06, virus mCMV-m06AA was generated by mutations L307A and L308A (amino acid positions in m06), replacing leucines with alanines. For this, nucleotides ^{6145}CTG CTC ^{6150} were substituted by ^{6145}GCT GCC^{6150} through *en-passant* mutagenesis of the BAC plasmid pSM3fr as described [28]. Briefly, a kanR cassette flanked by homologous viral sequences was amplified from plasmid pori6K-RIT [29] using the oligonucleotides m06_AA_for gct atg ttg ttc tac acc tac ggc cgc gag ccg cta gct aga gct gcc aag cga tac ggc aag cag ctc gcg acg cat cgt ggc cgg atc tc and m06_AA_rev ccg tcc gcg ggg atg cgg acg gcc gcg agc tgc ttg ccg tat cgc ttg gca gct cta gct agc. The resulting product was transformed into GS1783 bacteria carrying pSM3fr. After Red recombination, arabinose-induced I-SceI expression, and a second round of Red recombination, BAC m06AA was purified and successful mutagenesis was confirmed by sequencing (GATC, Koblenz, Germany). After BAC DNA purification, recombinant mCMVs were reconstituted by transfection of the DNA into MEF and propagated for six passages until residual BAC sequences were lost as verified by PCR [30].

Sequence alignment

Sequence alignments of m06 protein sequences derived from published mCMV strains with the following GenBank numbers were performed with ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalo/) [31]. Smith strain (GU305914.1), K181 (AM886412.1), WP11a (AM236108.1), WP15B (EU579860.1), G3e(AM236107.1), G4 (AM236105.1), N1 (AM236106.1) C4A (AM236099.1), C4B (AM236100.1), C4C (AM236101.1), C4D (AM236102.1), C7A (AM236103.1), s02 (MH118557.1), s17 (MH118558.1), s88 (MG957497.1), NO7 (HE610454.1), AA18d (HE610451.1).

Cytofluorometric analysis of MHC-I cell surface expression

At 16 h after infection, MEF were stained for cell surface MHC-I expression with PE-labeled monoclonal antibody mouse anti-mouse H-2 K^d (BD Pharmingen, catalog no. 553566) (FL-2) and for intracellular expression of m164/gp36.5 with a rabbit antiserum α-m164/gp36.5 directed against a C-terminal peptide [16, 32] and Alexa Fluor488-conjugated goat-anti-rabbit IgG (Invitrogen, catalog. no. A11008) (FL-1). Analysis was performed with a Beckman Coulter FC500 cytofluorometer and CXP software, version 2.2 (Beckman Coulter).

Quantitation of activated, IFN-γ-secreting CD8 T cells

An enzyme-linked immunospot (ELISpot) assay was performed to quantitate CD8 T cells that secrete IFN-γ in response to antigenic peptide presentation on MEF infected with the indicated viruses. Responder cells were either polyclonal and polyspecific ex vivo CD8 T cells immunomagnetically purified directly from spleen or lymph node cell populations of BALB/c (MHC-I $K^dD^dL^d$) mice 7 days after intraplantar/footpad infection with 10^5 PFU of mCMV, strain Smith, or were cells of viral epitope-specific but still polyclonal cytolytic T lymphocyte lines (CTLL) IE1-CTLL and m164-CTLL specific for the L^d-presented IE1 peptide YPHFMPTNL [33, 34] and the D^d-presented m164 peptide AGPPRYSRI [35], respectively. The assay was described in greater detail previously ([34–36] and references therein).

CLSM immunofluorescence analysis

The localization of viral protein m06/gp48 and of markers for subcellular structures was visualized by CLSM (confocal laser scanning microscope) immunofluorescence analysis, essentially as described previously [32]. Primary antibodies included monoclonal α-MHC-I rat antibody (Santa Cruz, catalog no. sc-59199), polyclonal α-TGN46 rabbit antibody (Novus Biologicals, catalog no. NBP1-03495), polyclonal α-LAMP1 rabbit antibody (Novus Biologicals, catalog no. NB120-19294), and monoclonal α-m06 mouse antibody (clone Croma 229; kindly provided by S. Jonjic, Rijeka, Croatia). Alexa-Fluor 468-conjugated goat anti-rabbit antibody (ThermoFisher scientific, catalog no. A11011), Alexa-Fluor 633-conjugated goat anti-mouse antibody (ThermoFisher scientific, catalog no. A21052), or Alexa-Fluor 546-conjugated goat anti-rat antibody (ThermoFisher scientific, catalog no. A11081) served as secondary antibodies for fluorescence staining. Cell nuclei were stained with Hoechst dye (ThermoFisher scientific, catalog no. H3570). Immunofluorescence was examined with a Zeiss Laser Scanning Microscope (LSM510).

Subcellular fractionation

10^7 MEF were either left untreated or were infected with the indicated viruses at an MOI of 4 for 8 h. Endosomal fractions were prepared and verified according to established protocols [37–39]. Briefly, cells were harvested and homogenized. A post-nuclear supernatant was prepared and adjusted to 40.6% sucrose, 3 mM imidazole, pH 7.4, loaded at the bottom of an SW60 tube, and overlaid sequentially with 35% and 25% sucrose solutions in 3 mM imidazole, pH 7.4, and homogenization buffer (HB; 8.5% sucrose, 3 mM imidazole, pH 7.4). The gradient was centrifuged for 90 min at 14,000 × *g*. Fourteen fractions were collected from the top of the gradient. Early endosomes (EE) accumulate at the 35%/25% sucrose interface (fraction 7) and late endosomes (LE) at the 25% sucrose/HB interface (fraction 4). The identity of both fractions was confirmed by immunoblotting for the endosomal markers Rab5 and Rab7 [38] (not shown) using Rab5 mAb D-11 (Santa Cruz, catalog no. sc-46692) and Rab7 mAb Rab7-117 (Sigma-Aldrich, St. Louis, MO, USA, catalog no. R8779), respectively. Viral protein m06 was detected with monoclonal α-m06 mouse antibody (clone Croma 229). The relative densitometric quantification was performed applying ImageJ gel analysis tool [40]. Statistical significance was tested by performing paired *t* test (GraphPad Prism version 6.07 GraphPad Software, San Diego California USA).

Results

The cargo sorting motif in m06 is dispensable for MHC-I downmodulation

Already the first experiment performed with cargo sorting motif mutant virus mCMV-m06AA mercilessly falsified the long-held hypothesis of a critical role of the dileucine motif in m06 for its function in reducing levels of cell surface

MHC-I, as shown by cytofluorometric analysis (Fig. 2). In accordance with previous findings [12, 25] deletion of gene *m06* in mutant virus mCMV-Δm06 prevented the m06-mediated component of MHC-I downmodulation from the cell surface of infected cells, regardless of whether or not MHC-I levels were in advance elevated by pre-treatment of the cells with IFN-γ [25]. It should be noted that m06 is the strongest downmodulator of overall cell surface MHC-I, whereas immune evasion protein m152 (p36/gp40) [41–43] contributes little [12, 13, 16, 25].

Deletion of the cargo sorting motif in m06 fails to fully restore the presentation of antigenic peptides

In accordance with a previous report [25], the absence of m06 after infection with mCMV-Δm06 facilitated the presentation of antigenic peptides by allowing some pMHC-I to reach the cell surface (Fig. 3). Notably, this occurred despite the presence of immune evasion molecule m152 (see above), which is the strongest inhibitor of antigenic peptide presentation to CD8 T cells by trapping freshly folded pMHC-I in an ER-Golgi intermediate compartment (ERGIC) [41, 44]. It must be noted that a number of earlier studies on antigen presentation after infection with a Δm06 mutant of mCMV, with the exception of [25], cannot serve for comparison, as the deletion of gene *m06* in that virus had inadvertently affected the expression of m152. This event during mutagenesis was not reproducible with independently generated Δm06 mutants [12]. A comparison between motif-sufficient virus mCMV-m06LL and motif-deficient virus mCMV-m06AA consistently revealed a statistically significant though, compared to m06 deletion, quantitatively minor improvement of antigenic peptide presentation. So, motif-mediated pMHC-I cargo disposal in lysosomes apparently is not the most critical step in the mechanism of immune evasion. The slight improvement of antigenic peptide presentation by motif inactivation was reproduced for defined

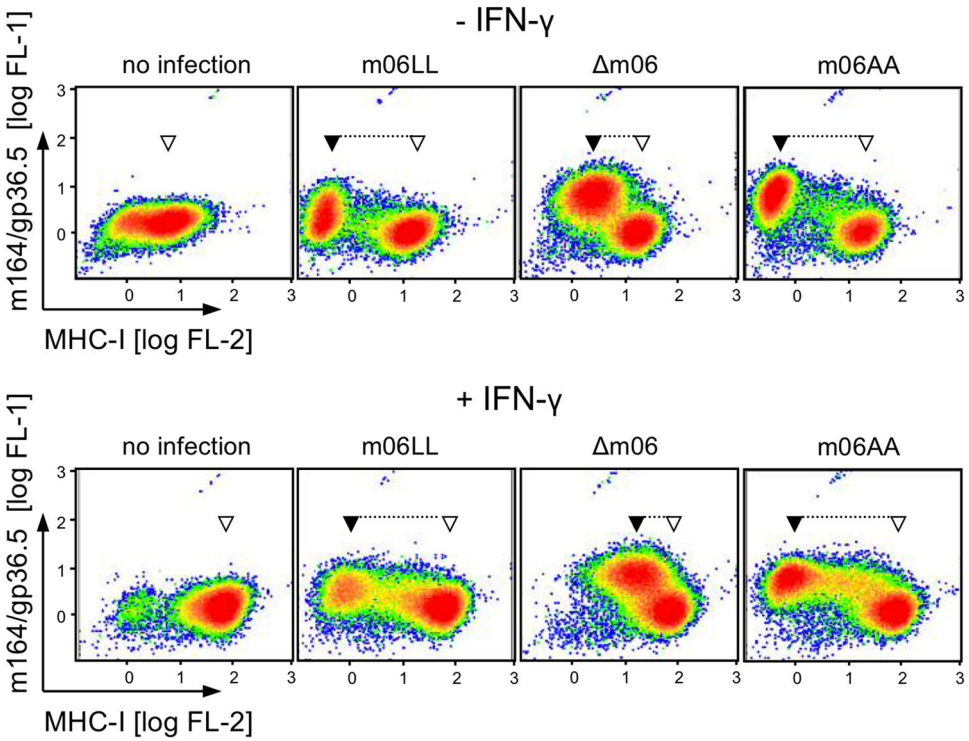

Fig. 2 Impact of the cargo sorting dileucine motif on MHC-I cell surface expression. Two-color cytofluorometric analysis of MHC-I molecule K^d cell surface expression (*abscissa* FL-2, PE fluorescence intensity) on untreated (upper panel − IFN-γ) or IFN-γ-pretreated (lower panel + IFN-γ) BALB/c (MHC-I $K^dD^dL^d$) MEF expressing ER-resident viral protein m164/gp36.5 (*ordinate* FL-1, Alexa Fluor488 fluorescence intensity) after infection for 16 h with the viruses mCMV-m06LL, mCMV-Δm06, and mCMV-m06AA. Data are displayed as color-coded density plots (with red and blue representing highest and lowest density, respectively). Note that in infected MEF cultures, a fraction of the cells is not permissive for productive infection and thus do not express the viral early (E) phase protein m164. These uninfected cells result in a peak population characterized by high MHC-I expression and uninfluenced by viral immune evasion molecules (open arrowhead). Compared to MEF in uninfected cultures, MHC-I expression on uninfected MEF present in infected cultures is enhanced by culture-intrinsic IFNs. Infected MEF form a second peak population that is characterized by expression of m164 and that responds to the expression of immune evasion molecules with reduced MHC-I display at the cell surface (closed arrowhead). Caliper rules highlight the degree of MHC-I downmodulation.

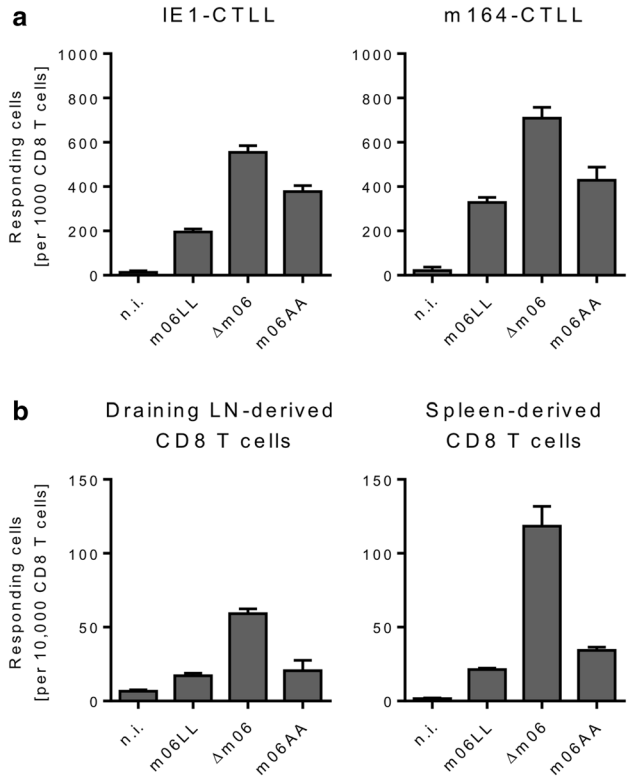

Fig. 3 Impact of the cargo sorting dileucine motif on the presentation of antigenic peptides. Quantitation of CD8 T cells sensitized in an ELISpot assay for secretion of IFN-γ by short-term co-culture with BALB/c (MHC-I $K^dD^dL^d$) MEF as stimulator cells infected with viruses mCMV-m06LL, mCMV-Δm06, and mCMV-m06AA. *n.i* no infection as negative control. Responder cells detecting MHC-I-presented viral peptides were **a** viral epitope-specific IE1-CTLL and m164-CTLL as well as **b** primed CD8 T cells isolated from the draining popliteal lymph node (LN) or the spleen. Bars represent most probable numbers determined by intercept-free linear regression analysis based on IFN-γ spot counts from triplicate cultures of graded numbers of responder cells seeded. Error bars indicate 95% confidence intervals

antigenic peptides IE1 (L^d-presented YPHFMPTNL) [33, 34] and m164 (D^d-presented AGPPRYSRI) [35], detected with respective CTLL, as well as for the full range of antigenic peptides of mCMV in BALB/c mice (MHC-I $K^dD^dL^d$) detected by polyclonal and polyspecific ex vivo CD8 T cells isolated from the draining lymph node and the spleen after priming by intraplantar infection (Fig. 3).

The cargo sorting motif in m06 mediates deviant MHC-I trafficking for lysosomal degradation

A role of the dileucine motif in m06 was so far concluded from *m06* transfection studies in cells deficient in adapter proteins AP-1A or AP-3A or both [22]. AP-1A and AP-3A both localize to the TGN. While AP-1A is thought to shuttle cargo between TGN and early endosomes (EE), AP-3A is thought to mediate cargo transport from TGN to lysosomes as well as from EE to late endosomes (LE)/lysosomes [22]. As a "connector" based on the dileucine motif, m06 feeds MHC-I cargo into the AP-1A/3A sorting pathways. Interestingly, free m06 that is not assembled with pMHC-I cargo, for instance, because pMHC-I is already bound to m04 (see above) or retained in the ERGIC by m152 (see above), is not sorted for lysosomal degradation but enters the cytosol for proteasomal degradation [45]. Perturbation of endosomal trafficking by mCMV, with focus on the role of motif-sufficient m06, has been thoroughly studied under conditions of cell infection as well as transfection by the group of P. Lucin ([46], reviewed in [47]). Upon infection, m06 was found to be retained in EE together with MHC-I molecules internalized from the cell surface, whereas m06-MHC-I complexes were not detected on the cell surface [46]. Importantly, another early (E) phase function of mCMV, thus independent of m06 and its dileucine motif, prevents MHC-I recycling to the cell surface by reorganization of the endosomal recycling compartment (ERC), blocking the transition of internalized MHC-I cargo from EE to the ERC ([48], for reviews see [47, 49]). This ERC blockade of MHC-I recycling is of utmost importance as it makes the fundamental difference between transfected cells, in which recycling is not blocked, and infected cells, in which recycling is blocked.

For validating the functionality of the dileucine motif in the context of infection, we have tested the localization of m06 by CLSM immunofluorescence analysis comparing cells infected with motif-sufficient virus mCMV-m06LL and motif-deficient virus mCMV-m06AA (Fig. 4). In the presence of the motif, m06 co-localized with MHC-I, but the complex did not detectably localize to the TGN, indicating that most m06-pMHC-I complexes have left the TGN. In contrast, after inactivation of the motif, detectable amounts of m06-pMHC-I complexes were found to stick in the TGN (Fig. 4a). Notably, however, not all m06-MHC-I complexes localized to the TGN in absence of the motif (Fig. 4a, purple arrows), suggesting either that the motif is not essential for leaving the TGN or, more likely, that complexes take a route via the cell surface reaching EE after internalization due to turnover. A step downstream in the TGN-endosome sorting pathway, m06 co-localized in the presence of the motif mainly with MHC-I and the LE/lysosome marker LAMP1, whereas after inactivation of the motif, m06-MHC-I complexes did not reach the LE/lysosomal compartment (Fig. 4b). This indicates a block in the transport of m06-MHC-I/pMHC-I from the TGN and EE compartments to the LE/lysosomal compartment in the absence of the motif.

For substantiating this conclusion further, subcellular fractionation of infected cells was performed to biochemically localize m06 (Fig. 5a, b). In accordance with the immunofluorescence images, m06 was found in fractions 7

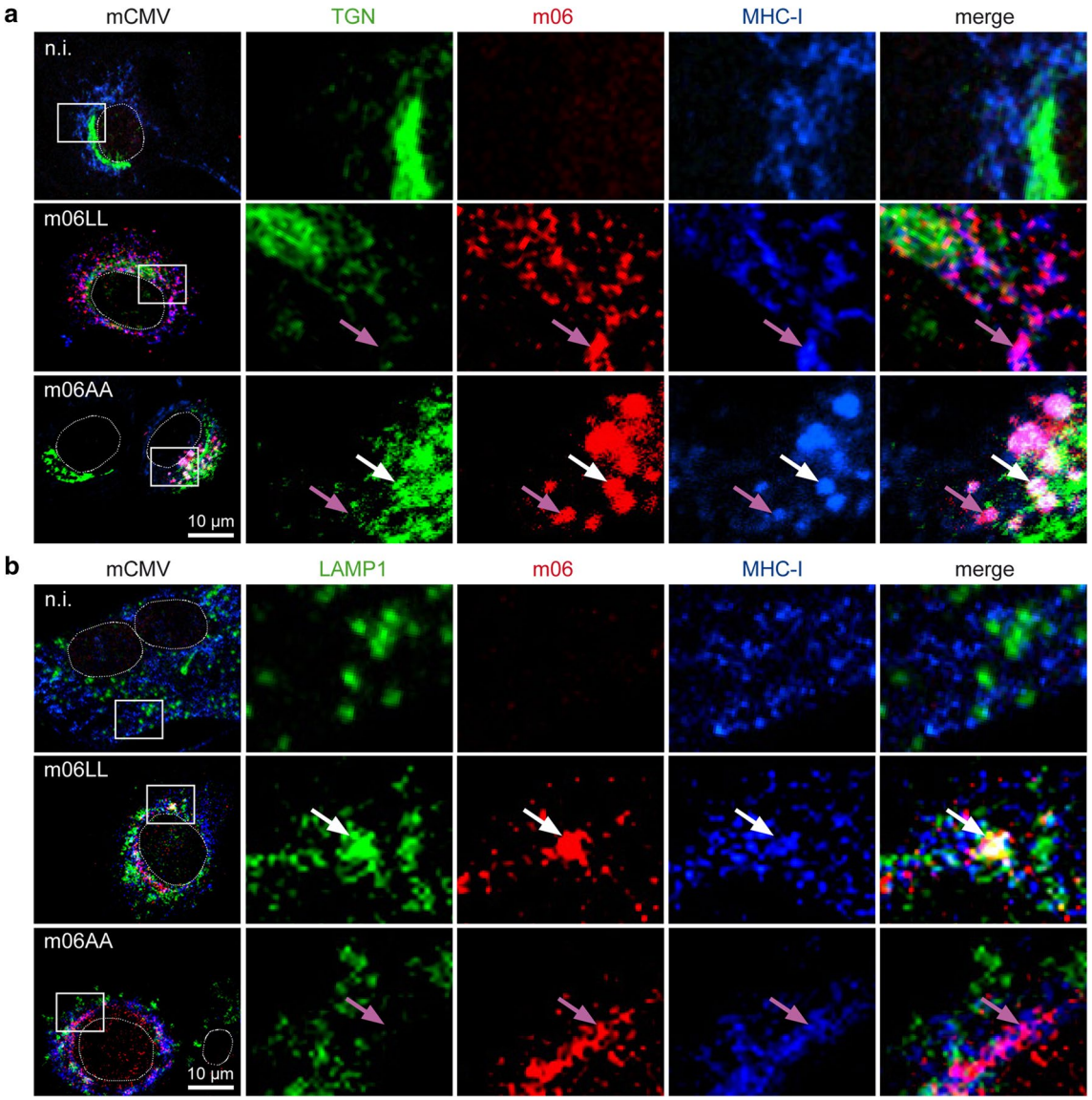

Fig. 4 Impact of the cargo sorting dileucine motif on the trafficking of m06-MHC-I complexes. **a** Co-localization of m06-MHC-I complexes and the TGN. **b** Co-localization of m06-MHC-I complexes and late endosomes (LE)/lysosomes marked by LAMP1. Shown are representative images taken from BALB/c MEF at 8 h after infection with motif-sufficient virus mCMV-m06LL and motif-deficient virus mCMV-m06AA. *n.i.* not infected. *Far left column* Overview images. Nuclei are demarcated for clarity by a dotted line. The boxed perinuclear region is resolved to greater detail in all corresponding images. White arrows point exemplarily to sites where m06-MHC-I complexes localize to TGN or to LE/lysosomes in **a** and **b**, respectively. Purple arrows point exemplarily to sites where m06-MHC-I complexes do not localize to TGN or LE/lysosomes in **a** and **b**, respectively

and 4, representing EE and LE, respectively, in the presence of the motif. In contrast, after inactivation of the motif, m06 failed to transit from EE to LE.

Correct folding of MHC-I complexes and transport with the constitutive vesicular flow require association of the MHC-I α-chain (heavy chain) and the β2m chain (light chain). Interestingly, a recent structural analysis of m06 binding to MHC-I revealed that m06 binds to a site located below the peptide-binding groove of the α-chain and partially overlapping with the site to where β2m binds [50]. Sterical competition between m06 and β2m weakens the binding of m06 to fully assembled MHC-I. As a consequence, absence of β2m will allow an even stronger binding of m06 to the α-chain. Accordingly, m06 binds to MHC-I also in the absence of β2m and, in the presence of the dileucine motif, the complex can transit from EE to LE for lysosomal degradation (Fig. 5c).

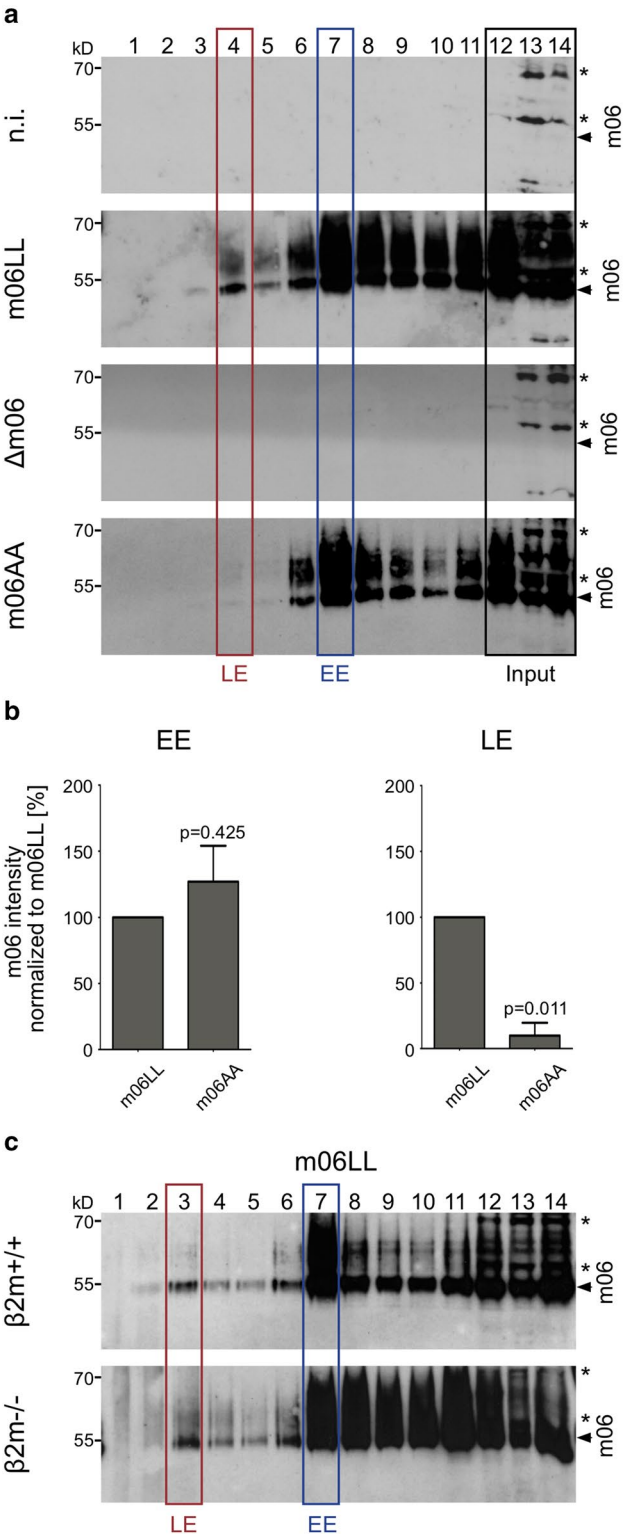

Fig. 5 The cargo sorting dileucine motif in m06 is required for the transition from EE to LE. **a** Representative Western blot analysis localizing m06 to subcellular fractions prepared from BALB/c MEF at 8 h after infection with viruses mCMV-m06LL, mCMV-Δm06, and mCMV-m06AA. *n.i.* not infected as negative control. EE and LE accumulated in fractions 7 and 4 are highlighted by blue and red frames, respectively. *Bands stained unspecifically with the α-m06 antibody as indicated by the presence in fractions from not infected cells and cells infected with m06 deletion mutant mCMV-Δm06. Arrowhead, m06-specific band. **b** Relative densitometric quantitation of signal intensities documenting the block of transport from EE to LE in absence of the cargo sorting dileucine motif was performed on three independent preparations. Signal intensities in the EE and LE fractions from cells infected with the motif-deficient virus mCMV-m06AA are shown as bars (mean values ± SEM, $n = 3$) normalized to the corresponding signal intensities from cells infected with the motif-sufficient virus mCMV-m06LL. The exact p value is given in each graph. **c** m06-guided class-I cargo sorting into the LE/lysosomal pathway does not require the β2m light chain of the MHC-I complex. MEF derived from β2m-expressing C57BL/6 (MHC-I K^bD^b) mice and from β2m knockout mice (on C57BL/6 genetic background) were infected for 8 h with motif-sufficient virus mCMV-m06LL. The m06 protein localizes to EE and LE accumulated in subcellular fractions 7 and 3, respectively

Synopsis

Collectively, our data have shown that the cargo sorting motif EPLARLL in the cytosolic tail of the mCMV immune evasion protein m06 (gp48) is functional also under conditions of cell infection in that it mediates the sorting of m06-MHC-I/pMHC-I complexes into the endosomal pathway, ending up in the lysosome for degradation. However, revising the view meanwhile published in numerous review articles, we show here that the motif, and thus the AP-1A/3A-mediated sorting to the lysosome for degradation, is not the essential mechanism by which m06 mediates downmodulation of cell surface MHC-I and evasion of target cell recognition by CD8 T cells recognizing viral peptide-presenting pMHC-I.

The current state of our knowledge on m06 in pMHC-I/MHC-I trafficking is sketched in Fig. 6. The formation of newly generated, viral peptide-folded pMHC-I takes place in the ER, where m06 binds to it as cargo transporter. The m06-pMHC-I complexes pass the Golgi apparatus and reach the TGN. Based on cell transfection studies performed with cells deficient in cellular adapter proteins AP-1A or AP-3A or both, the dileucine motif was found to link the complexes to AP-1A for sorting them from the TGN to EE, as well as to AP-3A for trafficking from the TGN directly to LE/lysosomes and from EE to LE/lysosomes for final disposal [22]. Importantly, pre-existing cell surface MHC-I, loaded with self-peptides, reach the EE by endocytosis in the course of MHC-I turnover, but recycling back to the cell surface via the ERC is blocked in mCMV-infected cells by an early (E) phase function unrelated to m06 and its sorting motif [47–49]. Finally, MHC-I, coming from the cell surface, and pMHC-I, coming from the TGN, both traffic from EE to LE/lysosomes for disposal. Together, these pathways of cargo sorting explain the loss of pre-existing cell surface MHC-I and

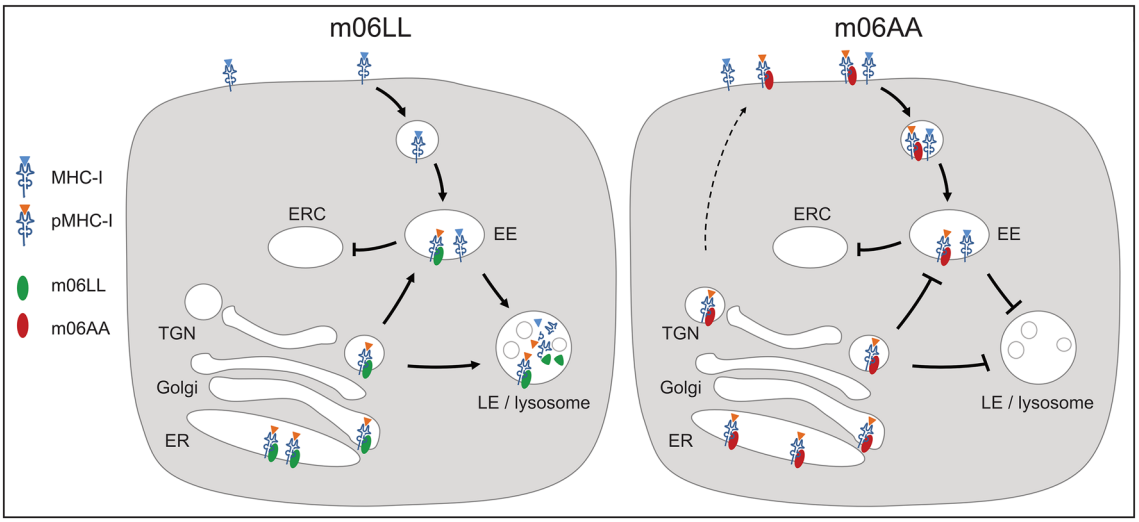

Fig. 6 Graphical synopsis of the role of the cargo sorting dileucine motif in m06-misdirected MHC-I trafficking. *EE* early endosome(s); *ER* endoplasmic reticulum; *ERC* endosomal recycling compartment; *LE* late endosome(s); *TGN* trans-Golgi network. Blue triangle self-peptide; red triangle foreign/viral peptide. The dashed arrow indicates a minor route of trafficking. For more detailed explanation, see the Synopsis in the main body of the text

the missing resupply with pMHC-I after infection of cells with motif-sufficient virus mCMV-m06LL (Fig. 6, left).

In absence of the motif, upon cell infection with mCMV-m06AA (Fig. 6, right), all LL-motif and AP-1A/3A-dependent sorting pathways are blocked, so that pMHC-I complexes can no longer transit from TGN to EE or LE/lysosomes, as proven by our localization studies, and are thus not degraded in the lysosome but, instead, are trapped in the TGN. In addition, internalized cell surface MHC-I is trapped in EE, as recycling via ERC is blocked (see above). As a consequence, over time, the cell surface becomes deprived of MHC-I molecules, just like in the presence of the motif after infection with mCMV-m06LL. As the only difference, as suggested by our functional assays, this "traffic jam" leads to cell surface escape of some m06-pMHC-I, or maybe also some free pMHC-I, complexes. The amount of these escapees must be low, as they did not show up in the cytofluorometric analysis of MHC-I cell surface expression. Only with the extremely sensitive assay of CD8 T cell activation, requiring only few pMHC-I complexes displayed at the cell surface, we observed a statistically significant but quantitatively minor increase in the number of CD8 T cells that responded in the absence of the sorting motif. We do not yet know for sure if the CD8 T cells detect only free pMHC-I or also m06-pMHC-I complexes. As a structural analysis has revealed binding of m06 to the MHC-I α-chain at a site below the peptide-binding groove that is formed by the α2 and α3 domains [50], and unless binding of m06 causes allosteric refolding, m06 should not sterically interfere with recognition of presented antigenic peptide. We, therefore, consider it possible that CD8 T cells can recognize also m06-pMHC-I complexes at the cell surface.

The full picture of mCMV interference with pMHC-I trafficking must include the molecules that compete with m06 for pMHC-I cargo, that is m04, which escorts pMHC-I to the cell surface, and m152, which retains pMHC-I in the ERGIC. Competition for the cargo, however, occurs at the stage of binding to pMHC-I in the ER and/or ERGIC, and is thus unlikely to be influenced by the sorting motif that mediates binding to cellular APs involved in endosomal sorting pathways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal experiments were approved according to German federal law §8 Abs. 1 TierSchG by the ethics committee of the Landesuntersuchungsamt Rheinland-Pfalz, permission number 177-07/G 14-1-015.

References

1. Rawlinson W, Farrell H, Barrell B (1996) Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol 70:8833–8849
2. Fink A, Blaum F, Babic Cac M, Ebert S, Lemmermann NA, Reddehase MJ (2015) An endocytic YXXΦ (YRRF) cargo sorting motif in the cytoplasmic tail of murine cytomegalovirus AP2 'adapter adapter' protein m04/gp34 antagonizes virus evasion of natural killer cells. Med Microbiol Immunol 204:383–394. https://doi.org/10.1007/s00430-015-0414-1
3. Kleijnen MF, Huppa JB, Lucin P, Mukherjee S, Farrell H, Campbell AE, Koszinowski UH, Hill AB, Ploegh HL (1997) A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. EMBO J 16:685–694. https://doi.org/10.1093/emboj/16.4.685
4. Kavanagh DG, Koszinowski UH, Hill AB (2001) The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment. J Immunol 167:3894
5. Lu X, Kavanagh DG, Hill AB (2006) Cellular and molecular requirements for association of the murine cytomegalovirus protein m4/gp34 with major histocompatibility complex class I molecules. J Virol 80:6048–6055. https://doi.org/10.1128/JVI.00534-06
6. Berry R, Vivian JP, Deuss FA, Balaji GR, Saunders PM, Lin J, Littler DR, Brooks AG, Rossjohn J (2014) The structure of the cytomegalovirus-encoded m04 glycoprotein, a prototypical member of the m02 family of immunoevasins. J Biol Chem 289:23753–23763. https://doi.org/10.1074/jbc.M114.584128
7. Reusch U, Muranyi W, Lucin P, Burgert HG, Hengel H, Koszinowski UH (1999) A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. EMBO J 18:1081–1091. https://doi.org/10.1093/emboj/18.4.1081
8. Reddehase MJ (2002) Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat Rev Immunol 2:831–844. https://doi.org/10.1038/nri932
9. Doom C, Hill AB (2008) MHC class I immune evasion in MCMV infection. Med Microbiol Immunol 197:191–204. https://doi.org/10.1007/s00430-008-0089-y
10. Hansen TH, Bouvier M (2009) MHC class I antigen presentation: learning from viral evasion strategies. Nat Rev Immunol 9:503–513. https://doi.org/10.1038/nri2575
11. Lemmermann NA, Böhm V, Holtappels R, Reddehase MJ (2011) In vivo impact of cytomegalovirus evasion of CD8 T-cell immunity: facts and thoughts based on murine models. Virus Res 157:161–174. https://doi.org/10.1016/j.virusres.2010.09.022
12. Lemmermann NA, Fink A, Podlech J, Ebert S, Wilhelmi V, Böhm V, Holtappels R, Reddehase MJ (2012) Murine cytomegalovirus immune evasion proteins operative in the MHC class I pathway of antigen processing and presentation: state of knowledge, revisions, and questions. Med Microbiol Immunol 201:497–512. https://doi.org/10.1007/s00430-012-0257-y
13. Wagner M, Gutermann A, Podlech J, Reddehase MJ, Koszinowski UH (2002) Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J Exp Med 196:805–816
14. Babic M, Pyzik M, Zafirova B, Mitrovic M, Butorac V, Lanier LL, Krmpotic A, Vidal S, Jonjic S (2010) Cytomegalovirus immunoevasin reveals the physiological role of "missing self" recognition in natural killer cell dependent virus control in vivo. J Exp Med 207:2663–2673. https://doi.org/10.1084/jem.20100921
15. Vidal S, Krmpotic A, Pyzik M, Jonjic S (2013) Innate immunity to cytomegalovirus in the murine model. In: Reddehase MJ (ed) Cytomegaloviruses: from molecular pathogenesis to intervention, vol II. Caister Academic Press, Norfolk, pp 192–214
16. Holtappels R, Gillert-Marien D, Thomas D, Podlech J, Deegen P, Herter S, Oehrlein-Karpi SA, Strand D, Wagner M, Reddehase MJ (2006) Cytomegalovirus encodes a positive regulator of antigen presentation. J Virol 80:7613–7624. https://doi.org/10.1128/JVI.00723-06
17. Pinto A, Munks MW, Koszinowski UH, Hill AB (2006) Coordinated function of murine cytomegalovirus genes completely inhibits CTL lysis. J Immunol 177:3225–3234
18. Lemmermann NA, Gergely K, Böhm V, Deegen P, Däubner T, Reddehase MJ (2010) Immune evasion proteins of murine cytomegalovirus preferentially affect cell surface display of recently generated peptide presentation complexes. J Virol 84:1221–1236. https://doi.org/10.1128/JVI.02087-09
19. Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, Bonifacino JS (2001) Signal-binding specificity of the mu4 subunit of the adapter protein complex AP-4. J Biol Chem 276:13145–13152. https://doi.org/10.1074/jbc.M010591200
20. Kielczewska A, Pyzik M, Sun T, Krmpotic A, Lodoen MB, Munks MW, Babic M, Hill AB, Koszinowski UH, Jonjic S, Lanier LL, Vidal SM (2009) Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response. J Exp Med 206:515–523. https://doi.org/10.1084/jem.20080954
21. Sgourakis NG, Natarajan K, Ying J, Vogeli B, Boyd LF, Margulies DH, Bax A (2014) The structure of mouse cytomegalovirus m04 protein obtained from sparse NMR data reveals a conserved fold of the m02-m06 viral immune modulator family. Structure 2:1263–1273. https://doi.org/10.1016/j.str.2014.05.018
22. Reusch U, Bernhard O, Koszinowski UH, Schu P (2002) AP-1A and AP-3A lysosomal sorting functions. Traffic 3:752–761
23. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH (1997) Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci USA 94:14759–14763
24. Wagner M, Jonjic S, Koszinowski UH, Messerle M (1999) Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J Virol 73:7056–7060
25. Fink A, Lemmermann NA, Gillert-Marien D, Thomas D, Freitag K, Böhm V, Wilhelmi V, Reifenberg K, Reddehase MJ, Holtappels R (2012) Antigen presentation under the influence of 'immune evasion' proteins and its modulation by interferon-gamma: implications for immunotherapy of cytomegalovirus infection with antiviral CD8 T cells. Med Microbiol Immunol 201:513–525. https://doi.org/10.1007/s00430-012-0256-z
26. Podlech J, Holtappels R, Grzimek NKA, Reddehase MJ (2002) Animal models: murine cytomegalovirus. In: Kaufmann SHE, Kabelitz D (eds) Methods in microbiology, vol 32. Immunology of infection. Academic Press, London, pp 493–525
27. Kurz S, Steffens HP, Mayer A, Harris JR, Reddehase MJ (1997) Latency versus persistence or intermittent recurrences: evidence for a latent state of murine cytomegalovirus in the lungs. J Virol 71:2980–2987
28. Tischer BK, Smith GA, Osterrieder N (2010) En passant mutagenesis: a two step markerless red recombination system. Methods Mol Biol 634:421–430. https://doi.org/10.1007/978-1-60761-652-8_30

29. Hammer Q, Rückert T, Borst EM, Dunst J, Haubner A, Durek P, Heinrich F, Gasparoni G, Babic M, Tomic A, Pietra G, Nienen M, Blau IW, Hofmann J, Na IK, Prinz I, Koenecke C, Hemmati P, Babel N, Arnold R, Walter J, Thurley K, Mashreghi MF, Messerle M, Romagnani C (2018) Peptide-specific recognition of human cytomegalovirus strains controls adaptive natural killer cells. Nat Immunol 19:453–463. https://doi.org/10.1038/s41590-018-0082-6
30. Lemmermann NA, Podlech J, Seckert CK, Kropp KA, Grzimek NK, Reddehase MJ, Holtappels R (2010) CD8 T-cell immunotherapy of cytomegalovirus disease in the murine model. In: Kabelitz D, Kaufmann SHE (eds) Methods in microbiology, vol 37. Immunology of infection. Academic Press, London, pp 369–420
31. Chojnacki S, Cowley A, Lee J, Foix A, Lopez R (2017) Programmatic access to bioinformatics tools from EMBL-EBI update: 2017. Nucleic Acids Res 45:W550–W553. https://doi.org/10.1093/nar/gkx273
32. Däubner T, Fink A, Seitz A, Tenzer S, Müller J, Strand D, Seckert CK, Janssen C, Renzaho A, Grzimek NK, Simon CO, Ebert S, Reddehase MJ, Oehrlein-Karpi SA, Lemmermann NA (2010) A novel transmembrane domain mediating retention of a highly motile herpesvirus glycoprotein in the endoplasmic reticulum. J Gen Virol 91:1524–1534. https://doi.org/10.1099/vir.0.018580-0
33. Reddehase MJ, Rothbard JB, Koszinowski UH (1989) A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. Nature 337:651–653. https://doi.org/10.1038/337651a0
34. Pahl-Seibert MF, Jülch M, Podlech J, Thomas D, Deegen P, Reddehase MJ, Holtappels R (2005) Highly protective in vivo function of cytomegalovirus IE1 epitope-specific memory CD8 T cells purified by T-cell receptor-based cell sorting. J Virol 79:5400–5413. https://doi.org/10.1128/JVI.79.9.5400-5413.2005
35. Holtappels R, Thomas D, Podlech J, Reddehase MJ (2002) Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2d haplotype. J Virol 76:151–164
36. Böhm V, Simon CO, Podlech J, Seckert CK, Gendig G, Deegen P, Gillert-Marien D, Lemmermann NA, Holtappels R, Reddehase MJ (2008) The immune evasion pardox: immunoevasins of murine cytomegalovirus enhance priming of CD8 T cells by preventing negative feedback regulation. J Virol 82:11637–11650
37. Aniento F, Gruenberg J (2004) Subcellular fractionation of tissue culture cells. Curr Protoc Protein Sci. 32:4.3.1–4.3.21. https://doi.org/10.1002/0471140864.ps0403s32
38. Scheffer K, Popa-Wagner R, Florin L (2013) Isolation and characterization of pathogen-bearing endosomes enable analysis of endosomal escape and identification of new cellular cofactors of infection. In: Bailer SM, Lieber D (eds) Methods in molecular biology, vol 1064. Virus–host interactions. Humana Press, Totowa, pp 101–113. https://doi.org/10.1007/978-1-62703-601-6_7
39. Gräßel L, Fast LA, Scheffer KD, Boukhallouk F, Spoden GA, Tenzer S, Boller K, Bago R, Rajesh S, Overduin M, Berditchevski F, Florin L (2016) The CD63-syntenin-1 complex controls post-endocytic trafficking of oncogenic human papillomaviruses. Sci Rep 6:32337. https://doi.org/10.1038/srep32337
40. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675
41. Ziegler H, Thale R, Lucin P, Muranyi W, Flohr T, Hengel H, Farrell H, Rawlinson W, Koszinowski UH (1997) A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. Immunity 6:57–66
42. Krmpotic A, Messerle M, Crnkovic-Mertens I, Polic B, Jonjic S, Koszinowski UH (1999) The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. J Exp Med 190:1285–1296
43. Fink A, Renzaho A, Reddehase M, Lemmermann NAW (2013) The p36 isoform of murine cytomegalovirus m152 protein suffices for mediating innate and adaptive immune evasion. Viruses 5:3171–3191. https://doi.org/10.3390/v5123171
44. Del Val M, Hengel H, Hacker H, Hartlaub U, Ruppert T, Lucin P, Koszinowski UH (1992) Cytomegalovirus prevents antigen presentation blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J Exp Med 172:729–738
45. Bubeck A, Reusch U, Wagner M, Ruppert T, Muranyi W, Kloetzel PM, Koszinowski UH (2002) The glycoprotein gp48 of murine cytomegalovirus proteasome-dependent cytosolic dislocation and degradation. J Biol Chem 277:2216–2224. https://doi.org/10.1074/jbc.M104178200
46. Kučić N, Tomaš MI, Mahmutefendić H, Blagojević G, Luči P (2012) Early endosomal retention of murine cytomegalovirus m06 protein. Croat Chem Acta 85:213–221. https://doi.org/10.5562/cca1816
47. Lučin P, Mahmutefendić H, Blagojević Zagorac G, Ilić Tomaš M (2015) Cytomegalovirus immune evasion by perturbation of endosomal trafficking. Cell Mol Immunol 12:154–169. https://doi.org/10.1038/cmi.2014.85
48. Karleuša L, Mahmutefendić H, Tomaš MI, Zagorac GB, Lučin P (2018) Landmarks of endosomal remodeling in the early phase of cytomegalovirus infection. Virology 515:108–122. https://doi.org/10.1016/j.virol.2017.12.001
49. Lučin P, Kareluša L, Blagojević Zagorac G, Mahmutefendić Lučin H, Pavišić V, Jug Vučko N, Lukanović Jurić S, Marcelić M, Lisnić B, Jonjić S (2018) Cytomegaloviruses exploit recycling rab proteins in the sequential establishment of the assembly compartment. Front Cell Dev Biol 6:165. https://doi.org/10.3389/fcell.2018.00165
50. Sgourakis NG, May NA, Boyd LF, Ying J, Bax A, Margulies DH (2015) A novel MHC-I surface targeted for binding by the MCMV m06 immunoevasin revealed by solution NMR. J Biol Chem 290:28857–28868. https://doi.org/10.1074/jbc.M115.689661

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