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CHAPTER 3

**HCMV-encoded US2 differentially affects
surface expression of MHC class I locus products
and targets membrane-bound,
but not soluble HLA-G1 for degradation**

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HCMV-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane bound, but not soluble HLA-G1 for degradation

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Human cytomegalovirus (HCMV) can elude cytotoxic T lymphocytes as well as NK cells by modulating surface expression of MHC class I molecules. This strategy would be most efficient if the virus would selectively down-regulate viral antigen-presenting alleles, while at the same time preserving other alleles to act as inhibitors of NK cell activation. We focussed on HCMV-encoded US2, which binds to newly synthesized MHC class I heavy chains and supports their dislocation to the cytosol for subsequent degradation by proteasomes. We studied the effect of US2 on surface expression of individual class I locus products using flow cytometry. Our results were combined with crystal structure data of complexed US2/HLA-A2/β2m and alignments of 948 HLA class I database sequences of the ER luminal region implicated in US2 binding. This study suggests that surface expression of all HLA-A, -G and most -B alleles will be affected by US2. Several HLA-B alleles and all HLA-C and -E alleles are likely to be insensitive to US2-mediated degradation. We also found that the MHC class I ER-luminal domain alone is not sufficient for degradation by US2, as illustrated by the stability of soluble HLA-G1 in the presence of US2. Furthermore, we showed that the membrane bound HLA-G1 isoform, but also tailless HLA-A2, are targeted for degradation. This indicates that the cytoplasmic tail of the MHC class I heavy chain is not required for its dislocation to the cytosol by US2.

A large proportion of the world population is carrier of human cytomegalovirus (HCMV), which usually gives rise to asymptomatic but lifelong infections. In immuno-compromised individuals, however, HCMV contributes to high morbidity and mortality rates. Besides this, HCMV is a notorious risk factor for congenital disorders if the mother undergoes primary infection during pregnancy ¹.

The host immune system is eluded by HCMV through a variety of defense mechanisms that it acquired while co-evolving with its host. Surface expression of antigen presenting MHC class I molecules, which play a central role in detection and elimination of infected cells, is affected by the concerted action of a set of HCMV unique short (US) region- encoded proteins (US2, US3, US6, US11) expressed along different stages of viral infection ²⁻⁶. Like US11, US2 induces proteolytic degradation of MHC class I molecules: immediately after their synthesis and translocation into the ER lumen, MHC class I heavy chains are transported back into the cytosol where they are deprived of their N-linked glycan and subsequently degraded by proteasomes ^{2,6,7}. When devoid of MHC class I surface expression, cells may become a target for NK cell lysis ⁸. Several mechanisms have been described by which HCMV can sidetrack NK cells, either by blocking expression of ligands that activate

NK cells or allowing expression of ligands that inhibit NK cell triggering. Several proteins encoded within the UL region of the HCMV genome (UL16, UL18, UL40) could protect from NK cell lysis ⁹. In addition, inhibitory signals to NK cells could be preserved by allowing selective surface expression of those MHC class I that are less efficient in viral antigen presentation ¹⁰.

Most host cells express HLA-A, -B, -C and -E alleles. HLA-A and -B alleles are likely most powerful in the presentation of viral antigens, which is supported by a very high degree of polymorphism in the world population for these alleles ¹¹. The contribution of HLA-C to immune control by cytotoxic T cells is not entirely clear. HLA-C alleles are less polymorphic and surface expression is generally lower than that observed for HLA-A and -B alleles, but HIV peptides displayed in the context of HLA-C have been reported ^{12,13}. HLA-E is low polymorphic, mainly presents peptides derived from signal sequences of MHC class I molecules, and is expressed in low amounts at the cell surface ¹⁰. HLA-E has also been reported to present peptides to specific T cell receptors on CD8+ T cells or NK CD8+ cells ^{14 15}. HLA-E and HLA-C could also serve important functions as inhibitory NK cell receptor ligands ^{16,17}.

HLA-G is found primarily on extravillous cytotrophoblast cells at the fetal-maternal interface and on a subpopulation of thymic epithelial cells¹⁸. These trophoblast cells express no HLA-A and -B alleles on their cell surface, only HLA-C, -E and -G. Different HLA-G isoforms have been described, of which only one, called HLA-G1, is expressed at the cell surface¹⁹. HLA-G1 has a unique feature: it has a relatively short cytoplasmic tail of 6 amino acids. Another isoform, soluble HLA-G1, has all domains required for stable complex formation with β 2m and peptide and is secreted²⁰. Membrane bound and soluble HLA-G both present viral peptides to CD8+ T cells²¹⁻²³. The soluble isoform also induces apoptosis of activated CD8+ T cells and inhibits CD4+ T cell proliferation²⁴⁻²⁶. It is unknown to what extent this potential T cell function is exploited, because only few macrophages and T and B immune cells are found at the implantation site. The population of lymphoid cells at this site predominantly consists of NK cells²⁷. Like other HLA class I molecules, HLA-G could also be an important modulator of cytokine production by NK cells. Interactions with activating NK receptors and stimulation of cytokine secretion by uterine NK cells could play a role in the placentation process. HLA-G could also serve as ligand for inhibitory NK receptors to prevent trophoblast lysis²⁷.

For efficient immune escape, HCMV should prevent the display of viral antigens by MHC class I molecules to cytotoxic T cells and at the same time preserve a substantial amount of ligands that can modulate NK cell activation. It would be beneficial to selectively down-regulate those MHC class I molecules that are most important for viral antigen presentation and keep other locus products as inhibitor of NK cell activation.

In general, HLA-A and -B alleles are believed to be down-regulated by HCMV, but detailed information is scarce. Pulse chase experiments suggested that US2 does not affect the stability of HLA-C, -G, and -E alleles^{28,29}.

In the present study, we evaluated the sensitivity of different MHC class I locus products to US2-mediated down-regulation using flow cytometry. Flow cytometrical analysis of MHC class I surface expression is most relevant with respect to T and NK cell interactions. Besides this, it can be a valuable complementation of pulse chase data as it could unravel or exclude other mechanisms than degradation that cause reduced surface expression.

We introduced different HLA class I alleles into a murine cell line, which also expresses human β 2m, and monitored the effect of US2 on surface expression of these molecules. To verify that our results were not species or cell type specific, we included experiments with human cell lines. We further investigated structural requirements for US2-induced HLA class I heavy chain degradation, making use of membrane-bound HLA-G1, which lacks most cytoplasmic tail residues, and soluble HLA-G1, which consists of ER-lumenal domains only. Our data will also show that US2 and US11 employ different strategies to target class I heavy chains for degradation.

MATERIALS AND METHODS

Cell lines

J26 cells (H-2^k murine Ltk⁻ cells expressing human β 2m)³⁰, the Phoenix amphotropic retroviral producer cell line and JEG-3 cells (both from the American Type Culture Collection, ATCC) were cultured in DMEM (Life Technologies Inc.). J26 cells expressing HLA-B7 (B*070201), B27 (B*270502) and Cw3 (Cw*030401) were described previously^{31,32}. U373-MG cells (ATCC) were cultured in RPMI 1640 medium (Life Technologies Inc.). All media were supplemented with 10% FCS (Greiner), 100 U/ml penicillin and 100 μ g/ml streptomycin and G418 (GibcoBRL).

Antibodies

The following anti-MHC class I mAbs were used for flow cytometry: 87G (HLA-G)²³, W6/32 (general HLA class I)³³, MA2.1 (HLA-A2)³⁴, MEM-E/06 (HLA-E; EXBIO Praha, Czech Republic), B1.23.2 (HLA-C)³⁵ and Y-3 (murine MHC class I; ATCC). PE-conjugated goat anti-mouse (gam), IgG (Jackson Immuno Research laboratories) was used as second Ab. The polyclonal antisera MR24 (directed against the α 3 domain of HLA-A*0201 and generated in rabbits using synthetic tetanus toxoid conjugated peptide PKTHMTHHAVSDHEA) and US2-N2 (directed against the N-terminus of US2 and generated in rabbits using synthetic tetanus toxoid conjugated peptide GITKAGEDALRPWKSTAK), as well as mAbs HCA2 (HLA-A, -G)³⁶, MEM-G/01 (HLA-G; EXBIO Praha, Czech Republic) and H68.4 (transferrin receptor; Zymed), were used for immunoprecipitations.

Construction of plasmids

Plasmid pLUMC9901 (encoding HLA-A*0201)³⁷, was used as template for the construction of short-tailed

HLA-A2 constructs containing 4 or 6 amino acid tails (-RRKS or -RRKSSD respectively). A cDNA fragment obtained from an HLA-Cw4 typed individual was used for subcloning into pcDNA3.1/V5/His-TOPO to generate the plasmid pCw*0304 (generous gift of B. van den Eynde). The HLA-Cw4 fragment was fully sequenced to determine the HLA-Cw*0304 allelic subtype. Plasmid pCw*0304 and pLUMC9901 were used for the construction of HLA-Cw3/HLA-A2 chimeras. For HLA-Cw3/tail A2, the HLA-Cw3 cytoplasmic tail was replaced with the corresponding HLA-A2 tail region (aa 310-342). The reverse was done for the construction of HLA-A2/tail Cw3. These constructs were generated applying the megaprimer method³⁸ and were subcloned into pcDNA3 (Invitrogen). Plasmid pHLA-A/E was generated by replacing the leader sequence of the HLA-E*01033-Gly¹⁰⁷ gene derived from cosmid 3.14³⁹ with the leader sequence of HLA-A2 and subcloning this into pcDNA3. Plasmid pcDNA-G1 (encoding HLA-G*01011) and soluble HLA-G1 cDNA have been described previously^{40,41}. Amplifications were performed using DNA polymerase Pwo (Eurogentec, Seraing, Belgium). All constructs were fully sequenced to verify the absence of unwanted mutations.

Transfection

J26 and U373 cells were transfected with the different MHC class I constructs using EffecteneTM Transfection Reagent (Qiagen). After 48 hours, stable transfectants were selected by adding 0.3 mg/ml G418 (GibcoBRL). Cells were sorted by flow cytometry for expression of the introduced cDNA as described⁴⁰.

Production of retrovirus and transduction

A retroviral vector expressing both US2 and EGFP was constructed by ligating a US2-encoding cDNA fragment into the pLZRS-*IRES*-EGFP vector⁴². Wild type or US2-*IRES*-EGFP constructs were transfected into the amphotropic Phoenix packaging cell line for the production of retrovirus, as described⁴⁰. Cells were transduced with retrovirus using retronectin (Takara, Japan) coated dishes.

Vaccinia virus infections

Cells were infected with wild type or US2-expressing recombinant vaccinia virus (generous gift of Dr. J. Yewdell) at a multiplicity of infection of 10 PFU/cell for 45-60 min in a small volume (~1.5 ml) of serum free culture medium at 37°C⁴³. After infection, a mix of conditioned and fresh culture medium was added.

Metabolic labeling of the infected cells was performed approximately 4½ h after infection.

Flow cytometry

Cell surface expression of MHC class I molecules as well as EGFP expression in cells transduced with retrovirus were analyzed using flow cytometry as described⁴⁰. J26-HLA-E and JEG-3 cells were stained in 3 steps to intensify the MHC class I staining: first with specific anti-MHC class I antibody, then with biotinylated-gam and finally with streptavidin-conjugated PE. Absence of MEM-E/06 binding to wild type J26 cells in flow cytometry (data not shown) excluded any possible cross reactivity with murine MHC class I. Data are collected from at least two independent experiments of which one representative experiment is shown. Independent measurements for MHC class I (PE) staining in EGFP positive cells differed with an average of 5%.

Metabolic labeling, immunoprecipitation and SDS-PAGE

Metabolic labeling, immunoprecipitations and SDS-PAGE were performed as described³⁷. In brief, cells were starved in MetCys⁻ medium at 37 °C, labeled with ³⁵S promix (Amersham), and chased in medium with excess amounts of L-cystine and L-methionine. Where indicated, media were supplemented with proteasome inhibitor ZL3H. Cells were lysed in a small volume of Nonidet-P40 lysis buffer, containing protease inhibitors leupeptin, AEBSF and ZL3H, for 30 min at 4°C. After centrifugation to remove cell debris, supernatant was transferred to a new tube to which 1/10 volume of 10% SDS and 1/10 volume of 0.1 M DTT was added. Samples were boiled for 5 min to further denature proteins. Next, the volume was increased 10 times with non-denaturing buffer (1% Triton X-100, 50 mM Tris HCl pH 7.4, 300 mM EDTA, 0.02%NaN₃) supplemented with protease inhibitors (leupeptin, AEBSF, ZL3H) and 10 mM iodoacetic acid. Immunoprecipitations were performed ≥ 2 h on precleared samples, with Abs pre-coupled to protein A/G sepharose beads. Samples were separated by SDS-PAGE and displayed via phosphor imaging (Bio-Rad Personal Molecular Imager FX).

RESULTS

Allelic differences in US2-mediated down-regulation of MHC class I surface expression.

We first investigated if US2 differentially affects surface expression of various MHC class I alleles.

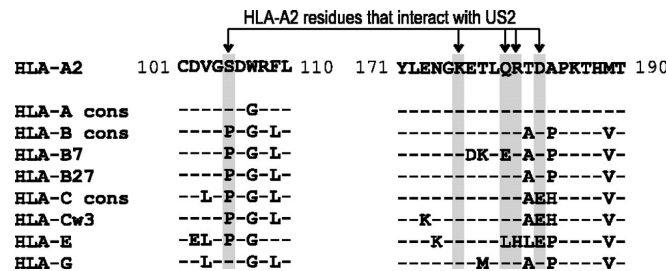


FIGURE 1. Overview of sequence variation within the US2 binding region of MHC class I locus products. Depicted is the region of HLA-A2 with residues directly involved in interaction with US2 marked in grey (according to crystal structure data from PUBMED Protein Data Base reference: 1IM3, deposited by Gewurz *et al.*⁴⁴). The US2 binding site of HLA-A2 has been aligned with corresponding regions of other alleles, and their locus (sub)group consensus sequences (cons) obtained from the IMGT/HLA Sequence Database⁴⁵.

Since alterations in surface display of MHC class I can directly affect the incidence of T and NK cell receptor interactions, we used flow cytometry for our investigation. The available crystal structure data on a soluble complex of US2/HLA-A2/ β 2m provided a starting point for the selection of representative locus products for our study⁴⁴. By aligning HLA class I sequences from the IMGT/HLA sequence database⁴⁵ to the region of US2-interaction described for HLA-A2 and looking at conserved residues, we found HLA-A2, -B7, -B27, -Cw3, -G and -E alleles to be good candidates (see Figure 1). These alleles all have an amino acid composition for the region implicated in US2 binding that is conserved within their locus (sub)group. HLA class I constructs were transfected into murine J26 cells to enable specific monitoring of introduced alleles by flow cytometry. J26 cells co-

express human β 2m to allow proper HLA class I complex formation. Cells were then transduced with retrovirus expressing US2-IRES-EGFP. Figure 2 shows that surface expression of HLA-A2, -B27 and -G was reduced by US2, to 12%, 39% and 7% respectively, compared to US2-negative cells. In contrast, HLA-B7, -Cw3 and -E are not or only slightly affected by US2 with 110%, 85% and 98% surface expression, respectively. Endogenously expressed H-2^k molecules were not downmodulated by US2. Control EGFP-expressing retrovirus had no effect on MHC class I cell surface expression, as shown here for HLA-A2.

Cytoplasmic tail of MHC class I heavy chains is not essential for US2-mediated down-regulation

We previously showed that residues at the extreme end of the cytoplasmic tail of class I heavy chains can

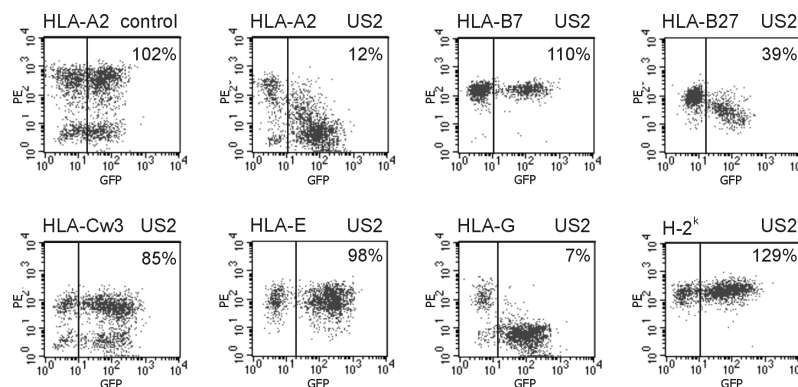


FIGURE 2. Selective US2-mediated down-regulation of HLA class I. Murine J26 cells, transfected with different plasmids encoding HLA class I and transduced with US2-IRES-EGFP encoding retrovirus, were analyzed using flow cytometry. The following MoAbs were used to stain the different HLA class I molecules: HLA-A2 (MA2.1), HLA-B7, -B27, and -Cw3 (W6/32), HLA-E (MEM-E/06), HLA-G (87G) or endogenously expressed H-2^k (Y-3), followed by PE-conjugated goat anti-mouse Ab (Y-axis). US2 positive cells are marked by EGFP expression (X-axis). The effect of US2 on surface staining of MHC class I was calculated by comparing mean PE fluorescence of EGFP negative (defined as 100%) and positive cells. As a control, J26-HLA-A2 cells were transduced with EGFP-expressing retrovirus to show that EGFP alone, does not affect MHC class I surface expression.

determine their sensitivity to down-regulation by US11⁴⁰. Others reported that a reduction of the tail to 4 amino acids ensures stability of HLA-A2 in the presence of either US11 or US2⁴⁶. HLA-G has a relatively short cytoplasmic tail of 6 amino acids⁴⁷, due to a premature stopcodon in exon 6, and was found to be insensitive to US2-mediated degradation²⁸. We decided to evaluate the contribution of the tail of class I heavy chains to US2-mediated down-regulation in more detail (Figure 3). By exchanging cytoplasmic tail regions of US2-sensitive (HLA-A2) and US2-insensitive (HLA-Cw3) class I molecules (see Figure 3A), we evaluated if the tail region could account for differences in sensitivity among locus products. Figure 3B shows that HLA-A2 with the tail of HLA-Cw3 was down-regulated as efficiently as HLA-A2 wt (both 12% surface expression in the presence of US2), while HLA-Cw3 with the tail of HLA-A2 and wild type HLA-Cw3 were almost equally resistant (with 75% and 85% surface expression). In parallel, we evaluated the effect of tail length on US2-sensitivity by reducing the size of the tail of HLA-A2 molecules (see Figure 3A) to 6 amino acids (similar in length to HLA-G) or 4 amino acids (identical to the construct tested previously⁴⁶). Figure 3B shows that both short-tailed HLA-A2 molecules were efficiently downmodulated by US2.

These data indicate that the tail of MHC class I heavy chains is not essential for US2-mediated down-regulation of cell surface expression.

HLA-G and short-tailed HLA-A2 are efficiently targeted for degradation by US2.

Downregulation of MHC class I surface expression can be accomplished by degradation, but also by retention in an intracellular compartment, as has been shown for US11⁴⁰. We investigated the underlying mechanism for down-regulation of short-tailed MHC class I molecules by US2. Since the tail region of the class I heavy chain is exposed to the cytosol it might serve an important function in the retrograde transport to the cytosol for subsequent proteasomal degradation. We investigated whether US2 affects the stability of HLA-G1 and short-tailed HLA-A2 (6 aa tail) in a pulse chase experiment (Figure 4). Recombinant vaccinia virus was used to introduce US2 into the cells. In parallel, a similar amount of cells was infected with wild type vaccinia virus. Immunoprecipitations were performed on denatured cell lysates to evaluate the effect of US2 on total amounts of HLA class I heavy chains, irrespective of their folding state.

We observed a destabilizing effect of US2 on wt HLA-A2 as well as on HLA-G1 and short-tailed HLA-A2 (upper panel Figure 4). In the presence of proteasome inhibitor ZL3H (lower panel) deglycosylated degradation intermediates could be observed in US2 expressing cells for all three HLA class I molecules.

Thus, HLA-G1 and short-tailed HLA-A2 are efficiently destabilized in US2⁺ cells, indicating that the cytosolic tail of MHC class I heavy chains is not required for US2-dependent dislocation and degradation.

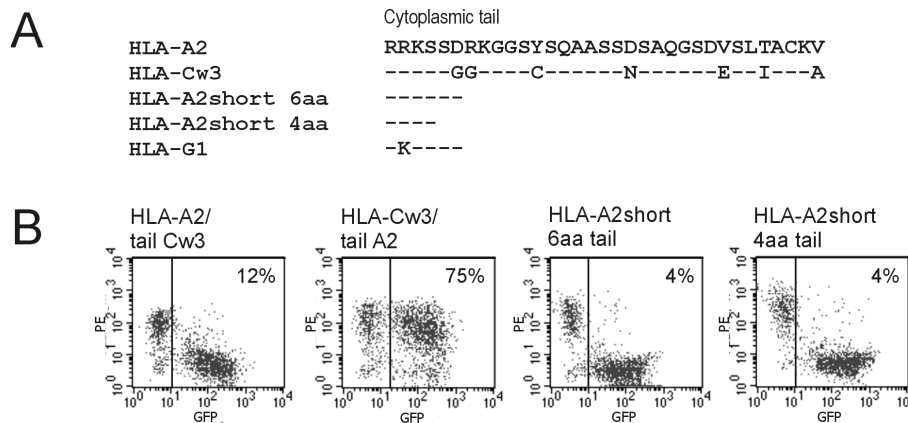


FIGURE 3. Cytoplasmic tail of MHC class I is not essential for US2-mediated down-regulation. **A)** Overview of amino acid sequences of the cytoplasmic tail regions that are exchanged in the HLA-A2/Cw3 chimeras and shortened for the HLA-A2 tail mutants. As a comparison, the HLA-G tail region is depicted. **B)** J26 cells were transfected with chimeras or tail mutants, transduced with US2-*IRES*-EGFP encoding retrovirus and analyzed using flow cytometry. MHC class I molecules are stained with W6/32 (chimeras) or MA2.1 (HLA-A2short), followed by goat anti-mouse-PE conjugate (Y-axis). US2 positive cells are marked by EGFP expression (X-axis).

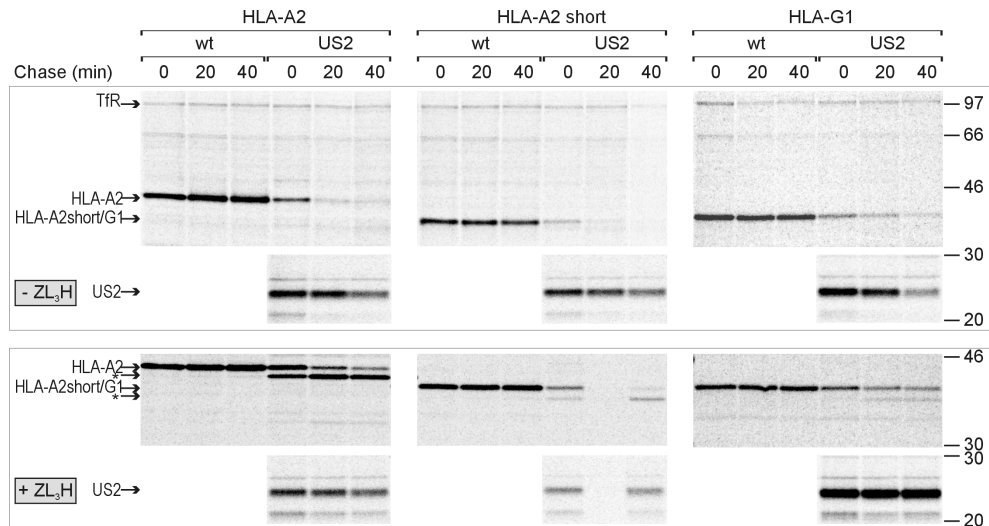


FIGURE 4. HLA-G and short-tailed HLA-A2 are efficiently degraded in US2 expressing cells. J26 cells expressing wt HLA-A2, HLA-A2short (6aa tail) or HLA-G were infected with wt or US2-encoding vaccinia virus. At 4½ hours post-infection, cells were metabolically labeled for 10 minutes and chased for 0, 20 or 40 minutes in the absence (upper panel) or presence (lower panel) of proteasome inhibitor ZL3H. Immunoprecipitations were performed on denatured cell lysates using the following antisera: MR24 (HLA-A2 wt, -A2short), HCA2 (HLA-G), H68.4 (transferrin receptor [TfR]) or US2-N2 (US2) Abs. Deglycosylated degradation intermediates are marked by an asterisk. The 20 min chase sample (+ZL3H, +US2) for HLA-A2short was lost during the procedure for this representative experiment. Molecular weight markers are given in kilo Daltons.

Evaluation of US2-mediated down-regulation of HLA-G in cell lines of human origin.

To ensure that our observation on HLA-G was not species or cell type specific, we included experiments in different cell lines of human origin (Figure 5).

First, U373 astrocytoma cells (expressing HLA-A2, -B18 and -Cw5), transfected with HLA-G and transduced with control or US2-*IRES*-EGFP encoding retrovirus, were analyzed by flow cytometry (Figure 5A). Comparable HLA-G surface expression was observed in control EGFP expressing cells and non-transduced cells, as determined with 87G (upper left histogram). This antibody is specific for HLA-G and does not cross-react with endogenous HLA class I, as wild type U373 cells remained PE negative when stained with this antibody (Figure 5A, lower left histogram). A reduction of HLA-G surface expression was seen in US2/EGFP expressing cells (to 45%). Down-regulation of HLA-G by US2 seems to be even more efficient than that of endogenous HLA-A2, stained by antibody MA2.1 (to 63%). This antibody preferentially recognizes HLA-A2 molecules and not HLA-B18 or -Cw5 in these U373 cells³⁴. MA2.1 did not cross-react with HLA-G in HLA-G1 expressing J26 cells (data not shown).

Note that wild type U373 cells expressing US2 demonstrated a stronger down-regulation of endogenous HLA-A2 molecules stained with MA2.1 (to 15%). This indicates that the transfection of an additional US2-sensitive class I heavy chain into cells affects the efficiency of down-modulation of the total pool of MHC class I. It is unlikely that the lower efficiency of MHC class I down-regulation in the U373-HLA-G cells is due to a lower level of US2 expression. The mean fluorescence value for EGFP, as a marker for US2 expression (not shown in figure 5A) was even higher in these cells (255) than in wild type U373 cells (194).

Secondly we tested the effect of US2 on HLA-G in the physiologically more relevant trophoblast cell line JEG-3 (Figure 5B). These cells naturally express HLA-G1, HLA-Cw4 and likely also low amounts of HLA-E, but lack HLA-A and HLA-B molecules at their cell surface. In cells transduced with US2 retrovirus, HLA-G surface expression was severely reduced (to 9%) whereas HLA-Cw4 surface expression remained stable. In this case, severity of HLA-G down-modulation was comparable to that observed for J26 cells transfected with HLA-G (to 7%, see Figure 2).

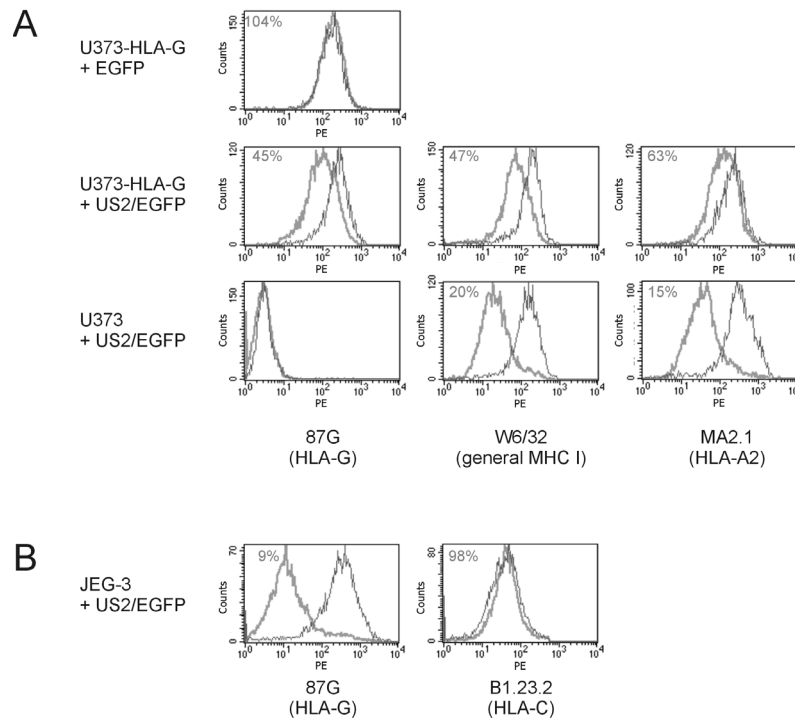


FIGURE 5. US2-mediated down-regulation of HLA-G in human cell lines. A) Wild type human U373 astrocytoma cells (HLA-A2, -B18, -Cw5), or U373 cells transfected with HLA-G were transduced with retrovirus (EGFP- or US2-IRES-EGFP) and analyzed by flow cytometry. Cells were stained with either 87G (HLA-G), MA2.1 (HLA-A2), or W6/32 (general anti-MHC class I) MoAbs, followed by goat anti-mouse PE conjugate. Shown are histograms representing transduced (thick grey line) and non-transduced (thin black line) cells. Surface expression is given as percentage of mean PE fluorescence in transduced cells compared to non-transduced cells. **B)** Similarly, JEG-3 cells expressing HLA-G1, -Cw4 (and likely also HLA-E) were transduced with retrovirus (US2-IRES-EGFP), stained with 87G (anti-HLA-G) or B1.23.2 (anti-HLA-C) and analyzed using flow cytometry.

Note that both cell lines expressed no other US2-sensitive MHC class I molecules besides HLA-G.

Together, these results show that down-regulation of short-tailed MHC class I molecules can be observed in different cell lines of human origin. These data further indicate that efficiency of down-regulation is influenced by amounts of US2-susceptible class I heavy chains present in the cells.

Soluble HLA-G1 is resistant to US2-mediated degradation

Interaction between US2 and class I heavy chains does not require transmembrane regions, as soluble trimeric HLA-A2/US2/ β 2m complexes could be crystallized⁴⁴. Binding of MHC class I molecules to a soluble form of US2 is not sufficient for their degradation⁴⁸. We investigated if interaction of US2 with a soluble form of MHC class I could induce its degradation. This is of clinical relevance, as

membrane-bound and soluble isoforms are generated by differential splicing of primary HLA-G mRNA transcripts²⁰. One of these isoforms is soluble due to retention of intron 4, which introduces a premature stopcodon (Figure 6A). This isoform possesses the ER-luminal α 1-3 domains required for interaction with US2. We compared its sensitivity to US2-mediated degradation to that of the membrane bound HLA-G isoform in pulse chase experiments (Figure 6B). In the absence of proteasome inhibitor, a progressive loss of membrane bound HLA-G1 and endogenous MHC class I molecules was observed in US2-expressing cells (upper panel). In contrast, soluble HLA-G1 remained stable throughout the chase (lower panel). In the presence of proteasome inhibitor, a deglycosylated degradation intermediate could be observed for both endogenous HLA molecules and membrane bound HLA-G1, but not for soluble HLA-G1.

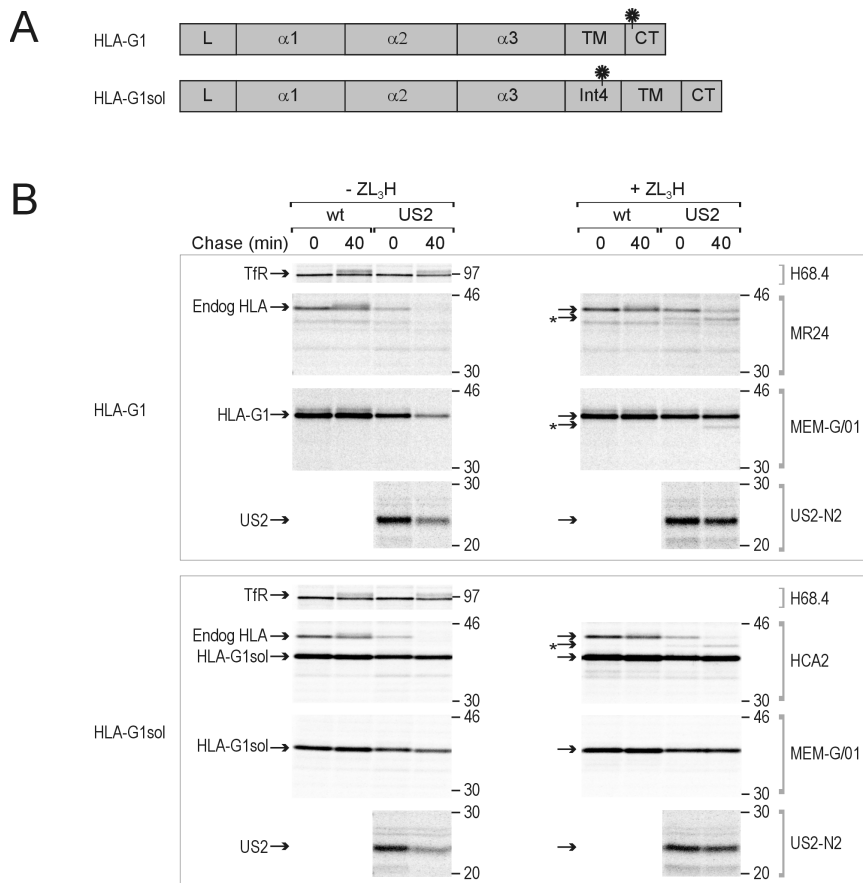


FIGURE 6. Soluble HLA-G1 is stable in US2-expressing cells. A) Depicted are two isoforms of HLA-G: membrane bound and soluble HLA-G1. HLA-G1 has a premature stopcodon (*) in exon 6 which allows translation of only 6 amino acids of the cytoplasmic tail (CT) region. Soluble HLA-G1 contains a premature stopcodon due to retention of intron 4, allowing translation of only ER-luminal $\alpha 1$ -3 domains. L=leader sequence, TM=transmembrane region **B)** U373 cells transfected with membrane bound or soluble HLA-G1 were infected with wt or US2-encoding vaccinia virus. At 4½ hours post-infection, cells were labeled for 10 minutes and chased for 0 or 40 minutes in the absence or presence of proteasome inhibitor ZL₃H. Immuno-precipitations were performed on denatured cell lysates with MR24 (endogenous HLA class I), MEM G/1 (HLA-G), H68.4 (Tfr) or US2-N2 (US2) Abs. Deglycosylated degradation intermediates are marked by an asterisk.

Evidently, soluble HLA-G1 can escape US2-mediated degradation although it possesses all ER-luminal $\alpha 1$ -3 domains required for binding US2.

DISCUSSION

HCMV encodes several proteins that interfere with cross talk between host cells and immune effector cells through modulation of surface expression of MHC class I molecules. The various MHC class I locus products can serve different immune functions. Some are more important for the presentation of viral

antigens, while others may mainly act as ligands for inhibitory NK receptors. The success of immune escape by HCMV through modulation of MHC class I surface expression is likely to be influenced by MHC class I allele specificity of the different HCMV US proteins. In this paper we focused on modulation of MHC class I expression by HCMV US2.

We first evaluated allelic differences in US2-mediated down-regulation of MHC class I cell surface expression. Previous studies on US2 mainly focused on the mechanism of interference with antigen presentation ^{2,7,46,48,49}. With the cell lines and

antibodies used in these studies it is difficult to deduce effects on individual MHC class I locus products. In general, HLA-A and -B alleles are believed to be down-regulated, but detailed information is scarce. Binding studies indicate that US2 associates with HLA-A2 and -Aw68, but no interaction could be detected with HLA-B7, -B27, -Cw4, or -E⁵⁰. Pulse chase experiments show that US2 does not affect the stability of HLA-C, -G, and -E alleles^{28,29}. It is, however, also important to specifically evaluate the effect on surface expression of MHC class I molecules, as this is most relevant with respect to T and NK cell interactions. MHC class I molecules that appear to be stable in pulse chase experiments can nevertheless be withheld from the cell surface via other mechanisms than degradation. US11, for example, can cause ER-retention of MHC class I molecules that can not be targeted for degradation⁴⁰. Flow cytometrical analysis of surface expression therefore is a valuable complementation of pulse chase data.

The available crystal structure of a soluble complex of US2/HLA-A2/β2m⁴⁴ provided us with a good starting point for the selection of representative class I locus products (Figure 1). We found that US2 down-regulates HLA-A2, -B27, and -G, but not HLA -B7, -Cw3, and -E alleles of this selection (Figure 2). Sequence variation in the region implicated in US2 binding (residues 101-110 and 171-190) was evaluated for those alleles present in the IMGT/HLA sequence database⁴⁵ that are fully sequenced for this region. Of the residues directly involved in interaction with US2, the residue at position 105 is either S or P, and both are found in US2 sensitive alleles. Out of 947 alleles, 946 have K176. Residues at position 105 and 176 are therefore unlikely to account for sensitivity differences between locus products. Among locus products that differ in sensitivity for US2, residues found at positions 180, 181 or 183 also differ. All 274 HLA-A and most of the 519 HLA-B alleles have residues Q180, R181 and D183 that are present in US2-sensitive HLA-A2 and HLA-B27. In contrast, US2-insensitive HLA-B7 has E180 instead of Q180, which is also found in other HLA-B alleles. Two other US2-insensitive locus products, HLA-Cw3 and HLA-E, have E183 instead of D183. This E183 is also found in 132 out of 133 HLA-C alleles and in all 6 HLA-E alleles. In addition, HLA-E alleles differ from US2-sensitive alleles at position 180 and 181, with their L180 and H181 residues. Among the residues that are not directly involved in US2 binding, but that are located around US2 binding residues, only little

sequence variation is found. Moreover, most of the sequence variation that is found here is unlikely to account for allelic differences, as they are found in both US2-sensitive and -insensitive alleles. Altogether, our findings suggest that US2 down-regulates all HLA-A, -G and most -B alleles and no HLA-C, or-E. In addition to HLA-B7 several other HLA-B isotypes, including HLA -B8, -B40, -B41, -B42, and -B48 are likely to be US2-resistant. These HLA-B isotypes are relatively common, as they are found in 25-30% of the Caucasian population (Frans Claas, personal communication). Since HLA-B alleles are very important for presentation of viral peptides to cytotoxic T cells, it might be advantageous for the host to possess such HLA-B alleles.

The finding that US2 down-regulates cell surface expression of HLA-G, which has a relatively short tail, is novel. A previous report claimed that HLA-G remains stable in the presence of US2²⁸. Resistance to US2-mediated down-regulation has been ascribed to absence of a full-length tail, as tailless HLA-A2 molecules were reported insensitive to US2-mediated degradation⁴⁶. When looking at the residues required for interaction with US2, the HLA-G locus product closely resembles HLA-A2 (see Figure 1A). The most striking difference between HLA-G and -A locus products is found in their cytosolic domains, as HLA-G alleles lack most cytosolic tail residues due to a premature stop codon in exon 6. Our flow cytometry experiments with murine cells showed that both HLA-G1 and tailless HLA-A2 molecules were withheld from the cell surface by US2 (Figures 2 and 3). When we investigated the mechanism underlying this reduction in cell surface expression, it became evident that HLA-G1 and tailless HLA-A2 molecules are targeted for degradation by US2 (Figure 4). These data reveal that the cytosolic tail is not required for US2-mediated dislocation and subsequent degradation. To exclude that cell- or species-specific factors account for our observations in the murine cell line, we also performed experiments in human JEG-3 trophoblast and U373 cell lines, with similar results (Figures 5 and 6). The observation that tailless HLA class I molecules are sensitive to US2-mediated degradation may have been missed in earlier reports as a result of a different experimental set up; The experiments shown by Schust et al.²⁸ are performed with porcine endothelial cells transfected with HLA-G. However, HLA-G expression in these cells was too poor to allow firm conclusions on possible stability changes as a result of US2 expression²⁸. Furthermore, our experiments with U373 cells (the same cell line used by Story et al.

⁴⁶⁾ indicate that expression of multiple, US2-sensitive class I products results in reduced efficiency of US2-mediated MHC class I down-regulation (Fig. 5). Down-regulation of HLA-G was more pronounced in murine J26 cells transfected with HLA-G or in naturally HLA-G expressing JEG-3 trophoblast cells. In contrast to the U373 cell line transfected with HLA-G, these cell lines express no other US2-sensitive MHC class I molecules than HLA-G. The effectiveness of down-modulation of HLA products likely also depends on expression levels of US2, which may vary between expression systems. US2-mediated degradation of tailless HLA-A2 molecules might have been revealed in previous experiments ⁴⁶ if the experiments had been performed in the presence of proteasome inhibitor. In case of a suboptimal degradation efficiency, a small amount of deglycosylated degradation intermediate already serves as indisputable evidence.

We also investigated if US2 could induce degradation of the soluble HLA-G1 isoform. This isoform consists of ER-luminal $\alpha 1-3$ domains only. It is known that interaction between US2 and HLA class I does not require transmembrane regions, as soluble US2/HLA-A2/ $\beta 2m$ complexes could be crystallized ⁴⁴. We have shown here that ER-luminal domains of HLA class I alone are nevertheless insufficient to allow US2-mediated degradation, as soluble HLA-G1 remained stable in the presence of US2 (Figure 6).

The observation that viruses use different proteins for interference with MHC class I expression is likely to improve the effectiveness of immune escape. Together they can affect a wider range of class I locus products. They may also act synergistically to reach a more dramatic down-regulation of a single locus product. The use of different strategies may also help to avoid saturation of a particular cellular pathway used by the virus to effectuate evasion. HCMV encodes several proteins that affect MHC class I surface expression during the course of infection, and they act through different mechanisms: a) by retaining MHC class I molecules in the ER (US3) ³, b) by blocking the Transporter associated with Antigen Processing (TAP)(US6) ^{4,5} c) by delaying MHC class I maturation (US10) ⁵¹, and d) by dislocating newly synthesized class I heavy chains to the cytosol for subsequent degradation by proteasomes (US2 and US11) ^{2,6}. When looking at the different mechanisms employed, US2 and US11 seem to act very similarly. Our data contribute to the understanding of the need of two proteins with, at first sight, similar functions.

Previous studies have already shown that US2 and US11 each interact with different sites on ER-luminal regions of the HLA class I heavy chain ^{40,44}. We showed here that they also differ in their requirements for cytosolic tail residues, as HLA-G1 and tailless HLA-A2 are efficiently targeted for degradation by US2. As shown before, this is not the case for US11 ^{28,40,46}. Interestingly, the requirement for cytosolic tail residues appears to be the reverse for the viral proteins themselves. Where tailless US11 can still target newly synthesized class I heavy chains for degradation, this is not the case for US2 ^{48,52}. Since US2 and US11 approach their target MHC class I molecules differently, they may affect different subsets of locus products. The difference in cytosolic tail requirements may also be accompanied by usage of different accessory proteins involved in the dislocation and degradation process.

Trophoblast membrane-bound and soluble HLA-G1 are believed to serve important immunological functions at the fetal-maternal interface. Our finding that membrane-bound, but not soluble HLA-G1, is sensitive to US2-mediated degradation may be of relevance in the context of HCMV infection during pregnancy.

It is unknown if viral antigen presentation could be effective at the fetal-maternal interface. Only few maternal CTLs are found here and during HCMV infection, T cell recognition of infected trophoblast cells will largely be prohibited due to absence or reduction of surface expression of antigen presenting MHC class I molecules: HLA-A and -B molecules are lacking in these cells and surface expression of HLA-G is reduced by both US2 (this paper), US3 ⁵³ and US6 ⁵⁴. In addition, HLA-C can be down-regulated by US3, US6 ⁵³ and US11 ⁵⁵ (our own unpublished results). HLA-E surface expression is not affected by US2 (this paper), US6 ⁵⁴ and US11 (our own unpublished results). We are unaware of data describing the effect of US3 on HLA-E expression.

NK cells are the predominant population of immune effector cells at the maternal-fetal interface and they serve several important functions at this site. By down-regulating most HLA class I locus products, many ligands for inhibitory receptors on NK cells will also be lost. Expression of HLA-E and UL16, UL18 and UL40 is not likely to fully compensate for the lack of inhibitory NK cell ligands ⁸. It is unclear if HCMV-infected trophoblast cells are at risk for NK cell attack *in vivo*, as the degree of protection from NK cell lysis

was shown to differ significantly between lab strains and clinical isolates ⁵⁶. HLA-G down-regulation by HCMV could also prevent interaction with activating NK cell receptors and prohibit uterine NK cell cytokine secretion, which is necessary for the placentation process.

We also demonstrated that soluble HLA-G1 is resistant to US2-mediated degradation. In a previous paper we showed that US11 has no effect on surface expression of membrane-bound HLA-G1 ⁴⁰, which makes it unlikely that US11 will prohibit secretion of the soluble HLA-G1 isoform. It remains to be established to what extent other US proteins could affect its secretion. Soluble HLA-G1 can induce apoptosis of activated T cells ²⁴. Cells secreting this HLA-G1 isoform could also modulate NK cell triggering.

Together, our results suggest that surface expression of all HLA-A, and -G, and most -B alleles will be down-regulated in the presence of US2. Several HLA-B alleles and all HLA-C and -E alleles will be resistant against US2-mediated degradation. Using HLA-G and tailless HLA-A2 we demonstrated that US2 does not require the cytosolic tail of class I heavy chains to initiate dislocation and degradation of these molecules. The MHC class I ER-luminal domain alone is not sufficient for US2-mediated degradation, as illustrated by the stability of soluble HLA-G in the presence of US2. Our data shed new light on the modulation of MHC class I expression on cells at the fetal-maternal interface in the context of HCMV infection. More research will be needed to evaluate the immunological consequences of these virus-induced changes in MHC expression.

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