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Expression of the Human Cytomegalovirus UL11 Glycoprotein in Viral Infection and Evaluation of Its Effect on Virus-Specific CD8 T Cells

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ABSTRACT

The human cytomegalovirus (CMV) UL11 open reading frame (ORF) encodes a putative type I transmembrane glycoprotein which displays remarkable amino acid sequence variability among different CMV isolates, suggesting that it represents an important virulence factor. In a previous study, we have shown that UL11 can interact with the cellular receptor tyrosine phosphatase CD45, which has a central role for signal transduction in T cells, and treatment of T cells with large amounts of a soluble UL11 protein inhibited their proliferation. In order to analyze UL11 expression in CMV-infected cells, we constructed CMV recombinants whose genomes either encode tagged UL11 versions or carry a stop mutation in the UL11 ORF. Moreover, we examined whether UL11 affects the function of virus-specific cytotoxic T lymphocytes (CTLs). We found that the UL11 ORF gives rise to several proteins due to both posttranslational modification and alternative translation initiation sites. Biotin labeling of surface proteins on infected cells indicated that only highly glycosylated UL11 forms are present at the plasma membrane, whereas less glycosylated UL11 forms were found in the endoplasmic reticulum. We did not find evidence of UL11 cleavage or secretion of a soluble UL11 version. Cocultivation of CTLs recognizing different CMV epitopes with fibroblasts infected with a UL11 deletion mutant or the parental strain revealed that under the conditions applied UL11 did not influence the activation of CMV-specific CD8 T cells. For further studies, we propose to investigate the interaction of UL11 with CD45 and the functional consequences in other immune cells expressing CD45.

IMPORTANCE

Human cytomegalovirus (CMV) belongs to those viruses that extensively interfere with the host immune response, yet the precise function of many putative immunomodulatory CMV proteins remains elusive. Previously, we have shown that the CMV UL11 protein interacts with the leukocyte common antigen CD45, a cellular receptor tyrosine phosphatase with a central role for signal transduction in T cells. Here, we examined the proteins expressed by the UL11 gene in CMV-infected cells and found that at least one form of UL11 is present at the cell surface, enabling it to interact with CD45 on immune cells. Surprisingly, CMV-expressed UL11 did not affect the activity of virus-specific CD8 T cells. This finding warrants investigation of the impact of UL11 on CD45 functions in other leukocyte subpopulations.

The genome of human cytomegalovirus (CMV) displays a remarkably large coding capacity. Careful reevaluation of the genomic information led to the conclusion that the genomes of CMV clinical isolates encode about 165 bona fide open reading frames (ORFs) (1, 2), and a recent ribosome profiling analysis of CMV-infected cells implied the presence of up to 750 translated ORFs (3). Interestingly, only 45 of the viral genes were found to be essential for replication in cell culture (4, 5), indicating that the majority of the CMV coding capacity is dedicated to accessory functions, for instance, to interference with various immune defense mechanisms of the host. The ability to modulate the immune response may be a prerequisite for CMV to establish a lifelong infection in its host (6) and to infect even cell types, such as macrophages and dendritic cells, that are central in orchestrating the antiviral immune response (reviewed in references 7 to 9). Moreover, in the rhesus monkey CMV model, it was shown that certain immunoevasins are required to allow reinfection of the seropositive host in the presence of the fully developed immune response (10). In healthy individuals, strong humoral and cell-mediated immunity to CMV, which holds the infection in check, is induced (6). Protective immunity has been especially ascribed to CD8 T cells (reviewed in reference 11), and in CMV-seropositive individuals it is often seen that up to 10% of this T cell subset is specific for CMV antigens (12). In immunocompromised patients and in neonates with an immature immune system, the delicate balance between host immunity and viral immunomodulation

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can, however, easily be disturbed, leading to serious disease, such as gastroenteritis, hepatitis, retinitis, or pneumonia (6, 13, 14).

CMV employs a series of strategies to modulate the host immune response, and for several viral immunomodulatory proteins, their mode of action has been investigated in detail (reviewed in references 15 to 19). One mechanism that protects infected cells from recognition and elimination by CD8 cytolytic T lymphocytes (CTLs) is interference with viral antigen presentation via the major histocompatibility complex class I (MHC-I) pathway. At least four viral proteins, US2, US3, US6, and US11, target this pathway by sequestering or degrading MHC-I molecules. More recently, it was reported that a virus genome-encoded microRNA targeting the aminopeptidase ERAP1 also contributes to escape from CTL recognition (20). However, evasion from CD8 T cell control is not absolute. There is evidence that the immunoevasins US2 and US11 only partially prevent the MHC-I presentation of peptides, for instance, those derived from the CMV antigens pp65 and IE1 (21, 22), and they do not seem to affect the transport or surface expression of some HLA molecules (23). One can therefore assume that CMV employs additional immunomodulatory proteins to dampen the activity of these immune effector cells.

A number of putative immunomodulatory CMV proteins have not yet been linked to a definite function. Examples are individual members encoded by the CMV RL11 gene family. The 14 members of this family are nonessential for virus replication *in vitro* (4, 5), as anticipated for immunomodulatory genes, and they are predicted to encode either type I transmembrane or secreted glycoproteins (2, 24, 25). Such viral proteins can potentially interfere with immunologically relevant host proteins within the secretory pathway and on the plasma membrane or in *trans* with molecules on the surface of immune effector cells recognizing infected cells. Most intriguingly, the RL11 proteins share an immunoglobulin-like domain, termed RL11D, which was proposed to be structurally related to a similar domain present within the known immunomodulatory E3 proteins of adenoviruses (24). Interestingly, several of the RL11 proteins display remarkable amino acid polymorphism between different clinical isolates (2, 24, 26, 27), implying that during evolution they were under diversifying selection pressure, possibly imposed by immune mechanisms and their polymorphic effector molecules. Some of the RL11 genes have been connected with immunomodulatory functions. The RL11 and RL13 proteins bind immunoglobulins and are considered to be virus genome-encoded Fc receptors (28–30). UL7 has been reported to display sequence similarity to a member of the signaling lymphocyte activation molecule family and apparently modulates the production of proinflammatory cytokines in myeloid cells (31). In addition, UL1 and UL13 may influence the cell tropism of CMV (32, 33).

UL11 was the first CMV gene for which a high interstrain variability has been reported (26). In a recent study, our group characterized the UL11 protein by expressing it independently of CMV infection (34). Following transduction of epithelial cells and fibroblasts with an adenoviral vector expressing UL11, we found that UL11 is exposed on the plasma membrane. Moreover, we showed that the membrane-bound UL11 as well as a soluble UL11-Fc protein can interact in *trans* with the receptor tyrosine phosphatase CD45 present on T cells. CD45 is expressed at a high abundance on T and B lymphocytes as well as on other leukocytes (35). The function of CD45 in T cells has been described to be that of a rheostat determining the activation threshold of these cells. By keeping the Lck kinase in an activated state, CD45 has a central role in the transduction of signals received by the T cell receptor (TCR). When T cells were incubated with a soluble UL11-Fc fusion protein at a relatively high concentration, their proliferation following stimulation with a TCR-specific antibody (Ab) was found to be diminished (34). This result suggested that the activity of CD45 may be reduced upon binding of UL11, thereby dampening the T cell effector function and, thus, possibly contributing to the protection of infected cells against CMV-specific T cells. This hypothesis remained to be tested, especially since little was known about the expression of UL11 during the CMV infection cycle.

In this study, we characterized the proteins generated from the UL11 ORF in CMV-infected cells and examined their posttranslational modification and subcellular localization. Using target cells infected with different CMV mutants, we tested a potential effect of UL11 on CMV-specific CTLs. We detected different UL11 proteins in the infected cells starting on day 3 postinfection (p.i.). Only a highly glycosylated UL11 form was present at the cell surface, while other UL11 forms remained intracellular, predominantly in the endoplasmic reticulum (ER). At the level at which UL11 is expressed in CMV-infected fibroblasts, it did not affect the effector function of CD8 T cells, as assessed by gamma interferon (IFN-γ) secretion. These results indicate that under the conditions examined, UL11 does not exert the expected immunomodulatory function during CMV infection of fibroblasts, as was supposed before. This implies that UL11 either has other functions or acts on other cell types.

MATERIALS AND METHODS

Cells. Retinal pigmented epithelial (hTERT-RPE-1 [RPE-1]) cells (Clontech) were propagated in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 medium buffered with 0.348% sodium bicarbonate. HEK293T cells (obtained from ATCC), MRC-5 fibroblasts (obtained from ECACC), and human foreskin fibroblasts (HFFs) were grown in DMEM. CMV-specific T cell clones were cultivated in RPMI 1640 medium supplemented with interleukin-2 (1,000 U/ml; Proleukin; Novartis) and restimulated every 2 weeks as described previously (23). Mini-lymphoblastoid cell lines (mLCLs) (36) were cultivated in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum (Biochrom), penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and 2 mM L-glutamine.

Generation of recombinant viruses. The CMV mutants used in this study are based on bacterial artificial chromosome (BAC)-cloned genomes of strains AD169 (37) and Merlin-UL128L^{TB40} (38), the latter of which is referred to here as HMPar. For generation of the HM11V5 mutant (Fig. 1), an ampicillin resistance gene-*sacB-lacZ* cassette (amplified with primers 5′-TTTCGAATACCGCAAAAGCTGTGGCTGCTCTGGCAGCATGACAAGCACGGCATCGTGCTCATCCCCCAAACCGATCTGCCTGTGACGGAAGATCACTTCG-3′ and 5′-AAGGCCTGTCCGCCGCGCCAGCTGGCACGGAGTTGGCGTTTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCCTGAGGTTCTTATGGCTCTTG-3′) was first inserted downstream of the UL11 ORF in the HMPar genome and subsequently replaced with the oligonucleotides 5′-GTGGCTGCTCTGGCAGCATGACAAGCACGGCATCGTGCTCATCCCCCAAACCGATCTGGGTAAGCCAATCCCTAACCCGCTCCTAGGTCTTGATTCTACG-3′ and 5′-GCACGGAGTTGGCGTTTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCTTACGTAGAATCAAGACCTAGGAGCGGGTTAGGGATTGGCTTACC-3′ by applying a recombineering technique described previously (32, 39). All other modifications, such as addition of sequences encoding enhanced green fluorescent protein (EGFP) or epitope tags (Fig. 1), were performed by *en passant* mutagenesis

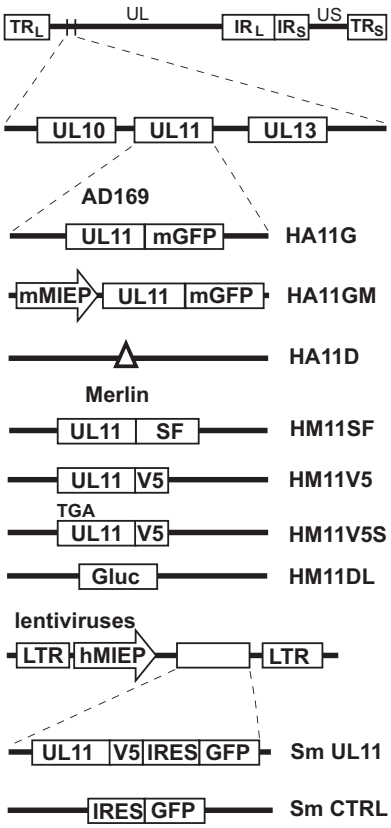

FIG 1 CMV mutants and lentiviral constructs used in this study. The scheme depicts the structure of the CMV genome (top) with the terminal repeat (TR), internal repeat (IR), unique long (UL), and unique short (US) sequences. The genomic region with the UL11 ORF and the genetic elements added, deleted (Δ), or replaced in the mutants with the indicated names are shown enlarged below. mGFP, monomeric green fluorescent protein; mMIEP, mouse CMV major immediate early promoter; SF, Strep-FLAG tag; V5, V5 epitope tag; TGA, replacement of the UL11 start codon with the stop codon TGA; Gluc, *Gaussia* luciferase. The mutants are based on the AD169 or the Merlin strain. The lentiviral vector Sm UL11 contains the long terminal repeats (LTRs), the human CMV MIEP (hMIEP), and the ORFs for the V5-tagged UL11 and for GFP separated by an internal ribosome entry site (IRES) element. The Sm CTRL vector encoding only GFP was used as a control.

in *Escherichia coli* strain GS1783 (40, 41) using PCR products comprising suitable homology regions and a kanamycin resistance (Kan^r) cassette with an adjacent I-SceI site. Following mutagenesis the Kan^r cassette was excised by I-SceI-stimulated homologous recombination as described previously (40). PCRs were performed with primers mGFP.for (5′-TGACAAGCACGGCATCGTGCTCATCCCTCAAACCGATCTGAGATCTGGAGCATCAGCAGTGAGCAAGGGCGAGGAGCTGTT-3′) and mGFP.rev (5′-TTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCTCACTTGTACAGCTCGTCCATGCCGA-3′) for amplifying the monomeric green fluorescent protein (mGFP) ORF, MIEP.for (5′-TTTCAAACCCACTGTTTGAATATAGGGACAGTCCCTACGGAACCTGAGAAAGGACGACGACGACAAGTAA-3′) and MIEP.rev (5′-TGAAAGGTAATGTACCTGAGCAGCATTCTACCACAGGTGATTTCCACATGCTGCAGCGAGGAGCTCTGCG-3′) for amplifying the major immediate early promoter of mouse CMV, ΔUL11.for (5′-CTACGGAACCTGAGAACATGTGGAAATCACCTGTGGTAGAGCAAGTCGCGTAGGAAATGACAAGGATGACGACGATAAG-3′) and ΔUL11.rev (5′-TTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCTCTACCACAGGTGATTTCCAGCCAGTGTTACAACCAATTAACC-3′) as well as Mut.for (5′-CTACGGAACCTGAGAACATGTGGAAATCACCTGTGGTAGATGACTGTTCAGGTACATTACCTTCAAGGATGACGACGATAAG-3′) and Mut.rev (5′-AAAGTACCTTTTCGCGATGAAAGGTAATGTACCTGAACAGTCATCTACCACAGGTGATTTCCAGCCAGTGTTACAACCAATTAACC-3′) for amplifying the Kan^r cassette in order to delete the UL11 ORF or to replace the ATG start codon by a stop codon, and UL11.for (5′-CTACGGAACCTGAGAACATGTGGAAATCACCTGTGGTAGAGCCACCATGGGAGTCAAAGTTCTGTT-3′) and UL11.rev (5′-TTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCGTTCATGTATGCGACTAGTC-3′) for amplifying the *Gaussia* luciferase ORF. For insertion of a Strep-FLAG tag, the Kan^r cassette amplified with primers TAP.for (5′-AAAGCTAGCGGAGAGGATTATAAAGATGATGATGATAAATGACAAGGATGACGACGATAAG-3′) and TAP.rev (5′-AAAGCTAGCTCCTTTCTCGAAGCCAGTGTTACAACCAATTAACC-3′) was first cloned into the NheI site of plasmid pDEST/C-SF-TAP (42), and the resulting plasmid was then used as the template for generating a PCR product with primers UL11SF.for (5′-TCTGGCAGCATGACAAGCACGGCATCGTGCTCATCCCCCAAACCGATCTGAAAGTGGTTCGATCCGCCAGC-3′) and UL11SF.rev (5′-GAGTTGGCGTTTCACAGTGATTTCATGCAATCATTTCCTACGCGACTTGCTCTAGATGCATGCTCGAGTCA-3′). Reconstitution of CMV mutants was done by transfection of RPE-1 cells with BAC DNA isolated from *Escherichia coli* cultures as described previously (43, 44). Preparation of viral stocks and titration by plaque assay were performed as reported previously (37).

An adenoviral vector expressing a UL11-Fc fusion protein was generated as follows. The sequence encoding the UL11 ectodomain of the Merlin strain was amplified with primers AL1.for (5′-TGGTGCTGAAGGGCGTGCAGTGCGAGGTGAAGCTGGTGCCACGCGGATCCATCAGCCTCCACGATGCCTG-3′) and AL1.rev (5′-CCCAGGAGTTCAGGTGCTGGGCACGGTGGGCATGTGTGAGTTTTGTCGACTGTAGCCAAGTGTTGGTGCT-3′) and cloned upstream of the sequences for the Fc region of human IgG1 present in a pCR3-based plasmid vector (45). The complete ORF was then amplified with primers 5′-AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTGGATCCGCCACCATGAACTTCGGGTTC-3′ and 5′-GGCGTGACACGTTTATTGAGTAGGATTACAGAGTATAACATAGAGTATAATATAGAGTATACAATAGTGACGTGGGATCCTCATTTACCCGGAGACAGGGAGAG-3′ and inserted into the genome of the adenovirus vector by homologous recombination as described previously (39).

The lentiviral vector Sm UL11 was constructed by cloning the UL11 ORF of Merlin optimized for human codon usage (Eurofins, Ebersberg, Germany) into plasmid pViF IRES GFP (46). HEK293T cells were cotransfected with the resulting vector and plasmids pMD.G VSV-G (47) and Sgpdelta2 (48) using a calcium phosphate-based protocol (49). The lentivirus particles were filtered through a 0.45-μm-pore-size filter, pelleted through a sucrose cushion (20% sucrose in PBS) by ultracentrifugation for 2 h at 75,000 × *g* and 4°C, and resuspended in DMEM. The titers of the virus preparations were determined by infection of HEK293T cells with serial dilutions and cytofluorometric measurement of green fluorescent protein (GFP) fluorescence. For production of stable cell lines, HEK293T cells were transduced with the lentiviruses at a multiplicity of infection (MOI) of 2 and passaged four times, followed by sorting of GFP-positive cells using a MoFlo XDP sorter (Beckman Coulter).

Abs. All animal experimentation was approved by the Ethics Committee for Biomedical Research of the University of Rijeka. The UL11-Fc protein used for immunization of BALB/c mice was purified by protein A affinity chromatography from the supernatant of RPE-1 cells transduced with the adenoviral vector as described previously (34). Stable hybridoma cell lines were generated by fusing SP2/0 myeloma cells with spleen cells of an immunized mouse, and after identification of a positive clone and recloning of UL11.1, Ab (isotype IgG1kappa) was purified by protein G affinity chromatography using an ÄKTAprime plus system (GE Healthcare). Abs specific for the following proteins and epitopes were used: IE1 (catalog number NEA-9221; PerkinElmer), pp65 (catalog number NB110-57244; Novus Biologicals), UL44 (kindly provided by Bodo

Plachter [University of Mainz, Mainz, Germany]), major capsid protein (a gift of Klaus Radsak [University of Marburg, Marburg, Germany]), HLA-I (clone W6/32; catalog number H1650; Sigma), CD45 (catalog number 21270453; ImmunoTools), CD8 (catalog number ABIN192069; Antibodies Online), FLAG M2 (catalog number F1804; Sigma), V5 (catalog number V8137, Sigma; catalog number R960-25, Invitrogen), p230 (catalog number 611280; Becton Dickinson), EEA1 (catalog number 610456; Becton Dickinson), calnexin (catalog number ab31290; Abcam), GFP (catalog number ab290; Abcam), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; catalog number 2118; Cell Signaling), and epidermal growth factor receptor (EGFR; catalog number 2232; Cell Signaling). Secondary Abs used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ab (catalog number P0448; Dako) and rabbit anti-mouse Ab (catalog number P0260; Dako) and phycoerythrin (PE)-conjugated goat anti-mouse Ab (catalog number 22549914; ImmunoTools) and Alexa Fluor 568-conjugated goat anti-mouse Ab (catalog number 11031; Molecular Probes). The mouse IgG1 isotype control Ab was obtained from ImmunoTools (catalog number 21275511).

Protein analysis. For biotin labeling of cell surface proteins, cells were washed three times with phosphate-buffered saline (PBS) and incubated in serum-free DMEM containing 0.5 mg/ml of EZ-link sulfo-NHS-LC-biotin (Thermo Scientific, Waltham, MA, USA) for 10 min at 37°C, followed by washing with ice-cold 100 mM glycine-PBS. Treatment of cells with MG-132 (5 μM; Calbiochem) and leupeptin (175 μM; Sigma) was done for 5 h. Cell lysates were prepared with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitor cocktail (Calbiochem). Proteins were precipitated by incubation of lysates cleared by centrifugation with V5 Abs coupled to agarose beads (Sigma) or streptactin-coated Sepharose (IBA, Göttingen, Germany) at 4°C for 2 h. After washing, proteins were eluted with either Roti-load 1 buffer (Roth, Karlsruhe, Germany) or, for treatment with endo-β-*N*-acetylglucosaminidase H (endo H), peptide-*N*-glycosidase F (PNGase F), neuraminidase, *O*-glycosidase, or deglycosylation enzyme mix (all from NEB), glycoprotein denaturing buffer, according to the manufacturers' instructions. Finally, proteins were separated by 10% SDS-PAGE and transferred to Hybond-ECL membranes (GE Healthcare) using a Trans-Blot semidry transfer cell (Bio-Rad). Membranes were incubated with an antigen pretreatment solution (catalog number 46640; Thermo Scientific), followed by blocking for 1 h at room temperature in Roti-Block solution (Roth), and were consecutively incubated with specific Abs overnight at 4°C and an appropriate HRP-conjugated secondary Ab for 1 h at 4°C. Signals were visualized by chemiluminescence using Super Signal West Femto maximum-sensitivity substrate (Thermo Scientific) or ECL Western blotting detection reagent (GE Healthcare) and an LAS 3000 imaging system (Fujifilm).

Flow cytometry. Cells were labeled with the soluble UL11-Fc or the Fc control protein (5 μg per 1×10^6 cells) or with the specific or isotype control Abs for 1 h at 4°C, followed by incubation with a secondary PE-conjugated Ab. Apoptotic and dead cells were excluded on the basis of staining with 7-aminoactinomycin D (Sigma). All Abs were diluted in PBS containing 6% goat serum (Sigma) and 2 mM EDTA. Measurements were performed on a Cytomics FC500 (Beckman Coulter) or LSR II (Becton Dickinson) flow cytometer and analyzed using FlowJo (TreeStar), CXP analysis, and Kaluza (Beckman Coulter) software. Fifty thousand cells were counted for each sample.

Immunofluorescence microscopy. HFFs cultured on glass coverslips and infected with CMV were fixed after 72 h with 3% paraformaldehyde for 20 min, followed by treatment with 50 mM NH_4Cl for 10 min. Then, the cells were permeabilized with 0.2% Triton X-100–PBS, and nonspecific binding sites and viral Fc receptors were blocked with 5% human serum-PBS for 10 min (Thermo Scientific). Cells were incubated with the primary Ab for 1 h and subsequently with a dye-conjugated secondary Ab for 1 h. Abs were diluted in 0.2% gelatin–PBS following the recommendations of the manufacturers. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). The coverslips were mounted on glass slides using Aqua-Poly/Mount medium (Polysciences Inc., Warrington, PA, USA). All steps were performed at room temperature. Images were taken with a Leica TCS SP2 confocal microscope.

T cell effector assay. CMV antigen-specific T cell clones (23) were restimulated every 2 weeks with autologous irradiated mLCLs expressing the pp65 or IE1 protein and mixed irradiated allogeneic peripheral blood mononuclear cells as described previously (23). The T cell clones were specific for the following epitopes: NLVPMVATV (NLV; derived from pp65, HLA-A*0201 restricted), VLEETSVML (VLE; derived from IE1, HLA-A*0201 restricted), and CRVLCCYVL (CRV; derived from IE1, HLA-C*0702 restricted). For the assay, MRC-5 fibroblasts were infected with CMV mutants at an MOI of 2 with enhancement by centrifugation at $700 \times g$ for 30 min and harvested by trypsinization at 78 h p.i. Untreated HEK293T cells, HEK293T cells transduced with the UL11 or control lentiviral vectors, and mock-infected MRC-5 cells were preincubated with the appropriate synthetic peptides (purchased from JPT, Berlin, Germany) at a final concentration of 10^{-6} M for 2 h at 37°C. Target cells were washed with RPMI 1640 medium and seeded into V-bottom 96-well plates (2×10^4 cells/well for MRC-5 cells and 3×10^4 cells/well for HEK293T cells), followed by cocultivation with CD8 T cells (1×10^4 cells/well) for 16 h. The supernatants were analyzed for the presence of IFN-γ by enzyme-linked immunosorbent assay (ELISA; Mabtech, Nacka, Sweden).

RESULTS

Expression of UL11 proteins during the lytic CMV infection cycle. In a previous study, we investigated the UL11 protein and its potential function independently of CMV infection (34). Since little was known about the properties of the protein(s) generated from the UL11 ORF in CMV-infected cells, we constructed mutants of the CMV strains AD169 and Merlin that encode UL11 versions C-terminally tagged with EGFP or with a Strep-FLAG or V5 epitope (Fig. 1). The recombinant genomes were generated in *Escherichia coli* by mutagenesis of CMV bacterial artificial chromosomes (BACs) employing a markerless homologous recombination-based technique (40, 41). The resulting mutants were termed HA11G, HM11SF, and HM11V5, respectively. Recent high-resolution analyses of the CMV transcriptome has shown that the level of expression of the ORFs encoding members of the RL11 protein family is rather low (3, 50). Therefore, we performed immunoprecipitation and took advantage of a high-sensitivity immunoblot assay to detect the protein(s) encoded by the UL11 ORF. Analysis of the proteins from cells infected with the Merlin strain mutants HM11SF and HM11V5 revealed two UL11-specific bands with molecular masses of about 50 and 70 kDa (Fig. 2A and B, lane 1). In cells infected with the AD169 strain-based HA11G mutant, we observed bands of ~70 and 100 kDa (Fig. 2D, top, lanes 4 to 6). The difference in the molecular masses of the UL11 proteins from the Merlin and AD169 strains reflects the different sizes of the V5 tag and of EGFP (1.4 and 27 kDa, respectively). In agreement with our previous results (34), these data suggest that UL11 is to various degrees posttranslationally modified by glycosylation when expressed in CMV-infected cells, since the calculated molecular mass of pUL11 is only 31 kDa.

Interestingly, we detected low levels of additional UL11 forms with apparent molecular masses of ~24 kDa (Fig. 2B, lane 1, band labeled with a closed circle) and 35 kDa (Fig. 2D, top, band labeled with a closed circle) in HM11V5- and HA11G-infected cells. Since these bands were rather faint, we reasoned that these UL11 forms may have a rapid turnover and asked whether proteasomal or lysosomal degradation was involved. After treatment with the

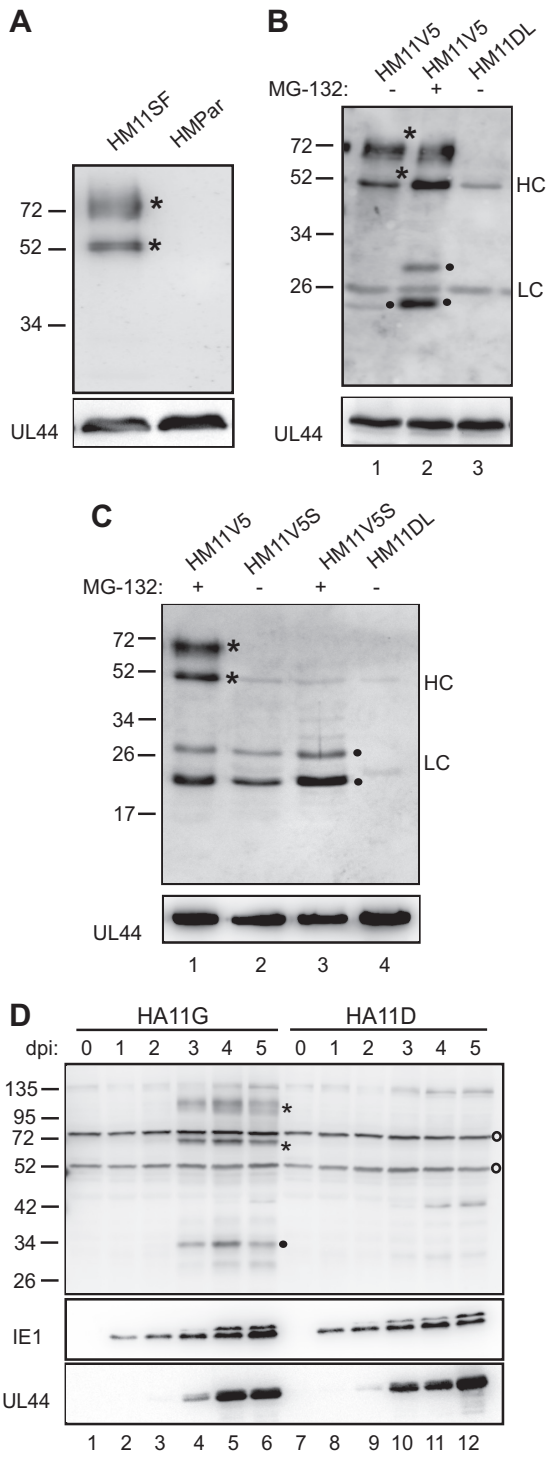

FIG 2 Expression of ORF UL11-encoded proteins in CMV-infected cells. (A) Lysates of HM11SF- or HMpar-infected cells were subjected to precipitation with streptactin-coated agarose beads, followed by immunoblotting with a FLAG tag-specific antibody. (B, C) Lysates of HM11V5-, HM11V5S-, or HM11DL-infected RPE-1 cells, which were kept with (+) or without (−) the proteasomal inhibitor MG-132, were subjected to immunoprecipitation and immunoblotting with a V5-specific antibody. High- and low-molecular-mass UL11 bands are indicated with asterisks and closed circles, respectively. Prior to immunoprecipitation, lysates were probed with an antibody specific for the CMV UL44 protein, serving as an infection and loading control. The reactivities of the heavy chain (HC) and light chain (LC) of the V5 Ab are indicated. (D) Human fibroblasts infected with the HA11G or HA11D mutant were harvested on the indicated day postinfection (dpi) and subjected to immunoblotting with a GFP-specific (top), an IE1-specific (middle), or a UL44-specific (bottom) Ab. UL11-specific bands are indicated as described in the legend to panel A, and the nonspecific reactivity of the GFP Ab is marked with open circles. The asterisks and closed circle are as described in the legend to panels B and C. The sizes of the marker bands (indicated to the left of each panel) are in kDa.

proteasome inhibitor MG-132 (but not after treatment with the lysosomal inhibitor leupeptin [data not shown]), the intensity of the ~24-kDa band was increased and another ~28-kDa band became visible (Fig. 2B, lane 2). One possibility was that the small bands reflect the products remaining within the cell upon putative cleavage of the UL11 full-length protein and shedding of its ectodomain. Notably, such a scenario applies to the adenovirus type 19a E3/49K protein, which shares some similarity with UL11 (51). An alternative explanation was that reinitiation of translation does occur within the UL11 ORF, as was recently observed for several CMV ORFs (3). To differentiate between these possibilities, we constructed the mutant HM11V5S, in which the ATG start codon of the UL11 ORF was mutated to the stop codon TGA (Fig. 1), and analyzed the expression of UL11 proteins in HM11V5S-infected cells. We found that both the ~50- and 70-kDa UL11 bands were no longer present, confirming that translation of the full-length UL11 ORF was abrogated (Fig. 2C, lanes 2 and 3); however, both the ~24- and ~28-kDa UL11 forms could still be observed. This finding strongly suggested that these UL11 forms result from protein synthesis that starts at initiation codons within the UL11 ORF (downstream of the first ATG codon) and argues against the hypothesis that they represent cleavage products originating from a full-length UL11 precursor protein.

Next, we sought to investigate the expression kinetics of the UL11 proteins during the CMV infection cycle. To this end, we analyzed the expression of the UL11-mGFP protein after infection of human fibroblasts with the AD169 HA11G mutant over 5 days postinfection (p.i.). The UL11 proteins were first detected on day 3 p.i. and remained present during the late phase of the infection cycle, with an apparent maximum of expression occurring at 4 days p.i. (Fig. 2D, top, lanes 4 to 6). This finding is in line with the result of a recent analysis of CMV mRNA expression, which suggested early-late kinetics for UL11 (3), and also with our measurement of UL11 promoter-driven luciferase activity using the Merlin strain-based mutant HM11DL (Fig. 1), in which the UL11 ORF is replaced by the ORF for *Gaussia* luciferase (data not shown). Taken together, the data show that different proteins originate from the UL11 ORF and become expressed during the late phase of the CMV infection cycle, although apparently at a low level, when considering that highly sensitive assays were needed for their detection.

The highly glycosylated but not the less glycosylated forms of UL11 are present at the cell surface. UL11 is predicted to be a type I transmembrane protein, and therefore, we assumed that the two high-molecular-mass UL11 bands whose molecular masses exceed the predicted molecular mass (34) resulted from differential glycosylation, as this is a common modification of proteins entering the secretory pathway (52). Following treatment of immunoprecipitated UL11 proteins with endo H, the ~70-kDa band shifted slightly, the ~50-kDa band virtually disappeared, and two new ~36- and 38-kDa bands became visible (Fig. 3A; compare

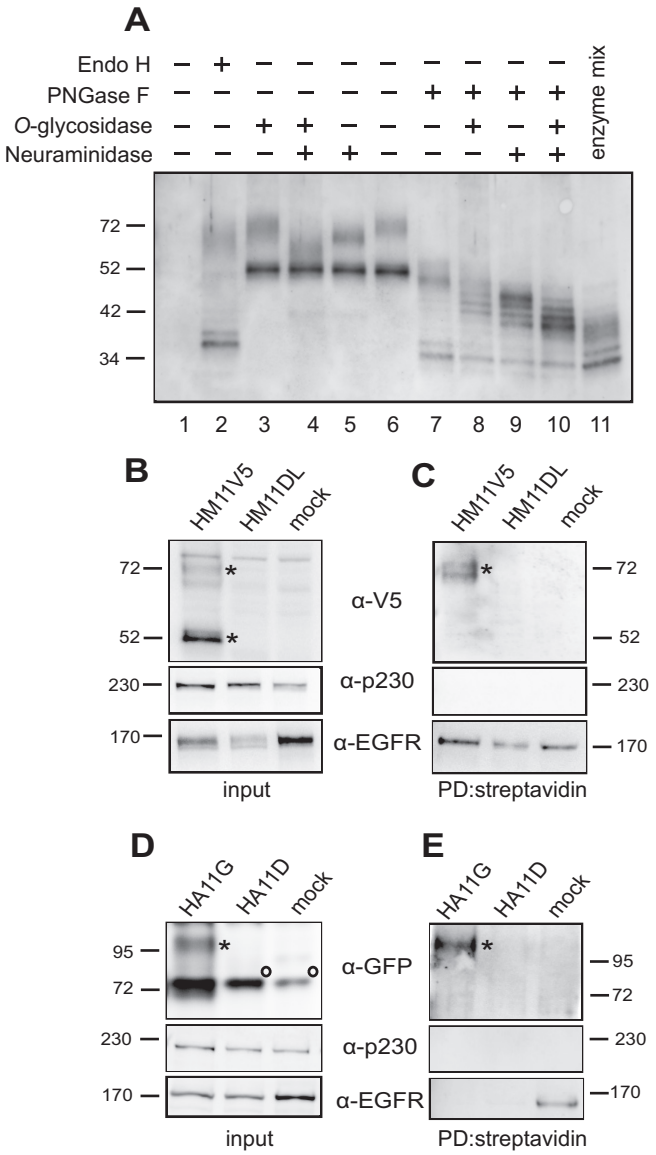

FIG 3 Posttranslational modification and surface expression of UL11 proteins. (A) Lysates of HM11DL-infected (lane 1) and HM11V5-infected (lanes 2 to 11) RPE-1 cells were subjected to immunoprecipitation with a V5-specific mouse Ab, and the eluted fractions were treated with endo H, PNGase F, *O*-glycosidase, neuraminidase, or a mix of deglycosylating enzymes or left untreated, as indicated, followed by immunoblotting with a V5-specific rabbit Ab. (B to E) Surface proteins of cells infected with the HM11V5, HM11DL, HA11G, or HA11D mutant were labeled with biotin and subjected to precipitation with streptavidin-coated beads, followed by immunoblotting with Abs specific for the V5 tag, GFP, p230, or EGFR. Input lanes show proteins of the whole-cell lysate before precipitation. The highly glycosylated UL11 form is marked with an asterisk, and the band resulting from the unspecific reactivity of the GFP Ab is indicated with a circle. The sizes of the marker bands (indicated to the left or right of each panel) are in kDa. PD, pulldown.

lanes 2 and 6). Upon PNGase F treatment, the ~70-kDa UL11 band was no longer detectable, the same ~36- and 38-kDa bands observed after endo H treatment appeared, and the new ~50-kDa bands probably originated from the ~70-kDa species (Fig. 3A, lane 7). These findings indicate that (i) the different UL11 forms are modified with *N*-linked glycans, (ii) the ~50-kDa UL11 forms are endo H sensitive, suggesting that they reside in the endoplasmic reticulum, and (iii) the ~70-kDa forms are partly endo H resistant, implying that they reached at least the medial Golgi compartment. The ~38-kDa band seen after endo H or PNGase F treatment as well as the ~50-kDa bands observed after PNGase F treatment suggested that many of the UL11 molecules carry additional modifications. Upon higher separation of the proteins, it became apparent that the ~50-kDa bands are indeed represented by two forms (data not shown), which also pointed to further modification and is consistent with the occurrence of the two low-molecular-mass bands seen for the endo H- or PNGase F-treated samples. The UL11 amino acid sequence contains a large threonine-rich stretch, which is a predicted substrate for *O*-glycosylation (26). Following treatment of the samples with *O*-glycosidase, there was no apparent change in the mobility of the different bands (Fig. 3A; compare lanes 3 and 6). This was not unexpected because, in agreement with the findings of other studies (53), the *O*-glycosidase applied can cleave only some of the unmodified core structures of *O*-linked glycans. Treatment with neuraminidase and subsequently with *O*-glycosidase led to a consecutive shift of the 70-kDa band (Fig. 3A, lanes 3 and 4), clearly indicating that UL11 is *O*-glycosylated and that these glycans and probably also the *N*-linked glycans were further modified with sialic acids. Combined treatment of samples with these glycosidases plus PNGase F as well as with a mix of different glycosidases (including β1-4-galactosidase and β-*N*-acetyl-glucosaminidase) revealed that the high-molecular-mass UL11 forms (~70-kDa band) are modified in a highly complex manner (Fig. 3A, lanes 7 to 11), pointing to the presence of *O*- as well as *N*-linked glycans.

The 24-kDa UL11 low-molecular-mass form was not affected by glycosidase treatment (data not shown). This is in agreement with the assumption that synthesis of this protein starts at an alternative initiation codon within the UL11 ORF, resulting in a protein that lacks a signal peptide and therefore cannot enter the secretory pathway.

Glycoproteins that acquire endo H resistance can potentially reach the plasma membrane (among other destinations) (52). In fact, previous reports (26, 34) suggested that UL11 is present at the cell surface. To examine which forms of the UL11 protein are expressed at the plasma membrane, we performed biotinylation of cell surface proteins of CMV-infected cells and analyzed the protein fraction precipitated with streptavidin-coated beads by immunoblotting. Only the ~70-kDa form of UL11 could be precipitated from lysates of cells infected with the HM11V5 mutant, although at the same time, the ~50-kDa version of the protein was clearly present in these cells (Fig. 3B and C). Consistent with this finding, only the highly glycosylated version of the UL11-mGFP fusion protein (~100 kDa) and not the ~70-kDa form was precipitated from lysates of cells infected with the HA11G mutant (Fig. 3D and E). The precipitated fractions also contained the EGFR, although in agreement with previous reports (54–56), the amount of the EGFR was reduced in CMV-infected cells, particularly in those cells infected with the strain AD169-based mutant. In contrast, the *trans*-Golgi compartment-resident membrane protein p230 (57) could not be precipitated, indicating that the plasma membrane remained intact during the labeling procedure and only surface proteins were exposed to biotin. To sum up, these data indicate that the highly glycosylated, endo H-resistant UL11 version (the ~70-kDa version for HM11V5 and the ~100-kDa version for HA11G) is present at the cell surface, whereas the less

glycosylated, endo H-sensitive UL11 forms reside inside the infected cells.

Subcellular localization of UL11. To further substantiate the findings about the surface expression of UL11, cells infected with the parental Merlin strain, a mutant expressing the V5-tagged UL11, or mutant HM11DL lacking the UL11 ORF were analyzed by flow cytometry using a monoclonal Ab that was generated by immunizing mice with a fusion protein comprising the UL11 ectodomain and the human IgG Fc part. This MAb turned out to be specific for the UL11 version of the Merlin strain only (data not shown). Immunostaining of CMV-infected cells can sometimes be troublesome due to binding of antibodies to the viral Fc receptors (29, 30), potentially leading to false-positive signals. Therefore, we employed several controls, including cells infected with the UL11-deficient mutant HM11DL as well as labeling with an isotype control antibody. The fluorescence-activated cell sorter (FACS) analysis indicated that cells infected with the parental Merlin strain or the HM11V5 mutant, but not HM11DL-infected cells, express distinct amounts of UL11 on the cell surface (Fig. 4A), confirming the result of the biotinylation experiment.

We next sought to determine the localization of UL11 proteins within the infected cells. To this end, we first applied confocal laser scanning microscopy to cells infected with the HA11G mutant (cf. Fig. 1), which expresses an UL11-mGFP fusion protein. The fluorescence signal of infected cells could hardly be distinguished from the autofluorescence of uninfected cells (data not shown), again indicating that the level of expression of UL11 in CMV-infected fibroblasts is rather low. To learn nevertheless about the subcellular distribution of UL11, the mutant HA11GM (Fig. 1), which expresses larger amounts of the UL11-mGFP protein due to insertion of the mouse CMV major immediate early promoter upstream of the UL11-mGFP ORF, was generated. By confocal microscopy, a predominantly cytoplasmic distribution of UL11-mGFP was seen in infected cells and a relatively faint staining was seen at the plasma membrane (Fig. 4B). The UL11-mGFP signal displayed substantial colocalization with calnexin (Pearson's correlation coefficient, 0.8), whereas there was no overlap with the p230 and EEA1 signals, which are markers for the *trans*-Golgi complex and for early endosomes, respectively. This result suggested that a portion of UL11 is present in the ER, which is consistent with the endo H sensitivity of the 50-kDa UL11 form (cf. Fig. 3A). In summary, we conclude that a portion of the UL11 proteins localizes to the ER and that a subfraction proceeds to the cell surface.

UL11 does not affect activation of CMV-specific CD8 T cells by infected cells. We have recently reported that a soluble UL11-Fc fusion protein can bind to CD45 on the surface of T cells, which resulted in functional impairment of these cells (34). It is well-known that CD45 has a central role in the transduction of signals received by the T cell receptor (35), and consequently, CD45-deficient cytotoxic T cells basically cannot respond to TCR stimuli (58). We therefore hypothesized that inhibition of CD45 function by UL11 binding would dampen the activity of CMV-specific CTLs. We chose CD8 CTL clones that were specific for different CMV epitopes, either the IE1 protein-derived peptides CRVLCCYVL- (CRV; HLA-C*0702 restricted) (23) and VLEET SVML (VLE; HLA-A*0201 restricted) (59) or the peptide NLVP MVATV (NLV; HLA-A*0201 restricted) derived from the pp65 protein (60). As expected, the CTL clones expressed CD45 at the cell surface, and they were able to bind the soluble UL11-Fc protein (Fig. 5A), which is in agreement with our previous results (34). To evaluate the effect of UL11, two pairs of CTL clones with CRV and NLV specificity were coincubated with human fibroblasts that had been infected for 78 h with the mutants HA11G and HA11D (Fig. 1), which encode and lack the UL11 ORF, respectively. We want to point out that both mutants also lacked ORFs US2 to US6 encoding immune evasins and were therefore expected to modulate HLA expression to a lesser extent than wild-type CMV (61). This characteristic of the mutants appeared to be a prerequisite for measuring the influence of UL11 on T cell activity, because many CMV-specific T cells are hardly activated when HLA levels are diminished (62, 63). Immunoblot analysis of cells infected with the two mutants confirmed the similar expression of the IE1 and pp65 proteins (Fig. 5B), from which the peptides recognized by the CTL clones are derived, and the levels of HLA class I molecules on infected cells were comparable (Fig. 5C). Moreover, labeling with an antibody to the major capsid protein confirmed a similar infection rate (Fig. 5D) and progression to the late infection phase in the majority of infected cells. After coincubating the CTLs and infected cells for 24 h, IFN-γ production was measured as a readout for T cell activation (Fig. 5E). Although each CTL clone has its own characteristics and responded to stimulation with CMV-infected or peptide-pretreated cells with the production of different amounts of IFN-γ, the IFN-γ levels did not differ whether UL11 was expressed in the infected cells or not (Fig. 5E). This result shows that under the conditions applied, the UL11 proteins expressed by CMV-infected fibroblasts did not affect the activation of CMV-specific CD8 T cells.

To test whether UL11 is able to influence CD8 T cells independently of other CMV gene products or soluble factors released from CMV-infected cells (64–66), we performed additional experiments in which we used the UL11-expressing lentiviral vector Sm UL11 and the corresponding control vector Sm CTRL (Fig. 1) to transduce HEK293T target cells. Immunoblotting confirmed the UL11 expression in Sm UL11-transduced cells (Fig. 6A), and flow cytometric analysis revealed higher levels of UL11 on their surface (Fig. 6B) than on CMV-infected fibroblasts (cf. Fig. 4A). Further analysis indicated that the amount of UL11 expressed in Sm UL11-transduced cells is approximately five times higher than the amount expressed in CMV-infected cells (data not shown). As shown in Fig. 6C, the levels of IFN-γ produced by the individual CRV- and VLE-specific T cell clones did not differ, regardless of UL11 expression in the target cells. Taken together, these data illustrate that neither CMV-expressed UL11 nor the protein expressed outside the context of CMV infection affected IFN-γ production by CD8 T cells.

DISCUSSION

In this work we report on the characterization of UL11-encoded proteins in CMV-infected cells and the effect of CMV-expressed UL11 on CD8 T lymphocytes that are specific for CMV antigens. Investigation into UL11 expression using two different CMV strains consistently revealed that the UL11 ORF gives rise to several proteins of different sizes, depending both on posttranslational modification and on the position of the translation start site. We provide evidence that only the highly glycosylated UL11 forms are present at the cell surface, whereas the less glycosylated UL11 forms are located in the ER. Analysis of IFN-γ secretion as a measure of the activity of the CD8 T cells indicated that neither the amount of UL11 produced in CMV-infected cells nor the higher

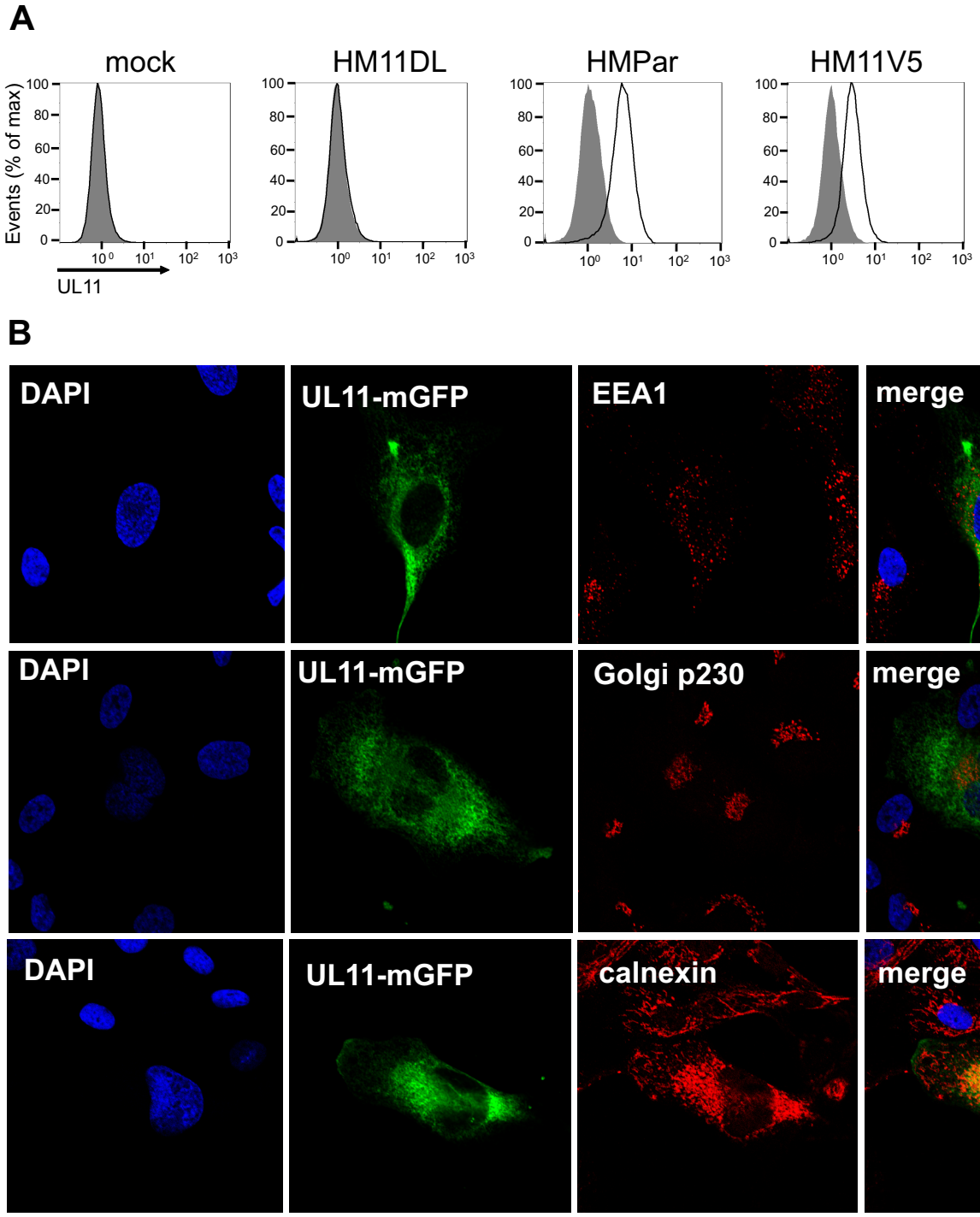

FIG 4 Localization of the UL11 proteins within CMV-infected cells. (A) RPE-1 cells either mock infected or infected with the indicated viruses were labeled with a UL11-specific MAb (black line) or the isotype control Ab (gray shading) and analyzed by flow cytometry. max, maximum. (B) Human fibroblasts infected with the HA11GM mutant were stained at 72 h p.i. with DAPI, and Abs specific for calnexin, Golgi compartment-resident protein p230, and EEA1 and fluorescence were visualized by confocal microscopy. Bars, 25 μm.

level obtained in cells transduced with a lentiviral vector affected the function of specific CTLs.

Many immunomodulatory CMV proteins seem to be expressed at a low abundance in infected fibroblasts, and thus, their detection can be a challenge. One prominent example is the viral MHC-I homolog UL18, which was therefore mainly studied upon expression by viral vectors or in transfected cells and whose function is still a matter of debate (67). An early report on global profiling of CMV gene expression using DNA microarrays failed to detect UL11 (68), and more recent studies of the CMV tran-

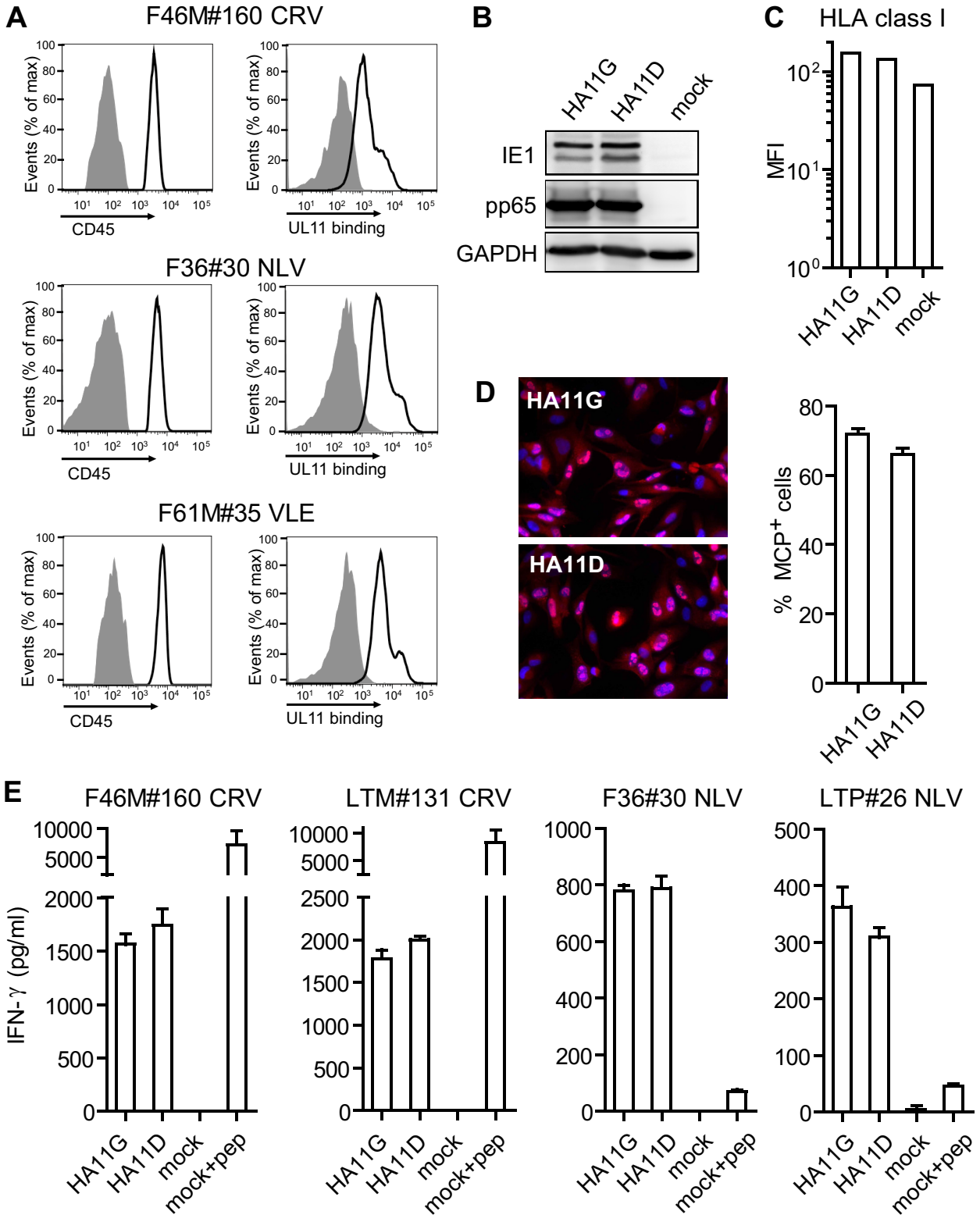

FIG 5 Analysis of UL11 expressed in CMV-infected fibroblasts on activation of CMV-specific T cell clones. (A) CD45 expression (left) and binding of the soluble UL11-Fc protein to the indicated CD8 T cell clones (right) were determined by flow cytometry upon labeling with a CD45 Ab (black line) or an isotype control Ab (gray shading) and upon incubation with the UL11-Fc protein (black line) or the Fc control protein (gray shading). (B) Immunoblot analysis for IE1 and pp65 proteins of MRC-5 cells infected with the indicated viruses at an MOI of 2 for 78 h. GAPDH served as a loading control. (C) MRC-5 cells either mock infected or infected with the indicated viruses, as described in the legend to panel B, were examined for surface expression of HLA class I molecules by flow cytometry, and mean fluorescence intensity (MFI) values are depicted. (D) MRC-5 cells infected as described in the legend to panel B were labeled with an antibody against the major capsid protein (MCP), and nuclei were stained with DAPI. Five random images were taken per setting, and the percentages of MCP-positive cells were determined. (E) MRC-5 cells infected with the HA11G or HA11D mutant were cocultivated with CD8 T cell clones specific for the CRVLCCVML (IE1-derived) or NLVPMVATV (pp65-derived) peptide. T cell reactivity was analyzed by measuring overnight IFN-γ secretion by ELISA. Mock-infected cells and cells loaded with peptides (pep) were used as negative and positive controls, respectively. Data show the means ± SDs for triplicate samples.

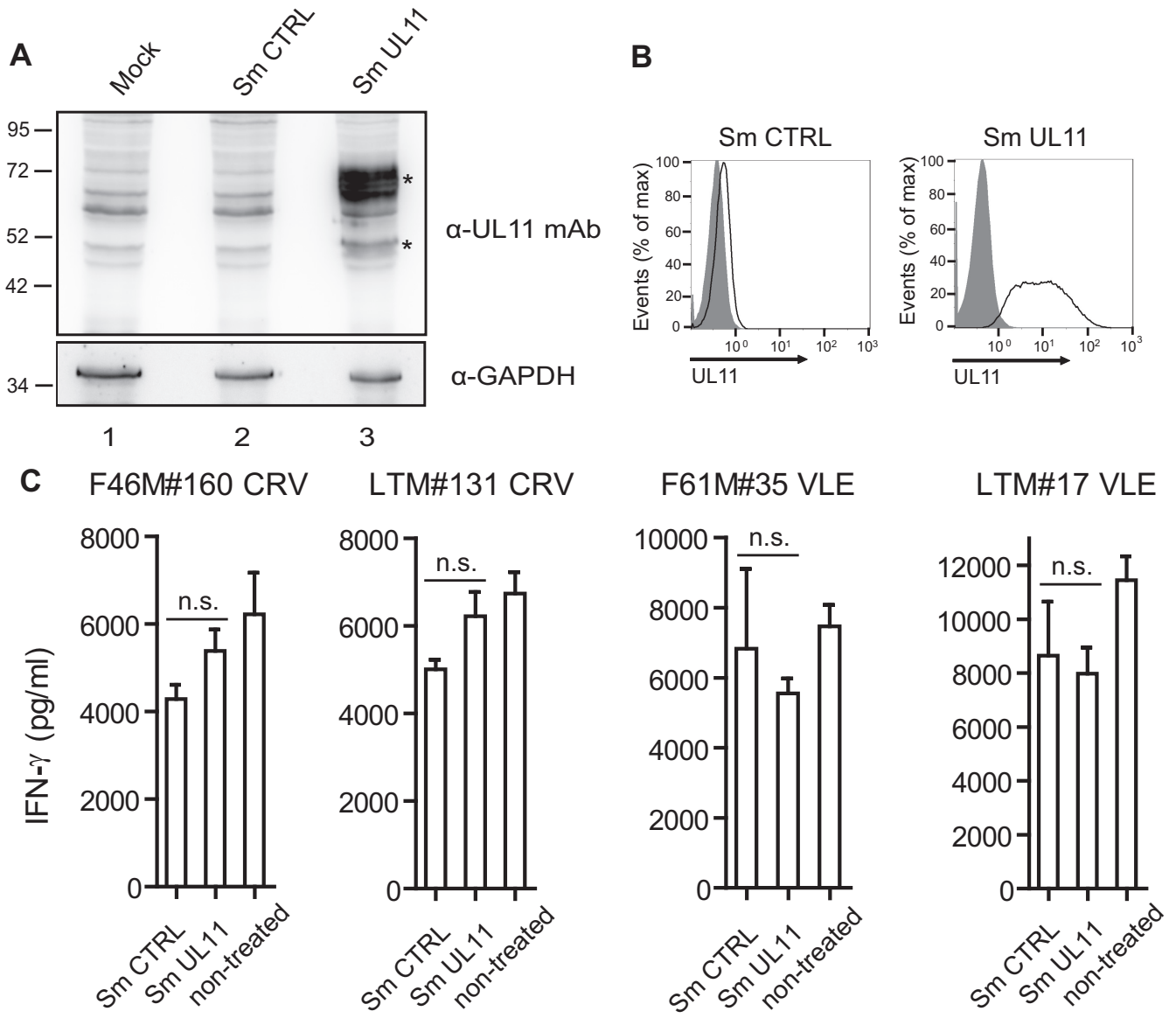

FIG 6 UL11 expressed in HEK293T cells does not affect activation of CMV-specific CD8 T cells. (A) Lysates of untreated HEK293T cells (lane 1) or cells transduced with lentiviral vectors Sm CTRL (lane 2) or Sm UL11 (lane 3) were subjected to immunoblotting with the UL11-specific monoclonal antibody (mAb). The highly and less glycosylated forms of UL11 are indicated with asterisks. The GAPDH signal served as a loading control. The sizes of the marker bands (indicated to the left) are in kDa. (B) Flow cytometric analysis of transduced HEK293T cells stained with the isotype control (gray shading) or the UL11-specific Abs (black line). (C) Untreated HEK293T cells or cells transduced with the indicated lentiviral vectors were preincubated with the IE1 protein-derived peptide CRVLCCVML (CRV) or VLEETSVML (VLE) and cocultivated with the indicated specific CD8 T cell clones. T cell reactivity was analyzed by measuring the overnight IFN-γ secretion by ELISA. Bars show the means ± SDs for triplicate samples. For clones F46M#160 CRV and F61M#35 VLE, one result of three independent experiments is shown, and for clones LTM#131 CRV and LTM#17 VLE, one result of two independent experiments is shown. Statistical analysis (two-tailed Mann-Whitney test) did not indicate significant differences. n.s., not significant.

scriptome revealed that the level of UL11 mRNAs synthesized is rather low (3, 50). By using a highly sensitive detection system and suitable CMV mutants, we could overcome this constraint, and for two different CMV strains, we found that several proteins are produced from the UL11 ORF in infected cells. The ~50- and 70-kDa UL11 proteins observed in cells infected with the Merlin UL11 V5 mutant correspond to the glycosylated UL11 versions that we detected before upon adenoviral expression of the UL11 ORF (34). Although there is evidence that CMV can alter the glycosylation machinery of infected cells (32, 69), we did not observe an apparent difference in the molecular masses of these UL11 species compared to those found in our previous study using an adenoviral vector. Newly detected were the low-molecular-mass bands of ~24 and 28 kDa, suggesting that these UL11 products are generated only in the context of CMV infection.

FACS analysis and biotinylation experiments indicated that a fraction of the UL11 proteins, namely, the ~70-kDa UL11 version, is present at the plasma membrane. This is supported by the fact that the ~70-kDa forms are endo H resistant, whereas the endo H sensitivity of the ~50-kDa UL11 species is in agreement

with its localization within the ER. It was important to examine the cell surface expression of UL11 in CMV-infected cells, because under the conditions previously applied (i.e., adenoviral transduction), the protein may have been overexpressed, resulting in the increased escape of UL11 to the plasma membrane. We indeed observed that upon overexpression the ~50-kDa version reaches the cell surface too (data not shown), suggesting that the capacity of the glycan-processing enzymes in the Golgi apparatus is exceeded. Surface expression of UL11 was also suggested by a previous report (26). With respect to that study, we raised the concern that the signal observed may have been confounded by the binding of the rabbit antibodies that were used to viral Fc receptors (29, 30). This cannot be determined in retrospect, but by including a UL11 deletion mutant and a specific mouse monoclonal antibody, we have now unambiguously demonstrated that the highly glycosylated UL11 forms reach the plasma membrane. Moreover, in another recent study that aimed at global investigation of the changes in host and viral proteins in CMV-infected cells (70), UL11 was also found at the cell surface and the UL11 expression kinetics was very similar to the one that we describe here.

Glycosylation can facilitate the folding of proteins and protect them from degradation by proteases (71). The latter point could be particularly important for proteins like UL11 that are exposed to the outside of cells and that can potentially be accessed by proteases present in blood. The glycans could also shield UL11 from binding by antibodies, which would disrupt the binding of UL11 to interaction partners and also render infected cells vulnerable to antibody-dependent cellular cytotoxicity. A fraction of the UL11 molecules, the ~50-kDa forms, was present in the ER, and at least two bands with slightly different molecular masses were observed. This could reflect the consecutive addition of *N*-linked glycans, suggesting the slow maturation of the protein and explaining the retention within the ER. For four out of seven putative *N*-glycosylation sites of the Merlin strain UL11 protein, a high propensity for glycosylation was predicted by the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc). Upon further maturation, most of the glycans acquired endo H resistance; however, some remained endo H sensitive, which could be due to steric hindrance and the lower accessibility for processing enzymes in the Golgi apparatus. We also found evidence for addition of sialic acids and for *O*-glycosylation of UL11. The latter was not unexpected, since a large threonine-rich stretch within UL11 was expected to represent a substrate for *O*-glycosylation. Overall, the UL11 proteins appear to be modified by glycosylation in a complex and probably heterogeneous manner.

At first sight, the detection of low-molecular-mass forms of UL11 suggested that the full-length UL11 version is proteolytically cleaved and that the resulting luminal domain is shed from infected cells, as has been reported for the adenovirus type 19a E3/49K protein (53), which shares sequence motifs with UL11 (24) and the ability to interact with CD45 (51). Cleavage and shedding have also been observed for UL7, another member of the CMV RL11 family (31). Our results show, however, that the small UL11 products originate from usage of alternative initiation codons for protein synthesis within the UL11 ORF, and we did not find evidence for cleavage of the full-length UL11 proteins. Usage of alternative translation start sites increases the repertoire of CMV proteins and probably the possibilities that the virus will interfere with cellular pathways. This viral coding strategy seems to be much more common (3) than was previously anticipated. The question for a potential function of the small cleavage products of the adenovirus type 19a E3/49K protein was posed recently (51). For the small UL11 proteins, this point has to await their more careful characterization, for instance, the precise definition of the initiation codons. The biogenesis of these UL11 proteins differs from that of the adenovirus type 19a E3/49K cleavage products, in that they are probably synthesized in the cytosol first and only subsequently associate with membranes, if at all. Localization within the cytosol is in agreement with the observed absence of glycosylation and degradation by the proteasome.

We did not find an effect of the UL11 proteins expressed in CMV-infected fibroblasts on the recognition and subsequent IFN-γ production by CTLs specific for peptides of the pp65 or IE1 antigens. Thus, the data do not support our hypothesis that surface-expressed UL11 dampens the activity of CMV-specific CTLs. This hypothesis was based on the observation that UL11 binds to CD45 and that soluble UL11 diminished T cell proliferation upon pan-specific stimulation with a TCR antibody (34). This result raises several questions: whether *in vitro* T cell assays reflect the situation *in vivo* is hard to address, since the interplay of CMV immune evasion mechanisms and possible countermeasures of the host, such as inflammation (21, 72), is difficult to mimic. Our experiments included pp65-specific and HLA-C-restricted IE1-specific CTL clones, which are relatively insensitive to viral interference with MHC-I antigen presentation (21–23), because for the evaluation of a potential effect of UL11, it was necessary to use CTL clones that respond with quantifiable activation. For the same reason, it was rather favorable that the CMV mutants used lacked the genes for the immune evasins US2 and US6. In fact, an HLA-A-restricted CTL clone specific for the IE1 peptide VLEET SVML was hardly activated upon incubation with CMV-infected cells (data not shown), because the remaining immune evasin expressed by the CMV mutants, US11, prevented the presentation of this peptide (22, 23). CTL clones with such characteristics could therefore not be used to address the UL11 effect, although such CTLs may be protective *in vivo* (73, 74). We conclude that UL11 expressed in CMV-infected fibroblasts cannot prevent the activation of specific CTLs.

One may argue that the absence of any effect of UL11 on CTLs is not unexpected, given the low level of surface expression. It will indeed be the subject of further work to examine the expression of UL11 in other cell types and under different infection conditions. Preliminary attempts to simulate inflammation by treating infected fibroblasts with various proinflammatory cytokines did not result in upregulation of UL11 expression (data not shown). Also, lentiviral transduction of target cells, which led to higher surface expression of UL11, did not impair the activity of the T cells. Arguably, CD45 is one of the most abundant proteins in T cells, and one may therefore expect that a similarly high level of UL11 is required to influence the function of CD45. On the other hand, it is known that CD45 must be excluded from the immunological synapse to allow transduction of signals received by the TCR (75, 76). Already small amounts of UL11 may therefore keep CD45 within the immunological synapse and thereby prevent T cell activation. Actually, the sheer size of the CD45 glycoprotein could interfere with the formation of contact between an MHC-I molecule and the TCR (77).

Notably, for the related adenovirus type 19a E3/49K protein, modulation of lymphocyte functions was observed only for the soluble protein and not for the membrane-resident version (51).

Similarly, our previous data reporting the interference of UL11 with T cell proliferation were obtained with soluble UL11. However, the facts that cross-linking of UL11-Fc was necessary to achieve inhibition and that membrane-resident UL11 bound to CD45 (34) suggested that UL11 present on the surface of CMV-infected cells would be able to influence specific T cells. Perhaps soluble UL11 (and E3/49K) can bind to CD45 in a different manner than membrane-bound UL11, and inhibition of CD45 may occur only under such conditions. However, as mentioned above, we did not find evidence for the release of soluble UL11 from infected fibroblasts, although this does not exclude the possibility that cleavage and shedding of UL11 may occur in other cell types.

One has to point out that CMV is able to infect a series of different cell types, including macrophages and dendritic cells, which are central in induction of the immune response. Also, CD45 is expressed not only in T cells but also in other lymphocytes, such as NK cells, and in myeloid cells. In this study, we did not see an effect of UL11 on the effector function of already primed memory T cells directed against CMV. This does not rule out the possibility of a role of UL11 upon activation of naive T cells by CMV-infected dendritic cells or perhaps an effect of UL11 on NK cells. Further work addressing the function of UL11 will therefore focus on other immune effector cells and not the least on those target cells of CMV that express CD45.

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I.G., E.E., S.A., A.M., and M.M. conceived and designed the experiments, I.G., E.E., S.A., L.S., R.S., and T.L.R. performed the experiments, I.G., E.E., S.A., M.D., T.L.R., S.J., A.M., and M.M analyzed the data, R.S., M.D., and U.K. contributed reagents, and I.G. and M.M. wrote the manuscript.

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