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MHC Class I–Subversive Gene Functions of Cytomegalovirus and their Regulation by Interferons–an Intricate Balance

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Abstract. Multiple glycoproteins of human cytomegalovirus (HCMV) encoded by the genes *US2, US3, US6* and *US11* interrupt the MHC class I pathway of antigen presentation at distinct checkpoints to avoid recognition of infected cells by cytotoxic CD8+ T lymphocytes. The action of cytokines like interferon (IFN)-γ, IFN-α/β and tumour necrosis factor α (TNF-α) compensate for the viral inhibition and restore antigen presentation in HCMV-infected cells. This finding was explained by their effects on cellular rather than viral genes and reflected by an increase in the production, assembly and maturation of MHC class I molecules resulting in an escape of MHC I from viral control. Here we reproduce the IFN-γ-mediated effect when MHC I-subversive gene functions of HCMV are tested in isolation, but the efficacy of IFN-γ to restore MHC I surface expression in *US2*-, *US6*- and *US11*-transfectants differs significantly. In addition, in HCMV-infected cells IFN-γ strongly affects the synthesis of the *US6*-encoded glycoprotein. Despite the capability of HCMV to block the interferon signaling pathway the IFN-γ driven enhancement of MHC class I and class II expression remains effective provided that cells are exposed to IFN-γ before infection. Our findings illustrate a complex interplay between host immune factors and viral immune evasion functions.

Key words: cytomegalovirus, antigen presentation, MHC, interferon, jak/stat pathway

Introduction

Cytomegaloviruses (CMV) constitute prototypes of the β-subgroup of the family of *Herpesviridae*. CMVs are characterized by their strict species specificity, a protracted replication cycle and their multiplication in a limited number of cell types. Both human (HCMV) and mouse (MCMV) CMVs share large DNA genomes of about 240 kbp encompassing more than 200 separate open reading frames (ORFs) which represent the highest herpesviral coding capacity. A core of genes located in the long sement between approximately 50 to 170 kb of the HCMV genome are closely related between cytomegaloviruses and also conserved in other herpesvirus families (1,2). A hallmark of CMV is the presence of extended virus-specific gene families that are tandemly arranged and cluster as homologous blocks with several members in the flanking regions of the CMV genomes (1,2).

CMVs are subjected to a tight immune control by cytotoxic histocompatibility complex (MHC) class I restricted CD8+ T lymphocytes (CTL) (3,4). CTL monitor the replication of intracellular pathogens such as viruses via a display mechanism mediated by MHC class I molecules (see Fig. 1) which are expressed in virtually all tissues. Peptides derived from viral proteins are presented at the cell surface by MHC class I molecules to CD8+ T cells which either destroy the virus-infected cell by cytotoxicity, secrete cytokines (e.g. IFN-γ), or both. MHC class I molecules are type I transmembrane glycoproteins of about 45 kDa. Noncovalent binding of a soluble 12 kDa light chain, β_2-microglobulin (β_2m) and peptide to the MHC class I heavy chain in the endoplasmic reticulum (ER) results in a stable MHC I complex able to leave the ER for transport to the cell

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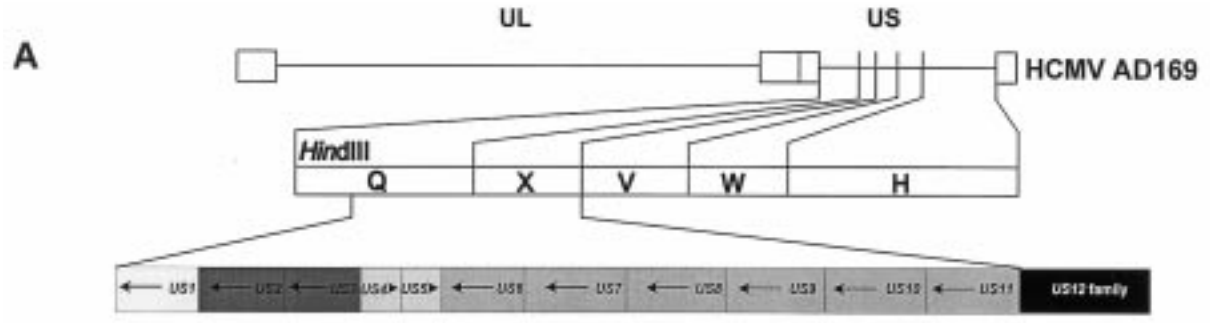

Fig. 1A. Structure of the human cytomegalovirus (HCMV) strain AD169 genome. The unique long (UL) and unique short (US) segments are flanked by reverted repeat sequences as indicated by the terminal boxes. The *US2* and *US6* gene families harbouring the MHC I regulating genes *US2* and *US3*, and *US6* and *US11*, respectively, are highlighted in the context of their neighboring genes *US1, US4, US5* and *US12* which belong to further HCMV gene families. The arrows represent the direction of transcription.

surface along the constitutive secretory pathway of the cell. In the MHC class I pathway of antigen presentation, peptides are generated by proteolytic cleavage in the cytosol. To encounter the peptide binding site of MHC class I molecules, peptides have to be imported into the ER by a specific peptide transporter, transporter associated with antigen processing, TAP, consisting of two subunits, TAP1 and TAP2 which are members of the ATP-binding cassette (ABC) transporter family (reviewed in 5). The transport of peptides by TAP requires two independent but coupled events. In the first step, the peptide is bound to the cytosolic face of TAP, before it is subsequently translocated in an ATP-dependent manner. The formation of trimeric MHC I complexes in the ER is assisted by sequential interactions with molecular chaperones which include calnexin, calreticulin and tapasin (6–8).

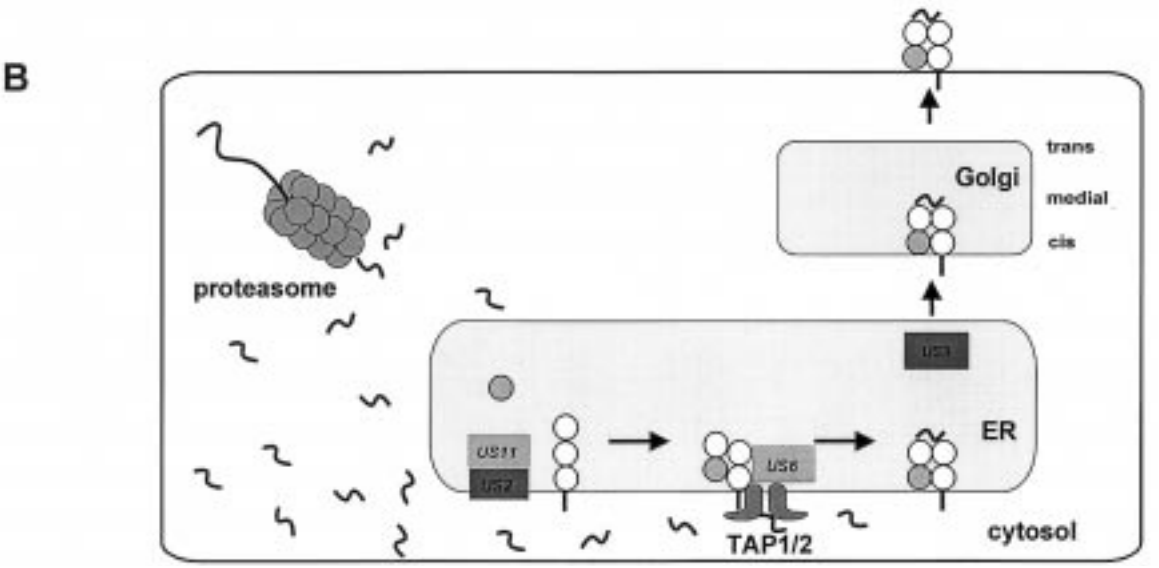

Fig. 1B. The major histocompatibility complex (MHC) class I pathway of antigen processing and presentation. *De novo* synthesized viral proteins or exogenous proteins derived from infecting virions are cleaved by the proteasome to produce peptides. Peptides are translocated across the membrane of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP1/2). In the ER lumen, peptide bind to MHC class I-β_2-microglobulin heterodimers (unfilled white and filled grey circles) to form ternary complexes. MHC class I complexes exit the ER, pass through the Golgi compartments and reach the cell surface to present the peptide to CD8 + cytotoxic T lymphoctes.

MHC Class I-Subversive Glycoproteins of HCMV are Members of the *US2* and *US6* Gene Families

The selective pressure of CD8 + T cell immunity, the extended time required for replication of the viral genome and the high number of potentially antigenic proteins expressed (> 200) may have led to the evolution of CMV genes that affect MHC class I molecules itself or components of the MHC class I pathway. After infection of fibroblasts both MCMV and HCMV abolish antigen presentation to MHC class I-restricted T cells *in vitro* (9–12). This effect is associated with a downregulation of MHC class I molecules without reducing the mRNA levels for MHC class I gene products (13–16). Although the loss of MHC class I surface expression is observed for both CMVs, the mode of interference with the MHC class I pathway differs thoroughly between HCMV and MCMV and the responsible genes have no homologs between both viruses (17–19).

In HCMV-infected cells MHC class I heavy chains are unstable and steady state levels of assembled MHC class I complexes are strongly decreased (14,15). The analysis of HCMV deletion mutants which had lost the MHC class I reduced phenotype in infected fibroblasts (20,21) guided the identification of four MHC I-subversive open reading frames (orf) within the short segment of the HCMV genome, i.e. *US2, US3, US6* and *US11* (20,22–29; see Fig. 1 and Table 1). The genes are members of two HCMV-specific gene families, *US2* and *US6* (1) coding for small type I transmembrane glycoproteins which are dispensable for virus replication *in vitro* and therefore referred as accessory glycoproteins (20,30). The common phenotype of transfectants expressing *US2, US3, US6* and *US11*, respectively, is the loss of MHC class I molecules on the cell surface, but the molecular mechanisms employed differ. The *US2* family is a clustered pair of two homologous genes, *US2* and *US3*, coding for short-lived glycoproteins of 24 kDa and 32/33 kDa, respectively. Comparing the *US2* and *US3* protein sequence reveals a homology of 23% and a similarity of 56% (25; Fig. 2a). Moreover, their sequences are significantly related to the members of the *US6* gene familiy as shown by the dendrogram depicted in Fig. 3. Therefore, it is tempting to speculate that the members of the *US2* and *US6* gene families have evolved from a common precursor but diverged over time to fulfill different tasks.

Studies of cells stably expressing *US2* provided insights into a novel intracellular pathway used by this HCMV protein to target MHC class I molecules to the cytosol for proteasomal destruction. Cell fractionation experiments demonstrated both a deglycosylated MHC class I heavy chain intermediate and a deglycosylated 20 kDa product of the US2 protein present in the cytosol (23). The physical removal of MHC class I molecules from the ER is ATP-dependent and sensitive to changes in the redox potential of the ER (31). Since both the MHC class I and the US2 intermediate were present in Sec 61p-immune complexes it was suggested that the retrograde transport of MHC I molecules involves the Sec 61p complex, the translocon (23). This is supported by genetic evidence form yeast linking the translocon to a general retrograde transport pathway for misfolded and abnormal proteins in the ER (32). While transcription of the *US2* gene in HCMV-infected cells starts from 3 to 6 h postinfection and is shut off in the late phase of infection (24), the *US3* gene is regulated by multiple copies of an 18-bp repeat present upstream of its promoter (33) resulting in transcription at immediate early times during 1–4 h postinfection which is shut off at early times after infection (25). The US3 protein is immunoprecipi-

Table 1. MHC I-subversive genes of HCMV

Orf*	Gene Family	Size (aa*)	Phase of Expression	Mechanism
US2	*US2*	199	early	SEC61-dependent dislocation of MHC class I heavy chains from the ER into the cytosol for proteolytic destruction
US3	*US2*	186	immediate-early	retention of MHC I complexes in the ER
US6	*US6*	183	early/late	inhibition of peptide translocation by TAP1/2
US11	*US6*	211	early	dislocation of MHC class I heavy chains from the ER into the cytosol for proteolytic destruction

*open reading frame
*amino acid

Fig. 2. Amino acid sequence alignment of the *US2* family members *US2* and *US3* (**A**) and the *US6* family members *US6* through *US11* (**B**). Numbers indicate the amino acid positions within the published amino acid sequences (1). Amino acids identical in more than 50% of the genes are highlighted. Points indicate artifical gaps introduced to achieve maximal amino acid matching.

tated with β_2m-associated peptide-loaded MHC class I molecules. In contrast to *US2*-expressing cells, *US3*-transfectants do not show a rapid degradation of MHC class I molecules but accumulate stable MHC class I complexes in the ER and prevent their transport to the cell surface (25,26). Since the expression of the *US3* gene is activated by cellular factors and independent of viral protein synthesis, one may speculate that the US3 glycoprotein is also able to limit presentation of viral peptides in cells nonproductively infected with HCMV.

Two of the six members of the *US6* gene family also interrupt the MHC class I pathway of antigen presentation, i.e. *US6* and *US11*. Another family member, the *US9*-encoded glycoprotein was shown to be implicated in the cell-to-cell spread of HCMV in polarized epithelial cells (34), indicating that the accessory glycoproteins of the *US6* family have diverse biological functions. While the overall sequence homology between the US6 polypeptides is in the range of about 25% and includes also *US2* and *US3* (Fig. 2 and 3), the *US6* family members are characterized by two areas of sequence homology (1). The core motif of the first region is defined as C(VY)X(DQKR)(7–10)WXXXGXF where the bracketed residues are alternatives and X stands for

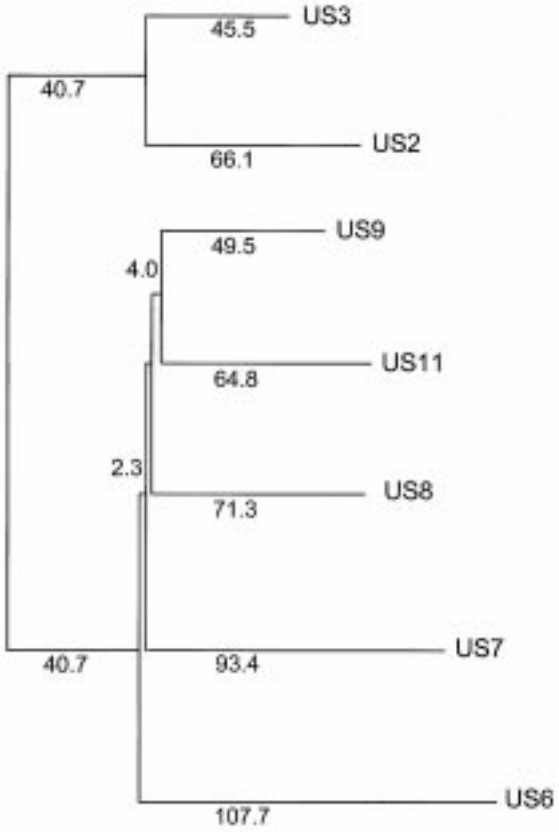

Fig. 3. Dendrogram showing the amino acid relatedness of the *US2* and *US6* gene families. The dendrogram was based on a multiple alignment of the complete amino acid sequences of the *US2* and *US6* gene family members using the CLUSTAL software (PC/GENE release 6.85, Intelligenetics Inc., CA). Horizontal distances are proportional to the relative sequence deviations between individual amino acid sequences and indicated as arbitiary values.

any residue. The motif of the second region is defined by cystein and proline residues: PCXXC(4–6)CXPWXP (1).

Phenotypically, the *US11*-encoded 33 kDa glycoprotein acts upon MHC class I molecules like US2 and dislocates nascent MHC class I molecules from the ER back to the cytosol where they are rapidly degraded (22). Remarkably, the expression kinetics of *US11* parallels that of *US2* (24,35), but their preference for MHC class I alleles differs as deduced from the fact that the US2 and US11 proteins exhibit a different ability to attack allelic forms of murine MHC class I heavy chains (36). On the other hand, both US2 and US11 leave out HLA-C and HLA-G histocompatibility antigens which escape from degradation (37). This might be due to the fact that natural killer (NK) cells are blocked by HLA-C and HLA-G alleles.

Unlike the US2, US3 and US11 proteins the *US6*-encoded 21 kDa glycoprotein (gpUS6) does not directly interact with MHC class I but shuts off the TAP1/2-mediated peptide transport into the ER (27–29). gpUS6 does not affect peptide binding to TAP1/2 but prevents the translocation step of the peptide ligand across the ER membrane. The US6 protein is found associated with the recently identified assembly complex consisting of TAP1, TAP2, MHC class I-β_2m, calreticulin and tapasin, and it binds also to calnexin (27). The inhibiton of peptide transport is accomplished despite the significantly augmented expression of TAP1 and TAP2 molecules in HCMV-infected fibroblasts (21). The expression kinetics of the US6 protein during permissive infection starts in the early phase and correlates with the inhibition of peptide transport. Detailed analysis of US6 transcripts revealed that transcription is driven from different initiation sites at early and late times postinfection, respectively (35). US6 synthesis reaches peak levels not before the late phase of infection when *US3, US2* and *US11* gene expression becomes almost silent (27).

Restoration of MHC I Functions by Cytokines

Complete escape from immune control would result in the uncontrolled replication of the virus. This would harm and finally kill the host and thus cease the dissemination of the virus. The efficacy of virus-specific CTL which can control CMV replication *in vivo* (3,4) indicated that the viral immune evasion mechanisms operate *in vivo* with a limited degree of effectiveness and suggested further that the antigen presentation function of CMV-infected cells is a matter of regulation. *In vitro* data provided evidence that certain cytokines, i.e. interferon γ (IFN-γ), type I interferons (IFN-α and IFN-β) as well as tumor necrosis factor-α (TNF-α) are able to restore antigen presentation and CTL recognition of fibroblasts infected with MCMV and HCMV, respectively (12,38). The cytokines compensate the MHC I inhibition by both viruses despite the fact that the mechanisms that are operative clearly differ. Among these cytokines, IFN-γ is most efficient in restoring antigen presentation of CMV-infected fibroblasts, but

type I interferons as well as TNF-α have also a significant effect (12). Two explanations of the effects on antigen presentation in CMV-infected cells are possible. First, all of these cytokines have been shown to exert strong inhibitory effects on CMV replication by inhibiting expression of late genes and nucleocapsid assembly (39), raising the possibility that the expression of MHC I-subversive genes can be suppressed by interferons. Alternatively, the effect could be explained by the fact that the factors influence cellular genes, i.e. stimulate MHC class I and β_2m gene expression (40–42). The potency of IFN-γ could be due to its ability to stimulate transcription of further genes, e.g. TAP1, TAP2, tapasin and MHC-encoded subunits of the proteasome (43) which might increase the generation and supply of viral peptides for MHC I assembly.

To address the first possibility, we tested modulation of MHC I expression by IFN-γ in stable transfectants expressing the HCMV-subversive genes *US2, US6* and *US11* in isolation. Fig. 4a and Fig. 4b show that *US2*-, *US6*- and *US11*-transfectants display a drastically reduced MHC class I surface density compared to untransfected control cells. Exposure of cells to graded concentrations of IFN-γ increases MHC class I expression in untransfected control cells in a dose-dependent order. The IFN-γ effect is reproduced in the presence of MHC I-subversive HCMV gene functions, albeit to an extent depending on the *US* gene expressed (Fig. 4A,B). After stimulation with IFN-γ, a surplus of MHC I molecules escapes from the control by the viral inhibitors and reaches the cell surface, where few MHC I molecules suffice for CTL recognition.

Next, we investigated whether IFN-γ displays effects on viral genes responsible for MHC class I downregulation. Pre-incubation of fibroblasts with IFN-γ increases the assembly of MHC class I complexes in cells infected with HCMV for 72 h dose-dependently reaching higher levels than mock-infected controls (12; Fig. 5a). At this time the *US6* gene is most abundantly expressed in HCMV-infected fibroblasts (27). We therefore tested whether the gpUS6-mediated inactivation of TAP1/2 is manifest under these conditions. Peptide translocation by TAP1/2 was found almost efficient as in mock-infected controls (F. Momburg and H. Hengel, data not shown). This is consistent with our finding that IFN-γ treatment strongly impairs gpUS6 synthesis in HCMV-infected cells (Fig. 5b). It will be interesting

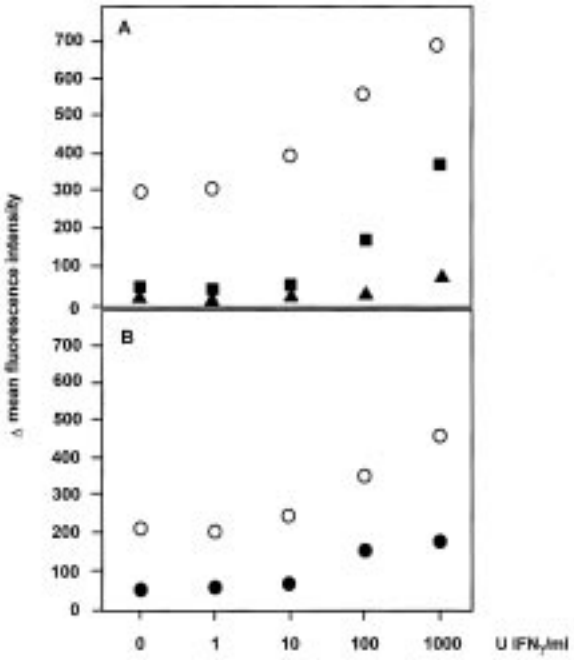

Fig. 4. Interferon-γ (IFN-γ)-mediated restoration of MHC class I surface expression in *US2* and *US11*-transfected LC-5 cells (**A**) and *US6*-transfected HeLa cells (**B**). Cytofluorometric analysis of MHC class I surface expression of cells transfected with pcDNAI-US2 (filled triangles), pcDNAI-US11 (filled rectangle) and pcDNAI-US6 (filled circles), respectively, and untransfected HeLa and LC-5 control cells (open symbols). Cells were incubated with graded doses of IFN-γ for 48 h before stained with MAb W6/32 recognizing human MHC class I molecules followed by FITC-labeled goat anti-mouse IgG antibodies. The data are given as mean fluorescence intensity values of W6/32-labeled cells minus control staining with the second antibody only.

to learn which of the *US6* transcription units are sensitive to IFN-γ and whether the expression of *US2, US3* and *US11* are also sensitive to IFN-γ, since transcription of these genes is under different control.

Remarkably, restoration of antigen presentation of fibroblasts strictly requires pretreatment of cells with cytokines before CMV infection, while IFN-γ had no effect on already infected cells (12,38). Likewise, the inhibition of CMV replication by IFN-γ critically depends on pre-exposure of cells before infection (39). These observations predicted recent reports demonstrating that CMVs interfere with the host cell response to IFNs (44,45).

Several findings from *in vivo* studies relate to the effects of cytokines on antigen presentation. First, the antiviral effector function of adoptively transferred $CD8^+$ CTL into MCMV-infected mice requires INF-γ

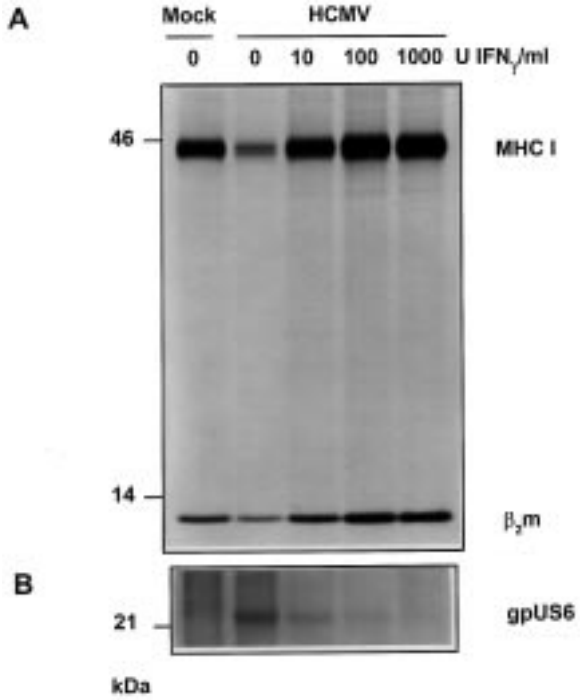

Fig. 5. Interferon-γ (IFN-γ)-pretreatment of fibroblasts before HCMV infection restores MHC class I assembly (**A**) and inhibits synthesis of the US6 glycoprotein (gpUS6) (**B**) in a dose-dependent fashion. Human foreskin fibroblasts were exposed to IFN-γ as indicated for 48 h before infected with HCMV AD169 (multiplicity of infection (moi) = 5) for 72 h. Cells were metabolically labeled with [^{35}S] methionine for 90 min and lysed in 1% NP 40 lysis buffer. All lysates used for immunoprecipitation were adjusted to ensure comparability in quantitative terms. MHC class I-β_2-microglobulin complexes were immunoprecipitated using MAb W6/32 and protein A sepharose, gpUS6 molecules were retrieved using a polyclonal rabbit antiserum raised against an US6 peptide. Immune complexes were eluted with sample buffer and analyzed by 11.5%–13.5% PAGE. Gels were dried and exposed to films for two days.

(38), compatible with the notion that this cytokine regulates antigen presentation of infected cells *in vivo*. In addition, the extraction of antigenic viral peptides from MCMV-infected organs demonstrated direct evidence for a pivotal role of IFN-γ *in vivo*. Efficient generation of antigenic peptides from viral proteins and the subsequent loading onto MHC class I molecules could be decreased by neutralization of INF-γ and restored in immunocompromised mice by INF-γ administration (46). The observation that IFN-γ is able to restore antigen presentation of adenovirus- and herpes simplex virus-infected cells which also subvert immunity by MHC I-reactive proteins (47,48) points to a more general role of IFN-γ to promote antiviral CD 8+ T cell effector functions against persisting viruses.

HCMV Interference with the Jak/Stat Pathway

MHC class II genes are constitutively expressed only in few cell types, i.e. B lymphocytes, dendritic cells and thymic epithelial cells. In MHC class II negative cells, IFN-γ is the most potent inducer of MHC class II transcription. IFNγ stimulates MHC class II gene expression by activating the Jak/Stat signal transduction pathway (49,50). In this pathway a cascade of events is inititated after IFN-γ binding to its receptor. This receptor is associated with the Janus kinases (Jaks) Jak1 and Jak2, both of which become phosphorylated upon IFN-γ binding, as well as the cytoplasmic tail of the IFN-γ receptor itself. Each phosphorylated IFN-γ-receptor chain forms a binding site for a member of the familiy of signal transducers and activators of transcription (Stats), Stat1α. After docking at the receptor, Stat1α is phosphorylated by the Jaks and migrates to the nucleus where it binds to specific sites present in promotors of IFN-γ-inducible genes. Both HCMV and MCMV disrupt the IFN-γ-mediated induction of MHC class II transcription through the Jak/Stat pathway and thus antigen presentation to CD4+ T cells (44,45; Fig. 6, lowest panel). Despite the common phenotype, the underlying viral mechanisms appear different. In contrast to HCMV, MCMV infection interferes with the induction of MHC class II genes at a stage downstream of Stat1α activation and nuclear translocation (45). In HCMV-infected cells levels of Jak1 are significantly decreased, obviously due to an HCMV-associated enhancement of Jak1 protein degradation (44). Since signal transduction by type I interferons is also Jak1-dependent, it is readily clear that HCMV interferes by this means also with IFN-α and IFN-β mediated responses (51). As found for MHC class I-restricted antigen presentation (12,38), preincubation of fibroblasts with IFNγ preserves induction of MHC class II gene expression in HCMV-infected fibroblasts with an efficiency almost comparable to mock-infected IFNγ-stimulated contol cells (Fig. 6). From this result one may speculate whether the HCMV genes which interfere with the IFN-γ driven induction of MHC

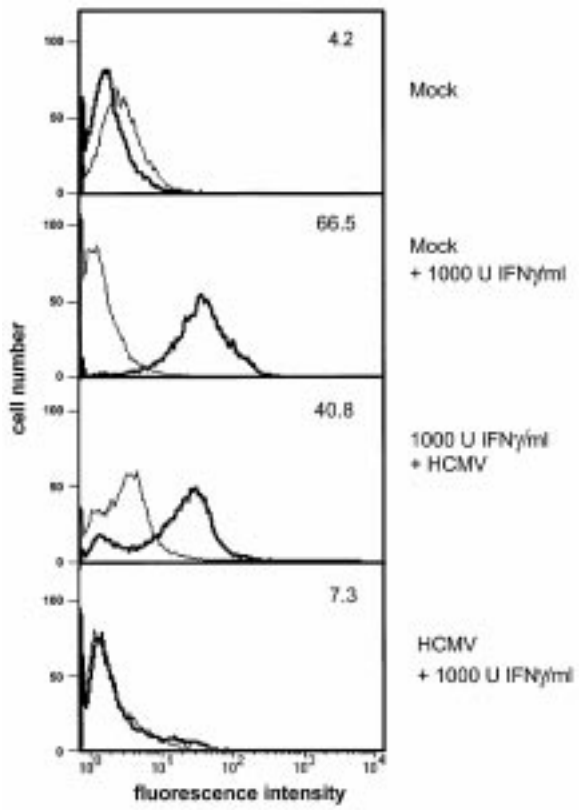

Fig. 6. Induction of MHC II molecules in HCMV-infected fibroblasts by preincubation with Interferon-γ (IFN-γ) before infection. Human foreskin fibroblasts were either mock treated (top panel) or exposed to 1000 U IFN-γ/ml for 96 h (second top panel) or exposed to 1000 U IFN-γ/ml for 24 h before infection with HCMV AD169 (multiplicity of infection (moi) = 5) for additional 72 h (second lower panel) or infected first with HCMV AD169 (moi = 5) before exposed to 1000 U IFN-γ/ml for 72 h. Cells were stained with MAb 2.06 recognizing human MHC class II molecules followed by FITC-labeled goat anti-mouse IgG antibodies (bold line) or with second antibody only (narrow line) and analyzed by cytofluometry. Mean fluorescence intensity values of MAb 2.06-labeled cells minus control staining with the second antibody only are given in each histogram.

class II transcription might be counterregulated by IFN-γ itself.

Conclusions and Perspective

The complete course of permissive HCMV infection is covered by the expression of MHC class I-subversive glycoproteins. They represent a paradigm for 'natural' immune modulators which have been highly adapted to their functions during the coevolution of CMVs with their hosts over millions of years. The viral inhibitors have proven to be valuable tools for the elucidation of molecular mechanisms in the MHC class I pathway of antigen presentation. The bewildering array of MHC class I-subversive genes in cytomegaloviral genomes may reflect the urgent need of these viruses to keep pace with the evolution of MHC class I genes as well as antagonistic effects mediated by cytokines. The intricate balance between host immune control and viral evasion ensures both the host's freedom from harmful disease manifestations and the need of CMVs to replicate sufficiently and to spread. The identification of the genetic basis for the subversion of the IFN response and MHC class II functions is a goal of prime importance for future research. It will be of interest to see whether one or multiple genes were used to prevent MHC class II expression. The number of cytomegaloviral genes affecting immune and cellular functions that have been identified to date probably represents just the tip of an iceberg. CMV genomes are promising sources for novel regulators for immune and nonimmune functions. Our knowledge about viral modulators has implications for the understanding of CMV biology, for the prevention of disease manifestations in patients at risk and for vaccine development.

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