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Current Opinion in Immunology
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Viral evasion of T cell immunity: ancient mechanisms offering new applications

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Upon infecting a host, viruses are confronted by a coordinated and multi-faceted immune response. Indeed, evolutionary combat between virus and host has contributed signally to the host's development of a formidable innate and adaptive immune defense arsenal, and to the virus' acquisition of effective means to evade it. Cytotoxic T lymphocytes play a key role in the elimination of virus-infected cells, which they detect through recognition of virus-derived peptides displayed at the cell surface in the context of MHC class I molecules. This highly sensitive recognition system is a prime target for immune evasion strategies deployed by many viruses, particularly large DNA viruses such as herpesviruses and poxviruses. Elucidation of the mode of action of the immune evasion proteins encoded by these viruses has not only provided new insights into viral pathogenesis, but has also led to the discovery of hitherto unknown cell biological and immunological phenomena. Moreover, viral immune evasion proteins constitute extremely useful tools to block defined stages of the MHC class I presentation pathway, not only for research purposes, but also for clinical applications.

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Current Opinion in Immunology 2011, **23**:96–103

This review comes from a themed issue on
Antigen processing
Edited by Peter van den Elsen and James McCluskey

Available online 9th December 2010

0952-7915

DOI 10.1016/j.coi.2010.11.005

Introduction

Cytotoxic T lymphocytes (CTLs) play an important role in the elimination of virus-infected cells, which they recognize through the detection of virus-derived peptides presented at the cell surface by MHC class I (MHC I) molecules. The viral peptides result from proteasomal degradation of proteins in the cytosol and are translocated by the Transporter associated with Antigen Processing (TAP) into the endoplasmic reticulum (ER), where they are loaded onto newly synthesized MHC I molecules. As an alternative to presentation of peptides derived from endogenously synthesized proteins, specialized antigen presenting cells, such as dendritic cells, may take up viral proteins from their environment and present peptides derived from those proteins to CTLs. This process of presenting exogenous antigens via MHC I molecules is known as cross-presentation; the routes by which exogenous antigens reach MHC I molecules are poorly understood. As will be discussed, viral inhibitors of antigen presentation are powerful instruments to decipher the routing of antigens in both direct and cross-presentation.

DNA viruses with a large genome coding capacity, such as herpesviruses, have proven particularly adept at preventing CTL recognition through the actions of dedicated immune evasion proteins. The family *Herpesviridae*, which is divided into the subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (whose members are often referred to as alphaherpesviruses, betaherpesviruses, and gammaherpesviruses, respectively), is estimated to have emerged roughly 400 million years ago [1]. The long co-evolution of these viruses with their host is believed to have contributed to extensive adaptation of these pathogens to their respective hosts.

The vast majority of the human population is infected with one or more of the eight known human herpesviruses, namely herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpesviruses 6 and 7 (HHV-6 and HHV-7), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) [2]. Herpesvirus infections usually cause only mild symptoms, but serious complications can arise, particularly in immunocompromised individuals. In addition, HCMV is the most common viral cause of congenital defects, and EBV and KSHV are associated with the development of several malignancies [3].

A hallmark of herpesviruses is their ability to establish lifelong infections despite the presence of a powerful immune response directed against these viruses. In addition, re-infections of immune individuals may occur, even by the same viral strain. These examples indicate that herpesviruses have acquired powerful immune evasion mechanisms. Evasion indeed occurs at multiple

levels, targeting both innate and adaptive branches of the immune response. Herpesviruses have been found to affect antiviral host responses by interfering with cytokine and chemokine signaling [4], by impairing the complement cascade [5], by preventing natural killer cell-mediated recognition and elimination of infected cells [6], and by inhibiting innate immune signaling by pattern-recognition receptors, such as Toll-like receptors and RIG-I-like receptors [7]. Furthermore, the past decades have witnessed the elucidation of diverse strategies by which herpesviruses manipulate the host adaptive immune response [8,9]. These include utilizing herpesvirus-encoded Fc receptors to inhibit antibody-mediated effector mechanisms [10] and subverting the MHC II [11] and, particularly, the MHC I antigen processing and presentation pathways.

Herpesviruses encode a wealth of proteins specifically interfering with MHC I-restricted antigen presentation. More recently, also poxviruses have been found to actively evade MHC I-restricted CTLs through a series of specialized gene products. The characteristics of these immune evasion proteins, their deployment during evolutionary combat, their remarkably distinct mechanisms of action, and their application as versatile tools in antigen presentation studies are discussed in this review.

The MHC I antigen presentation pathway is a prime target for viral immune evasion

Herpesvirus immune evasion strategies appear to target each step of the MHC I presentation pathway (Figure 1). The gammaherpesviruses EBV and KSHV encode the latency-associated proteins EBNA1 and LANA, respectively, which interfere with their own translation and with their proteasomal degradation, thereby reducing the generation of antigenic peptides [12,13,41]. Alphaherpesviruses and gammaherpesviruses express shutoff proteins that block host protein synthesis, with new MHC I molecules being among the host proteins that are affected [14–18]. The Viral Inhibitor of Heavy Chain Expression or VIHCE protein of rhesus CMV (RhCMV) specifically inhibits the synthesis of MHC I heavy chains in a signal peptide-dependent fashion through a yet enigmatic mechanism [19•].

Levels of MHC I molecules are also targeted in a more selective fashion, for example by the HCMV-encoded proteins US2, US10, and US11 [20–22], mouse cytomegalovirus (MCMV) glycoprotein (gp) 48, and murine gammaherpesvirus 68 (MHV68) mK3, which accelerate the degradation of MHC I molecules [8,9,23]. An alternative strategy to reduce display of peptide-presenting MHC I complexes at the surface of infected cells involves intracellular retention (by HCMV US3 and MCMV gp40) or increased endocytosis (by KSHV kK3/kK5 and EBV BILF1) [8,9,23]. At the cell surface, MCMV gp34 interferes with recognition of peptide-loaded MHC I complexes by cytotoxic T cells [23]. Finally, many herpesviruses encode proteins that specifically block ER import of antigenic peptides by TAP. As will be discussed below, this class of immune evasion proteins demonstrates an unexpected diversity, both in structure and mode of action. Recently, also cowpox virus has been found to encode molecules specifically eluding MHC I-restricted CTL recognition. The CPX V203 protein interferes with intracellular trafficking of MHC I molecules by sequestering them in the ER [24••]. CPX V012 prevents peptide transport by TAP, thereby representing the first TAP inhibitor outside the herpesvirus family [25••,26••,27].

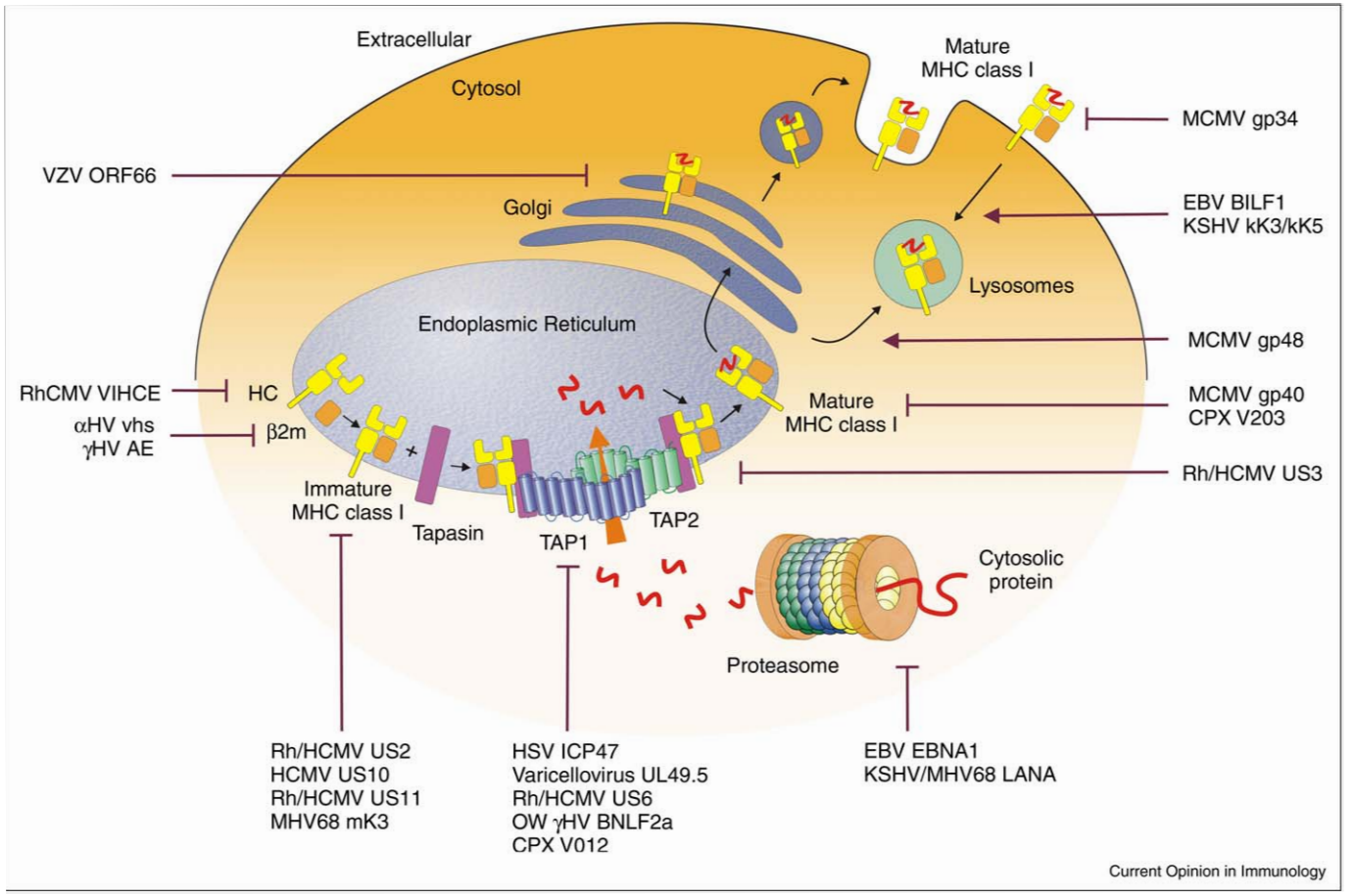
The expanding repertoire of viral evasion molecules not only creates a valuable toolbox for immunological studies, but also reveals new insights into normal functions of the cell and the immune system in particular.

Viral immune evasion: lessons in immunology and cell biology

Research into the mechanisms underlying viral evasion strategies has led to the discovery of previously unknown cellular processes. This is illustrated by the HCMV proteins US2 and US11, which target MHC I molecules for proteasomal degradation [20,21]. The MHC I heavy chains (HC) are properly translocated into the ER, as inferred from their N-linked glycosylation, but subsequently are redirected to the cytosol, where they are degraded through the ubiquitin–proteasome pathway. The retrograde movement of the MHC I HC, termed 'dislocation' (as opposed to translocation), appears to occur via a constitutive pathway in the cell that plays a role in many important cellular processes, including protein quality control, the release of proteins from the ER in the context of the Unfolded Protein Response (UPR) [28], and also antigen (cross-)presentation (discussed below). The US2 and US11-mediated dislocation of MHC I HC serves as a paradigm of ER protein dislocation and degradation and has facilitated the characterization of many features of this process. These include the involvement of the translocon or Sec61 complex as a channel mediating transport of at least certain categories of dislocation substrates [20], the role of the proteasome–ubiquitin system, and the discovery of hitherto unknown constituents of this protein degradation pathway, among which the AAA ATPase p97, members of the derlin family, and ER-resident ubiquitin E3 ligases [28,29].

Recent studies indicate that protein dislocation plays a role in antigen presentation as well, allowing proteins to migrate from intracellular compartments, such as phagosomes, and the secretory pathway back to the cytosol, where they are degraded into peptides by the proteasome [30,31••,32••,33]. The peptides derived from dislocated ER proteins are re-imported into the ER by TAP for MHC I-restricted presentation. Additionally, TAP

Figure 1



Herpesvirus interference with MHC class I antigen presentation. Herpesviruses encode a large variety of proteins that interfere with MHC I antigen presentation to prevent elimination of the infected cell by CTLs. EBV EBNA1 and KSHV/MHV68 LANA avoid their own proteasomal degradation, thus limiting the generation of antigenic peptides. The VIHCE protein of RhCMV inhibits the translation of MHC I HC in a signal-peptide dependent manner. Viral host shut-off proteins, such as the UL41 homologs of alphaherpesviruses (αHV) like HSV, BHV-1 and PRV, and the alkaline exonucleases of the gammaherpesviruses (γHV) EBV (BGLF5), KSHV (SOX) and MHV68 (ORF37), degrade host mRNAs, thereby reducing protein levels of HC and β2m. HCMV US10, RhCMV/HCMV US2 and US11 and MHV68 mK3 direct immature MHC I molecules to the cytosol where they are degraded. Transport of peptides through TAP is inhibited by HSV ICP47, varicellovirus UL49.5 homologs, RhCMV/HCMV US6, Old World primate gammaherpesvirus (OW γHV) BNLF2a homologs and CPX V012. RhCMV/HCMV US3 interferes with the function of tapasin, thereby preventing proper peptide loading of MHC I. MCMV gp40 and CPX V203 inhibit transport of mature MHC I molecules out of the ER. VZV ORF66 retains mature MHC I complexes in the *cis*/medial Golgi. MCMV gp48 interferes with MHC I trafficking by directing these molecules from the Golgi to lysosomes. MCMV gp34 prevents cytotoxic T lymphocyte-induced lysis by associating with MHC I molecules at the cell surface. Finally, EBV BILF1 and KSHV kK3 and kK5 increase the endocytosis and lysosomal degradation of cell surface MHC I molecules.

complexes are present in phagosomal compartments, facilitating translocation of peptides into those compartments for (cross-)presentation by MHC I molecules [30,31••,34••]. In a number of studies, dislocation of proteins was found to involve the Sec61 complex, analogous to HCMV US2 and US11-mediated retrotranslocation of MHC I HC [30,31••,33].

The role of the dislocation pathway in antigen processing is further illustrated by tyrosinase, a melanocyte differentiation protein that is a target for melanoma-reactive T cells [35]. Glycoproteins that are dislocated from the ER back to the cytosol lose their N-linked glycans in a reaction that is catalyzed by a cytoplasmic N-glycanase. This deglycosylation involves the deamidation of asparagines, changing these amino acids into aspartic acid. The presence of an aspartic acid residue within a T cell epitope of tyrosinase at a position where the native protein carries an asparagine residue witnesses the involvement of the cytosolic N-glycanase, and thus ER-to-cytosol dislocation, as an essential step in the processing of this particular epitope [36]. Deamidation has been found for several other T cell epitopes, including epitopes derived from HIV-1 env, hepatitis C virus E1, and lymphocytic choriomeningitis virus (LCMV) GP1 [36], suggesting a wide role for protein dislocation in antigen presentation.

Another important cellular process that is manipulated by viruses in many ways is the ubiquitin system. Herpesviruses code for E3 ubiquitin ligases, such as HSV ICP0, KSHV kK3 and kK5, and MHV68 mK3, but also for numerous de-ubiquitinating enzymes. The intriguing interplay between viruses and the ubiquitin system has been reviewed by Isaacson and Ploegh [37]. An example of an interesting, novel phenomenon discovered in the context of herpesvirus immune evasion pertains to ubiquitin conjugation by the E3 ligases mK3 and kK3/kK5 of MHV68 and KSHV, respectively. These proteins catalyze the ubiquitination of cytoplasmic domains of MHC I HC, resulting in the proteasomal degradation (in case of MHV68) or endocytosis (in case of KSHV) of MHC I molecules [8]. When the amino acid residues that are ubiquitinated were identified within the MHC I HC, it was found that in addition to the common ubiquitin acceptor lysine, also cysteine, serine, and threonine residues were ubiquitinated [38••,39••,40•]. These findings implicate a novel chemical mechanism of substrate ubiquitination via ester linkages. Most probably, cysteine, serine, and threonine ubiquitination is used more widely by the cell. The functional implications of this particular type of conjugation are yet to be determined.

Viral immune evasion proteins: valuable tools in antigen presentation studies

EBV EBNA1 and KSHV LANA carry large repeats of glycine-alanine and serine-proline residues, respectively, that hamper proteasomal degradation of these viral gene products [13,41]. When transferred to other proteins, the repeats effectively block degradation of these proteins *in cis*. Thus, they can be used to inhibit the first stage of the MHC I presentation pathway, namely the generation of antigenic peptides by proteasomes [41,42]. EBNA1 has also been instrumental in uncovering the role of autophagy in antigen presentation. Using EBNA1 as a model antigen, autophagy was found to allow MHC II-mediated presentation of peptides derived from a nuclear or cytosolic protein [43••,44••].

Herpesvirus-encoded TAP inhibitors have been particularly useful in cross-presentation studies aiming to unravel the routes by which exogenous antigens are loaded onto MHC I molecules. A 35-residue peptide encompassing the TAP-inhibiting domain of HSV ICP47 has been shown to be endocytosed and to subsequently block TAP function [31••]. Since ICP47 interferes with the binding of peptides to cytosolic domains of TAP, this finding implies that extracellular proteins can reach the cytosol of cells, possibly via the Sec61 dislocation pathway (discussed above) [30,45]. The ICP47 peptide inhibits both direct and cross-presentation, implicating the involvement of TAP in cross-presentation.

HCMV US6 inhibits TAP by interacting with ER-luminal domains of TAP. Exposure of DCs to a soluble form of US6 completely abrogates cross-presentation, confirming the essential role for TAP in cross-presentation [34••,46]. At the same time, these experiments show the existence of a connection between the extracellular milieu and TAP-containing compartments and illustrate the contribution of phagosomes and pinosomes to presentation of exogenous antigens by DCs. Selective deposition of US6 into early endosomes using a US6-transferrin fusion protein identified these endosomes as loading compartments for cross-presented peptides derived from soluble antigens [32••].

The herpesvirus-encoded TAP inhibitors each act in a unique way, targeting different stages of the peptide translocation cycle (summarized in Table 1). ICP47 and EBV-encoded BNLF2a block binding of substrates to the cytosolic peptide binding site of TAP; US6, BNLF2a, and the UL49.5 proteins of equine herpesvirus 1 and 4 affect the association of ATP with the nucleotide binding domains of TAP; US6 and the UL49.5 proteins of all TAP-inhibiting varicelloviruses interfere with conformational transitions required for translocation of peptides over the ER membrane, and UL49.5 of bovine herpesvirus 1 and related ruminant varicelloviruses target TAP for proteasomal degradation [47–54,55•]. Recently, the cowpox protein CPX V012 was identified as a TAP inhibitor [25••,26••]. The mechanism by which this protein blocks TAP-mediated peptide transport has not yet been resolved.

Table 1

Comparison of the mechanisms by which virus-encoded proteins specifically interfere with TAP-mediated peptide transport

Virus	Inhibitor	Structural features	Mechanism of TAP inhibition	Refs.
HSV-1/2	ICP47	Cytosolic protein	Interference with peptide binding	[47,48]
BHV-1	UL49.5	Type I membrane protein	Conformational alterations and degradation of TAP1/2	[54,55•]
EHV-1/4	UL49.5	Type I membrane protein	Conformational alterations and interference with ATP binding	[54,55•]
PRV	UL49.5	Type I membrane protein	Conformational alterations	[54,55•]
HCMV	US6	Type I membrane protein	Conformational alterations and interference with ATP binding	[49–51]
EBV	BNLF2a	Tail-anchored protein	Interference with ATP and peptide binding	[52,53]
CPX	V012	Type II membrane protein	Unknown	[25••,26••]

The viral TAP inhibitors can be used to block the peptide translocation cycle at different stages and therefore are valuable tools to study the molecular biophysics of peptide translocation by TAP. In addition, viral TAP inhibitors may be used to freeze TAP at intermediate stages of the translocation cycle, which may be informative when resolving the crystal structure of the peptide transporter [56,57]. Elucidation of the molecular mechanisms underlying viral TAP inhibition will facilitate the use of TAP inhibitors for selective immune suppression in the context of, for example, auto-immune diseases and tissue and organ transplantation. In addition, this knowledge may aid the development of substances blocking other ABC transporters, for example those responsible for multiple drug resistance in bacterial and cancer cells.

In vivo implications of viral stealth technology

Recently, several interesting studies have highlighted the importance of viral immune evasion mechanisms in the life cycle of viruses *in vivo*. In addition, animal studies have been initiated to explore the applications of viral immune evasion proteins for the rational design of novel strategies for vaccine development, cancer treatment, transplant protection, and gene therapy.

Using RhCMV as a model, Hansen *et al.* have shown that the evasion molecules encoded by the US region of this virus (e.g. US2, US3, US6, and US11) are dispensable for primary infection and persistence of the virus, but are essential for superinfection of a CMV-immune host [58••]. The *in vivo* relevance of viral T cell immune evasion proteins has been demonstrated for two other viruses as well, namely cowpox virus and MHV68. An elegant study by Byun and colleagues demonstrated the biological function of two cowpox virus-encoded inhibitors of MHC I-restricted antigen presentation, CPX V012 and CPX V203 [25••]. Deletion of the genes encoding these evasion proteins restored MHC I surface expression and T cell stimulation *in vitro* and reduced virulence in mice, thus demonstrating the significance of these immune evasion proteins in one of the virus' natural host species. For MHV68, deletion of the MHC I inhibitor mK3 increased CTL responses to lytic viral proteins, without major effects on viral replication. By contrast, removal of mK3 resulted in decreased latent viral loads, suggesting a role for immune evasion in amplifying the latent reservoir for this herpesvirus [59].

The capacity of herpesviruses to superinfect immune hosts, and to induce and maintain strong T cell immunity makes these viruses promising vaccine vectors. The potential of this approach has been demonstrated in a landmark study by Hansen *et al.*, who used RhCMV as a carrier virus for immunization against simian immunodeficiency virus infection [60••].

Alternative applications of viral stealth technology in the fields of transplant protection and cancer therapy have been reviewed by Horst *et al.* [61]. An interesting example is the use of viral TAP inhibitors to induce T cells recognizing an alternative peptide repertoire carried by tumor cells with antigen processing defects [62••]. MHC I molecules of tumor cells with deficiencies in the MHC I presentation pathway carry a repertoire of subdominant T cell epitopes, named T cell Epitopes associated with Impaired Peptide Processing (TEIPP). T cells directed against TEIPP can be selected *in vitro* using antigen presenting cells expressing viral TAP inhibitors such as the bovine herpesvirus 1 UL49.5 protein. These TEIPP-specific T cells protect mice against the outgrowth of TAP-deficient lymphomas and fibrosarcomas [63•].

Viral stealth technology also holds promise in gene therapy, providing tools to improve the survival of cells expressing a transgene, which often represents a neoantigen (reviewed by Horst *et al.*) [61].

Conclusions

Large DNA viruses, such as herpesviruses and poxviruses, dedicate a considerable part of their genome to immune evasion. The MHC I antigen processing and presentation pathway appears to be a prime target for viral evasion, illustrating the evolutionary pressure exerted by $CD8^+$ CTLs during co-evolution of these viruses with their hosts. Viral immune evasion proteins target virtually every step in the MHC I pathway and therefore constitute powerful tools for antigen processing and presentation research. The power of this approach has been demonstrated in studies in which TAP inhibitors have been used

to uncover important, basic features of cross-presentation. Elucidation of the mechanisms underlying viral immune evasion strategies reveals essential, hitherto unknown cell biological and immunological processes that have been discovered by viruses as effective targets for immune evasion millions of years ago.

Acknowledgements

We thank Dr. Bryan Griffin for his valuable contributions to this manuscript. We gratefully acknowledge financial support by the Dutch Cancer Foundation (grant RUL 2005-3259), the Dutch Diabetes Research Foundation, the Netherlands Scientific Organization (NWO, Vidi 917.76.330), and the UK Medical Research Council.

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The E3 ligase kK3, encoded by Kaposi's sarcoma-associated herpesvirus, catalyzes the ubiquitination of MHC class I heavy chains. In this study, Caldwell and Coscoy demonstrate that in addition to lysine residues, also cysteine residues can be ubiquitinated. In the case of kK3, this results in endocytosis and degradation of the MHC class I heavy chains.

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The mouse gamma herpesvirus protein mK3 is a viral RING-CH-type E3 ligase that specifically targets nascent major histocompatibility complex I heavy chains (HC) for degradation, thus blocking the immune detection of virus-infected cells. Wang *et al.* show that this reaction involves ubiquitination of the cytoplasmic domain of the MHC class I heavy chains. In addition to lysine residues, also cysteine, threonine and serine residues appear to be ubiquitinated through a novel reaction that involves ester/thiolester bonds. Since mK3 has numerous cellular and viral homologs, it will be of considerable interest to determine the pervasiveness of this novel form of ubiquitination.

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Wang *et al.* identify Ube2j2 as the primary cellular ubiquitin conjugating enzyme or E2 recruited by the mK3 E3 ligase, and show that this E2–E3 pair is capable of catalyzing ubiquitination of lysine and serine residues of substrates. Ube2j2-mK3 preferentially promotes ubiquitination of hydroxylated amino acids via ester bonds, even when lysine residues are present on the substrate.

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Paludan *et al.* show that the Epstein-Barr virus Nuclear Antigen 1 (EBNA1) gains access to the MHC class II antigen presentation pathway via autophagy. The authors demonstrate that inhibition of lysosomal acidification decreases recognition of EBNA1 by specific $CD4^+$ T cells, indicating a role for lysosomal processing after autophagy in T cell recognition of long-lived endogenous antigens, including nuclear proteins.

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In this study, Leung *et al.* show that cytoplasmic expression of EBNA1 increases the efficiency of processing of $CD4^+$ T cell epitopes through autophagy. Nuclear expression strongly reduces EBNA1 recognition by the same T cells, explaining the limited level and range of EBNA1 $CD4^+$ T cell epitopes naturally displayed on EBV-infected cells.

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When the mechanism of TAP inhibition by varicellovirus UL49.5 proteins was investigated, unexpected differences were observed among the various homologs. UL49.5 of bovine herpesvirus 1 blocks essential conformational transitions of the TAP complex and targets TAP for proteasomal degradation. This is not observed for the UL49.5 protein of equine herpesvirus 1 and 4. The latter inhibit the binding of ATP to TAP. The UL49.5 protein of pseudorabies virus does not destabilize TAP, nor interferes with ATP binding, but freezes TAP in a translocation-incompetent state.

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Using rhesus cytomegalovirus as a model, Hansen *et al.* show that superinfection of immune animals requires evasion of $CD8^+$ T cell immunity, mediated by the homologs of the human CMV immune evasion proteins US2, 3, 6, and 11, which all interfere with antigen presentation via MHC class I molecules. Unexpectedly, these evasion proteins are neither required for primary infection, nor establishment of persistent infection.

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Cytomegaloviruses are very effective inducers of life-long effector memory T cell responses. Hansen *et al.* have used rhesus CMV as a vector for the expression of simian immunodeficiency virus (SIV)-encoded Gag, Ref-Tat-Nef, and Env. The recombinant RhCMV persistently infected rhesus macaques, and maintained effective SIV-specific $CD4^+$ and $CD8^+$ effector memory T cell responses, regardless of pre-existing immunity against RhCMV. The vaccinated animals showed increased resistance to acquisition of progressive SIV infection.

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In this study, Van Hall *et al.* identify a unique category of CTLs that recognize an alternative repertoire of peptide epitopes emerging in MHC class I molecules at the surface of cells with impaired function of TAP, tapasin, or the proteasome. These peptides, called T cell epitopes associated with impaired peptide processing (TEIPP), are derived from self antigens and can be exploited for immune intervention against processing-deficient tumors through adoptive T-cell transfer or peptide vaccination.

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Chambers *et al.* have further explored the concept of TEIPP-targeted therapy using a dendritic cell (DC)-based cellular vaccine. Impairment of TAP function in DCs induced the presentation of endogenous TEIPP antigens by MHC class I molecules. Immunization with these DCs protected mice against the outgrowth of TAP-deficient lymphomas and fibrosarcomas. TEIPP antigens could be successfully induced in wild-type DCs by introducing the viral TAP inhibitor UL49.5. These results imply that immune intervention strategies with TAP-inhibited DCs could be developed for the treatment of antigen processing-deficient cancers in humans.