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Cowpox Virus Evades CTL Recognition and Inhibits the Intracellular Transport of MHC Class I Molecules

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Orthopoxviruses evade host immune responses by using a number of strategies, including decoy chemokine receptors, regulation of apoptosis, and evasion of complement-mediated lysis. Different from other poxviral subfamilies, however, orthopoxviruses are not known to evade recognition by CTL. In fact, vaccinia virus (VV) is used as a vaccine against smallpox and a vector for eliciting strong T cell responses to foreign Ags. and both human and mouse T cells are readily stimulated by VV-infected APC in vitro. Surprisingly, however, CD8+ T cells of mice infected with cowpox virus (CPV) or VV recognized APC infected with VV but not APC infected with CPV. Likewise, CD8+ T cells from vaccinated human subjects could not be activated by CPV-infected targets and CPV prevented the recognition of VV-infected APC upon coinfection. Because CD8+ T cells recognize viral peptides presented by MHC class I (MHC I), we examined surface expression, total levels, and intracellular maturation of MHC I in CPV- and VV-infected human and mouse cells. Although total levels of MHC I were unchanged, CPV reduced surface levels and inhibited the intracellular transport of MHC I early during infection. CPV did not prevent peptide loading of MHC I but completely inhibited MHC I exit from the endoplasmic reticulum. Because this inhibition was independent of viral replication, we conclude that an early gene product of CPV abrogates MHC I trafficking, thus rendering CPV-infected cells “invisible” to T cells. The absence of this immune evasion mechanism in VV likely limits virulence without compromising immunogenicity.


After the eradication of variola (VAR), the cause of smallpox, the main orthopoxviruses naturally infecting humans are zoonotic infections by monkeypox virus (MPV) and cowpox virus (CPV) (1). Despite their names, the animal reservoirs of CPV and MPV are predominantly rodents. For instance, CPV spreads from wild rodents to domesticated animals, pets, and humans (2). Genomic comparisons suggest that CPV is an ancestor of other orthopoxviruses (3), including VAR, which is now considered a bioterrorist threat. This ancestral role of CPV is supported by the observation that CPV contains the most complete set of immune modulators, of which many are mutated and/or deleted in vaccinia virus (VV) (4). It is likely that these mutations and deletions contribute to its diminished virulence and success as an attenuated vaccine against smallpox (5) CPV is thus an excellent model for identifying immune modulators of orthopoxviruses and their relationship to viral virulence.

Both the humoral and cellular components of adaptive immunity are considered important for the long-lasting protection of vaccinates or primary infection by poxviruses (6). Poxvirus-specific Ab titers are stable for the entire human lifespan and T cell immunity is maintained for decades (7, 8). Therefore, orthopoxviruses mainly infect unvaccinated individuals. The outcome of primary infections depends on the efficiency of the immune response and viral virulence, which is related to the virus’ ability to counteract innate and adaptive immunity (9). Poxviruses encode multiple classes of immune modulators, including inhibitors that act within infected cells, e.g., to inhibit apoptosis, or that prevent the attraction of lymphocytes to the site of infection (9). Poxviral genomes differ widely in their encoded immune modulators and none of the immune modulators are found in all poxviruses. For instance, the genera Capripoxvirus, Leporipoxvirus, Suipoxvirus, and Yatapoxvirus encode gene products that interfere with MHC class I (MHC I)-mediated Ag presentation to CTLs that are not conserved in orthopoxviruses (10).

The presumed absence of MHC I regulators in orthopoxviruses is surprising, because the presentation of virus-derived peptides to CTL is an important immune defense against viruses. During infection, viral peptides are produced in the cytosol by the proteasomal destruction of predominantly defective translation products (11). These peptides are then imported into the endoplasmic reticulum (ER) by the dedicated peptide transporter TAP and trimmed to 8- to 9-mers by the endoplasmic reticulum (ERAP) (12, 13). Empty MHC I heterodimers are retained in the ER by a chaperone complex, including the dedicated chaperone tapasin, until high affinity peptides that fit the highly polymorphic peptide binding groove of the H chain are obtained, followed by intracellular transport to the cell surface (14). MHC I-peptide complexes are thus crucial for T cell surveillance of infection. Many viruses, particularly large DNA viruses such as herpesviruses and adeno viruses, encode proteins that specifically interfere with this pathway by inhibiting the assembly or maturation of MHC I, blocking TAP, or removing MHC I from the cell surface (10, 15–17). Examples for TAP inhibitors are ICP47 of the HSV, US6 of the CMV, and UL49.5 of varicella viruses (18–20). The cytomegaloviral proteins
US2 and US11 destroy newly synthesized MHC I, whereas the human CMV protein US3, the murine CMV protein M152, and the adenovirus protein E19 retain assembled complexes of MHC I in the ER (15, 21). The above mentioned poxvirus genera as well as some \( \gamma-2 \) herpesviruses encode RING-type ubiquitin ligases of the K3-family that ubiquitinate and efficiently remove MHC class I from the surface of infected cells (10).

In contrast, the inhibition of Ag presentation has not been described for orthopoxviruses, and VV was observed to only moderately down-regulate MHC-I (22). Indeed, VV is widely used as a vector to deliver and present Ag both in vitro and in vivo (23). However, because VV lacks many immunomodulatory genes, it seemed possible that more virulent orthopoxviruses might encode factors that inhibit Ag presentation. To address this question, we examined antiviral T cell responses against cells infected with CPV and VV. These studies revealed that CPV-infected cells evade recognition by CPV-specific T cells, whereas the same T cells are able to recognize VV-infected targets. The inability of T cells to recognize CPV-infected cells correlated with an efficient inhibition of MHC I maturation observed in CPV-infected murine and human cells. During the early phase of infection, peptide loaded MHC I molecules are efficiently retained in the ER by CPV but not VV. Therefore, we conclude that CPV encodes a novel immune modulator that prevents MHC I Ag presentation.

Materials and Methods

Viruses and reagents

VV (Western Reserve strain) and CPV (Brighton Red strain) were grown and titrated by plaque assay on BSC40 or Vero cells. Cells were grown in DMEM or RPMI 1640 (A20) supplemented with 10% FBS, 1% penicillin/streptomycin, and \( \gamma \)-glutamine (2 mM) and infected at a multiplicity of infection (MOI) of 5. Ab W6/32 was from American Type Culture Collection; antisera K455 was a gift from P. Peterson (R. W. Johnson Pharmaceutical Research Institute, San Diego, CA); Abs to transferrin receptor, CD44, and CD166 were obtained from BD Biosciences; CD3 (clone SP34-M) was from Beckman Coulter; CD14 (clone RMO52) was from Immunotech; and CD20 (clone HI47) was from Caltag Laboratories. Mouse Abs to CD8 (clone 5H10) were from BD Pharmingen; CD8\(^+\) (clone 2ST8.5H7) was from Beckman Coulter; CD14 (clone RMO52) was from Immunotech; and CD20 (clone HI47) was from Caltag Laboratories. Mouse Abs to CD8\(^+\) (clone SH10) were from Caltag Laboratories, and IFN-\( \gamma \) (clone XMG1.2) and TNF-\( \alpha \) (clone MP6-XT22) were from BD Pharmingen. A biotinylated rabbit polyclonal Ab against orthopoxvirus proteins (anti-vaccinia) was from ViroStat, and conjugated secondary Abs and PE-conjugated or Pacific Blue-conjugated streptavidin were from Molecular Probes. Endoglycosidase H (EndoH) was from Roche, and brefeldin A (BFA) was from ICN Pharmaceuticals.

Human subjects

Each VV-immune subject provided informed written consent before signing research authorization forms that complied with the U.S. Health Insurance Portability and Accountability Act (HIPPA) in addition to a medical history questionnaire. These studies were approved by the Institutional Review Board of Oregon Health and Science University.

Animals

BALB/c and C57BL/6 mice were bred at Oregon Health and Science University or were purchased from The Jackson Laboratory. All animal experiments were reviewed and approved by the Oregon Health and Science University institutional animal care and use committee.

Flow cytometry

MHC I and other cellular markers were measured as described (24). Briefly, cells mock infected or infected with CPV or VV were washed with PBS twice and treated with EDTA (2 mM) in Tris-Cl (pH 7.4) to detach the cells from tissue culture plates. After a brief spin, cells were washed in ice-cold PBS and fixed in 2% paraformaldehyde on ice for 20 min. Cells were then washed twice in ice-cold PBS and 0.5 \( \times \) 10\(^6\) cells were used for staining and were analyzed using a FACScalibur flow cytometer (BD Biosciences). For intracellular staining, a 0.4 M glycine solution was added after fixation to quench the reaction. Cells were then permeabilized with 0.1% Triton X-100 for 10 min on ice before staining.

Intracellular cytokine staining (ICCS) of human PBMC was performed as described (7). Briefly, PBMC were infected with VV, CPV (sucrose gradient-purified intracellular mature virus) at different MOI values as noted in the figure legends or left uninfected at 37°C with 6% CO2 in RPMI containing 20 mM HEPES, \( \gamma \)-glutamine, antibiotics, and 5% heat-inactivated FBS. After 12 h of culture, BFA was added at a final concentration of 2 \( \mu \)g/ml for an additional 6 h. Approximately 10% of the cells were taken off for orthopoxvirus staining (see below). The remaining cells were stained overnight at 4°C with Abs specific for CD8\(^+\) and CD4. Cells were fixed, permeabilized, and stained intracellularly using Abs to IFN-\( \gamma \) and TNF-\( \alpha \). Samples were analyzed on a LSRII cytometer (BD Biosciences), acquiring ~1 million events per sample. Data were analyzed using FlowJo software (Tree Star) and a live cell gate was performed using forward and side scatter characteristics. The number of IFN-\( \gamma \)-TNF-\( \alpha \) T cells was quantitated after first gating on live CD8\(^+\) CD4\(^-\) cells, and the number of IFN-\( \gamma \)-TNF-\( \alpha \)-events from uninfected cultures was subtracted. To measure intracellular staining of human PBMC for orthopoxviruses, cells were stained overnight at 4°C with CD14-specific Abs. Cells were fixed, permeabilized, and stained intracellularly using a biotinylated rabbit polyclonal Ab to orthopoxvirus Ags (anti-vaccinia; ViroStat) followed by washing and staining with streptavidin-Pacific Blue. Samples were analyzed on a LSRRII cytometer and data were analyzed using FlowJo software. A live cell gate was performed using forward and side scatter characteristics and the number of orthopoxvirus-infected cells was quantitated after subtracting nonspecific binding events from uninfected cultures.
Mice were obtained from The Jackson Laboratory and infected i.p. with 2 × 10⁵ PFU of VV or CPV. Spleens were collected on day 8 postinfection for analysis of antiviral T cell responses as described (25), and 10⁵ splenocytes were mixed with 5 × 10⁴ A20 or MC57 cells that were uninfected or infected overnight. After 6 h of incubation with the target cells in the presence of BFA, cells were stained for CD8 and then fixed, permeabilized, and stained intracellularly for IFN-γ and TNF-α. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Immunoblots and immunoprecipitation

For immunoblotting, cell lysates were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was then blocked by 5% fat-free milk in TBS-Tween 20 (0.2%) and probed with the Abs K655 or anti-calreticulin. For immunoprecipitation, infected or uninfected HeLa cells were starved for 1 h beginning at 3 h postinfection (hpi) and metabolically labeled for 20 min with [35S]cysteine-[35S]methionine (Amersham Biosciences) (100 μCi/10⁵ cells). After labeling, the cells were washed twice with PBS and either harvested immediately or chased in regular medium supplemented with excess unlabeled methionine and cysteine for the indicated times. Cells were lysed in PBS containing 1% Nonidet P-40. The cell lysate was precleared with 30 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 2 h followed by incubation with W6/32 for 2 h. The immune complexes were captured by incubation with 30 μl of protein A/G beads. The precipitate was washed 3× with 0.1% Nonidet P-40 and then incubated in PBS containing 1% Nonidet P-40. The eluate was incubated with or without EndoH overnight. Upon boiling in Laemmli SDS sample buffer, samples were separated by 10% SDS-PAGE and detected by autoradiography. For CD4 molecules, the same protocol was used with some modifications; cell line that was used was HeLa expressing CD4 constitutively (26), the pulse was done for 30 min, and all of the samples were treated with EndoH.

To measure MHC I thermostability, lysates were incubated at either 0°C or at 37°C for 1 h before immunoprecipitation.

Results

CPV and VV-specific CTL are stimulated by target cells infected with VV, but not CPV

To study the T cell responses to orthopoxviruses, we infected BALB/c and C57BL/6 mice with either CPV or VV and analyzed the splenic CD8⁺ T cell response against CPV- or VV-infected target cells 8 days later (Fig. 1). CD8⁺ T cells from VV-infected BALB/c mice (H-2d haplotype) recognized VV-infected targets and produced IFN-γ (Fig. 1A) (25). In contrast, <1% of VV-induced CD8⁺ T cells responded to CPV-infected targets. Because CPV and VV infected A20 cells at similar rates (data not shown), this suggested that either the immunodominant epitopes of CPV were different from those of VV or that cross-reactive CPV peptides (27, 28) were not effectively presented. To distinguish between these two possibilities, we examined the responsiveness of antiviral CD8⁺ T cells from CPV-infected mice. If different immunodominance profiles were involved, then one would expect to observe higher IFN-γ responses by CPV-specific T cells stimulated with CPV-infected targets than when stimulated with VV-infected targets. Instead, we observed that CPV-specific CD8⁺ T cells were 10-fold more capable of recognizing VV-infected targets than CPV-infected targets (Fig. 1A). Similarly, CD8⁺ T cells from VV- or CPV-infected C57BL6 mice (H2-b haplotype) responded to VV-infected targets but did not recognize CPV-infected targets (Fig. 1B) despite >90% infection of MC57 target cells by either virus. In addition, there was no significant difference in viral titers obtained from cells infected with either virus (data not shown). This lack of virus-specific recognition of CPV-infected cells was not limited to IFN-γ production, because antiviral TNF-α responses were also similarly inhibited (data not shown). Thus, our data indicate that CPV, but not VV, evades MHC I-restricted T cell recognition.

CTL from vaccinated human subjects recognize VV-, but not CPV-infected targets

Rodents represent the natural reservoir for CPV and it was thus possible that this stealth mechanism was restricted to the evasion of rodent T cells. To determine whether CPV evades recognition by human T cells, we examined the activation of orthopoxvirus-specific T cells obtained from subjects who had recently received the smallpox vaccine (vaccinia). The first step in these experiments was to measure VV and CPV infection of primary human cells. This was accomplished by staining PBMC at 18 h postinfection for
intracellular expression of orthopoxvirus Ags using polyclonal anti-orthopoxvirus Abs (Fig. 2A). The CD14<sup>+</sup>/H11001 monocyte is the main cell type in human PBMC that are infected by orthopoxviruses and we found that both VV and CPV readily infected monocytes at similar frequencies. (Fig. 2A and data not shown). To measure virus-specific T cell recognition of VV- or CPV-infected cells, PBMC were cultured in medium alone or infected with the indicated amounts of virus for 18 h with BFA added for the last 6 h to increase intracellular accumulation of antiviral cytokines (7). In these experiments, we used VV at an optimized dose (MOI of 0.3) and CPV at the same dose (MOI of 0.3). Alternatively, we mixed VV with CPV at a VV:CPV ratio of 10:1, 1:1, or 1:10 (CPV MOI of 0.03, 0.3, or 3.0, respectively). To measure antiviral T cell responses, we pregated on CD8<sup>+</sup>/H9252CD4<sup>+</sup>/H11002 T cells and quantitated the number of T cells that expressed both IFN-γ/H9253 and TNF-α/H9251 (Fig. 2B and C) after 18 h of stimulation with either VV, CPV, or a mixture of VV and CPV. VV-infected cells induced substantial CD8<sup>+</sup> T cell responses, whereas the T cell response against CPV-infected cells was reduced by >100-fold. Moreover, the immune evasive effect of CPV was dominant over VV as shown by staining with an orthopoxvirus-specific Ab (Fig. 3A, right panel). Also, supernatants from CPV- and VV-infected HeLa cells showed comparable levels of virus release (data not shown). We examined whether CPV infection caused CPV down-regulates cell surface levels of MHC I

The most likely explanation for the observed evasion of T cell stimulation by CPV is that CPV-derived Ags are not displayed to T cells by MHC I. To examine whether CPV interferes with MHC I surface expression, we performed flow cytometry to determine MHC I levels. Indeed, MHC I surface levels were significantly reduced in CPV-infected cells compared with uninfected or VV-infected HeLa cells (Fig. 3A, left panel). In contrast, cell surface levels of CD44 (Fig. 3A, center panel), CD166, and transferrin receptor (data not shown) were not affected. Decreased MHC I was not caused by more effective infection by CPV compared with that by VV as shown by staining with an orthopoxvirus-specific Ab (Fig. 3A, right panel). Also, supernatants from CPV- and VV-infected HeLa cells showed comparable levels of virus release (data not shown). We examined whether CPV infection caused
down-regulation of murine MHC I by infecting the mouse fibroblast cell line MC57 with VV or CPV and staining for cell surface K\(^b\) with the mAb Y3. Similarly as in human cells, CPV infection caused a decreased level of MHC I at the cell surface, whereas VV infection had little effect (Fig. 3B, left panel). This result was specific, because cell surface levels of murine CD44 molecules remained unchanged (Fig. 3B, center panel). Moreover, murine cells were equally infected with CPV and VV (Fig. 3B, right panel).

To test the kinetics of MHC I down-regulation during CPV replication, we infected HeLa cells with CPV and measured MHC I surface levels at different time points post infection. As shown in Fig. 3C, down-regulation of MHC I was observed as early as 4 h after infection, with levels further decreasing during the remaining time points. In contrast, infection with VV did not cause any significant change in the levels of class I molecules during these periods of time (data not shown). The kinetics of MHC I down-regulation indicated that early gene product(s) of CPV might be responsible. Therefore, we examined whether blocking late gene expression by the addition of cytosine arabinoside (Ara-C) would restore MHC I surface levels. However, as shown in Fig. 3D, MHC I down-regulation by CPV occurred despite the presence of Ara-C. We conclude that a viral early gene is responsible for diminishing MHC I surface levels during infection with CPV.

To examine whether the reduction of MHC I expression occurred exclusively on infected cells, we measured MHC I levels by flow cytometry at 16 h after infection with either high (1.0) or low (0.1) MOI. At MOI of 1.0, nearly all cells were infected and displayed reduced MHC I levels (Fig. 4). At a lower MOI, CPV-positive cells displayed either normal or reduced MHC I levels. However, upon treatment with Ara-C to prevent virus replication, all CPV-positive cells displayed reduced MHC I levels at an MOI of 0.1. The latter observation is consistent with reduced MHC I expression being restricted to cells that were directly infected with input virus. The CPV-infected cells that display normal levels of MHC I in the absence of Ara-C were therefore likely caused by secondary spread of virus after the infection of cells at a low MOI. Because reduced MHC I levels are not detected before 4 hpi (Fig. 3C), any recently infected cells caused by secondary spread of virus in the cultures will likely display normal MHC I levels because they have not yet had time for MHC I down-modulation to occur.

**CPV does not reduce total levels of MHC I**

To determine whether the reduced MHC I surface levels were caused by a reduction of total MHC I, we examined steady state levels. At 16 hpi there was no significant change of MHC I or of calreticulin, used as a control, detected by immunoblot from CPV-infected cells compared with control (Fig. 5A). Similarly, intracellular staining of MHC I did not show reduced amounts in CPV-infected cells compared with uninfected cells (Fig. 5B). Therefore, we conclude that the reduction of MHC I surface levels is not due to specific or global inhibition of protein expression or accelerated protein degradation.

**CPV inhibits intracellular trafficking of MHC class I molecules**

The findings that MHC I surface levels, but not total MHC I, were reduced suggested that CPV mislocalizes MHC I. Therefore, we followed the fate of newly synthesized MHC I molecules using pulse-chase labeling and immunoprecipitation. Uninfected HeLa cells or cells infected with VV or CPV for 4 h were metabolically labeled for 20 min followed by various chase periods. MHC I was immunoprecipitated using the conformation-specific Ab W6/32, which recognizes assembled MHC I. Half of the precipitated material was treated with EndoH to monitor the maturation of MHC I glycan residues upon passage through the Golgi. In uninfected cells, MHC I molecules rapidly exited the ER as reflected by the acquisition of EndoH resistance (Fig. 6A). In VV-infected cells MHC I molecules exited the ER with similar kinetics. However, there was less MHC I recovered at the end of the chase period (see Discussion). In contrast, the apparent m.w. of MHC I molecules in CPV-infected cells was unaltered in both untreated and EndoH-treated samples throughout the labeling and chase period. Because the shift in mobility reflects the modification of oligosaccharide side chains as MHC I leaves the ER.
and traffics through the Golgi network en route to the cell surface, we conclude that MHC I molecules remain in the ER in CPV-infected cells. In infected cells, several coprecipitating bands are also recovered (Fig. 6A, arrowheads) and the implications for this is discussed later. To confirm that the inhibition of trafficking was MHC I-specific, we immunoprecipitated CD4 molecules from pulse/chase labeled in HeLa cells stably transfected with CD4. Asterisks indicate non-specific proteins. Arrowheads indicate coprecipitating proteins in virus-infected samples. Material was incubated with Endo H. Asterisks indicate nonspecific proteins.

CPV infection does not affect the thermostability of MHC I

MHC I molecules are retained in the ER by the specific chaperone tapasin until they acquire high affinity peptides (30). Therefore, the retention of MHC I in the ER could be the result of a lack in peptide loading, as observed for viruses that inhibit the peptide import by TAP (16, 17). Alternatively, CPV could prevent the exit of peptide-loaded MHC I molecules, a mechanism used by viral "anti-chaperones" (15). The binding of high affinity peptides to MHC I results in a complex that is more stable than "empty" MHC I heterodimers (31). To determine whether CPV infections cause a defect in the peptide loading of MHC class I molecules, we therefore determined the thermostability of MHC I molecules in the lysates of CPV-infected HeLa cells. As a positive control, we used HeLa cells infected with a recombinant adenovirus expressing the HSV-1 protein ICP47 (Ad-ICP47), which blocks peptide translocation into the lumen of the ER by TAP (16, 17). Lysates from uninfected, CPV-, or Ad-ICP47-infected HeLa cells were incubated at 4°C or 37°C for 1 h followed by immunoprecipitation with W6/32 (Fig. 7). In uninfected cells, MHC I molecules remain assembled and, thus, W6/32 reactive at the elevated temperature. In contrast, heteromeric immature MHC I was recovered from AdICP47-infected lysates kept at 4°C but not at 37°C. The observation that less MHC I was immunoprecipitated from Ad-ICP47-infected cells after 180 min of "chase" is due to the turnover of empty MHC I molecules as reported (32). Thus, TAP inhibition by ICP47 retained empty MHC I molecules in the ER that are ultimately degraded by the proteasome. Different from ICP47, however, the immature MHC I molecules recovered from CPV-infected cells were resistant to temperature challenge. (The decreased amounts of MHC I is likely due to actively replicating CPV competing for the label). In summary, these experiments show that CPV infection does not affect the peptide loading of MHC I.

Discussion

We report that CPV evades recognition by T cells from VV-immunized humans or from mice infected with VV or CPV. Because T cells of CPV-infected mice are able to recognize VV-infected target cells but not CPV-infected target cells, we conclude that this evasion is not due to a lack in cross-reactive T cells but represents an active immune evasion process. Consistent with an active process, it is our observation that CPV but not VV prevents the intracellular transport of MHC I molecules, suggesting that a CPV-encoded gene that is absent or nonfunctional in VV is responsible. Although intracellular trafficking of MHC I molecules was completely blocked by CPV, ER exit was unhampered by VV. However, we recovered reduced amounts of labeled MHC I over time from VV-infected cells. In parallel, we observed several unknown protein bands coprecipitating at the end of the chase period in both VV- and CPV-infected cells, but not in uninfected cells, suggesting that MHC I formed a complex with poxviral proteins. Although we have not identified these proteins, it is likely that these are virion proteins because MHC I is abundantly present in VV particles (33). Reduced recovery of MHC I in VV-infected cells is thus likely due to the release of virus particles. Because ER-retained MHC I in CPV-infected cells was not reduced during the chase, it is likely that the newly synthesized MHC I molecules do not leave the ER.
not assemble with cowpox viral proteins and that the virion proteins are coprecipitated by unlabeled, preexisting MHC I. Although this still needs to be verified experimentally, our data clearly show a dramatic reduction of MHC I transport by CPV but not by VV. The conclusive demonstration that ER-retention of MHC I renders CPV-infected cells invisible to T cells will have to await the identification and deletion of this open reading frame. However, the highly efficient retention of MHC I molecules that occurs very early during infection by CPV renders it highly likely that MHC retention is indeed the cause for CTL evasion.

Although such ER retention of MHC I molecules has been observed for other viruses, it has not been described for any poxvirus. The prototype MHC I retention molecule is the adenovirus protein E19, which links MHC I to the cellular vesicle retrieval machinery (reviewed in Ref. 15). The human CMV US3 protein also retains MHC I (24). However, E19 and US3 also affect MHC I maturation indirectly by binding to tapasin (34, 35) or by US3-mediated degradation of protein disulfide isomerase (36). Although thermostability assays suggest that CPV does not prevent MHC I peptide loading, MHC I molecules are more stable toward the end of the chase period, suggesting a slowdown of the loading process (Fig. 6). Further studies will be required to clarify the exact mechanism of retention.

This is the first report showing that orthopoxviruses prevent T cell recognition and inhibit the intracellular transport of MHC I. This finding suggests that a CPV-encoded gene that is absent or nonfunctional in VV is responsible. Previously, it was shown that VV moderately down-regulated MHC I (22). Similarly, we observed that VV completely shut down MHC I expression at 24 hpi (data not shown). However, this was equally observed for CPV and thus unlikely responsible for the observed immune evasion phenotype.

This stealth mechanism did not appear to greatly impair the induction of T cell responses to CPV in vivo, because VV-infected targets stimulated a high percentage of T cells from CPV-infected mice (Fig. 1, A and B). Similar observations have been reported for T cells from murine CMV-infected mice that were unable to recognize murine CMV-infected cells in vitro unless viral immune modulators had been deleted from the viral genome. Nevertheless, mice infected with deletion viruses displayed an immune response that was virtually identical with that of wild type-infected mice (37). A possible interpretation of these results as well as of ours is that the induction of T cell responses occurs indirectly via cross-presentation of infected cell material by APCs, a process that is different from the recognition of infected cells by activated T cells.

MHC I evasion might play a role in CPV virulence as exemplified in the myxoma model. Rabbits infected with a myxoma virus lacking the MHC I-degrading ubiquitin ligase, M153R (26), showed considerably fewer secondary lesions than wild type, suggesting that viral dissemination was reduced (38). It is thus likely that reducing T cell recognition aids the dissemination of a cell-associated virus. VV is deficient in its ability to disseminate from the primary site of inoculation in healthy adults, whereas more virulent viruses such as MPV and VAR rapidly disseminate throughout the body. Although we do not know whether VAR escapes CTL recognition, we observed that the CTL of patients infected with MPV during the 2003 U.S. outbreak are largely unable to recognize MPV-infected targets but are activated by VV-infected targets (E. Hammarlund and M. K. Slifka, unpublished observations). It is thus highly likely that the immune evasion mechanism described here is conserved among orthopoxviruses but is lost from the genome of VV. An alternative explanation is that VV originates from a virus that lacks the ability to retain MHC class I. Although the origin of VV is not entirely clear, recent evidence suggests that VV is much more closely related to horsepox virus (HSPV) than to either CPV or MPV (39). Moreover, experimental infection of horses with VV can produce the same clinical symptoms as those of HSPV (40). Additional evidence that HSPV might be the ancestor of VV comes from E. Jenner’s original studies in which the vaccine that he used was from HSPV, referred to as the “grease” infection of horses (for a recent discussion see Ref. 41). Thus, it will be interesting to determine whether HSPV is able to retain MHC I molecules or, akin to VV, lacks the ability to evade the cellular immune response.

Abs play a major role in controlling orthopoxvirus infection through the neutralization of free virus as well as through other potential mechanisms (42, 43). For this reason, evasion of the host T cell response is likely a key attribute of the cell-associated viremia observed following infection with disseminating orthopoxviruses, because this simultaneously provides evasion from antiviral T cells as well as the circulation of neutralizing Abs. Understanding the mechanisms involved with the pathogenesis of virulent orthopoxvirus infection will be critical for developing better vaccines and antiviral therapies, and the discovery of orthopoxvirus immune evasion of antiviral T cell responses opens a new field of investigation.

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