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MULTIPLE VIRAL LIGANDS NATURALLY PRESENTED BY DIFFERENT CLASS I MOLECULES IN TRANSPORTER ANTIGEN PROCESSING-DEFICIENT VACCINIA VIRUS-INFECTED CELLS

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ABSTRACT

The transporter associated with antigen processing (TAP) delivers the viral proteolytic products generated by the proteasome in the cytosol to the endoplasmic reticulum lumen that are subsequently recognized by cytotoxic T lymphocytes (CTL). However, several viral epitopes have been identified in TAP-deficient models. Using mass spectrometry to analyze complex human leukocyte antigen (HLA)-bound peptide pools isolated from large numbers of TAP-deficient vaccinia virus-infected cells, we identified eleven ligands naturally presented by four different HLA-A, -B, and -C class I molecules. Two of these ligands were presented by two different HLA class I alleles, and thus, thirteen different HLA/peptide complexes were formed simultaneously in the same vaccinia-infected cells. In addition to the high-affinity ligands, one low-affinity peptide restricted by each of the HLA-A, -B and -C class I molecules was identified. Both high- and low-affinity ligands generated long-term memory CTL responses against vaccinia virus in an HLA-A2 transgenic mouse model. The processing and presentation of two vaccinia virus-encoded HLA-A2-restricted antigens could take place via proteasomal and non-proteasomal pathways, which were blocked in infected cells with chemical inhibitors specific for different subsets of metalloproteinases. These data have implications for the study of the effectiveness of early empirical vaccination with cowpox virus against smallpox disease.

INTRODUCTION

The eradication of smallpox, a disease caused by variola major virus, was made possible by early empirical, cross-protective vaccination with cowpox virus and later through the massive worldwide production and administration of vaccinia virus (VACV) (17). The *Orthopoxvirus* vaccinia is a widely used tool for research and vaccine development (46), and recent concerns about bioterrorism and emerging infectious diseases have elicited renewed interest in VACV and other poxviruses (30). Vaccination induces a strong humoral response leading to viral clearance, and the role of cellular responses in this cross-protection is well documented (20),(55). In recent years, studies in both vaccinated humans and human histocompatibility complex (HLA)-transgenic mouse models have allowed the identification of more than 70 VACV-derived epitopes restricted by various HLA molecules (reviewed in (31),(29)).

In cellular immunity, the recognition and killing of infected cells by $CD8^+$ cytolytic T lymphocytes (CTLs) first requires viral proteins to be proteolytically degraded (73). Antigen processing generates short peptides that are translocated to the endoplasmic reticulum lumen by transporter associated with antigen processing (TAP) and then assembled with newly synthesized β2-microglobulin and HLA class I heavy chain. Despite initial assumptions that the multi-catalytic and ubiquitous proteasome was the only protease fully capable of generating peptide ligands for presentation on HLA class I molecules, several studies have demonstrated that a growing number of alternative pathways contribute to endogenous antigen processing (reviewed in (12),(26)).

Individuals with mutations in the TAP gene that generate non-functional TAP complexes have been described (reviewed in (8)). Patients with this HLA

class I deficiency may appear asymptomatic for long periods of their lives. TAP-deficient patients do not seem particularly susceptible to viral infections or neoplasms. Therefore, their immune systems must be reasonably efficient, and antibodies, NK cells, $CD8^+$ γδ T cells, and the reduced cytolytic $CD8^+$ αβ T subpopulation that is specific for TAP-independent antigens may all contribute to immune defenses that protect against severe viral infections in these individuals. In addition, several strains of viruses, including cowpox virus (1), have found ways to obstruct TAP expression or function in order to prevent CTLs from identifying infected cells (reviewed in (35)); therefore, the TAP-independent pathways must be important for killing cells infected with these viruses. The identification of TAP-independent epitopes conserved among orthopoxviruses could also be relevant to the study of the mechanisms of early empirical vaccination against smallpox disease performed with cowpox virus.

Although TAP-independent viral epitopes are known (reviewed in (35)), there is a marked absence of methodical studies of TAP-independent epitopes/ligands restricted by different HLA molecules in cells infected with a single virus. Moreover, in all previous studies, very limited antigen processing capacity has been reported in TAP-deficient cells. Therefore, is only one TAP-independent ligand/epitope restricted by a single HLA molecule exposed on the cell membrane surface, as suggested by these studies? Conversely, could a TAP-deficient cell bind several viral ligands to different HLA molecules simultaneously? We are interested in the identification of viral ligands presented by several common HLA antigens in TAP-deficient infected cells. Using immunoproteomic analysis, we compared HLA ligands isolated from large numbers of healthy or VACV-infected cells. This study identified eleven TAP-

independent, naturally processed ligands from eight different VACV proteins in infected cells that were mostly conserved among the *Orthopoxviridae* family, including cowpox virus.

MATHERIALS AND METHODS

Mice

H-2 class I knockout HLA-A*0201-transgenic mice (19) were bred in our animal facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish government regulations (accreditation number 28079-34A). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Health "Carlos III" (Permit Number: PI-283). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell lines

T2 cells, a line of TAP-deficient human cells that express HLA-A2, -B51, and -Cw1 class I molecules on their surface (57), were transfected with HLA-B27 (a gift from Dr. David Yu, University of California, Los Angeles, CA). The mouse cell lines RMA (TAP positive) and RMA-S (TAP negative) were transfected with HLA-A*0201 α1α2 domains, and the mouse H-2D^{b} α3 transmembrane and cytoplasmic domains have been previously described (51). The RMA-S transfectant cells expressing HLA-B*2705 have been previously described (66). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 5 μM β-mercaptoethanol.

Synthetic peptides.

Peptides were synthesized in a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and purified by reverse-phase HPLC. The

correct molecular mass of the peptides was established by MALDI-TOF MS, and their correct composition was determined by quadrupole ion trap microHPLC.

Inhibitors.

Brefeldin A (BFA) and all protease inhibitors were purchased from Sigma-Aldrich, except for leupeptin (Amersham-UBS), pepstatin (Boehringer Mannheim), Z-VAD.fmk (Enzyme System Products, CA, USA), $(z\text{-}LL)_2$ ketone (Merck), and lactacystin (Dr. E. J. Corey, Harvard University). The specificity and activity of all inhibitors used in this study were summarized in Table 1. For control of activity of the protease inhibitors, RMA-HLA-A*0201 cells (1×10^8) were disrupted by sonication for 15 min at 4ºC, and centrifuged as previously reported (39). A supernatant aliquot corresponding to 1×10^7 cells was directly frozen (non-degraded control). Equivalent aliquots were incubated in the presence of individual inhibitors at 200 μM and digestion by cellular proteases was allowed for 5 days at 37ºC in PBS. Inhibitors were renewed daily. A sample incubated without inhibitors was taken as the degraded control. After SDS-PAGE separation and Coomassie Blue staining of these samples, the overall protein content of each lane was quantitated by densitometry with the TINA 2.09e program (Isopenmeβgeräte, GmbH, Germany). % Inhibition of protein degradation caused by each inhibitor was calculated as follows: 100 x (sample with inhibitor – degraded)/(non-degraded – degraded).

Infection of the T2-B27 cell line by VACV

1-3 x 10^9 T2-B27 cells were infected with VACV at a multiplicity of infection of 10 plaque-forming units/cell in 10 ml, incubated for 2 h at 37ºC and then washed. The cells were then cultured for 24 h and stained with the Omnitope antiserum-FITC that recognizes VACV purified virions. Samples were analyzed by FACS. Later the cells were frozen.

Isolation of HLA-bound peptides.

HLA-bound peptides were isolated from 4 x 10^{10} healthy or VACV-WR-infected T2-B27 transfectant cells. Cells were lysed in 1% CHAPS (Sigma), 20 mM Tris/HCl buffer, and 150 mM NaCl, pH 7.5, in the presence of a protease inhibitor cocktail (23). HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following mAbs, used sequentially: PA2.1 (anti-HLA-A2) (50), ME1 (anti-HLA-B27) (13), and W6/32 (specific for a monomorphic HLA class I determinant) (5) (Figure 1). HLA-bound peptides were eluted at room temperature with 0.1% aqueous trifluoroacetic acid (TFA), separated from the large subunits and concentrated with a Centricon 3 column (Amicon, Beverly, MA) exactly as previously described (23).

Electrospray-ion trap mass spectrometry analysis.

Peptide mixtures recovered after the ultra-filtration step were concentrated using Micro-Tip reversed-phase columns (C_{18}, 200 µl, Harvard Apparatus, Holliston, MA) (23). Each C_{18} tip was equilibrated with 80% acetonitrile in 0.1% TFA, washed with 0.1% TFA, and then loaded with the

peptide mixture. The tip was then washed with an additional volume of 0.1% TFA, and the peptides were eluted with 80% acetonitrile in 0.1% TFA. Peptide samples were then concentrated to about 18 µl using vacuum centrifugation (23).

HLA class I peptides immunoprecipitated with each HLA-specific mAb were analyzed in three different HPLC runs by µLC-MS/MS using an Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) fitted with a capillary HPLC column (Eksigent, Dublin, CA) (23). The peptides were resolved on homemade Reprosil C18 capillary columns (75 micron ID) (24) with a 7-40% acetonitrile gradient for 2 h in the presence of 0.1% formic acid. The seven most intense masses that exhibited single-, double-, and triple-charge states were selected for fragmentation from each full mass spectrum by CID.

Database searches.

Sequest 3.31 (Thermo-Fisher) (14) was used for peak-list generation of the µLC-MS/MS data. The peaks were identified by using Proteome Discoverer 1.0 SP1 (Thermo-Fisher), combining the results of Sequest 3.31 and Bioworks Browser 3.3.1 SP1 (Thermo-Fisher) (14), and using the human and virus parts of the NCBI database (Jan 2009), which includes 656,486 proteins. The search was not limited by enzymatic specificity, the peptide tolerance was set to 0.005 Da, and the fragment ion tolerance was set to 0.5 Da (23)'(42). This search was not limited by any methodological bias (selection of individual protein, use of HLA consensus scoring algorithms, etc.). Identified peptides were selected if the following criteria were met: Sequest Xcorr >1.4 for singly, >2.2 for doubly,

194 and >2.9 for triply charged peptides; P(pep) less than 1 x 10^{-3}; and mass
195 accuracy of 0.005 Da (23)'(42). When the MS/MS spectra fitted more than one
196 peptide, only the highest scoring peptide was analyzed. No peptides were
197 found in a search of a reversed database. The purpose of the filtering criteria
198 was to identify candidate vaccinia virus peptide MS/MS scans for further
199 manual inspection to determine whether the MS/MS fragment ion fingerprint
200 matched the identified peptide sequence. In addition, the corresponding
201 synthetic peptide was made, and its MS/MS spectrum was used to confirm the
202 assigned sequence.
203
204 *MHC/peptide stability assays.*
205
206 The following synthetic peptides were used as controls in complex
207 stability assays: KPNA2 (GLVPFLVSV, HLA-A2-restricted) (22), Flu NP
208 (SRYWAIRTR, HLA-B27-restricted) (67), HBV HBc_{19-27} (LPSDFFPSV, HLA-
209 B51-restricted) (7), CMV $pp65_{7-15}$ (RCPEMISVL, HLA-Cw1-restricted) (32), and
210 C4CON (QYDDAVYLK, HLA-Cw4-restricted) (16). Either RMA-S transfectants
211 or T2 cells expressing low amounts of MHC class I on the cell surface were
212 incubated at 26°C for 16 h in RPMI 1640 medium supplemented with 10% heat-
213 inactivated fetal bovine serum (FBS). This allows the expression of empty MHC
214 class I molecules (without antigenic peptide) that are stable only at 26ºC, but
215 not at 37ºC, on the cell membrane. The cells were washed and incubated for
216 2 h at 26 °C with various concentrations of peptide in the same medium.
217 Afterward, the cells were kept at 37 °C and collected for flow cytometry after
218 4 h. This assay allows for the internalization of empty MHC class I molecules

and can therefore discriminate between bound and unbound peptides. HLA expression levels were measured using the Abs monoclonal PA2.1 (anti-HLA-A2), monoclonal ME1 (anti-HLA-B27), polyclonal H00003106-B01P (specific for HLA-B class I molecules) (Abnova, Taipei, Taiwan), and polyclonal SC-19438 (specific for HLA-C class I molecules) (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (40). Samples were acquired on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). Cells incubated without peptides had peak fluorescence intensities close to background staining with secondary Ab alone. The fluorescence index (FI) was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. The binding of peptides was also expressed as the EC_{50}, which is the molar concentration of the peptides producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 µM.

Magnetic antigen cell separation (MACS)

Mouse T lymphocytes were isolated by the depletion of non-T $CD8^+$ cells (negative selection) using the T $CD8a^+$ Cell Isolation Kit (Miltenyi Biotec GmbH, Gladbach, Germany) according to the manufacturer's specifications. The purity of the cell preparations recovered after negative selection was verified by fluorescence-activated cell sorting (FACS) and found to be higher than 95% for $CD8^+$ T lymphocytes (56).

Ex vivo intracellular cytokine staining (ICS)

Intracellular cytokine staining assays were performed as described previously (58). Purified $CD8^+$ T lymphocytes were obtained from HLA-A*0201 transgenic mice up to 30 days (memory) post i.p. infection with 1 x 10^7 PFU VACV-WR, stimulated for 2 h with either RMA or RMA-S HLA-A*0201 cells infected with VACV-WR, and incubated overnight in the presence of 5 µg/ml BFA. Later, cells were incubated with FITC-conjugated anti-CD8 mAb (ProImmune, Oxford, UK) for 30 min at 4°C, fixed with Intrastain kit (DakoCytomation, Glostrup, Denmark) reagent A, and incubated with PE-conjugated anti-IFN-γ mAb (BD PharMingen, San Diego, CA) in the presence of Intrastain kit permeabilizing reagent B for 30 min at 4°C. Events were acquired and analyzed as MHC/peptide stability assays.

T cell lines, cytotoxicity assays, and ICS

Polyclonal SIINFEKL or VACV peptide-monospecific CTLs were generated by immunizing mice i.p. with 1 x 10^7 PFU VACV-$OVA_{257–264}$ encoding the miniprotein MSIINFEKL or VACV-WR as previously described (44),(39), respectively. Splenocytes from immunized mice were re-stimulated *in vitro* with mitomycin C-treated spleen cells pulsed with 10^{-6} M of the respective peptide and cultured in α-MEM supplemented with 10% FBS, 1x10^{-7} M peptide and 1% 2-ME. Recombinant human interleukin-2 for the long-term propagation of peptide-specific CTL lines was generously provided by Hoffmann-LaRoche. The RMA-HLA-A*0201 cells were used as target cells in standard 4 h cytolytic assays (39).

ICS assays to detect the recognition of infected cells by polyclonal CTL cell lines were performed as previously described (9). CTL lines were stimulated for 4 h in the presence of 5 µg/ml BFA and target cells that had been infected with VACV or VACV-OVA$_{257–264}$ overnight. When protease inhibitors were used, all drugs were added 15 min before the virus and kept at a 5-fold higher concentration during the 1 hr adsorption period than throughout the infection. After washing the virus inoculum, the inhibitors were kept at the concentrations indicated for the individual experiments. The inhibitors were not toxic at the indicated concentrations since they not affect either antigen presentation both the A10L$_{688-696}$ and A17L$_{9-17}$ epitopes (see below) or VACV infection when the Omnitope antiserum with specifity for VACV proteins from purified virions (ViroStat Inc., Portland, ME) was used (Supplemental Fig. 1). The ICS with polyclonal CTL was performed similarly to *ex vivo* ICS.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed as published (44). Spleens were obtained, erythrocytes were removed, and HLA-A*0201 splenocytes were split in two populations and labeled with either a high concentration (5 µM) or a low concentration (0.5 µM) of CFSE. After washing excess CFSE, CFSE^{high} spleen cells were pulsed with 10^{-6} M VACV peptides for 30 min at 37°C. Excess peptide was washed at least twice, and CFSE^{high} peptide-pulsed cells were mixed with equal numbers of CFSE^{low} cells. A total of 8×10^6 cells of the spleen cells mixed suspension were i.p. injected into each HLA-A*0201-transgenic mouse, which had been infected or not with i.p. 1×10^7 PFU VACV-WR 7 days

earlier. Two days later, the peritoneal cavity was lavaged, and spleens were extracted, and the cells were analyzed by flow cytometry to measure in vivo killing, using a FACSCanto flow cytometer. Data were analyzed using CellQuest Pro 2.0 software. Specific lysis was calculated as published (44) according to the formula: [1 − (ratio unprimed/ratio primed) × 100], where the ratio unprimed is $\%CFSE^{low}/\%CFSE^{high}$ cells remaining in control uninfected recipients, and the ratio primed is $\%CFSE^{low}/\%CFSE^{high}$ cells remaining in experimental infected recipients.

RESULTS

VACV-specific CD8$^+$ T cells recognize TAP-deficient HLA-A*0201-transfected cells.

As the first step in the study of TAP-independent HLA-restricted responses to vaccinia virus, HLA-A*0201 transgenic mice were immunized with the virus. Next, the VACV-specific CD8$^+$ response was evaluated using by intracellular cytokine staining (ICS) assays. A strong *ex vivo* response (50.1 ± 9.4% of CD8+ cells secreted IFNγ) specific for this virus was detected in TAP$^+$ target cells (Fig. 2, left panel). Additionally, a small fraction of VACV-specific CD8$^+$ T lymphocytes recognized infected TAP-deficient cells (3.8 ± 0.8% of CD8+ cells secreted IFNγ, Fig. 2, right panel). These data indicate the existence of a TAP-independent antigen processing pathway(s) of some vaccinia virus epitopes in infected cells that could be recognized by specific CD8$^+$ T lymphocytes.

Physiological processing generates three different viral HLA-A2 ligands in human TAP-deficient vaccinia-infected cells.

HLA-A2-bound peptide pools were isolated from large numbers of either healthy or VACV-infected human TAP-deficient cells (110 ± 20 of MFI in VACV infected cells vs 15 ± 5 in healthy cells stained with an anti-VACV antiserum). These peptide mixtures were subsequently separated by reverse-phase HPLC and analyzed by mass spectrometry. Using bioinformatics tools, three

fragmentation spectra present in the VACV-infected HLA-A2-bound peptide pool, but absent from the control uninfected pool, were resolved with high confidence parameters as peptides of vaccinia virus proteins. A human proteome database search failed to identity any of these spectra as human protein fragments, confirming the viral origin of these peptides. The first ion peak, with an *m/z* of 926.4, was assigned to the viral amino acid sequence MLDDFSAGA, spanning residues 9-17 of the A17L protein of vaccinia virus (Supplemental Fig. 2, upper panel). In addition, two different ion peaks at *m/z* 974.6 and 514.8 were assigned to peptides of the same viral protein. These ion peaks corresponded to SPEGEETII (Supplemental Fig. 2, medium panel) and ILDRIITNA (Supplemental Fig. 2, lower panel) peptides, which span residues 614-623 and 688-696, respectively, of the A10L protein. Virtually all significant fragments of the three MS/MS spectra were assigned as daughter ions of the putative peptidic sequences (Supplemental Fig. 2). This theoretical assignment was confirmed by identity with the MS/MS spectrum of the corresponding synthetic peptide (Supplemental Fig. 2). Therefore, these results indicate that a total of three TAP-independent HLA-A2 ligands were endogenously processed and presented in the VACV-infected cells.

Binding affinity of TAP-independent vaccinia virus ligands for the A*0201 molecule.

The classical anchor motifs for HLA-A*0201 binding, Leu or Met at position 2 (P2) and aliphatic C-terminal residues (SYFPEITHI database: http://www.syfpeithi.de (53)), were present in two of the three detected TAP-

independent viral ligands. In contrast, the ligand $A10L_{614-623}$ presented Pro at P2, although it was co-immunoprecipitated with an HLA-A2-specific mAb and thus could be an unusual HLA-A2-restricted ligand. To confirm that HLA-A*0201 was the MHC class I molecule that presented these ligands, MHC/peptide complex stability assays were performed using TAP-deficient RMA-S cells transfected with the HLA-A*0201 molecule (Fig. 3). The two viral ligands with HLA-A2 anchor motifs, $A10L_{688-696}$ and $A17L_{9-17}$, bound to HLA-A*0201 class I molecules with EC_{50} values in the range commonly found among other natural high-affinity ligands (Fig. 3B). In contrast, the HLA affinity was substantially lower for the $A10L_{614-623}$ ligand, as the absence of HLA-A2 anchor motifs suggested; therefore, this peptide must be considered a low-affinity ligand. These data confirm that all ligands detected in vaccinia virus-infected cells were endogenously presented in association with the A*0201 molecule.

Three viral HLA-B*2705 ligands were endogenously processed in human TAP-deficient vaccinia virus-infected cells.

To date, about 60 human TAP-independent MHC class I ligands are known (35)-(70), and these are mostly restricted by HLA-A2 and derived from the cleavage of signal sequences generated by the SPase complex. The A*0201-restricted CTL epitopes of vaccinia virus that we detected in TAP-deficient cells could therefore be exceptional, and TAP-independent antigen processing pathways might be unable to generate vaccinia virus peptides that could bind to other HLA class I molecules. HLA-B27, which was previously described as an allele with high TAP dependency (2), was used to obtain

peptide pools from either healthy or VACV-infected TAP-deficient cells, as used to identify HLA-A*0201 ligands. Again, three fragmentation spectra present in the VACV-infected, HLA-B27-bound peptide pool, but absent from the control uninfected pool, were also resolved as peptides of vaccinia virus proteins. The human proteome database search failed to identify these spectra as human protein fragments, confirming the viral origin of these HLA-B27-bound peptides. The first ion peak, with an *m/z* of 428.8, was assigned to the viral amino acid sequence SRGYFEHMKK, spanning residues 867-876 of the A10L protein of vaccinia virus (Supplemental Fig. 3, upper panel), indicating that antigen processing of this protein could generate several viral ligands bound to two different HLA class I molecules. The second ion peak, at *m/z* 567.6, was assigned to the peptidic sequence YRLQGFTNAGIVAYK (Supplemental Fig. 3, medium panel), which spans residues 16-30 of the K2L protein. Finally, the third ion peak, at *m/z* 471.8, corresponded to a WQTMYTN peptide (Supplemental Fig. 3, lower panel), which spans residues 53-59 of the B8R protein. Supplemental Fig. 3 shows that all significant fragments of these three MS/MS spectra were assigned as daughter ions of the putative peptidic sequences. As HLA-A2 ligands, these assignments were confirmed by identity with the MS/MS spectrum of the corresponding synthetic peptide (Supplemental Fig. 3). In addition, the $K2L_{16\text{-}30}$ ligand was also identified as a molecular ion at *m/z* +2 (Supplemental Fig. 5). Collectively, these results indicate that a similar number of TAP-independent ligands were endogenously processed and presented by HLA-A2 or –B27 class I molecules in the same vaccinia virus-infected cells.

Binding affinity of TAP-independent vaccinia virus ligands for the B*2705 molecule.

The $A10L_{867-876}$ and $K2L_{16-30}$ (but not $B8R_{53-59}$) peptides have the known anchor motifs for HLA-B*2705 binding Arg at P2 and basic or aliphatic C-terminal residues (SYFPEITHI database (53)). As the $B8R_{53-59}$ peptide was also co-immunoprecipitated with an HLA-B27-specific mAb, it could be an unusual HLA-B27-restricted ligand. To confirm that HLA-B*2705 is the MHC class I molecule that presents these ligands, MHC/peptide complex stability assays were performed using TAP-deficient RMA-S cells transfected with the HLA-B*2705 molecule (Figure 4). The two viral ligands with HLA-B27 anchor motifs, $A10L_{867-876}$ and $K2L_{16-30}$, bound to HLA-B*2705 class I molecules with EC_{50} values to similar those of other natural high-affinity ligands (Fig. 4B). In contrast, the HLA affinity was substantially lower for the $B8R_{53-59}$ ligand, as suggested by the absence of HLA-B27 anchor motifs, and this peptide could be considered a low-affinity ligand. These data confirm that the ligands detected in vaccinia virus-infected cells were endogenously presented in association with the B*2705 molecule. In summary, either HLA-A*0201 or B*2705 class I molecules can bind both high- and low-affinity ligands derived from different vaccinia virus proteins.

Six vaccinia virus ligands were endogenously presented by HLA-B51 and/or –Cw1 class I molecules in human TAP-deficient cells.

Given that several TAP-independent viral ligands were identified in association with either HLA-A*0201 or B*2705 class I molecules (see above), we investigated the possibility of new vaccinia virus ligands being presented by other HLA class I molecules expressed in the same T2 cells. As in the previous two analyses, six fragmentation spectra were found in the VACV-infected peptide pool that were absent from the uninfected control pool, and these spectra were also resolved as peptides of vaccinia virus proteins. Furthermore, the human proteome database search also failed to identify these spectra as human protein fragments, confirming the viral origin of these HLA-bound peptides. Supplemental Figures 4 and 5 show the experimentally obtained MS/MS spectra and their respective assignments. Each putative peptidic sequence was confirmed by identity with the MS/MS spectrum of the corresponding synthetic peptide (Supplemental Fig. 4 and 5). Five of these peptides were new viral sequences: IAMKRTLLEL (D5R $_{148\text{-}157}$), LPFGSLGI (A50R $_{294\text{-}301}$), IPSPGIMLV (C11R $_{101\text{-}110}$), MLDDFSAGAGVLDKDL (A17L $_{9\text{-}25}$), and DGLIIISI (D8L $_{112\text{-}119}$). Surprisingly, the K2L$_{16\text{-}30}$ ligand previously detected in association with HLA-B*2705 (Supplemental Figure 3) was also immunoprecipitated in the third round with the W6/32 Ab. As HLA-A2, - B51, and –Cw1 present peptides with similar anchor motifs (SYFPEITHI database, (53)), HLA/peptide complex stability assays were performed to confirm that the sequential immunoprecipitation was performed correctly and to exclude the possibility that residual HLA-A2-bound ligands that were not fully immunoprecipitated with the PA2.1 (anti-HLA-A2) Ab in the first round would be immunoprecipitated in the third round with the W6/32 Ab (specific for a monomorphic HLA class I determinant). Figure 5A shows that, in contrast to the

positive control KPNA2 peptide, binding of HLA-A2 complexes to these six ligands was not detected. Therefore, these viral ligands do not bind to HLA-A2, which validates the experimental strategy used. In addition, to identify the HLA restriction of these ligands, new HLA/peptide complex stability assays using TAP-deficient T2 cells with specific anti-HLA-B or -C Abs were performed. The numbers of HLA-peptide surface complexes induced by the IAMKRTLLEL (D5R$_{148-157}$), LPFGSLGI (A50R$_{294-301}$), and IPSPGIMLV (C11R$_{101-110}$) synthetic peptides were similar to those induced by a well-known HLA-B51 ligand, HBV HBc$_{19-27}$ (Fig. 5B), using the anti-HLA-B Ab, indicating that these peptides were restricted by the HLA-B51 class I molecule. HLA stabilization was detected using anti-HLA-C (Fig. 5C) Ab with IPSPGIMLV (C11R $_{101-110}$), MLDDFSAGAGVLDKDL (A17L $_{9-25}$), and DGLIIISI (D8L $_{112-119}$), indicating that these peptides are restricted by the HLA-Cw1 allele. In addition, the K2L$_{16-30}$ ligand also bound to HLA-B*2705 (Supplemental Fig. 3 and Figure 4) was positive in their binding to HLA-Cw1 class I molecule justifying their dual immunoprecipitation (Figure 5). Five of these six viral ligands bound to HLA-B51 (Fig. 5D) or –Cw1 (Fig. 5E) class I molecules with EC$_{50}$ values similar to those of other natural high-affinity ligands. The only exception was the K2L$_{16-30}$ peptide, which showed a substantially lower affinity for HLA-Cw1, indicating that this peptide could be considered a low-affinity ligand (Fig. 5E).

Thirteen natural peptide-HLA class I complexes were formed simultaneously in the same infected TAP-deficient cells.

A total of eleven viral ligands were identified bound to the four HLA class I molecules expressed in the T2 cells (summarized in Table 2). Similar numbers of ligands (3 or 4) were identified in association with HLA class I molecules previously described as having low (HLA-A2), high (HLA-B27) or unknown (HLA-B51 and –Cw1) TAP dependency. The same N-terminal core peptide, MLDDFSAGA, was found in two different ligands, $A17L_{9-17}$ and $A17L_{9-25}$, bound to HLA-A2 and –Cw1, respectively. Ten sequences were new vaccinia HLA ligands. Most were restricted by a single HLA allele, but two ligands, $K2L_{16-30}$ and $C11R_{101-110}$, were found to be associated with two different alleles, HLA-B27 and –Cw1 or HLA-B51 and –Cw1, respectively. This implies that thirteen different natural peptide-HLA class I complexes were formed simultaneously in the same infected TAP-deficient cells.

Conservation of ligands among the *Orthopoxvirus* family.

The sequences of eleven vaccinia virus ligands identified in the WR strain were compared with homologs derived from various poxviruses. This comparison included the Copenhagen and MVA strains of VACV, two strains of human poxvirus (variola major and variola minor), and other mammalian poxviruses, such as camelpox, cowpox, ectromelia virus, horsepox, monkeypox, rabbitpox, and taterapox. This study revealed the high degree of conservation of the ligands among orthopoxviruses (Table 3). Ten of these eleven ligands are almost fully conserved in the variola major and minor viruses, with a minor substitution in the P7 position of the $C11R_{101-110}$ sequence. Only 60% of the previously described TAP^+ vaccinia virus epitopes are conserved in the variola proteome (49),(52),(45) , indicating that TAP-

independent vaccinia ligands are more highly conserved than TAP-dependent epitopes between immunogenic and pathologic poxviruses.

Low hydrophobicity in TAP-independent vaccinia virus ligands.

Eight TAP-independent epitopes, restricted by four different HLA class I molecules, were previously characterized in the Epstein-Barr virus (EBV) CTL response (reviewed in (37)). For this virus, only peptides from the BRLF1 and LMP2 proteins with high hydrophobicity are TAP independent (36),(37). Therefore, we tested this correlation in our vaccinia virus system. The hydrophobicity of all TAP-independent EBV epitopes was up to 2.1 on the grand average of hydropathicity (GRAVY) scale, with the maximum value found for the EBV LMP2 LLWTLVVLL peptide (Table 4). All three HLA-B*2705 ligands and the $A10L_{614-623}$ HLA-A*0201-restricted ligand identified in our study were hydrophilic, with negative GRAVY values (Table 4). Only the vaccinia virus HLA-Cw1-restricted epitope $D8L_{112-119}$, with a GRAVY value of 2.1, was hydrophobic. The other six ligands showed low positive GRAVY values, indicating low hydrophobicity (Table 4). The GRAVY mean of these eleven vaccinia ligands was only 0.3, which was very different from the EBV epitopes and more similar to the TAP-dependent vaccinia epitopes (Table 4). These results show that hydrophobicity is not a necessary condition for the TAP-independent presentation of ligands/epitopes from other viruses such as vaccinia.

Recognition of HLA-A*0201 ligands by specific $CD8^+$ T cells in HLA transgenic mice immunized with vaccinia virus.

To study the immunogenicity of the identified HLA-A*0201 viral ligands, transgenic HLA-A*0201-positive mice were immunized with VACV. Later, physiological measure of the functional in vivo activity of $CD8^+$ T lymphocytes against HLA-A2 viral ligand identified by mass spectrometry was carried out. HLA-A*0201-transgenic mice eliminated VACV peptide-pulsed $CFSE^{high}$ cells when they had been previously immunized but not with uninfected control VACV (Fig. 6). In addition, up to 30 days post-immunization, polyclonal CTL lines were generated that were monospecific for each HLA-A2 viral ligand identified by mass spectrometry. These CTL lines specifically recognized peptide-pulsed cells (Fig. 7), indicating that the HLA ligands were all A*0201-restricted CTL epitopes and that they were simultaneously recognized as part of the long-term memory response to vaccinia virus. Also, these $CD8^+$ effector lines also specifically recognized VACV-infected cells (Figure 7D).

Several attempts to induce CTL responses in HLA-B*2705 transgenic mice against the viral B*2705-restricted ligands identified were unsuccessful. This was also the case for the influenza NP epitope previously described in flu-infected $B27^+$ humans (67) that we used as a CTL-positive control. The mouse model used carried HLA-B27 and endogenous murine H2 class I molecules (27), in contrast to the HLA-A2 transgenic mouse knockout used previously for these mouse molecules (51). This may reduce the efficiency of HLA-restricted Ag-specific responses to undetectable levels (10) although the T cell repertoire as limiting factor could not be discarded. Unfortunately, HLA-B27 transgenic mice deficient for H2 class I expression (10) were not available. Therefore, the

545 study of HLA-B*2705-restricted CTL responses against vaccinia virus ligands
546 was not feasible.
547
548 **Three different antigen processing pathways were involved in**
549 **the presentation of A10L$_{688-696}$ and A17L$_{9-17}$ epitopes in infected TAP-**
550 **sufficient cells.**
551
552 To study the antigen processing pathways involved in the endogenous
553 generation of the A10L$_{688-696}$ and A17L$_{9-17}$ viral epitopes, we investigated the
554 presentation of these epitopes to specific CTL in the presence of diverse
555 protease inhibitors in VACV-infected TAP-sufficient cells.
556 First, to demonstrate that these HLA-A2-restricted epitopes require
557 endogenous processing, their presentation was analyzed in the presence of
558 BFA. This drug blocks class I export beyond the cis-Golgi compartment (38),
559 thus preventing the surface expression of newly assembled HLA class I-peptide
560 complexes from endogenous origin (Table 1 summarizes the specificity of all
561 inhibitors used). The complete inhibition of specific lysis in both specific CTL
562 lines caused by the addition of BFA during vaccinia infection (Fig. 8A)
563 demonstrated that the two relevant epitopes were indeed generated from
564 proteins endogenously processed in infected cells.
565 Lactacystin (LC), a *Streptomyces* metabolite (18),(48) was used to study
566 the involvement of the proteasome in the presentation of these epitopes. This
567 drug had no effect on the specific recognition of target cells infected with VACV
568 by A10L$_{688-696}$- or A17L$_{9-17}$-specific CTLs (Fig. 8A). Although the proteasome

may be involved in the antigen processing of these epitopes, these data suggest that the LC-inhibitable proteasome activity is not absolutely required.

Because several endogenous TAP-independent HLA-2 class I ligands are derived by cleavage of the signal sequences generated by the signal peptidase (SPase) complex (35)'(70) and because no specific inhibitor of this enzymatic activity was available, the involvement of SPase complex in $A17L_{9-17}$ ligand production could not be studied directly. SPase-processed peptides need further cleavage by signal peptide peptidase (SPPase) (69)'(68)'(41). Therefore, the involvement of SPPase in antigen presentation was tested by treating target cells with the SPPase-specific inhibitor $(z\text{-}LL)_2$ ketone (69)'(68){. Similarly to LC, the inhibition of specific presentation was not detected with this drug (Fig. 8A).

To identify proteases distinct from the proteasomes that could contribute to antigen processing of both HLA-A2 epitopes, experiments with several specific protease inhibitors were performed. Leupeptin (LEU) {Umezawa, 1976 19523 /id}, pepstatin (PEPST) (33)'(65) and 1,10-phenanthroline (PHE) (33)'(64) were initially tested because they specifically inhibit different protease families (Table 1), thereby covering a wide range of proteases. Figure 8A shows that these three inhibitors had no effect on the specific recognition of target cells infected with VACV. In addition, because the activity of ERAAP, an enzyme involved in antigen processing (59)'(72), is not fully blocked by PHE at the concentration used in this study, leucinethiol (LeuSH) (Table 1) (61) was also included. Like the other protease inhibitors, LeuSH did not inhibit the recognition of infected cells (Fig. 8A), indicating that ERAAP cannot be involved in the generation of these TAP-independent epitopes.

In summary, the inhibitors used did not block the presentation of the two viral epitopes tested. The two most likely explanations for these results are as follows. First, these epitopes could be processed by protease(s) that not were blocked by the collection of inhibitors used in Figure 8A. This explanation is not likely because the inhibitors were chosen to cover a wide range of protease classes. Alternatively, these epitopes could be processed in parallel by different proteases independently, meaning that the different antigen processing pathways would need to be inhibited at the same time to see an effect. To test this hypothesis, the effects of combinations of several inhibitors on antigen presentation in vaccinia infected cells were tested.

Because most class I epitopes are generated by proteasome activity, the presentation of these epitopes was analyzed in the presence of LC, together with each other inhibitor in turn. Figure 8B shows that the combination of LC with $(z\text{-}LL)_2$ ketone, LEU, or PEPST did not block presentation in infected cells. In contrast, a partial block of presentation was observed in target cells treated with LC and PHE (Fig. 8B). These results demonstrate that the $A10L_{688\text{-}696}$ and $A17L_{9\text{-}17}$ peptides were processed by both metalloproteases and proteasomes in vivo. Additionally, the antigen presentation of both epitopes was partially inhibited by the combination of LC and LeuSH, indicating that ERAAP or other metallo-aminopeptidases were involved in the generation of these viral epitopes. Metallopeptidases can be divided into aminopeptidases, endopeptidases, carboxypeptidases, and carboxy-dipeptidases, among others, based on their cleavage mechanism (reviewed in (54)). Some of these groups can be distinguished by the use of different specific inhibitors (summarized in Table 1). To more precisely identify the metallopeptidase group involved in both

618 A10L$_{688-696}$ and A17L$_{9-17}$ antigen processing, target cells were infected with
619 VACV and treated with a mixture of LC and different specific inhibitors (Table
620 1). The caspase-1-specific inhibitor z-VAD.fmk was also included in view of the
621 sensitivity of this cysteine protease to PHE. Remarkably, none of the different
622 combinations of inhibitors tested prevented antigen presentation to specific
623 CTLs (Fig. 8B). Because phosphoramidon is able to inhibit bacterial
624 endopeptidases but does not block all metallo-endopeptidases of mammalian
625 origin, the most likely explanation for our results is that mammalian metallo-
626 endopeptidases that are not blocked by phosphoramidon are involved in the
627 antigen processing of these viral epitopes. Over 100 different well-characterized
628 higher vertebrate metallo-endoproteases are resistant to this drug (6); therefore,
629 positive identification of the peptidase involved the processing of TAP-
630 independent vaccinia epitopes awaits further characterization.
631 To demonstrate that two different metalloproteases, amino and endopeptidases,
632 were independently involved in the generation of the A10L$_{688-696}$ and A17L$_{9-17}$
633 epitopes, their presentation was analyzed in the presence of a mixture of PHE
634 and LeuSH. The incomplete block detected in presentation indicates that
635 aminometalloproteases and metallo-endopeptidases are independently needed
636 to process both the A10L$_{688-696}$ and A17L$_{9-17}$ epitopes (Fig. 8B). Lastly, the
637 recognition of infected cells by specific CTL was abrogated in the presence of
638 three inhibitors: LC, PHE and LeuSH (Fig. 8B). To exclude that the inhibitory
639 effects of LC, PHE or LeuSH were due to toxic effects on target cells or on rVV
640 replication, rather than to a specific block of the respective proteases, similar
641 experiments in the same target cells to those shown in Fig. 8B were performed
642 in parallel using the VACV-OVA$_{257–264}$ which codes for the miniprotein

643 MSIINFEKL. Specific recognition by SIINFEKL-specific CTL of target cells
644 infected with the VACV-OVA$_{257–264}$ virus (64 ± 11 % of CD8^{+} cells secreted
645 IFNγ) was detected. In contrast, VACV-OVA$_{257–264}$-infected target cells
646 incubated with all combinations of these three inhibitors were efficiently
647 recognized by SIINFEKL-specific CTL and no inhibition was detected (hatched
648 bars in Fig. 8B). These data indicate that inhibition of the A10L$_{688-696}$ and A17L$_{9-}$
649 $_{17}$ epitopes by addition of LC, PHE and LeuSH drugs is formally due to specific
650 blockage of the respective proteases and not to a block in rVV replication or
651 other toxic effects.

652 In summary, the proteasome, aminometalloproteases, and metallo-
653 endopeptidases are all independently involved in the antigen processing of both
654 A10L$_{688-696}$ and A17L$_{9-17}$ epitopes. The existence of identical neighboring
655 residues around both the A10L$_{688-696}$ and A17L$_{9-17}$ sequences (Fig. 8C) could
656 explain the similarity of the antigen processing pathways identified in these two
657 different HLA-restricted epitopes.
658

DISCUSSION

The results reported here show an exceptional diversity of TAP-independent ligands, with eleven ligands processed and presented as part of thirteen different HLA/peptide complexes simultaneously in VACV-infected cells. All three of the identified HLA-A2 ligands generated long-term CTL memory responses against vaccinia virus in a transgenic mouse model. Proteasomal and non-proteasomal pathways were involved in the processing and presentation of two vaccinia virus-encoded HLA-A2-restricted antigens.

Identification of viral HLA ligands by mass spectrometry analysis contributes to a better understanding of the cellular antiviral immune response. However, the mass spectrometry strategies to identify such ligands have not become routine because of the difficulty in selecting and identifying the very limited number of viral sequences among the large number of self-peptides bound to HLA class I molecules. Therefore, the number of studies on viral HLA ligands is still limited. To date, only two studies have identified vaccinia virus ligands by mass spectrometry (45),(25). Currently, we identified three to four TAP-independent ligands that were processed and presented by each of the four class I molecules expressed in infected cells. Neither HLA-specific elution nor CTL responses have previously been reported in TAP^+ cells for ten of the ligands identified in our study. However, as all three TAP-independent HLA-A*0201 ligands also generated responses in TAP^+ HLA-A*0201-transgenic mice, these epitopes must also be generated in wild-type cells.

We detected HLA ligands from viral gene products expressed in the three gene expression temporality clusters of the viral life cycle (Supplemental Table 1), early (A50R, B8R, C11R, and D5R), early/late (K2L), and late (A10L, A17L, and D8L), in agreement with a previous study investigating the CTL response in the same virus strain (52). Similar to the TAP^+ response, the TAP-independent response also sampled proteins from the entire viral life cycle of the VACV WR strain. Other authors reported the absence of presentation of peptides derived from late viral Ags to specific T cells by infected mouse target cells (28),(45), but a different VACV strain was used in their studies. The sequences we identified were derived from eight different vaccinia virus proteins (Supplemental Table 1). Five of these proteins present signal sequence or transmembrane domains in their respective amino acid sequences and are therefore accessible to HLA-containing compartments where they could be processed by resident proteases. In contrast, for three vaccinia proteins, A10L, A50R, and D5R, no obvious TAP-independent antigen presentation could be predicted. However vaccinia DNA replication is associated with the cytoplasmic side of the rough ER, and when the replication proceeds, ER membranes are recruited (reviewed in (60)). Late in infection, the ER envelope is disassembled, and these membranes are recycled to the ER (reviewed in (60)). During this complex process that includes deep membrane reorganization, some molecules of A10L, A50R, and D5R proteins could gain access to a TAP-independent antigen processing pathway.

In most cases, the identified natural MHC class I ligands had the canonical anchor motifs, and their respective antigenicity and MHC class I binding affinity were correlated. This suggests that only high-affinity peptides are recognized by CTLs and that epitopes of low affinity may be

immunologically irrelevant. We have demonstrated that cytotoxic responses are targeted against both high- and low-affinity HLA-A*0201 ligands of VACV; therefore, the TCRs of individual CTLs specific for low-affinity epitopes must display a compensatory high affinity. In addition, it has been reported that low-affinity self-peptides in autoimmunity (reviewed in (15)) or tumor peptides (3) generate specific responses. These findings suggest that the range of peptides that can generate CTLs is broader than formerly considered and that the role of low-affinity epitopes in antiviral responses must be evaluated in future studies.

Different MHC class I alleles have different TAP dependencies. The widespread HLA-A2 allele is considered to be the least TAP dependent (63). Alleles such as HLA-B7 and –B8 can also bind ligands that are dependent on mechanisms other than TAP transport, while other MHC class I molecules, including HLA-A3, -A24, and –B27, have been described as mainly TAP dependent (2). In the present report, several TAP-independent ligands were identified for alleles with different TAP requirements. Thus, the overall expression of MHC class I molecules with endogenous bound peptides is not indicative of specific TAP-independent cellular responses, which must be studied individually in each specific virus.

Although TAP-independent viral epitopes are known (reviewed in (12),(26),(35)), only one epitope has been identified as the target of a specific antiviral CTL response in most of the cases studied. No systematic studies of TAP-independent pathways with a single virus and different HLA molecules have been reported. The exception is EBV, for which CTLs from different donors recognize several viral epitopes from two different viral proteins restricted by several HLA class I molecules in TAP-negative cell backgrounds

731 (reviewed in (37)). Here, we report a second case with eleven ligands from eight
732 different viral proteins presented by four different HLA class I molecules in the
733 same TAP-deficient vaccinia-infected cells. The simultaneous presentation of
734 this broad complexity of viral peptide/MHC complexes can help to explain why
735 TAP-deficient patients do not seem particularly susceptible to viral infections
736 and may appear asymptomatic for long periods of their lives. In addition, the
737 existence of multiple TAP-independent ligands in two very different viruses, a
738 gamma-herpes virus and an orthopoxvirus, suggests that these pathways could
739 be an extended, but perhaps secondary, mechanism that forms part of the
740 multiple layers of defense against viral infection and that the significance of
741 these alternative processing pathways *in vivo* needs to be further studied.

742 The involvement of the following proteases in the processing of
743 endogenously synthesized antigens, independently of the classical proteasome
744 pathway, has been reported previously (reviewed in (11)). In the present report,
745 two HLA-A2-restricted epitopes could be processed in parallel by amino
746 metallo-peptidases as well as metallo-endoproteases and a classic proteasomal
747 pathway.

748 In addition to the A17$L_{9\text{-}17}$ epitope previously described in TAP-sufficient
749 cells (4), two distinct peptide epitopes recognized by human HLA-A2-restricted
750 $CD8^+$ T cells and eight HLA-B27, -B51, and/or –Cw1 ligands were newly
751 detected in this study. Nine of the viral ligands identified are conserved in all
752 three vaccinia virus strains, seven orthopoxviruses (including cowpox virus) and
753 two variola strains. In contrast, only 60% of the previously described vaccinia
754 virus epitopes are identical in the variola proteome (49)'(52)'(45)**.** Therefore,
755 TAP-independent vaccinia ligands are more highly conserved than TAP-

dependent epitopes between immunogenic and pathologic poxviruses and could be more specific targets for immunization. Cowpox virus, the first component of early vaccines, specifically inhibits TAP-dependent peptide translocation(1); therefore, TAP-independent epitopes conserved between variola and this virus are probably responsible for the initial cross-protection. Additionally, truncated gene fragments similar to TAP-blocking CPXV12 protein are widely found in poxviral genomes (Poxvirus Bioinformatics Resources Center, http://www.poxvirus.org) and frequently represent loss-of-function phenotypes, but this “genetic debris” might acquire entirely new, unanticipated functions. Therefore, the conservation of epitopes between different vaccine strains and pathogens is relevant for vaccine design and suggests that ligands from TAP-independent pathways could be interesting candidates to be included in immunization protocols. This is also important with respect to bioterrorism because some countries did not participate in the WHO smallpox eradication program, and at the present time, no information about the elimination of their samples of the pandemic virus has been reported.

Computational methods for predicting MHC-peptide binding are increasingly being used to identify epitopes for vaccine design and to monitor T cell responses (reviewed in (34),(43)). The prediction of MHC-peptide binding is far from perfect, as our results indicate. The prediction by bioinformatics tools of HLA class I molecules presenting the nine vaccinia viral ligands identified in current study showed several different mistakes, including incomplete HLA identification ($K2L_{16-30}$ and $C11R_{101-110}$ ligands), ambiguous HLA restriction ($D5R_{148-157}$ and $A17L_{9-25}$ ligands) or the wrong assignation ($A10L_{614-623}$ and $D8L_{112-119}$ ligands) (Table 5). In summary, more than half of these ligands

showed inconsistencies between the computational predictions and experimentally detected HLA restriction. These results reveal the limitations of predictive methods for identifying natural MHC class I ligands and T cell epitopes. The current analytical algorithms may not be sufficiently accurate and should be used with caution.

Collectively, the results in the current report highlight the importance of analyzing natural peptides that result from the endogenous processing of viral proteins and demonstrate the complexity and plasticity of MHC-peptide interactions. This analysis is of fundamental importance for gaining a detailed understanding of MHC class I-restricted immunity and future vaccine design.

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Table 1

Specificity and activity of the inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference	Concentration	% Inhibition of degradation [a]
Brefeldin A	BFA	Vesicle transport	(71),(47)	5 μg/ml	ND
Lactacystin	LC	Proteasome	(18),(48)	10 μM	ND
$(z\text{-}LL)_2$ ketone	$z\text{-}LL_2$	Signal peptide peptidase	(69),(68)	100 μM	13 ± 4
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	(65)	100 μM	38 ± 18
Pepstatin	PEPST	Aspartic proteases	(33),(65)	100 μM	50 ± 5
1-10 Phenanthroline	PHE	Metalloproteases and caspase-1	(33),(64)	50 μM	ND
Leucinethiol	LeuSH	Metallo-aminopeptidases including ERAAP	(61)	30 μM	ND
Benzyl Succynil Acid	BENZ	Metallo-carboxypeptidases A & B	(33)	100μM	-10 ± 8
Captopril	CAPT	ACE and ACE-like proteases	(33)	100 μM	25 ± 2
Phosphoramidon	PHOSP	All bacterial metallo-endopeptidases but few of mammalian origin	(21),(33)	100 μM	15 ± 4
z-VAD.fmk	z-VAD	Caspases	(62)	100 μM	ND [b]

[a] Activity of these inhibitors was measured as their ability to prevent proteolytic degradation in cellular extracts as in reference (39). The amount of protein still present after incubation in the case of the degraded control sample was considered as 0 % inhibition of degradation, and the non-degraded unincubated sample was taken as 100 % inhibition. Data are mean of two independent experiments. Negative values indicate that there was enhanced degradation in the presence of the compound. ND, not done.

[b] The compound was found to block apoptosis (data not shown).

Table 2

Summary of HLA molecules bound by vaccinia virus epitopes

Ligand	Sequence	mAb used[a]	HLA-A2	HLA-B27	HLA-B51	HLA-Cw1
A17L $_{9-17}$	MLDDFSAGA	PA2.1	+[b]	ND	ND	ND
A10L $_{614-623}$	SPEGEETII	PA2.1	+	ND	ND	ND
A10L $_{688-696}$	ILDRIITNA	PA2.1	+	ND	ND	ND
A10L $_{867-876}$	SRGYFEHMKK	ME1	ND[c]	+	ND	ND
B8R $_{53-59}$	WQTMYTN	ME1	ND	+	ND	ND
K2L $_{16-30}$	YRLQGFTNAGIVAYK	ME1 / W6-32	-	+	-	+
D5R $_{148-157}$	IAMKRTLLEL	W6-32	-	ND	+	-
A50R $_{294-301}$	LPFGSLGI	W6-32	-	ND	+	-
C11R $_{101-110}$	IPSPGIMLV	W6-32	-	ND	+	+
A17L $_{9-25}$	MLDDFSAGAGVLDKDL	W6-32	-	ND	-	+
D8L $_{112-119}$	DGLIIISI	W6-32	-	ND	-	+

[a] The mAbs used for the sequential immunoprecipitations were PA2.1 (specific for HLA-A2), ME1 (specific for HLA-B27), and W6/32 (specific for a monomorphic HLA-A, B, C determinant).
[b] A significant difference ($P < 0.001$) compared with the negative controls.
[c] ND, not done.

Table 3

Conservation of viral HLA ligands in several orthopoxviruses

Poxvirus[a]	**A17L$_{9-17}$** **(HLA-A2)**	**A10L$_{614-623}$** **(HLA-A2)**	**A10L$_{688-696}$** **(HLA-A2)**	**A10L$_{867-876}$** **(HLA-B27)**	**B8R$_{53-59}$** **(HLA-B27)**	**K2L$_{16-30}$** **(HLA-B27, -Cw1)**
VACV WR	MLDDFSAGA	SPEGEETII	ILDRIITNA	SRGYFEHMKK	WQTMYTN	YRLQGFTNAGIVAYK
VACV Copenhagen	---------	---------	---------	----------	-------	---------------
VACV MVA	---------	---------	---------	----------	-------	---------------
Variola major	---------	---------	---------	----------	-------	---------------
Variola minor	---------	---------	---------	----------	-------	---------------
Camelpox	---------	---------	---------	----------	-------	---------------
Cowpox	---------	---------	---------	----------	-------	---------------
Ectromelia virus	---------	---------	---------	----------	-------	---------------
Horsepox	---------	---------	---------	----------	-------	---------------
Monkeypox	---------	---------	---------	----------	-------	---------------
Rabbitpox	---------	---------	---------	----------	-------	---------------
Taterapox	---------	---------	---------	----------	-------	---------------

Poxvirus[a]	**D5R$_{148-157}$** **(HLA-B51)**	**A50R$_{294-301}$** **(HLA-B51)**	**C11R$_{101-110}$** **(HLA-B51, -Cw1)**	**A17L$_{9-25}$** **(HLA-Cw1)**	**D8L$_{112-119}$** **(HLA-Cw1)**
VACV WR	IAMKRTLLEL	LPFGSLGI	IPSPGIMLV	MLDDFSAGAGVLDKDL	DGLIIISI
VACV Copenhagen	----------	--------	---------	----------------	--------
VACV MVA	----------	--------	---------	----------------	--------
Variola major	----------	--------	------V--	----------------	--------
Variola minor	----------	--------	------V--	----------------	--------
Camelpox	----------	--------	------V--	----------------	--------
Cowpox	----------	--------	------V--	----------------	--I--VA-
Ectromelia virus	----------	--------	---------	----------------	--------
Horsepox	----------	--------	---------	----------------	--------
Monkeypox	----------	--------	---L--V--	----------------	--I---A-
Rabbitpox	----------	--------	------V--	----------------	--------
Taterapox	----------	--------	------V--	----------------	--------

[a] The sequences used were obtained from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 4

TAP-independent ligands/epitopes and their hydrophobicity.

Ligand/epitope	Protein	Virus	Hydrophobicity[a]	Reference
LLWTLVVLL	LMP2	EBV	2.9	(37)
CLGGLLTMV	LMP2	EBV	2.1	(37)
Mean ± SD of 8 TAP^- epitopes		EBV	2.5 ± 0.2	(37)
MLDDFSAGA	$A17L_{9-17}$	Vaccinia	0.4	This study
SPEGEETII	$A10L_{614-623}$	Vaccinia	-0.6	This study
ILDRIITNA	$A10L_{688-696}$	Vaccinia	0.8	This study
SRGYFEHMKK	$A10L_{867-876}$	Vaccinia	-1.7	This study
WQTMYTN	$B8R_{53-59}$	Vaccinia	-1.2	This study
YRLQGFTNAGIVAYK	$K2L_{16-30}$	Vaccinia	-0.1	This study
IAMKRTLLEL	$D5R_{148-157}$	Vaccinia	0.7	This study
LPFGSLGI	$A50R_{294-301}$	Vaccinia	1.5	This study
IPSPGIMLV	$C11R_{101-110}$	Vaccinia	1.6	This study
MLDDFSAGAGVLDKDL	$A17L_{9-25}$	Vaccinia	0.3	This study
DGLIIISI	$D8L_{112-119}$	Vaccinia	2.1	This study
Mean ± SD of 11 TAP^- ligands		Vaccinia	0.3 ± 1.2	This study
		P value	< 0.0001	
Mean ± SD of 79 TAP^+ epitopes		Vaccinia	0.4 ± 1.0	(45),(25)

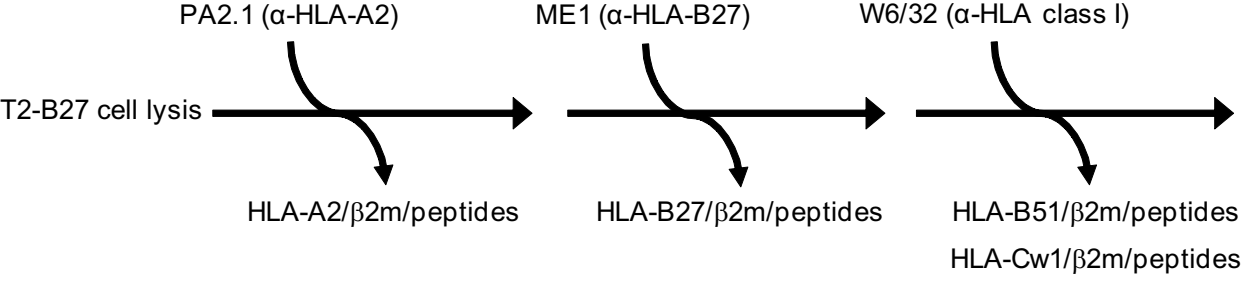
[a] as measured on the grand average of hydropathicity (GRAVY) scale (ProtParam tool, ExPASy Proteomics Server, http://www.expasy.ch).

Table 5

Comparison between predicted and experimentally detected binding of vaccinia virus ligands to HLA molecules

Ligand	Sequence	HLA	
		Predicted [a]	Experimentally detected
A17L $_{9-17}$	MLDDFSAGA	-A2	-A2
A10L $_{614-623}$	SPEGEETII	-B51	-A2
A10L $_{688-696}$	ILDRIITNA	-A2	-A2
A10L $_{867-876}$	SRGYFEHMKK	-B27	-B27
B8R $_{53-59}$	WQTMYTN	-B27	-B27
K2L $_{16-30}$	YRLQGFTNAGIVAYK	-B27	-B27 / -Cw1
D5R $_{148-157}$	IAMKRTLLEL	-B51 / -Cw1	-B51
A50R $_{294-301}$	LPFGSLGI	-B51	-B51
C11R $_{101-110}$	IPSPGIMLV	-B51	-B51 / -Cw1
A17L $_{9-25}$	MLDDFSAGAGVLDKDL	-A2 / -Cw1	-Cw1
D8L $_{112-119}$	DGLIIISI	-B51	-Cw1

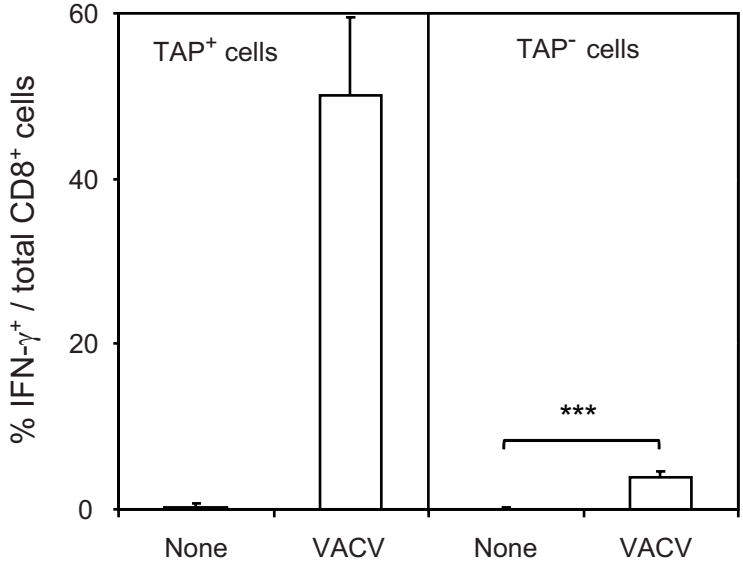
[a] From the SYFPEITHI (http://www.syfpeithi.de), BIMAS (http://www-bimas.cit.nih.gov), and IEDB (http://www.immuneepitope.org) databases.



Lorente et al. Figure 1

Diagram of sequential immunoprecipitation.

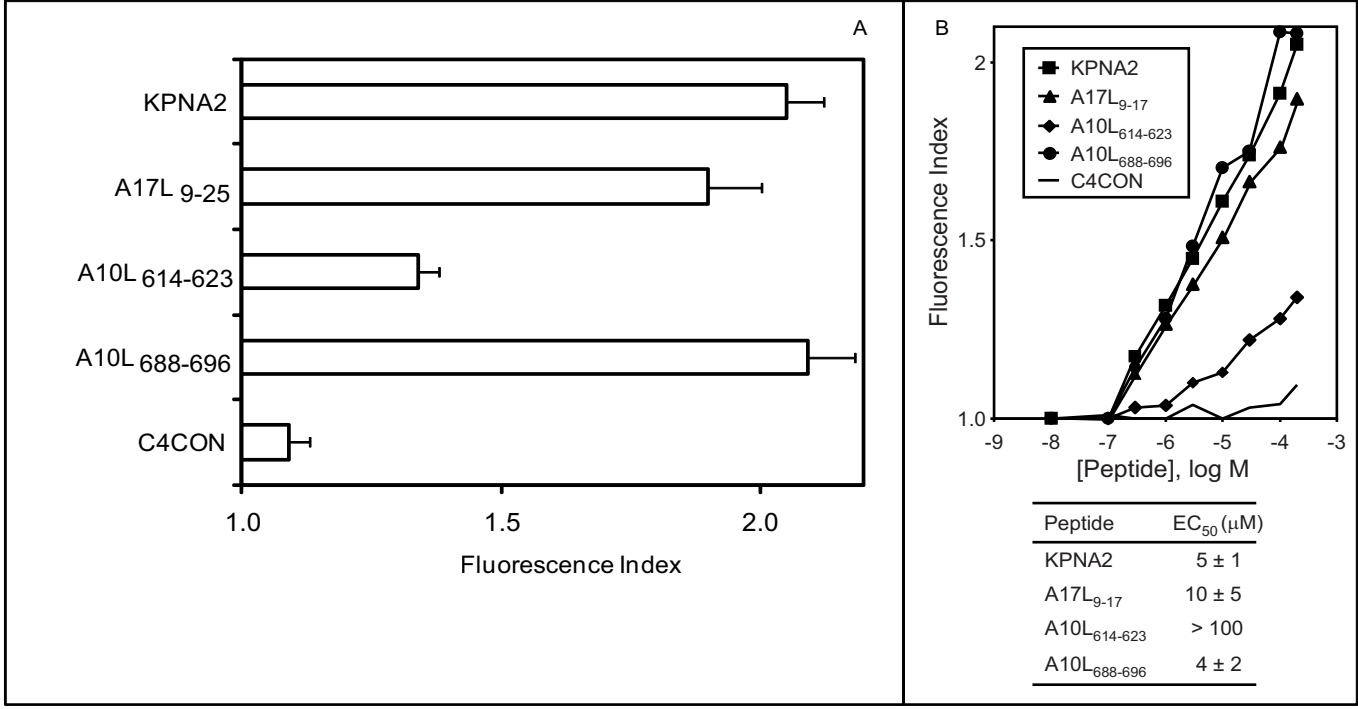
4 x 10^{10} healthy or VACV-infected T2-B27 transfectant cells were lysed. HLA-peptide complexes were isolated via affinity chromatography of the soluble fraction of cell extracts with the following mAbs, used sequentially: PA2.1 (anti-HLA-A2), ME1 (anti-HLA-B27), and W6/32 (specific for a monomorphic HLA class I determinant).



Lorente et al. Figure 2

Recognition of TAP$^+$ and TAP$^-$ cell lines by VACV-specific CD8$^+$ T lymphocytes.

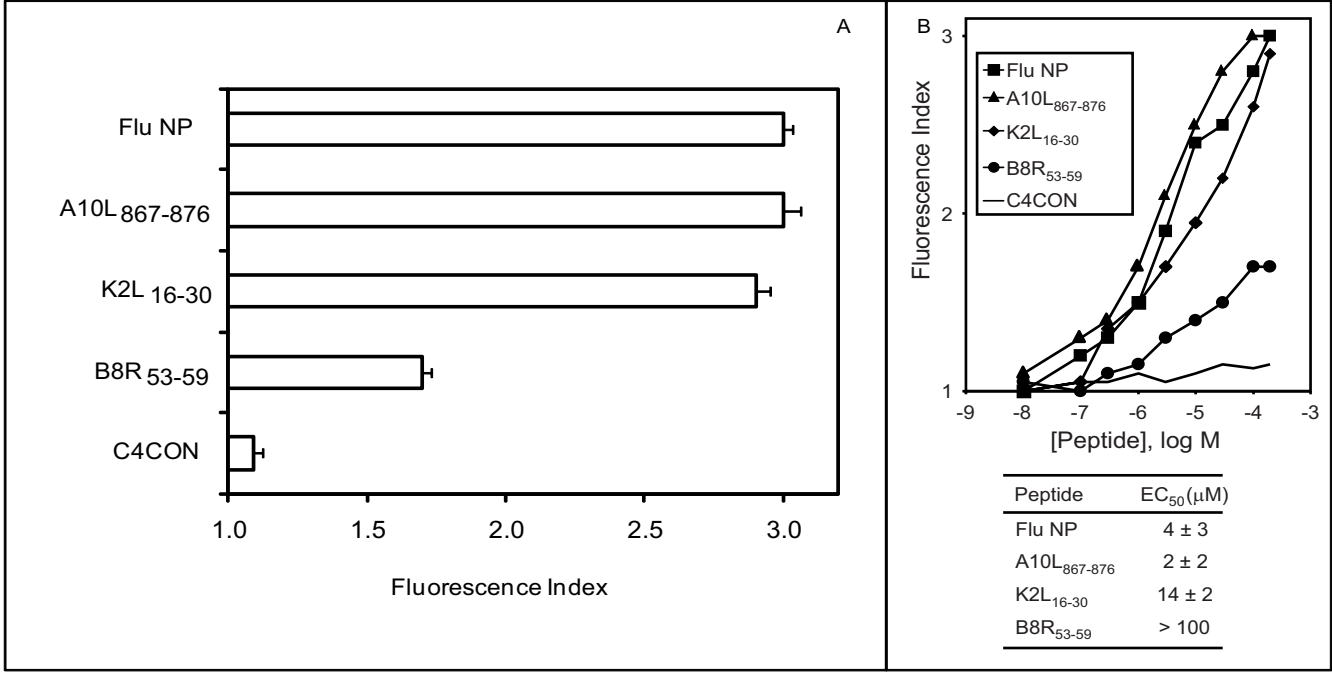
HLA-A*0201 TAP$^+$ (RMA, left panel) and TAP$^-$ (RMA-S, right panel) cells were infected with VACV at a multiplicity of infection of 40 plaque-forming units/cell and analyzed by ICS for CD8$^+$ T cell activation. The results are calculated as the mean of three or four independent experiments ± SD. *** Significant P values ($P < 0.0001$).



Lorente et al. Figure 3

HLA-A*0201 stabilization assay with synthetic VACV ligands.

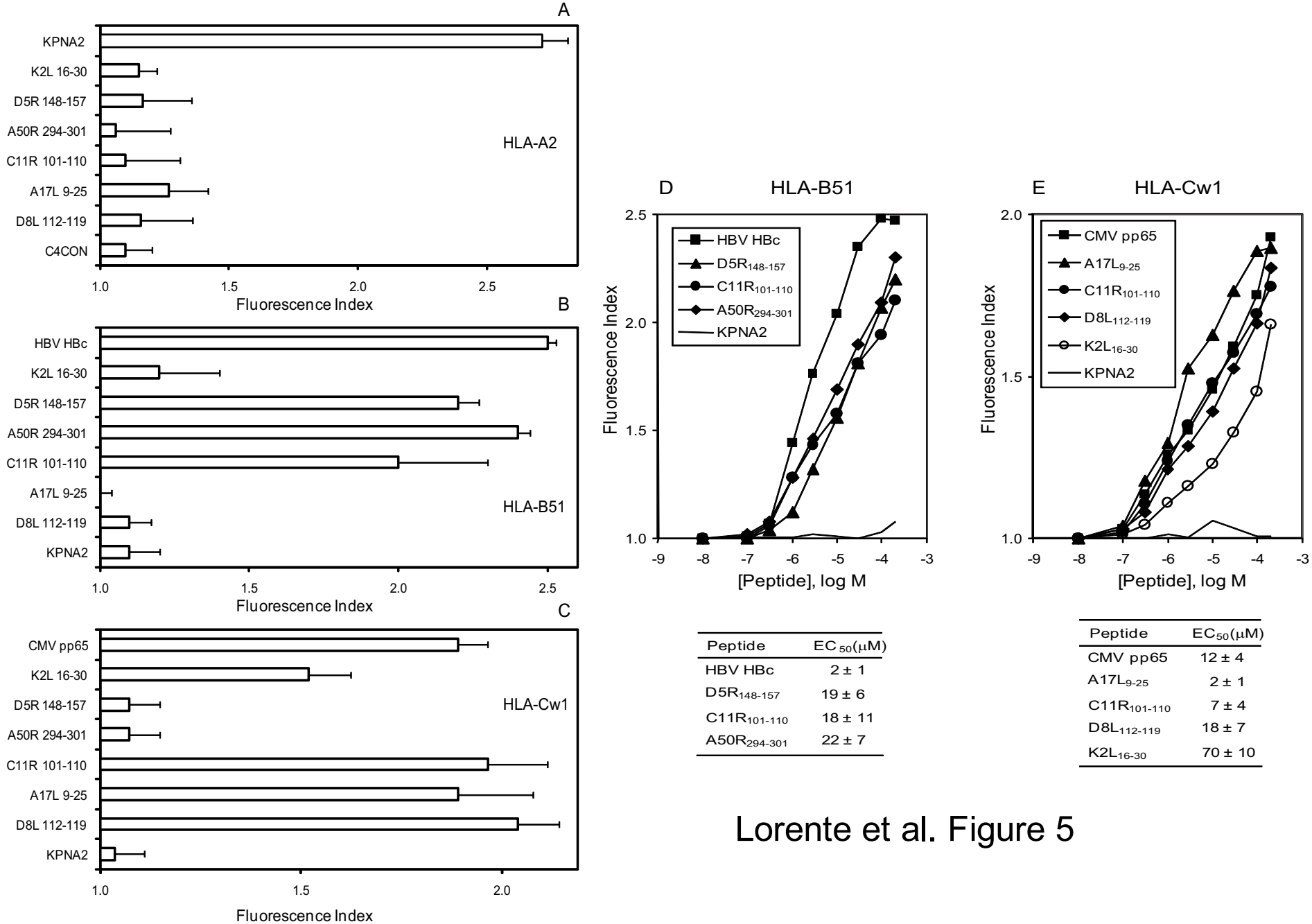
The stability of HLA-A*0201/peptide complexes on the surface of RMA-S transfectant cells was measured by flow cytometry. The indicated peptides were used at 200 μM (panel A). The mAb used was the monoclonal PA2.1. Panel B: The titration curves for synthetic VACV $A17L_{9-17}$ (triangles), $A10L_{614-623}$ (diamonds), and $A10L_{688-696}$ (circles) peptides with HLA-A*0201 are depicted. The C4CON (solid line) and KPNA2 (squares) peptides were used as negative and positive controls, respectively. The results, calculated as the fluorescence index (panel A), are the mean of three or four independent experiments. The calculated EC_{50} values ± SD are shown in panel B.



Lorente et al. Figure 4

HLA-B*2705 stabilization assay with synthetic VACV ligands.

The stability of HLA-B*2705/peptide complexes on the surface of RMA-S transfectant cells measured by flow cytometry. The indicated peptides were used at 200 μM (panel A). The mAb used was the monoclonal ME1. Panel B: The titration curves for synthetic VACV A10L$_{867-876}$ (triangles), K2L$_{16-30}$ (diamonds), and B8R$_{53-59}$ (circles) peptides with HLA- B*2705 are depicted. The C4CON (solid line) and Flu NP (squares) peptides were used as negative and positive controls, respectively. The results, as in Figure 2, are the mean of four independent experiments.



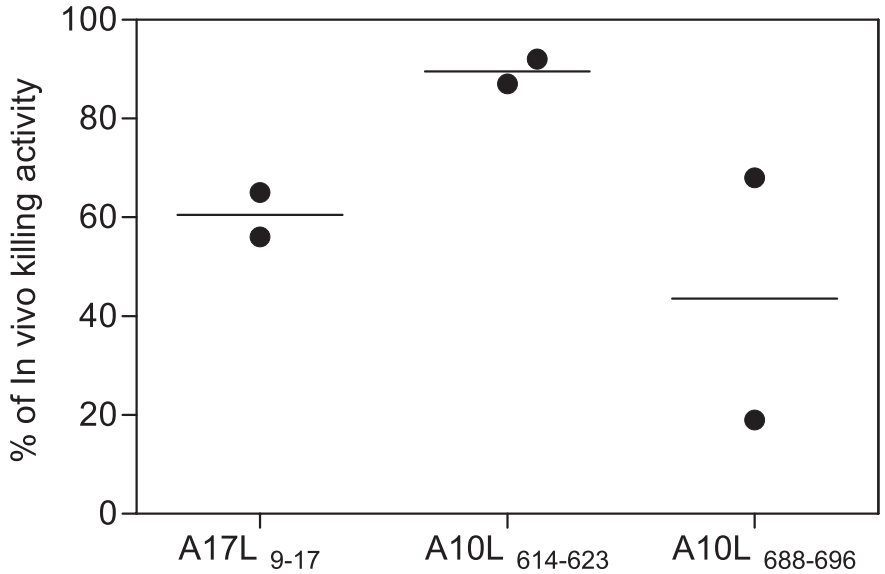
Peptide	EC_{50}(μM)
HBV HBc	2 ± 1
$D5R_{148-157}$	19 ± 6
$C11R_{101-110}$	18 ± 11
$A50R_{294-301}$	22 ± 7

Peptide	EC_{50}(μM)
CMV pp65	12 ± 4
$A17L_{9-25}$	2 ± 1
$C11R_{101-110}$	7 ± 4
$D8L_{112-119}$	18 ± 7
$K2L_{16-30}$	70 ± 10

Lorente et al. Figure 5

HLA class I stabilization assay of synthetic VACV ligands.

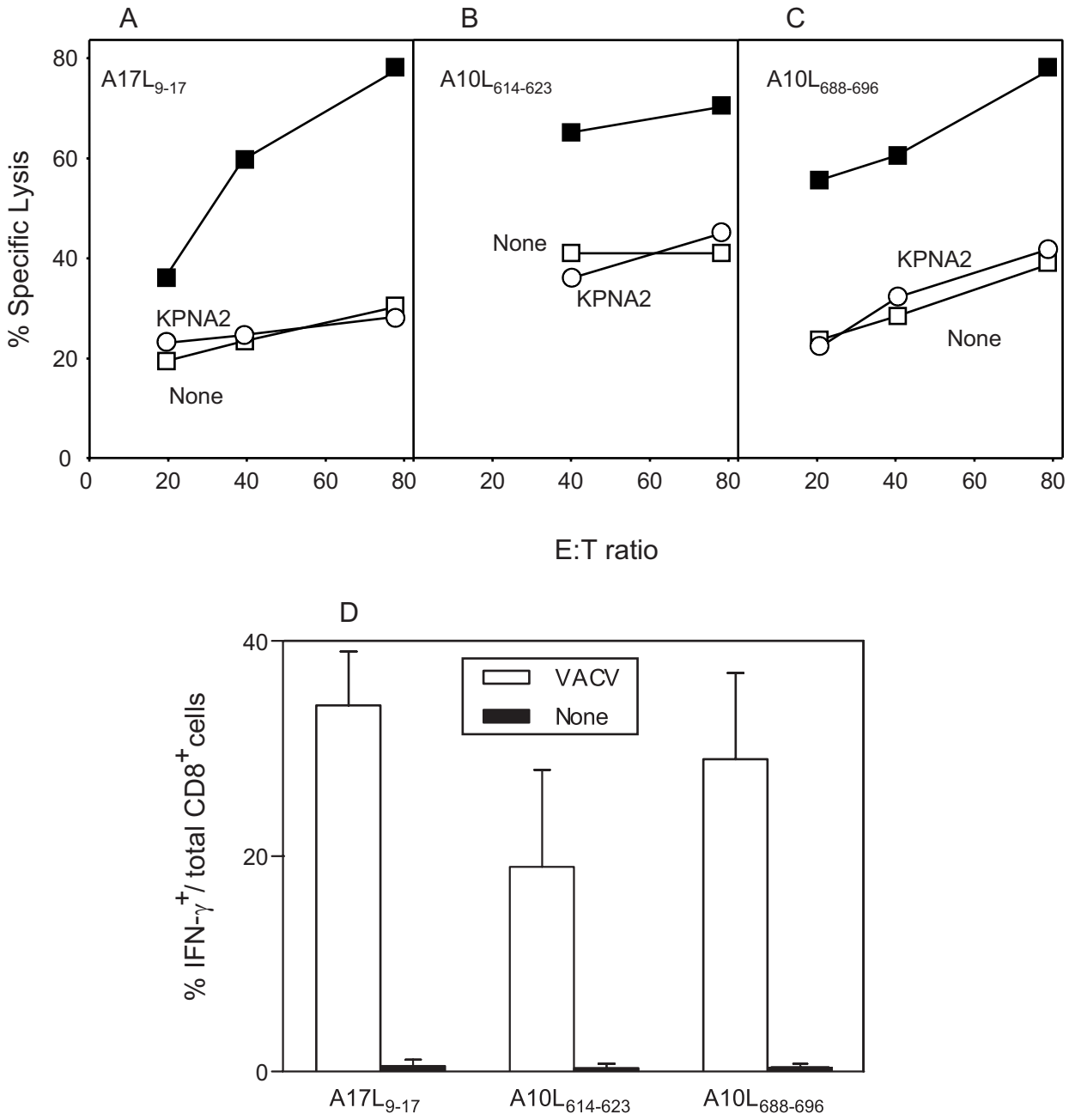
The stability of HLA-A2 (panel A), -B51 (panel B), and -Cw1 (panel C) at the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. Titration curves for the indicated synthetic VACV peptides, immunoprecipitated with W6/32 Ab, with HLA-B51 (panel D) or –Cw1 (panel E) are shown. The C4CON peptide was used as negative control (solid line). The HBV HBc_{19-27} and CMV $pp65_{7-15}$ peptides were used as positive controls for binding to the HLA-B51 and –Cw1 alleles, respectively. The Abs used were monoclonal PA2.1 (anti-HLA-A2, panel A), polyclonal H00003106-B01P (anti-HLA-B class I molecules, panel B, D), and polyclonal SC-19438 (anti-HLA-C class I molecules, panel C, E). The results, as in Figure 2, are the mean of four to six independent experiments.



Lorente et al Figure 6

In vivo killing activity of VACV peptide-specific CTLs in HLA-A*0201-transgenic mice.

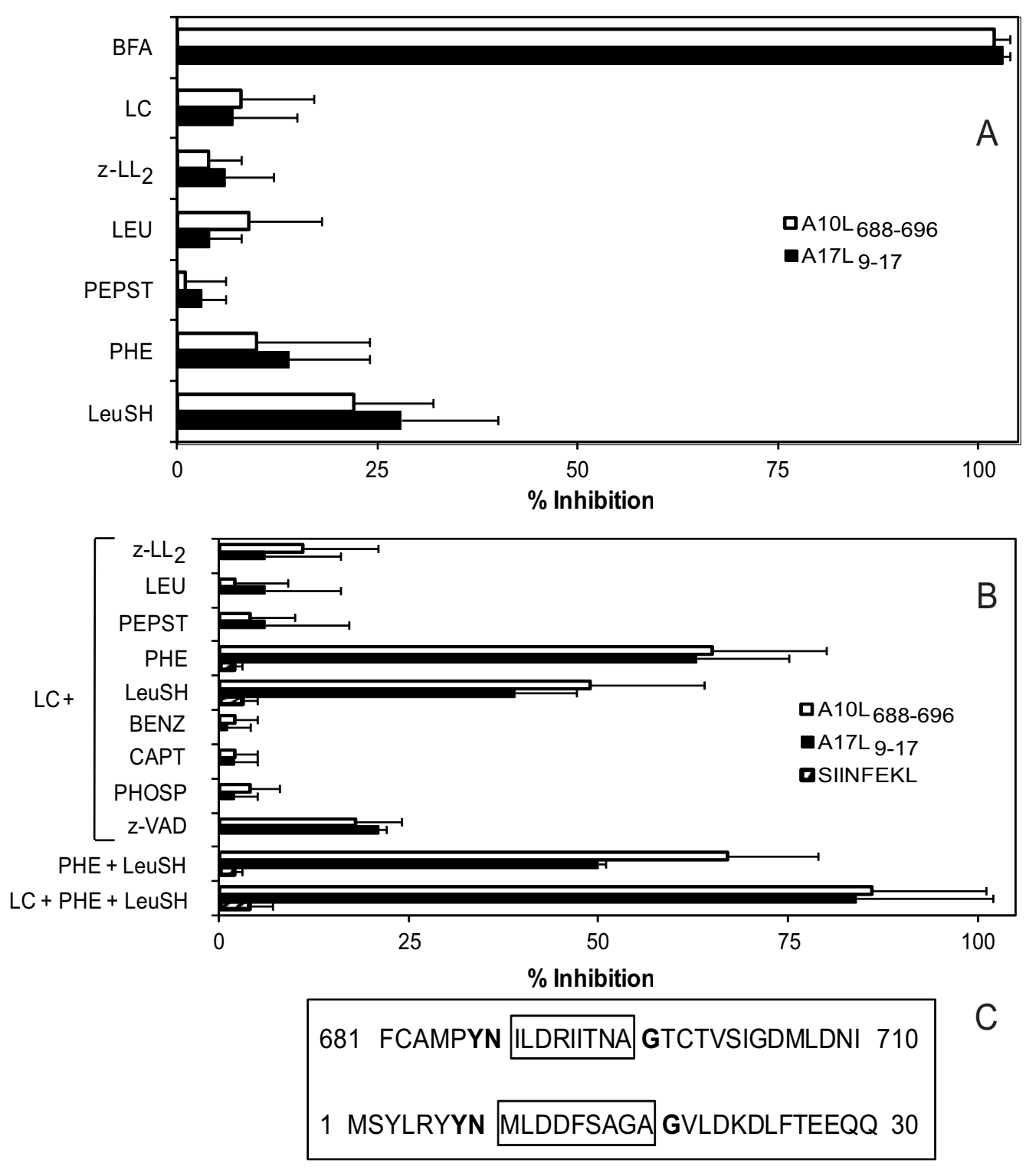
HLA-A*0201-transgenic mice were infected i.p. with VAVC. One week later, they were injected i.p. with syngeneic splenocytes either unpulsed and labeled with low CFSE (none) or pulsed with the indicated VACV peptides and labeled with high CFSE. Two days later, remaining CFSE-labeled target cells in the peritoneal cavity were lavaged and analyzed by flow cytometry. Percent in vivo lysis values of individual animals (circles) ± SD (line) are shown.



Lorente et al. Figure 7

Recognition of VACV epitopes presented by the HLA-A*0201 class I molecule by CTLs.

RMA-HLA-A*0201 target cells pre-pulsed with 10^{-5} M $A17L_{9-17}$ (panel A), $A10L_{614-623}$ (panel B), and $A10L_{688-696}$ (panel C) synthetic peptides were tested in a cytolytic assay with their respective memory peptide-specific CTL lines obtained from HLA-A*0201-transgenic mice immunized up to 30 days before with VACV (upper panel). The KPNA2 peptide was used as negative control (open circles).The data are the mean of at least three independent experiments. HLA-A*0201 TAP^+ cells were infected with VACV at a multiplicity of infection of 40 plaque-forming units/cell and analyzed by ICS for $CD8^+$ T cell activation with the HLA-A*0201 memory peptide-specific CTL lines (lower panel). The data are the mean of 4-6 independent experiments.



Lorente et al. Figure 8

Recognition of target cells infected in the presence of inhibitors.

RMA-HLA-A*0201 target cells infected for 16 hr with VACV or VACV-OVA$_{257–264}$ at a multiplicity of infection of 40 plaque-forming units/cell were treated with the indicated inhibitors. An ICS assay was used to test for recognition by A10L$_{688-696}$- (open bars), A17L$_{9-17}$- (closed bars) or SIINFEKL-specific (hatched bars) CTLs. The percentage of specific inhibition obtained by the addition of the indicated single (panel A) or mixture of two or three (panel B) inhibitors was calculated as:

$$\% \text{ Specific Inhibition} = 100 - \frac{[(\text{ICS VACV + Inhibitor}) - \text{ICS without infection}]}{\text{ICS VACV} - \text{ICS without infection}} \times 100$$

and the data displayed is the mean ± SD of 4-6 independent experiments. Panel C: the sequence of the neighboring residues to the epitopes in A10L and A17L proteins. The sequence of each epitope is boxed. Identical residues in both sequences are bolded.

Supplemental Table 1

Major characteristics of HLA ligand-containing vaccinia proteins [a]

Protein	Name	Expression	Signal sequence	Transmembrane domains	Encapsidated	pI	Mw	PTM [b]	Essential to VACV
A10L	P4a precursor	Late	None	0	Yes, in core	6.1	102		NI [c]
A17L	IMV membrane protein	Late	Putative	4	Yes, in membrane	4.4	23	Phosphorylation	Yes
A50R	DNA ligase	Early	None	0	No	7.9	63		No
B8R	IFN-gamma receptor	Early	Putative	0	No	5.5	31		NI
C11R	EGF growth factor	Early	Putative	1	No	6.4	16		NI
D5R	NTPase	Early	None	0	No	6.8	90		Yes
D8L	Carbonic anhydrase	Late	None	1	Yes, in membrane	8.8	35		NI
K2L	Serpin 1,2,3	Early/Late	Putative	0	No	9.0	42		NI

[a] All information was obtained from the Poxvirus Bioinformatics Resources Center (http://www.poxvirus.org).

[b] PTM, post-translational modification.

[b] NI, no information.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. VACV infection of the RMA-HLA-A*0201 cell line in presence of different protease inhibitors.

RMA-HLA-A*0201 target cells infected for 16 hr with VACV at a multiplicity of infection of 40 plaque-forming units/cell were treated with the indicated inhibitors. A mock infected control was included as negative control. The cells were stained with the Omnitope antiserum-FITC that recognizes VACV purified virions. Samples were analyzed by FACS. The results, calculated as fluorescence index ± SD, are the mean of 4 independent experiments. The fluorescence index was calculated as the ratio of mean channel fluorescence of the sample to that of the control incubated without VACV. All VACV-infected conditions (with and without inhibitors) show significant P values ($P < 0.01$) versus mock infected controls. In contrast, all inhibitor conditions show non significant P values versus VACV-infected control without an inhibitor.

Figure S2. Identification of three HLA-A*0201 ligands in infected cell extracts by mass spectrometry.

MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 926.4 (upper left panel), *m/z* 974.6 (medium left panel), and *m/z* 514.8 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The vertical axis represents the relative abundance of the parental ion and each fragmentation ion detected. The horizontal axis corresponds to the m/z region in which significant daughter ions were detected. Ions generated by the fragmentation are detailed, and the sequence deduced from the indicated fragments is shown in the upper left box of each panel.

Figure S3. Identification of three HLA-B27 ligands in infected cell extracts by mass spectrometry.

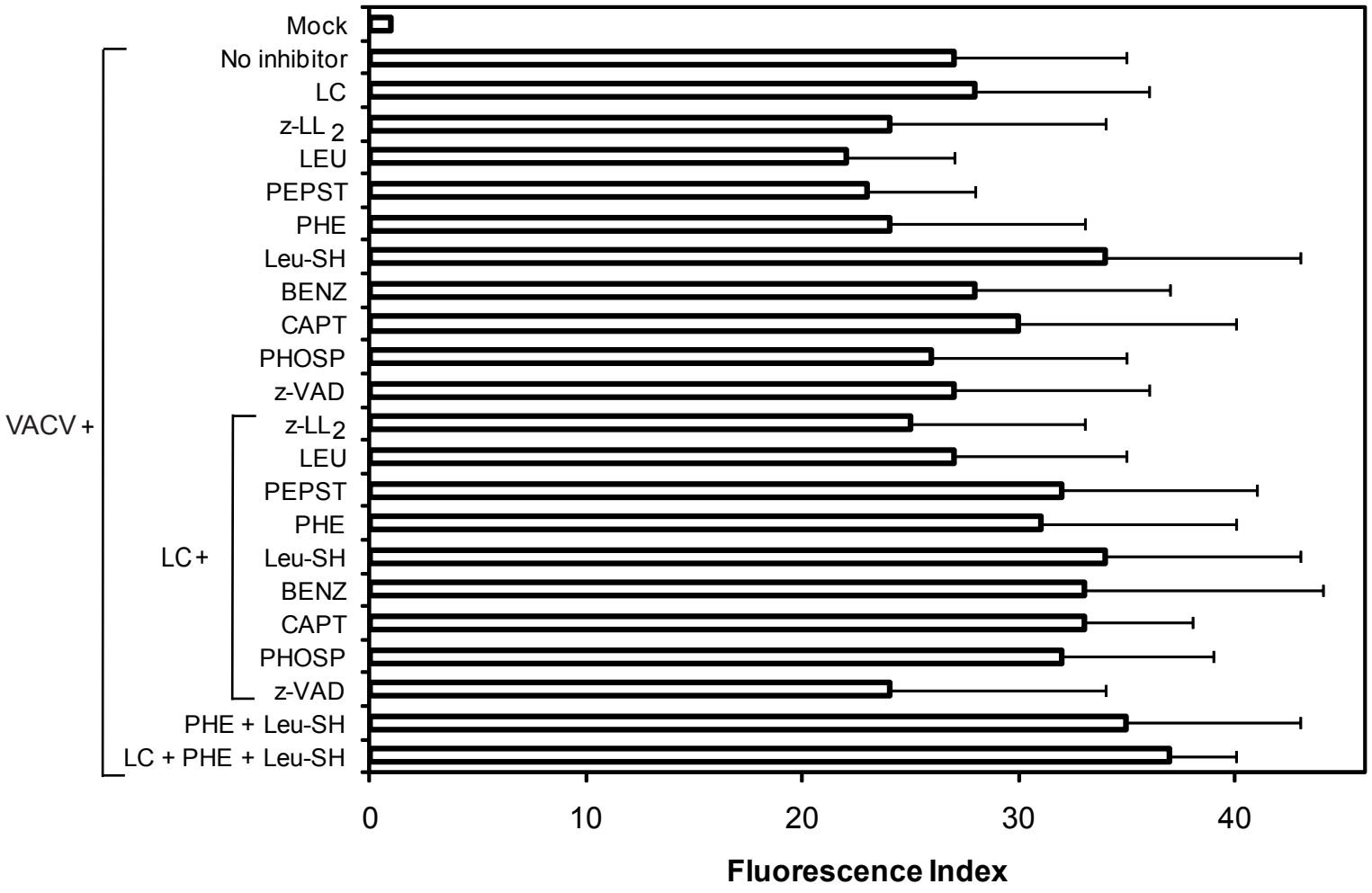
MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 428.2 (upper left panel), *m/z* 567.6 (medium left panel), and *m/z* 472.1 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.

Figure S4. Identification of three HLA ligands in infected cell extracts by mass spectrometry (I).

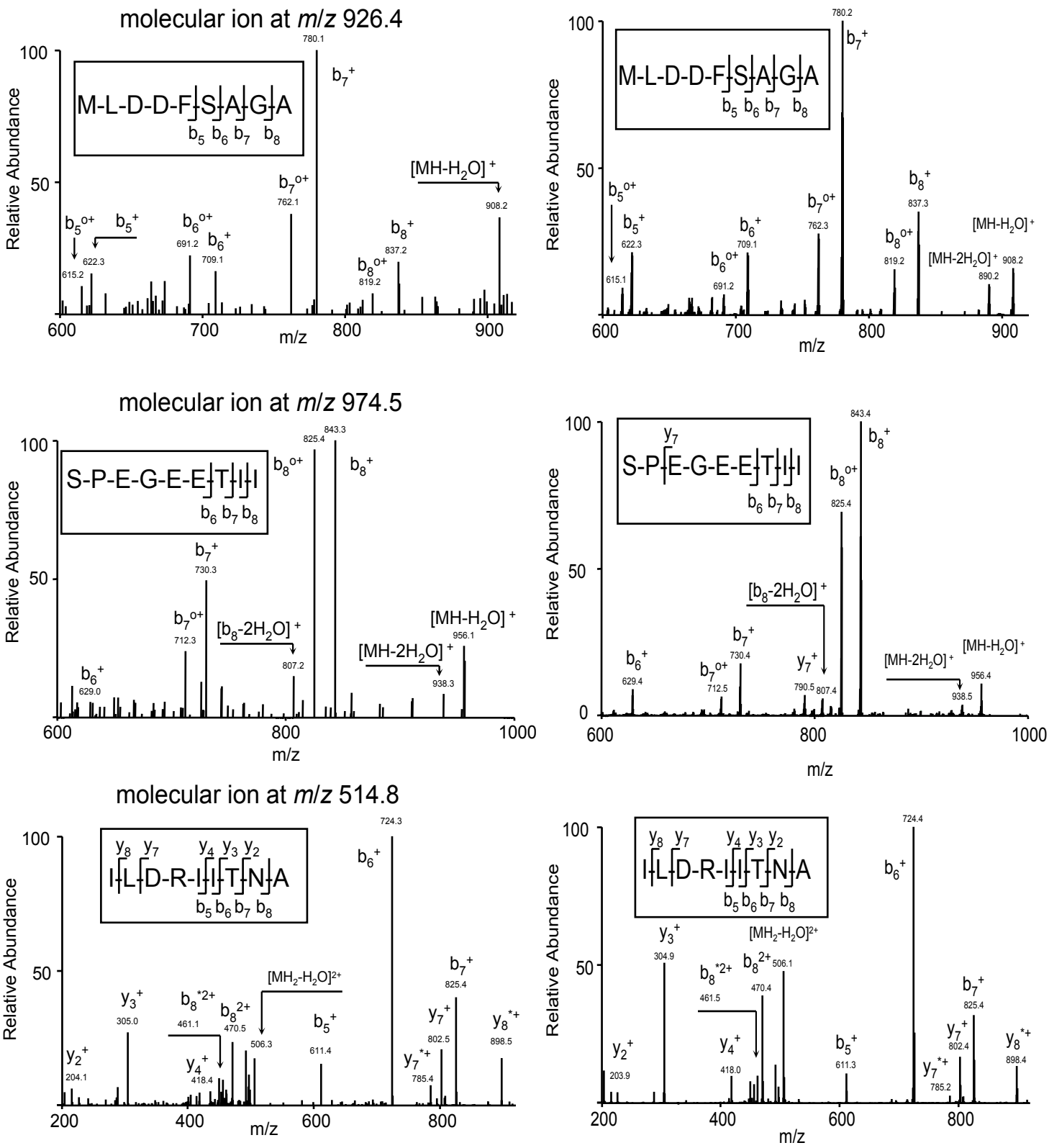
MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 594.4 (upper left panel), *m/z* 803.5 (medium left panel), and *m/z* 926.5 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.

Figure S5. Identification of three HLA ligands in infected cell extracts by mass spectrometry (II).

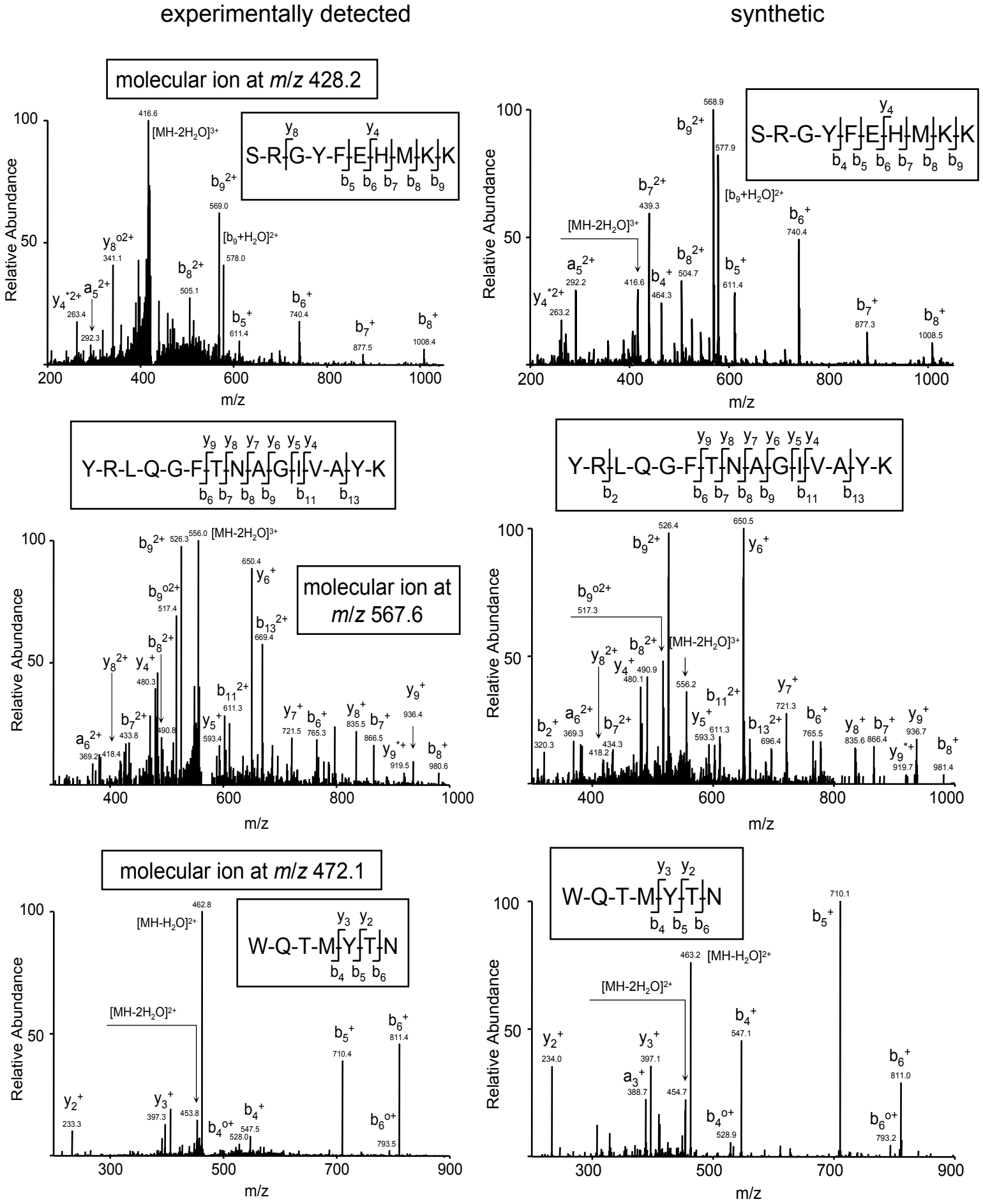
MS/MS fragmentation spectra obtained from quadrupole ion trap mass spectrometry of the ion peaks at *m/z* 843.5 (upper left panel), *m/z* 833.9 (medium left panel), and *m/z* 851.0 (lower left panel) from the VACV-infected cell extract and the corresponding synthetic peptide (right panels). The axes are as described in Figure S1.



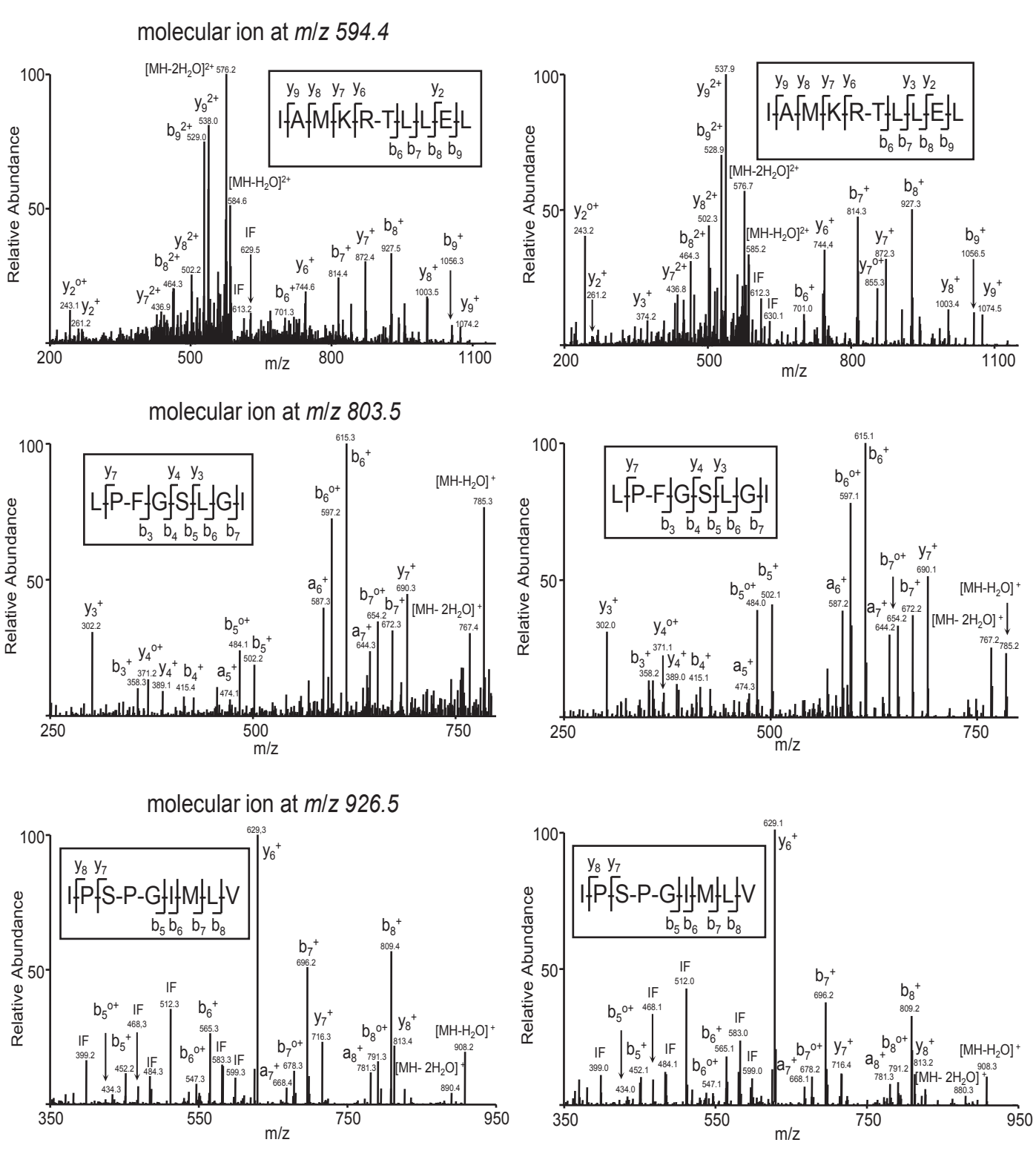
Lorente et al. Figure S1



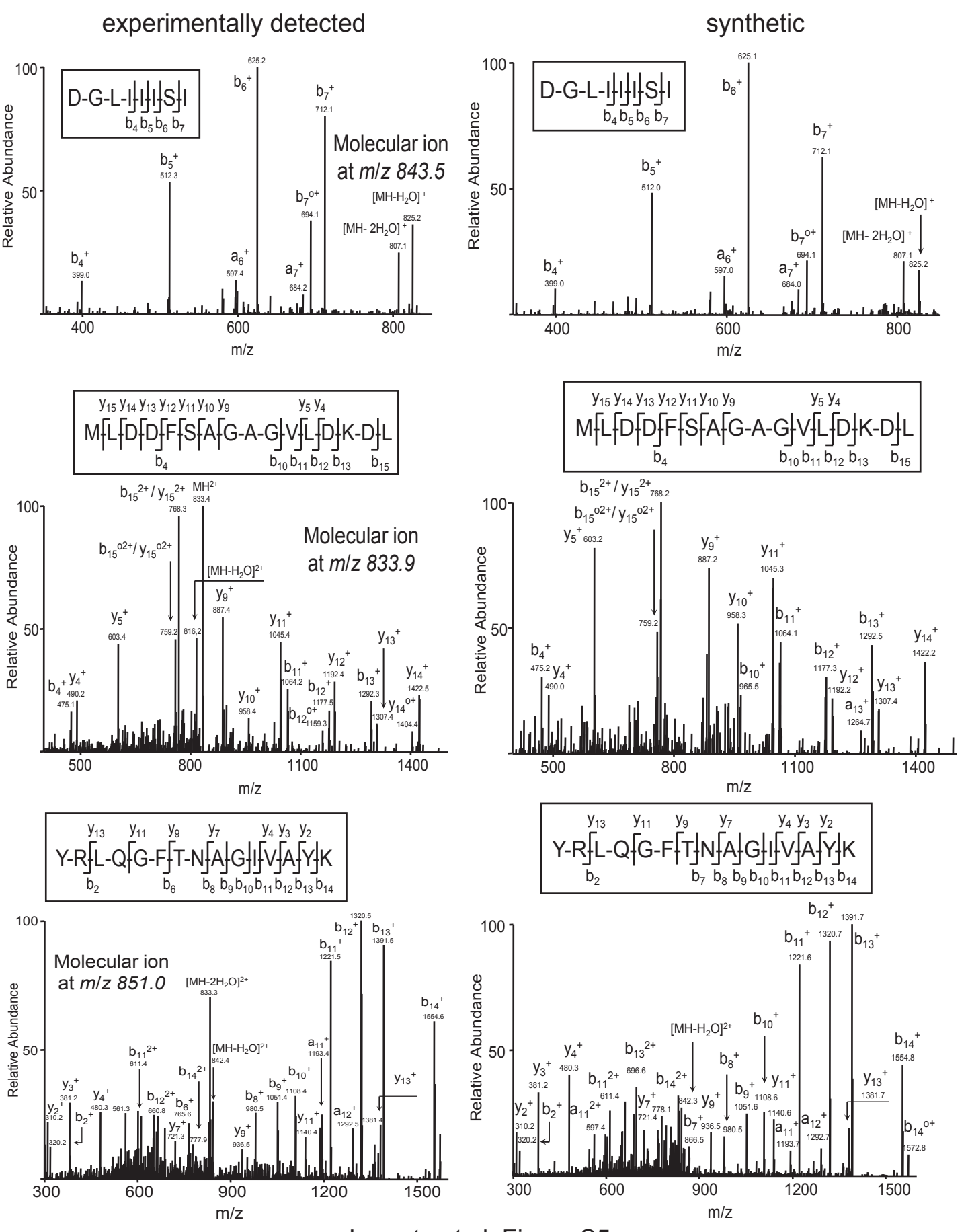
Lorente et al. Figure S2



Lorente et al. Figure S3



Lorente et al. Figure S4



Lorente et al. Figure S5