A Viral, Transporter Associated with Antigen Processing (TAP)-independent, High Affinity Ligand with Alternative Interactions Endogenously Presented by the Nonclassical Human Leukocyte Antigen E Class I Molecule*

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Background: Individuals with nonfunctional transporter associated with antigen processing (TAP) present ligands generated by TAP-independent processing pathways associated with classical HLA class I molecules.

Results: A vaccinia virus ligand is efficiently presented by nonclassical HLA-E using alternative interactions.

Conclusion: Nonclassical HLA-E presents viral ligands.

Significance: This expands the role of HLA-E as an antigen-presenting molecule.

The transporter associated with antigen processing (TAP) enables the flow of viral peptides generated in the cytosol by the proteasome and other proteases to the endoplasmic reticulum, where they complex with nascent human leukocyte antigen (HLA) class I. Later, these peptide–HLA class I complexes can be recognized by CD8+ lymphocytes. Cancerous cells and infected cells in which TAP is blocked, as well as individuals with unusable TAP complexes, are able to present peptides on HLA class I by generating them through TAP-independent processing pathways. Here, we identify a physiologically processed HLA-E ligand derived from the D8L protein in TAP-deficient vaccinia virus-infected cells. This natural high affinity HLA-E class I ligand uses alternative interactions to the anchor motifs previously described to be presented on nonclassical HLA class I molecules. This octameric peptide was also presented on HLA-Cw1 with similar binding affinity on both classical and nonclassical class I molecules. In addition, this viral peptide inhibits HLA-E-mediated cytolysis by natural killer cells. Comparison between the amino acid sequences of the presenting HLA-E and HLA-Cw1 alleles revealed a shared structural motif in both HLA class I molecules, which could be related to their observed similar cross-reactivity affinities. This motif consists of several residues located on the floor of the peptide-binding site. These data expand the role of HLA-E as an antigen-presenting molecule.

CD8+ cytolytic T lymphocyte-mediated recognition and killing of virally infected cells first requires proteolytic degradation of viral proteins by the proteasome and other cytosolic proteases (1). This degradation generates short peptides of 8–11 amino acids, which are then translocated to the endoplasmic reticulum lumen by transporter associated with antigen processing (TAP),2 where they assemble with newly synthesized HLA class I heavy chain and β2-microglobulin.

Humans and mice with mutations in the TAP gene that generate nonfunctional TAP complexes have been described (2, 3). This TAP deficiency implies reduced functionality of the CD8+ population, but TAP-deficient patients are not particularly susceptible to viral infections or neoplasms. Thus, TAP-independent HLA class I loading pathways may be sufficient to control these diseases and allow these individuals to live with only a decreased susceptibility to chronic respiratory bacterial infections. In addition, several strains of viruses have specific mechanisms to block TAP expression or to prevent CD8+ lymphocytes from identifying infected cells (reviewed in Ref. 4); therefore, the TAP-independent pathways must also be important for killing cells infected with these viruses.

Early administration of the cowpox virus, which encodes for a TAP-blocking protein (5), was the inspiration for the massive worldwide cross-protective vaccination by vaccinia virus (VACV) that eradicated pandemic smallpox, a disease caused by the variola major virus (6). The Orthopoxvirus vaccinia is a widely used tool for research and vaccine development (7). Currently, bioterrorism and emerging infectious diseases have elicited renewed interest in VACV and other poxviruses (8). VACV administration generates a strong humoral response leading to viral clearance, and the role of cytotoxic T lymphocyte responses in this cross-protection is well documented (9, 10). During the last several years, studies in both HLA-transgenic mouse models and vaccinated humans have identified more...
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than 70 VACV-derived epitopes presented by various HLA molecules (11, 12).

HLA-E is a nonclassical class I molecule that binds morphic signal peptides derived from classical HLA class I proteins. This complex is the ligand of innate receptors expressed mainly by natural killer cells and thereby regulates lymphocyte activity (13). Several recent studies have indicated that HLA-E complexed with pathogen-derived peptides could be recognized by CD8+ T cells (14). In addition, binding to HLA-E has been demonstrated for some viral peptides that were previously thought to bind the classical HLA-A2 class I molecule (15, 16).

In a previous study using mass spectrometry to analyze HLA-bound peptide pools isolated from large numbers of TAP-deficient VACV-infected cells, we identified eleven ligands that were naturally presented by four different HLA-A, -B, and -C class I molecules (17). Of these, six were obtained by immunoprecipitation with the mAb W6/32, which is specific for a nonmorphic HLA class I determinant (18). Later, to identify the HLA restriction of these ligands, HLA-peptide complex stability assays were performed using the TAP-deficient T2 cells with specific anti-HLA mAbs (17). Two of these VACV ligands were endogenously presented by HLA-B51 in human TAP-deficient cells, and another three were presented by HLA-Cw1 class I molecules. In addition, one VACV ligand, C11R101-110, was presented by both classical HLA-B51 and -Cw1 class I molecules in infected cells. Because the mAb W6/32 used in the HLA immunoprecipitation recognizes a conformational epitope on human HLA class I molecules, including the nonclassical HLA-E allele (19), some of these six VACV ligands could also have additional binding ability and could be presented by the HLA-E allele in T2 TAP-deficient VACV-infected cells. In the present study, we explore a possible role for HLA-E in presenting some of the previously described TAP-independent VACV ligands.

EXPERIMENTAL PROCEDURES

Cell Lines—T2 cells are a line of TAP-deficient human cells that express HLA-A2, -B51, and -Cw1 class I molecules on their surface (20). The 721.221 cells are a HLA-A, -B, and -C null human line that express HLA-E on their surface (21). Both cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 5 μM β-mercaptoethanol. The NK3.3 natural killer cells were cultured in α-minimal essential medium supplemented with 100 units/ml recombinant human IL-2 and 25% FBS (22). Recombinant human IL-2 was generously provided by Hoffmann-La Roche for the long term propagation of NK3.3 cell line.

Synthetic Peptides—Peptides were synthesized with a peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and were purified by reverse phase HPLC. The monosubstituted Ala analogues of VACV D8L peptide (DGLIIISI) were named according to the position of the substituted residue. Thus, A3 refers to the octamer of sequence DGAIISI. The correct molecular mass of the peptides was established by MALDI-TOF MS, and their correct composition was determined by MS/MS on a quadrupole ion trap micro-HPLC.

HLA-Peptide Stability Assays—The following synthetic peptides were used as controls in HLA-peptide complex stability assays: KPNAA2 (GLVPFLVSV, HLA-A2-restricted) (23), HBV HBE19–27 (LPSDFFSV, HLA-B51-restricted) (24), CMV pp657–15 (RCPEMISVL, HLA-Cw1-restricted) (25), the leader peptide of HLA (VMAPRALLL, HLA-E-restricted), and C4CON (QYDDAVYLLK, HLA-Cw4-restricted) (26).

The T2 line of TAP-deficient cells expresses low amounts of classical MHC class I on the cell surface. For classical HLA-A2, -B51, and -Cw1 class I stability assays, T2 cells were incubated at 26 °C for 16 h in RPMI 1640 medium supplemented with 1% heat-inactivated FBS. This allows the expression of empty MHC class I molecules that lack antigenic peptide and are only stable on the cell membrane at 26 °C and not at 37 °C. Later, the cells were washed and incubated for 2 h at 26 °C with various concentrations of peptide in the same medium. The cells were then kept at 37 °C and collected for flow cytometry after 4 h (27). This assay allows for the internalization of empty HLA class I molecules and can therefore discriminate between bound and unbound peptides.

For HLA-E stability assays, T2 cells were incubated with peptides for 6 h at 37 °C in culture medium before immunofluorescent staining as described previously (15). This treatment enhances cell surface expression of HLA-E class I molecules bearing specific HLA-E-bound peptides (15).

HLA expression levels were measured using the following Abs: monoclonal 3D12 (anti-HLA-E) (28), monoclonal PA2.1 (anti-HLA-A2) (29), 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 (30). The samples were assayed on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). The cells incubated without peptide had peak fluorescence intensities similar to the background staining observed with the secondary Ab alone or isotypic controls. The fluorescence index was calculated as the ratio of the mean channel fluorescence of the sample to that of control cells incubated without the peptides. Peptide binding was also expressed as EC50, which is defined as the molar concentration of the peptide producing 50% of the maximum fluorescence obtained at a concentration range between 0.001 and 100 μM.

T Cell Line and Cytotoxicity Assays—Cytotoxicity assays were performed using the 721.221 cell line as target (T) cells and NK3.3 cell line as effector (E) cells. Before performing the assay 1 × 105 target cells were incubated overnight at 26 °C either in the absence or in the presence of the indicated peptides at 100 μM. A 2-h 51Cr release assay was performed as previously described (21). Specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The spontaneous release never exceeded 20%.

Statistical Analysis—To analyze statistical significance, an unpaired Student t test was used. p values < 0.001 were considered to be significant.

Molecular Dynamics: Starting Structures—The native leader peptide HLA-E-binding peptide was taken from chains A, B, and C of the 3bzf Protein Data Bank file. The 3bzf112-119 peptide bound to HLA-E model was built with the MODELLER9v7 program using the 3bzf Protein Data Bank file as template. The
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protonation states of the ionizable groups for the three systems were calculated using the H+ + server (31) (32). The positions of hydrogen atoms, standard atomic charges, and radii for all the atoms were assigned according to the ff03 force field (33). The complexes were immersed in cubic boxes of TIP3P water molecules that were large enough to guarantee that the shortest distance between the solute and the edge of the box was more than 13 Å (34). Counter ions were also added to maintain electroneutrality. Three consecutive minimizations were performed: the first involved only hydrogen atoms, the second involved only the water molecules and ions, and the third involved the entire system.

Simulation Details—The initial minimized structures, prepared as stated before, were simulated in the NPT ensemble using Periodic Boundary Conditions and Particle Mesh Ewald to treat long range electrostatic interactions. The systems were then heated and equilibrated in two steps. The first step involved 200 ps of MD heating the whole system from 100 to 300 K, and the second involved equilibration of the entire system during 1.0 ns at 300 K. The equilibrated structures were the starting points for the 10-ns MD simulations at constant temperature (300 K) and pressure (1 atm). The SHAKE algorithm was used to keep bonds involving hydrogen atoms at their equilibrium length, allowing a 2-fs time step for the integration of Newton’s equations of motion. ff03 and TIP3P force fields, as implemented in AMBER 10 package, were used to describe the proteins, the peptides, and the water molecules, respectively. Sample frames at 20-ps intervals from the molecular dynamics trajectory were subsequently used for analysis.

Interaction Energies Analysis—Effective binding free energies between the peptides and HLA-E were estimated using the MM-GB-SA approach as implemented in the AMBER10 package (35). The MM-GB-SA method approaches the free energy of binding as a sum of a molecular mechanics (MM) interaction term, a solvation contribution thorough a generalized Born (GB) model, and a surface area (SA) contribution to account for the nonpolar part of solvation. In addition, to better characterize peptide-protein interactions, an energy decomposition analysis in a pairwise fashion (between the peptides residues and HLA-E residues) was performed using a cutoff of 5 Å from the peptides. Polar contribution to solvation free energies were calculated with GB, whereas nonpolar were estimated to be proportional to the area lost upon binding using the linear combinations of pairwise overlaps (LCPO) method to calculate accessible surface areas (36). These calculations were performed for each snapshot from the simulations using the appropriate module within AMBER 10 package.

RESULTS

VACV D8L112–119 Is a Noncanonical HLA-E Ligand—To explore the potential role of HLA-E as an antigen-presenting molecule of TAP-independent VACV ligands, HLA-peptide complex stability assays were performed using TAP-deficient T2 cells with an anti-HLA-E Ab. Fig. 1 shows that in contrast to a control HLA-E ligand, the leader peptide of HLA, the induction of complexes with five of the six VACV peptides tested were not detected. Thus, these viral ligands do not bind to HLA-E. In contrast, the D8L112–119 synthetic peptide induced similar numbers of HLA-peptide-surface complexes as the positive control HLA-E ligand (Fig. 1). The consensus peptide-binding motif for HLA-E is Met, Leu, or Gln at peptide position 2 (P2); Leu, Ile, Val, or Pro at P7; and Leu, Glu, or Phe C-terminal residues (37, 38). Thus, the D8L112–119 octamer DGLIIIS is an unusual VACV ligand presented by HLA-E class I molecules.

Identical Binding Affinity to Classical HLA-Cw1 and Nonclassical HLA-E Class I Molecules for the Viral D8L112–119 Peptide—The D8L112–119 peptide was previously described as a HLA-Cw1-restricted ligand (17). Because the mAb 3D12 used in the current study for HLA-E binding cross-reacts with some HLA-C class 1 molecules, although not with HLA-Cw1 (39), HLA-peptide complex stability assays were performed using T2 cells incubated with a natural high affinity HLA-Cw1 ligand, the CMV pp65 peptide (25), and stained with the anti-HLA-E mAb 3D12 to exclude HLA-Cw1 cross-reactivity. In this case, induction of HLA complexes with the CMV pp65 peptide was not detected (data not shown). Thus, the mAb 3D12 does not bind to HLA-Cw1.

In addition, the relative affinity of D8L112–119 to both HLA-E and Cw1 class I molecules was evaluated. This peptide bound to HLA class I molecules in the range commonly found among natural ligands (Fig. 2). This octamer efficiently stabilized
HLA-E (Fig. 2, left panel) and HLA-Cw1 (Fig. 2, right panel) expression on cells, with an EC50 for MHC binding of 14 ± 3 and 18 ± 7 μM, respectively, confirming their dual presentation. These EC50 values are similar to those of the other natural high affinity ligands used as positive controls. In summary, the VACV D8L112-119 octamer is a TAP-independent high affinity ligand presented by both classical and nonclassical class I molecules.

The D8L112-119 Peptide Is Not a HLA-A2 or -B51 Ligand—Some viral HLA-E-binding ligands were previously described as HLA-A2-restricted epitopes (15, 16). In addition, HLA-A2 and -Cw1 class I molecules present some peptides with similar anchor motifs (SYFPEITHI database (40)). Thus, HLA-peptide complex stability assays were performed to study possible HLA-A2 cross-reactivity of D8L112-119 ligand. Fig. 3 (upper panel) shows that induction of HLA-A2 complexes with the vaccinia D8L112-119 peptide was not detected, in contrast to a control HLA-A2 ligand, the KPNA2 peptide. Thus, this viral ligand does not bind to HLA-A2.

Because the HLA-B51 was also expressed in the T2 cell line, and HLA-B51 and -Cw1 class I molecules present some peptides with similar anchor motifs (SYFPEITHI database (40)), the possible HLA-B51 cross-reactivity of D8L112-119 ligand was examined. HLA-peptide complex stability assays were performed in which HLA-B51 molecules were stained in the presence of the vaccinia D8L112-119 peptide. In contrast to a control HLA-B51 ligand, the HBV HBC peptide, induction of complexes with the vaccinia D8L112-119 peptide was not detected (Fig. 3, lower panel). In summary, this viral ligand binds to HLA-E and -Cw1 but not to HLA-A2 or -B51 alleles.

The Viral D8L112-119 Peptide Inhibits Cytolysis Mediated by Natural Killer Cells—Because HLA-E/peptide-complexes are recognized by different NK cell receptors mediating either activating or inhibitory signals, we studied whether the binding of D8L112-119 ligand to HLA-E alters cytolytic activity of natural killer cells. Thus, 721.221 HLA-E cells were incubated with the viral peptide, and a standard 51Cr release assay was performed. As indicated in Fig. 4, incubation of target cells with the D8L112-119 peptide reduced their susceptibility to NK3.3-mediated cytotoxicity (65 ± 9% of specific inhibition) to a similar extent as was obtained with the positive control peptide (71 ± 6% of specific inhibition), whereas incubation of 721.221 cells with an irrelevant peptide had no effect on cytotoxic function of NK3.3 cells.

VACV D8L112-119 Uses Alternative Interactions to the Anchor Motifs Previously Described for Its Presenting HLA-E Class I Molecule—The crystal structure of HLA-E, in complex with the peptide VMAPRALLL, has been previously described (41). In contrast to classical HLA class I molecules, where the peptides that bind different allotypes are anchored by two primary spec-
ificity pockets in the binding groove, HLA-E possesses five main anchor sites at the P2, P3, P6, P7, and P9 positions (Fig. 5A). The pockets accommodating the side chains at the P2, P7, and P9 positions are deep, but those at the P3 and P6 positions are relatively shallow. The A pocket interacts with the P1 Val residue (Fig. 5A). The P2 and P3 side chains are directed into pockets on opposite sides of the groove to the deep B and the shallow D pockets. The superficial C pocket interacts with the P6 Ala residue, and the P7 residue is accommodated into E pocket. Last, the side chain of P9 is buried in the F pocket.

Modeling of the VACV D8L112–119 octamer peptide in complex with HLA-E was based on the existing x-ray structure of the VMAPRALLL-HLA-E complex (Fig. 5). Two alternative conformations with similar HLA-E interaction energies were predicted. The first (A model) keeps the interaction of P1 N-terminal residue with the A pocket but loses contact with the C°/H9024 residue with the F pocket (Fig. 5B). In contrast, in the second conformation (B model), the lateral chain of the N-terminal P1 residue is buried in the B pocket, and contacts between the side chain of P8 and the F pocket are conserved (Fig. 5C). The P6 residue is an anchor residue in both alternative conformations, although it interacts with different pockets: the C pocket in the A model (Fig. 5B) and the E pocket in the B model (Fig. 5C). These different models predicted mutually exclusive interactions with either the P3 residue and the D pocket (A model; Fig. 5B) or the P5 residue and the C pocket (B model; Fig. 5C).

To test the two alternative models, new HLA-peptide complex stability assays were performed using monosubstituted Ala analogues of D8L112–119 peptide. Substituting Ala with the P6 Ile residue, which could serve as anchor motif in either of the two conformations, abolished interactions between the HLA and the viral octamer (Fig. 6). Additionally, substituting the P3 Leu with Ala abrogated peptide binding to HLA-E molecule, suggesting that this residue serves as an anchor motif as it does in the A model (Fig. 5B). In contrast, exchanging Ala with either P5 Ile or P8 Ile residues, which could be additional anchor residues for HLA molecule in the B model but were absent in the A model as suggested by the modeling of Fig. 5 (B and C), had no effect on the stabilization of HLA-E molecules (Fig. 6). In summary, the analysis of HLA-D8L112–119 interactions using monosubstituted Ala analogues indicated that this viral peptide bound to HLA-E using only the two anchor residues P3 Leu and P6 Ile, consistent with the A model but not consistent with the B model.

DISCUSSION

The results reported here show that the 112–119 octamer derived from the vaccinia D8L protein is efficiently presented by the MHC class I molecule HLA-E using alternative interactions with the anchor motifs previously described for this non-classical MHC class I molecule. In addition to the its role in the presentation of monomorphic signal peptides derived from the classical HLA class I proteins to the innate receptors (13), HLA-E was identified as a restriction element for bacteria (42, 43).
and some viral peptides from EBV (15), HCV (16), HIV (44), and influenza (15). Moreover, most of these viral, but not bacterial, peptides are capable of binding to both classical HLA-A2 and nonclassical HLA-E class I molecules (15, 16), suggesting that HLA-E may bind, at least in part, a repertoire of viral pathogen antigens comparable with HLA-A2 despite the large sequence differences found. Comparison between the HLA-E and HLA-A2 sequences shows 42 amino acid differences in residues located in the peptide binding groove, with 15 conservative and 27 nonconservative changes (Table 1). In our study, the vaccinia ligand showed HLA cross-reactivity with HLA-Cw1 but not HLA-A2 class I molecules. Sequence analysis between HLA-E and HLA-Cw1 class I molecules reveals a lower degree of differences than HLA-A2 with 14 conservative and 20 nonconservative amino acid changes in the residues facing the antigen-binding site (Table 1). Fourteen residues are identical in HLA-E and HLA-Cw1 and are not conserved in the HLA-A2 sequence, but only six of them are facing the peptide binding groove: three in the β sheets (Ser-24, Leu-95, and Trp-97) and three in the α helix (Arg-62, Ala-71, and Glu-152) (Fig. 7). When similar sequence analysis between HLA-E and HLA-B51, which did not cross-react with the D8L viral ligand, was carried out, only four residues located in the peptide binding groove (Ser-24, Ala-71, Leu-95, and Trp-97) were observed to be identical, but they were not conserved in the HLA-A2 or -B51 class I molecules (Table 1 and Fig. 7). All four of these residues match the minimum desetope conserved between HLA-E and HLA-Cw1 but absent in HLA-A2 or -B51 and thus are possibly responsible for the cross-reactivity of the D8L112–119 ligand. In addition, although HLA-A2, -B51, and -Cw1 present some peptides with similar P2 and PΩ anchor motifs, overlapping peptide repertoires are not described (SYFPEITHI database (40)). Thus, the cross-reactivity identified in the current report

**TABLE 1**

Polymorphic residues among HLA class I molecules

| Residuesa | 1 | 10 | 20 | 30 | 40 | 50 | 60 |
|-----------|---|----|----|----|----|----|----|
| **HLA-E** | GSHSLKYFHTSVSRPGREFISVGYVDDTQFVRFDNDAAASPRMVPRAQPMEQEGSEYW | | | | | | |
| **HLA-Cw1** | C | M | F | S | GE | V | P |
| **HLA-A2** | MR | F | A | S | Q | E |
| **HLA-B51** | MR | Y | AM | A | TE | I | P |

| Residuesa | 61 | 70 | 80 | 90 | 100 | 110 | 120 |
|-----------|----|----|----|----|-----|-----|-----|
| **HLA-E** | DRETRSDTQAIFRVNLTRLRGGYYNQSEAGSHTLOWMHCGELGPDRRFLRGYEQFYADG | | | | | | |
| **HLA-Cw1** | QKYKQR | TD | S | N | C | G | L | D | Y |
| **HLA-A2** | G | KVKAHSH | D | G | V | R | Y | DV | S | W | H | Y |
| **HLA-B51** | N | Q | KTN | TY | E | IALR | W | T | Y | DV | G | L | HN | Y |

| Residuesa | 121 | 130 | 140 | 150 | 160 | 170 |
|-----------|-----|-----|-----|-----|-----|-----|
| **HLA-E** | KDYLTLNEDLRSTAVDAAIQSEQKSNDASEAEHQRAYLEDTCEWELHKYLEKGKETL | | | | | |
| **HLA-Cw1** | IA | A | TQR | WEA | R | QR | G | RR | N |
| **HLA-A2** | IA | K | A | M | TTKH | WEA | HV | QL | G | RR | N |
| **HLA-B51** | IA | S | A | TQR | WEA | R | QL | GL | RRH | N |

*α1 and α2 HLA-E residues were compared with HLA-Cw1, -A2, or -B51 class I Molecules. Blanks indicate identity with the HLA-E reference sequence. The identical residues identified between HLA-E and HLA-Cw1 but not HLA-A2 or -B51 molecules (minimum desetope) and proposed as contributing to a shared structural motif that could confer peptide presenting similarities to HLA-E and HLA-Cw1 are in bold type. The sequences are from the IMGT/HLA Database.*
between HLA-E and -Cw1, an allele not clustered into HLA-A2 supertype, expands the range of possible HLA-E cross-reactivity and indicates that several other HLA class I viral ligands different from HLA-A2-restricted epitopes could be presented in association with HLA-E. Thus, future studies analyzing the HLA-E peptide repertoire under infection conditions with different viruses are needed.

Some studies have shown cross-reactivity of epitopes between very different MHC class I molecules. Cross-reactivity between multiple HLA-B alleles (HLA-B7, -B27, -B40, -B54, -B55, and -B56) that differ by ~20 residues facing the antigen-binding site has been widely reported (45). Additionally, interspecies cross-reactivity of viral ligands, shared by a human and a rhesus macaque, a rhesus macaque and a mouse, a human and a mouse, and two different chimpanzee MHC class I molecules have been described (46–49). These pairs of cross-reactive MHC molecules are very different and have marked differences in the sequence and structure of the peptide-binding groove. Dual reactivity of CD8+ T cell clones reflected presentation of structurally related peptides by two HLA class I and II molecules: HLA-B27 and HLA-DR2 (50). These findings and the results reported in the current study show the complexity and plasticity of interactions in MHC-peptide complexes.

Our study includes one distinct difference from the previous viral classical and nonclassical HLA cross-reactivity reports (15, 16, 44); the D8L112–119 ligand was isolated from TAP-deficient vaccinia virus-infected cells, and thus this viral ligand was naturally processed by a TAP-independent pathway previous to its presentation by HLA-E. Only the HCMV gpUL-40-derived ligand is currently known to assemble with HLA-E via a TAP-independent mechanism (51). This peptide exactly matches the leader sequence peptides of various HLA class I alleles and is able to substitute for the natural leader peptides from HLA-E produced by TAP that are blocked by the protein US6 in HCMV-infected cells (52). Therefore, this TAP-independent antigen presentation was previously reported as a viral mechanism to bypass the normal HLA-E loading system that evolved to occlude NK cell recognition of infected cells, whereas most HCMV epitopes remain in the cytosol without any possibility of entering the endoplasmic reticulum. Unlike this previously described tolerogenic peptide, D8L112–119 could be recognized by CD8+ T cells in the same manner as vaccinia virus-encoded HLA-A2-restricted epitopes generated in the same TAP-deficient infected cells (17), allowing it to contribute to host defense against viral infection. Lastly, the lack of polymorphism of the HLA-E gene in humans suggests that D8L112–119 could be a universal epitope, requiring future studies to understand the HLA-E-restricted response of this viral peptide.

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REFERENCES
1. Kloetzel, P. M., and Ossendorp, F. (2004) Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. Curr. Opin. Immunol. 16, 76–81
2. Cerundolo, V., and de la Salle, H. (2006) Description of HLA class I- and CD8-deficient patients. Insights into the function of cytotoxic T lymphocytes and NK cells in host defense. Semin. Immunol. 18, 330–336
3. Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L., and Tonegawa, S. (1992) TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4+8+ T cells. Cell 71, 1205–1214
4. Voldby Larsen, M., Nielsen, M., Weinzierl, A., and Lund, O. (2006) TAP-independent MHC class I presentation. Curr. Immunol. Rev. 2, 233–245
Natural HLA-E Ligand in TAP™ Vaccinia-infected Cells

5. Alhanova, D., Edwards, D. M., Hammarlund, E., Schulz, I. G., Horst, D., Wagner, M. J., Upton, C., Wiertz, E. J., Slifka, M. K., and Fruh, K. (2009) Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition. Cell Host Microbe 6, 433–445

6. Fenner, F., Henderson, D. A., Arita, I., Jezek, Z., and Ladnyi, I. (1988) Smallpox and Its Eradication, World Health Organization, Geneva, Switzerland

7. Moss, B. (1991) Vaccinia virus. A tool for research and vaccine development. Science 252, 1662–1667

8. Kennedy, R. B., Osvyannikova, I., and Poland, G. A. (2009) Smallpox vaccines for biodefense. Vaccine 27, Suppl. 4 D73–D79

9. Foulds, R. E., Duma, R. J., and Escober, M. R. (1972) Vaccinia vaccinatum and its relationship to impaired immunological responsiveness. Am. J. Med. 52, 411–420

10. Redfield, R. R., Wright, D. C., Jones, T. S., Brown, C., and Burke, D. S. (1987) Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N. Engl. J. Med. 316, 673–676

11. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Wagner, M. J., Upton, C., Wiertz, E. J., Slifka, M. K., and Fruh, K. (2009) Cowpox virus inhibits the transporter associated with antigen processing to evade T cell recognition. Cell Host Microbe 6, 433–445

12. Kennedy, R., and Poland, G. A. (2007) T-cell epitope discovery for variola and vaccinia viruses. Rev. Med. Virol. 17, 93–113

13. van Hall, T., Oliveira, C. C., Joosten, S. A., and Ottenhoff, T. H. (2010) The other Janus face of QA-1 and HLA-E: Diverse peptide repertoires in times of stress. Microbes Infect. 12, 910–918

14. Pietra, G., Romagnani, C., Manzini, C., Moretta, L., and Mingari, M. C. (2010) The emerging role of HLA-E restricted CD8+ T lymphocytes in the adaptive immune response to pathogens and tumors. J. Biomed. Biotechnol. 2010, 907092

15. Ulbrecht, M., Modrow, S., Srivastava, R., Peterson, P. A., and Weiss, E. H. (1992) Characterization of peptides bound to the class I MHC molecule HLA-A2 by mass spectrometry. J. Immunol. 149, 443–453

16. Lorente, E., Infantes, S., Barnea, E., Beer, I., Garcia, R., Lasala, F., Jiménez, M., Vilches, C., Lemmonier, F. A., Admon, A., and López, D. (2012) Multiple viral ligands naturally presented by different class I molecules in transporter antigen processing-deficient vaccinia virus-infected cells. J. Virol. 86, 527–541

17. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Williams, A. F., and Ziegler, A. (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. Cell 14, 9–20

18. Braud, V., Jones, E. Y., and McMichael, A. (1997) The human major histocompatibility complex class Ibeta molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur. J. Immunol. 27, 1164–1169

19. Salter, R. D., and Cresswell, P. (1986) Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. EMBO J. 5, 943–949

20. Lorente, E., García, R., Mir, C., Barriga, A., Lemmonier, F. A., Ramos, M., and López, D. (2012) Role of metalloproteases in vaccinia virus epitope processing for transporter associated with antigen processing (TAP)-independent human leukocyte antigen (HLA)-B7 class I antigen presentation. J. Biol. Chem. 287, 9990–10000

21. Carbone, E., Ruggiero, G., Terrazzano, G., Palomba, C., Manzo, C., Fontana, S., Spits, H., Kärre, K., and Zappacosta, S. (1997) A new mechanism of NK cell cytotoxicity activation. The CD40-CD40 ligand interaction. J. Exp. Med. 185, 2053–2060

22. Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevlik, N., Cox, A. L., Appella, E., and Engelhard, V. H. (1992) Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 255, 1261–1263

23. Berton, R., Sidney, J., Fowler, P., Chesnut, R. W., Chisari, F. V., and Sette, A. (1997) Human histocompatibility leukocyte antigen-antigen-binding supermo-tifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. J. Clin. Invest. 100, 503–513

24. Kondo, E., Akatsuka, Y., Kuzushima, K., Tsujimura, K., Asakura, S., Tajima, K., Kagami, Y., Kodera, Y., Tanimoto, M., Morishima, Y., and Taka-hashi, T. (2004) Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. Blood 103, 630–638

25. Fan, Q. R., Garboczi, D. N., Winter, C. C., Wagtmann, N., Long, E. O., and Wiley, D. C. (1996) Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule. Proc. Natl. Acad. Sci. U.S.A. 93, 7178–7183

26. Infantes, S., Lorente, E., Barnea, E., Beer, I., Cragnolini, J., García, R., Lasala, F., Jiménez, M., Admon, A., and López, D. (2010) Multiple, non-conserved, internal viral ligands naturally presented by HLA-B27 in human respiratory syncytial virus-infected cells. Mol. Cell. Proteomics 9, 1533–1539

27. Lee, N., Llano, M., Carretero, M., Ishitani, A., Navarro, F., López-Botet, M., and Geraghty, D. E. (1998) HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc. Natl. Acad. Sci. U.S.A. 95, 5199–5204

28. Parham, P., and Bodmer, W. F. (1978) Monoclonal antibody to a human histocompatibility alloantigen, HLA-A2. Nature 276, 397–399

29. Duan, Y., Wu, C., Chowdhury, S., Lee, M. C., Xiong, G., Zhang, W., Yang, R., Cieplak, P., Luo, R., Lee, T., Caldwell, J., Wang, J., and Kolmant, P. (2003) A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. J. Comput. Chem. 24, 1999–2012

30. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Skell, W. C. (1983) Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 79, 926–935

31. Still, W. C., Tempczyk, A., Hawley, R. C., and Hendrickson, T. Y. (1990) Semianalytical treatment of solvation for molecular mechanics and dynamics. J. Am. Chem. Soc. 112, 6127–6129

32. Weiser, I., Shenkin, P. S., and Still, W. C. (1999) Approximate atomic surfaces from linear combinations of pairwise overlaps. J. Comput. Chem. 20, 217–230

33. Miller, J. D., Weber, D. A., Ibegbu, C., Pohl, J., Altman, J. D., and Jensen, P. E. (2003) Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2. J. Immunol. 171, 1369–1375

34. Ravindranath, M. H., Pham, T., El-Awar, N., Kaneku, H., and Terasaki, P. I. (2004) Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. Web-tools validate the immunogenic epitopes of HLA-B27 in human respiratory syncytial virus-infected cells. J. Immunol. 173, 5852–5862
43. Joosten, S. A., van Meijgaarden, K. E., van Weeren, P. C., Kazi, F., Geluk, A., Savage, N. D., Drijfhout, J. W., Flower, D. R., Hanekom, W. A., Klein, M. R., and Ottenhoff, T. H. (2010) *Mycobacterium tuberculosis* peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PloS Pathog.* 6, e1000782

44. Nattermann, J., Nischalke, H. D., Hofmeister, V., Kupfer, B., Ahlenstiel, G., Feldmann, G., Rockstroh, J., Weiss, E. H., Sauerbruch, T., and Spengler, U. (2005) HIV-1 infection leads to increased HLA-E expression resulting in impaired function of natural killer cells. *Antivir. Ther.* 10, 95–107

45. López, D., García-Hoyo, R., and López de Castro, J. A. (1994) Clonal analysis of alloreactive T cell responses against the closely related B*2705 and B*2703 subtypes. Implications for HLA-B27 association to spondyloarthropathy. *J. Immunol.* 152, 5557–5571

46. Hickman-Miller, H. D., Bardet, W., Gilb, A., Luis, A. D., Jackson, K. W., Watkins, D. I., and Hildebrand, W. H. (2005) Rhesus macaque MHC class I molecules present HLA-B-like peptides. *J. Immunol.* 175, 367–375

47. Sette, A., Sidney, J., Bui, H. H., del Guercio, M. F., Alexander, J., Loffredo, J., Watkins, D. I., and Mothé, B. R. (2005) Characterization of the peptide-binding specificity of Mamu-A*11 results in the identification of SIV-derived epitopes and interspecies cross-reactivity. *Immunogenetics* 57, 53–68

48. Infantes, S., Lorente, E., Cragnoiini, J. I., Ramos, M., García, R., Jiménez, M., Iborra, S., Del Val, M., and López, D. (2011) Unusual viral ligand with alternative interactions is presented by HLA-Cw4 in human respiratory syncytial virus-infected cells. *Immunol. Cell Biol.* 89, 558–565

49. Cooper, S., Kowalski, H., Erickson, A. L., Arnett, K., Little, A. M., Walker, C. M., and Parham, P. (1996) The presentation of a hepatitis C viral peptide by distinct major histocompatibility complex class I allotypes from two chimpanzee species. *J. Exp. Med.* 183, 663–668

50. López, D., Barber, D. F., Villadangos, J. A., and López de Castro, J. A. (1993) Cross-reactive T cell clones from unrelated individuals reveal similarities in peptide presentation between HLA-B27 and HLA-DR2. *J. Immunol.* 150, 2675–2686

51. Tomasec, P., Braud, V. M., Rickards, C., Powell, M. B., McSharry, B. P., Gadola, S., Cerundolo, V., Borysiewicz, L. K., McMichael, A. J., and Wilkinson, G. W. (2000) Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287, 1031

52. Griffin, B. D., Verweij, M. C., and Wiertz, E. J. (2010) Herpesviruses and immunity. The art of evasion. *Vet. Microbiol.* 143, 89–100

53. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329, 506–512

54. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) The foreign antigen-binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329, 512–518