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 increased 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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[2] The abbreviations used are: TAP, transporter associated with antigen processing; HLA, human leukocyte antigen; VACV, vaccinia virus; Ab, antibody.

than 70 VACV-derived epitopes presented by various HLA molecules (11, 12).

HLA-E is a nonclassical class I molecule that binds monomorphic 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 monomorphic 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, C11R$_{101-110}$, 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 DG$\underline{A}$IIISI. 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: KPNA2 (GLVPFLVSV, HLA-A2-restricted) (23), HBV HBc$_{19-27}$ (LPSDFFPSV, HLA-B51-restricted) (24), CMV pp65$_{7-15}$ (RCPEMISVL, HLA-Cw1-restricted) (25), the leader peptide of HLA (VMAPRALLL, HLA-E-restricted), and C4CON (QYDDAVYLK, 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 10% 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 EC$_{50}$, which is defined as the molar concentration of the peptides 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 D8L$_{112-119}$ peptide bound to HLA-E model was built with the MODELLER9v7 program using the 3bzf Protein Data Bank file as template. The

protonation states of the ionizable groups for the three system 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 with 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 D8L$_{112-119}$ synthetic peptide
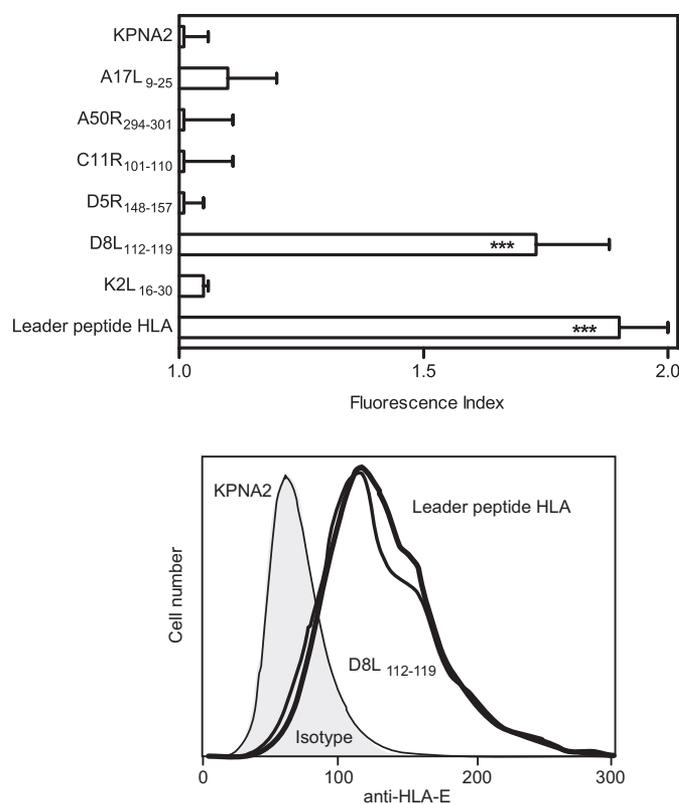

FIGURE 1. **HLA-E stabilization with synthetic VACV ligands.** The stability of HLA-E-peptide complexes on the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 peptide and the leader peptide of HLA were used as negative and positive controls, respectively. The mAb 3D12 was used for staining. The results, calculated as fluorescence index values ± S.D., are the means of four to five independent experiments. ***, significant p values ($p < 0.001$). A representative experiment was depicted in the *bottom panel*. *Shaded histogram*, isotypic control; *thin line*, KPNA2 peptide; *medium line*, D8L 112–119; *thick line*, leader peptide of HLA.

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 D8L$_{112-119}$ octamer DGLIIISI 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 D8L$_{112-119}$ 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 I 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 D8L$_{112-119}$ 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
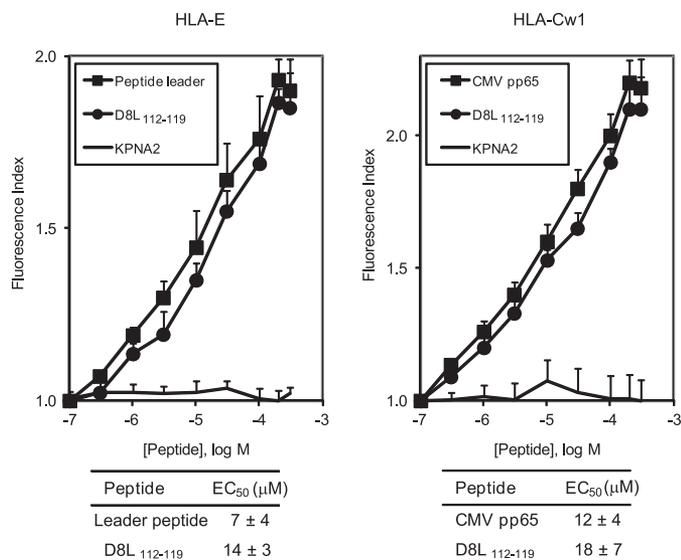
FIGURE 2. **Binding affinity to HLA-E and -Cw1 of VACV D8L$_{112–119}$ synthetic peptide.** The synthetic peptide VACV D8L$_{112–119}$ (*circles*) was titrated bound to HLA-E (*left panel*) or HLA-Cw1 (*right panel*) on T2 TAP-deficient cells, and stabilization of HLA was measured by flow cytometry. The KPNA2 peptide was used as a negative control (*solid line*). The leader peptide of HLA and CMV pp65$_{7–15}$ were used as positive controls (*squares*) for binding to the HLA-E and -Cw1 alleles, respectively. The Abs used were monoclonal 3D12 (anti-HLA-E, *left panel*), and polyclonal SC-19438 (anti-HLA-C class I molecules, *right panel*). The data calculated as EC$_{50}$ values ± S.D. are shown below and are the means of three to five independent experiments.

HLA-E (Fig. 2, *left panel*) and HLA-Cw1 (Fig. 2, *right panel*) expression on cells, with an EC$_{50}$ for MHC binding of 14 ± 3 and 18 ± 7 μM, respectively, confirming their dual presentation. These EC$_{50}$ values are similar to those of the other natural high affinity ligands used as positive controls. In summary, the VACV D8L$_{112–119}$ octamer is a TAP-independent high affinity ligand presented by both classical and nonclassical class I molecules.

The D8L$_{112–119}$ 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 D8L$_{112–119}$ ligand. Fig. 3 (*upper panel*) shows that induction of HLA-A2 complexes with the vaccinia D8L$_{112–119}$ 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 D8L$_{112–119}$ ligand was examined. HLA-peptide complex stability assays were performed in which HLA-B51 molecules were stained in the presence of the VACV ligand. In contrast to a control HLA-B51 ligand, the HBV HBc peptide, induction of complexes with the vaccinia D8L$_{112–119}$ 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.

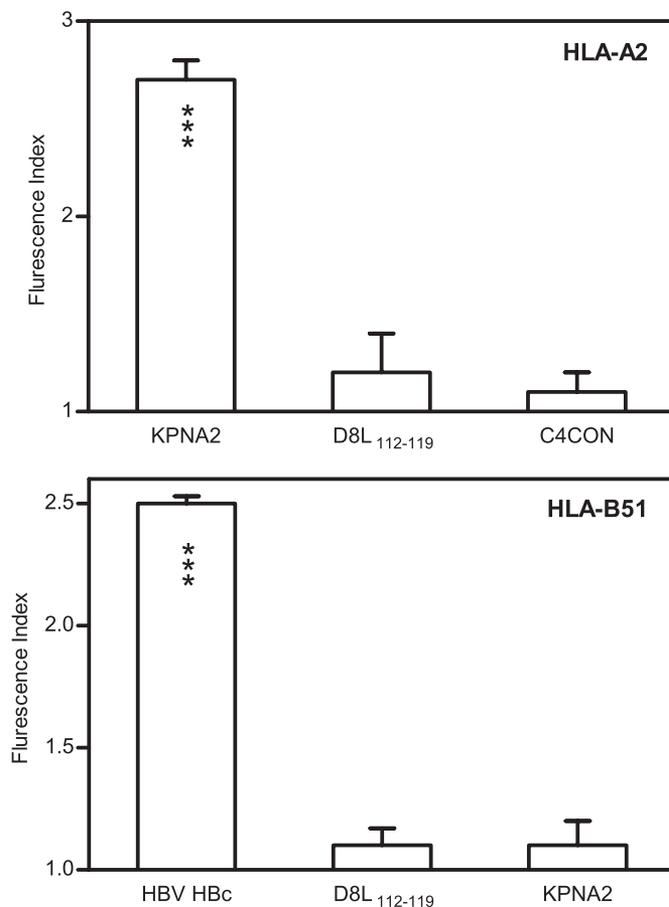FIGURE 3. **HLA-A2 and -B51 stabilization assay with synthetic VACV D8L$_{112–119}$ ligand.** The stability of HLA-A2-peptide (*upper panel*) or HLA-B51-peptide (*lower panel*) complexes on the surface of T2 TAP-deficient cells were measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 and the HBV HBc peptides were used as positive controls for binding to the HLA-A2 and -B51 alleles, respectively. The C4CON and the KPNA2 peptides were used as negative controls for binding to the HLA-A2 and -B51 alleles, respectively. The Abs used were monoclonal PA2.1 (anti-HLA-A2, *upper panel*) and polyclonal H00003106-B01P (anti-HLA-B class I molecules, *lower panel*). The results, calculated as in Fig. 1, are the mean of four independent experiments. ***, significant p values ($p < 0.001$).

The Viral D8L$_{112–119}$ 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 D8L$_{112–119}$ 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 ^{51}Cr release assay was performed. As indicated in Fig. 4, incubation of target cells with the D8L$_{112–119}$ peptide reduced their susceptibility to NK3.3-mediated cytotoxicity ($65 \pm 9\%$ of specific inhibition) to a similar extent as was obtained with the positive control peptide ($71 \pm 6\%$ of specific inhibition), whereas incubation of 721.221 cells with an irrelevant peptide had no effect on cytotoxic function of NK3.3 cells.

VACV D8L$_{112–119}$ 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-
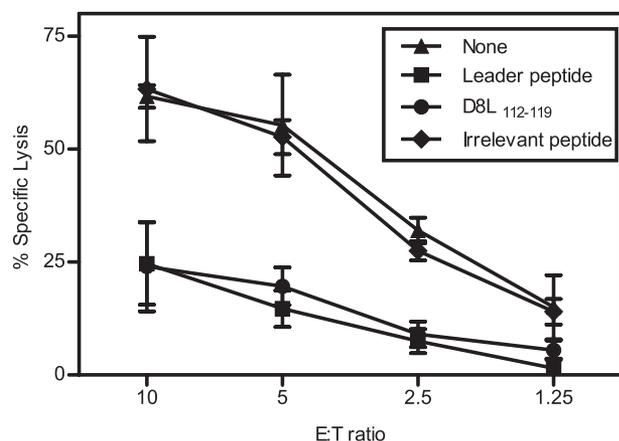
FIGURE 4. **VACV D8L$_{112-119}$ synthetic peptide inhibits HLA-E-mediated cytotoxicity by natural killer cells.** 721.221 target cells prepulsed with 100 μM of the indicated synthetic peptides were tested in a standard cytolytic assay for natural killer cytotoxicity with NK3.3 cells. The leader peptide of HLA (*squares*) and an irrelevant peptide (*diamonds*) were used as positive and negative controls, respectively. The data are the means of three independent experiments $\pm$ S.D. ***, significant *p* values (*p* < 0.001) were found between no peptide or irrelevant peptide *versus* leader peptide of HLA or D8L$_{112-119}$.

ificity pockets in the binding groove, HLA-E possesses five main anchor sites at the P2, P3, P6, P7, and P9 positions (Fig. 5*A*). 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. 5*A*). 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 D8L$_{112-119}$ 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 CΩ residue with the F pocket (Fig. 5*B*). 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. 5*C*). 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. 5*B*) and the E pocket in the B model (Fig. 5*C*). These different models predicted mutually exclusive interactions with either the P3 residue and the D pocket (A model; Fig. 5*B*) or the P5 residue and the C pocket (B model; Fig. 5*C*).

To test the two alternative models, new HLA-peptide complex stability assays were performed using monosubstituted Ala analogues of D8L$_{112-119}$ 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. 5*B*). In contrast, exchanging Ala with either P5 Ile or P8 Ile residues, which could be additional anchor res-
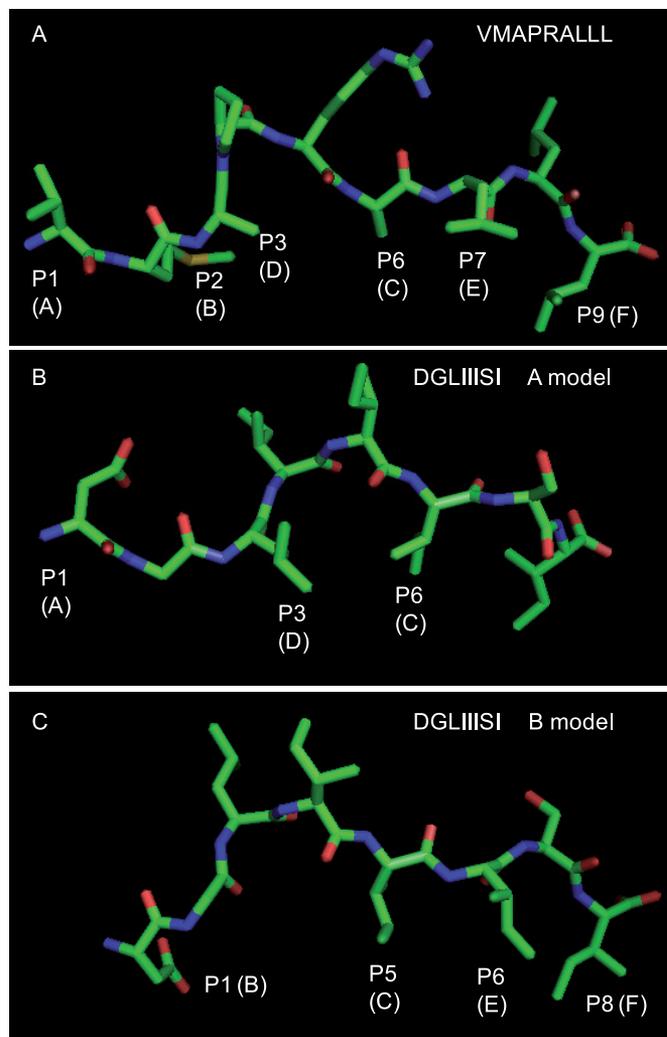
FIGURE 5. **Modeling of HLA-E-bound conformations of VACV D8L$_{112-119}$ peptide.** The backbone atoms of the indicated HLA-E-bound peptides are displayed as *ribbon tubes* (*A*, VMAPRALLL; *B* and *C*, DGLIIISI in two possible conformations: A model and B model, respectively). The atoms are represented by sticks using the following color scheme: *blue*, nitrogen; *red*, oxygen; and *green*, carbon. The peptide residues that interact with the indicated HLA-E pockets are designated. The HLA-E protein is not displayed. The figure was prepared using the PyMOL program.

idues 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-D8L$_{112-119}$ 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 nonclassical 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

FIGURE 6. HLA stabilization assay with monosubstituted Ala analogues of VACV D8L$_{112-119}$ synthetic peptide. Stability at the cell surface of HLA-E on the cell surface of T2 TAP-deficient cells was measured by flow cytometry. The indicated peptides were used at 200 μM. The KPNA2 p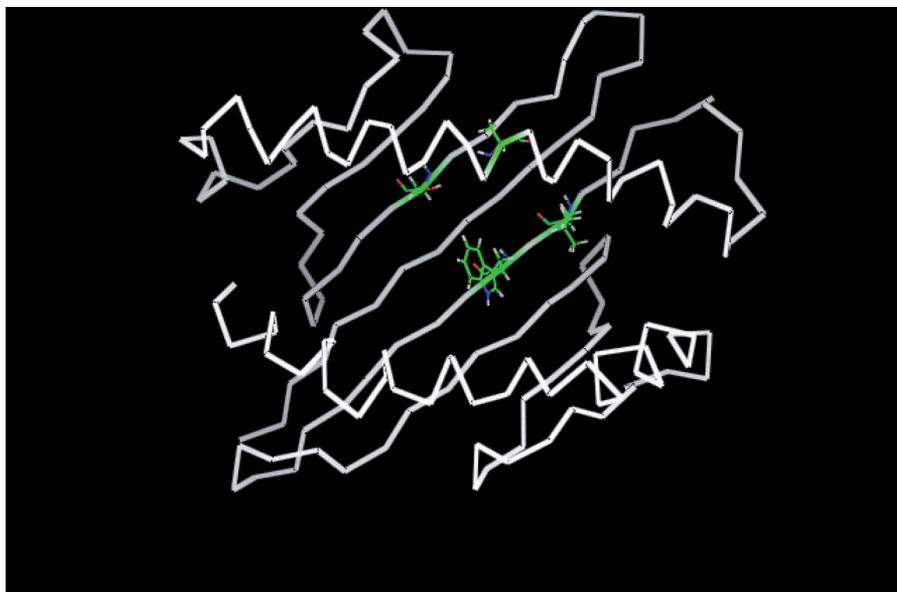eptide was used as negative control. The mAb 3D12 was used for staining. The data, calculated as in Fig. 1, are the means of three or four independent experiments. ***, significant p values ($p < 0.001$).

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 D8L$_{112-119}$ 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

	Residues[a]					
	1	10	20	30	40	50	60
HLA-E	GSHSL**K**YFHTSVSRPGRGEPRFI**S**VGYVDDTQFVRFDNDAASPRMVPRAPWMEQEGSEYW
HLA-Cw1	C	M	F		S	GE	V	P
HLA-A2		MR	F	A	S	Q	E
HLA-B51		MR	Y AM	A	S	TE	I	P

	61	70	80	90	100	110	120
HLA-E	DRETRSARDTAQIFRVNLRTLRGYYNQSEAGSHT**LQW**MHGCELGPDRRFLRGYEQFAYDG
HLA-Cw1	QKYKRQ	TD	S	N	C	D	G L	D Y
HLA-A2	G	KVKAHS	TH	D G	V R Y	DV S W	H Y
HLA-B51	N Q	KTNT	TY E	IALR	W T Y	DV	G L	HN Y

	121	130	140	150	160	170
HLA-E	KDYLTLNEDLRSWTAVDTAAQISEQKSNDASEAEHQRAYLEDTCVEWLHKYLEKGKETL
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

[a] 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.

FIGURE 7. **Structural similarities between HLA-E and HLA-Cw1 but not with HLA-A2 or-B51.** The amino acid sequence of the $\alpha 1$ and $\alpha 2$ domains of HLA-E (*white backbone*) was compared with the sequence of the equivalent domains of HLA-A2, -B51, and -Cw1 class I molecules using the same alignment used by Bjorkmann *et al.* (53, 54). 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 between HLA-E and HLA-Cw1 are depicted. The atoms of these four residues are represented by sticks using the following color scheme: *blue*, nitrogen; *red*, oxygen; and *green*, carbon. The viral peptide is not displayed. The figure was prepared using the PyMOL program.

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 $D8L_{112-119}$ 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, $D8L_{112-119}$ 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 $D8L_{112-119}$ could be a universal epitope, requiring future studies to understand the HLA-E-restricted response of this viral peptide.

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