# The immunogenicity of a viral cytotoxic T cell epitope is controlled by its MHC-bound conformation

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**Thousands of potentially antigenic peptides are encoded by an infecting pathogen; however, only a small proportion induce measurable CD8$^+$ T cell responses. To investigate the factors that control peptide immunogenicity, we have examined the cytotoxic T lymphocyte (CTL) response to a previously undefined epitope ($^{77}$APQPAPENAY$^{86}$) from the BZLF1 protein of Epstein-Barr virus (EBV). This peptide binds well to two human histocompatibility leukocyte antigen (HLA) allotypes, HLA-B*3501 and HLA-B*3508, which differ by a single amino acid at position 156 ($^{156}$Leucine vs. $^{156}$Arginine, respectively). Surprisingly, only individuals expressing HLA-B*3508 show evidence of a CTL response to the $^{77}$APQPAPENAY$^{86}$ epitope even though EBV-infected cells expressing HLA-B*3501 process and present similar amounts of peptide for CTL recognition, suggesting that factors other than peptide presentation levels are influencing immunogenicity. Functional and structural analysis revealed marked conformational differences in the peptide, when bound to each HLA-B35 allotype, that are dictated by the polymorphic HLA residue 156 and that directly affected T cell receptor recognition. These data indicate that the immunogenicity of an antigenic peptide is influenced not only by how well the peptide binds to major histocompatibility complex (MHC) molecules but also by its bound conformation. It also illustrates a novel mechanism through which MHC polymorphism can further diversify the immune response to infecting pathogens.**

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Abbreviations used: LCL, lymphoblastoid cell line; MFI, mean fluorescence intensity; v.d.w., van der Waals.

The CD8$^+$ T cell response to an infecting pathogen is generally focused toward a limited subset of antigenic peptides presented on the surface of infected cells. Furthermore, a hierarchy of immunodominance that is maintained in unrelated individuals is often observed between those peptides that are the targets of CTL recognition (1). There appear to be three major factors that control the immunogenicity of a foreign peptide: the specificity of the antigen processing machinery, the peptide binding preferences of MHC class I molecules, and limitations in the diversity of the TCR repertoire (1). How these parameters focus the CTL response toward a limited number of determinants within an antigen is not completely understood.

The dominant factor controlling the magnitude of the CTL response to a foreign peptide is the quantity of peptide presented on the surface of the APC. MHC class I molecules show strict binding specificity because of the high level of polymorphism concentrated in the antigen-binding cleft (2). The pockets (A–F) of the peptide-binding groove vary in their depth, electrostatic potential, and hydrophobicity, thereby determining the individual specificity of the peptide–MHC interaction (3). For most MHC alleles, two of these pockets display a marked preference for one or two amino acids,

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termed anchor residues, at certain positions within the peptide. For example, the common class I molecule HLA-B*3501 prefers peptide ligands, with proline as a dominant anchor residue at position 2 (P2) and tyrosine (or less commonly phenylalanine, methionine, leucine, or isoleucine) at PΩ (the COOH terminus) (4, 5). Peptide amino acids at other secondary anchor positions can also influence allele-specific binding (6).

Although immunodominance of antigenic CTL epitopes usually correlates with the abundance of peptide presented on the surface of the APC (7), there have been several reports where this is clearly not the case (8, 9). In these instances, the major factors controlling immunodominance have been proposed to be limitations and bias in the TCR repertoire imposed by thymic or peripheral selection (1, 10–12). There has also been a suggestion that some immunodominant determinants may be intrinsically more immunogenic because of an innate propensity to interact with TCRs, perhaps through the orientation or nature of side chains available for interaction (1); however, no evidence for this theory has been presented to date.

We have defined a CTL epitope ($^{77}$APQPAPENAY$^{86}$, referred to as APQP) from the BZLF1 or Z EBV replication activator protein of EBV that binds well to both HLA-B*3501 and HLA-B*3508, two closely related molecules that differ by a single amino acid at position 156 ($^{156}$Leucine vs. $^{156}$Arginine, respectively). This epitope was found to be immunogenic in individuals expressing HLA-B*3508 but, unexpectedly, no response could be detected in HLA-B*3501$^+$ EBV-exposed individuals, indicating that factors other than the level of peptide presentation are influencing immunogenicity. Structural analysis revealed major differences in the peptide conformation when bound to each HLA-B35 allotype but, surprisingly, there were no important differences in the MHC class I heavy chain conformation. These data indicate that T cell responsiveness to a foreign peptide can be influenced by its MHC-bound conformation.

## RESULTS

### The influence of a single MHC amino acid difference on the immunogenicity of a CTL epitope from BZLF1

A highly immunogenic CTL epitope (BZLF1 54–64, EPLPQGQLTAY) that binds to HLA-B*3501 has recently been identified from the BZLF1 antigen of EBV (13, 14). A close examination of the BZLF1 protein sequence revealed a 10-mer sequence (BZLF1 77–86, APQPAPENAY) downstream from this previously defined 11-mer epitope that also conforms to the HLA-B35 peptide-binding motif (4, 5). A peptide corresponding to this 10-mer sequence was tested for recognition by a CTL line raised from an HLA-B35$^+$ EBV-sero$^+$ donor (SB) by in vitro stimulation of PBMCs with the peptide for 10 d. Our earlier studies have shown that this stimulation protocol with EBV peptides only induces a measurable in vitro CTL response in donors who have been previously exposed to the antigenic determinant through prior infection with EBV, and any peptide-specific CTL lysis observed in these assays reflects a substantial in vivo CTL response in these individuals (unpublished data). This CTL line recognized autologous PHA blasts (SB) only after pretreatment with the APQP peptide, thereby demonstrating that this sequence includes an EBV CTL epitope (Fig. 1 A). To determine if the HLA-B35 allele expressed by donor SB (HLA-B*3508) is responsible for presenting this

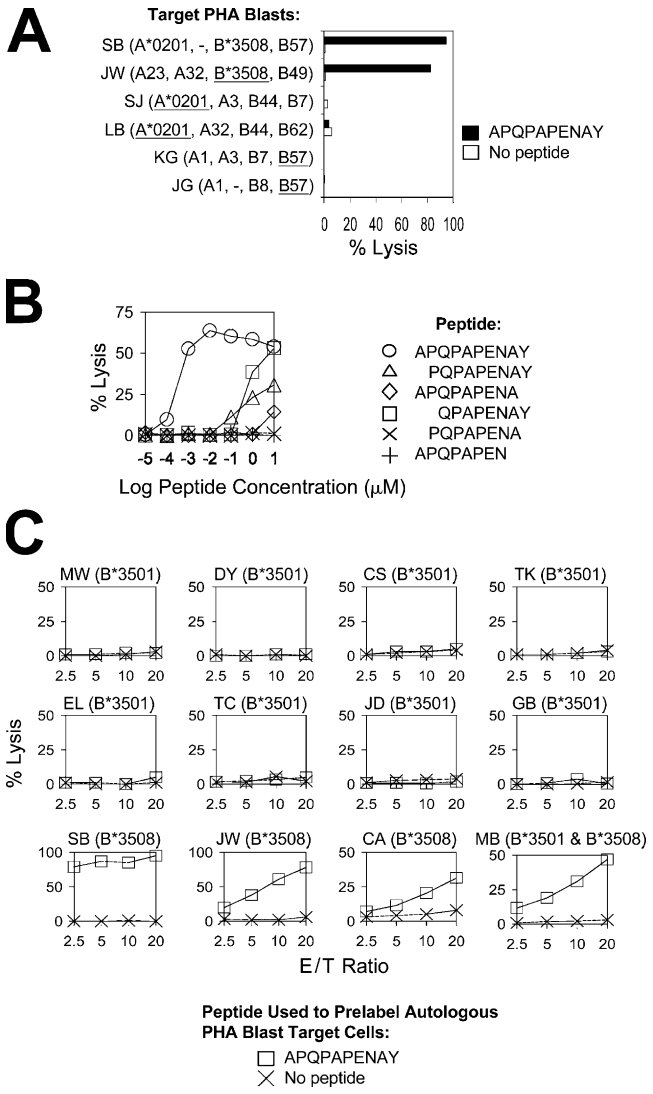


**Figure 1. Identification of a CTL epitope from the BZLF1 antigen of EBV that is immunogenic in HLA-B*3508$^+$ individuals.** (A) Cytotoxicity by a CTL line raised against the APQP peptide of autologous PHA blast target cells (SB) and PHA blasts sharing one HLA allele with the CTLs (underlined). The target cells were tested with or without the addition of 0.1 μM of the APQP peptide. E/T ratio = 10:1. (B) Peptide dose-response cytotoxicity assay using a CTL line from donor SB (HLA-B*3508$^+$) raised against the APQP peptide, and PHA blast target cells also from donor SB. E/T ratio = 10:1. (C) CTL recognition at a variety of E/T ratios by polyclonal T cell lines raised against the APQP peptide from PBMCs from eight HLA-B*3501$^+$ donors, three HLA-B*3501$^+$ donors, and one HLA-B*3501/-B*3508 coexpressing donor. The target cells were autologous PHA blasts that were pretreated with 0.1 μM of the APQP peptide or left untreated.

EBV epitope, cells expressing a range of other HLA alleles were also included as targets for this CTL line. PHA blasts sharing HLA-B*3508 with donor SB were recognized efficiently by the CTLs only after the addition of the APQP peptide, whereas target cells sharing other class I alleles with donor SB were not lysed, confirming that this peptide is presented by HLA-B*3508 (Fig. 1 A).

Because the APQP 10-mer peptide includes an 8-mer sequence that also conforms to the HLA-B35 binding motif, experiments were conducted to determine the minimal length of this CTL epitope. All possible 8- and 9-mer peptides from within the APQP 10-mer sequence were tested for recognition by a CTL line raised from donor SB. This dose-response experiment clearly shows that the 10-mer peptide is recognized most efficiently, with high levels of lysis induced with peptide concentrations down to 0.001 μM (Fig. 1 B), suggesting that APQPAPENAY is the naturally presented epitope.

To determine if other EBV-sero$^+$ HLA-B35$^+$ donors respond to this BZLF1 epitope, CTL lines were raised against the APQP peptide using PBMCs from a panel of such donors to test for reactivity with the peptide. The donors expressed either HLA-B*3501 or HLA-B*3508, two allotypes of the HLA-B35 serotype that diverge by a single amino acid difference at position 156. Surprisingly, this micropolymorphism appeared to have a major impact on responsiveness to the APQP epitope, because no evidence of a response to the peptide could be seen with these cytotoxicity assays in any of eight HLA-B*3501$^+$ donors. In contrast, three donors expressing the much less common HLA-B*3508 responded well to the peptide, as did a donor positive for both HLA-B*3501 and HLA-B*3508 (Fig. 1 C).

### Peptide conformation, not peptide binding efficiency, controls the immunogenicity of a viral CTL epitope

To determine if these dramatic differences in responsiveness to this 10-mer epitope in individuals expressing HLA-B*3501 or HLA-B*3508 are related to how well the peptide associates with each HLA-B35 subtype, MHC–peptide binding assays were conducted. These assays used the T2 cell line transfected with either HLA-B*3501 or HLA-B*3508. These antigen-presenting mutant cells express stable class I HLA molecules on their surface upon addition of exogenous HLA binding peptide (15). As positive controls for these experiments, peptides known to be highly immunogenic EBV epitopes in HLA-B*3501$^+$ or HLA-B*3508$^+$ individuals were used for comparison. These included the BZLF1 11-mer EPLPQGQLTAY (13, 14), which is immunogenic in HLA-B*3501$^+$ individuals, and the unusually long 13-mer peptide LPEPLPQGQLTAY was included for HLA-B*3508 (13, 16). An earlier study has shown that this 13-mer epitope binds to HLA-B*3508 more efficiently than the completely overlapping EPLPQGQLTAY 11-mer peptide. Consistent with this observation, EBV-exposed individuals expressing HLA-B*3508 show a strong CTL response to the 13-mer epitope in preference to the overlapping 11-mer peptide (13, 16).

As shown in Fig. 2 A, the APQP peptide stabilized the expression of both HLA-B*3501 and HLA-B*3508 on the surface of T2 cells, indicating efficient binding to each class I molecule. In comparison to the immunogenic 11- or 13-mer BZLF1 epitopes, the APQP 10-mer bound with similar efficiency to both HLA-B*3501 and HLA-B*3508.

To investigate the possibility that the APQP peptide may bind unstably to HLA-B*3501 in comparison to an EBV epitope that is immunogenic in the context of this class I molecule, peptide–MHC dissociation rates were examined on living cells. T2.B*3501 cells were loaded with either APQP or the highly immunogenic EPLPQGQLTAY peptide and were then washed and assessed for B*3501 surface expression levels at various time points. The data show that the two peptides dissociate from HLA-B*3501 at essentially similar rates (Fig. 2 B), indicating that the contrasting responses to the APQP epitope in individuals expressing HLA-B*3501 or HLA-B*3508 is unrelated to peptide–MHC binding stability.

To determine if the T cells that respond to the APQP peptide in HLA-B*3508$^+$ donors have the capacity to recognize the peptide bound to HLA-B*3501, peptide-specific

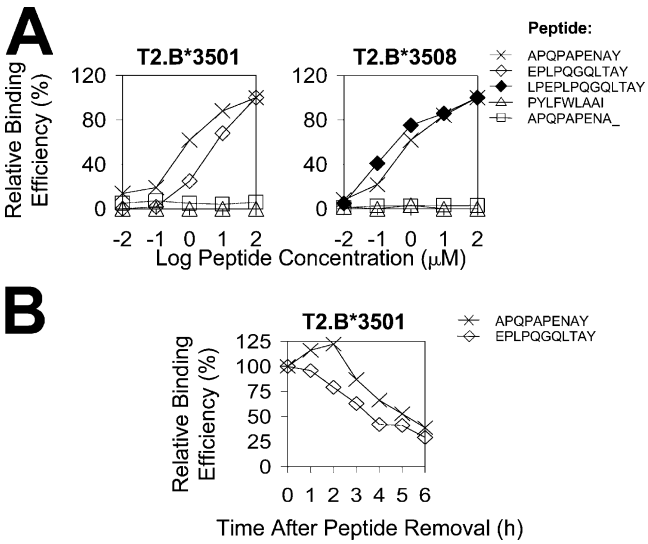


**Figure 2. The APQP peptide binds efficiently to both HLA-B*3501 and HLA-B*3508.** (A) Peptide–MHC binding assay comparing APQP with other peptides for their ability to stabilize HLA-B*3501 or HLA-B*3508 expression on T2 cells that had been transfected with these HLA genes. These other peptides included EPLPQGQLTAY, an EBV epitope immunogenic in HLA-B*3501$^+$ individuals (tested only with T2.B*3501), and LPEPLPQGQLTAY, an EBV epitope immunogenic in HLA-B*3508$^+$ individuals (tested only with T2.B*3508). As negative controls, the HLA-A24/-A23–binding EBV epitope PYLFWLAAI and a truncated version of the APQP peptide (APQPAPENA_) were also included. The T2.B*3501 data were calculated relative to the EPLPQGQLTAY peptide used at 100 μM, and the T2.B*3508 data were calculated relative to the LPEPLPQGQLTAY peptide used at 100 μM. (B) Peptide–MHC dissociation rates were examined for the APQP and the EPLPQGQLTAY peptides using T2.B*3501. Data were calculated relative to EPLPQGQLTAY at time 0.

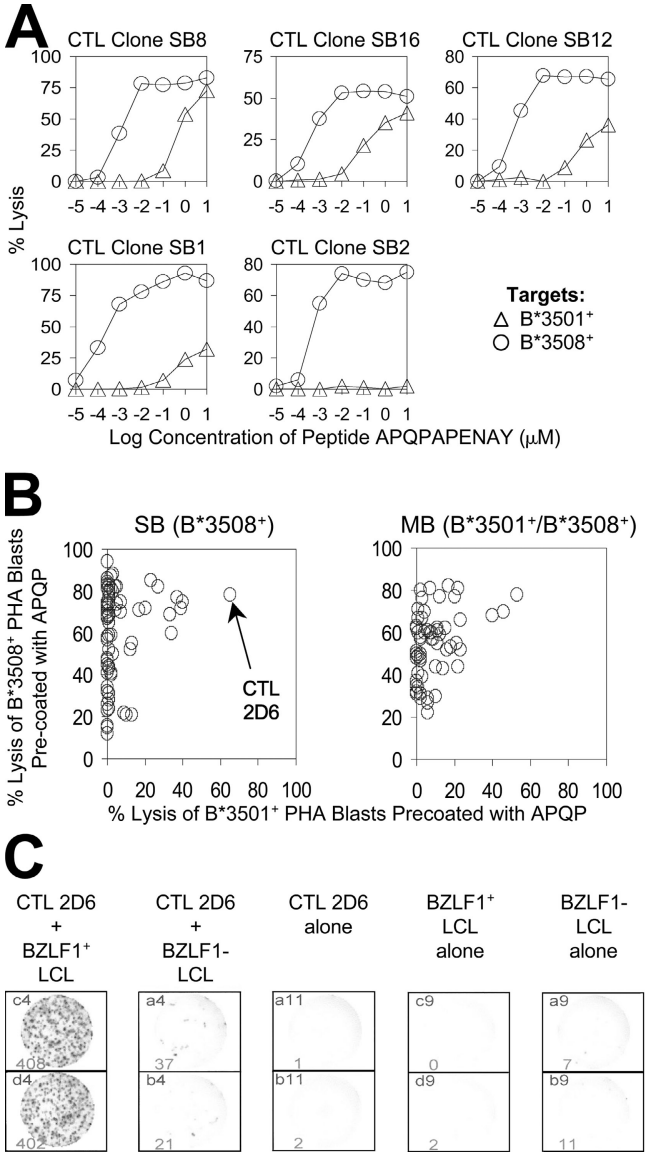


**Figure 3. CTL recognition of the APQP peptide in the context of HLA-B*3508 or HLA-B*3501.** (A) Peptide dose-response cytotoxicity assay using five APQP-specific CTL clones from donor SB (HLA-B*3508+), and PHA blast target cells expressing either HLA-B*3501 or HLA-B*3508. E/T ratio = 2:1. (B) Multiple CTL microcultures were established from the HLA-B*3508+ donor SB and the HLA-B*3501/-B*3508 coexpressing donor MB by stimulating PBMCs at limiting dilution with the APQP peptide. On day 13, the microcultures were screened for cross-recognition of the peptide (used at 0.02 μg/ml) presented on either HLA-B*3501+ or HLA-B*3508+ PHA blasts. The data are from CTL microcultures raised from responder PBMC concentrations from which <50% of the wells produced CTLs specific for the stimulator EBV epitope; thus, most were likely to have been generated from a single peptide-specific CTL. Data from any CTL microcultures that killed the PHA blast target cells without peptide addition was discarded, and data are only shown for microcultures that displayed considerable lysis of one or both of the peptide-coated target cells. (C) The CTL microculture 2D6 that could efficiently recognize exogenously added APQP peptide in the context of HLA B*3501 was used in an IFN-γ ELISPOT assay to determine if this epitope is naturally presented on EBV-infected cells expressing this HLA allele. The target cells were HLA B*3501+ LCLs carrying either the WT EBV genome (BZLF1+ LCL) or an EBV genome that had been rendered incapable of lytic cycle entry by disruption of the *BZLF1* gene (BZLF1− LCL). The number of spots per well is shown.

CTL clones were raised from an HLA-B*3508+ donor and screened for recognition of the epitope presented on target cells expressing either HLA-B*3501 or HLA-B*3508 (Fig. 3 A). These peptide dose-response cytotoxicity assays show that five out of five CTL clones from donor SB recognized the peptide several orders of magnitude more efficiently in the context of self–HLA-B*3508 compared with HLA-B*3501, suggesting that the peptide binds with distinct conformations on these closely related HLA molecules. To examine this same issue with a broader range of APQP-specific T cells, multiple CTL microcultures were generated from donors SB (HLA-B*3508+) and MB (HLA-B*3501+ and HLA-B*3508+) by stimulating PBMCs at a limiting dilution with the APQP peptide. On day 13, the microcultures were screened for cross-recognition of the peptide presented by either HLA-B*3501+ or HLA-B*3508+ PHA blast target cells (peptide concentration = 0.02 μg/ml). The data shown in Fig. 3 B are from CTL microcultures raised from responder PBMC densities from which <50% of the wells produced CTL specific for the EBV epitope; thus, most were likely to have been generated from a single peptide-specific CTL. Consistent with data presented in Fig. 3 A, the vast majority of these CTLs recognized the APQP peptide much more efficiently when bound to HLA-B*3508+ target cells compared with HLA-B*3501+ target cells. These data indicate that the HLA-B*3501–APQP and HLA-B*3508–APQP structures are quite distinct targets for T cell recognition.

To determine if the APQP peptide is processed and presented from endogenously expressed viral antigen in HLA-B*3501+ cells, the single T cell microculture from donor SB (HLA-B*3508+) that could efficiently cross-recognize the exogenously added peptide presented by HLA-B*3501 (referred to as CTL 2D6; Fig. 3 B) was used as a probe for HLA-B*3501–APQP complexes. Because only a small proportion of cells in lymphoblastoid cell lines (LCLs) express the BZLF1 lytic antigen of EBV, it was impossible to use conventional cytotoxicity assays to test for T cell recognition of endogenously expressed antigen. Instead we used a recently described method (17) that measures IFN-γ release in an ELISPOT assay where the effector T cells were challenged with LCLs expressing HLA-B*3501 and infected with either WT EBV (BZLF1+ LCLs) or a recombinant EBV in which the *BZLF1* gene was deleted (BZLF1− LCLs). As shown in Fig. 3 C, the 2D6 CTLs responded strongly to the BZLF1+ LCLs but not the BZLF1− LCLs, demonstrating that the APQP peptide is processed from endogenously expressed antigen and presented by HLA-B*3501 at sufficient levels for T cell activation. This data supports the notion that factors other than peptide presentation levels control the immunogenicity of this viral CTL epitope.

To examine levels of the APQP peptide processed and presented from endogenously expressed viral antigen in

HLA-B*3501$^+$ versus HLA-B*3508$^+$ cells, we acid eluted class I–bound peptides from EBV-infected cells expressing each of these HLA alleles and subjected extracts to HPLC fractionation. As mentioned in the previous paragraph, only a small proportion of cells in LCLs express the BZLF1 antigen, which posed a potential problem for these experiments. Because levels vary slightly between different cell lines, we used flow cytometry and an mAb for BZLF1 to test several HLA-B*3501$^+$ and HLA-B*3508$^+$ LCLs from our stored panel, and those expressing the highest levels were selected for further analysis (HLA-B*3501$^+$ LCL, 7.3% BZLF1$^+$; B*3508$^+$ LCL, 3.8% BZLF1$^+$). Ionizing radiation has been shown to increase the amount of viral reactivation in LCLs (18); thus, to ensure that levels of BZLF1 expression were sufficient for the peptide elution experiments, the LCLs were γ-irradiated (200 rads) and incubated at 37°C overnight before peptide recovery. This procedure nearly doubled the percentage of cells in our cell lines that were positive for BZLF1 expression (HLA-B*3501$^+$ LCL, 12.3% BZLF1$^+$; B*3508$^+$ LCL, 6.5% BZLF1$^+$). Additional control experiments using ELISPOT assays similar to those shown in Fig. 3 C showed that processing and presentation of the APQP peptide were not inhibited by the irradiation step (unpublished data).

The HPLC fractions from extracts from the two cell lines that were predicted to include the APQP peptide (based on a parallel HPLC run with synthetic APQP peptide) were tested at varying dilutions for recognition by an APQP-specific CTL clone (Fig. 4, A and B). To allow an estimate of the concentration of APQP in each fraction, synthetic APQP was tested in parallel for CTL recognition at varying concentrations (Fig. 4 C). Half-maximum lysis was observed with 48 pg/ml of synthetic peptide (Fig. 4 C), and similar levels of lysis were recorded when HLA-B*3501 fraction 32 was used at a dilution of 1:32 (Fig. 4 A), suggesting that the concentration of APQP in this fraction was ~1,540 pg/ml. Similar extrapolation with data from the other fractions allowed an estimate of the APQP concentration in each (Fig. 4 D). The total amount of APQP peptide eluted off each cell line (from ~$8 \times 10^8$ cells) was estimated at 90 pg for HLA-B*3501 and 40 pg for B*3508. Because there were approximately twice as many BZLF1-expressing cells in the original HLA-B*3501$^+$ population, these data suggest that similar levels of the APQP peptide are presented by virus-infected cells expressing these highly homologous HLA alleles, indicating that other factors must control the immunogenicity of this EBV T cell epitope. Further analysis by liquid chromatography–mass spectrometry of the peptide repertoire in fraction 32 from the B*3501$^+$ LCLs confirmed that the APQP 10-mer peptide was present (not depicted).

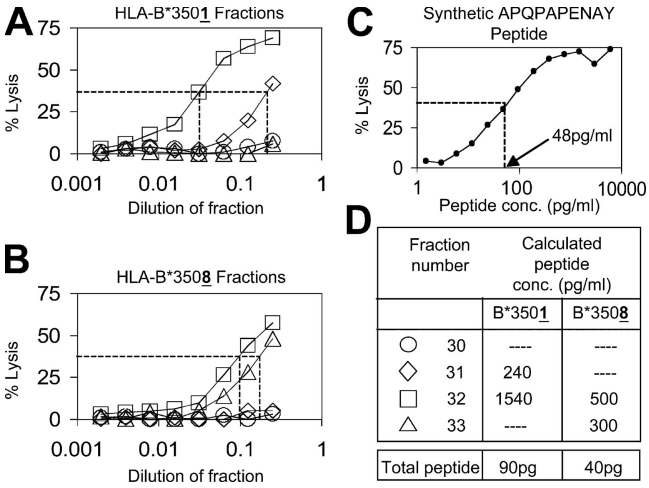


**Figure 4. EBV-infected cells expressing HLA-B*3501 or HLA-B*3508 present similar levels of the APQP peptide after endogenous processing.** Approximately $8 \times 10^8$ LCLs from the HLA-B3501$^+$ donor MW or the HLA-B3508$^+$ donor CA were irradiated and incubated overnight to enhance expression of BZLF1 (donor MW, 12.3% of cells BZLF1$^+$; donor CA, 6.5% of cells BZLF1$^+$). Cells were then lysed in 0.5% TFA, homogenized, and subjected to ultrafiltration and HPLC fractionation. Fractions 30–33 from (A) the HLA-B*3501$^+$ LCL and (B) the HLA-B*3508$^+$ LCL that were predicted to include the APQP peptide (based on a parallel HPLC run with synthetic APQP peptide) were tested at varying dilutions for their ability to sensitize HLA-B*3508$^+$ target cells to lysis by an APQP-specific CTL clone (see panel D for the graph legend; E/T ratio = 2:1). The broken lines at the points of inflection on the dose-response curves mark the fraction dilution that led to 37% lysis. Toxicity controls consisting of target cells incubated with fractions in the absence of CTLs were negative (not depicted). (C) To allow an estimate of the concentration of the APQP peptide in each fraction, synthetic APQP was tested in parallel for CTL recognition at varying concentrations. Half-maximum lysis (37%) was measured at a synthetic peptide concentration of 48 pg/ml. (D) The concentration of synthetic peptide that led to 37% lysis (48 pg/ml) was divided by the fraction dilution that also led to 37% lysis to give an estimate of the amount of APQP in each fraction and a total amount of the peptide eluted from each cell populations.

## Structural analyses

To determine the effect that the polymorphic residue 156 has on peptide conformation, we determined the structures of HLA-B*3501 and HLA-B*3508 bound to APQP to 2.0 Å ($R_{factor}$, 21.3%; $R_{free}$, 23.3%) and 1.8 Å ($R_{factor}$, 20.9%; $R_{free}$, 23.4%), respectively (Table I). These two binary complexes crystallize in the same space group under identical conditions with isomorphous unit cells and, importantly, the bound epitopes do not participate in crystal contacts. Accordingly, conformational differences that are observed between these structures can be attributed to the polymorphic amino acid at position 156. The electron density for the bound peptides and contacting residues was very clear in the HLA-B*3501-APQP and the HLA-B*3508-APQP structures (Fig. 5). The analysis below links changes in peptide conformation to the local effect of the polymorphism at position 156.

Like the previously determined HLA-B35 structure (19), the APQP epitope is bound in a slightly bulged mode, with the N and COOH termini anchored in the A and F pockets, respectively. 10 out of the 12 direct H bonds between the peptide and HLA-B*3508 are located within these pockets (Table II), the majority of which are conserved between all solved HLA B35 structures (16, 19–21). The P1-Cα/PΩ-Cα distance in the HLA-B*3508–APQP complex compares

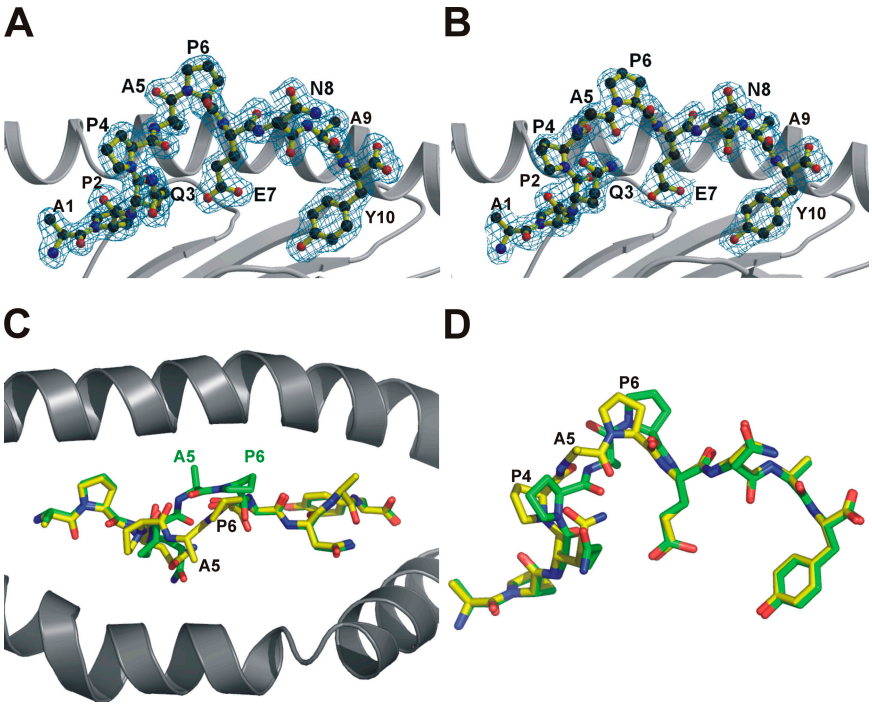


**Figure 5. High resolution structures of HLA-B*3501 and HLA-B*3508 presenting APQPAPENAY show that the peptides are presented in different conformations.** Structures of APQP complexed to (A) HLA-B*3508 and (B) HLA-B*3501. For clarity, the α2 helix has been removed. 2Fo–Fc electron density, displayed in mesh format, clearly shows the accurate modeling of peptide residues. (C and D) Superposition of the APQP peptides presented by HLA-B*3501 (yellow) and HLA-B*3508 (green) show a dramatic difference in peptide presentation, including (C) a switch from the P5-Ala Cβ group pointing toward the α1 helix (HLA-B*3508) to the α2 helix (HLA-B*3501) and (D) a change from cis–P6-Pro to trans–P6-Pro.

closely with that observed in the previously determined HLA-B35 complexes (19, 20). The binding pockets for P2-Pro and PΩ-Tyr, primary anchor residues for HLA-B35, are conserved between this structure and that previously described (20, 21). Positions P4-Pro, P5-Ala, P6-Pro, P8-Asn, and P9-Ala of the peptide are solvent exposed and thus represent possible TCR contact points. Interestingly, only 21.4% of the 10-mer is solvent exposed when bound to HLA-B*3508, compared with 25% for the previously described nonameric HLA-B35 complex. This may be attributed to the apparent secondary anchor residue in the HLA-B*3508–APQP complex, where the P7-Glu side chain points down toward the antigen-binding cleft, forming an H bond with Tyr 74 and salt bridging to Arg 97 and the polymorphic Arg 156. This tethering of the central-bulged region is distinct from several previously determined bulged peptides where the central bulge participates minimally in contacts with the antigen-binding cleft (16, 21).

In HLA-B*3508, Arg 156 forms an integral part of an unusual charged cluster of residues, with its guanadinium group stacking antiparallel to the guanadinium group of Arg 97. Interestingly, the high-resolution structure has enabled us to visualize discrete mobility of the Arg 97 and Arg 156 residues, creating two conformations of these residues (Fig. 6 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050864/DC1). The Arg 97–Arg 156 interaction is flanked by two salt-bridging residues: Asp 114, a residue located within the E-pocket, and P7-Glu from the peptide. The extended conformation of the aliphatic moiety of Arg 156 is further stabilized by van der Waals (v.d.w.) interactions with Leu 126, Trp 133, and Val 152. In one conformation, Arg 156 also forms a direct H bond with the P3-Gln (Fig. 6 A). The two conformations of Arg 156 and Arg 97 in HLA-B*3508 do not affect the conformation of the solvent-exposed residues of the APQP peptide where no such discrete mobility was observed.

In HLA-B*3501, the positively charged Arg at position 156 is replaced by the hydrophobic Leu residue that forms v.d.w. contacts with Trp 133, Val 152, and the aliphatic moiety of Asp 114 and unfavorable v.d.w. interactions with the charged Arg 97; Leu 156 does not contact the peptide (Fig. 6 B). To avoid unfavorable interactions with the hydrophobic Leu 156, the carboxylate moiety of Asp 114 rotates away to form a more favorable interaction with Arg 97. Accommodating this polymorphic residue at position 156 requires minimal changes in the HLA-B*3501 heavy chain (the heavy chains in the two complexes are virtually identical; Cα root mean square deviation = 0.25 Å$^2$). However, the polymorphism at position 156 greatly affects the conformation of the peptide. Somewhat surprisingly, in the HLA-B*3501–APQP complex, the P7-Glu/Arg 97 salt bridge is maintained despite the loss of the Arg 156 salt bridge. How-

**Table I.** Data collection and refinement statistics

| Statistics | HLA-B*3501 | HLA-B*3508 |
|---|---|---|
| Data collection | | |
| Temperature (K) | 100 | 100 |
| Space group | $P2_12_12_1$ | $P2_12_12_1$ |
| Cell dimensions (a,b,c) (Å) | 50.81, 81.56, 110.38 | 50.83, 81.53, 110.73 |
| Resolution (Å) | 50–2 | 50–1.8 |
| Total number of observations | 115,720 | 158,895 |
| Number of unique observations | 28,293 | 43,173 |
| Multiplicity | 4.1 | 3.7 |
| Data completeness (%) | 88 (82.9) | 99.1 (97.7) |
| Number of data $>2\sigma_I$ | 72.4 | 81.7 |
| $I/\sigma_I$ | 21.4 (3.9) | 31.7 (2.82) |
| $R_{merge}$[a] (%) | 9.7 (42) | 4.3 (49.5) |
| Refinement | | |
| Nonhydrogen atoms | | |
| Protein | 3160 | 3185 |
| Water | 342 | 516 |
| Resolution (Å) | 2 | 1.8 |
| $R_{factor}$[b] (%) | 21.3 | 20.85 |
| $R_{free}$[b] (%) | 23.33 | 23.42 |
| Rms deviations from ideality | | |
| Bond lengths (Å) | 0.007 | 0.005 |
| Bond angles (°) | 1.43 | 1.24 |
| Impropers (°) | 25.05 | 24.96 |
| Dihedrals (°) | 0.98 | 0.88 |
| Ramachandran plot (%) | | |
| Most-favored region | 90.7 | 91.8 |
| Allowed region | 9.3 | 8.2 |
| B-factors (Å$^2$) | | |
| Average main chain | 29.52 | 27.02 |
| Average side chain | 31.86 | 29.73 |
| Average water molecule | 43.04 | 42.55 |
| Rmsd of bonded Bs | 1.76 | 1.76 |

[a]$R_{merge} = \Sigma \mid I_{hkl} - < I_{hkl} > \mid / \Sigma I_{hkl}$.
[b]$R_{factor} = \Sigma_{hkl} \mid \mid F_o \mid - \mid F_c \mid \mid / \Sigma_{hkl} \mid F_o \mid$ for all data except ~4% that were used for $R_{free}$ calculation.

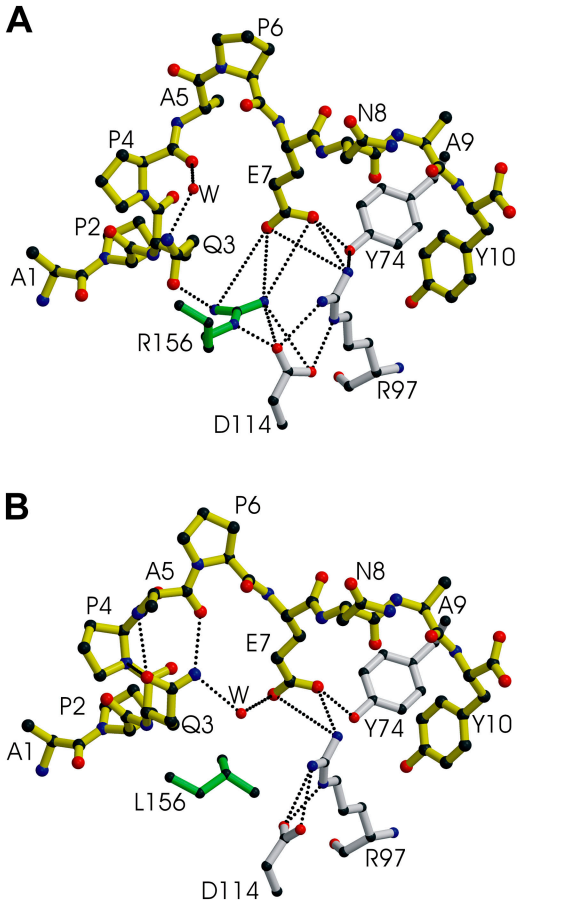


**Figure 6. Local impact of the 156 polymorphism on peptide presentation.** (A) An extensive network of H bonding is seen involving Arg 156 in HLA-B*3508. Particularly important is the interaction with P3-Gln. (B) The polymorphic residue, Leu 156, makes no direct contacts with the peptide in HLA-B*3501. The side chain of P3-Gln interacts instead by pushing P4-Pro toward the α1 helix and pulling P5-Ala and P6-Pro in the direction if the α2 helix. Particularly evident is the switch from cis–P6-Pro to trans–P6-Pro. Residues are in ball-and-stick format. Polar interactions are depicted as dotted lines. The polymorphic residue is green, the peptide is yellow, and other MHC heavy chain residues are shown in gray.

ever, it is the loss of the heavy chain interaction with the P3-Gln that considerably affects the peptide conformation. This polymorphism-induced change in peptide interactions culminates in a dramatic shift of residues P4-Pro, P5-Ala, and P6-Pro of the peptide (Fig. 5, C and D). The side chain of P3-Gln within the HLA-B*3501–APQP complex swings upwards, forming H bonds to P5-Ala$^N$ and P5-Ala$^O$. To accommodate the new conformation of the P3-Gln, the P4-Pro is pushed away toward the α1 helix of HLA-B*3501. The movement of the P4-Pro also includes a dramatic flip in the main chain carbonyl group P4-Pro peptide main chain, which then results in a marked shift in the main chain conformation of P5-Ala, such that the P5-Ala Cβ groups are 6.5 Å apart in the HLA-B*3501 and HLA-B*3508 structures. This change in peptide conformation also moves the P5-Ala and P6-Pro closer toward the α2 helix of HLA-B*3501 (Fig. 5 D) and results in a switch from cis–P6-Pro to trans–P6-Pro. These conformational changes are important enough to drastically reduce recognition of the HLA-B*3501–APQP complex by the majority of T cells raised against HLA-B*3508–APQP.

### CTLs specific for the APQP epitope interact with peptide residues that are oriented differently when bound to HLA-B*3501 and HLA-B*3508

To investigate why these peptide structural differences had such a major impact on CTL recognition, three APQP-specific CTL clones raised from the HLA-B*3508$^+$ donor SB

**Table II.** APQPAPENAY peptide contacts with HLA-B*3501 and HLA-B*3508

| Peptide residue | MHC residue | Type of bond | 08/01 |
|---|---|---|---|
| P1 | | | |
| $Ala^{O}$ | Tyr $159^{O\eta}$ | H bond | |
| $Ala^{N}$ | Tyr $171^{O\eta}$, Tyr $7^{O\eta}$ | H bond | |
| Ala | Met 5, Tyr 7, Tyr 159, Trp 167, Tyr 171 | v.d.w. | |
| P2 | | | |
| $Pro^{O}$ | Arg $62^{N\varepsilon}$, Arg $62^{N\eta 2}$ | water mediated | |
| Pro | Tyr 7, Tyr 9, Asn 63, Phe 67, Tyr 99, Tyr 159 | v.d.w. | |
| P3 | | | |
| $Gln^{N}$ | Tyr $99^{O\eta}$ | H bond | |
| $Gln^{O\varepsilon 1}$ | P7 $Glu^{O\varepsilon 2}$ | water mediated | 01 |
| $Gln^{N\varepsilon 2}$ | P4 $Pro^{O}$ | water mediated | 08 |
| $Gln^{O\varepsilon 1}$ | Arg $156^{N\eta 1}$ | H bond | 08(1) |
| $Gln^{O}$ | Asn $70^{N\delta 2}$, Tyr $9^{O\eta}$, P7 $Glu^{O\varepsilon 2}$ | water mediated | |
| Gln | Tyr 159, Tyr 99, Ile 66 | v.d.w. | |
| Gln | Arg 156, Gln 155 | v.d.w. | 08 |
| P4 | | | |
| Pro | Tyr 159 | v.d.w. | |
| Pro | Ile 66, Leu 163 | v.d.w. | 01 |
| P5 | | | |
| Ala | Ile 66 | v.d.w. | 08 |
| P6 | | | |
| Pro | Thr 69 | v.d.w. | 08 |
| P7 | | | |
| $Glu^{N}$ | Asn $70^{O\delta 1}$, Thr $73^{O\gamma 1}$, P7 $Glu^{O}$ | water mediated | 01 |
| $Glu^{O}$ | Thr $73^{O\gamma 1}$ | water mediated | 08 |
| $Glu^{O\varepsilon 1}$ | Arg $97^{N\eta 1}$ | salt bridge | |
| $Glu^{O\varepsilon 1}$ | Tyr $74^{O\eta}$ | H bond | |
| $Glu^{O\varepsilon 1}$ | P8 $Asn^{O}$ | water mediated | |
| $Glu^{O\varepsilon 1}$ | Arg $97^{N\eta 1}$ | water mediated | 08 |
| $Glu^{O\varepsilon 1}$ | Arg $97^{N\eta 2}$ | salt bridge | 08(2) |
| $Glu^{O\varepsilon 1}$ | Arg $156^{N\eta 2}$ | salt bridge | 08(1) |
| $Glu^{O\varepsilon 2}$ | Arg $97^{N\eta 2}$ | salt bridge | 08(2) |
| $Glu^{O\varepsilon 2}$ | Arg $97^{N\eta 1}$ | salt bridge | |
| $Glu^{O\varepsilon 2}$ | Arg $156^{N\eta 2, N\eta 1}$ | salt bridge | 08(1) |
| Glu | Thr 73, Tyr 74, Asn 70, Tyr 9, Arg 97 | v.d.w. | |
| P8 | | | |
| $Asn^{N}$ | Arg $156^{N\eta 1, N\eta 2}$ | water mediated | 08 |
| $Asn^{O}$ | Arg $97^{N\eta 1}$ | water mediated | |
| $Asn^{O}$ | Arg $97^{N\eta 2}$ | water mediated | 01 |
| Asn | Trp 147, Lys 146, Ala 150 | v.d.w. | |
| Asn | Val 152 | v.d.w. | 08 |
| P9 | | | |
| $Ala^{O}$ | Trp $147^{N\varepsilon 1}$ | H bond | |
| Ala | Thr 73, Ser 77, Glu 76, Trp 147, Lys 146 | v.d.w. | |
| PΩ | | | |
| $Tyr^{N}$ | Ser $77^{O\gamma}$ | H bond | |
| $Tyr^{O}$ | Lys $146^{N\zeta}$, Asn $80^{O\delta 1}$ | H bond | |
| $Tyr^{OXT}$ | Thr $143^{O\gamma 1}$, Tyr $84^{O\eta}$ | H bond | |
| $Tyr^{O\eta}$ | Ser $116^{O\gamma}$ | H bond | |
| Tyr | Ser 77, Thr 143, Leu 81, Trp 147, Tyr 74, Tyr 123, Ser 116, Ile 95, Arg 97, Lys 146, Tyr 84, Asn 80 | v.d.w. | |

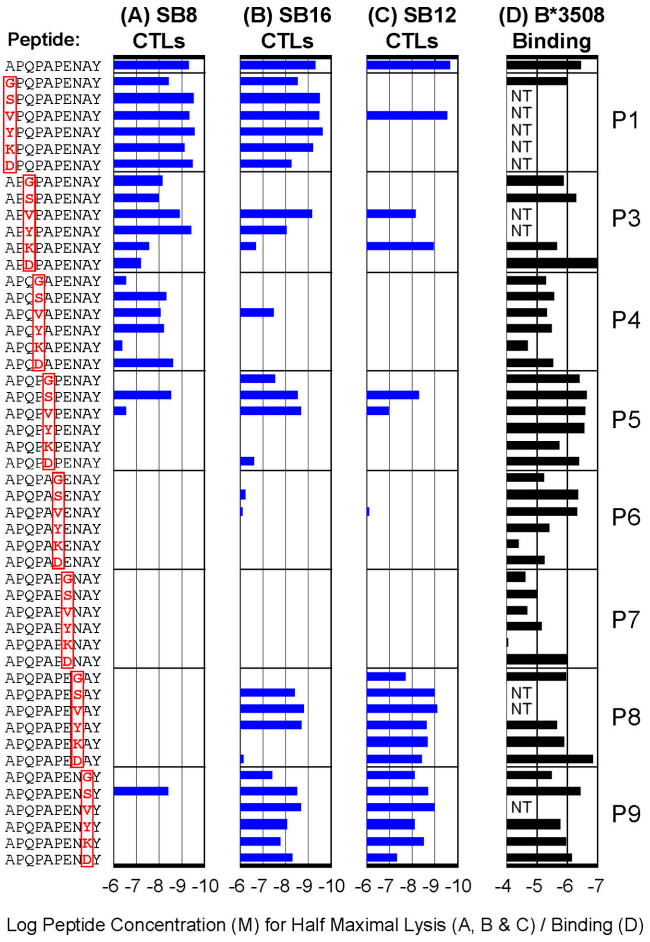


**Figure 7. The impact of single amino acid substitutions within the APQP peptide on CTL recognition and HLA-B*3508 binding.** The APQP-specific CTL clones SB8 (A), SB16 (B), and SB12 (C) were tested for recognition of a panel of altered peptide ligands into which single amino acid substitutions were introduced (E/T ratio = 2:1). A range of peptide concentrations were used in these chromium release assays, and the concentration required for half-maximum lysis was calculated from this dose-response data. (D) MHC–peptide binding assays were also conducted by testing each peptide at a range of concentrations for its ability to stabilize HLA-B*3508 expression on the surface of the antigen-processing mutant T2 cell line. The concentration of peptide required for half-maximum HLA-B*3508 stabilization was calculated. Peptides that were well recognized by a CTL clone were not tested (NT) for MHC binding.

were tested for their ability to tolerate single amino acid substitutions throughout the length of the peptide (Fig. 7, A–C). To aid with interpretation of this data, peptide–MHC binding assays were also performed on selected peptides that were not well recognized by any of the CTL clones. These binding assays measured stabilization of HLA-B*3508 molecules on the surface of the T2.B*3508 cell line after exogenous peptide addition, and the concentration of peptide required for half-maximum stabilization of HLA-B*3508 was then calculated (Fig. 7 D). Considering that the primary anchor residues at P2 and P10 were maintained within all the

analogues, a surprising number of amino acid substitutions at other positions within the peptide reduced binding to HLA-B*3508. Consistent with the structural analysis, the glutamate at P7 appears to be a particularly important secondary anchor residue for this peptide because most substitutions at this position resulted in reduced MHC binding.

A total of 48 analogues of the 10-mer peptide were tested for CTL recognition over a range of concentrations using chromium release assays, and the concentration of peptide required for half-maximum lysis was calculated (Fig. 7, A–C). Each CTL clone displayed a unique pattern of fine specificity for the 48 analogues, indicating that each expresses a different TCR. CTL recognition by the SB8 CTL clone was particularly affected by amino acid replacements toward the COOH terminus of the peptide (Fig. 7 A), whereas the SB16 clone was focused toward central residues, with recognition less affected by substitutions at the extremities of the peptide (Fig. 7 B). The SB12 CTL clone was particularly tolerant of substitutions toward the COOH terminus, suggesting that the highly exposed peptide residues at P8 and P9 do not contact the TCR expressed by this clone (Fig. 7 C). It is notable that all three CTL clones have difficulty recognizing peptides with substitutions at P5 and P6, suggesting that the alanine and proline residues at these positions within the EBV peptide might interact closely with the antigen receptors of these clones. Therefore, these data help explain why these clones fail to recognize the APQP peptide bound to B*3501 because the side chains of the residues at P5 and P6 are oriented quite differently when bound to B*3501 and B*3508.

## DISCUSSION

Polymorphism at the MHC locus enhances immune defense across the population by ensuring wide variation in the T cell response to infecting pathogens through presentation of a broad array of target epitopes (22, 23). This report has demonstrated another mechanism through which MHC polymorphism can diversify the immune response to an infecting pathogen. Thus, polymorphic MHC residues can markedly affect peptide binding conformation as well as MHC–peptide binding affinity, and this can have a major impact on the T cell response. Although previous studies have also demonstrated peptide structural alterations induced by MHC polymorphism (24, 25), none have shown that such changes can influence a peptide-specific immune response to this extent.

Our data demonstrate that a single residue polymorphism between HLA-B*3501 and HLA-B*3508 controls responsiveness to the APQP epitope through a mechanism unrelated to peptide–MHC binding efficiency/stability (Fig. 2). Furthermore, HLA-B*3501$^+$, EBV-infected lymphoblasts clearly present the APQP epitope from endogenously expressed antigen with similar efficiency to HLA-B*3508$^+$ LCLs (Fig. 3 C and Fig. 4), ruling out differences in epitope processing within EBV-infected cells. It should be noted, however, that the response to this epitope could be initiated by cross-priming through dendritic cells that could process and present a different repertoire of EBV peptides compared with infected B cells. Nonetheless, direct stimulation by EBV-infected B cells is likely to have the major role in restimulating and maintaining these T cell populations during persistent infection. We also consider it highly unlikely that polymorphic antigen processing genes are influencing this response in our study subjects because our HLA-B*3501$^+$ donors were all unrelated and the HLA-B*3508$^+$ donors were from two unrelated families from different ethnic groups.

Another factor with the potential to influence the immunogenicity of a T cell epitope is immunodomination whereby the T cell response to an immunodominant determinant suppresses the response to another epitope (26, 27). It was therefore feasible that HLA-B*3501, but not HLA-B*3508, presents another unknown EBV epitope that is highly immunogenic, thereby suppressing the response to APQP in HLA-B*3501$^+$ individuals. Arguing against this possibility are data presented in Fig. 1 C and Fig. 3 B showing that the HLA-B*3501/-B*3508–coexpressing donor MB responds to the APQP epitope.

The potential role of peptide conformation in controlling the differential responsiveness to this epitope became apparent after functional assays demonstrated that APQP-specific CTL clones from an HLA-B*3508$^+$ donor were unable to recognize this peptide efficiently in the context of HLA-B*3501 (Fig. 3 A). Thus, although these two closely related HLA molecules present this identical peptide sequence very efficiently, they appear to be structurally distinct from the perspective of many T cells. This conclusion was strengthened by our analysis of multiple CTL microcultures, most of which recognized the peptide much more efficiently when presented on HLA-B*3508$^+$ target cells compared with HLA-B*3501$^+$ targets. It is notable that one of the donors used for this analysis coexpressed HLA-B*3501 and HLA-B*3508, yet there was no indication that T cells had been selected for the ability to cross-recognize the peptide on each of these HLA molecules (Fig. 3 B). Subsequent structural analysis confirmed major conformational differences between the peptide bound to HLA-B*3501 and HLA-B*3508 resulting from direct peptide interactions with the polymorphic MHC residue (Fig. 5 and Fig. 6). Furthermore, the conformational differences were particularly striking at highly exposed peptide residues that were shown to be important TCR contact residues for three out of three APQP-specific CTL clones (Fig. 7).

There are several possible mechanisms through which such peptide conformational differences could influence immunogenicity. It is possible that thymic and postthymic selection influence the relative number of naive T cells with the capacity to recognize each complex. For example, there may be an abundantly presented self-peptide that binds to both HLA-B*3501 and HLA-B*3508 that negatively selects CTLs with the potential to recognize the HLA-B*3501–bound conformation of the viral peptide or that positively

selects for T cells that recognize the viral peptide presented by HLA-B*3508 but not HLA-B*3501. Alternatively, certain self-peptides may be presented preferentially by HLA-B*3508 that positively select T cells specific for HLA-B*3508–APQP (11, 12). In any case, the unique conformation of the APQP peptide when bound to HLA-B*3508 appears to be critical for recognition by most of the selected T cells and is, therefore, a major controlling influence over the immunogenicity of this EBV epitope.

Another possible explanation for our data, unrelated to TCR repertoire differences between HLA-B*3501$^+$ and HLA-B*3508$^+$ individuals, is that the conformation of this EBV epitope on HLA-B*3508 is intrinsically more immunogenic through structural features that enable it to interact favorably with a higher frequency of TCRs (1). It is now clear that certain antigenic peptides present challenging target structures for TCR recognition and are recognized by a very limited TCR repertoire (16, 28–30). It is therefore not unreasonable to expect that some peptide conformations will present docking surfaces complementary to a higher proportion of the TCR repertoire compared with others. Although it is impossible to decipher the exact mechanisms underlying the phenomenon, this study clearly demonstrates that peptide conformation can have a dramatic impact on the immunogenicity of an MHC–peptide complex and could therefore play a major role in controlling determinant selection and immunodominance in other T cell responses.

## MATERIALS AND METHODS

**Cell lines.** LCLs were established by exogenous transformation of peripheral B cells with EBV and were maintained in growth medium (RPMI 1640 with 10% FCS). The mutant LCL × T lymphoblastoid hybrid cell line, 174 × CEM.T2 (referred to as T2 cells) (31), expressing either HLA-B*3501 (T2.B*3501) or HLA-B*3508 (T2.B*3508) was also used in this study and has been described elsewhere (13, 32). PHA blasts were generated as previously described (10). Blood donors used in this study were healthy laboratory staff selected for particular HLA alleles and prior exposure to EBV as assessed by standard virus-specific antibody tests.

**CTL cultures.** CTL clones were generated by agar cloning as previously described (10) after initial stimulation of PBMCs for 1 h with 0.1 μM of the APQP peptide. Clones were maintained with biweekly restimulation with rIL-2 and the γ-irradiated (8,000 rads) autologous LCLs that had been prelabeled with the APQP peptide at 0.1 μM for 1 h and washed three times. Short-term CTL bulk cultures were also used as effectors in cytotoxicity assays. These were generated by culturing 2 × $10^6$ PBMCs/2-ml well in growth medium with autologous PBMCs that had been precoated for 1 h with 0.1 μM of the APQP peptide (responder/stimulator = 2:1). Cultures were supplemented with 20 U/ml rIL-2 on day 3, split on day 7, and analyzed on day 10. CTL cultures were tested in duplicate or triplicate for cytotoxicity in the standard 5-h chromium release assay. In brief, CTLs were assayed against $^{51}$Cr-labeled LCL or PHA blast targets that were pretreated with synthetic peptide (Mimotopes Ltd.) or left untreated. The mean spontaneous lysis for target cells in culture medium was always <20%, and the variation from the mean specific lysis was <10%.

Short-term CTL microcultures were generated by limiting dilution as follows: PBMCs were distributed in roundbottom microtiter plates in growth medium at cell numbers ranging from $10^3$ to 4 × $10^4$ cells/well. Approximately 5 × $10^4$ γ-irradiated (2,000 rads) autologous PBMCs that had been preincubated for 1 h with 1 μM of the APQP peptide were added to each well to give a total volume of 100 μl. Cultures were fed on days 4, 7, and 10 with 50 μl of medium supplemented with 20 U of rIL-2 and 30% supernatant from MLA-144 cultures. On day 13, each CTL microculture was split into multiple replicates and used as effectors in a standard 5-h $^{51}$Cr-release assay against target PHA blasts that had been treated with the APQP peptide or left untreated.

**HLA class I peptide binding assays.** To assess peptide binding to the different HLA-B35 subtypes, T2.B*3501 and T2.B*3508 cells were incubated in AIM V serum-free medium (Invitrogen) with various concentrations (0.01, 0.1, 1, 10, and 100 μM) of peptides at 26°C for 14–16 h, followed by incubation at 37°C for 2–3 h and staining for HLA-B35 surface expression. To determine peptide dissociation rates, T2.B*3501 cells were pulsed with 100 μM of each peptide at 26°C for 14–16 h, followed by incubation at 37°C for 2–3 h. Cells were then either stained immediately for HLA-B35 surface expression (0 h) or were washed three times and incubated at 37°C for 1–6 h before staining. HLA-B35 surface expression was measured by a flow cytometer (FACSCalibur; Becton Dickinson) using an mAb to HLA-Bw6 (SFR8 Bw6). Data were expressed relative to the mean fluorescence intensity (MFI) measured using 100 μM of a reference peptide that was known to bind to HLA-B*3501 or HLA-B*3508, using the following formula: ([MFI with test peptide − MFI without peptide addition] × 100) / (MFI with reference peptide [at 100 μM] − MFI without peptide addition).

**ELISPOT assays.** IFN-γ ELISPOT assays were performed using cytokine capture and detection reagents according to the manufacturer's instructions (Mabtech). In brief, anti–IFN-γ antibodies were coated on the wells of a 96-well nitrocellulose plate, and duplicate wells were seeded with 1,000 CD8$^+$ T cells and 50,000 target cells. Two LCLs raised from an HLA B*3501$^+$ individual were used as target cells in these experiments: one carried a WT B95.8 virus genome (BZLF1$^+$ LCL), and the other carried a B95.8 genome that had been rendered incapable of lytic cycle entry by disruption of the *BZLF1* gene (BZLF1$^-$ LCL; a gift from A. Hislop and A. Rickinson, University of Birmingham, UK) (17). After incubation for 16 h, captured IFN-γ was detected with a biotinylated anti–IFN-γ antibody followed by development with streptavidin–horseradish peroxidase complex and chromogenic substrate, and spots were counted using an automated plate counter (AID).

**Expression, purification, and crystallization of HLA-B35 alleles with APQPAPENAY.** Soluble HLA-B*3501 and HLA-B*3508 molecules (residues 1–276) and full-length β2-microglobulin (residues 1–99) were expressed, refolded with the APQP peptide, purified, and concentrated to 10 mg/ml as previously described (33). Crystals were obtained by the hanging drop vapor diffusion technique. Block-shaped crystals grew within 4 d in a condition containing 0.2 M ammonium acetate and 16% wt/vol PEG 3350 (100 mM cacodylate, pH 7.6) at 4°C.

**X-ray data collection and structure determination.** Crystals were soaked in reservoir solution containing increasing increments of glycerol as a cryoprotectant (5, 10, and 15%) and then flash frozen before data collection. Data were collected on an in-house radiation source and was processed and scaled using the HKL suite (34).

The HLA-B35 complex structures were refined from an HLA-B*3501 structure that was previously determined in our laboratory (unpublished data). The model was manually built using the program "O" (35) and improved through multiple rounds of refinement using the CNS suite (36). The progress of refinement was monitored by $R_{factor}$ and $R_{free}$ values. Rigid-body refinement and simulated annealing were used in the first instance, but in later rounds energy minimization and B-individual refinement were used to improve the quality of the model. See Table I for the final refinement and model statistics. The structures have been deposited in the Protein Data Bank under accession no. 2AXF (HLA-B*3508–APQP) and 2AXG (HLA-B*3501–APQP).

**Purification of HLA complexes and peptide analysis.** Purification of HLA molecules was performed from ∼8 × $10^8$ HLA-B*3501$^+$ LCLs (do-

nor MW: HLA A1, A3, B8, and B*3501) and HLA-B*3508$^+$ LCLs (donor CA: HLA A30, A32, B42, and B*3508) that had been irradiated (200 rads) and incubated at 37°C overnight to enhance BZLF1 expression (18). These LCLs were selected for these experiments on the basis of relatively high levels of BZLF1 expression as measured using flow cytometry and a mouse mAb to BZLF1 (BZ.1). Peptides were recovered from cell lysates after treatment with 0.5% TFA and acid extraction from the cell pellet as described elsewhere (37). After ultrafiltration (3-kD cutoff) and preconcentration, peptides were collected in 50-μl fractions as described previously (38). Peptides were separated by RP-HPLC using a SMART system HPLC (GE Healthcare) with a vydac C18 column (1 mm [inside diameter] × 25 cm) and eluted using an optimized linear gradient of acetonitrile in aqueous 0.09% TFA. Peptide fractions were used in chromium release assays to sensitize HLA-B*3508$^+$ LCL target cells for lysis by a CTL clone from the B*3508$^+$ donor SB. In brief, 5 μl of each undiluted or diluted fraction was added to 15 μl of target cells for 90 min before CTL addition. To test for toxicity, each fraction was also added to chromium-labeled target cells without CTL addition, but no toxicity was detected, even at the lowest dilution (1:4; unpublished data). HLA-B*3501 fraction 32 was also characterized by liquid chromatography–mass spectrometry using an Ultra ion trap mass spectrometer coupled to an 1100 nanoLC (both Agilent Technologies) as described previously (38, 39) and found to contain the APQP 10-mer peptide after MS/MS fragmentation of the $[M+2H]^{2+}$ species of target mass m/z = 529.2 D (unpublished data).

**Online supplemental material.** Fig. S1 shows an alternate conformation of the polymorphic residue 156 in the HLA-B*3508–APQP complex. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050864/DC1.

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## REFERENCES

1. Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17:51–88.
2. Parham, P., and T. Ohta. 1996. Population biology of antigen presentation by MHC class I molecules. *Science.* 272:67–74.
3. Saper, M.A., P. Bjorkman, and D.C. Wiley. 1991. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6A resolution. *J. Mol. Biol.* 219:277–319.
4. Hill, A.V., J. Elvin, A.C. Willis, M. Aidoo, C.E. Allsopp, F.M. Gotch, X.M. Gao, M. Takiguchi, B.M. Greenwood, A.R. Townsend, et al. 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. *Nature.* 360:434–439.
5. Falk, K., O. Rotzschke, B. Grahovac, D. Schendel, S. Stevanovic, G. Jung, and H.G. Rammensee. 1993. Peptide motifs of HLA-B35 and -B37 molecules. *Immunogenetics.* 38:161–162.
6. Ruppert, J., J. Sidney, E. Celis, R.T. Kubo, H.M. Grey, and A. Sette. 1993. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell.* 74:929–937.
7. Chen, W., S. Khilko, J. Fecondo, D.H. Margulies, and J. McCluskey. 1994. Determinant selection of major histocompatibility complex class I–restricted antigenic peptides is explained by class I–peptide affinity and is strongly influenced by nondominant anchor residues. *J. Exp. Med.* 180:1471–1483.
8. Busch, D.H., I.M. Pilip, S. Vijh, and E.G.P. Am. 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity.* 8:353–362.
9. Crotzer, V.L., R.E. Christian, J.M. Brooks, J. Shabanowitz, R.E. Settlage, J.A. Marto, F.M. White, A.B. Rickinson, D.F. Hunt, and V.H. Engelhard. 2000. Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. *J. Immunol.* 164:6120–6129.
10. Burrows, S.R., S.L. Silins, D.J. Moss, R. Khanna, I.S. Misko, and V.P. Argaet. 1995. T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. *J. Exp. Med.* 182:1703–1715.
11. Messaoudi, I., J.A. Guevara Patino, R. Dyall, J. LeMaoult, and J. Nikolich-Zugich. 2002. Direct link between mhc polymorphism, T cell avidity, and diversity in immune defense. *Science.* 298:1797–1800.
12. Nikolic-Zugic, J., and M.J. Bevan. 1990. Role of self-peptides in positively selecting the T-cell repertoire. *Nature.* 344:65–67.
13. Green, K.J., J.J. Miles, J. Tellam, W.J.M. van Zuylen, G. Connolly, and S.R. Burrows. 2004. Potent T cell response to a class-I-binding 13-mer viral epitope and the influence of HLA micropolymorphism in controlling epitope length. *Eur. J. Immunol.* 34:2510–2519.
14. Saulquin, X., C. Ibisch, M.A. Peyrat, E. Scotet, M. Hourmant, H. Vie, M. Bonneville, and E. Houssaint. 2000. A global appraisal of immunodominant CD8 T cell responses to Epstein-Barr virus and cytomegalovirus by bulk screening. *Eur. J. Immunol.* 30:2531–2539.
15. Stuber, G., G.H. Leder, W.T. Storkus, M.T. Lotze, S. Modrow, L. Szekely, H. Wolf, E. Klein, K. Karre, and G. Klein. 1994. Identification of wild-type and mutant p53 peptides binding to HLA-A2 assessed by a peptide loading-deficient cell line assay and a novel major histocompatibility complex class I peptide binding assay. *Eur. J. Immunol.* 24:765–768.
16. Tynan, F.E., N.A. Borg, J.J. Miles, T. Beddoe, D. El-Hassen, S.L. Silins, W.J.M. van Zuylen, A.W. Purcell, L. Kjer-Nielsen, J. McCluskey, et al. 2005. High resolution structures of highly bulged viral epitopes bound to the major histocompatability class I: implications for T-cell receptor engagement and T-cell immunodominance. *J. Biol. Chem.* 280:23900–23909.
17. Pudney, V.A., A.M. Leese, A.B. Rickinson, and A.D. Hislop. 2005. CD8$^+$ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. *J. Exp. Med.* 201:349–360.
18. Ferrieu, C., B. Ballester, J. Mathieu, and E. Drouet. 2003. Flow cytometry analysis of gamma-radiation-induced Epstein-Barr virus reactivation in lymphocytes. *Radiat. Res.* 159:268–273.
19. Menssen, R., P. Orth, A. Ziegler, and W. Saenger. 1999. Decamer-like conformation of a nona-peptide bound to HLA-B*3501 due to non-standard positioning of the C terminus. *J. Mol. Biol.* 285:645–653.
20. Smith, K.J., S.W. Reid, D.I. Stuart, A.J. McMichael, E.Y. Jones, and J.I. Bell. 1996. An altered position of the α2 helix of MHC class I is revealed by the crystal structure of HLA-B*3501. *Immunity.* 4:203–213.
21. Miles, J.J., D. Elhassen, N.A. Borg, S.L. Silins, F.E. Tynan, J.M. Burrows, A.W. Purcell, L. Kjer-Nielsen, J. Rossjohn, S.R. Burrows, and J. McCluskey. 2005. CTL recognition of a bulged viral peptide involves biased TCR selection. *J. Immunol.* 175:3826–3834.
22. Germain, R.N., and D.H. Margulies. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 11:403–450.
23. Lawlor, D.A., J. Zemmour, P.D. Ennis, and P. Parham. 1990. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Annu. Rev. Immunol.* 8:23–63.
24. Hulsmeyer, M., M.T. Fiorillo, F. Bettosini, R. Sorrentino, W. Saenger, A. Ziegler, and B. Uchanska-Ziegler. 2004. Dual, HLA-B27 subtype–dependent conformation of a self-peptide. *J. Exp. Med.* 199:271–281.
25. Luz, J.G., M. Huang, K.C. Garcia, M.G. Rudolph, V. Apostolopoulos, L. Teyton, and I.A. Wilson. 2002. Structural comparison of allogeneic and syngeneic T cell receptor–peptide–major histocompatibility complex complexes: a buried alloreactive mutation subtly alters peptide presentation substantially increasing Vβ interactions. *J. Exp. Med.* 195:

1175–1186.
26. Lewicki, H.A., G. von Herrath, C.F. Evans, J.L. Whitton, and M.B. Oldstone. 1995. CTL escape viral variants. II. Biologic activity in vivo. *Virology*. 211:443–450.
27. van der Most, R.G., R.J. Concepcion, C. Oseroff, J. Alexander, S. Southwood, J. Sidney, R.W. Chesnut, R. Ahmed, and A. Sette. 1997. Uncovering subdominant cytotoxic T-lymphocyte responses in lymphocytic choriomeningitis virus-infected BALB/c mice. *J. Virol.* 71: 5110–5114.
28. Stewart-Jones, G.B., A.J. McMichael, J.I. Bell, D.I. Stuart, and E.Y. Jones. 2003. A structural basis for immunodominant human T cell receptor recognition. *Nat. Immunol.* 4:657–663.
29. Kjer-Nielsen, L., C.S. Clements, A.W. Purcell, A.G. Brooks, J.C. Whisstock, S.R. Burrows, J. McCluskey, and J. Rossjohn. 2003. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. *Immunity*. 18:53–64.
30. Turner, S.J., K. Kedzierska, H. Komodromou, N.L. La Gruta, M.A. Dunstone, A.I. Webb, R. Webby, H. Walden, W. Xie, J. McCluskey, et al. 2005. Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific $CD8^+$ T cell populations. *Nat. Immunol.* 6:382–389.
31. Salter, R.D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J.* 5:943–949.
32. Khanna, R., S.L. Silins, Z. Weng, D. Gatchell, S.R. Burrows, and L. Cooper. 1999. Cytotoxic T cell recognition of allelic variants of HLA B35 bound to an Epstein-Barr virus epitope: influence of peptide conformation and TCR-peptide interaction. *Eur. J. Immunol.* 29:1587–1597.
33. Macdonald, W., D.S. Williams, C.S. Clements, J.J. Gorman, L. Kjer-Nielsen, A.G. Brooks, J. McCluskey, J. Rossjohn, and A.W. Purcell. 2002. Identification of a dominant self-ligand bound to three HLA B44 alleles and the preliminary crystallographic analysis of recombinant forms of each complex. *FEBS Lett.* 527:27–32.
34. Rossmann, M.G., and C.G. van Beek. 1999. Data processing. *Acta Crystallogr. D Biol. Crystallogr.* 55:1631–1640.
35. Jones, T.A., J.Y. Zou, S.W. Cowan, and M. Kjeldgaard. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta. Crystallogr. A.* 47:110–119.
36. Brunger, A.T., P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, et al. 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 54:905–921.
37. Purcell, A.W. 2004. Isolation and characterization of naturally processed MHC-bound peptides from the surface of antigen-presenting cells. *Methods Mol. Biol.* 251:291–306.
38. Purcell, A.W., J.J. Gorman, M. Garcia-Peydro, A. Paradela, S.R. Burrows, G.H. Talbo, N. Laham, C.A. Peh, E.C. Reynolds, J.A. Lopez de Castro, and J. McCluskey. 2001. Quantitative and qualitative influences of tapasin on the class I peptide repertoire. *J. Immunol.* 166:1016–1027.
39. Macdonald, W.A., A.W. Purcell, N. Mifsud, L.K. Ely, D.S. Williams, L. Chang, J.J. Gorman, C.S. Clements, L. Kjer-Nielsen, D.M. Koelle, et al. 2003. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. *J. Exp. Med.* 198:679–691.