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Promiscuous CTL Recognition of Viral Epitopes on Multiple Human Leukocyte Antigens: Biological Validation of the Proposed HLA A24 Supertype

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Multiple HLA class I alleles can bind peptides with common sequence motifs due to structural similarities in the peptide binding cleft, and these groups of alleles have been classified into supertypes. Nine major HLA supertypes have been proposed, including an A24 supertype that includes A*2301, A*2402, and A*3001. Evidence for this A24 supertype is limited to HLA sequence homology and/or similarity in peptide binding motifs for the alleles. To investigate the immunological relevance of this proposed supertype, we have examined two viral epitopes (from EBV and CMV) initially defined as HLA-A*2301-binding peptides. The data clearly demonstrate that each peptide could be recognized by CTL clones in the context of A*2301 or A*2402; thus validating the inclusion of these three alleles within an A24 supertype. Furthermore, CTL responses to the EBV epitope were detectable in both A*2301 and A*2402 individuals who had been previously exposed to this virus. These data substantiate the biological relevance of the A24 supertype, and the identification of viral epitopes with the capacity to bind promiscuously across this supertype could aid efforts to develop CTL-based vaccines or immunotherapy. The degeneracy in HLA restriction displayed by some T cells in this study also suggests that the dogma of self-MHC restriction needs some refinement to accommodate foreign peptide recognition in the context of multiple supertype alleles. The Journal of Immunology, 2003, 171: 1407–1412.

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iruses-infected human cells are recognized by CD8+ T cells through antigenic viral protein fragments of 8–12 aa in length that are presented on the cell surface in association with HLA class I molecules. These peptide fragments are derived through protein degradation, peptide transport to the endoplasmic reticulum, peptide-HLA binding, and export of peptide-HLA complexes to the cell surface (1). HLA molecules are extremely polymorphic, with much of the polymorphism concentrated within binding pockets that engage specific anchor residues of peptide ligands (2–6). HLA-binding peptides contain position-specific amino acids that interact with these binding pockets of the HLA. Sequencing of naturally processed peptides coisolated with purified HLA class I molecules has led to the identification of peptide binding motifs for many different HLA alleles and revealed that different alleles are characterized by distinct ligand specificities (7).

More recent studies have demonstrated a degree of degeneracy in HLA-peptide binding, whereby multiple class I alleles can recognize common sequence motifs (referred to as supermotifs) due to homology of amino acids within the major binding pockets of the peptide binding cleft, and these groups of alleles are referred to as HLA supertypes. Based on these HLA structural similarities and overlapping peptide binding motifs, nine major HLA supertypes have been proposed (8). For example, the A2 supertype includes HLA A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*6802, and A*6901 (9). These alleles recognize peptides with small or aliphatic hydrophobic residues (L, I, V, M, A, T, or Q) at position 2 and L, I, V, M, A, or T at the C-terminal position. The biological significance of several HLA supertypes has also been demonstrated, with reports of individual peptides binding to multiple HLA alleles and T cell recognition of such peptides presented by the different class I alleles (10–17). The HLA-A24-supertype was proposed in 1999 by Sette and Sidney (8) to include A*2301, A*2402, and A*3001 on the basis of similarities between the published peptide binding motifs for A*2402 (18, 19) and A*3001 (20) and homology with two peptides known to bind to A*2301 (21, 22). The alleles A*2403, A*2404, A*3002, and A*3003 were also tentatively included within the A24 supertype on the basis of possessing identical or conservatively similar amino acid residues surrounding the likely B- and F-pockets in these alleles compared with A*2301, A*2402, and A*3001. Thus, an A24 supermotif was proposed to include Y or F (or, less frequently, W, L, V, I, M, or T) at peptide position 2 and F or I (or, less frequently, Y, W, L, or M) at the C terminus. No further evidence in support of this proposed A24 supertype has been published to date. The A24 supertype is highly represented in different ethnic groups, such as Caucasians (23.9%), Chinese (40.1%), and Japanese (58.6%) (8). Therefore, biological validation of this supertype and identification of antigenic peptides from viral Ags
with the capacity to bind promiscuously across the supertype could promote efforts to develop epitope-based vaccines or immunotherapy. The present report has addressed this issue by examining two viral epitopes (from EBV and CMV) initially identified as HLA-A*2301-binding peptides. The data clearly demonstrate that these peptides are recognized by T cells in association with different alleles of the A24 supertype, and in the case of the EBV peptide, CTL responses are directed toward this single epitope in both A*2301$^+$/A*2402$^+$ individuals. CTL clones were isolated with promiscuous HLA restriction, displaying a capacity to recognize the peptides in association with HLA A*2301 or A*2402. Together these data confirm the immunological relevance of grouping at least these three alleles within an A24 supertype.

Materials and Methods
Establishment and maintenance of cell lines
Lymphoblastoid cell lines (LCLs)$^a$ were established by exogenous transformation of peripheral B cells with EBV derived from the supernatant of the B95.8 cell line and were maintained in growth medium containing 10% FCS (RPMI 1640). Some HLA-typed LCLs were also obtained from the European Collection of Cell Cultures. PHA blasts were generated by stimulating PBMCs with PHA (Sigma-Aldrich, Sydney, Australia), and after 3 days, growth medium containing supernatant from the MLA-144 cell line (American Type Culture Collection, Manassas, VA) and rIL-2 was added. PHA blasts were pulsed with biweekly replacement of rIL-2 and MLA-144 supernatant (PHA free) for up to 8 wk. The blood donors used in this study were healthy laboratory staff selected for particular HLA alleles and prior exposure to either EBV or CMV as assessed by standard virus-specific Ab tests. All cell lines were regularly screened for mycoplasma contamination.

CTL clones were generated by agar cloning as follows. PBMCs (2 $\times$ 10$^7$) were stimulated with 2 ml of growth medium with autologous cells presenting a viral epitope. These stimulator cells were either PBMCs pre-coated with the CMV peptide AYAQKIFKIL (1 $\mu$M for 1 h, responder/stimulator ratio of 2:1) or gamma-irradiated (8000 rad) LCLs (responder/stimulator ratio of 50:1) to raise clones against the CMV peptide PLYFWLAAI. After 3 days, cells were dispersed and seeded in 0.35% agarose (Seaplaque; BioWhittaker Molecular Applications, Rockland, ME) containing RPMI 1640, 20% FCS, 25% supernatant from MLA-144 cultures, and rIL-2 (20 U/ml). Colonies were harvested after a further 3–5 days and were amplified in culture with biweekly restimulation with rIL-2, MLA-144 supernatant, and the gamma-irradiated (8000 rad) autologous LCL stimulators. CTL microcultures were pre-loaded with the CMV peptide AYAQKIFKIL for use with clones initially raised against this peptide. CTL clone CSIC7 has been described previously (21).

Short term CTL microcultures were generated by limiting dilution as follows. PBMCs were distributed in round-bottom microtiter plates in growth medium at cell numbers ranging from 10$^5$ to 5 $\times$ 10$^5$ cells/well. Approximately $5 \times 10^7$ gamma-irradiated (2000 rad) autologous PBMCs that had been pulsed with the EBV peptide PLYFWLAAI (1 $\mu$M for 1 h), were added to each well to give a total volume of 100 $\mu$l. Cultures were fed on days 4 and 7 with 20% of medium supplemented with 20 U of rIL-2 and 25% (v/v) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into four replicates and used as effectors in a standard 5-h $^{51}$Cr release assay against target PHA blasts (HLA-A*2301$^+$ or A*2402$^+$) that had been treated with the peptide PLYFWLAAI or left untreated.

Cytotoxicity assay
CTL clones were tested in duplicate for cytotoxicity in the standard 5-h chromium release assay. Briefly, CTLs were assayed against $^{51}$Cr-labeled LCL or PHA blast targets that were pretreated with synthetic peptide and washed or left untreated. Peptides were synthesized by Mimotopes (Clayton, Australia). Toxicity testing of all peptides was performed before use by adding peptide to $^{51}$Cr-labeled PHA blasts in the absence of CTL effectors. In some experiments target cells were infected with recombinant vaccinia virus at a multiplicity of infection of 10:1 for 1 h at 37°C. After overnight infection, cells were washed with growth medium, incubated with $^{51}$Cr for 60 min, and used as targets in a $^{51}$Cr release assay. Recombinant vaccinia virus constructs encoding the latent membrane protein 2A (LMP2A) Ag of EBV (Vacc.LMP2A), the IE1 Ag of CMV (Vacc.IE1), and a vaccinia virus construct made by insertion of the pScI1 vector alone and negative (thymidine kinase (Vacc.TK$^-$)) have been previously described (23, 24). A beta scintillation counter (Topcount Microplate; Packard Instrument, Meriden, CT) was used to measure $^{51}$Cr levels in assay supernatant samples. The mean spontaneous lysis for target cells in culture medium was always <20%, and the variation about the mean specific lysis was <10%.

Results
The LMP2A Ag of EBV is a target for recognition by CTLs both in patients with acute infectious mononucleosis and in healthy virus carriers (25). In a previous investigation we identified a nonamer peptide from this Ag, PLYFWLAAI, that was recognized in association with HLA A*2301 by a CTL clone (CSIC7) raised from an A*2301$^+$ individual (21). To investigate the possibility that this EBV peptide could be presented by other members of the proposed HLA A24 supertype, the CSIC7 CTL clone was tested for recognition of multiple HLA-mismatched target cells that had been treated with the PLYFWLAAI peptide or left untreated. As shown in Fig. 1A, not only could the CTL clone lyse peptide-pulsed target cells expressing the HLA A*2301 allele that the clone was selected against, it was also capable of recognizing the peptide in association with the non-self molecule HLA A*2402. The CTLs could not cross-recognize the peptide on A*2403, A*3002, or A*3003, which have also been tentatively included within the proposed A24 supertype (8). This could be due to a lack of peptide binding to these alleles or to nonrecognition by the TCR of the peptide bound to these HLA molecules.

![Figure 1. Promiscuous HLA restriction by CTL clones raised from HLA A*2301$^+$ individuals against an EBV or a CMV epitope. A, Lysis by CTL clone CSIC7 of target cells pretreated with the EBV peptide PLYFWLAAI or left untreated; B, lysis by CTL clone JW29.2 of target cells pulsed with the CMV peptide AYAQKIFKIL or left untreated. Target cells were LCLs expressing a range of HLA alleles, as shown in the figure. The peptide concentration was 10 $\mu$M, and the E:T cell ratio was 2:1.](http://www.jimmunol.org/Downloadedfrom)
During a recent investigation aimed at identifying new CMV T cell epitopes using IFN-γ ELISPOT assays, a 10-aa peptide (AYAQKIFKIL) from the IE1 Ag was identified as an immunogenic sequence in two HLA A*2301+ CMV-exposed individuals (data not shown). CTL clones were raised from each of these individuals against the AYAQKIFKIL peptide and screened for cross-recognition of the peptide on a range of HLA-mismatched LCLs, as was done with the EBV-specific CTL clone. The data shown in Fig. 1B are for clone JW29.2, and very similar results were obtained with the second clone (data not shown). As with the EBV-specific CTLs, these CMV-specific T cells exhibited promiscuous HLA restriction, recognizing the AYAQKIFKIL peptide in association with either self-HLA-A*2301, or the non-self-HLA A*2402. No lysis was observed through other HLA alleles, such as A*2403 or A*3003, although peptide-pulsed target cells expressing A*3002 were recognized at low levels by the clones.

Since the promiscuous HLA restriction observed with EBV-specific CTLs was demonstrated with only one clone, experiments were conducted to determine how frequently this pattern of cross-reactivity is displayed. CTL microcultures were raised at limiting dilution against the PYLFWLAAI peptide by stimulating PBMCs from an HLA A23+ EBV-sero+ individual with the peptide. All CTL cultures reactive toward the EBV peptide were capable of recognizing the peptide in the context of self-HLA-A*2301 or non-self-HLA-A*2402 (Fig. 2), indicating that these two peptide-HLA complexes share considerable structural similarity. Notably, HLA A*2301 and A*2402 differ by just four amino acids, and two of these differences are located at positions unlikely to influence either peptide binding or TCR interactions. However, the conserved mismatch at amino acid position 151 (Arg in A*2301 and His in A*2402), which is located at the docking surface of the HLA has the potential to influence TCR interactions. Furthermore, the mismatch at the α2 helix position 156 (Leu in A*2301 and Gin in A*2402), which projects into the peptide binding groove, could theoretically influence peptide binding or conformation (26).

These data demonstrate indirectly that the PYLFWLAAI epitope binds to multiple alleles of the A24 supertype, raising the possibility that this peptide is immunogenic in individuals expressing A*2402. Two CMV-sero+ donors who expressed HLA A*2402 were also tested for CTL responses to the AYAQKIFKIL peptide using the IFN-γ ELISPOT assay; however, no responses were detected, suggesting that this peptide is nonimmunogenic in A*2402+ individuals (data not shown). It was not possible to assess CTL responses to these EBV and CMV epitopes in A*3001+ individuals because donors expressing this HLA allele were unavailable for this study.

To determine whether these CTL clones raised from A*2301+ and A*2402+ individuals displayed degeneracy in HLA restriction against target cells presenting endogenously processed viral peptide, target cells were infected with recombinant vaccinia virus A*2402+ donors

**FIGURE 2.** Cross-reactivity by multiple CTL cultures raised from an HLA A*2301+ donor with an EBV peptide presented in association with HLA A*2301 or A*2402. CTL microcultures were raised against peptide PYLFWLAAI from the EBV-seropositive donor PC (HLA A*2301, A25, B21, -) using a responder PBMC concentration from which less than half the wells produced peptide-specific CTLs. Data from all wells that showed significant recognition of the peptide in association with A*2301 are shown. The target cells were HLA A*2301+ (vertical axis; other class I alleles, A3, B35, and B44) or A*2402+ (horizontal axis; other class I alleles, A29, B44, and B61) PHA blasts that had been pretreated with peptide PYLFWLAAI (0.1 μM) or left untreated.

**FIGURE 3.** Promiscuous HLA restriction by CTLs raised from HLA A*2402+ individuals against the EBV epitope PYLFWLAAI. A, CTL microcultures were raised against peptide PYLFWLAAI from the EBV-seropositive donors WT (HLA A*2402, A3, B14, B60), MB (HLA A*2402, A1, B7, B58), MS (HLA A*2402, A1, B7, B8), and DM (A*2402, A29, B44, -) using a responder PBMC concentration from which less than half the wells produced peptide-specific CTLs. The target cells were HLA A*2301+ (and A3, B35, and B44) or A*2402+ (and A29, B44, and B61) PHA blasts that had been pretreated with peptide PYLFWLAAI (0.1 μM) or left untreated. B, Lysis by CTL clone AM9 of target cells pretreated with the EBV peptide PYLFWLAAI or left untreated. Target cells were LCLs expressing a range of HLA alleles as shown in the figure. The peptide concentration was 10 μM, and the E:T cell ratio was 2:1.
constructs encoding the LMP2A Ag of EBV, the IE1 Ag of CMV, or a vaccinia virus construct made by insertion of the pSC11 vector alone and negative for thymidine kinase (Vacc.TK-). As shown in Fig. 4A, the A*2301+ CTL clone CSIC7 displayed classical self-HLA-restriction when the target EBV peptide was processed endogenously. In contrast, the EBV-specific AM9 CTL clone cross-recognized the LMP2A Ag expressed in A*2402+ or A*2301+ target cells (Fig. 4B). Furthermore, the A*2301+ CMV-specific CTL clone JW29.2 demonstrated a capacity to kill IE1-expressing cells with this same subset of HLA A24-supertype alleles (Fig. 4C).

As a final comprehensive analysis of the degeneracy in HLA restriction of these CTL clones, dose-response experiments were conducted using a wide range of synthetic peptide concentrations to treat the target cells. The two A*2301+ clones clearly recognized their target peptides most efficiently when presented on self-HLA-A*2301, although cross-recognition of the target EBV or CMV peptides on the non-self-HLA A*2402 was clearly displayed at relatively high peptide concentrations (Fig. 5, A and C). In contrast, the HLA restriction of CTL clone AM9 was highly promiscuous, with very similar dose-response curves obtained with target cells expressing self-HLA-A*2402 or non-self-HLA-A*2301 (Fig. 5B).

**Discussion**

This study has indirectly demonstrated that the AYAQKIFKIL epitope from CMV and the PYLFWLAAI epitope from EBV can bind promiscuously to HLA A*2301 and A*2402, thereby validating at the biological level that these alleles can be grouped together into an A24 supertype as proposed by Sette and Sidney (8). The structural basis for this degeneracy revolves around the identity or homology of amino acid residues surrounding the B- and F-pockets of these HLA alleles. HLA A*2301 and A*2402 are particularly closely related within their peptide binding grooves, sharing identical residues surrounding the B- and F-pockets (B-pocket: S9,M 45,E 63,K 66,V 67,H 70; F-pocket: N77,I 80,A 81,Y 116). The most convincing evidence for the inclusion of A*2301 and A*2402 within an A24 supertype was provided by studies involving the EBV epitope PYLFWLAAI. Using a CTL clone from an A*2402+ individual and dose-response analysis with synthetic peptide, this viral peptide was shown to be presented to these two alleles with similar efficiencies. Furthermore, target cells expressing each of these HLA alleles and the LMP2A Ag through recombinant vaccinia virus infection were lysed by this clone, demonstrating presentation of the PYLFWLAAI peptide at significant levels in association with A*2301 and A*2402 after endogenous processing. Memory responses to this epitope were also detectable in healthy EBV carriers expressing either A*2301 or A*2402. Supporting evidence for the inclusion of A*2301 and A*2402 within an A24 supertype was provided by data generated with the two A*2402+ CTL clones.

The broad immunogenicity of the PYLFWLAAI epitope has potentially important practical implications for EBV vaccine development. The LMP2A Ag of EBV is of particular interest in this context.
context because it is one of the few EBV Ags expressed in malignancies such as nasopharyngeal carcinoma and Hodgkin’s disease (27). Since it is well established that immunization with whole viral proteins does not elicit an efficient CTL response, interest has been directed toward epitope-based vaccines, particularly with oncogenic viruses such as EBV, in which individual viral genes have the potential to initiate tumorigenic processes. A major potential obstacle to developing such vaccines is the large number of epitopes potentially required to achieve broad population coverage, given the extreme degree of polymorphism in HLAs. Before this investigation, the PYLFWLAAI epitope was thought to be immunogenic in only HLA A*2301 individuals, an HLA allele with a gene frequency of only 1.3% of Caucasians and 0.6% of Asians (28). Our data demonstrate that this peptide also stimulates a CTL response through the much more common allele A*2402 (gene frequency of 6.6% in Caucasians and 18.9% in Asians). This study has therefore highlighted a much broader potential population coverage of this single LMP2A epitope if included in a CTL-based EBV vaccine.

The cross-reactivity of the AM9 CTL clone with the EBV peptide presented at very limiting concentrations on A*2301 or A*2402, shown clearly in Fig. 5B, suggests exceptional degeneracy at the level of both peptide binding and TCR recognition. The mechanism by which the TCR expressed by AM9 tolerates these major differences will only be determined by crystal structure analysis, but presumably it can be triggered by these distinct complexes without making major contacts with the side chains of these divergent α helix residues. It is relevant to point out that the AM9 CTL clone was raised from an Indonesian donor who expressed both A*2402 and the rare allele A*2407. It is possible that broadly reactive CTLs have been preferentially selected in this donor for their capacity to cross-recognize the EBV peptide in association with each of these alleles. It is also notable that human CTL clones raised against foreign peptides often display cross-reactivity with alloantigens and are therefore tolerant of amino acid differences between the selecting self-HLA allele and the allo-HLA target Ag (29–33).

Degenerate peptide binding to multiple HLA alleles that are grouped together as an HLA supertype is now a widely accepted phenomenon, and this report has confirmed the validity of the A24 supertype. It is probably less well excepted that, as a consequence of this degeneracy in peptide binding, along with additional degeneracy at the level of TCR recognition, the T lymphocytes with αβ TCRs are not always self-MHC restricted. The dogma that defines recognition of foreign peptides by αβ T cells is that it only occurs in the context of a presenting self-MHC protein. Clones such as AM9 can clearly recognize the same foreign peptide with similar efficiency in association with a self-HLA or some non-self-HLAs. Several other examples of CTL clones with very promiscuous HLA restriction have been described in which the degeneracy is observed with low concentrations of exogenous peptide or after endogenous peptide processing (13, 15, 16). The interaction between a TCR and an MHC occurs within a limited area on the top surface of the MHC molecule and, as illustrated with the AM9 CTL clone, αβ TCRs can accommodate a degree of polymorphism in the exposed α helix residues between the restricting MHC and foreign MHC Ags that present the same peptide. Self-MHC restriction is controlled at the level of both peptide-MHC and TCR-MHC binding, and since MHC molecules differ primarily in amino acids that bind peptide rather than at positions predicted to be directly accessible to TCRs (34), the specificity of peptide binding is likely to be a more important factor. Despite this polymorphism, peptides do commonly bind to multiple MHC Ags, and it is therefore likely that a significant proportion of αβ T cells can recognize foreign peptides on self- and non-self-MHC Ags and are therefore not strictly self-MHC restricted.

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References


1412 HLA-A24 SUPERTYPE-RESTRICTED T CELL RECOGNITION

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