Published in final edited form as:
Eur J Immunol. 2007 September ; 37(9): 2419–2433. doi:10.1002/eji.200737365.

Extensive HLA class I allele promiscuity among viral CTL epitopes

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Summary

Promiscuous binding of T helper epitopes to MHC class II molecules has been well established, but few examples of promiscuous class I restricted epitopes exist. To address the extent of promiscuity of HLA class I peptides, responses to 242 well-defined viral epitopes were tested in 100 subjects regardless of the individuals' HLA type. Surprisingly, half of all detected responses were seen in the absence of the originally reported restricting HLA class I allele, and only 3% of epitopes were recognized exclusively in the presence of their original allele. Functional assays confirmed the frequent recognition of HLA class I-restricted T cell epitopes on several alternative alleles across HLA class I supertypes and encoded on different class I loci. These data have significant implications for the understanding of MHC class I restricted antigen presentation and vaccine development.

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Conflict of Interest
The authors have no conflicting financial interests.

Keywords

CD8 T cells; HIV; Immune Responses; Antigen Presentation/Processing; Bioinformatics

Introduction

HLA class I restricted cytotoxic T lymphocytes (CTL) recognize a trimeric complex composed of the MHC-encoded class I heavy chain, the β_2-microglobulin light chain and a short antigenic peptide derived from the degradation of intracellular proteins. This peptide plays a pivotal role in the stabilization of the trimeric complex and is necessary for its egress to the cell surface. The repertoire of peptides that bind to MHC class I molecules is determined largely by the tertiary structure of the MHC class I heavy chain, where variable, discontinuous resides located largely in the α_1 and α_2 domains determine the allele-specific epitope binding pockets [1–3]. These pockets allow for the selective binding of peptides that fulfill the structural requirements required for high-affinity binding, thereby determining the allele-specific binding motif [4].

These binding motifs were initially identified in studies where peptides eluted from surface-expressed class I molecules were sequenced and aligned [4]. The over-representation of functionally similar amino acids in corresponding locations of the eluted peptides further supported that specific anchor residues were required for binding to specific alleles. These binding motifs have allowed for the development of computational algorithms that predicted a number of peptides derived from pathogens that would be capable of binding to given alleles. Subsequent experimental detection of immune responses to these predicted peptides and HLA binding studies validated the algorithms in many cases and provided further experimental evidence for the validity of allele-specific binding motifs [5–9].

Although the individual HLA loci are among the most genetically diverse genes in the human genome, with over 1000 alleles described to date, many of the HLA class I heavy chains are structurally similar. In cases where this similarity conserved the structure of the epitope binding pockets, it has allowed for the grouping of HLA alleles into supertypes, defined as clusters of alleles with potentially similar binding motifs [10–12]. Currently, there are nine HLA supertypes that have been well described for HLA-A and –B alleles, for some of which sharing of individual epitopes has been experimentally demonstrated [11,13,14].

Given the relatively well-established class I binding motifs for at least some alleles, and in strong contrast to the well-established binding promiscuity for MHC class II-restricted epitopes [15–21], binding of epitopes across a broad spectrum of HLA class I alleles has been considered the exception rather than the rule [22,23]. Yet, some similarities between different binding motifs exist, and promiscuous presentation of epitopes to a certain degree could be expected [11–13]. Indeed, a number of individual CTL epitopes have been shown to be presented in the context of more than one HLA class I allele [22–30]. However, the described instances have largely been limited to epitopes shared by alleles that are assigned to the same HLA supertype which, by definition, share similar binding motifs. While some of these data point to the possibility that peptide binding to HLA class I molecules may be more promiscuous than previously thought, no study to date has systematically addressed the extent of class I epitope promiscuity despite its significant implications for a better understanding of HLA class I-restricted antigen presentation and vaccine development.

Here, we determined the HLA class I promiscuity of previously described, well-defined CTL epitopes by testing responses in 100 subjects to a set of 242 HIV- and EBV-derived

CTL epitopes, regardless of the individual's HLA type. Remarkably, half of all positive responses were detected in individuals not expressing the originally described restricting HLA allele, indicating that epitope presentation and CTL recognition must occur frequently in the context of alternative HLA class I alleles. While some of the alternative restrictions were confirmed experimentally, most alternative restricting allele(s) were identified by statistical methods, showing presentation by up to seven different alleles for at least some epitopes. Overall, this study demonstrates that T cell responses against previously defined HLA class I-restricted CTL epitopes can frequently occur in the context of more than one restricting allele, and documents an unprecedented degree of HLA class I epitope promiscuity.

Results

Epitope-specific responses detected in the absence of described restricting HLA alleles

One hundred and three HIV-infected subjects were tested against 162 optimally defined, HIV-derived CTL epitopes previously described in the Los Alamos HIV Immunology Database [31]. Additionally, fifty of these HIV-infected subjects, as well as fifty HIV negative subjects, were screened with a set of 80 well-defined, EBV-derived CTL epitopes by IFN-γ ELISpot assay for positive responses, regardless whether they expressed the described restricting HLA class I allele [32]. The tested cohort showed a diverse HLA allele distribution that covered all 46 HLA class I alleles described to restrict the tested HIV- and EBV-derived CTL epitopes. Thus, each epitope was tested at least once in the context of its original HLA restriction. One HIV-infected subject without detectable responses to the HIV epitopes and eight subjects without detectable EBV responses were excluded from further analyses.

Among the HIV-derived, epitope-specific responses, an average of 14 positive responses (range 1–57) per person were detected. On average, only 38% of a subject's HIV-specific responses were directed against epitopes for which the individual actually expressed the described restricting allele (Fig. 1a), indicating that the epitopes can be recognized in the context of additional, alternative alleles. Based on the fact that alleles assigned to the same HLA supertype share similarities in their binding motifs, one would expect such alternative alleles be mostly class I alleles belonging to the same HLA class I supertype as the originally described, restricting allele. To test this, for each response that was detected in the absence of the original restricting allele, the HLA type of the responding subject was examined to determine if responses may have been restricted by an allele of the same HLA supertype as the originally described allele. Alleles were assigned to supertypes based on binding assays, published motifs or structural prediction (based on the amino acid composition of the binding pockets), and all assignments are summarized in Supplementary tables 1 and 2. Surprisingly, on average only 29% of all responses could be attributed to the presence of a supertype-matched allele, leaving another 33% of all responses unaccounted for. While the mere presence of a supertype-matched allele does not necessarily mean that the response was indeed restricted by this allele, these data indicate that in this cohort at least one third of all HIV epitope-specific responses were presented in the context of alternative HLA alleles that were outside the original restricting allele's HLA supertype.

A similar pattern emerged when responses to the 80 EBV-derived CTL epitopes were assessed (Fig. 1b). In the 100 subjects tested, an average of seven responses (range 1–37) were detected per person, and the original allele was present for 58% of these responses. Only 10% of responses could be explained by the presence of a supertype-matched allele in the responding subjects, again leaving a third (32%) of all EBV-specific responses detected in the absence of both the originally described, restricting allele and a supertype-matched allele (Fig. 1b). Importantly, the magnitude of epitope-specific responses detected in the

presence or absence of the original allele were comparable (Fig. 1c and d, $p > 0.05$ for HIV as well as EBV epitopes). In addition, all positive responses were defined on the basis of stringent criteria for positivity (see methods) and were substantially above the background that was seen when testing 20 HIV-negative controls, indicating that responses detected in the absence of the original allele were not due to weak and unspecific background activity in the ELISpot assay. Furthermore, independent data on repetitive-well ELISpot screenings showed that the false-positive discovery rate was less than 1% (data not shown). Together, the data demonstrate that peptides presented in the context of alleles other than the original restricting allele are able to elicit immune responses of similar magnitude to responses generated when the peptide is presented on the originally described allele.

Although short, optimally defined HLA class I restricted epitopes (typically 9mers) were used in the present screenings, the observed promiscuity could in theory be due to CD4 T cell recognition of these antigens. However, when 48 epitope-specific responses were assessed by intracellular cytokine staining, 47 were found to be mediated exclusively by CD8 T cells (supporting Fig. S1). This is in line with previous data demonstrating that CD8 T cells mediate more that 95% of responses detected by ELISpot in patients from a similar cohort of chronically HIV infected individuals [33]. Thus, these data demonstrate that 50% of all epitope-specific, CD8 T cell-mediated responses to the tested HIV and EBV derived epitopes were detected in individuals not expressing the originally described restricting HLA class I allele. Since these responses were CD8 T cell mediated, and thus likely restricted by at least one alternative HLA class I allele expressed by the responding individual, the data reveal an unexpected and extensive HLA promiscuity in CTL epitope recognition.

Most epitopes show promiscuous presentation

For the vast majority (94%) of all HIV- and EBV-derived epitopes tested, responses were detected in at least one individual not expressing the original allele (Table 1). In addition, 27 HIV-derived and 14 EBV-derived epitopes were never targeted by individuals expressing the originally described allele, although most of these (36 of 41, 88%) elicited responses in individuals not expressing the original allele, suggesting that lack of recognition was not due to a faulty peptide preparation. While some of these peptides were restricted by rare alleles and thus may have been tested in the presence of the original allele only once, they also included epitopes restricted by common alleles such as HLA-A02, -A03 or –B35 (data not shown). This indicates that low allele frequency in the cohort was not solely responsible for a lack of detectable responses in the context of the originally described allele.

Of the 153 HIV and 75 EBV peptides for which the data indicated promiscuous recognition, 117 HIV (72%) and 49 EBV peptides (61%) were targeted in the context of at least two alternative alleles, since there was no single HLA class I allele common to epitope responders not expressing the original allele. Even with this conservative estimate, assuming that the most frequent allele in the epitope responders is a restricting allele, these data show that the vast majority of the epitopes tested can be presented by at least three different HLA class I molecules, including the originally described one. To more reliably estimate the number of potentially restricting alternative class I alleles on a single epitope level, the HLA allele distribution among epitope responders who did not express the originally described restricting HLA class I allele was analyzed. A comprehensive search algorithm was used to determine the minimal number of different HLA class I alleles needed to cover the HLA allele representation found in the group of all epitope responders, including the subjects expressing the original allele. The minimal number of alleles needed for complete HLA coverage for HIV and EBV epitope responders varied from one to seven alleles (median 3 alleles for both HIV and EBV). Table 2 lists those HIV-derived epitopes for which the T cell reactivity data and the HLA types of the responding individuals suggested six or more different alternative alleles. Interestingly, six of these nine epitopes are located in regions of

the HIV proteome that previous studies identified as hot spots of CTL recognition [34]. Of note, the applied algorithm did not determine the most probable alternative alleles, but the minimum number of alleles necessary to explain the observed reactivity, and alleles listed in Table 2 are therefore enriched for highly prevalent HLA alleles such as HLA-A02. Two additional algorithms were developed to determine probable alternative alleles (see below).

Epitopes can be presented on alleles encoded by different loci

In order to experimentally demonstrate promiscuous recognition of a specific epitope, individuals with a response directed against the highly promiscuous HIV Nef-derived YT9 epitope (YFPDWQNYT) were analyzed and the restricting HLA alleles determined. This epitope had previously been described to be restricted by HLA-B37 and -B57 [35]. However, of the 42 subjects responding to YT9, only eight expressed either HLA-B37 or -B57, indicating that presentation on multiple alternative HLA class I alleles was likely.

HLA restriction analyses were performed in three subjects, identifying HLA-A29, -B35 and -Cw06 as additional alternative HLA restriction elements for this epitope (Fig. 2). Peptide titrations using single-residue truncated peptide variants of the YT9 epitope confirmed in all but one case that the YT9 9-mer peptide was the most active, shortest and therefore the “optimal” epitope [36]. The exception was the case where YT9 was presented on HLA-B35, and where the embedded, N-term truncated peptide FT8 showed the strongest response, consistent with the presence of a proline residue in position 2 of the new epitope and fulfilling the binding motif of HLA-B35. Although this example highlights the need to consider the presence of embedded epitopes in the apparent epitope promiscuity, these data show that, together with the previously established restriction elements on two HLA-B alleles, identical epitopes can be presented by alleles belonging to all three HLA class I loci.

Experimental confirmation of promiscuous presentation of identical epitopes

To address whether in most cases the identical (i.e. same length) optimal epitope was presented on the alternative allele(s), standard peptide titration analyses using single amino acid truncations and extensions of the tested epitopes were performed on a subset of promiscuous epitopes. Peptide truncations and extensions were synthesized for 70 HIV epitopes and tested in a total of 121 samples. These analyses focused mostly on responses to epitopes of identical length in subjects mounting the response in the absence of the original allele (Fig. 3, top panel for 12 examples), but also confirmed the possible targeting of shorter epitopes (Fig. 3, bottom panel), highlighting the need to carefully assess peptide sequences for the presence of embedded epitopes. Besides demonstrating that the same-length epitopes were presented in the absence of the original allele, the titration data also indicate that responses restricted by alternative alleles can be of similar functional avidity as when presented on the original allele [37], thus further supporting the potential *in vivo* relevance of these responses.

Promiscuous epitopes are processed and presented in the absence of original alleles

Finally, to demonstrate the physiological relevance of epitope-specific responses detected in the absence of the original allele, their natural processing and presentation were analyzed. To this end, dendritic cells (DC) were transfected with mRNA encoding the full-length nef sequence and co-cultured with autologous PBMC from HIV-infected subjects for 12 days. The specificity of the expanded cells was then determined by ELISpot and ICS analyses. As shown in Fig. 4 for subject L8 29 (HLA-A02 / 33, B35 / 57, Cw04 / 07), CD8 T cells that expanded in response to endogenously processed Nef antigen recognized the nominal A24-RW8 optimal synthetic peptide even though the donor was HLA A24-negative. These data demonstrate that promiscuous epitopes can be naturally processed from precursor protein

sequences and effectively presented in the context of the alternative allele(s) emerging from our analyses.

Prediction of alternative restricting class I alleles

While the above data demonstrate extensive HLA class I promiscuity by virtue of detecting responses in individuals not expressing the originally described restricting HLA class I types, the identification of specific alternative allele(s) presenting these epitopes would help to refine allele- and HLA supertype-specific binding motifs and to identify promiscuous epitopes for potential inclusion in vaccines. Thus, two algorithms were used to predict the most likely alternative restricting element(s) and to potentially identify allele pairs or groups of alleles that frequently share epitopes. Despite the distinct nature of the two strategies, the results were robust and produced largely overlapping associations between specific epitopes and their potential alternative restricting alleles. Common to both approaches, the HLA class I allele frequencies in the group of epitope responders and non-responders were compared. HLA class I alleles that were over-represented among the epitope responders compared to the non-responders were considered likely alternative restricting alleles for the epitope under investigation. Both approaches allowed ranking the associations based on their p-values, but due to the very large number of comparisons (requiring an uncorrected $p < 7.7\times10^{-5}$ when applying Bonferroni correction), q-values were also computed, since the standard correction for multiple tests is likely too conservative for these p-values, given that for each response that cannot be explained by a known restricting HLA- element, some alternative HLA molecule must be presenting the epitope. The most probable associations between epitopes and possible alternative restricting alleles ($p \leq 0.002$ in both approaches) are summarized in Table 3. Seven of these thirty-three associations remained significant even after Bonferroni correction. These analyses point to those class I alleles that are most enriched among the subjects responding to the epitope under analysis and who do not carry the known presenting allele. Importantly, as highlighted by the recognition of an embedded epitope in Fig. 2, each allele-epitope association was examined for the presence of a potential embedded peptide sequence within the tested epitope that fulfilled the binding motif of the alternative allele (or its supertype in the absence of an allele-specific binding motif). Conservatively, associations suggestive of the presence of such embedded shorter epitopes were excluded from Table 3 and are shown in Table 4 instead. The applied binding motifs were collected from Marsh [38] and SYFPEITHI [39] and are summarized including supertype motifs [11] in Supporting Table S3.

A total of 255 additional associations were identified by both algorithms not reaching the same statistical power as described above. Statistically robust associations for these promiscuous epitopes often may have been missed due to the small numbers of individuals tested who express the alternative allele(s). Nevertheless, these associations still identify the most likely alternative restricting alleles, as in each responding subject, one allele must be presenting the epitope in the absence of the known presenting molecule. These data are summarized in Supporting Table S4 and include associations that fulfill the following requirements: $p < 0.1$ in the LANL approach and $p < 0.05$ in the MSR approach. Together, even after applying these exclusion criteria, the results show that testing 100 individuals each against previously described HIV and EBV CTL epitopes provided sufficient data to predict more than 300 HLA class I alleles that can likely serve as alternative presenting alleles for these epitopes.

Multiple epitopes can be shared between pairs of original and alternative alleles

The associations between epitopes and alternative alleles described in Table 3 show that there is no clear trend as to the relationships between original and alternative alleles (some pairs belong to the same supertypes, but many pairs are across loci). It therefore seems that

additional restricting elements cannot be predicted based on the original allele, but will depend mostly on the epitope under consideration. Nonetheless, a closer analysis of Supporting Table S3 revealed that there are certain allele pairs that frequently share epitopes. Although such sharing of epitopes was previously proposed for alleles belonging to the same supertype, the present analyses clearly indicate that epitope promiscuity extends far beyond alleles of the same HLA supertype (Fig. 1). In particular, analyzing the 16 pairs of alleles that were identified to share three or more epitopes (Table 5), the data show that more HLA allele pairs sharing multiple epitopes belong to different loci (7 of 16, 44%) than to the same supertype (5 of 16, 31%). While the p-values for many of the associations in Table 5 lose significance after correction for multiple comparisons, the fact that several epitopes are shared between specific alleles lends further support to the biological relevance of the observed epitope sharing across HLA loci.

Discussion

Promiscuous antigen presentation has been considered a hallmark of MHC class II-restricted T helper cell responses [15–19], while few reports have shown CTL epitope recognition in the context of two or more unrelated HLA class I molecules [22,23]. However, defining the extent of MHC class I promiscuity could significantly improve our understanding of HLA function and facilitate vaccine development, as fewer epitopes would be needed to cover a larger portion of specific host populations [40,41]. The present report provides statistical and experimental evidence for an extensive degree of HLA promiscuity among viral CTL epitopes, which extends well beyond currently defined HLA supertypes and across HLA-A, -B and -C loci. The data also shed new light on responses seen in the absence the described restricting HLA allele, which may have oftentimes been dismissed as unspecific reactivity rather than being considered relevant immune responses to promiscuously presented epitopes [42].

Remarkably, 230 of 242 (95%) epitopes tested elicited a response in at least one individual not expressing the original restricting allele. In fact, conservative estimates predicted a minimum of three different alleles presenting each epitope tested, with some epitopes requiring up to seven different HLA alleles to cover the group of epitope responders. Surprisingly, the majority of potential alternative alleles were not matched to the same HLA supertype or even the same locus as the original restricting allele, demonstrating that promiscuous presentation of epitopes is not restricted to a few, closely related HLA alleles but rather is a widespread phenomenon. Moreover, a comparison of the minimal number of alternative alleles for epitopes with originally known HLA-A, -B or -C restriction did not show statistically significant differences, suggesting that most epitopes can be presented on other alleles, regardless of their initially defined restriction. These conclusions are in line with previously published data assessing HIV-specific T cell responses using overlapping peptides sets spanning all HIV proteins [34]. In that case, many of the 410 peptides included in the test set were recognized by dozens of subjects (with up to 50% of all tested, genetically diverse individuals responding to single 18mers), supporting extensive promiscuity of the limited number of optimal epitopes that can be contained in the 18mer peptide sequence.

In order to determine the statistically most probable alternative presenting HLA allele for each tested epitope, the present study employed two complementary biometric approaches. Overall, more than 600 associations were identified by one or the other approach, with 303 emerging from both analyses as likely alternative restricting elements. Importantly, the associations presented in Table 3 excluded instances where potentially embedded epitopes were presented on an alternative allele by taking into consideration known binding motif information. For many cases, experimental *in vitro* data confirmed the optimal length of the

epitopes presented on the alternative alleles, both for identical and embedded epitopes. Depending on the intended use of such promiscuous epitopes, these epitope/allele combinations may require experimental demonstration that the precise same epitope is indeed being presented on the allele(s) that emerge from the biometric analyses. This will also need to include predictions that relax currently known binding motifs, and will help to further refine currently incompletely characterized binding motifs. Together, using two complementary algorithms, and considering the existence of potentially embedded epitopes and HLA linkage disequilibrium effects, our data shown that the emerging alternative restricting allele assignments are likely of physiological relevance and will provide guidance for future epitope identification.

Remarkably, only one sixth (52 of 303) of all identified associations between original and alternative restricting elements occurred between alleles that fell into the same HLA supertype (Supporting Table S4), indicating that epitope sharing across alleles in different HLA class I supertypes is frequent. However, further analyses also indicated that the present data do not warrant alternative classifications of HLA class I alleles into allelic groups that are guided by functional rather than sequence or binding motif similarities, as has been proposed for HLA class II alleles [20]. Nevertheless, the currently available data may help to better define the structural flexibility of different epitope-binding pocket interactions and to confirm previous studies showing that residues flanking the traditional binding positions can also affect the ability of specific epitopes to bind to their restricting HLA alleles(s) [10, 43–45]. Epitopes that produced high statistical likelihood in both of the biometric approaches, yet did not fulfill the known binding motifs of the alternative alleles and did not indicate the presence of an embedded epitope, may be of special interest in this regard.

The present study did not systematically exclude the possibility that some responses in the absence of the original allele could have been due to CD4 T-helper cell mediated recognition of epitopes presented on HLA class II alleles. However, confirming nearly 50 epitope-specific responses by flow cytometry showed that 95% of the tested epitopes were indeed recognized by CD8 T cells (supporting Fig. S1). This is in line with the observation that the use of short, generally 9-mer, peptides is unlikely to elicit HLA class II restricted T cell responses [39, 46], and the fact that most of the tested subjects were chronically HIV infected with a low prevalence of detectable CD4 T cell responses [17]. Similarly, while recent studies report a role for HLA-E in the presentation of viral [47, 48] and bacterial [49, 50] peptides to CD8 T cells, it is unlikely that the observed reactivities were due to common epitope presentation on HLA-E alleles, given the near-monomorphic nature of HLA-E which would dominate the statistical analyses that identify alleles in more diverse loci [49].

Virus-specific CTL responses against highly variable pathogens have been described to leave characteristic HLA footprints on the viral sequence [51,52]. As a consequence, promiscuous epitope presentation could exert immune pressure through multiple alleles, complicating such imprinting analyses. For example, a statistically significant association between HLA-Cw03 and a sequence polymorphism located at position four of the known HLA-Cw08 epitope KAAVDLSHFL was previously described [51], and HLA-Cw03 was predicted to be an alternate presenting allele in this study (Table 3). Similarly, our more recent analyses of HLA-driven viral evolution and effects of phylogenetic founder effects have identified additional instances where immune pressure through multiple alleles may contribute to the observed footprints [14,53]. Obviously, this also bears on the question of how individuals expressing combinations of original and alternative alleles will be able to control viral replication as, from a functional standpoint, these individuals may be considered “functionally homozygous” and may progress more rapidly to AIDS [54]. The present data will allow us to address these questions for an extensive number of epitopes and

allele combinations, and should help to gain further insight into mechanisms of immune control of HIV in particular, and other viral pathogens in general.

Materials and Methods

Study subjects

One hundred three HIV-infected individuals were recruited from a previously described, largely non-Caucasian cohort in the Boston area and tested using 162 optimal HIV epitopes [34]. All but one of these subjects had detectable HIV-specific responses using the peptide set described below. Fifty of these individuals, as well as fifty HIV negative subjects were tested for responses against a set of 80 EBV-derived CTL epitopes. Of these 100 individuals, 92 mounted at least one detectable EBV-specific response. HLA typing was performed by SSP-PCR followed by sequence-based typing where allele-specific subtypes fell into more than one HLA supertype (HLA-A68 and –B15 subtypes). Twenty HIV-negative controls were tested as well using all HIV-derived peptides and showed no reactivity above the employed stringent cut-off of positive responses (see below). All human subject protocols were approved by the Partners Human Research Committee, and all subjects provided written informed consent prior to enrollment.

Assessment of T cell responses

Peripheral blood mononuclear cells (PBMC) were separated from whole blood by density gradient centrifugation and used in direct *ex vivo* ELISpot assays as described [34]. Briefly, 100,000 to 150,000 PBMC/well were plated in 96-well polyvinylidene difluoride plates (Millipore, Bedford, MA), which had been pre-coated overnight with 2 μg/ml of anti-IFN-γ monoclonal antibody (mAb) 1-D1K (Mabtech, Stockholm, Sweden). Each peptide was added at a final concentration of 14 μg/ml. For six negative control wells, cells were incubated in medium alone. PHA (Sigma-Aldrich, St. Louis, MO) was added at a concentration of 1.8 μg/ml to serve as a positive control. Plates were developed using 0.5 μg/ml of biotinylated anti-IFN-γ Mab 7-B6-1 (Mabtech) followed by a 1:2000 dilution of streptavidin-coupled alkaline phosphatase (Mabtech) and the color reagents NBT and BCIP (BioRad, Hercules, CA). Spots were counted using an AID ELISpot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and results were expressed as spot forming cells (SFC) per million input cells. The peptide set consisted of 162 optimally defined HIV-derived CTL epitopes included in the 2001 edition of the Los Alamos National Laboratory HIV Immunology Database CTL epitope list [31]. The set of EBV-derived CTL epitopes has largely been described [32]. However, described EBV epitopes of more than 12 residues in length were excluded from the present study as they may contain multiple epitopes and are likely not of optimal length. Thresholds for positive responses were defined as at least 5 spots per well (regardless of input cell number), and responses exceeding “mean of negative wells plus 3 standard deviations” or “3 times mean of negative wells”, whichever was highest. At least 6 negative wells were included per subject, and the average of all negative wells in the HIV+ subjects was 6 SFC/million PBMC.

Intracellular cytokine staining and peptide titrations

Intracellular cytokine staining to determine the phenotype of responding cells was performed using monoclonal antibodies anti-CD3-PerCP, –CD4-PE, –CD8-APC and –IFN-γ-FITC (all BD Biosciences, San Jose, CA). Cells were stimulated for 6 hours using appropriate peptides as well as CD28 and CD49d costimulatory antibodies (BD). Brefeldin A was added during the final 5 h of incubation at 10μg/ml. Stimulated cells were stored at 4°C overnight, and stained, fixed and permeabilized using BD Cytofix/Cytoperm (BD) according to the manufacturer’s instructions. Alternative restricting HLA alleles were confirmed using the same assay with peptide-pulsed partly HLA-matched antigen presenting

cells as described [55]. Peptide-stimulated T cell lines were generated from patient's PBMC and used after 2 weeks of *in vitro* culture. The optimal epitope length was determined using freshly isolated, unexpanded PBMC from individuals expressing the alternative alleles. Cells were stimulated in ELISpot plates with single-residue truncated and extended peptides added in serial 10-fold dilutions ranging from 100 μg/ml to 10 pg/ml [55]. Half-maximal stimulatory antigen doses (SD50%) were determined as the peptide concentration needed to achieve half-maximal response rates. The shortest peptide with the lowest SD50% was considered the optimal epitope [36].

Biometric prediction of alternative alleles

Two complementary statistical approaches were developed to predict potential alternative restricting alleles. In the first approach, used at Los Alamos National Laboratory (LANL), prediction of alternative alleles was based on one-sided Fisher's exact test for each HLA allele present in the individuals under study. First, for each epitope, responders expressing the originally described restricting allele were excluded from the analysis. For the remaining individuals, contingency tables tallied whether the individual was an epitope responder or non-responder and whether the individual carried or did or did not carry the allele in question [56]. Statistically significant allele-overrepresentation among epitope responders was considered predictive of epitope presentation on the respective allele. No predicted allele was assigned for epitopes when all contingency tables resulted in $p > 0.1$. For the remaining epitopes, the first tier analysis was repeated after excluding all the responders who expressed either the original allele or the allele that had the lowest p-value in the first tier analysis. A third tier analysis was performed analogously. Associations were then tested for the presence of embedded peptide sequences fulfilling the binding motif of the alternative allele(s) within the epitope sequence. Since individuals with the original allele were excluded from the analysis, this prevented potential associations to emerge that were due to linkage disequilibrium between the original allele and the emerging alternative allele rather than true promiscuous presentation.

The second approach was established at Microsoft Research (MSR) as described in detail elsewhere [57]. Briefly, a generative model of the data was used wherein each patient allele, without excluding individuals expressing the originally described restricting allele, was considered an independent cause of a possible response in that patient. If a responding patient's alleles included an allele already known to present the epitope, the remaining alleles of the patient were given less credit for the observed response. This identified the most overrepresented alleles among epitope responders compared to the entire cohort and no a priori limit was set on the number of overrepresented alleles per epitope (up to five were found). By including individuals expressing the original allele in the analysis, this approach also identified potential mis-assigned original HLA restrictions in cases where, despite testing large numbers of individuals expressing the original allele, the original restriction did not emerge as a likely presenting allele. Both p-values and q-values were computed for this approach [58].

The compilation of the most probable alternative alleles was based on the following criteria: $q < 0.2$ for the MSR approach, which resulted in 54 associations. These were compared to the 54 associations with the lowest p-values from the LANL approach, and the intersecting group (Table 3) was considered to contain the most probable associations.

A comprehensive search algorithm developed by MSR was used to find the minimum number of HLA alleles including known restrictions that were needed to cover all reactions for each peptide. All combinations of up to five alleles were searched. If full coverage was not achieved with five alleles, a (greedy) forward selection algorithm was used to complete the search. At each step in forward selection, the allele with the greatest increase in coverage

was added until complete coverage of the responder HLA types was achieved. Of note, the search algorithm used for this analysis determines the minimal number of alleles necessary to explain the observed reactivity, not the most probable alleles, and is thus a conservative estimate of the true HLA class I promiscuity of these epitopes.

Natural processing of promiscuous epitopes in nef-transfected dendritic cells

Dendritic cells were transfected with mRNA encoding full-length HIV Nef sequence, or were mock transfected, and co-cultured with autologous PBMC from chronic HIV+ subjects as described [59]. After 12 days co-culture, the specificity of the expanded T cells was determined by ICS and ELISpot assay while proliferation was assessed by CFSE staining as described [59].

Additional statistical analyses

All averages are presented as mean values unless otherwise stated. Graphpad Prism 4 was used for Wilcoxon matched pairs and Mann-Whitney tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been funded in whole or in part with Federal funds from the NIH under contract N01-Al-15422 (C.B.), R01-A1-067077 (C.B.) and R21-AI-055421-01-A1 (K.Y.).

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Figure 1. Distribution of recognized epitopes based on recognition in the presence or absence of matched HLA alleles

Percent of responses targeting a) HIV and b) EBV epitopes per patient recognized in the presence of the original allele (left column, "(HLA)-matched"), in the absence of the original but presence of an allele in the same HLA supertype as the original allele (middle column, "ST matched"), and in the absence of both the original and a supertype-matched allele (right column, "unmatched"). For each c) HIV and d) EBV epitope, the median magnitude of responses in the presence of the original allele (left column), in the presence of a supertype matched (middle column), or in the absence of both the original and a supertype-matched allele (right column) is shown.

Figure 2. Presentation of YFPDWQNYT (YT9) on multiple HLA class I alleles

HLA restriction assays by intracellular cytokine staining (ICS) were performed as described [55]. Partly HLA matched antigen presenting cells were pulsed with optimal epitopes (black bars) or unpulsed (grey bars) and incubated with peptide-stimulated T cell lines. Percentages of IFN-γ-producing CD8 T cells are indicated on the X-axis; HLA alleles matching the donors' HLA type are indicated for each cell line on the Y-axis. Right hand panels show peptide titration analyses performed as described in Figure 3 to confirm the length of the optimal CTL epitope presented.

Figure 3. Optimal epitope definition in individuals not expressing the original HLA allele

Individuals not expressing the known restricting HLA allele were tested for recognition of single amino acid truncated and extended peptides encompassing previously defined optimal epitopes. Peptides were added to wells of ELISpot plates in serial 10-fold dilutions ranging from 100μg/ml to 10pg/ml and incubated with 100,000 of directly *ex vivo* isolated PBMCs. The magnitude of responses is indicated as spot forming cells (SFC) per 10^6 input PBMC. The optimal epitope was defined as the shortest peptide requiring the lowest concentration to achieve half-maximal magnitude of response. Data for 12 optimal epitope definitions are show representing well-documented promiscuous epitopes and their original restriction. The first 12 epitopes were shown to be shared between different alleles, while the last two epitopes represent examples of epitopes that were predicted to be embedded based on their binding motif and indeed identified a shorter peptide version as the most potent sequence.

Figure 4. T cells recognize naturally processed promiscuous epitopes on Nef-transfected dendritic cells

Dendritic cells were transfected with mRNA encoding full-length HIV Nef sequence, or were mock transfected, and co-cultured with autologous PBMC from HIV-infected subjects. After 12 days of co-culture, the specificity of the expanded T cells from subject L8 29 were tested by ELISpot for responses targeting the promiscuous epitope RYPLTFGW. Responses were assessed using either overlapping peptides containing the optimal epitope (NF18: NYTPGPGIRYPLTFGWCF and RV15: RYPLTFGWCFKLVPV) or using the described optimal epitope itself (RW8: RYPLTFGW).

Table 1

Frequent recognition of viral CTL epitopes in the absence of the originally described restricting HLA class I allele.

	HIV	EBV
Total number of epitopes tested	162	80
Epitopes targeted exclusively in the presence of the original allele	4	5
Epitopes never targeted in the presence of the original allele	27	14
Epitopes targeted in the absence of the original allele	153	75
Epitopes targeted by at least three different alleles	125	57
Median number of HLA alleles potentially restricting each epitope[a)]	3 (1 – 7)	3 (1 – 4)

a) based on the minimal number of alleles needed to provide coverage for the observed responses, including the original allele as a necessary assignment; numbers in parentheses indicate range

Table 2

Most promiscuously presented HIV epitopes

Epitope	Origin	Known restriction(s)	Additional alleles[a)]
AVDLSHFLK	Nef	A03, A11	A02, A23, A30, B44
QAISPRTLNAW	p24	A25	A23, A68, B44, Cw04, Cw07
QVPLRPMTYK	Nef	A03, A11	A02, A33, B44, Cw07
RAIEAQQHL	gp41	Cw03, Cw15	A02, A30, B58, Cw04
RLRDLLLIVTR	gp41	A03, A31	A02, A74, B44, Cw04
RYPLTFGW	Nef	A24	A01, A02, A23, Cw04, Cw07
TPQDLNTML	p24	B07, B42, B53, B81	A02, A24, B14
YFPDWQNYT	Nef	B37, B57	A01, A02, A23, Cw02, Cw06
YPLTFGWCY	Nef	B18	A02, A11, A23, A24, Cw04

*a)*Additional alleles required for HLA coverage of epitope responders

Table 3

Associations between viral CTL epitopes and alternative HLA class I alleles

pathogen	peptide sequence	original HLA	# subjects[a]	average magnitude [b]	predicted alternative HLA	# subjects[c]	average magnitude [d]	p-value LANL	p-value MSR	q-value MSR
HIV	AEQASQDVKNW	B44	16/31	458	B45	5/7	295	2.6E-04	1.6E-04	0.03
HIV	FPRIWLHGL	B07 B81	4/11 2/4	435 85	B42	4/6	430	1.6E-04	1.8E-04	0.03
HIV	GHQAAMQML	B39	0/2	0	A03	4/13	237	2.0E-04	1.6E-03	0.20
HIV	GHQAAMQML	B39	0/2	0	B38	4/4	525	1.2E-04	1.7E-05	0.00
HIV	GPGHKARVL	B07	2/11	327	B40	6/7	245	**1.7E-07**	**5.1E-08**	0.00
HIV	HPVHAGPIA	B07	3/11	451	B55	2/2	777	7.9E-04	2.8E-04	0.05
HIV	IEELRQHLL	B40	2/7	575	B37	3/3	346	1.3E-04	1.9E-05	0.00
HIV	IVLPEKDSW	B57	1/8	2550	B58	4/14	315	2.6E-04	5.4E-05	0.02
HIV	KAAVDLSHFL	Cw08	5/7	412	Cw03	7/13	674	3.4E-04	1.1E-04	0.03
HIV	KEKGGLEGL	B40	4/7	322	B49	6/9	693	**5.8E-07**	**1.5E-07**	0.00
HIV	KQNPDIVIY	A30	5/14	126	A6801	3/4	303	9.2E-04	6.0E-04	0.09
HIV	KYKLKHIVW	A24	4/12	454	A23	5/21	300	1.5E-03	1.1E-03	0.15
HIV	PIQKETWETW	A32	3/7	359	A24	7/12	516	2.0E-03	4.8E-05	0.01
HIV	QASQEVKNW	B53 B57 Cw04	12/15 2/8 16/34	1148 910 1188	B58	7/14	1468	1.8E-04	707E-05	0.03
HIV	QVPLRPMTYK	A03 A11	8/13 5/8	670 564	A34	4/4	597	1.4E-04	2.8E-05	0.01
HIV	RYLKDQQLL	A24	6/12	407	A23	10/21	735	**1.6E-06**	**1.1E-06**	0.00

pathogen	peptide sequence	original HLA	# subjects[a)]	average magnitude [b)]	predicted alternative HLA	# subjects[c)]	average magnitude [d)]	p-value LANL	p-value MSR	q-value MSR
HIV	SEGATPQDL	B40	2/7	250	B44	11/31	569	**2.4E-05**	**8.1E-06**	0.00
HIV	SPRTLNAWV	B07	2/11	305	B42	3/6	233	1.2E-03	6.8-04	0.10
HIV	TAFTIPSI	B51	1/10	760	B40	5/7	294	3.0E-04	1.3E-04	0.03
HIV	TAFTIPSI	B51	1/10	760	B35	8/16	525	9.4E-04	1.4E-05	0.00
HIV	TPQVPLRPM	B07	8/11	183	B42	5/6	698	**2.4E-06**	**5.4E-07**	0.00
HIV	WASRELERF	B35	5/16	136	B53	5/15	206	5.1E-05	1.8E-04	0.03
HIV	YPLTFGWCY	B18	1/2	510	B53	12/15	700	**1.7E-06**	**3.8E-06**	0.00
HIV	YPLTFGWCY	B18	1/2	510	B35	9/16	855	3.9E-04	4.8E-05	0.01
EBV	FRKAQIQGL	B27	1/7	80	Cw06	10/22	448	**2.9E-06**	**8.3E-06**	0.03
EBV	GQGGSPTAM	B1501	0/3	0	B40	5/11	194	7.9E-04	2.4E-04	0.09
EBV	LDFVRFMGV	B37	1/2	260	B35	9/16	499	9.6E-03	2.0E-04	0.08
EBV	LEKARGSTY	B1501	0/3	0	A23	3/12	443	1.6E-03	3.9E-04	0.13
EBV	LEKARGSTY	B1501	0/3	0	B40	5/11	268	1.7E-03	6.4E-04	0.15
EBV	LPCVLWPVL	B07	0/15	0	B45	3/5	1083	2.6E-04	7.2E-05	0.03
EBV	RPPIFIRRL	B07	9/15	405	Cw02	4/5	142	2.0E-03	6.0E-04	0.15
EBV	RVRAYTYSK	A03	2/11	500	A30	5/10	897	7.9E-05	4.8E-05	0.03
EBV	WTLVVLLI	B1516	0/3	0	A24	5/15	280	1.3E-03	1.1E-03	0.19

[a)] # subjects expressing the original allele responding out of total number of subjects expressing the allele

[b)] magnitude in SFC/million PBMCs

[c)] # subjects expressing the alternative allele responding out of total number of subjects expressing the allele

d) magnitude on alternative allele was calculated excluding subjects also expressing the original allele

Table 4

Potentially embedded CTL epitopes

pathogen	peptide sequence[a)]	original HLA	# subjects[b)]	average magnitude [c)]	predicted alternative HLA	# subjects[d)]	average magnitude [e)]	p-value LANL	p-value MSR	q-value MSR
HIV	AEQASQDVKNW	B44	16/31	458	B53	11/15	427	7.1E-05	2.2E-05	0.00
HIV	ERYLKDQQL	B14	3/4	193	A24	7/12	241	6.2E-05	6.5E-06	0.00
HIV	ERYLKDQQL	B14	3/4	193	A23	10/21	489	2.1E-04	3.0E-04	0.05
HIV	HTQGYFPDWQ	B57	1/8	300	A29	4/9	395	1.9E-03	7.9E-04	0.10
HIV	IEIKDTKEAL	B40	1/7	390	B08	4/8	595	8.9E-06	2.7E-06	0.00
HIV	IYQEPFKNLK	A11	1/8	420	A24	9/12	582	1.5E-05	2.3E-06	0.00
HIV	KRWIILGLNK	B27	3/5	2063	B35	5/16	306	1.8E-03	1.0E-04	0.03
HIV	KTKPPLPSVKK	A03	1/13	580	B42	3/6	293	4.8E-04	2.4E-04	0.04
HIV	QAISPRTLNAW	A25	1/1	50	B57	7/8	1133	7.3E-05	3.8E-05	0.01
HIV	RRWIQLGLQK	B27	0/5	0	B35	8/16	236	3.8E-05	1.7E-06	0.00
HIV	TPQDLNTML	B07 B42 B81 B53	4/11 5/6 1/4 2/15	1550 1756 1000 695	B14	3/4	1690	3.3E-04	1.4E-04	0.03
HIV	TQGYFPDWQNY	B1501	1/3	1090	A23	9/21	176	1.3E-04	1.0E-04	0.03
HIV	TQGYFPDWQNY	B1501	1/3	1090	A29	7/9	882	1.3E-04	1.4E-05	0.00
HIV	VPVWKEATTTL	B35	4/16	165	A23	8/21	216	1.5E-04	1.6E-05	0.00
EBV	YPLHEQYGM	B35	9/16	972	A80	2/2	630	7.3E-04	1.0E-04	0.04

[a)] potential embedded epitopes are underlined

[b)] # subjects expressing the original allele responding out of total number of subjects expressing the allele

c) magnitude in SFC/million PBMCs

d) # subjects expressing the alternative allele responding out of total number of subjects expressing the allele

e) magnitude on alternative allele was calculated excluding subjects also expressing the original allele

Table 5

Frequently shared allele pairs

pathogen	peptide	original HLA	alternative HLA	p-value LANL	p-value MSR	q-value MSR
EBV	LIPETVPYI	A02	A80	2.6E-02	3.8E-03	0.41
EBV	LLSAWILTA	A02	A80	4.1E-02	4.1E-03	0.42
EBV	LLWAARPRL	A02	A80	2.8E-02	9.6E-03	0.58
EBV	SLVIVTTFV	A02	A80	4.1E-02	1.7E-02	0.71
EBV	FMVFLQTHI	A02	B35	3.6E-02	7.1E-04	0.16
EBV	ILIYNGWYA	A02	B35	1.5E-02	8.0E-06	0.03
EBV	LLWAARPRL	A02	B35	6.8E-02	7.5E-05	0.03
EBV	VLQWASLAV	A02	B35	4.1E-02	1.5E-05	0.03
HIV	YTAFTIPSI	A02	B35	1.5E-02	9.0E-04	0.12
EBV	LLDFVRFMGV	A02	B45	4.5E-02	1.7E-02	0.71
EBV	LLSAWILTA	A02	B45	1.2E-02	6.3E-03	0.50
EBV	LTAGFLIFL	A02	B45	4.4E-02	1.5E-02	0.68
EBV	SVRDRLARL	A02	B45	4.3E-02	2.7E-03	0.38
HIV	RLRPGGKKKY	A03	B1516	8.5E-02	1.5E-02	0.63
HIV	ERILSTYLGR	A03	B1517	2.0E-02	3.7E-03	0.32
HIV	QIYPGIKVR	A03	B1517	4.0E-02	4.0E-03	0.34
HIV	TVYYGVPVWK	A03	B39	4.5E-02	8.2E-03	0.47
HIV	RLRDLLLIVTR	A03	B39	3.3E-02	4.5E-03	0.35
HIV	GHQAAMQML	B39	A03	2.0E-03	1.6E-03	0.20
HIV	TLYCVHQRI	A11	B1516	4.1E-02	1.2E-02	0.55
HIV	PLRPMTYK	A11	B1517	3.9E-02	1.7E-02	0.66
HIV	TLYCVHQRI	A11	B1517	4.0E-02	2.0E-02	0.72
HIV	QIYPGIKVR	A11	B1517	4.0E-02	4.0E-03	0.34
HIV	QIYAGIKVK	A11	B1517	4.1E-02	4.9E-03	0.36
HIV	KYKLKHIVW	A24	A23	1.5E-03	1.1E-03	0.15
HIV	RYLKDQQLL	A24	A23	1.6E-06	1.1E-06	0.00

pathogen	peptide	original HLA	alternative HLA	p-value LANL	p-value MSR	q-value MSR
HIV	RYPLTFGW	A24	A23	6.2E-03	2.0E-02	0.72
HIV	ETINEEAAEW	A25	B53	2.1E-03	1.3E-03	0.16
HIV	QAISPRTLNAW	A25	B53	9.2E-03	2.2E-02	0.73
HIV	EPVDPRLEPW	B53	A25	4.5E-02	1.9E-02	0.71
HIV	HPVHAGPIA	B07	B35	4.4E-03	3.1E-05	0.01
EBV	QPRAPIRPI	B07	B35	1.3E-02	1.2E-02	0.62
HIV	TVLDVGDAY	B35	B07	1.4E-02	2.3E-02	0.73
HIV	TPYDINQML	B07	B35	2.1E-02	5.0E-03	0.36
HIV	RPMTYKAAV	B07	B39	2.1E-02	4.4E-03	0.35
HIV	SPRTLNAWV	B07	B39	4.1E-02	2.0E-02	0.72
HIV	TPQVPLRPM	B07	B39	2.3E-02	4.5E-03	0.35
HIV	TPQDLNTML	B07	B39	4.6E-02	2.3E-02	0.73
HIV	FPRIWLHGL	B07	B42	1.6E-04	1.8E-04	0.03
HIV	GPGHKARVL	B07	B42	3.5E-03	7.8E-04	0.10
HIV	HPRVSSEVHI	B07	B42	4.1E-02	3.7E-02	0.88
HIV	IPRRIRQGL	B07	B42	4.3E-03	2.9E-03	0.29
HIV	RPMTYKAAL	B07	B42	4.3E-03	2.9E-03	0.29
HIV	RPMTYKAAV	B07	B42	9.2E-03	4.9E-03	0.36
HIV	RPNNNTRKSI	B07	B42	6.1E-02	1.8E-02	0.69
EBV	RPQKRPSCI	B07	B42	4.1E-02	1.2E-02	0.62
HIV	SPAIFQSSM	B07	B42	3.0E-02	1.4E-02	0.59
HIV	SPRTLNAWV	B07	B42	1.2E-03	6.8E-04	0.10
HIV	TPQDLNTML	B07	B42	-------	-------	-------
HIV	TPQVPLRPM	B07	B42	2.4E-06	5.4E-07	0.00
HIV	FPRIWLHGL	B07	B81	-------	-------	-------
HIV	SPAIFQSSM	B07	B81	2.4E-02	1.8E-02	0.69
HIV	SPRTLNAWV	B07	B81	4.2E-02	1.2E-02	0.55
HIV	TPQDLNTML	B07	B81	-------	-------	-------

pathogen	peptide	original HLA	alternative HLA	p-value LANL	p-value MSR	q-value MSR
HIV	EIYKRWII	B08	B50	4.2E-02	4.0E-03	0.34
HIV	FLKEKGGL	B08	B50	6.2E-02	4.9E-02	0.94
HIV	WPTVRERM	B08	B50	2.0E-02	4.0E-03	0.34
HIV	IVNRNRQGY	A30	B27	4.1E-03	2.6E-03	0.27
HIV	GRRGWEALKY	B27	A30	5.5E-02	1.4E-02	0.61
EBV	HRCQAIRK	B27	A30	3.0E-02	1.7E-02	0.71
EBV	LRGKWQRRYR	B27	A30	5.2E-02	4.2E-02	1.01
EBV	RKIYDLIEL	B27	A30	3.1E-03	1.6E-03	0.22
EBV	RRARSLSAERY	B27	A30	2.7E-02	1.5E-02	0.68
EBV	RRIYDLIEL	B27	A30	6.8E-03	5.0E-03	0.49
HIV	RRWIQLGLQK	B27	A30	5.0E-02	2.2E-02	0.73
EBV	FRKAQIQGL	B27	B50	4.6E-02	3.4E-03	0.41
EBV	HRCQAIRK	B27	B50	3.3E-02	8.3E-03	0.54
EBV	RRARSLSAERY	B27	B50	3.1E-02	7.5E-03	0.54
HIV	HTQGYFPDWQ	B57	B58	7.4E-03	4.3E-03	0.35
HIV	ISKKAKGWF	B57	B58	5.1E-02	5.3E-03	0.37
HIV	IVLPEKDSW	B57	B58	2.6E-04	5.4E-05	0.02
HIV	KAVRLIKFLY	B57	B58	-------	-------	-------
HIV	QASQEVKNW	B57	B58	1.8E-04	7.7E-05	0.03
HIV	TSTLQEQIGW	B57	B58	-------	-------	-------

------- indicate previously known shared peptides, therefore no p-values were assigned