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Sampling of MHC class I-associated peptidome suggests relatively looser global association of HLA-B*5101 with peptides¹

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Summary

We have analyzed peptides associated with six human MHC class I allomorphs expressed by the U937 cell line. Peptides were isolated by mild acid elution or by MHC class I immunoprecipitation using W6/32 monoclonal antibody. A total of 85 peptides were sequenced by mass spectrometry and their putative binding alleles were assigned using bioinformatic tools. Only three peptides isolated by the two approaches were identical, suggesting that the approaches may yield distinct, partially overlapping peptide populations. Mild acid treatment-derived peptides had overall characteristics suggestive of relatively lower affinity of binding for MHC class I. Interestingly, a large proportion of putative HLA-B*5101-binding peptides was found among the mild-acid treatment eluted peptides, and to a lesser degree in the affinity-purified peptide pool. These results suggest that HLA-B*5101 may bind a potentially large pool of peptides with relatively lower affinity. We suggest that lower affinity of peptide binding may be the basis for inefficient tolerance to HLA-B*5101-binding self-peptides, a predisposing factor for the development of Behçet disease.

Keywords

HLA; antigen processing/presentation; self-peptides; autoimmunity

Introduction

The MHC class I molecules are expressed on the surface of most nucleated cells and display short peptide fragments generated by proteolytic degradation of intracellular proteins. Peptide fragments are generated in the cytoplasm by the action of the proteasome and are then transported into the endoplasmic reticulum by the transporter associated with antigen processing, where they bind to the nascent MHC class I molecules [1]. At any given time, single cells display between 1000 and 10000 different peptides [2], collectively termed peptidome [3]. The MHC class I-associated peptidome is continuously scanned by CD8⁺ T cells in peripheral lymphoid tissues for the presence of foreign antigens that indicate presence of intracellular pathogens. Recognition of a foreign antigen triggers the destruction of the infected cells by antigen-specific effector CD8⁺ T cells. In the absence of infections all peptide displayed by MHC class I are derived from self-proteins. Qualitative and/or quantitative

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alterations of component(s) of the self-peptide pool by transformation can also induce cytotoxic CD8⁺ T cell responses that form the basis of antigen-specific anti-tumor responses [4]. MHC class I bound self-peptides may also serve as targets for tissue destruction in autoimmune diseases [5] and organ rejection following transplantation [6].

Besides these roles in eliciting antigen-specific effector CD8⁺ T cell responses (protective or destructive), peptide/MHC class I complexes have several important roles in the physiology of T cells. Engagement of the T-cell receptors with self peptide/MHC complex is the basis for TCR repertoire formation in the thymus and thymocyte maturation [7]. Further, homeostasis of naïve T cells and maintenance of functional competence of memory T cells in the periphery depends on the constant engagement with self-peptide/MHC complexes [8]. In addition, self-peptide/MHC complexes may modulate the strength of immune responses to foreign antigens [9]. In addition to T cells, self-peptidome can impact other systems as well. For example, the function of NK cells is inhibited by self-peptide/MHC class I complexes through the engagement of killer inhibitory receptors [10]. Finally, MHC class I-bound peptides influence mating preferences in mice [11].

Given these diverse roles of peptide/MHC class I complexes, it is important to determine the rules of formation of the peptidome, as well as the individual components that may exhibit important biologic functions. This task is complicated by the complexity of the peptidome, with individual peptides being presented in low copy numbers, ranging from 10-1000 per cell [12]. In this study we isolated and characterized 85 different endogenous MHC class I bound peptides from undifferentiated U937 cells, associated with any one of the six MHC Class I alleles expressed by this cell line. Peptides isolated by mild acid-elution from the cell surface displayed distinct characteristics, different from peptides isolated by immunoprecipitation of MHC class I. The former peptide set contained peptides with relatively looser association with MHC class I. The differential distribution of MHC class I alleles as putative binders of peptides in the two sets suggests fundamental differences between MHC class I alleles in self peptidome binding. This may explain the association of certain MHC class I alleles with autoimmune diseases.

Materials and Methods

Cell lines and reagents

The human monocytic leukemia cell U937 was cultured in stirrer flasks (Wheaton Science Products, Millville, NJ, USA) on Cellgro™ stirrers (Barnstead International, Dubuque, IA, USA) in complete RPMI-1640 medium containing 25 mM HEPES and supplemented with 10 % fetal calf serum, 2mM L-glutamine, 1mM sodium pyruvate, 4.5 g/l glucose and 10mM Penicillin/Streptomycin at an initial cell density of 1×10^6 cells/ml in a 5% carbon dioxide atmosphere. The murine hybridoma cells (HB-95) producing monoclonal antibody W6/32 (mouse anti human MHC Class I) reactive with HLA-A, B and C were obtained from ATCC (Manassas, Virginia, IL, USA). The hybridoma cells were grown in serum free SFM4MAB cell culture media (Hyclone Laboratories Inc. Logan, Utah, USA) supplemented with 10mM Penicillin/Streptomycin, 0.1 mM hypoxanthine, 0.016 mM thymidine, 1.0 mM sodium pyruvate at an initial cell concentration of 2×10^5 cells/ml.

Protease cocktail was obtained from Roche Molecular Biochemicals, Indianapolis, IN, USA. HPLC solvents, Tri Fluoro Acetic Acid (TFA) and acetonitril were obtained from Sigma, St. Louis, MO, USA. EZ-Link-sulfo-NHS biotin was from Pierce, Rockford, IL, USA. RPMI 1640, Penicillin/Streptomycin from Biosource, Camarillo, CA. Hypoxanthine and thymidine were from ATCC (Manassas, Virginia, USA). L-Glutamine and NuPAGE gel from Invitrogen, San Diego, CA, USA. N-octyl-beta-D-glucopyranoside CALBIOCHEM, La Jolla, CA, USA. Dynabeads Protein G from Dynal Biotech Inc. Lake Success, NY, USA. Nitrocellulose

membrane was from Bio-Rad, Hercules, CA, USA. ECL-Plus purchased from Amersham Biosciences, Piscataway, NJ, USA.

Mild acid elution of peptides

MHC class I bound peptides were acid extracted from the surface of U937 cell line as described [13]. Briefly, up to 4×10^9 cells were washed three times with PBS and incubated with 0.1M citrate-phosphate buffer pH 3.0 for one minute. The cells were then centrifuged for 10 min at 10,000xg and the supernatant passed through the 5kDa cutoff filter and lyophilized. Peptides were reconstituted in 0.1% TFA and subjected to reverse-phase HPLC purification.

Affinity purification of MHC class I and peptide elution

A one-liter culture of hybridoma cells HB-95/W6/32 were grown in serum free media until the cell concentration reached 2×10^9 . Cells were harvested and supernatant was precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$. Protein precipitate was resuspended and anti MHC class I antibodies (IgG) were purified using a Protein G column. The purified protein was analyzed using a 4–20% NuPAGE gel stained with Coomassie Blue.

A total of 4×10^9 U937 cells, grown in log phase up to a concentration of $1 \times 10^8/\text{L}$, were harvested and washed twice in PBS, pH 8.0. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 30 mM n-octyl-beta-D-glucopyranoside and protease inhibitor cocktail). The lysate was incubated on ice for 1h and centrifuged for 10 min at 10,000xg. After sample centrifugation at 100,000xg for 1 hour, supernatants were immunoprecipitated with the W6/32 monoclonal antibody and Protein G-coated magnetic beads. The optimal ratio between IgG to MHC molecules was previously determined by immunoprecipitation of biotinylated U937 cells, and plotting the immunoblot intensity of biotinylated $\beta 2\text{m}$ molecules versus concentrations of IgG (2–5 μg of IgG / 10^7 U937 cell lysate-data not shown). The magnetic beads were then washed 4 times with 300 μl of buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.3 mM n-octyl-beta-D-glucopyranoside. Subsequently, the beads were rinsed 3 times with 300 μl of low salt buffer (20 mM Tris-HCl, pH 8.0) to remove residual salts and detergents. The immunoprecipitates were treated with 10% acetic acid, boiled for 5 minutes, centrifuged through an Ultrafree-CL filter with 5kDa cutoff, and lyophilized by vacuum centrifugation.

HPLC and Mass Spectrometry Analysis

The lyophilized peptides were resuspended in (A) $\text{H}_2\text{O}/0.1\%$ TFA and analyzed by reverse-phase HPLC using a C18 column (Vydac, Hesperia, CA; 4.6 x 250 mm) and Beckman 126 System Gold, equipped with programmable solvent module and variable wavelength detector 165 (Beckman Coulter, Inc., Fullerton, CA, USA). The analog signal was converted to digital signal using a single channel A/D converter and data acquisition software using PeakSimple Chromatography software (SRI Instruments, Torrance, CA, USA). The gradient was made by combining solution (A) with solution (B) of acetonitril/0.1% TFA at a flow rate of 1 ml/min. The reconstituted samples in solution (A) were then applied to the column and 0.31 min fractions were collected from a linear 1h gradient running from 0–60% of solution (B). The eluted peptide peaks were detected at 214 nm. Microsequencing for all fractions was done by use of MALDI-TOF-TOF (Applied Biosystems, Foster city, CA). HPLC fractions eluted between 10 and 40 min were submitted each time for Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-TOF) for sequencing. Individual masses were assigned to peptides with strong MS/MS signals and ion scores equal or greater than 40. Peptide sequencing revealed the presence of multiple peptides in each fraction. Peptide identification was performed using the GPS system followed by BLAST.

HLA-Genotyping of U937 cells

HLA-genotyping was carried out using the PEL-FREEZ ABC SSP unitary kit (Dynal Biotech, LLC, Brown Deer, WI) according to the manufacturer's instructions.

HLA allele binding assignment

The binding scores of each peptide to one of the six alleles expressed by U937 cells was obtained by using a profile-based prediction algorithm [14], accessible at <http://mif.dfci.harvard.edu/Tools/rankpep.html>. Each peptide received a numerical score for binding to any of the six alleles. For some alleles it is possible to query peptides of different lengths (e.g. nine, ten and eleven amino acid long for HLA-A*03), while in some cases there is a general allele query and a more specific one as well (e.g. HLA-A*03 and HLA-A*0301). In these cases, all possible scores were examined and the best was retained. The numerical scores were expressed as the percent of the best possible score for binding of a peptide to a given allele, supplied by the provider. Shown in Tables 1 and 2 are the two best scores for each peptide. The allele with the best relative score is considered the source of presentation of particular peptide.

Peptide binding

Peptide binding was assessed as described [15]. Peptides synthesized using FITC-labeled εNH₂-lysine at position four were custom purchased from GenScript Corporation (Scotch plains, NJ). Peptides were HPLC purified and >95% pure as assessed by mass spectrometry. U937 cells (1×10⁶/well) were incubated for 10 min on ice with various concentrations of FITC-conjugated peptides in 150 μl of PBS in a 96 well polypropylene plates followed by three washes in PBS, resuspended in 300 μl PBS and immediately analyzed by Flow cytometry.

Results

General characteristics of mild acid-eluted peptides

MHC class I-binding peptides can be isolated by direct acid elution from cells or by immunoprecipitation of MHC class I followed by peptide elution. To analyze the spectrum of MHC class I-bound peptides of U937 cells we performed peptide elution by both methods. Isolated peptides were subsequently analyzed by matrix-assisted laser desorption mass spectrometry (MALDI-TOF-TOF). Typical sequencing results are shown in Fig. 1. The length distribution for the acid eluted peptides ranged from 7 to 28. A total of 64 peptides were isolated and sequenced (Table 1). 33 peptides (52%) were 8–12 aa long, 28 (44%) were larger peptides, while 3 (4%) peptides were 7aa in length. We found several nested sets of peptides (for examples see Table 1, peptides #5 and 6, 7 and 8, 12 and 13, 16 and 17, 20 and 21, 24 and 25, 26–28, 31 and 32, 35–37, 38 and 39, 41 and 42, 43 and 44, 45 and 46, 47 and 48, 49 and 50, 57 and 58, 60 and 61), which are more characteristic for MHC class II than for MHC class I molecules [16]. In contrast to MHC class II, the ends of MHC class I peptide binding groove are closed and only peptides of certain length can fit without reducing the biophysical properties of binding. Nevertheless, the nested set of peptides were recently described for MHC class I bound peptides [17], and likely result from partial proteolysis.

General characteristics of affinity-purified peptides

Because of the relatively high frequency of longer peptides, as well as few peptides shorter than optimal for binding to MHC class I, we suspected that by using mild acid elution we may have isolated a subset of peptides enriched for low-affinity binding to MHC class I. To test whether the size of eluted peptides was dependent on conditions of isolation, peptides were isolated by affinity purification using an antibody specific for all human class I allomorphs (W6/32) and then analyzed by mass spectrometry in an identical manner. As expected, the

frequency of peptides longer than 12aa was reduced to 3 out of a total of 21 (14%), while peptides shorter than 9aa were not detected at all (Table 2). Even though reduced in frequency, the longer peptides were still detected. One particular peptide (#13; Table 2) was isolated and identified in two separate experiments. Only three peptides identical in their sequences were common for both techniques. These were peptides derived from elongation factor 1- α 1, placental calcium-binding protein (calvasculin) and 60S ribosomal protein L15. The minimal overlap between the mild acid eluted and affinity purified MHC class I-eluted peptides suggests that characteristics of peptides isolated by the two approaches may differ.

MHC class I allele binding assignment

To predict MHC class I allele binding to peptides we first identified all alleles present in U937 cells by HLA genotyping. This analysis revealed presence of two alleles for each HLA-A (A*0301 and A*3101), HLA-B (B*1801 and B*5101) and HLA-C (Cw*0102 and Cw*0702) locus (data not shown). To assign particular peptides to given alleles we used a profile based prediction algorithm [14]. Binding of long peptides to MHC class I has been reported only sporadically [18–23], hence the rules for their binding to class I molecules are not well defined. Therefore the assignment of the MHC class I allele binding was done only for peptides of 12aa or less. For peptides 7aa and 8aa in length, the optimal binding allele was searched with N-terminal and C-terminal 3aa extensions (or 4aa N-terminal extension for peptides located at the C-terminus) obtained from the original proteins by blast search. Two top scores for each peptide are shown in Tables 1 and 2. The allele with the highest binding score is hereafter considered as the presenting MHC molecule. We believe that these HLA assignment results are accurate for two reasons. First, four out of five putative HLA-B*1801-binding peptides were confirmed in a recent study that identified peptides binding to soluble HLA-B*1801 [17]. Second, all affinity purified peptides and 72.4% of mild acid-eluted peptides with assigned HLA allele contained at least one or both anchor residues (double underlined in Tables 1 and 2) defining the motifs for binding to the particular allele [24,25]. These motifs are: L, V or M at position 2 and K, Y, or F at position 9 (HLA-A*03); R at position 9 and auxilliary anchors I, V, Y, or F at position 2 and/or F, L, Y, or W at position 3 (HLA-A*31); E at position 2 and F or Y at position 9 (HLA-B*18); A, P, or G at position 2 and V or I at position 9 (HLA-B*51); A, or L at position 2 and L at position 9 (HLA-Cw*01); and Y, F, or L at position 9 and auxilliary anchors Y or P at position 2, I, V, Y, L, M or F at position 5 and/or V, I, L, or M at position 6 (HLA-Cw*07).

Overall, peptides with scores indicative of binding to all MHC class I alleles were isolated by both mild acid elution and affinity purification. However, the alleles were not equally represented (Table 3). The most mild acid-eluted peptides appeared to bind to HLA-B*5101 (eight), followed by HLA-A*3101 (seven). On the other end of the spectrum, no peptides appeared to have been derived from HLA-B*1801. In the affinity purified peptide set, however, the distribution of putative binding alleles was more balanced between the HLA-A and HLA-B molecules, with the exception of HLA-A*0301. In addition, peptides binding to either of the HLA-C molecules were not abundant. The average binding scores for each individual allele were higher in the affinity-purification-derived peptides than in the mild acid elution-derived group (Table 3). In addition, the proportion of peptides with scores obtained for a fraction of the identified peptide (“mismatched peptides”) is much higher in the mild acid-eluted-group (Table 3). The implication of the “mismatch” is that the binding score for the entire peptide would be lower than the most optimal one. The lower average binding scores and higher proportion of the “mismatched peptides” suggest that peptides eluted by mild-acid treatment bind to the HLA alleles with generally lower affinity. In turn, the relative abundance of peptides binding to the particular allele derived by mild acid elution or affinity purification suggests the overall relative affinity of that allele for binding U937-derived peptides. For example, HLA-B*1801 appears to bind peptides with relatively high affinity since five HLA-B*1801-binders

were identified in the affinity purification-derived peptides, while none were assigned from the mild acid elution-derived group. Conversely, HLA-B*5101 appears to be a poor peptide binder since the proportion of peptides assigned to this allele is significantly reduced in the group of affinity purification-derived peptides.

Protein source of the peptides

The sequenced peptides were found to originate from a variety of proteins. Two questions seem to be the most relevant in this context. First, is abundance of peptide presentation by MHC class I in the function of the level of protein expression? The U937 cell line is very convenient model to address this question because protein expression 2D gel data has been made available on the web (www.expasy.org). Of the 22 proteins, which were shown to be highly expressed, only three proteins were the source of MHC class I-bound peptides: actin, glyceraldehyde-dehydrogenase and peptidyl-prolyl cis-trans isomerase A. Interestingly, all peptides from these proteins were identified by the mild acid elution method. No peptide sequence homology for any of the other highly expressed proteins of U937 was found.

Second, are MHC class I-binding peptides preferentially derived from ribonucleoproteins, as suggested recently by the study on HLA-B*1801 peptidome [17]. Seven of the 21 affinity purified peptides (33.3%) were derived from nucleoproteins (Fig. 2). These results suggest that ribonucleoproteins indeed preferentially serve as a source of MHC class I-binding peptides. Interestingly, among the mild acid-eluted peptide group, the frequency of ribonucleoproteins as a source is significantly lower (3 out of 37 <13aa long peptides- 8.1%) suggesting that lower affinity binding peptides are rarely produced from ribonucleoproteins. The converse is true for membrane associated proteins. Only one of the seven ribonucleoprotein-derived peptides binds to HLA-B*1801. The remaining peptides bind to HLA-A*3101 (4 of 7), HLA-Cw*0102 (1 of 7), while the binding HLA for one peptide 18aa long has not been assigned. Therefore, the distribution of allele binding of ribonucleoprotein-derived peptides is relatively unbiased (with most peptides binding neither to the “well binding allele HLA-B*1801, nor the poor binding HLA-B*5101).

HLA-B*1801- binding synthetic peptide with low binding score binds better than HLA-B*5101-binding peptide with high binding score

An implication of the above findings is that peptide binding to certain HLA class I alleles (i.e. HLA-B*1801) may be relatively strong, while relatively weak to the others (HLA-B*5101). Furthermore, relatively high binding scores can be obtained with peptides binding to both alleles, and yet the numbers of peptides binding to HLA-B*1801 or HLA-B*5101 is severely reduced in the peptide set obtained by mild acid elution or affinity purification, respectively. An assumption can therefore be made that binding to HLA-B*1801 of peptides with relatively poor binding scores may be of higher affinity than binding to HLA-B*5101 of peptides with relatively high binding scores. To test this hypothesis we identified from the literature a highly-scoring (score: 77.1) HLA-B*5101-binding peptide [26], and a poorly scoring HLA-B*1801-binding peptide from the present study (peptide number 3 from Table 2; score: 28.1). These two peptides were chosen because both have lysine at position 4, whose side chain can be labeled with FITC, and the binding of labeled peptides to U937 cells can be comparatively assessed. Indeed, U937 cells stained brighter in the presence of equimolar concentrations of HLA-B*1801-binding peptide (Fig. 3).

Discussion

We have analyzed characteristics of peptides eluted from six MHC class I molecules expressed by the U937 cell line. This cell line was chosen because it is frequently used model for epitope identification from infectious agents and because of existing considerable data on intracellular

protein composition. Peptides were simultaneously isolated either by mild acid elution from living cells, or by affinity purification using antibody with specificity for a common epitope of MHC class I. Very little overlap in obtained peptide sequences indicated that the two peptide subsets have broadly different characteristics. Several lines of evidence, discussed in more detail below, indicate that mild acid elution produces a peptide subset that binds to MHC class I with lower affinity. Assignment of binding MHC class I molecules to individual peptides revealed preferential representation: HLA-B*1801 was represented mostly in the peptides isolated by affinity purification, whereas HLA-B*5101, and to a lesser degree HLA-Cw*0102 and HLA-Cw*0702 were preferentially represented among the peptides obtained by mild acid elution. These findings suggest that the latter group of MHC class I molecules binds peptides with reduced affinity, relative to other MHC class I molecules.

There are several differences between the self-peptide sets obtained by mild acid elution or MHC class I immunoprecipitation. First, the former peptide set was more heterogeneous with respect to the peptide size and contained more peptides longer and shorter than optimal for binding to MHC class I. Although there is no direct proof that these peptides actually bind MHC class I, prior evidence for binding of unusually long peptides to MHC class I [18–23], together with heavy bias of the HLA-binding scores for alleles expressed by the U937 cells (data not shown), suggest that these peptides are associated with MHC class I. Second, the same peptide population contained more examples of nested sets of peptides. Both of these characteristics are suggestive of lower binding affinity of the former peptide set. Further support for this notion is provided by predictive scores of binding, that were generally lower in the former set. In addition, the scores in the former set were more frequently assigned to peptides that were one or more amino acid shorter than the actual sequence obtained by mass-spectrometry analysis, indicating that these scores for the actual (longer) peptides should in effect be even lower. Collectively, these data make a strong case in favor of less tight association with MHC class I of peptides which were obtained by mild acid elution.

Interestingly, significant portion of mild acid-eluted peptides represent C-terminal sequences of proteins. Fourteen of the total 64 sequenced peptides from eleven proteins were derived from the C-terminal portion, in contrast to none of the peptides in the affinity-purified group. Eight of the fourteen C-terminal peptides were either shorter or longer than optimal length. These data suggests that this form of processing for MHC class I presentation may not be best suited for generation of optimal MHC class I binding peptides. The C-terminal epitope generation, as originally described, involves the action of ER-resident amino terminal peptidase cleaving the C-terminus of the ER-resident proteins [27]. Subsequently, it was suggested that C-terminal peptides can be liberated from proteins with virtually any subcellular localization [28]. Indeed, the eleven donor proteins in our study were membrane, cytoplasmic, mitochondrial, ribosomal and vacuolar proteins. Furthermore, the higher abundance of C-terminal peptides in the mild acid-eluted group suggests that processing C-termini of proteins yields in general peptides with suboptimal capacity for binding to MHC class I. Most likely, this is the reason why C-terminal processing was originally discovered in transporter associated with antigen processing-deficient cells.

The finding that mild acid-eluted peptides have a lower binding affinity for MHC class I than the affinity-purified peptides, together with the differences in allele-binding assignments in the two groups of peptides provided an insight into the relative efficiency of distinct MHC class I alleles in binding peptides. The biggest differences were seen with HLA-B*1801 and HLA-B*5101, which were preferentially found as binding alleles in the affinity-purified- or mild acid-eluted groups of peptides, respectively. This observation indicates that HLA-B*1801 may bind peptides with higher affinity than other alleles expressed by the U937 cells, whereas HLA-B*5101 may be on the other end of the spectrum of binding affinity for U937 cell derived self-peptides. Affinity and stability of binding of peptides to MHC molecules can be determined

on an individual peptide basis, and binding to HLA-B*5101 was previously found to be lower than individual peptide binding to closely related HLA-B*3501 [29]. Our results extend these findings suggesting that peptide binding to HLA-B*5101 is globally of low affinity relative to not only to closely related but also different HLA molecules. Direct binding assays suggested that even the worst scoring HLA-B*1801-binding peptide has a higher affinity than the relatively well scoring HLA-B*5101-binding peptide (Fig. 3), albeit the binding of peptides to other HLA alleles in this assay has not been excluded. The structural basis for the low affinity peptide binding may be a small size of the A pocket in the HLA-B*5101 that accommodates only very small amino acids and forces a non-standard zig-zag peptide conformation with the peptide N- and C-termini protruding out of the groove more than in other HLA molecules [30].

HLA-B*5101 allele expression is associated with Behçet disease [31,32], a multisystemic inflammatory disorder possibly of autoimmune nature [33,34]. The linkage is very suggestive of involvement of peptide presentation function, since Behçet disease is not associated with HLA-B*5201, which differs by only two amino acids located in the pocket B of the peptide-binding groove [34]. However, although the general characteristics of HLA-B*5101 and other related allomorphs have been studied [26,35], the identity of peptide(s) responsible for development of the Behçet disease is not known. Responses to Herpes Simplex Virus, streptococcal and other bacterial antigens have been proposed as a triggering event for subsequent cross-reactive responses to self-antigens [33,34]. Peptides derived from thymidilate synthase, guanine-nucleotide binding protein b subunit-like protein 12.3, or yeast protein UBC5 have been proposed as possible autoantigens involved in the pathogenesis of Behçet Disease [33]. The sequence of the first of these three peptides has been identified in this study (Table 2, peptide #20). T cell activation and oligoclonal expansions of T cells are frequently found in Behçet Disease patients, but high individual variability of dominant V β subsets makes a single underlying antigen or auto-antigen involvement unlikely [36]. A unifying hypothesis linking potentially multiple (auto)antigens to the selective involvement of HLA-B*5101 in the pathogenesis of the disease is missing.

Our findings of generally decreased peptide binding to HLA-B*5101 may provide the missing link. Tolerance to self-peptide/MHC complexes is most efficiently established for self-peptides with high affinity binding for MHC [37]. Peptides that bind with low affinity are unable to establish tolerance, but are also unable to induce immune responses. However, peptides with intermediate affinity are poor at inducing tolerance, but are capable of inducing the immune responses. In fact, this group of peptides is considered for immunotherapy approaches against cancer. The same group, of course, carries the potential of initiating autoimmune responses. Thus, lower affinity binding of peptides by HLA-B*5101 may predispose development of distinct autoimmune responses, which could explain presumed multiple specificities in Behçet Disease patients. The question remains, how T cell responses to different antigen-specificities can produce the single clinical entity of Behçet disease? Presumably, different autoantigens may have different tissue distribution and would thus favor different individual pathology. This is to a degree indeed the case, as Behçet disease may present with relatively heterogeneous symptomatology [33,34]. In addition, animal models of autoimmunity have clearly demonstrated that an autoimmune response to ubiquitous autoantigen can lead to localized pathology [38], further complicating the relationship between the localization of causative autoantigen and the clinical outcome of the disease.

Assessing the binding of a population of different peptides to MHC class I is technically difficult because of the individual variability in peptide binding. MHC-binding prediction algorithms provide a tool for initial estimates of the binding abilities of distinct MHC molecules. The present study provides an example of how prediction algorithms together with different methodologies of isolating MHC class I-binding peptides, followed by mass

spectrometry sequencing, can provide this needed information on binding of peptidomes to distinct MHC class I molecules. Because MHC alleles are associated with a number of different types of diseases [39], this information may be important for understanding the pathogenesis of the MHC-associated diseases, as here postulated for the association of HLA-B*5101 with Behçet disease.

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Figure 1.

Peptide isolation and typical mass spectrometry analysis. A-B) Reverse phase HPLC separation of MHC Class I bound peptides, isolated from U937 cells. Peptides were obtained by mild acid elution (A) or from HLA molecules purified by immunoprecipitation (B). Peptides bound to MHC Class I were released by acid treatment and passed through 5kDa cutoff filter. The peptide mixtures were separated on a C18 column (4.6x250mm) using a 0–60% acetonitril in 0.1% TFA vol/vol) gradient run over 1h at a flow rate of 1ml/min. C) A mass spectrometer spectrum for the peptide sequence of 60S Ribosomal protein L26 fractionated at t~23min and sequenced by use of matrix-assisted laser desorption mass spectrometry (MALDI-TOF-TOF).

Figure 2. Subcellular distribution of protein donors of MHC class I-associated peptides. A) Peptides isolated by mild acid elution. B) Peptides isolated by affinity purification.

Figure 3.

Binding of fluoresceinated peptides to U937 cells. A) Representative histograms of U937 cells incubated in the absence or presence of HLA-B*1801- (sequence: DEFKIGELF) or HLA-B*5101- (LPEKDSWTV) binding peptides (1×10^{-3} M). B) Graph displaying mean fluorescence intensity of U937 cells incubated with indicated concentrations of HLA-B*1801- or HLA-B*5101-binding peptides.

Characteristics of peptides obtained by mild acid-elution. Bolded peptides were also found by affinity purification. Underlined are the sequences that scored best for HLA-binding. For peptides shorter than nine amino acids neighboring residues from either sides were added to search for the HLA-binding, and the ones that contributed to the binding score are indicated in parenthesis and separated from the actual sequence obtained by mass spectrometry. Double-underlined are residues conforming to previously identified binding motifs (see text for explanation).

Table 1

Peptide Number	Protein name	Sequence	Peptide length (aa)	Ion score	Calculated mass	Observed mass	HLA-binding assignment (score)
1	Actin (Gamma)	RVAP <u>EEHPVL</u>	10	58	1146.626465	1146.609253	Cw*0102(61.43); B*5101 (38.16)
2	ADP-ribosylation factor 1	DWLSNQLRNQK	11	73	1401.723267	1401.721924	A*0301(55.97); A*31 (43.59)
3	Calcyelin	(AL)....LYNEAL <u>KG</u>	8	63	907.4882813	907.4818726	A*0301(62.41); A*31 (53.85)
4	Calcyelin	PLDQAI <u>GLL</u>	9	63	939.5509033	939.541626	Cw*0102(11.43)
5	Calcyelin	DRNKDOEVNF	10	56	1264.591553	1264.580078	Cw*0702(37.84)
6	Calcyelin	LDRNKDOEVN	10	45	1230.6073	1230.605835	B*5101(30.26)
7	Calpactin I light chain	VVHMKQK <u>GKK</u>	10	49	1182.713867	1182.71936	A*0301(55.64); A*31 (46.15)
8	Calpactin I light chain	FVVHMKQK <u>GKK</u>	11	51	1329.782349	1329.772461	A*0301(58.96); A*31 (46.15)
9	Calgizzarin	IGGLAMACHDSF	12	60	1221.539063	1221.52417	B*5101(24.34)
10	Cofilin, non-muscle isoform	MLPDKDCRYA	10	48	1211.554688	1211.61853	B*51(54.42); Cw*0102 (24.29)
11	Deoxyuridine 5'-triphosph. nucleotidohydrolase	RIFYPEIIE	9	53	1195.599243	1195.592407	A*3101(63.01); A*0301 (41.35)
12	Elongation factor 1-alpha 1	SDYPLGRF	9	47	1051.52063	1051.515503	Cw*0701(20.27); B*18 (17.98)
13	Elongation factor 1-alpha 1	FSDYPLGRF	10	67	1198.589111	1198.573853	A*31(26.50); Cw*0701 (20.27)
14	Fructose-bisphosphate aldolase A	(SL)....FVSNHAY	7	48	837.388916	837.3813477	A*0301(47.37); A*3101 (38.15)
15	Fructose-bisphosphate aldolase A	TADDRVNP <u>CI</u> GG	12	40	1217.557861	1217.557983	Cw*0102(25.00); B*5101 (21.71)
16	Galectin-1	VKLDPDGYEFKFPNRLNL	17	48	2050.111816	2050.207031	NA
17	Galectin-1	TVKLDPDGYEFKFPNRLNL	18	40	2151.159668	2151.258057	NA
18	Glucose-6-phosphate isomerase	IKQOREARVQ	10	50	1255.7229	1255.738403	B*5101(31.58); A*03 (22.55)
19	Glyceraldehyde 3-phosphate dehydrogenase	(DL)....MAHMASKE*	8	45	904.4014893	904.3967285	A*03(62.75); A*31(53.85)
20	Glyceraldehyde 3-phosphate dehydrogenase	YDNEFGYSNRVVDL	14	78	1690.77063	1690.73877	NA
21	Glyceraldehyde 3-phosphate dehydrogenase	ISWYDNEFGYSNRVVDL	17	92	2076.966064	2076.969482	NA
22	Glyceraldehyde 3-phosphate dehydrogenase	QERDPSKIKWGDAGAEY	17	122	1949.935059	1949.892944	NA
23	10 kDa heat shock protein (Hsp10)	FRDGDILGKYVD	12	80	1397.705933	1397.699097	Cw*0702(32.43); B*5101 (21.71)
24	Macrophage capping protein	LGERPVQHREVOGNE <u>SD</u>	17	52	1949.942261	1950.013672	NA
25	Macrophage capping protein	LGERPVQHREVOGNE <u>SDLF</u>	19	99	2210.094727	2210.056885	NA
26	Macrophage migration inhibitory factor	(ERL)....RISPDRVY	8	41	1005.547546	1005.540588	A*31(54.70); B*51(48.30)
27	Macrophage migration inhibitory factor	RLRISPDRVY	10	39	1274.732666	1274.719238	A*0301(27.61); B*51 (22.22)

Peptide Number	Protein name	Sequence	Peptide length (aa)	Ion score	Calculated mass	Observed mass	HLA-binding assignment (score)
28	Macrophage migration inhibitory factor	LAERLRISPDK VY	13	51	1587.896484	1587.881836	NA
29	Macrophage migration inhibitory factor	IVNTNVPRASVPDGF LSEL	19	78	2028.075928	2028.056641	NA
30	Macrophage migration inhibitory factor	TQQLAQA TGKPPQYIAVHVVPDQL	24	50	2602.398682	2602.343506	NA
31	Mannose-6-phosphate receptor binding protein	(S)....VQQRQEQ	8	42	1043.522827	1043.552612	A*03(54.89); B*18(46.49)
32	Mannose-6-phosphate receptor binding protein	DVASVQQRQEQSY	14	59	1665.782593	1665.781494	NA
33	NSFL1 cofactor p47 (p97 cofactor)	FIVDARPA MAATSF	14	45	1496.756592	1496.731689	NA
34	Nuclear protein ZAP3 (ZAP13)	(WDR)....DYGRPLDE	8	50	964.4370117	964.5213623	A*0301(27.30); Cw*0102(21.79)
35	Peptidyl-prolyl cis-trans isomerase A	VDGEPLGRVSFEL	13	50	1417.732056	1417.721313	NA
36	Peptidyl-prolyl cis-trans isomerase A	DIAVDGEPLGRVSF	14	59	1474.75354	1474.729492	NA
37	Peptidyl-prolyl cis-trans isomerase A	IAVDGEPLGRVSFEL	15	53	1601.853271	1601.857422	NA
38	Peptidyl-prolyl cis-trans isomerase A	ILKHTGPGILSMANAGPNTNGSQF	24	110	2425.229248	2425.177979	NA
39	Peptidyl-prolyl cis-trans isomerase A	FLKHTGPGILSMANAGPNTNGSQF	25	44	2572.297607	2572.261719	NA
40	Phosphatidylethanolamine-binding protein	PVDLSKWSGPLSL	13	97	1398.762695	1398.767212	NA
41	Placental calcium-binding protein	FE GE PPDKQPRKK	12	73	1476.795654	1476.801514	A*31(43.59); B*51(38.78)
42	Placental calcium-binding protein	FFEGFPDKQPRKK	13	57	1623.864136	1623.835815	NA
43	Placental calcium-binding protein	MSNLDSNRDNEVD	13	49	1508.628174	1508.631958	NA
44	Placental calcium-binding protein	MSNLDSNRDNEVDF	14	66	1655.696533	1655.693848	NA
45	Profilin I	VNITPAEVGVLYGKDRSSF	19	94	1988.081055	1988.04541	NA
46	Profilin I	AAVPGKTFVNITPAEVGVLYGKDRSSF	27	130	2759.508789	2759.43042	NA
47	Profilin I	KEGVHGGLINKKCYEMASHLRSSQY	25	41	2904.471924	2904.578125	NA
48	Profilin I	MGKEGVHGGLINKKCYEMASHLRSSQY	27	60	3092.533691	3092.452148	NA
49	Proteasome act. complex subunit 2 (PA28beta)	YREL PQ KIYL	11	46	1453.856567	1453.854248	Cw*0102(51.79); Cw*0702(45.95)
50	Proteasome act. complex subunit 2 (PA28beta)	LYREL PQ KIYL	12	65	1566.940552	1566.933838	Cw*0102(51.79); Cw*0702(45.95)
51	Pyruvate kinase, M1 isozyme	(TF)....LEHMCRL	7	46	901.4382324	901.432373	Cw*0702(34.68)
52	40S ribosomal protein S28	LESEREARRLR	11	42	1414.787231	1414.779785	A*31(52.14); B*5101(35.53)
53	60S acidic ribosomal protein P2	MRYVASYLL	9	55	1115.591675	1115.591187	Cw*0702(32.43); B*5101(28.95)
54	60S ribosomal protein L15	ATYGKPVHH	9	50	1009.521362	1009.526062	A*31(29.91); A*03(29.41)
55	Small nuclear ribonucleoprotein Sm D1	FIL PS LSPLD TL	12	45	1343.745605	1343.746582	B*51(40.58); A*3101(32.56)
56	Synaptic vesicle membrane pr. VAT-1 homolog	LARTWVWQF	9	40	1221.616333	1221.69519	B*51(11.56)
57	Synaptic vesicle membrane pr. VAT-1 homolog	VSGVVARLLAL	11	40	1097.703979	1097.750122	B*51(45.41); Cw*0102(30.00)

Peptide Number	Protein name	Sequence	Peptide length (aa)	Ion score	Calculated mass	Observed mass	HLA-binding assignment (score)
58	Synaptic vesicle membrane pr. VAT-1 homolog	<u>L</u> VSGVVVARLL <u>AL</u>	12	40	1210.788086	1210.833374	B*51(45.41); Cw*0102 (30.00)
59	Transaldolase	<u>I</u> SPFVGRILDW	11	40	1302.720459	1302.778198	A*31(28.19); Cw*0702 (26.58)
60	Transgelin 2	WEGKNMACVQR	11	51	1321.613892	1321.685547	A*31(40.17); B*18(27.63)
61	Transgelin 2	WEGKNMACVQRTL	13	66	1535.745728	1535.807861	NA
62	Transketolase	FGIDRDAIAQAVRGITKA*	19	69	2015.139526	2015.222778	NA
63	Vacuolar ATP synthase subunit G 1	DIRPEIHENYRING	14	55	1725.866577	1725.852661	NA
64	Zinc finger protein 236	EEEEHS...(DR)	7	50	888.3217163	888.2773438	A*31(40.17); B*18(31.58)

Table 2

Peptides obtained by MHC class I immunoprecipitation. Bolded peptides were also found by affinity purification. Underlined are the sequences that scored best for HLA-binding. Double-underlined are residues conforming to previously identified binding motifs (see text for explanation).

Peptide Number	Protein name	Peptide sequence	Peptide length (aa)	Ione score	Calculated Mw (Da)	Observed Mw (Da)	HLA-binding assignment (score)
1	Acyl-CoA desaturase 1	LPLRLFLII	9	56	1097.74450	1097.76538	B*51(74.15)
2	Chromatin assembly factor I subunit C	<u>EE</u> RVINEEY	9	50	1180.54797	1180.51867	B*18(34.65); Cw*0702 (20.27)
3	DNA-dep. protein kinase catal. subunit	<u>DE</u> FKIGELF	9	50	1097.55126	1097.56286	B*18(28.07)
4	Elongation factor 1-alpha 1	<u>FSD</u>YPP<u>LG</u>RF	10	41	1198.58911	1198.59143	A*31(26.50); Cw*0702 (20.27)
5	Histone H1.2	SLAALKKALAAAGYDVEKNNRIKLGK	28	66	2942.71484	2942.63501	NA
6	Histone H1.3	KVAGAAATPK	9	48	842.50933	842.50152	A*03(75.49); A*31(69.23)
7	Human heat shock protein HSP 90 beta	IDIPNPQERTLT	14	54	1622.91113	1622.91638	NA
8	Microsomal signal peptidase	<u>VP</u> YIGIVT	9	61	974.59204	974.54852	B*5101(74.34); Cw*0102 (19.29)
9	Placental calcium-binding protein	<u>FE</u>GFPDKQPRKK	12	40	1476.79565	1476.81176	A*31(43.59); B*5101 (26.32)
10	Polymyositis/scleroderma autoantigen	<u>DE</u> YDFYRSE	9	38	1241.51086	1241.53002	B*18(35.53); Cw*0702 (24.77)
11	26S proteasome non-ATPase regulatory subunit 7	<u>RIG</u> KVGNQK	9	47	999.60571	999.60681	A*03(49.02); A*31(40.17)
12	40S ribosomal protein S26	<u>DAY</u> VLPLKLYVKL	12	44	1421.84021	1421.80920	A*31(53.85); A*0301 (50.38)
13	40S ribosomal protein S30	KVHGSLARAGKVRGOTPK	18	84	1890.11425	1890.14758	NA
14	60S ribosomal protein L15	<u>AT</u>YGKPVHH	9	51	1009.52136	1009.52252	A*31(29.91); A*03(29.41)
15	60S ribosomal protein L18a	<u>RIF</u> APNHVYAK	11	62	1251.73193	1251.73999	Cw*0102(52.14); A*3101 (44.65)
16	60S ribosomal protein L23a	<u>RL</u> RRQPKYPRK	11	37	1497.92358	1497.94116	A*31(65.81); A*0301 (27.07)
17	60S ribosomal protein L26	<u>DEV</u> QVVRGHY	10	72	1201.59594	1201.56811	B*18(43.86)
18	60S ribosomal protein L29	<u>RLA</u> YIAHPK	9	62	1068.63122	1068.61950	A*31(70.94); A*03(55.88)
19	U5 small nuclear ribonucleoprotein	<u>SE</u> LELRVF	9	49	1139.60949	1139.60278	B*18(26.32); B*51(17.69)
20	Thymidylate synthase	<u>DA</u> HIYLNHI	9	50	1095.55810	1095.56201	B*5101(32.89); B*18 (24.56)
21	Tubulin epsilon chain	<u>DEF</u> PEVYRF	9	54	1201.55236	1201.56945	Cw*0702(59.01); B*18 (38.60)

Table 3
Comparison of predictive binding of peptides isolated by mild acid elution or MHC class I precipitation to six HLA alleles.

HLA allele	Mild acid elution				Affinity purification			
	Total # of peptides	# of peptides with score/length * mismatch	# of peptides with no anchor residues	Average binding score	Total # of peptides	# of peptides with score/length * mismatch	# of peptides with no anchor residues	Average binding score
A03 *	4	3	1	49.55	2	0	0	62.26
A31 *	7	5	2	40.50	6	4	0	48.43
B18 *	0	0	0	-	5	1	0	33.69
B51 *	8	6	4	35.45	3	0	0	60.46
Cw01	5	4	1	40.28	1	1	0	52.14
Cw07	4	2	0	30.74	1	0	0	59.01
Total	28	20 (71.4%)	8 (28.6%)	37.24	18	6 (33.3%)	0	48.67

* Score/length mismatch is defined by HLA-binding scores assigned to peptides either shorter or longer than the actual sequence obtained by mass spectrometry (see tables I and II for examples).