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Discovery of Naturally Processed and HLA-Presented Class I Peptides from Vaccinia Virus Infection using Mass Spectrometry for Vaccine Development

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Abstract

An important approach for developing a safer smallpox vaccine is to identify naturally processed immunogenic vaccinia-derived peptides rather than live whole vaccinia virus. We used two-dimensional liquid chromatography coupled to mass spectrometry to identify 116 vaccinia peptides, encoded by 61 open reading frames, from a B-cell line (homozygous for **HLA class I** A*0201, B*1501, and C*03) after infection with vaccinia virus (Dryvax). Importantly, 68 of these peptides are conserved in variola, providing insight into the peptides that induce protection against smallpox. Twenty-one of these 68 conserved peptides were 11 amino acids long or longer, outside of the range of most predictive algorithms. Thus, direct identification of naturally processed and presented HLA peptides gives important information not provided by current computational methods for identifying potential vaccinia epitopes.

Keywords

vaccinia virus; HLA class I; antigen presentation; smallpox vaccine

1. Introduction

Despite the success of the smallpox (vaccinia) vaccine in eradicating smallpox world-wide, the targets of cell-mediated immunity (CD8+ and CD4+ T cell epitopes) are mostly unknown. From an adaptive immunity standpoint, the most important requirement for an immunogenic smallpox vaccine is the ability of the immune system to process and present specific peptides

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to T cells in the context of specific human leukocyte antigen (HLA) molecules. Antigen specificity of both CD8+ and CD4+ T cells is strongly controlled by HLA and other gene polymorphisms, and the immunogenicity (and ultimately the efficacy) of vaccines are dependent on particular HLA allelic polymorphisms [1] that restrict which peptides can be bound and hence presented [2,3]. Both class I and class II HLA polymorphisms control antigen and peptide epitope specificity of T cell responses and have been associated with differences in smallpox vaccine response outcome [4-6]. Therefore, the immune response to smallpox vaccine is determined in large part by multigenic influences controlling the presentation of specific sets of peptides [7]. The existence of HLA allelic "supertypes" (for example, the HLA-A2, A3, B7, and B62 supertypes), which are functionally defined by their ability to bind similar sets of, and in some cases, identical peptides [8-10], allow for the identification of "promiscuous" or shared **presented** peptides that possess enormous potential for future peptide-based smallpox vaccine development.

To date, the majority of known T-cell epitopes from vaccinia have been discovered using predictive computer algorithms, followed by assays that measure immunoproliferative response to synthesized peptides of the predicted sequences [11-13]. While algorithms have **some success** at predicting peptides that bind to HLA, there **are still gaps** in being able to predict which peptides will be produced by the proteasome and transported to HLA **class I** molecules in the endoplasmic reticulum via the transporter associated with antigen processing (TAP), and which exogenous peptides will be displayed on the HLA class II molecules [14].

Alternatively, mass spectrometry (MS) has been used for directly sequencing peptides presented by HLA molecules from infected cells, as pioneered by Hunt and co-workers [15], and has been used to identify naturally processed class I and class II epitopes from pathogens [16-20]. An imposing challenge of this approach has been the complexity of the peptide mixture isolated from HLA molecules and the often relatively low abundance of HLA ligands of pathogenic origin. Another targeted approach to identifying HLA ligands of pathogenic origin takes advantage of both predictive algorithms and direct identification by MS. The 'predict-calibrate-detect' strategy uses predictive algorithms to generate target sequences predicted to be epitopes. MS methods are then optimized for peptide sequences predicted by the HLA-binding algorithm [21-23].

Recently, proteomics-driven advances in MS sensitivity and speed of acquiring data-directed MS/MS data, along with automated data processing, database searching, and statistical evaluation of large sets of search results have provided an unprecedented ability to **rapidly** characterize the range of peptides presented by the immune system. This approach has the potential advantages of being able to detect post-translationally modified ligands, or ligands of atypical length or binding motif.

In previous research studying measles virus, we identified HLA class II (DRB1*0301) bound measles-derived peptides using orthogonal dimensions of liquid chromatography to separate the complex pool of peptides, followed by tandem mass spectrometry (MS/MS) to generate peptide sequence information [24,25]. This "reverse immunogenetics" approach represents a promising solution to the identification of vaccine candidates for many diseases [20,26,27], including vaccines against agents of bioterrorism, such as smallpox.

The goal of the present study was to identify the range of vaccinia virus-derived peptides presented by HLA-A*0201 (A2 supertype), HLA-B*1501 (B62 supertype), and HLA-C*03 alleles using orthogonal chromatography separations and MS/MS, with high mass accuracy measurement of precursor ions, similar to methods applied in previous work that identified class II peptides from measles virus [25]. In addition, we compared a set of vaccinia-derived peptides, identified after natural infection with vaccinia, with those predicted by computer

algorithms. We believe the identification of these naturally processed and presented peptides resulting from vaccinia virus infection complements predictive methods and will aid in understanding the immune process, perhaps leading to effective, integral components of a new generation of vaccines based upon cocktails of synthetic peptides.

2. Materials and Methods

2.1. Cell culturing and vaccinia virus infection

Epstein-Barr virus (EBV)-transformed B cells (European Collection of Cell Cultures – ECACC, number 86052111, Salisbury, Wiltshire, UK), homozygous for the HLA-A*0201, B*1501, and C*03 were of human origin and used as APC. The New York City Board of Health (NYCBOH, Dryvax®) vaccine-strain of vaccinia virus was cultured in HeLa cells in Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum (FCS; Life Technologies, Gaithersburg, Maryland). Cells were infected with live vaccinia virus at a multiplicity of infection (moi) of 0.1 PFU/cell for 2 hours and further maintained for 24-30 hrs in RPMI-1640 supplemented with 8% FCS. These uninfected and vaccinia-infected cells (approximately 1×10^9 cells each) were used for obtaining cell lysates for the class I HLA molecules purification.

2.2. Isolation of HLA-associated peptides from uninfected and vaccinia-infected cells

Class I HLA molecules were purified from homozygous B cells using an immunoaffinity approach, and their associated peptides were extracted as previously described [28,29]. In brief, cells were lysed with a buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1% CHAPS and protease inhibitors (1 mM Pefabloc SC, Roche Applied Science, Indianapolis, IN). The clarified supernatants were passed over a protein A-sepharose 4B (Sigma) column containing the monoclonal antibody (mAb) W6/32 specific for HLA-A, B and C [30,31]. The HLA molecules (1.2 mg/mL) were dissociated from their bound class I peptides using 0.2 N acetic acid, pH 2.7 and peptides were separated from the HLA by filtration through a 10-kDA molecular mass cutoff filter (Millipore, Bedford, MA).

2.3. Strong cation exchange fractionation

Strong cation exchange (SCX) fractionation of the sample was performed after desalting the peptide pool with a reversed phase 1mm by 8mm peptide trap (Michrom BioResources, Auburn, CA). SCX chromatography used a gradient of 5 mM KH_2PO_4, pH 3.0 to 0.4 M KCl of 5 mM KH_2PO_4, pH 3.0. Acetonitrile was added to each mobile phase to 20% by volume. Desalted peptides were loaded onto a polysulfoethyl aspartamide column (Michrom BioResources) at 0.5% mobile phase A and a gradient was developed to 20% B over 20 minutes at a flow rate of 200 μL/min, then from 20% B to 80% B over the next 10 minutes. Two minute fractions were collected; each fraction was vacuum centrifuged to dryness before analyzing the fractions by nLC-MS/MS.

2.4. LTQ-Orbitrap nLC-MS/MS analyses

Automated nLC-MS/MS analyses were performed on a commercial linear ion trap-Fourier transform hybrid mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, Waltham, MA r), interfaced to a nano-scale liquid chromatograph and autosampler (Eksigent NanoLC 1D, Dublin, CA), using a 15cm by 75 μm i.d. column packed with Magic C_{18AQ} (5 μm particles, 200 Å pore size, Michrom BioResources). The autosampler loaded 5-20 μL onto a 0.25μL pre-column (Optimize Technologies, Oregon City, OR), custom-packed with Magic C_8, 5μm, 300 Å (Michrom BioResources). Mobile phase A consisted of water/acetonitrile/formic acid (98/2/0.2 by volume) and mobile phase B was acetonitrile/water/formic acid (90/10/0.2 by volume). A 90 minute LC method employed a gradient from 2-40% B over 60 minutes,

followed by a second segment to 90% B at 85 minutes, with a column flow of 0.4μL/min. A third pump was used to load peptides from the autosampler to the pre-column, with 0.05% TFA and 0.15% formic acid in water at 15μL/min.

SCX fractions were analyzed multiple times by nLC-MS/MS using data-dependant acquisition of tandem mass spectra. The first experiment targeted singly charged precursors between 750 and 1500 on the *m/z* (molecular mass *m* divided by charge *z*) scale. A second experiment targeted doubly and triply charged precursors between *m/z* 375 and 750, consistent with MHC Class I peptides which are predominantly 9-11 amino acids long.

The LTQ-Orbitrap was operated in a data-dependant mode, first acquiring an Orbitrap survey scan with 60000 resolving power (FWHM at *m/z* 400), a target cell population of 1×10^6 ions, and a maximum ion fill time of 300 ms. The preview Fourier transform was used to select the five most abundant ions for MS/MS experiments in the LTQ. LTQ MS/MS spectra were acquired with a 2.5 mass unit isolation width, target ion population of 1×10^4 ions, one microscan, maximum ionization fill time of 100 ms, normalized collision energy of 35%, activation Q of 0.25, and activation time of 30 ms. Once ions were selected for MS/MS, they were subsequently excluded for 45 seconds. The exclusion window was 1 *m/z* below, and 1.6 *m/z* above the exclusion mass.

2.5. Peptide identification from MS/MS data

Database searching was performed using the SWIFT workflow tool developed in-house. SWIFT coordinates the generation of database search files (using extract_msn software from ThermoFisher Scientific), initiates database searches using MASCOT, Sequest, and X!Tandem search engines, and integrates these search results using Scaffold (Ver. 1_07_00, Proteome Software, Portland, OR). Database searches were done against a subset of the SwissProt database (January, 2007) obtained using the Bioworks 3.2 (Thermo Fisher) database utility to select human, bovine, and vaccinia proteins. Bovine proteins were included in the database since cell cultures were supplemented with fetal calf serum. Database searches were performed with a precursor mass tolerance of 7 parts-per-million (ppm), fragment ion mass tolerance of 0.6 mass units, and without any protease specificity. Single oxidation on methionine residues was considered as a variable modification. The database was appended with decoy protein entries consisting of randomized proteins sequences (MASCOT utility) for estimating the false positive rate resulting in a database of 43,400 entries (including the randomized decoy entries). Results from all analyses of all SCX fractions were combined by Scaffold and exported to an Excel spreadsheet.

The Scaffold program (ProteomeSoftware) was used to combine search results from all of the LC-MS/MS analyses and to calculate peptide identification probabilities using Scaffold's implementation of PeptideProphet [32,33]. An export function within Scaffold (Spectrum Report) was used to create a text file of all search results passing a lenient filter threshold of 80% protein probability, with at least 1 peptide identified above an 80% peptide probability threshold; 23,800 search results met those criteria, 1,521 of these were from decoy peptides. Additional filtering was then applied to this dataset while estimating the false-positive rate (FPR) of identifications from the incidence of identifications from the decoy database using the formula: 2×# matches to decoy peptides / (number of true positives + number of false positives) as described by Elias and Gygi [34]. The FPR was calculated as a function of thresholds for the following scoring parameters: Sequest cross correlation score (XCorr), the difference between the top two normalized cross-correlation scores (ΔC_n), Mascot Ion Score, and mass error of the precursor mass.

3. Results

3.1. HLA class I peptide identification by tandem mass spectrometry

Figure 1 provides an overview of the protocol used to sequence HLA class I peptides isolated from B-cells after infection with vaccinia. Sixteen strong cation exchange fractions were analyzed by nano-scale liquid chromatography coupled with tandem mass spectrometry (nLC-MS/MS) on the LTQ-Orbitrap. Two data sets were acquired, each using multiple injections as described in the methods sections. The initial data set was acquired with external mass calibration, followed by a second data set collected with internal mass calibration using lock masses at *m/z* 391.2843 and *m/z* 445.1200 as described by Olsen *et al*. [35]. These analyses generated 214,800 MS/MS spectra that were searched against the human, bovine, and vaccinia subset of proteins in the SwissProt database (January 2007 version).

Peptide sequencing by mass spectrometry involves matching MS/MS fragmentation spectra against theoretical fragmentation spectra calculated for any peptide sequence in the database within a tolerance window of the molecular weight of the peptide as measured by the mass spectrometer. As a result, every database search will return a result, and each of these matches to a sequence must be evaluated for their validity. A variety of scoring metrics exist from which a threshold is established for accepting or rejecting the search result from any MS/MS spectrum. The goal of that threshold is to minimize the number of incorrect or random matches that are accepted (false positives) while also trying to minimize the rejection of correct sequence matches (false negatives). Most of these scoring criteria have been developed within the context of identifying peptides generated from cleavage of proteins with trypsin. Trypsin cleaves on the C-terminal side of arginine and lysine and this cleavage specificity greatly reduces the list of candidate peptide sequences that are matched against the experimental spectra. The basic C-terminus of tryptic peptides provided by the Arg and Lys side chains, favorably directs fragmentation during MS/MS, influencing the scores from the database search results.

HLA class I peptides are not constrained to a basic C-terminal amino acid. For most common alleles, hydrophobic amino acids predominate in the C-terminal position, though basic residues such as Lys, Arg, Pro, or His, are often found somewhere in the C-terminal half of the peptide. Also by not requiring Lys or Arg as the C-terminal amino acid from the database, greatly increasing the number of candidate sequences whose theoretical fragmentation spectrum must be matched against the experimental fragmentation spectrum. Because of these differences we implemented the use of decoy database entries during the search as described by Elias and Gygi [34]. For each protein in the database an additional entry is created with its amino acid sequence randomized, and labeled as such, in its accession identifier. During the database search these decoy proteins compete against authentic proteins for the best match to experimental spectra. Search results that identify peptides from a decoy protein are known to be incorrect. The rate of matches to peptides from the decoy proteins is used to estimate the false positive rate as described below.

Search results are summarized in Table 1 at the estimated 1% and 5% FPR. 5,915 MS/MS spectra were identified at the 1% FPR (30 matches against decoy peptides), representing 2,731 unique sequences. 65 of these peptides were unique to vaccinia virus, originating from 44 vaccinia virus proteins. At the 5% FPR, the number of matched spectra increased from 5,915 to 12,819 (313 matches against decoy peptides), 5,601 of which were unique sequences. Of these 5,601 unique sequences, 116 peptides originated from 61 vaccinia proteins.

The naturally processed and presented vaccinia peptides we identified are listed in Table 2. The peptides are sorted by the open reading frame (ORF) they originated from and are marked whether they were identified within database search scoring criteria that characterized the 5% FPR (shaded background) or the 1% FPR (unshaded). Vaccinia peptide sequences identified

at the 5% or better FPR have been selected for additional studies to characterize their immunogenic properties.

Figure 2 shows the distribution of peptide lengths and putative allele from the vaccinia subset (Figure 2a) as well as all class I peptides identified at the 5% FPR rate (Figure 2b). As expected, the majority of the peptides are 9-mers in both the full set of peptides as well as the vaccinia subset. However, there are a significant number of peptides longer than 11 amino acids in the full set of identified peptides as well as in the vaccinia subset.

Since the W6/32 antibody used to isolate the HLA-peptide complexes has affinity for each of the major HLA class I alleles, the list of identified peptides reflects the allotypes of the host cell line; in this case HLA-A*0201, B*1501, and C*03. As a first approximation, we associated identified peptides with an allele by comparing the C-terminal amino acid to the reported binding motifs at the P9 position [36]. We assigned peptides terminating in L or V to A*0201, and peptides terminating in F or Y to B*1501, while designating the remaining peptides as “not assigned”. Figure 2c and 2d shows an estimate of the distribution of peptides among the A and B alleles, and while our method of associating a peptide with its putative allele is rudimentary, it clearly shows a significant subset of the peptides being presented by the B allele. There is also an unknown contribution of peptides from the HLA-C allele, although the density of HLA-C molecules on the surface of cells has been reported as being 6-fold less than that of the A and B alleles [37,38].

The vaccinia peptides identified also bear evidence of the genetic heterogeneity of the Dryvax strain [39]. For example, two forms of the same peptide from ORF A44L were identified: IQYPGSKIKGNAY from the Copenhagen strain, and IQYPGSEIKGNAY from the Western Reserve and Ankara strains of vaccinia. At first glance these two sequences were flagged as redundant identities of the same peptide, since they vary only in one amino acid residue resulting in a mass difference of one dalton. However, these sequences were assigned from two distinctly different peptides with IQYPGSEIKGNAY identified from SCX fractions 4 and 5, and IQYPGSKIKGNAY identified from SCX fractions 14-16. Figure 3a and 3b shows the annotated MS/MS spectra for the two peptides. Orbitrap spectra for each precursor mass (inset) illustrate the difference in mass for the two peptides (0.5 on the *m/z* axis, where *z*=2 as shown by the isotope spacing). Fragment ions are observed from the N-terminal end of the peptide (B-ions, highlighted in red) and from the C-terminal end of the peptide (Y-ions, highlighted in blue), while the y- and b-ions highlighted within the dashed box delineate the sequence difference between the two peptides. Table 2 contains other instances where peptides were identified that were unique to specific vaccinia strains.

Additionally, annotated MS/MS spectra of all vaccinia peptide sequences that were identified at the 5% FPR criteria, but did not meet the criteria for a 1% FPR, are included in the supplemental information. An Excel spreadsheet is also included as supplemental information that contains the entire search dataset used for calculating false positive identification rates.

3.2. Comparison of directly identified vaccinia class I peptides to predictive algorithms

The vaccinia protein sequences represented by this set of peptides were submitted to three algorithms for predicting potential epitopes according to their predicted alleles. Results from the NetMHC, [40,41] BIMAS, [42] and SYFPEITHI [43] algorithms are shown in Table 2. The NetMHC algorithm uses a neural network approach to determine likely epitopes from protein sequence, calculates a predicted binding affinity to HLA molecules, and classifies this binding as being strong (IC_{50} < 50 nM), weak (IC_{50} > 50 nM and < 500 nM), or less than weak (IC_{50} > 500 nM), (http://www.cbs.dtu.dk/services/NetMHC/, accessed Jan. 4, 2008). Figure 4 summarizes how the peptides we directly identified by MS are predicted by the NetMHC algorithm to bind with HLA molecules. Twenty seven (22%) of the vaccinia peptides identified

by MS were predicted by the algorithm to be strong binders with another 49 (41%) predicted to be weak binding. Twenty four peptides identified by MS/MS were not predicted by the algorithm, 23 because of excess length.

Additionally, we compared our list of identified vaccinia peptides to vaccinia epitopes, from all alleles, contained in the Immune Epitope Database and Analysis Resource (IEDB, http://www.immuneepitope.org/home.do, accessed Jan. 4, 2008) [44]. From our list of 116 directly identified peptides, 23 were an exact match to an IEDB database record, while another 7 peptides were contained within a vaccinia epitope from the IEDB database. These results demonstrate both the complementary information provided by direct identification of epitopes by MS, and the limitations inherent in relying solely on computer algorithms for understanding the spectrum of peptides presented by natural infection.

4. Discussion

While very effective, the Dryvax vaccine has the highest rate of serious adverse events of any Food and Drug Administration (FDA)-approved vaccine [45-47]. Because of the risks associated with administration of the current live viral smallpox vaccine, and the large percentage of the population for whom this live virus vaccine is contraindicated, public health interests would best be served by the development of an efficacious recombinant or peptide-based vaccine. One approach for developing a safer smallpox vaccine is to utilize naturally processed immunogenic vaccinia-derived peptides rather than live whole vaccinia virus.

Recombinant vaccinia viruses have been created from several strains of vaccinia virus. In the U.S. most recombinant viruses have been made from either the NYCBOH strain or a mouse neuroadapted derivative, the Western Reserve (WR) vaccinia strain. Several recombinants have been made from the Copenhagen and Lister vaccinia strains, which are more pathogenic in animals than the NYCBOH strain [48]. In general, genes located in the center of the virus genome are conserved between orthopoxviruses and are important for virus replication [49].

In this study we identified a set of naturally processed and HLA (A*0201, B*1501, C*03)-presented peptides, derived from the Dryvax virus strain (**NYCBOH**), which are encoded by 61 vaccinia virus genes. Since there are approximately 230 ORFs in vaccinia, multiple viral proteins can be potentially targeted by human cellular (CTL) immune responses. In fact, some of these gene products have been demonstrated to be immunogenic in both humans and a mouse model, including A6L, A8R, A10L, A23R, D12L, E9L, J6R, and M1L [4,6,12,14,50].

Seven of the peptides we have identified after infection with vaccinia virus have previously been reported to have immunogenic properties, including ILDDNLYKV (ORF G5R) which has been classified as an immunodominant epitope [14,51]. The other peptides are: KLFTHDIML (D12L), ILSDENYLL (A6L), KIDYYIPYV (E2L), FLTSVINRV (F12L), NLFDIPLLTV (F12L), and GLLDRLYDL (O1L). All seven of these peptides were predicted to be strong binding peptides by the NetMHC algorithm. We also identified the naturally processed peptide SQIFNIISY (ORF A17L) that is homologous to QIFNIISYI (A17L). QIFNIISYI was reported by Assersson *at al*., to be one of 15 immunodominant A*0201-restricted epitopes [14]. SQIFNIISY is predicted by NetMHC to have an IC_{50} value of 74nM for the B*1501 allele, making some form of this sequence a potentially useful epitope across multiple alleles. As shown in Table 2, 30 of the 61 vaccinia proteins were represented by a single class I peptide, while the remainder of the vaccinia proteins were represented by two to seven peptides. These data provide the first direct evidence of multiple naturally processed vaccinia peptides, resulting from natural viral infection, in the context of A2 and B62 HLA supertypes.

A goal of future work will be to study cellular (CTL) class I-restricted immune responses *in vitro* to identified peptides in unvaccinated and previously smallpox vaccinated subjects for evidence of peptide recognition by T cells. Ostrout *et al.* [4] recently described T cell memory IFN-γ responses to theoretical HLA-A2 epitopes in humans vaccinated against smallpox. This study revealed six peptides encoded by 14L, G1L, A8R, 18R, D12L, and H3L ORFs that were identical for vaccinia (Copenhagen), variola major (Bangledesh 1975) and modified vaccinia Ankara (MVA) strains [4]. In addition, Oseroff *et al*. [12] identified a total of 48 computational epitopes derived from 35 vaccinia proteins, some of which (B8R, D1R, D5R, C10L, C19L, C7L, F12, and O1L) were recognized in an *ex vivo* Elispot assay in multiple donors of different HLA types. Immunogenic peptide identification for poxviruses is just beginning, and it is likely that as more peptides are identified, a growing number of new vaccinia antigens that contain multiple epitopes will be identified [20]. In this regard, MS techniques, such as those described here, will further facilitate the search for unknown naturally processed peptides from poxvirus proteins. Sequence analysis of poxvirus genomes has improved our knowledge of the structure and function of poxviral genes and increased our understanding of host-virus interactions [49].

Several lines of evidence indicate that in humans a class I-mediated T cell response is sufficient to provide protection against a poxviral challenge [52-54], justifying initial research that focused on class I-related activation. However, recent studies indicate an important role for humoral immunity (neutralizing antibodies) as well as CD4+ T cells in protection against poxvirus infection [55,56]. Recently two HLA-DR1-restricted computer-predicted epitopes on vaccinia virus A24R and D1R enzyme proteins were described, that are conserved among poxviruses [5]. This study identified five new peptides from the A24R enzyme protein, which is involved in mRNA synthesis. Although the involvement of CD8+ T lymphocytes in the immune response to smallpox virus has been demonstrated, naturally processed vaccinia virus-derived peptides presented in the context of several HLA class I molecules, have not yet been identified. Our results show that approximately 60% of the peptides identified in this study are conserved from vaccinia to variola. If found to be immunodominant, these peptides may allow for monitoring of cellular immune responses induced by licensed smallpox immunization and **the** development of new poxvirus **multiepitope**-based vaccines. Future studies are warranted to investigate the value of these naturally processed and HLA-presented class I vaccinia-derived peptides for immunostimulatory responses, T cell recognition, and HLA restriction. Finally, our study demonstrates that computer-based algorithms are insufficiently robust to predict the full repertoire of naturally presented pathogen-derived peptides, in this case failing to predict 20% of the peptides identified by MS.

The technical challenges of comprehensively identifying the HLA ligandome [57] by mass spectrometric methods are the same challenges being faced in proteomics in terms of number of components, their range of relative abundance, and how the abundance of components change as a function of disease. Proteomics continues to drive significant improvements in sensitivity and throughput of MS-based methods that are immediately applicable to immunology. Recent reviews have speculated on what future role enhanced mass spectrometric capabilities will play in immunology [58,59]. We believe that as larger data sets become available, predictive algorithms will be used to rank the potential immunological significance of directly identified HLA ligands for further study, as well as provide additional insight into ligands that are exceptions to known binding motifs [19,58,59]. As stated by Kessler and Melief, “It is expected that the rapidly increasing power of mass spectrometric techniques will have a tremendous impact on the unraveling of the cancer-specific HLA-bound ‘ligandome’. The identification of HLA class I ligands by reverse immunology-based predictions may eventually be bypassed by direct identification of cell surface-presented peptides with mass spectrometry”[59].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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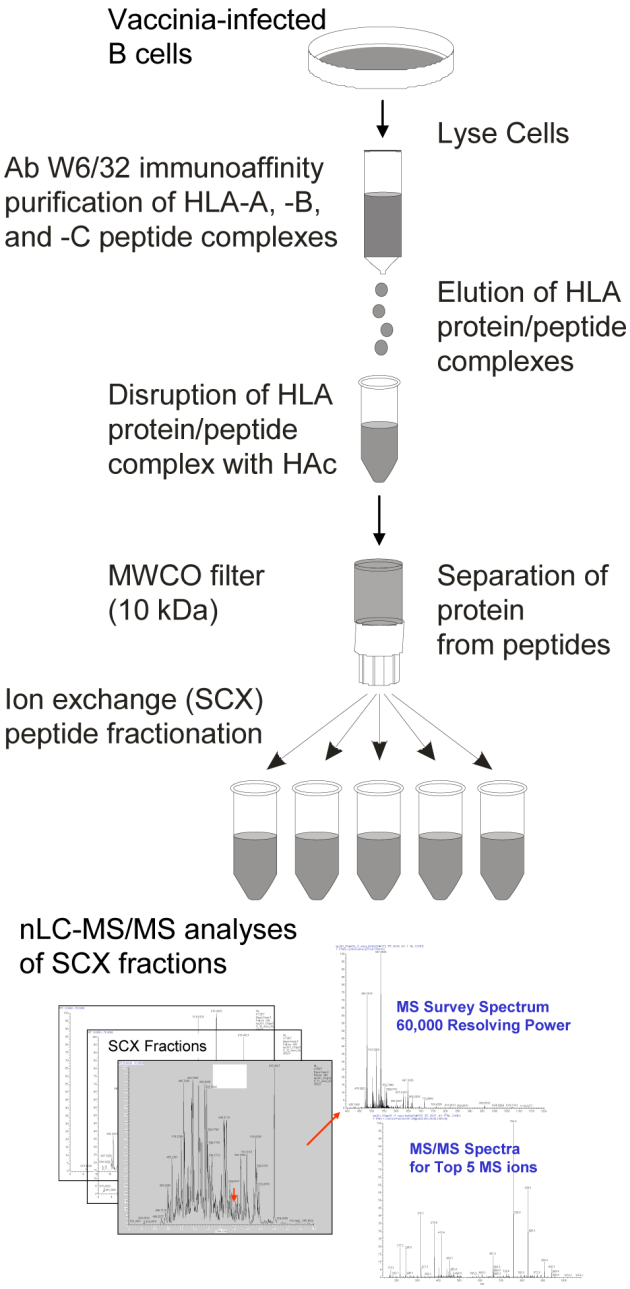

Figure 1.
Overview of protocol for isolating and identifying HLA class I peptides from B cells infected with vaccinia virus.

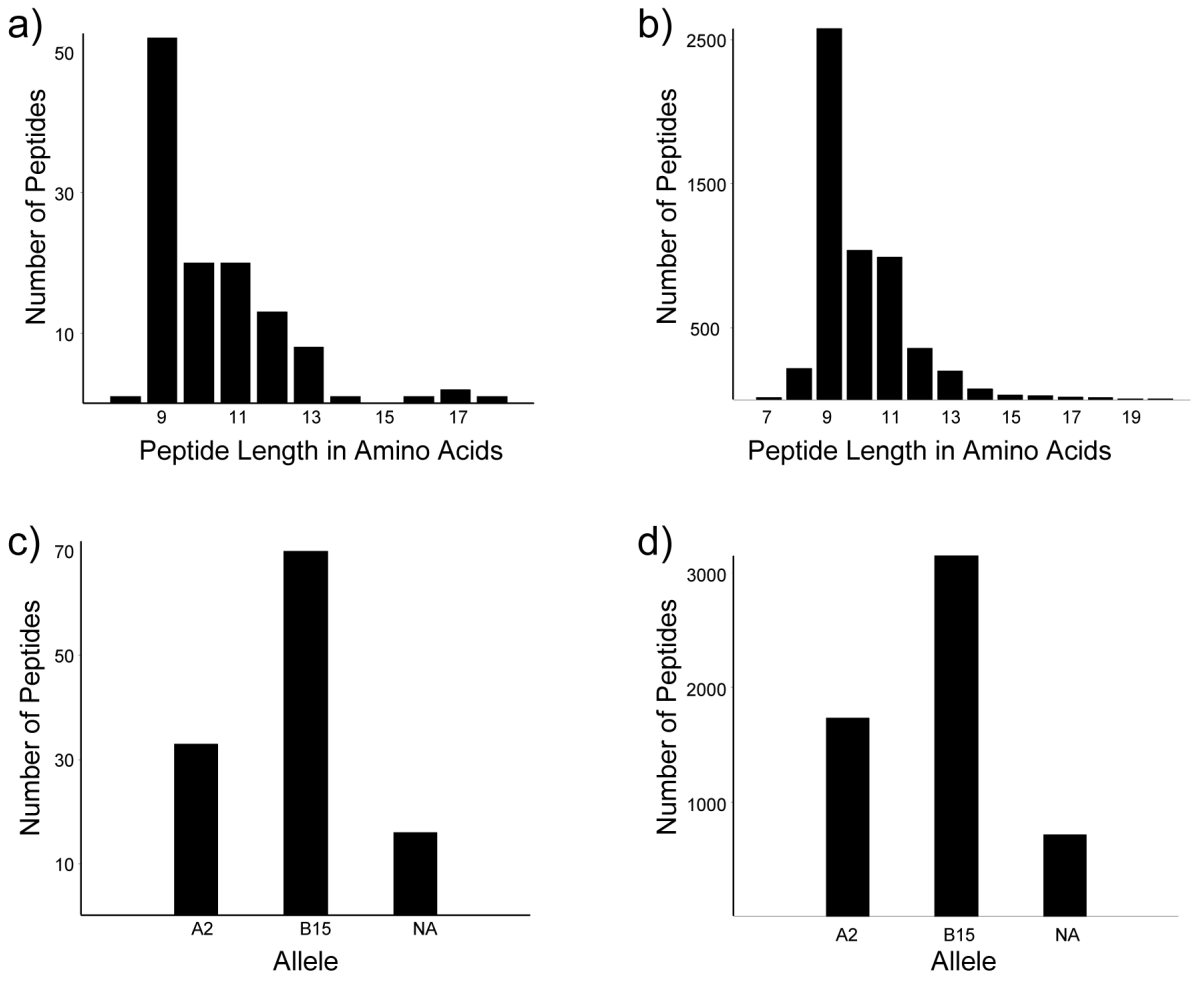

Figure 2.

Distribution of HLA peptide amino acid length for a) vaccinia peptides and b) all identified peptides; putative sorting of peptides by binding allele for c) vaccinia peptides, and d) all identified peptides. Peptides classified by allele using their C-terminal amino acid: L or V assigned to A*0201, F or Y assigned to B*1501, and all other peptides marked as NA (not assigned).

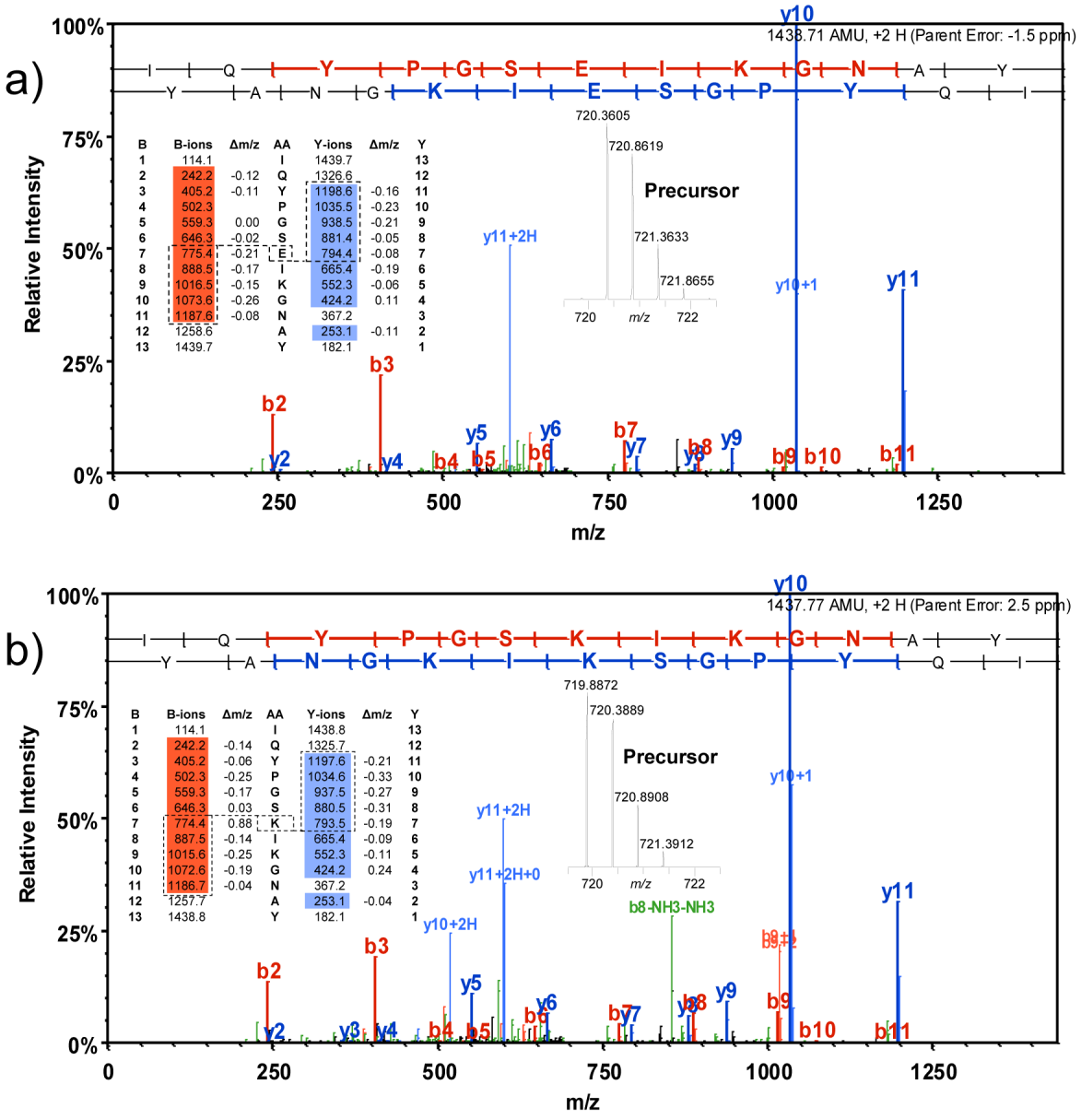

Figure 3.

Figure 3a shows the MS/MS spectrum of the vaccinia peptide IQYPGSEIKGNAY found in SCX fractions 4 and 5, as annotated by Scaffold, including mass accuracy of the precursor mass in parts-per-million (ppm). A table of the fragment ions matched and the experimental error of the fragment ions is included. The Orbitrap survey spectrum of the precursor ion is shown. Figure 3b shows the same information for the vaccinia peptide IQYPGSKIKGNAY as identified from SCX fractions 14-16. Note the different precursor mass, as well as concomitant changes to fragment ion masses consistent with the change in amino acid E (Glu) to K (Lys).

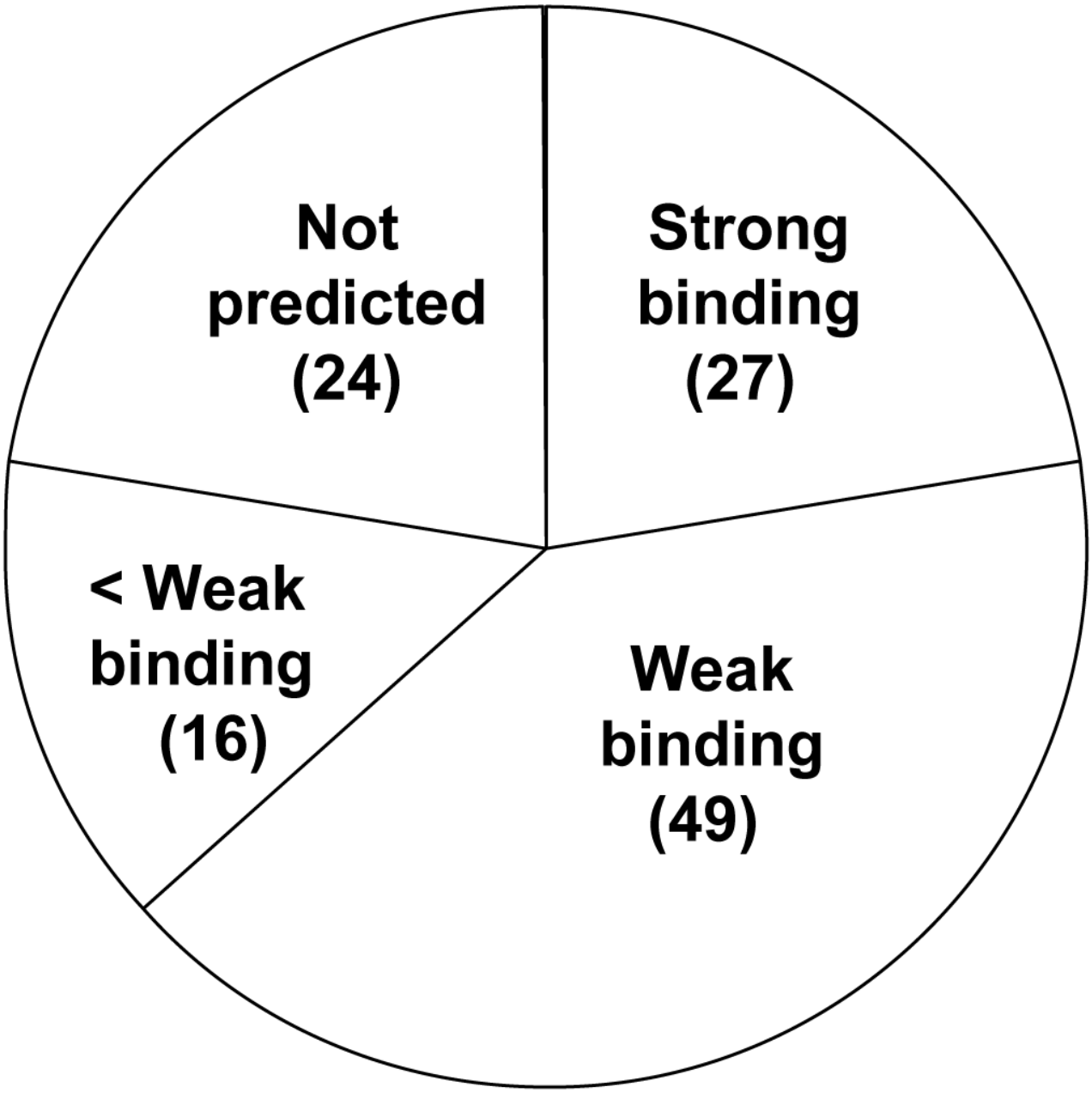

Figure 4.
Vaccinia epitopes directly identified by MS/MS are classified by predicted HLA-binding strength as determined by the netMHC algorithm at the Center for Biological Sequence Analysis, Technical University of Denmark, (http://www.cbs.dtu.dk/services/NetMHC/. Peptide sequences with calculated IC_{50} values < 50 nM were classified as strong binding, IC_{50} values between 50 nM and 500 nM were classified as weak binding, and IC_{50} > 500 nM were classified as non-binding peptides.

Table 1

Summary of peptides identified by two-dimensional liquid chromatography and tandem mass spectrometry.

	1% FPR[a]	5% FPR[a]
# MS/MS spectra identified[b]	5,915	12,819
# Unique sequences[c]	2,731	5,601
# Unique vaccinia sequences[d]	65	116
# Vaccinia proteins represented[e]	44	61

[a] Database search results summarized at the 1% false positive rate (FPR) and the 5% FPR.

[b] Number of tandem mass spectrometry (MS/MS) search results surpassing scoring thresholds that characterize the 1% and 5% FPR. Includes results for peptides identified in multiple strong cation exchange fractions, multiple MS/MS spectra from the same precursor m/z, at multiple charge states, and from multiple database entries containing the identified sequence.

[c] Number of unique peptide sequences identified, from all species represented in the database (human, bovine, vaccinia proteins).

[d] Number of peptide sequences identified that are unique to vaccinia proteins.

[e] Number of vaccinia proteins represented by the identified vaccinia peptides.

Table 2

Class I peptides from vaccinia virus identified by two-dimensional liquid chromatography and tandem mass spectrometry after vaccinia infection of human B cells.

Peptide Sequence[a]	ORF	Vaccinia Strain[b]	Other Pox Viruses[c]	Putative Allele[d]	BIMAS[e]	CBS IC50 (nM)[f]	SYFPEITHI Prediction[g]
[ox]MQKFTILEY[h]		A,C,V	Vr,Cm,Cw,Mn	B*1501	288.0	65	23
ILIRGIINV	A ORF T	C,V	None	A*0201	271.9	26	30
AQITTDDLVKSY	A10L	C,V	Vr,Cw,Mn,Ra	B*1501			
VQAVTNAGKIVY	A12L	A,C,V	Vr,Cm,Cw,Mn,Ra	B*1501			
SQIFNIISY	A17L	C,V	Vr,Cm,Cw,Mn	B*1501	96.0	74	9
S[ox]MADVSIKTNSV	A1L	A,C,V	Cm,Cw	A*0201			
ALDEKLFLI	A23R	A,C,T,V	Vr,Cm,Cw,Mn	NA	228.2	6	27
HMIDKLFYV	A23R	A,C,T,V	Vr,Cm,Cw,Mn	A*0201	513.8	2	26
LLFEDIIQNEY	A23R	A,C,T,V	Vr,Cm,Cw,Mn	B*1501		114	
FTVNIFKEV	A24R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	A*0201	8.4	195	16
GDKFTTRTSQKGTVAY	A24R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501			
ILYDPETDKPY	A24R	A,C,T,V	Vr,Cm,Cw,Fw,Mn,Ra	B*1501		81	
TTRTSQKGTVAY	A24R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501			
VIINSTSIF	A24R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501	10.0	223	13
LTREMGFLVY	A29L	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501	17.4	132	15
TVINEDIVSKLTF	A29L	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501			
TLRFLEKTSF	A31R	C,V	Vr,Cm,Cw,Mn	B*1501	72.0	507	11
QIDVRDIKY	A35R	C,V	Cm,Cw	B*1501	1.8	12810	16
SIMDFIGPYI	A35R	C,V	Cm,Cw,Mn,Ra	NA	119.7	11	21
VQIDVRDIKY	A35R	C,V	Cm,Cw	B*1501	52.8	131	23
YIIGNIKTV	A35R	C,V	Cm,CwRa	A*0201	101.2	143	29
IQYPGSEIKGNAY	A44L	A,V	Cm,Cw	B*1501			
IQYPGSKIKGNAY	A44L	C	Ra	B*1501			
IQYPGSKIKGNAYF	A44L	C	Ra	B*1501			
KISNTTFEV	A44L	A,C,V	Cm,Cw,Mn,Ra	A*0201	194.1	10	23
LLISADDVQEIRV	A44L	A,C,V	Vr,Cm,Cw,Mn,Ra	A*0201			
TLYDISPGHVYA	A44L	A,C,V	Cm,Cw,Mn,Ra	NA			
YPGSKIKGNAY	A44L	C	Ra	B*1501		8183	
VIRNEVNDTHY	A46R	C,V	Vr,Cm,Cw,Ra	B*1501		476	
FQQKVLQEY	A48R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501	160.0	112	21
IVIEAIHTV	A48R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	A*0201	97.6	53	27
VAYAAAKGASM	A48R	A,C,T,V	Cm,Cw,Ra	NA		14028	
YAAAKGASM	A48R	A,C,T,V	Cm,Cw,Ra	NA	0.3	4895	17
I[ox]MNNPDFKTTY[h]	A49R	C,V	Vr,Cw	B*1501		97	
ILQNRLVYV	A52R	C,V	Cw	A*0201	1495.7	13	28
VQKQDIVKLTVY	A52R	C,V	Cw	B*1501			
ILSDENYLL	A6L	C,V	Vr,Cm,Cw,Mn,Ra	A*0201	148.9	10	24
KLFNEDLSSKY	A7L	A,C,T,V	Vr,Cw,Mn	B*1501		183	
LIQEIVHEV	A7L	A,C,T,V	Vr,Cm,Cw,Mn	A*0201	153.3	18	29
LVIENDSQF	A8R	A,C,V	Vr,Cm,Cw	B*1501	1.1	215	19
KLYKSGNSHIDY	B12R	A,C,V	Cw,Ra	B*1501			
RVFAPKDTESVF	B12R	A,C,V	Cw,Ra	B*1501			
KVSAQNISF	B13R	C,V	Vr,Cm,Cw,Mn,Ra	B*1501	2.4	117	7
GQLYSTLLSF	B15R	C,V	Vr,Cm,Ra	B*1501	96.0	106	21
IQFMHEQGY	B1R	A,C,V	Vr,Cm,Cw,Mn	B*1501	52.0	106	21
LQYAPRELLQY	B1R	A,C,V	Vr,Cm,Cw,Mn	B*1501		78	
HCYL[ox]MNEGFES	B21R,C15L	C	Cw	NA		39041	
TLLDHIRTA	B22R,C16L	C	Cw,Ra	NA	34.7	84	23
[ox]MLINYL[ox]MLL[i]	C11R	A,T	None	A*0201	83.5	4	27

Peptide Sequence[a]	ORF	Vaccinia Strain[b]	Other Pox Viruses[c]	Putative Allele[d]	BIMAS[e]	CBS IC50 (nM)[f]	SYFPEITHI Prediction[g]
KIKDDFQTVNF	C12L	C,V	Vr,Cm,Cw,Mn	B*1501		731	
KIYGSDSIEF	C12L	C,V	Vr,CwMn	B*1501	14.4	229	12
RQFYNANVL	C2L	C,T,V	Cm,Cw,Ra	A*0201		6960	11
YV^{ox}MGGVYTTY	C2L	C,T,V	Cm,Cw,Ra	B*1501	6.3	68	23
ILKINSVKY	D12L	A,C,V	Vr,Cm,Mn	B*1501	187.2	309	22
KLFTHDIML	D12L	A,C,V	Vr,Cm,Mn	A*0201	276.6	15	22
KLSDSKITV	D13L	A,C,T,V	Vr,Cm,Cw,Mn	A*0201	998.1	21	24
VLSLELPEV	D13L	A,C,T,V	Vr,Cm,Cw,Mn	A*0201	271.9	14	28
ILVPNINILKI	D6R	A,C,T,V	Vr,Cm,Cw,Mn	NA		78	
I^{ox}MSESYTLKEV	D6R	A,C,T,V	Vr,Cm,Cw,Fw,Mn,Ra	A*0201		46	
RLKPLDIHY	D8L	A,C,T,V	Cm,Cw,Ra	B*1501	172.8	135	23
YAIDVSKVKPL	E10R	C	Vr,Cm,Cw,Mn	A*0201		1337	
KIDYYIPYV	E2L	C,V	Vr,Cm,Cw,Mn,Ra	A*0201	169.4	2	24
GKASQNPSK^{ox}MVY	E5R	C,D	Cw,Ra	B*1501			
IGKASQNPSK^{ox}MVY	E5R	C,D	Cw,Ra	B*1501			
KLFSDISAI	E5R	C,D,V	Vr,Cm,Cw,Ra	NA	310.7	8	25
SQNPSK^{ox}MVY	E5R	C,D	Cw,Ra	B*1501	52.8	88	22
LARLGLVL	E6R	C,V	Vr,Cm,Cw,Mn,Ra	A*0201			
GSFSGRYVSY	E8R	C,V	Vr,Cm,Cw,Mn,Ra	B*1501	1.2	204	15
KQKFPYEGGKVF	E9L	A,C,V	Vr,Cm,Cw,Ra	B*1501			
LLLETKTILV	E9L	A,C,V	Vr,Cm,Cw,Mn,Ra	A*0201	1793.7	11	26
IQHRQQLELAY	F11L	C,P	Ra	B*1501		83	
IQKDINITH	F11L	C,P	Vr,Cm,Cw,Ra	NA	0.0	42108	4
MLTEFLHYC	F11L	C,P	Vr,Cw,Mn,Ra	NA	1664.5	105	17
SLSNLDFRL	F11L	C,P	Cw,Mn,Ra	A*0201	123.9	30	23
FLTSVINRV	F12L	C	Vr,Cm,Cw,Mn,Ra	A*0201	735.9	5	25
LF^{ox}MDEIDHESY[h]	F12L	C,P	Vr,Cm,Cw,Mn,Ra	B*1501		566	
NLFDIPLLTV	F12L	C,P	Vr,Cm,Cw,Mn,Ra	A*0201	2426.7	7	29
VQILMKTANNY	F12L	C	Vr,Cm	B*1501		106	
KQISISTGVLY	F16L	C	Vr,Cm,Cw,Mn	B*1501		85	
RVKQISISTGVLY	F16L	C	Vr,Cm,Cw,Mn	B*1501			
IL^{ox}MDNKGLGV	F1L	A,C,P,T,V	Cw	A*0201	1793.7	8	26
IL^{ox}MDNKGLGVRL	F1L	A,C,P,T,V	Cw	A*0201			
RQLPTKTRSY	F1L	A,C,P,T,V	Vr,Cm,Cw,Mn,Ra	B*1501	96.0	80	25
QLIYQRIYY	F2L	C,P,V	Vr,Cm,Cw,Mn,Ra	B*1501	26.4	250	21
ILKSEIEKATY	G4L	C,V	Cw	B*1501		374	
SLKDVLVSV	G5.5E	A,C,T,V	Vr,Mn	A*0201	23.0	18	30
ILDDNLYKV	G5R	C	Vr,Cm,Cw,Mn,Ra	A*0201	446.0	4	30
VPLPCQL^{ox}MY	G5R	C	Vr,Cm,Cw,Mn,Ra	B*1501	2.8	18814	12
ILIEIIPKI	H4L	A,C,V	Vr,Cm,Cw,Mn,Ra	NA	167.2	4	31
ITNKADTSSF	H5R	C,V	Vr	B*1501	2.6	276	10
IIKEDISEY	H7R	A,C,T,V	Vr,Cm,Cw,Mn,Ra	B*1501	42.9	171	19
NTIDKSSPL	I1L	A,C,T,V	Mn	A*0201	1.2	3795	18
TQFNFNGHTY	I1L	A,C,P,T,V	Cm,Cw,Mn	B*1501	88.0	70	22
YSKKFQESF	I1L	A,C,P,T,V	Cm,Cw,Mn	B*1501	6.0	185	11
KLLLGELFFL	J3R	A,C,V	Vr,Cm,Cw,Mn	A*0201	20297.3	7	27
LQKGHNKFPVNF	J4R	C,V	Vr,Cm,Cw,Mn	B*1501			
VVIGNTLIKY	J6R	A,C,V	Vr,Cw,Mn,Ra	B*1501	2.9	242	22
Y^{ox}MIERFISF[h]	J6R	A,C,V	Vr,Cm,Cw,Mn,Ra	B*1501	2.6	49	11
K^{ox}MIIEKHVEY	K1L	C,V	None	B*1501	2.4	103	16
^{ox}MIIEKHVEY	K1L	C,V	None	B*1501	13.2	154	18
SLLFIPDIKL	K1L	C,V	Vr,Cw,Mn,Ra	A*0201	79.0	61	25
SQFDDKGNTALY	K1L	C,V	Cm,CwMn,Ra	B*1501			
VLLDDAEIAK^{ox}M	K1L	V	Cm,Cw,Mn,Ra	NA		55	

Peptide Sequence[a]	ORF	Vaccinia Strain[b]	Other Pox Viruses[c]	Putative Allele[d]	BIMAS[e]	CBS IC50 (nM)[f]	SYFPEITHI Prediction[g]
VLLDDAEIAK^{ox}MII[h]	K1L	V	Cm,Cw,Mn,Ra	NA			
KLVGKTVKV	K3L	C,V	Cm,Cw,Mn	A*0201	243.3	54	30
YLFDYPHFEA	K3L	V	None	NA	2010.7	8	20
ITYPKALVF	K6L	C,V	Cw,Mn	B*1501	4.1	168	11
MMIDDFGTARGNY	K6L	C,V	None	B*1501			
L^{ox}MKFDDVAIRY	K7R	A,C,V	Cw	B*1501		147	
RLYKEL^{ox}MKF[h]	K7R	A,C,V	Cm,Cw,Ra	B*1501	40.0	763	20
IINDKGKQY	M1L	C,V	Vr,Cw,Mn,Ra	B*1501	14.3	423	18
QAIEPSGNNY	M1L	C,V	Cw,Ra	B*1501	2.2	146	12
ILFRMIETY	N1L	C,V	Vr,Cw	B*1501	57.2	208	24
HIIKEFMTY	N2L	C,V	Vr,Cm,Cw,Mn,Ra	B*1501	11.0	797	18
SIIAILDRF	N2L	V	Vr,Cm,Cw,Ra	B*1501	20.0	3775	16
GLLDRLYDL	O1L	C,V	Cm,Cw,Ra	A*0201	745.4	10	29

[a] Peptides without shading were identified at a 1% false positive identification rate (1% FPR); shaded peptides were identified within scoring criteria characteristic of a 5 % FPR. Peptide sequences in boldface are predicted to be strong binders (IC_{50} < 50 nanoM) by the epitope prediction algorithm at: http://www.cbs.dtu.dk/services/NetMHC/ (accessed Jan.4, 2008). ^{ox}M denotes that the peptide contains an oxidized methionine (not considered in calculation of IC_{50} values).

[b] Epitope is common to the following vaccinia strains: A=Ankara, C=Copenhagen, D=Dairen I, P=L-IVP, T=Tian Tan, V= Western Reserve as listed in the SwissProt database.

[c] Epitope also found in the following common poxviruses: Vr=variola, Cm=camelpox, Cw=cowpox, Fw=fowlpox, Mn=monkeypox, Ra=rabbitpox, None=not found in other poxviruses as reported in the NCBI nr database (http://www.ncbi.nlm.nih.gov/).

[d] Putative association of peptide with A*0201 or B*1501 alleles based upon the C-terminal amino acid: amino acids V and L classified as A2; amino acids F and Y classified as B15; NA = not assigned.

[e] Score is proportional to predicted affinity. Bioinformatics and Molecular Analysis Section, National Institute of Health, http://www-bimas.cit.nih.gov/molbio/hla_bind/

[f] Center for Biological Sequence Analysis, Technical University of Denmark, http://www.cbs.dtu.dk/services/NetMHC/

[g] Score is proportional to predicted affinity. Department of Immunology, University of Tübingen, http://www.syfpeithi.de/

[h] Peptide was identified with oxidized and non-oxidized forms of methionine

[i] Peptide was identified in four oxidative forms: without methionine oxidation, oxidized at the N-terminal methionine, oxidized at the position-7 methionine, and oxidized at both methionines.