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In Silico-Accelerated Identification of Conserved and Immunogenic Variola/Vaccinia T-Cell Epitopes

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Abstract

Epitopes shared by the vaccinia and variola viruses underlie the protective effect of vaccinia immunization against variola infection. We set out to identify a subset of cross-reactive epitopes using bioinformatics and immunological methods. Putative T-cell epitopes were computationally predicted from highly conserved open reading frames from seven complete vaccinia and variola genomes using EpiMatrix. Over 100 epitopes bearing low human sequence homology were selected and assessed in HLA binding assays and in T-cell antigenicity measurements using PBMCs isolated from Dryvax-immunized subjects. Experimental validation of computational predictions illustrates the potential for immunoinformatics methods to identify candidate immunogens for a new, safer smallpox vaccine.

Keywords

smallpox; T-lymphocyte epitopes; epitope mapping

1. Introduction

Over 200 years ago, Edward Jenner developed vaccination against variola virus using the related poxvirus vaccinia, enabling a worldwide effort that culminated in the eradication of smallpox 1979. In wake of the September 11, 2001 terror attacks, fears of deliberate dissemination of variola in an unprotected world population prompted the United States government to stockpile vaccine for the civilian population. Licensed smallpox vaccines, such

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as Dryvax (Wyeth) and WetVax (Aventis Pasteur) effectively protect against infection but are contraindicated for about 20% of the US population because they are associated with a broad range of adverse events. For example, dermal complications including vaccinia necrosum, a progressive skin condition with case-fatality rates of 75% to 100% among persons with cellular immunodeficiency, were observed during the global campaign to eradicate smallpox [1]. Eczema vaccinatum, a complication among eczema patients, was associated with case-fatality rates of up to 10% overall and 30% to 40% in children younger than two years of age. Moreover, inadvertent inoculation may result in wider spread when vaccinia is transferred from the vaccination site to another location on the vaccinee or to another person [2]. As a result, smallpox vaccination is contraindicated in persons who have eczema, active acute, chronic, or exfoliative skin conditions that disrupt the epidermis, HIV/AIDS, autoimmune conditions, cancer, radiation treatment, or immunodeficiencies.

We set out to design a safer smallpox vaccine that will provide protection to a greater proportion of the US population. Because epitopes provide the minimal essential information needed to trigger a protective immune response, epitope-driven vaccines represent a logical approach to vaccine development that obviates the risks inherent in live vaccines. Our approach to discovering T-cell epitopes is based on the advent of fully sequenced poxvirus genomes coupled with the availability of immunoinformatics tools that can rapidly identify potentially immunogenic and protective poxvirus sequences. In light of the well known fact that vaccinia immunization protects against variola infection, our studies focused on the subset of epitopes that are common to both poxvirus strains (Figure 1). Here, we report the results of the first steps in the vaccine design process whereby computationally identified epitopes were validated in vitro and ex vivo in order to select epitopes to be later tested as a prototype vaccine in a human leukocyte antigen (HLA) transgenic mouse model.

2. Materials and Methods

2.1. Immunoinformatics

Seven complete poxvirus genomes were downloaded from GenBank: four vaccinia strains (Tian Tian, Accession AF095689; Western Reserve, Accession AY243312; Copenhagen, Accession M35027; Ankara, Accession U94848) and three variola (Variola major, strain India 1967, Accession X69198; Variola major, strain Bangladesh 1975, Accession L22597; Variola minor, strain Garcia 1966, Accession Y16780).

EpiMatrix, a matrix-based epitope mapping algorithm, was used to identify Class I and II HLA epitopes in vaccinia and variola open reading frame (ORF) sequences [3,4,5]. For Class I epitope identification, 9-mer and 10-mer sequences were scored for potential binding to six supertype Class I alleles (A*0101, A*0201, A*0301, A*2402, B*0702 and B*4403 alleles) that cover >90% of humans in five major human population groups [6]. For Class II epitope identification, potential binding of 9-mer sequences was scored for eight archetypical Class II alleles (DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501) that are expected to cover over 95% of any given human population [7]. EpiMatrix raw scores were normalized with respect to a score distribution derived from a very large set of randomly generated peptide sequences. The resulting “Z” scores are normally distributed and directly comparable across alleles. Any peptide scoring above 1.64 on the “Z” scale (approximately the top 5% of any given peptide set) has a significant chance of binding to the MHC molecule for which it was predicted. Peptides scoring above 2.32 on the scale (the top 1%) are extremely likely to bind; most well known T-cell epitopes fall within this range of scores.

Vaccinia/variola ORF and epitope homology were evaluated using Conservatrix, a sequence alignment algorithm that searches a dataset for matching segments. Criteria for conservation significance were application dependent and are described in the Results section. Epitopes were

evaluated for homology with human sequences using the BLAST algorithm [8]. Clusters with no more than 7 matches per 9-mer frame were selected for further study.

For ORFs conserved across all vaccinia/variola strains, HLA Class II epitopes were further analyzed for clustering and among all other ORFs, extended immunogenic consensus sequences (ICS) were developed. Regions of HLA Class II epitope density were discovered using ClustiMer, an algorithm that uses a statistical function to identify sequences which contain more predicted epitopes than would be found by chance alone [9,10]. Clusters whose sum of significant EpiMatrix Z-scores exceeded a value of 10 after subtracting the expected sum of scores for a random sequence of equal length, were considered for further study. ICS were built by EpiAssembler, an algorithm that maximizes epitope density in a 20-25 amino acid stretch by assembling potentially immunogenic 9-mers to be identically positioned as they are in their native protein sequences [11].

2.2. Peptide Synthesis

Peptides were manufactured using 9-fluoronylmethoxycarbonyl (Fmoc) chemistry by SynPep (Dublin, CA) and by New England Peptide (Gardner, MA). Peptides were purified to >80% as ascertained by analytical reversed phase HPLC. Peptide mass was confirmed by MALDI-TOF mass spectrometry.

2.3. HLA Binding Assays

2.3.1. Class I Assay—Class I A2 and B7 peptides were assayed for HLA binding using a quantitative "sandwich" ELISA, as described previously [12]. HLA class I A2 or B7 molecules were incubated at a concentration of ~2 nM together with 25 nM human β2 microglobulin (β2m) and an increasing concentration of test peptides at 18°C for 48 h. HLA molecules were then captured on a 96-well plate coated with the pan-specific anti-human MHC class I mouse monoclonal antibody W6/32, and HLA–peptide complexes incubated with horseradish peroxidase-conjugated anti-human β2m conformational-specific polyclonal detection antibody (Dako P0174) and signal enhancer (Dako Envision). Plates were developed by colorimetric reaction and absorbances measured at 450 nm using a Victor2 Multilabel ELISA reader. Based on a standard curve, absorbance measurements were converted to the concentration of HLA–peptide complexes using a standard curve, and plotted against the concentration of test peptide used in the assay. The concentration of peptide required to half-saturate the HLA was determined. At the limiting HLA concentration used, the half-saturation value approximates the equilibrium dissociation constant value (K_D).

2.3.2. Class II Assay—Class II HLA binding assays were performed as previously described [13]. Briefly, in 96-well plates, non-biotinylated test peptide at 100 μM competed for binding to purified DR1 (50 nM) against biotinylated influenza hemagglutinin 306-318 standard peptide (0.1 μM) for 24 hours at 37°C. DR1 molecules were then captured on ELISA plates using pan anti-Class II antibodies (L243, anti-HLA-DR), developed by addition of streptavidin-europium and read on a time-resolved fluorescence (TRF) plate reader. Percent inhibition of biotinylated peptide binding was calculated. Peptides that inhibited competitor by >50% were considered DRB1*0101 binders.

2.4. Human PBMC T Cell Assay

2.4.1. Study Subjects—Twenty-two healthy adults, ages 18 to 29 years and vaccinated with Dryvax, were recruited for blood draws at the Saint Louis University Center for Vaccine Development. Donors were vaccinated between two and three years before blood draws. Donor HLA types (Class I and II) were determined using One Lambda Micro SSPTM High Resolution HLA class I and II kits at the Hartford Hospital Transplant Immunology Laboratory. Human subject studies were performed in accordance with NIH regulations and with the approval of

the Independent Review Consulting (EpiVax) and Saint Louis University institutional review boards.

2.4.2. PBMC Isolation and Culture—PBMCs were isolated from whole blood by centrifugation over a Ficoll cushion. PBMCs were seeded in 12-well tissue culture plates at 10×10^6 cells/well and stimulated with pools of Class I or Class II peptides in RPMI supplemented with 10% human AB serum, L-glutamine, gentamicin (Invitrogen), at 37°C under a 5% CO_2 atmosphere. 10 U/mL IL-2 and 20 ng/mL IL-7 (R&D Systems) were added to each of the wells. Cells were fed every 2 days by half media replacement containing the same concentration of cytokines. Seven to twenty days post-stimulation, PBMCs were collected and washed in preparation for antigen re-stimulation to measure cytokine secretion measurements by enzyme-linked immunospot (ELISpot) assay.

2.4.3. ELISpot Assay—Interferon-gamma ELISpot assays were performed using kits purchased from Mabtech and performed according to the manufacturer's specifications. Individual target peptides were added at 10 μg/mL to triplicate wells containing 250,000 PBMCs (in RPMI1640 with 10% human AB serum) and incubated for twenty to forty-eight hours at 37°C under a 5% CO_2 atmosphere. Triplicate wells were plated with phytohemagglutinin (PHA; 10 μg/mL) and CEF peptide pool (2 μg/mL) as positive controls and six wells with no peptide were used for background determination. Results were recorded by ZellNet Consulting, Inc. using a Zeiss high resolution automated ELISpot reader system and companion KS ELISpot software. In general, responses are considered positive if the number of spots is at least two times background and greater than 20 spots per one million cells over background (1 response over background per 50,000 PBMCs). Results are recorded as the average number of spots over background and adjusted to spots per one million cells seeded.

3. Results

3.1. In silico epitope mapping

3.1.1. Class I HLA—1,472 open reading frames from 4 vaccinia and 3 variola virus genomes were computationally screened for conserved Class I MHC epitopes using EpiMatrix (see Methods for details). First, each protein sequence was parsed into 9-mer and 10-mer sequences, each overlapping the next by 8 or 9 amino acids, respectively, for a total of 369,394 9-mers and 367,922 10-mers. Using Conservatrix to discover unique, identical peptides conserved across all vaccinia and variola strains, we narrowed down the Class I smallpox immunome to 27,158 9-mers and 26,287 10-mers. Each of these peptides was then scored for Class I HLA motif matches to the A*0101, A*0201, A*0301, A*2402, B*0702 and B*4403 alleles. More than 1000 EpiMatrix hits (Z-score > 1.64; top 5% of scores) per allele were discovered (data not shown). The top 100 hits for each allele were subject to a BLAST search against the human genome to exclude epitopes that may be recognized as self (with a cutoff of no more than 7 identities in a 9-mer sequence), and the top 40 A2 and 20 B7 peptides in a list of ascending human homology were selected for experimental validation (Figures 2 and 3).

3.1.2. Class II HLA—Two strategies to identify conserved and immunogenic Class II MHC epitopes were pursued to maximize the likelihood of discovering protective vaccine immunogens. First, an ORF-by-ORF sequence comparison was performed with the Copenhagen vaccinia strain selected as the standard for alignment because it contains the most ORFs of all the strains under consideration. 107 of the 262 ORFs in Vaccinia Copenhagen had matching ORFs in all six alternate strains with at least 80% identity within the first 200 amino acids. These ORFs in Vaccinia Copenhagen were computationally screened using EpiMatrix and ClustiMer to identify epitope dense regions containing sequences predicted to bind

multiple Class II HLA alleles (DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501). 272 epitope clusters were identified, each bearing at least 90% sequence identity across all seven strains and a cluster score of 15 or above. The sequences were then analyzed by the BLAST algorithm for human homology. Epitope clusters were ranked first by lowest human homology with no more than 7 matches in a 9-mer frame accepted, and then by cluster score. The top 24 epitope clusters were selected for in vitro confirmation (Figure 4). In addition, a 25^{th} cluster was selected for maximal potential immunogenicity, regardless of human homology.

In a second, separate computational screen, ORFs excluded from the investigation above were analyzed using Conservatrix to find identical 9-mers in at least six strains, where minimally three were vaccinia-derived and two variola. 5,781 peptides were discovered, each then scored for binding affinity to a panel of 8 HLA Class II alleles (see above) using EpiMatrix. 786 unique 9-mers were EpiMatrix hits and subsequently input into the EpiAssembler algorithm to identify sets of overlapping, conserved and promiscuous epitopes, termed immunogenic consensus sequence" (ICS) T helper epitopes. 74 ICS with cluster scores greater than 15 were identified and analyzed for human homology using BLAST. Epitope clusters were ranked first by lowest human homology, as above, and then by cluster score. The top 25 ICS were selected for in vitro validation (Figure 4).

3.2. In vitro validation of computational predictions

3.2.1. Class I HLA binding assay—EpiMatrix-predicted epitopes were assessed for their HLA binding potential in binding assays using soluble HLA. Affinities of HLA*A2 and *B7 epitope peptides for their respective HLA were assessed. We found that 100% of the 40 selected A2 epitopes identified by EpiMatrix bound A2 (Figures 2 and 5). 31 bound with very high affinity (1-5 nM K_D), 4 with high affinity (6-25 nM K_D) and 5 at moderate affinity (26-500 nM K_D). Of 20 B7 peptides assayed, 14 (70%) bound B7, 2 with very high affinity, 2 with high affinity, and 10 with moderate affinity (Figures 3 and 5).

3.2.2. Class II HLA binding assay—Class II epitopes, at a peptide concentration of 100 μM, were screened for binding HLA DRB1*0101 in a competition binding assay using soluble HLA. Percent inhibition of competitor peptide was used to estimate test peptide affinity. 21 of 50 peptides bound with high affinity (75%-100% inhibition), 2 peptides with moderate (50%-75% inhibition) and 5 peptides with weak affinity (30%-50%). In total, 28/50 (56%) of peptides tested bound DR1, as expected of a set of sequences that were predicted to cover a HLA diverse population, not only DRB1*0101 carriers.

3.3. Ex vivo validation of computational predictions

We validated EpiMatrix-predicted epitopes in measurements of antigen-specific T-cell responses in 22 human subjects, ages 18-29 years old, who received Dryvax 2 to 3 years before blood draw. PBMCs were stimulated with pools of smallpox epitopes for 7-20 days and re-stimulated with individual epitopes and epitope pools in an IFNγ ELISpot assay for 20-48 hours. Response frequency among subjects ranged from 10 to 100% for individual Class II epitopes. Responses were observed to 41 of 50 (82%) Class II epitopes, with an average of 36% positive responses per subject (Figure 6 and Figure 7, top). All subjects exhibited a robust response to pooled Class II peptides. Responses plotted according to gene expression temporality [14,15] reveal no preponderance of T-cell reactivity in a single group of antigens.

For Class I epitopes, antigenicity was detected for 17 of 40 (43%) HLA-A*0201 (Figure 7, bottom left) and 5 of 20 (25%) B*0702 epitopes (Figure 7, bottom right). Per subject, responses to A2 epitopes averaged 7% and to B7 epitopes 10%.

4. Discussion

Using immunoinformatics methods, we scanned vaccinia- and variola-conserved sequences for HLA Class I and Class II epitopes and then validated the predictions in HLA binding assays and in humans vaccinated with Dryvax. Our approach to epitope prediction was to discover the intersection of vaccinia and variola genome sequences that give rise to CD8 and CD4 T cell-mediated protection against variola as conferred by vaccination with vaccinia. CD8 responses play an important role in containing orthopoxvirus infections and may be a critical correlate of protection after re-exposure [16,17]. CD4 responses are critical for robust CD8 T cell proliferation and function and for their differentiation into memory cells in vaccinia infection [18,19]. Moreover, CD4 responses provide required help to B cells to produce antibodies that are necessary and sufficient to protect against orthopoxvirus challenge [20]. Hence, we set out to discover potential HLA Class I and Class II variola/vaccinia protective determinants using computational methods as a high throughput method for scanning large orthopoxvirus genomes without bias to time of expression or protein function.

4.1. CD4+ T-cell epitopes

We identified 50 HLA Class II epitopes conserved in vaccinia and variola genomes. Half were predicted with a requirement that all epitopes be conserved in all genomes analyzed and the other half with the more relaxed requirement that sequences be conserved in at least 3 vaccinia and 2 variola genomes only out of a total of seven genomes analyzed. We discovered that >80% of the epitopes were antigenic, with multiple responses observed in all Dryvax vaccinees tested, illustrating the effectiveness of a predictive approach. Unexpectedly, we found that, with the exception of the Tian Tian strain, the ICS sequences were more highly conserved, contained more EpiMatrix hits and produced greater numbers of spot forming cells in interferon-gamma ELISpot assays than the sequences derived from the more traditional alignment based-approach. Higher ELISpot numbers may be attributed to the relatively higher concentration of high scoring 9-mers in the ICS epitopes, to greater sequence conservation or to both factors.

In two related studies, Koelle and co-workers used a non-predictive, experimental screen to identify CD4+ T cell-antigenic open reading frames and/or protein fragments in the vaccinia proteome [21,22]. As in those studies, we observed a broad CD4+ T cell response to vaccinia antigens expressed both early and late in infection, with a majority of the responses to proteins expressed at the early stage. Because the predictive approach triages sequences for ex vivo validation, only 29 open reading frames are represented by the selected Class II promiscuous epitopes in this study. Among these 29, 23 (79%) were observed by Koelle and co-workers to be antigenic. Considering that the predictive approach significantly limits the part of an open reading frame that is used to measure antigenicity, this finding illustrates the advantage immunoinformatics provides for rapidly identifying antigenic open reading frames and focusing in on a segment that is immunoreactive. Notably, only one of these open reading frames (L4R) is recognized by 100% of subjects in the non-predictive study, whereas here, 3 of 16 subjects (19%) responded to 4020_II_L4R. While the predictive method is useful for selecting immunogenic proteins, it is important to note that a single epitope from a highly immunogenic protein will not necessarily be recognized by all individuals tested. Differences in the frequency of recognition of a single epitope versus the large protein sequences evaluated by Koelle and co-workers are possibly due to the limited number of L4R epitope sequences assayed in the present study, the limited number of subjects in the studies (5 in [21], 12 in [22], 16 here), the HLA types of the subjects, or differences in the methods used for measuring antigenicity (proliferation vs. ELISpot).

4.2. CD8+ T-cell epitopes

We selected 40 A2 and 20 B7 supertype epitopes conserved in the vaccinia and variola genomes using immune-informatics methods and evaluated them for T cell reactivity ex vivo. Of the 60 epitopes tested, 17 A2 epitopes and 5 B7 epitopes elicited IFN-gamma responses in ex vivo ELISpot; thus, one in three predicted sequences was confirmed. Only one of these 22 Class I restricted antigenic epitopes, 5019_A2_I8R, has been reported beforehand [23]. This is especially noteworthy as several published reports identify A2 vaccinia epitopes [23,24,25, 26]. Particularly, Sette and co-workers performed an extensive study of HLA Class I restricted vaccinia responses using immuno-informatics methods [24]. Starting with a list of approximately 2000 A2 and B7 supertype epitopes, 14 A2 and 5 B7 epitopes were found to be antigenic in Dryvax vaccinees, a ratio of roughly one in 100. None of these epitopes are identical to the sequences reported here, although two are found in common open reading frames (E2L, A2; J6R, B7). In a study similar in design, Kazura and co-workers reported 6 new A2 epitopes reactive in persons vaccinated against smallpox. Two are common to protein antigens reported here (G1L, I8R) and one is identical in sequence, as mentioned above. Factors that may have contributed to a lack of concordance between these studies include the different epitope prediction tools used by each group and the limited numbers of subjects sampled. Nevertheless, like the previously published studies, the breadth of vaccinia-induced immune response is shown here by ex vivo responses for epitopes derived from 18 different open reading frames. In addition, we note that the number of epitopes recognized in this study is far fewer than we observed in previous epitope mapping studies of HIV using EpiMatrix [27]. This may be a natural outcome of the larger size of the genome, compared to the genome of HIV, which gives rise to more complex CD8+ T-cell epitope hierarchies [28]. Alternatively, class I epitopes may not be as critical for protection from poxviruses as previously believed. We plan to evaluate the relative contributions of class I and class II epitopes to protection from vaccinia in a challenge study.

4.3. Future studies

The broader goal of this study is to identify epitopes for incorporation into a new smallpox vaccine that is safer than previously licensed smallpox vaccines. The use of epitopes overcomes potential safety concerns associated with vaccinating with live vaccinia virus. In addition, multiple epitopes derived from more than one antigen can be packaged into a relatively small delivery vehicle. Furthermore, epitope-based vaccines appear to be capable of inducing more potent responses than whole protein vaccines [29], and they sidestep the propensity for the immune system to focus on a single immunodominant epitope by simultaneously targeting multiple dominant and subdominant epitopes [30,31]. This latter feature is particularly significant because of the great breadth of the antiviral response [21,22,32]. It should be noted that the field of immuno-informatics is new, and few epitope-driven vaccines for infectious pathogens have reached the stage of efficacy trials in humans, although several have been shown to be effective in animal models.

In summary, we are in the process of developing an epitope-based vaccine based on intersecting sets of epitopes derived from the variola and the vaccinia genomes; this article describes our progress along the pathway to that goal. We believe that our immunoinformatics-driven smallpox vaccine development approach may have several advantages over other approaches: (1) rapidity (the mapping of epitopes and confirmation using human PBMC was accomplished in less than 18 months); (2) safety (the entire protein is not used, thus the recipient is not exposed to live vaccinia virus, which may be associated with side effects); and (3) broad immunogenicity (delivery of multiple epitopes, derived from multiple proteins, recognized in the context of many different MHC). We also believe that the methods described here, which will lead to the development of a multi-epitope smallpox vaccine, may be a step in the right direction for the development of a range of safer, more effective biodefense vaccines.

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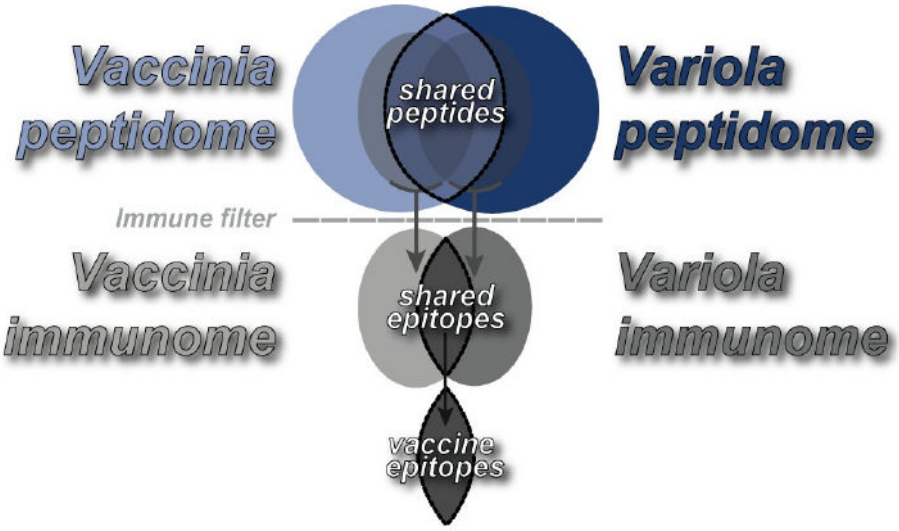

Figure 1.

In silico approach to identification of smallpox vaccine candidates. Immunoinformatics tools identify potential T-cell epitopes from large viral genome datasets, such as variola and the related vaccine strain vaccinia. Here, informatics methods were used to delineate the intersection of vaccinia and variola immunogenic epitope sets, which contain sequences that provide T cell-mediated protection against variola. Conserved sequences among 3 variola and 4 vaccinia genomes were assessed for immunogenic potential using the T-cell epitope mapping algorithm EpiMatrix. We selected 110 epitopes for vaccine design, of which 50 were promiscuous Class II HLA epitopes, 40 were Class I HLA A2 and 20 were Class I B7. 60% of the epitopes were derived from regulatory factors, 24% from hypothetical and unknown proteins and16% from structural proteins.

PeptideID_HLA_ParentProtein	Expression of Parent Gene	Amino Acid Sequence	Genome Y16780: ORF Prefix:	Genome: X69198 ORF prefix:	Genome: U94848 ORF prefix:	Genome: M35027 ORF prefix:	Genome: L22579 ORF Prefix:	Genome: AF095689 ORF prefix:	Genome: AY243312 ORF prefix:	EpiMatrix A2 Score	Kd HLA A2
5001_A2_A16L	Late	YLGPRVCWL	54720.1	49061.1	96467.1	48138.1	60868.1	34010.1	89415.1	4.2	3
5002_A2_A46R	Early	GLFDFVNFV	54759.1	49099.1	96538.1	48177.1	60901.1	34057.1	89451.1	4.1	1
5003_A2_F10L	Late	ALNDFDFSQV	54634.1	48975.1	96420.1	48026.1	60782.1	33901.1	89328.1	3.9	1
5004_A2_D6R	Early	KLLKMVTSV	54696.1	49037.1	96515.1	48105.1	60844.1	33975.1	89390.1	3.8	1
5005_A2_A9L	Late	KLRPNSFWFV	54713.1	49054.1	96461.1	48128.1	60861.1	34000.1	89407.1	3.8	2
5006_A2_F10L	Late	KLLSHFYPAV	54634.1	48975.1	96420.1	48026.1	60782.1	33901.1	89328.1	3.8	1
5007_A2_E6R	Late	YLVSNFPQHV	54647.1	48988.1	96487.1	48044.1	60795.1	33918.1	89341.1	3.7	2
5008_A2_A14L	Late	LMIGNYFSGV	54718.1	49059.1	96465.1	48136.1	60866.1	34008.1	89412.1	3.7	2
5009_A2_D11L	Late	KLGGLCSYIV	54701.1	49042.1	96451.1	48110.1	60849.1	33981.1	89395.1	3.7	11
5010_A2_A32L	Late	NLLKMPFRMV	54739.1	49080.1	96476.1	48158.1	60887.1	34037.1	89434.1	3.7	111
5011_A2_A32L	Late	YIWPNHINFV	54739.1	49080.1	96476.1	48158.1	60887.1	34037.1	89434.1	3.7	1
5012_A2_A12L	Late	AMDGQIVQAV	54716.1	49057.1	96463.1	48134.1	60864.1	34006.1	89410.1	3.7	2
5013_A2_E8R	NK	SLYKGPIPV	54649.1	48990.1	96489.1	48047.1	60797.1	33920.1	89343.1	3.6	1
5014_A2_E5R	Early	ALLLYMFPNL	54646.1	48987.1	96486.1	48042.1	60794.1	33916.1	89340.1	3.6	28
5015_A2_A23R	Early	SLDHTVFPSL	54727.1	49068.1	96525.1	48147.1	60875.1	34019.1	89422.1	3.6	1
5016_A2_I2L	Late	KLYAAIFGV	54656.1	48997.1	96434.1	48057.1	60804.1	33930.1	89350.1	3.6	1
5017_A2_H6R	Early & Late	FLYNFWTNV	54689.1	49030.1	96509.1	48093.1	60837.1	33965.1	89383.1	3.6	7
5018_A2_E6R	Late	YLDGQLARL	54647.1	48988.1	96487.1	48044.1	60795.1	33918.1	89341.1	3.5	1
5019_A2_I8R	Early & Late	KLLLWFNYL	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	3.5	117
5020_A2_E2L	Early	YLPKVLYNNV	54643.1	48984.1	96427.1	48039.1	60791.1	33912.1	89337.1	3.5	1
5021_A2_I1L	Late	RLYDYFTRV	54655.1	48996.1	96433.1	48056.1	60803.1	33929.1	89349.1	3.5	1
5022_A2_A31R	Early	SLNRTIVTKV	54738.1	49079.1	96527.1	48157.1	60886.1	34036.1	89433.1	3.5	109
5023_A2_H4L	Late	NLYDLFFNTL	54687.1	49028.1	96448.1	48091.1	60835.1	33963.1	89381.1	3.5	10
5024_A2_J3R	Early	ILNPVASSL	54680.1	49021.1	96504.1	48083.1	60828.1	33954.1	89374.1	3.5	3
5025_A2_F13L	Late	YIASFCCNPL	54637.1	48978.1	96422.1	48031.1	60785.1	33905.1	89331.1	3.5	1
5026_A2_A3L	Late	VMGSAVHSPV	54707.1	49048.1	96457.1	48118.1	60855.1	33991.1	89401.1	3.4	4
5027_A2_E2L	Early	YLSSWTPVV	54643.1	48984.1	96427.1	48039.1	60791.1	33912.1	89337.1	3.4	1
5028_A2_G1L	Late	VMTPSPFYTV	54663.1	49004.1	96440.1	48065.1	60811.1	33938.1	89357.1	3.4	1
5029_A2_G5R	Early	YLAKLTALV	54667.1	49008.1	96493.1	48069.1	60815.1	33942.1	89361.1	3.4	1
5030_A2_G1L	Late	YLYETYHLI	54663.1	49004.1	96440.1	48065.1	60811.1	33938.1	89357.1	3.4	1
5031_A2_A32L	Late	ILLCQTYRHV	54739.1	49080.1	96476.1	48158.1	60887.1	34037.1	89434.1	3.3	16
5032_A2_I7L	Late	NLLCHIYSL	54661.1	49002.1	96439.1	48063.1	60809.1	33936.1	89355.1	3.3	1
5033_A2_A21L	Late	RLNKNFICV	54725.1	49066.1	96470.1	48142.1	60872.1	34014.1	89419.1	3.3	2
5034_A2_I8R	Early & Late	ILKSLGFKV	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	3.3	69
5035_A2_H3L	Late	FLTGTFVTA	54686.1	49027.1	96447.1	48090.1	60834.1	33961.1	89380.1	3.3	1
5036_A2_J6R	Early	YLYQPCDLL	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	3.3	1
5037_A2_J1R	Late	KLFNKVPIV	54678.1	49019.1	96502.1	48081.1	60826.1	33952.1	89372.1	3.3	3
5038_A2_D10R	Late	FLDPNSGNGL	54700.1	49041.1	96518.1	48109.1	60848.1	33980.1	89394.1	3.3	1
5039_A2_A38L	NK	IMVSEHFSL	54747.1	49088.1	96477.1	48168.1	60893.1	34047.1	89441.1	3.3	1
5040_A2_A24R	Early	FTFSNVCESV	54728.1	49069.1	96526.1	48148.1	60876.1	34020.1	89423.1	3.3	1

Figure 2.

Characteristics of selected A2 peptides. The peptide IDs and amino acid sequences are shown followed by their gene expression temporality. Here, “early and late” refers to early and late post-replication phase expression. The accession numbers for the corresponding ORFs within 3 variola and 4 vaccinia genomes are listed. In addition, the EpiMatrix A2 Z-score and K_D (nM) are presented.

PeptideID_HLA_ ParentProtein	Expression of Parent Gene	Amino Acid Sequence	Genome Y16780: ORF Prefix: CAB	Genome: X69198 ORF prefix: CAA	Genome: U94848 ORF prefix: AAB	Genome: M35027 ORF prefix: AAA	Genome: L22579 ORF Prefix: AAA	Genome: AF095689 ORF prefix: AAF	Genome: AY243312 ORF prefix: AAO	EpiMatrix A2 Score	Kd HLA B7
5101_B7_L4R	Late	FPRSMLSIF	54676.1	49017.1	96500.1	48079.1	60824.1	33951.1	89370.1	4.0	4
5102_B7_L3L	Late	IPRTNIVFSV	54675.1	49016.1	96444.1	48078.1	60823.1	33950.1	89369.1	3.9	294
5103_B7_D6R	Early	SPITNTPNTL	54696.1	49037.1	96515.1	48105.1	60844.1	33975.1	89390.1	3.9	108
5104_B7_A24R	Early	RPPSFYKPL	54728.1	49069.1	96526.1	48148.1	60876.1	34020.1	89423.1	3.9	28
5105_B7_C10L	Early	TPICGGKIKL	54598.1	48944.1	96402.1	47986.1	60750.1	33858.1 34092.1	89289.1 89488.1	3.8	11,052
5106_B7_E9L	Early	IPRLLRTFL	54650.1	48991.1	96430.1	48049.1	60798.1	33922.1	89344.1	3.8	2
5107_B7_A9L	Late	RPNSFWFVV	54713.1	49054.1	96461.1	48128.1	60861.1	34000.1	89407.1	3.7	47
5108_B7_D6R	Early	LPPHPSIVKV	54696.1	49037.1	96515.1	48105.1	60844.1	33975.1	89390.1	3.6	non
5109_B7_I8R	Early & Late	LPRIALVRL	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	3.6	21,632
5110_B7_I8R	Early & Late	SPISLRYGSI	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	3.6	60
5111_B7_G1L	Late	TPSPFYTVM	54663.1	49004.1	96440.1	48065.1	60811.1	33938.1	89357.1	3.6	14
5112_B7_D11L	Late	RPGSLQHQSL	54701.1	49042.1	96451.1	48110.1	60849.1	33981.1	89395.1	3.6	19
5113_B7_D11L	Late	TPPERRYVNV	54701.1	49042.1	96451.1	48110.1	60849.1	33981.1	89395.1	3.5	26,808
5114_B7_A10L	Late	LPRVVGGKTV	54714.1	49055.1	96462.1	48129.1	60862.1	34001.1	89408.1	3.5	226
5115_B7_A26L	Late	SPMYLWFNV	54733.1	49074.1	96471.1	48151.1	60881.1	34031.1	89428.1	3.5	304
5116_B7_J6R	Early	RPNSTFTNKL	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	3.5	140
5117_B7_A7L	Late	FPKQTIQTPI	54711.1	49052.1	96460.1	48124.1	60859.1	33997.1	89405.1	3.5	33
5118_B7_L2R	Early	CPAILRPLI	54674.1	49015.1	96499.1	48077.1	60822.1	33949.1	89368.1	3.4	117
5119_B7_O2L	Late	CPFCRNALDI	54654.1	48995.1	96432.1	48055.1	60802.1	33928.1	89348.1	3.4	non
5120_B7_D6R	Early	TPNTLGHII	54696.1	49037.1	96515.1	48105.1	60844.1	33975.1	89390.1	3.4	8,371

Figure 3.

Characteristics of selected B7 peptides. The peptide IDs and amino acid sequences are shown followed by their gene expression temporality. Here, “early and late” refers to early and late post-replication phase expression. The accession numbers for the corresponding ORFs within 3 variola and 4 vaccinia genomes are listed. In addition, the EpiMatrix HLA B7 Z-score and K_D (nM) are shown.

PeptideID HLA ParentProtein	AA SEQUENCE	Expression Temporality	Y16780: CAB	X69198: CAA	U94848: AAB	M35027: AAA	L22579: AAA	AF095689: AAF	AY243312: AAO	EpiMatrix Class II Cluster Score	Sum DRB1*0101 SCORES	DRB1*0101 % inhibition
4000_II_D11L	GTNIWYSNSNRLMSINR	Late	54701.1	49042.1	96451.1	48110.1	60849.1	33981.1	89395.1	15.1	2.4	90
4001_II_D6R	KKLLYLKFKTKETNRIYSI	Early	54696.1	49037.1	96515.1	48105.1	60844.1	33975.1	89390.1	21.9	2.2	46
4002_II_B1R	LDAVIRANNNRLPKRS	Early	54770.1	49110.1	96545.1	48194.1	60910.1	34067.1	89462.1	18.4	3.8	7
4003_II_E2L	PEKLYLFKPRTVAPLDLIST	Early	54643.1	48984.1	96427.1	48039.1	60791.1	33912.1	89337.1	16.7	3.6	94
4004_II_A24R	ICDFVTDFRRRKRMGFFGN	Early	54728.1	49069.1	96526.1	48148.1	60876.1	34020.1	89423.1	16.4	0.0	0
4005_II_A8R	GAVINQMVNTVLITVYEKLQLVIE	Early	54712.1	49053.1	96520.1	48127.1	60860.1	33999.1	89406.1	17.5	4.2	86
4006_II_I5L	MQSLKFNRAVTIFKYIGLFIYIP	Late	54659.1	49000.1	96437.1	48061.1	60807.1	33934.1	89353.1	18.8	2.8	18
4007_II_D5R	DTAVYRRKTTLRVVGTRKNPNCDT	Early	54695.1	49036.1	96514.1	48102.1	60843.1	33972.1	89389.1	19.6	4.1	30
4008_II_E6R	DADIVLNRHAITMYDKILSYIY	Late	54647.1	48988.1	96487.1	48044.1	60795.1	33918.1	89341.1	16.9	0.0	83
4009_II_D12L	IDTMRIYCSLFKNVRLLKCVSDSWL	Early	54702.1	49043.1	96452.1	48113.1	60850.1	33985.1	89396.1	19.2	1.8	14
4010_II_E8R	IYNILFWFKNTQFDITKH	NK	54649.1	48990.1	96489.1	48047.1	60797.1	33920.1	89343.1	17.6	2.1	*
4011_II_I2L	WGWYWLIIIFFIVLILLLLIYLYLKVVW	Late	54656.1	48997.1	96434.1	48057.1	60804.1	33930.1	89350.1	40.1	11.0	96
4012_II_B15R	LTEYIYWSSYAYRNRQCAGQLYS	Early	54784.1	49123.1	96554.1	48212.1	60922.1	34084.1	89475.1	18.1	3.6	96
4013_II_A20R	LKELLSLYKSLRFSDSAAIEKY	Early	54724.1	49065.1	96523.1	48143.1	60873.1	34015.1	89420.1	16.3	4.0	0
4014_II_A23R	NQPWIKTISKRMRVDIINHSIVT	Early	54727.1	49068.1	96525.1	48147.1	60875.1	34019.1	89422.1	18.4	2.1	53
4015_II_L3L	LVRSRKAVGFPLLKAAKRISHGSM	Late	54675.1	49016.1	96444.1	48078.1	60823.1	33950.1	89369.1	19.0	4.4	54
4016_II_H1L	MDKKSLYKYLLLRSTGDMHKA	Early & Late	54684.1	49025.1	96446.1	48088.1	60832.1	33959.1	89378.1	25.1	6.0	91
4017_II_A24R	GVFYRPLHFQYVSYSNFILHRL	Early	54728.1	49069.1	96526.1	48148.1	60876.1	34020.1	89423.1	16.8	4.3	98
4018_II_L3L	GEMFVRSQSSTIIV	Late	54675.1	49016.1	96444.1	48078.1	60823.1	33950.1	89369.1	22.4	4.6	91
4019_II_J3R	KLPYQGQLKLLLGELFFLSKL	Early & Late	54680.1	49021.1	96504.1	48083.1	60828.1	33954.1	89374.1	18.1	4.4	0
4020_II_L4R	LSIFNIVPRTMSKYELELI	Late	54676.1	49017.1	96500.1	48079.1	60824.1	33951.1	89370.1	15.7	5.6	0
4021_II_A18R	VSEVVSNMRKMIESKRPLYITLH	Early	54722.1	49063.1	96522.1	48140.1	60870.1	34012.1	89417.1	19.1	1.8	2
4022_II_J3R	FYNLGMIIKWMLIDGRHHDPIL	Early	54680.1	49021.1	96504.1	48083.1	60828.1	33954.1	89374.1	16.3	2.0	0
4023_II_A7L	GDDIVRLRTTSDIIQFVN	Late	54711.1	49052.1	96460.1	48124.1	60859.1	33997.1	89405.1	17.4	1.7	6
4024_II_L2R	RPLIRLFIDILLFVIVIYIF-TVRLVSRNYQMLLAL	Early	54674.1	49015.1	96499.1	48077.1	60822.1	33949.1	89368.1	54.5	5.8	45
4026_II_J6R	YKYFIDLGLLMRMERKLSDKI	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	28.9429	5.87	7
4027_II_J6R	TGSQYYFSMLVARSQSTDIVC	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	26.7694	7.16	97
4028_II_C10L	PVTEDDYKFLSRLVLYAKSQS	Early	54598.1	48944.1	96402.1	47986.1	60750.1	33858.1 34092.1	89289.1 89488.1	26.2054	3.4	0
4029_II_J6R	NDVDSNFVVAMRHLSLAGLLS	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	25.626	6.05	45
4030_II_A32L	NLLKMPFRMVLTGGSGSGKTI	Late	54739.1	49080.1	96476.1	48158.1	60887.1	34037.1	89434.1	25.553	5.55	*
4031_II_A4L	EIGLKSQESYYQRQLREQLARD	Early & Late	54708.1	49049.1	96458.1	48120.1	60856.1	33994.1	89402.1	25.1661	4.66	17
4032_II_N2L	VSILNKYKPVYSYVLYENVLY	Early	54617.1	48958.1	96408.1	48002.1	60765.1	not found	89308.1	23.3434	2.9	100
4033_II_D10R	NKFFEVIFFVGRISLTSDQII	Late	54700.1	49041.1	96518.1	48109.1	60848.1	33980.1	89394.1	23.019	1.69	0
4034_II_D10R	SSIISQIIKYNRRLAKSIICE	Late	54700.1	49041.1	96518.1	48109.1	60848.1	33979.1	89394.1	22.9169	2.17	5
4035_II_I8R	EFLHNYILYANKFNLTLPEDL	Early & Late	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	22.6904	3.71	29
4036_II_J6R	ASNQVKFYFNKRLNQLTRIRQ	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	20.7991	1.87	0
4037_II_J6R	AGYKVNPTELMYILGTYGQQR	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	19.7261	4.3	88
4038_II_F12L	YETIEILRNYLRLYIILARNE	Early	54636.1	48977.1	96421.1	48029.1	60784.1	not found	89330.1	19.5805	3.63	98
4039_II_G6R	SIIFINYTMSLTSHLNPSIEK	Early & Late	54669.1	49010.1	96495.1	48070.1	60817.1	not found	89363.1	18.3891	4.16	94
4040_II_A26L	KFKTLNIYMITNVGQYILYIV	Late	54733.1	49074.1	96471.1	48151.1	60881.1	34031.1	89428.1	18.2497	7.53	91
4041_II_B18R	GYTALHYYYLCLAHVYKPGEC	Early	54789.1	49128.1	96556.1	48217.1	60925.1	34089.1	89478.1	17.3236	4.94	25
4042_II_J6R	GSIQDEIVAAYSLFRIQDLCL	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	17.0822	3.66	0
4043_II_G6R	GYLSAKVYMLENIQVMKIAAD	Early & Late	54669.1	49010.1	96495.1	48070.1	60817.1	not found	89363.1	16.8695	6.61	*
4044_II_D4R	DKFFIQLKQPLRNKRVCVCGI	Early	54694.1	49035.1	96513.1	48100.1	60842.1	not found	89388.1	16.1557	2.3	95
4045_II_G8R	VFYRGAENIVFNLPVSKVKSC	NK	54671.1	49012.1	96496.1	48074.1	60819.1	not found	89365.1	16.0997	2.14	97
4046_II_F15L	PFHFQQPQFQYLLPGFVLTCI	Early	54639.1	48980.1	96424.1	48034.1	60787.1	not found	89333.1	15.9793	6.89	95
4047_II_J6R	KNNMIRSYIVARRKDQTARSV	Early	54683.1	49024.1	96506.1	48086.1	60831.1	not found	89377.1	15.9253	2	0
4048_II_J6R	SVNKFKFGAASTLKRATFGDN	Early	54683.1	49024.1	96506.1	48086.1	60831.1	33957.1	89377.1	15.8127	4.77	99
4049_II_A44L	SYDMFNLLLMKPLGIEQGSRI	Early	54755.1	49096.1	96479.1	48175.1	60899.1	34055.1	89449.1	15.6538	6.86	94
4050_II_I8R	SLPRIALVRLHSNTILKSLGF	Early & Late	54662.1	49003.1	96491.1	48064.1	60810.1	33937.1	89356.1	15.5166	4.62	96

Figure 4.

Characteristics of selected Class II peptides. The peptide IDs and amino acid sequences are shown followed by their gene expression temporality. Here “early and late” refers to early and late post-replication phase expression. The accession numbers for the corresponding ORFs within 3 variola and 4 vaccinia genomes are listed. The top 25 epitopes were discovered in the first Class II epitope screen and the bottom 25 in the second (see Results). In addition, the EpiMatrix Class II cluster score, the cluster sum of DRB1*0101 scores and % inhibition of competitor peptide binding to DRB1*0101 at 100 μM epitope peptide are shown. Asterisks refer to peptides that were not assayed for binding.

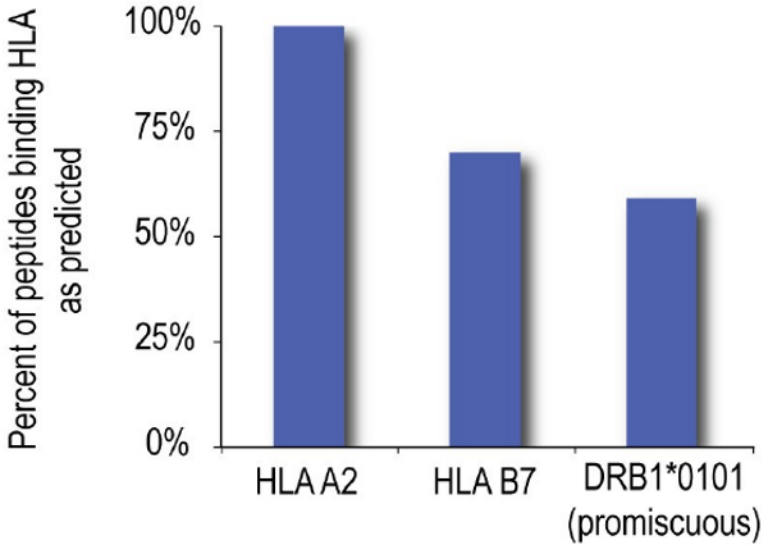

Figure 5.

In vitro validation of computationally identified epitopes. Predicted epitopes were assayed for binding to individual HLA alleles, A2, B7 and DRB1*0101. The percent of predicted epitopes that bound these alleles is shown. Class II epitopes were selected for predicted binding to multiple HLA alleles (see Methods), not solely to DRB1*0101.

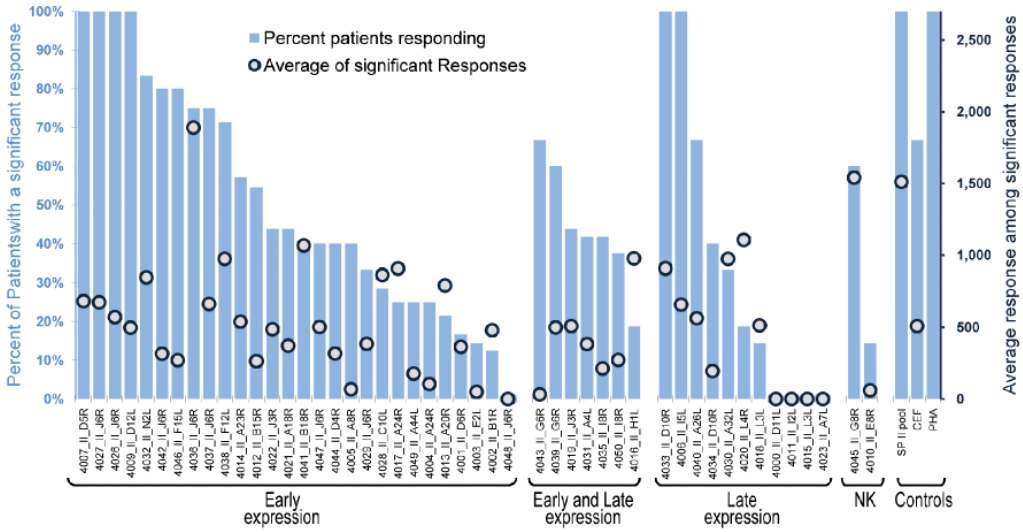

Figure 6.

Ex vivo validation of computationally identified Class II HLA epitopes. Predicted epitopes were assayed for T cell reactivity by IFN-γ ELISpot assay using PBMCs isolated from Dryvax-vaccinated donors. Epitopes are grouped according to their timing of expression as parts of whole proteins in the poxvirus life cycle. The “early and late” designation refers to early and post-replication late expression. The percentage of subjects who responded to individual epitopes in descending order (bars) and the average spot forming cells per million PBMCs for each epitope (circles) are illustrated.

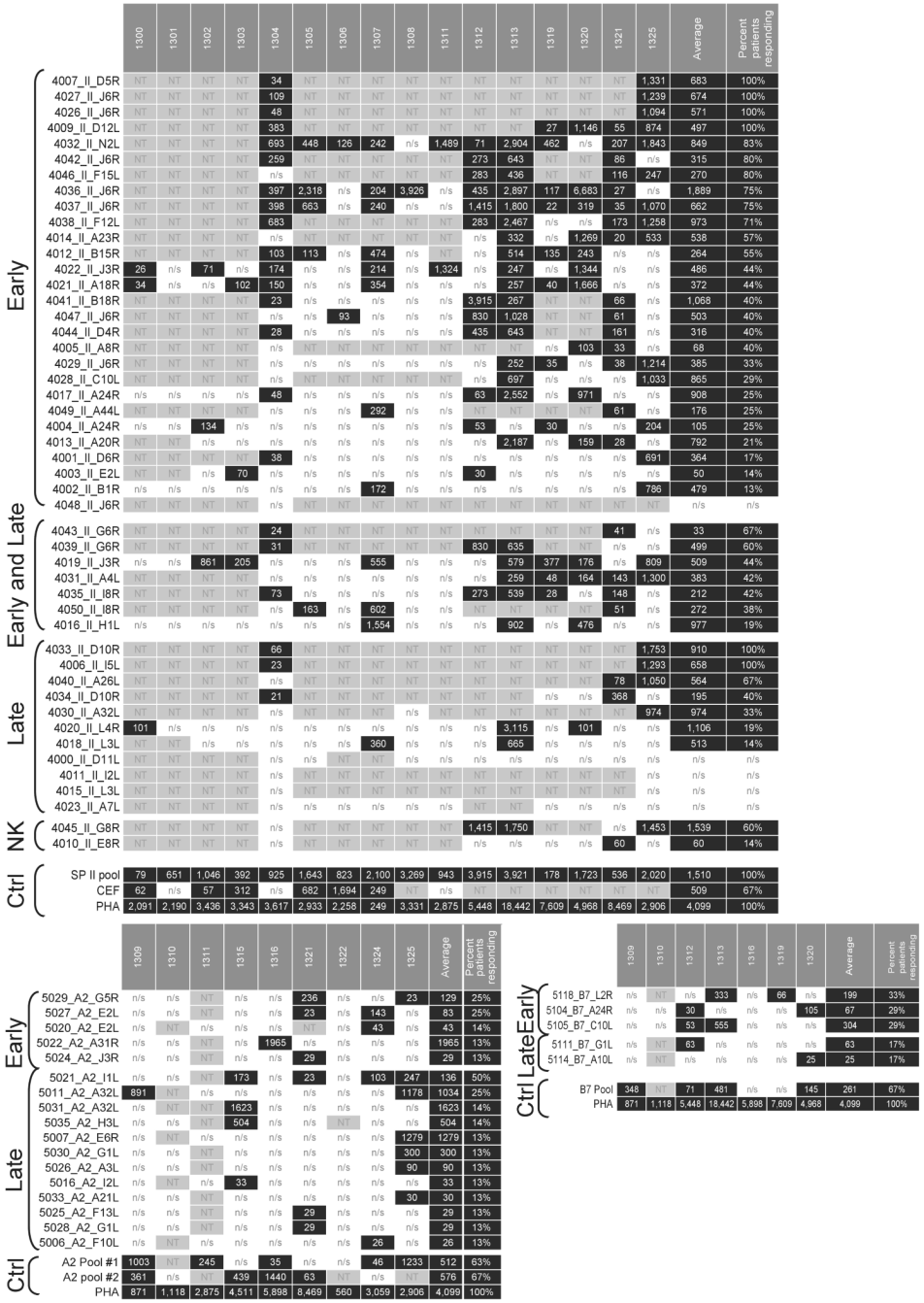

Figure 7.

IFN-γ ELISpot responses to predicted Class I and Class II epitopes in Dryvax vaccinated human subjects. The numbers of spot forming cells (over background) per million PBMCs that secrete IFN-γ in response to individual and pooled Class I and Class II epitopes, as well as PHA and CEF are presented. For simplicity, non-significant results are denoted by n/s and missing data are omitted (not tested, NT). Significant results are highlighted in black. An ELISpot response was considered positive if two criteria were met: (1) spot-forming cells per million PBMC were at least 20 over background; (2) spot-forming cells (SFC) per million PBMC were at least two-fold over background. Column headers: human subject ID code, average responses and

percent of subjects responding. Row labels: peptide ID. Epitopes are grouped according to their timing of expression as parts of whole proteins in the poxvirus life cycle.