Research Article

EPIPOX: Immunoinformatic Characterization of the Shared T-Cell Epitome between Variola Virus and Related Pathogenic Orthopoxviruses

Magdalena Molero-Abraham,¹ John-Paul Glutting,¹ Darren R. Flower,² Esther M. Lafuente,¹ and Pedro A. Reche¹

¹School of Medicine, Unit of Immunology, Complutense University of Madrid, Pza. Ramón y Cajal, s/n, 28040 Madrid, Spain

²School of Life and Health Sciences, University of Aston, Aston Triangle, Birmingham B4 7ET, UK

Correspondence should be addressed to Pedro A. Reche; parecheg@med.ucm.es

Received 26 June 2015; Revised 8 September 2015; Accepted 1 October 2015

Academic Editor: Jacek Tabarkiewicz

Copyright © 2015 Magdalena Molero-Abraham et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Concerns that variola viruses might be used as bioweapons have renewed the interest in developing new and safer smallpox vaccines. Variola virus genomes are now widely available, allowing computational characterization of the entire T-cell epitome and the use of such information to develop safe and yet effective vaccines. To this end, we identified 124 proteins shared between various species of pathogenic orthopoxviruses including variola minor and major, monkeypox, cowpox, and vaccinia viruses, and we targeted them for T-cell epitope prediction. We recognized 8,106, and 8,483 unique class I and class II MHC-restricted T-cell epitopes that are shared by all mentioned orthopoxviruses. Subsequently, we developed an immunological resource, EPIPOX, upon the predicted T-cell epitome. EPIPOX is freely available online and it has been designed to facilitate reverse vaccinology. Thus, EPIPOX includes key epitope-focused protein annotations: time point expression, presence of leader and transmembrane signals, and known location on outer membrane structures of the infective viruses. These features can be used to select specific T-cell epitopes suitable for experimental validation restricted by single MHC alleles, as combinations thereof, or by MHC supertypes.

1. Introduction

Smallpox was a devastating contagious disease that ravaged humankind for millennia, wiping out entire civilizations [1]. The disease was caused by two types of variola virus (VARV), major and minor, which differed greatly in their average mortality rates: 30% versus 1%, respectively. VARV major was the most prevalent form [2, 3]. Systematic vaccination against smallpox began in the early 19th century but the disease lingered until the World Health Organization (WHO) initiated worldwide vaccination campaigns in 1967. The last case was reported in Somalia in 1977 and in May 1980 the WHO declared that smallpox had been eradicated, ceasing vaccination [1, 2]. Eradication was facilitated because there are no animal reservoirs for the virus, as it only infects humans [4].

VARV belongs to the *Orthopox* genus of the *Poxviridae* family, consisting of large double-stranded DNA viruses that

replicate in the cytoplasm of infected cells [5, 6]. Poxviruses are large and complex with ~250 genes and a multistage life cycle, producing different infective forms including intracellular mature virions (IMV) and extracellular enveloped virus (EEV) [5, 6]. Humans can be infected by several poxviruses; the closest to VARV that are also pathogenic to humans are vaccinia (VACV), cowpox (CPXV), and monkeypox (MPXV) viruses [7, 8]. The primary reservoir of MPXV is rodents [9], while CPXV has the broadest animal reservoir range of all poxvirus, including cats, dogs, elephants, and rodents [10]. Historically, VACV has been considered to emerge after repeated passages from an ancestral CPXV [11]. However, phylogenetic studies question that view and there are some speculations that VACV could be a horsepox virus (HPXV) [12]; yet both, the host and origin of VACV, remain unknown [13]. VACV and CPXV infections in humans are generally mild and self-limiting and can induce cross-protective

immunity [14]. The observation that CPXV sufferers did not get smallpox led Edward Jenner in 1798 to introduce a method of vaccination through scarifications with *Variolae Vaccinae*, Latin, for CPXV [15]. Immunization with CPXV was eventually displaced by VACV vaccine, which was used subsequently for global smallpox vaccination [12].

As smallpox was eradicated and vaccination ceased, the global population has become increasingly susceptible to both smallpox and zoonosis by orthopoxviruses [8, 9]. People under 30 have no immunity against these viruses and VACV-induced immunity is waning in those that were vaccinated [16]. Despite recommendations by the WHO, stockpiles of smallpox virus had never been destroyed and there are concerns that unregistered stocks could be used as a weapon of bioterrorism [17]. Several features make smallpox a major terrorist threat. It replicates easily, is aerosolizable, and is highly contagious before, during, and after disease onset. Moreover, smallpox is lethal and disfiguring and has already been used as a biological weapon in North America during the French and Indian Wars [18]. Thus, there is renewed interest in the development of vaccines against smallpox, particularly safer ones, since immunization with VACV can result in serious adverse events and it is considered risky in immunocompromised or immune-suppressed individuals [19].

Immune protection against orthopoxviruses requires both B and T cells [20] but the relevance of T cells is paramount. CD8 T cells are required to eliminate infected cells, while help by CD4 T cells is essential to elicit effective humoral responses [21]. Thus, people with dysfunctional humoral responses (e.g., agammaglobulinemia) can be vaccinated with VACV, while those with loss of T cells cannot as they can suffer severe disease [22]. T-cell immune responses are triggered by the recognition of foreign peptides bound to cell surface-expressed major histocompatibility complex (MHC) molecules, also known as human leukocyte antigens, HLA, in humans. CD4 T cells recognize peptides presented by MHC class II (MHC II) molecules while CD8 T cells recognize peptides presented by MHC class I (MHC I) molecules.

Advances in both immunology and genomic analysis offer new possibilities for eliciting immune protection without the requirement for live-virus vaccination and attendant complications. The identification of HLA class I and class II restricted T-cell epitopes (CD8 and CD4 T-cell epitopes, resp.) from poxviruses may allow us to develop safe and yet immunogenic peptide-based vaccines. Here, we describe the identification of protein antigens that are shared between several pathogenic orthopoxviruses, including VARV, MPXV, CPXV, and VACV, and T-cell epitopes that are identical in all selected proteins. This information was used to create a freely accessible web resource, EPIPOX: URL <http://imed.med.ucm.es/epipox/>, intended to facilitate the design of epitope-based vaccines against orthopoxviruses.

2. Materials and Methods

2.1. Orthopoxvirus Sequences and Experimentally Defined T-Cell Epitopes. In this study, we used the entire proteomes of 8 orthopoxviruses: VARV major, strain Bangladesh-1975,

TABLE 1: Orthopoxviruses used in this study.

Virus	Strain	ACC	Genes
VARV major	Bangladesh-1975	L22579	189
VARV major	India-1967	NC_00161	197
VARV minor	Garcia-1966	Y16780	206
MPXV	Zaire-96-I-16	NC_003310	191
CPXV	Brighton Red	AF482758	218
VACV	Copenhagen	M35027	262
VACV	Tian Tan	AF095689	243
VACV	Ankara*	U94848	157

* Modified strain that has lost the ability to replicate; VARV: variola virus; MPXV: monkeypox virus; CPXV: cowpox virus; VACV: vaccinia virus.

GenBank Accession: GB: L22579; VARV major, strain India-1967, GB: NC_00161; Variola major minor, strain Garcia-1966, GB: Y16780; Monkeypox virus, strain Zaire-96-I-16, GB: NC_003310; Cowpox virus strain, strain Brighton Red, GB: AF482758; Vaccinia virus, strain Copenhagen, GB: M35027; Vaccinia virus, strain Tian Tan, GB: AF095689; Vaccinia virus, strain Ankara, GB: U94848. The proteomes were obtained from the various translation features of the relevant GenBank genomic records using BIoPERL [23] (Table 1).

We also used experimentally defined poxvirus-specific HLA I and HLA II-restricted T-cell epitopes that were retrieved from the IEDB [24] and EPIMHC [25] databases. We only considered unique T-cell epitope sequences with a size of 9 amino acids that were reported to be identified in humans infected with orthopoxviruses or who were vaccinated. We provide a list of experimentally defined T-cell epitopes as supplementary material in Additional File S1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2015/738020>.

2.2. Protein Sequence Analyses and Annotations. We took VARV major, strain Bangladesh-1975, as the reference for subsequent sequence analyses. We identified proteins with leader signals using SIGNALP [26] and transmembrane regions using TMHMM [27]. We identified protein orthologs using BLAST [27]. Briefly, we first BLAST the reference proteins against formatted databases of each of the remaining orthopoxvirus proteomes. We performed BLAST searches with default settings and considered only the description of the first hit and the corresponding alignment. Subsequently, we selected those protein searches that gave hits in each of the proteomes with identities greater than 60% and identified the corresponding orthologs. We used BIoPERL to parse BLAST hits [23].

Information on the temporal expression of VACV genes was kindly provided by Dr. Lefkowitz from the Poxvirus Bioinformatics Resource Center [28]. The information consisted on annotations identifying those genes that are expressed early (E), intermediate (I), and late (L) during the life cycle of VACV. This information is provided as supplementary material in Additional File S2. In addition, we identified, from the data provided by Dr. Lefkowitz, gene products associated with the outer membranes of VACV IMV

FIGURE 1: EPIPOX database structure. EPIPOX is a relational database consisting of three main tables: *peptides*, *predictions*, and *proteins*. Table names are boxed with double lines. For each table, we show their fields and boxed with single lines the fields that work as table keys. For fields taking discrete nominal values, we show them between square brackets.

and EEV infective forms, as well as those proteins that are part of the VACV virion or CORE. This information is also included as supplementary material in Additional File S2. Protein annotations obtained for VACV were transferred to protein orthologs.

2.3. Prediction of T-Cell Epitopes. We predicted MHC I and MHC II peptide binding to anticipate potential CD8 and CD4 T-cell epitopes, respectively. Specifically, we predicted peptide-MHC binding from VARV Bangladesh proteins that are shared between all selected orthopoxviruses using 32 HLA I- and 33 HLA II-allele specific position-specific scoring matrices (PSSMs) [29–31]. For a given protein, we considered the top 2% and 4% of scoring peptides to constitute HLA I- and HLA II-binding peptides, respectively. We only predicted binding for peptides of nine residues; most HLA I-restricted peptides are 9 residues in length and while HLA II-restricted peptides vary in length (9–22 amino acids) they have a core of 9 residues that anchor the peptide in the binding groove of HLA II molecules [30, 32]. We also used N-gram language models to identify whether peptides can be generated from the source antigen by proteasomal cleavage [33]. This information is only relevant to HLA I-binding peptides, since most peptides presented by MHC I are derived from antigens degraded by the proteasome [34].

2.4. Database Building and Web Server Implementation. Predicted T-cell epitopes and obtained protein annotations were incorporated into a POSTGRES relational database. The database consists of 3 tables (*peptides*, *predictions*, and *proteins*) that are linked through unique keys (Figure 1). Briefly, table *predictions* contains peptide sequences and their MHC restriction elements; table *peptides* includes the peptide molecular weight, its protein accession number, and whether the peptide is cleaved by the proteasome; and table *proteins* contains gene product information including temporal expression (E: early, I: intermediate, and L:late), location in the virus (IMV, EEV, and CORE), and the existence of leader and transmembrane regions. We also developed a web front end or GUI to allow ready access to EPIPOX. Behind the interface is a Python script that handles database queries through underlying SQL. The EPIPOX resource is

implemented on an Apache Web server under the Mac OSX operating system.

3. Results and Discussion

3.1. Epitope-Vaccine Design against Orthopoxviruses. T-cell adaptive immunity is required for clearance of poxviruses during infection and/or vaccination and can also contribute to protective immunity from subsequent exposures [35, 36]. Moreover, peptides corresponding to VACV-specific CD8 T-cell epitopes can confer protection to mice subjected to lethal VACV challenges [37]. Fueled by the need to develop safer smallpox vaccines, such knowledge has led to the recent identification of many VACV-specific T-cell epitopes [37, 38]. These T-cell epitopes are deposited haphazardly in various specialized databases, including IEDB [24], EPIMHC [25], TEPIDAS [39], and AntiJen [40]. Of relevance for epitope-vaccine design, CD8 T-cells target primarily early and nonstructural gene products [41, 42]. CD4 T cells target late and the most abundant genes products (IMV and EEV membrane proteins and CORE proteins), as do antibodies [42, 43]. While some of the identified VAVC-specific T-cell epitopes are conserved in VARV a rational approach to identifying all potential T-cell epitopes eliciting cross-protective immunity is still required.

3.2. Shared Orthopoxvirus Proteins for Cross-Protective Immunity. Nearly all orthopoxviruses can protect against challenge with another orthopoxvirus [14]. This exquisite cross-protective immunity is likely a result of direct antigenic similarity between poxviruses. Therefore, prior to defining potential T-cell epitopes we identified shared antigens between pathogenic orthopoxviruses. Identification of shared antigens is also relevant to reducing the experimental burden associated with T-cell identification. Human pathogen orthopoxviruses have large genomes encompassing over 180 open reading frames (ORF) with the exception of VACV Ankara strain, which has only 157 genes and lacks the ability to replicate [44] (Table 1). Using VARV major, strain Bangladesh-1975, as a reference, we identified 124 ORFs that are shared between 8 different complete genomes from several orthopoxviruses, including VARV minor, CPXV, MPXV, and several VACV strains (Additional File S3). Despite the

TABLE 2: Orthopoxvirus proteins contributing to cross-protective immunity.

VACC: GI ORF	VARV: GI ORF	MPXV: GI ORF	CPXV: GI ORF	LOC ¹ /EXP ² /TM ³ /LD ⁴
335424 LIR	438991 MIR	17974993 MIR	20153082 V099	IMV/late/yes/no
335455 D8L	439016 F8L	17975018 E8L	20153106 V119	IMV/late/yes/no
335500 A27L	439052 A31L	17975052 A29L	20153143 V156	IMV/late/no/no
335508 A33R	439057 A36R	17975058 A35R	20153149 V162	EEV/early/yes/no
335549 B5R	439084 B6R	17975080 B6R	20153177 V190	EEV/#/yes/yes
335438 H3L	439004 I3L	17975006 H3L	20153094 V107	IMV/late/yes/no
335477 A10L	439032 A11L	17975034 A11L	20153122 V135	CORE/#/no/no
335341 C7L	438926 D11L	17974926 D10L	20153015 V028	U/early/no/no

Table shows GenBank identification numbers (GI) and open reading frame names (ORF) for VACC (strain Copenhagen), VARV (strain Bangladesh-1975), MPXV (strain Zaire-I-16), and CPXV (strain Brighton Red). ¹LOC: location, ²EXP: temporal expression, ³TM: transmembrane, and ⁴LD: leader signal. NS: nonstructural gene. #: information not available. U: unknown. List of proteins was obtained from [45]. Annotations 1, 2, 3, and 4 obtained as indicated elsewhere in Section 2.

criterion for selection being 60% identity, all 124 selected proteins have an average identity $\geq 85\%$ as shown in Additional File S3. These proteins are prime candidates to induce cross-protective immunity although they need to be targeted by the immune system. Interestingly, within the selected proteins there are 8 known immunogens that conferred $>60\%$ protection to VACV in animal models (Table 2) [45]. Six of these immunogens are IMV or EEV proteins carrying transmembrane regions and/or are being late gene products. Interestingly, among the selected 124 proteins we found 26 additional proteins with transmembrane regions that could also be prime vaccine subunits candidates (Table 3). Some of these proteins also have leader signal sequences (Table 3). Viral proteins with leader sequences follow the cell secretory pathway and are thus also important targets to consider for vaccine design [46, 47].

3.3. T-Cell Epitome from Pathogenic Orthopoxvirus Proteins. We targeted the shared orthopoxvirus proteins for T-cell epitopes prediction using 32 and 33 HLA I- and HLA II-specific profile matrices (details in Material and Methods). The alleles targeted for peptide binding prediction are shown in Additional File S4. We selected these alleles because there are experimental peptide-binding data for them, which is required to make accurate peptide-MHC binding predictors [48]. Incidentally, these HLA alleles are frequently expressed in the general population and targeting them for epitope prediction permits the development of epitope-based vaccines covering the entire population. These HLA allelic variants can have overlapping peptide-binding repertoires and can be clustered accordingly in supertypes [49, 50]. Selecting promiscuous peptide-binders to multiple HLA molecules facilitates the development of vaccines with a minimum number of peptides [49–51].

We predicted a total of 18726 HLA I-restricted and 32722 HLA II-restricted orthopoxvirus specific T-cell epitopes, all being identical between all orthopoxviruses considered in this study. In Additional File S4 we provide numbers of T-cell epitopes predicted by each HLA-specific profile used in this study. We predicted more CD4 than CD8 T-cell epitopes because we used a more permissive peptide-binding threshold for MHC II molecules (4% of top scoring peptides)

than for MHC I molecules (2% of top scoring peptides) since peptide-binding prediction to MHC II molecules is considerably less accurate than to MHC I molecules [46]. Interestingly, we identified only 8106 unique HLA I-restricted T-cell epitope sequences and a few more (8483) unique HLA II-restricted T-cell epitope sequences. Therefore, there is a considerable overlap between the peptide binding repertoires of HLA molecules, which is larger for HLA II molecules than for HLA I molecules. HLA I-restricted peptides bound on average to 2.3 distinct HLA I molecules, while HLA II-restricted peptides bound on average to 3.8 distinct HLA II molecules. This is due to the fact that peptide-binding to MHC II molecules is more degenerate than to MHC I molecules [29, 30]. In Additional File S5, we provide all distinct predicted peptides with the HLA molecules that they were predicted to bind. Interestingly, there is also some overlap between HLA I- and HLA II-restricted peptides. In particular, we find that there are 2452 peptides that are predicted to be restricted by both HLA I and HLA II molecules. Thus, in total the predicted T-cell epitome consisted of just 14137 unique sequences among all predicted T-cell epitopes.

We compared the predicted T-cell epitome with experimentally defined poxvirus-specific HLA-restricted T-cell epitopes deposited in the IEDB [24] and EPIMHC [25]. We retrieved 170 HLA I and 9 HLA II-restricted T-cell epitopes meeting our criteria (see Additional File S1) but we only considered for comparison 85 HLA I- and 8 HLA II-restricted T-cell epitopes that we identified here to be conserved in all orthopoxviruses considered in this study. Of those, 72 HLA I- and 6 HLA II-restricted T-cell epitopes were found within our predicted T-cell epitome. Moreover, we predicted the experimentally verified restriction element in $> 80\%$. The experimentally determined and shared epitopes that were not predicted (a minority) either are restricted by noncovered alleles or were simply not predicted. In Table 4, we summarize the data showing the verified and predicted HLA restriction elements. In sum, we readily predicted most of the experimentally verified T-cell epitopes. Considering that on average 10% of predicted T-cell epitopes can be experimentally verified [52], we shall expect that there are many more valid T-cell epitopes remaining to be validated within the T-cell epitome predicted in this study.

TABLE 3: Shared orthopoxvirus proteins with transmembrane and/or leader sequences.

VARV GI ORF	MPXV GI ORF	CPXV GI ORF	VACV GI ORF	IDEN ¹ (%)	TM ²	LEAD ³	EXP ⁴	LOCATION ⁵
GI:439084 B6R	GI:17975080 B6R	GI:20153177 V190	GI:335549 B5R	93.1	Yes	Yes	L	EEV membrane*
GI:439016 F8L	GI:17975018 E8L	GI:20153106 V119	GI:335455 D8L	94.7	Yes	No	L	IMV membrane*
GI:438990 H9R	GI:17974992 G10R	GI:20153081 V094	GI:335423 G9R	98.1	Yes	No	L	U
GI:438919 D4R	GI:17974919 D3R	GI:20153007 V020	GI:335333 C11R	88.8	Yes	Yes	U	U
GI:439085 B7R	GI:17975081 B7R	GI:20153178 V191	GI:335550 B6R	93.1	Yes	No	U	U
GI:439035 A14L	GI:17975037 A14L	GI:20153125 V138	GI:335483 A13L	88.6	Yes	No	L	IMV membrane
GI:438946 C8L	GI:17974949 C10L	GI:20153036 V049	GI:335366 F4L	97.6	Yes	No	E	U
GI:438977 K5L	GI:17974979 I5L	GI:20153068 V081	GI:335409 I5L	94.9	Yes	No	L	IMV membrane
GI:438967 E8R	GI:17974969 F7R	GI:20153058 V071	GI:335395 E8R	97.4	Yes	No	L	U
GI:439004 I3L	GI:17975006 H3L	GI:20153094 V107	GI:335438 H3L	95.8	Yes	No	L	IMV membrane*
GI:439014 F6R	GI:17975016 E6R	GI:20153104 V117	GI:335453 D6R	99.0	Yes	No	L	U
GI:439003 I2R	GI:17975005 H2R	GI:20153093 V106	GI:335437 H2R	99.2	Yes	No	L	U
GI:439056 A35L	GI:17975057 A34L	GI:20153148 V161	GI:335506 A32L	98.1	Yes	No	L	U
GI:439000 L5L	GI:17975002 L5L	GI:20153090 V103	GI:335433 J5L	98.1	Yes	No	L	U
GI:439058 A37R	GI:17975059 A36R	GI:20153150 V163	GI:335509 A34R	98.1	Yes	No	L	EEV membrane
GI:438991 M1R	GI:17974993 M1R	GI:20153082 V095	GI:335424 L1R	99.2	Yes	No	L	IMV membrane*
GI:439057 A36R	GI:17975058 A35R	GI:20153149 V162	GI:335508 A33R	93.0	Yes	No	E	EEV membrane*
GI:438951 C13L	GI:17974954 C15L	GI:20153041 V054	GI:335373 F9L	97.5	Yes	No	L	U
GI:439038 A17L	GI:17975040 A17L	GI:20153129 V142	GI:335486 A16L	97.0	Yes	No	L	U
GI:438974 K2L	GI:17974976 I2L	GI:20153065 V078	GI:335405 I2L	99.3	Yes	No	L	U
GI:439008 I7R	GI:17975010 H7R	GI:20153098 V111	GI:335442 H7R	95.2	Yes	No	L	U
GI:438982 H3L	GI:17974984 G2L	GI:20153073 V086	GI:335414 G3L	95.8	Yes	No	L	U
GI:439042 A22L	GI:17975044 A21L	GI:20153134 V147	GI:335490 A21L	96.9	Yes	No	U	U
GI:439059 A38R	GI:17975061 A38R	GI:20153152 V165	GI:335512 A36R	92.3	Yes	No	E, L	EEV membrane
GI:439031 A10L	GI:17975033 A10L	GI:20153121 V134	GI:335476 A9L	89.0	Yes	Yes	E, L	U
GI:439033 A12R	GI:17975035 A12R	GI:20153123 V136	GI:335481 A11R	98.5	Yes	No	L	U
GI:439036 A15L	GI:17975038 A15L	GI:20153126 V139	GI:335484 A14L	97.8	Yes	No	L	IMV membrane
GI:439067 A46R	GI:17975066 A43R	GI:20153159 V172	GI:335522 A43R	92.3	Yes	Yes	U	U
GI:439039 A18L	GI:17975041 A18L	GI:20153130 V143	GI:335487 A17L	98.0	Yes	No	L	IMV membrane
GI:439077 J7R	GI:17975076 B2R	GI:20153172 V185	GI:335539 A56R	82.1	Yes	Yes	E, L	EEV membrane
GI:439062 A41L	GI:17975063 A40L	GI:20153155 V168	GI:335516 A38L	94.7	Yes	Yes	U	U

Table shows GenBank identification numbers (GI) and open reading frame names (ORF) for VARV: strain Bangladesh-1975, MPXV: strain Zaire-96-I-16, CPXV: strain Brighton Red, and VACV: strain Copenhagen. ¹IDEN: average identity between the selected proteins. ²TM: transmembrane. ³LEAD: leader signal. ⁴EXP: temporal expression (E: early, I: intermediate, and L: late). ⁵LOCATION: location. *Proteins known to induce protective immunity (see Table 2). Annotations were obtained as indicated elsewhere in Section 2. U: information not found.

3.4. EPIPOX Database and Web Server. We developed a relational database based upon the predicted T-cell epitome and a web-based resource to facilitate online access and to query the database. We named this resource EPIPOX and made it available for free public use (URL: <http://imed.med.ucm.es/epipox/>). EPIPOX is a *de facto* analysis pipeline of viral T-cell epitomes. The content of the EPIPOX database is organized in three tables (*peptides*, *predictions*, and *proteins*) (Figure 1). The table *predictions* contains all predicted T-cell epitopes, consisting of 18726 HLA I- and 32722 HLA II-restricted peptides, each identified by its sequence and restriction element. Peptide sequences in this table are not unique as each peptide can bind to numerous HLA I molecules. Peptide sequences are, however, unique in the table *peptides*. This table contains 14137 sequences comprising the whole predicted epitome regardless of the restriction elements. Antigen annotations in EPIPOX are

found within the table *proteins* (Figure 1). We only included annotations that are relevant to epitope vaccine design, such as temporal expression of gene products and location in relevant structures of the virus such as the EEV and IVM membranes and CORE. Early expressed proteins and highly expressed proteins are generally thought to be more immunogenic, particularly with regard to CD8 T cells [53, 54]. On the other hand, highly abundant late proteins that are located in membrane structures of the poxvirus appear to be the main focus of the antibody and CD4 T-cell response [42, 43]. In the table *proteins*, we also provide annotations on whether the proteins have transmembrane region or leader signal sequence, as proteins with these features often interact with host cells and are important targets for subunit vaccine design [46, 47].

The EPIPOX web interface (Figure 2) allows querying of the database combining any annotation field in the database,

TABLE 4: Experimentally identified T-cell epitopes within the shared T-cell epitome predicted from pathogenic orthopoxvirus proteins.

(a)

CD8 T-cell epitopes	VARV GI	VARV ORF	Experimental HLA I restriction	HLA-A0201	HLA-A0202	HLA-A0203	Predicted HLA I restriction	HLA-A0205	HLA-A0206	HLA-A6802
ALMRIAIV	439013	F5R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLLSLFSTL	439056	A35L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLAKLTALV	438985	H5R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
NLLCHISYL	438979	K7L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
IVIEAHTV	439072	J2R	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
SLSAIIRV	439004	I3L	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLDGQLARL	438965	E6R	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLPEVISTI	438988	H7L	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
TYNDHIVNL	439072	J2R	HLA-A2301	HLA-A2301	HLA-A2402	HLA-A2403	HLA-A2403	HLA-A2405	HLA-A2407	HLA-A6802
RPPSFVKPL	439046	A25R	HLA-B7	HLA-B0702	HLA-B3501	HLA-B5101	HLA-B5101	HLA-B5301	HLA-B5401	HLA-A6802
ALDEKFLI	439045	A24R	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
FPYEGGKVF	438968	E9L	HLA-B0702	HLA-B0702	HLA-B1502	HLA-B3501	HLA-B3501	HLA-B5101	HLA-B5301	HLA-A6802
RLDYFTRV	438973	K1L	HLA-A0201, HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
ILDDNLKVV	438985	H5R	HLA-A0201, HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
LLSYVVVVV	439009	F1R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
FLIDLAFLI	438960	E1L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
FPRSMLSIF	438994	M4R	HLA-B07:02	HLA-B0702	HLA-B3501	HLA-B4402	HLA-B4402	HLA-B5301	HLA-B5401	HLA-A6802
YLFDFVISL	438996	L1R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLKLIIEPV	439009	F1R	HLA-A0201, HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
SPSNHILL	439025	A4L	HLA-B07:02	HLA-B0702	HLA-B3501	HLA-B5101	HLA-B5101	HLA-B5301	HLA-B5401	HLA-A6802
YPSNKNYEI	439033	A12R	HLA-B07:02	HLA-B0702	HLA-B3501	HLA-B5101	HLA-B5101	HLA-B5301	HLA-B5401	HLA-A6802
MLMETWFFI	439007	I6R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
ILNPVASSL	438998	L3R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
FPSVFNPFI	438968	E9L	HLA-B0702	HLA-B0702	HLA-B3501	HLA-B5101	HLA-B5101	HLA-B5301	HLA-B5401	HLA-A6802
KYQSPNIF	439043	A21R	HLA-A24, HLA-class I	HLA-A2401	HLA-A2402	HLA-A2403	HLA-A2403	HLA-A2405	HLA-A2407	HLA-A6802
YLFGGFSTL	438980	K8R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLYETVHLI	438981	H1L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
VLYNGVNYL	439009	F1R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
LIQETVHEV	439029	A8L	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
VELGSGNSF	439043	A21R	HLA-B3701	HLA-B3701	HLA-B1502	HLA-B4402	HLA-B4402	HLA-B5301	HLA-B5401	HLA-A6802
RMIAISAKV	438934	P1L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
FILGIIITV	439036	A15L	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
LLSKNTFYI	438981	H1L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
RPRDAIRFL	438961	E2L	HLA-B0702	HLA-B0702	HLA-B3501	HLA-B5101	HLA-B5101	HLA-B5301	HLA-B5401	HLA-A6802
KLFNKVPIV	438996	L1R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
SLFMILCTR	438979	K7L	HLA-A0301, HLA-A1101	HLA-A1101	HLA-A3101	HLA-A3301	HLA-A3301	HLA-A3501	HLA-A3601	HLA-A6802
ILNDEQLNL	439029	A8L	HLA-A0201	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
YLLGDSDSV	439038	A17L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
QLMYALEPR	438985	H5R	HLA-A0301, HLA-A1101	HLA-A3101	HLA-A3301	HLA-A3501	HLA-A3501	HLA-A3601	HLA-A3701	HLA-A6802
LMDENTYAM	439066	A45R	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
GLLLGCFWV	438955	C17L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802
LLSHFYPAV	438952	C14L	HLA-A2	HLA-A0201	HLA-A0202	HLA-A0203	HLA-A0203	HLA-A0205	HLA-A0206	HLA-A6802

(a) Continued.

CD8 T-cell epitopes	VARV GI	VARV ORF	Experimental HLA I restriction	Predicted HLA I restriction
NLFTFLHEI	439045	A24R	HLA-class I	HLA-A0201 HLA-A0203 HLA-A0206
GLDFVNFV	439070	A49R	HLA-A2, HLA-A0201	HLA-A0202 HLA-A0203 HLA-A0206
YLGPVVCWL	439038	A17L	HLA-A2	HLA-A0202 HLA-A0203 HLA-A0205
ILKSLGFKV	438980	K8R	HLA-A2	HLA-A0202 HLA-A0203 HLA-A0205
DEVASTHDW	439025	A4L	HLA-B4403	HLA-B4402 HLA-B5801 HLA-Cw0304
FLVIATNAM	439029	A8L	HLA-A2, HLA-A0201	HLA-A0206 HLA-A0101 HLA-A0205
LSDLKKTII	439040	A19R	HLA-A1, HLA-class I	HLA-A0101 HLA-A0205 HLA-B1501
LLYFKVFGI	439019	NIL	HLA-A2	HLA-A0202 HLA-A0203 HLA-A0206
SSNIMSESY	439014	F6R	HLA-A0101, HLA-A3002	HLA-A0101 HLA-B5801 HLA-A6802
DTRGIFSAY	439032	A1IL	HLA-A26, HLA-class I	HLA-A0207 HLA-A0205 HLA-A0207
QIDVEKKIV	439029	A8L	HLA-A0201	HLA-A0203 HLA-A0206 HLA-Cw0702
YLFRCVDAV	439030	A9R	HLA-A2	HLA-A0205 HLA-A0207 HLA-B0801
KIEDLINQL	438973	K1L	HLA-A0201	HLA-A0203 HLA-A0206 HLA-Cw0702
FTIDFKLY	439009	F1R	HLA-A1, HLA-A2601, HLA-A2902	HLA-A0101 HLA-B0801 HLA-A0207
WLKIKRDYL	439074	J4R	HLA-B0801	HLA-A0202 HLA-A0203 HLA-B4402
AINVEKIEL	473688	L6R	HLA-A0201	HLA-A0205 HLA-A0207 HLA-B4402
RIFVRVYV	439007	I6R	HLA-A2	HLA-A0203 HLA-A0205 HLA-B4402
SIIDLIDEY	438942	C4R	HLA-B1501	HLA-A0203 HLA-A0205 HLA-B4402
EERTIFLDY	439009	F1R	HLA-B4403	HLA-A0203 HLA-A0205 HLA-B4402
ILSDENYLL	439028	A7L	HLA-A2, HLA-A0201	HLA-A0203 HLA-A0205 HLA-B4402
HISALKRRY	439027	A6R	HLA-A0101, HLA-A2902	HLA-A0203 HLA-A0205 HLA-B4402
FLNISWFYI	438968	E9L	HLA-A2, HLA-A02:01	HLA-A0203 HLA-A0205 HLA-B4402
SEVKFKYVL	439002	IIL	HLA-B44	HLA-A0203 HLA-A0205 HLA-B4402
KLLWFNYL	438980	K8R	HLA-A2	HLA-A0203 HLA-A0205 HLA-B4402
YIDISDVKV	438973	K1L	HLA-A0201	HLA-A0203 HLA-A0205 HLA-B4402
VWINNSWKF	439013	F5R	HLA-A24, HLA-A2301, HLA-A2402	HLA-A0203 HLA-A0205 HLA-B4402
VLPDFDIKL	439014	F6R	HLA-A0201	HLA-A0203 HLA-A0205 HLA-B4402
VETSISDYY	439043	A2IR	HLA-B3701	HLA-A0203 HLA-A0205 HLA-B4402
SQIIDISLR	439022	A1L	HLA-A0301, HLA-A1101	HLA-A0203 HLA-A0205 HLA-B4402
HDVYGVSNF	439045	A24R	HLA-B4403	HLA-A0203 HLA-A0205 HLA-B4402

(b)

CD4 T-cell epitope	VARV GI	VARV ORF	Experimental HLA II restriction	Predicted HLA II restriction
FLIDLAFLI	438960	EIL	HLA-class II	DRB1*0101 DRB1*0301 DRB1*0401 DRB1*1302 DRB1*1502 DRB3*0101 DRB5*0101
IHWQIISSE	439072	J2R	DRB1*04:05	DRB1*0311 DRB1*0401 DRB1*1304 DRB3*0101
YIDAVVSRL	439021	N3L	DRB1*15:01	DRB1*0405 DRB1*0406 DRB1*0410 DRB1*1304 DRB3*0101
LMIDENTYAM	439066	A45R	HLA-class II	DRB1*0901 DRB1*1103 DRB3*0202
IDAVVSRL	439021	N3L	DRB1*15:01	DRB1*1103 DRB1*1201 DRB3*0202
RMIAISAKV	438934	P1L	HLA-class II	DRB1*1201 DRB1*1501 DRB3*0202

T-cell epitopes in this table are a subset of those provided in Additional File S1.

Databases
>> EpiPOX

EPIPOX DATABASE SEARCH

This resource contains potential T cell epitopes from 124 proteins that are shared between pathogenic orthopoxviruses including variola (VARV) major and minor, monkeypox (MPXV), cowpox virus (CPXV) and vaccinia (VACV) viruses. The system uses as reference VARV major, strain Bangladesh-1975

T cell epitopes can be retrieved by distinct criteria, including HLA restriction, temporal expression and several protein features.

SEARCH: SELECT HLA RESTRICTION AND VARV PROTEINS

HLA	Protein	Supertypes
☐ AND ☒ OR -- All Class I -- HLA_A0201 HLA_A0202 HLA_A0203 HLA_A0205 HLA_A0206 HLA_A0207 HLA_A0301 HLA_A1 HLA_A1101 HLA_A2301 HLA_A2402 HLA_A2403 HLA_A2405	All 439072 J2R 439041 A20L 438962 E3L 438944 C6L 438941 C3L 438927 D12L 439016 F8L 473688 L6R 438981 H1L 439067 A46R 439070 A49R 439044 A23R 438920 D5L	☐ A2: (A*0201 A*0202 A*0203 A*0205 A*0206) ☐ A3: (A*3301, A*1101, A*3101, A*0301, A*6801) ☐ B7: (B*5301, B*3501, B*0702, B*5401, B*5101)

LIMIT SEARCH RESULTS

SEQ:		
LEADER: ?		All
TRANS: ?		All
EXPRESS: ?		All E: Early I: Intermediate L: Late
LOCATION: ?		All CORE IMV EEV
PCT OPT: ?		All
CLEAVED: ?		All

Reset
Search

FIGURE 2: EPIPOX input page. The input page of EPIPOX is divided in two main sections for intuitive use. In the first part (SEARCH), users select HLA molecules and proteins to retrieve T-cell epitopes (multiple selection is allowed) while in the second part the user can limit the search output according to various criteria. These criteria include temporal expression of gene products (E: early; I: intermediate; L: late), location of proteins in relevant structures of the virus (CORE, IMV, and EEV), and the presence of leader and transmembrane regions. In addition, users can select only those peptides with a relative score above some selectable value. HLA-specific profiles used to score T-cell epitopes can reach a maximum score, which is used to set the relative score in percentage of each peptide. For HLA I-restricted epitopes, users can also restrict the search to those epitopes potentially generated by the proteasome.

as described above. For intuitive use, the interface is divided in two main sections. In the first section (SEARCH), users select proteins and restriction elements for epitope retrieval. In this section, EPIPOX also provides the option to query the database for promiscuous T-cell epitopes binding to three HLA I supertypes (A2, A3, and B7). The alleles belonging to these supertypes are present in 88% of the population regardless of their ethnic groups. Selecting promiscuous peptides restricted by these 3 supertypes facilitates maximizing the population coverage of vaccines with minimum numbers of peptides [49, 50, 55]. In the second section (LIMIT), users can select annotation criteria to restrict the results.

As an example, in Figure 3, we show the page resulting from a sample query consisting of promiscuous peptides from CORE binding to the A2 supertype. From the EPIPOX output, users can also access additional information available from the Virus Pathogen Resource database (Figure 3) [56].

EPIPOX is related somewhat to certain existing databases. On the one hand, it shares features with generic epitope databases such as EPIMHC [25], AntiJen [40], and IEDB [24] and on the other hand it shares features with poxvirus genome annotation-orientated databases such as the *Poxviridae* database [28] (no longer operating) and the Virus Pathogen Resource (<http://www.viprbrc.org/>) [56].

>> EPIPOX Database										
SOURCE GI	SOURCE NAME	MTX	SEQ	CLASS	CLEAVE	PCT OPT	LOCATION	EXPRESS	LEADER	TRANS
47 results found										
439025	A4L	HLA_A0201	ILASILSV	1	Yes	82.46	CORE	Late	No	No
Protein: Major core protein 4b										
Protein Information: ²				SEQ						
(1)				(2)						
Protein Name:				Major core protein 4b						
UniProtKB Accession:				P33818						
GenBank Protein Accession:				AAA60855.1						
GenBank Protein GI:				439025						
Source:				GenBank						
Protein Sequence:				View Sequence						
Comment:				homolog of vaccinia virus CDS A3L (major core protein p4b); putative						
Keywords:				Complete proteome; Reference proteome; Virion						
438994	M4R	HLA_A0206	ALISKYAGI	1						
438994	M4R	HLA_A0207	ALISKYAGI	1						
438994	M4R	HLA_A6802	ALISKYAGI	1	Yes	63.37	CORE	Late	No	No
439025	A4L	HLA_A0201	MIRKAIMNI	1	No	69.3	CORE	Late	No	No
439025	A4L	HLA_A0202	MIRKAIMNI	1	No	63.27	CORE	Late	No	No
439025	A4L	HLA_A0203	MIRKAIMNI	1	No	54.84	CORE	Late	No	No
439025	A4L	HLA_A0205	MIRKAIMNI	1	No	65.28	CORE	Late	No	No
439025	A4L	HLA_A0206	MIRKAIMNI	1	No	57.47	CORE	Late	No	No
439034	A13L	HLA_A0201	QLKNLLAQI	1	Yes	77.19	CORE	Late	Yes	No
439034	A13L	HLA_A0202	QLKNLLAQI	1	Yes	65.31	CORE	Late	Yes	No
439034	A13L	HLA_A0203	QLKNLLAQI	1	Yes	68.82	CORE	Late	Yes	No
439034	A13L	HLA_A0205	QLKNLLAQI	1	Yes	53.47	CORE	Late	Yes	No
439034	A13L	HLA_A0206	QLKNLLAQI	1	Yes	61.49	CORE	Late	Yes	No
439034	A13L	HLA_A0207	QLKNLLAQI	1	Yes	47.32	CORE	Late	Yes	No
439034	A13L	HLA_A6802	QLKNLLAQI	1	Yes	48.26	CORE	Late	Yes	No

FIGURE 3: EPIPOX result page. The figure shows a slice of the output resulting from promiscuous CORE protein peptides binding to the A2 supertype. The output consists of a tabulated list, with information on each of the fields of the search query (columns). From field *SOURCE NAME* (1), users can access proteins from the Virus Pathogen Database (<http://www.viprbrc.org/>) (1) and by clicking on the epitope sequence, field *SEQ* (2), users will get the amino acid sequence of the protein showing the peptide in bold (2).

This later resource contains information on virus sequences, functional annotations and epitopes derived from IEDB [24]. However, the Virus Pathogen Resource does not allow selection of epitopes or antigens by criteria that are relevant to epitope vaccine design. In fact, EPIPOX is the only dedicated immunologic resource that has been designed to facilitate the rational selection of epitopes and antigens for subunit vaccine design.

4. Conclusions and Future Development

The availability of the VARV genomes enables the use of predictive tools that reveal entire T-cell epitomes and facilitate the development of epitope-based vaccines. However, in large and complex viruses, such as VARV, the potential T-cell epitome can be so sizeable that it will challenge experimental validation. Therefore, in this work we applied a rational strategy to limit the list of potential T-cell epitopes. First, we reduced the number of antigens by half by simply selecting those that are conserved among pathogenic orthopoxviruses related to VARV. Second, we enriched the antigens with annotations such as temporal expression and location. Lastly, we created a resource and *de facto* analysis pipeline (EPIPOX) with which to interrogate the resulting T-cell epitome and enable users to select immunologically relevant subsets of T-cell epitopes suitable for experimental validation.

We expect that this work and EPIPOX will be instrumental in developing safer smallpox vaccines and thereby in preventing zoonosis caused by other orthopoxviruses, including MPXV, which is also a potential terrorist bioweapon. In the future, we plan to enhance EPIPOX with validated and/or experimentally determined epitopes, upgrade protein annotations with functional information, and include additional features such as TAP transport [57], ERAAP cleavage [58], and T-cell epitope immunodominance. In sum, we would expect EPIPOX to establish itself as a facilitating resource of true utility in *inter alia* immunoinformatic characterization of viral genomics and computational reverse vaccinology.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors want to specially thank Dr. Ellis L Reinherz for his continuous support, inspiration, and future collaborative work. The authors also wish to thank *Immunotek*, SL, for financial support of MMA, and the Spanish Department of Science at MINECO for supporting the group's research through Grants SAF2006:07879, SAF2009:08301, and BIO2014:54164-R to Pedro A. Reche. Finally, The authors

wish to acknowledge Hong Zhang for programming assistance.

References

- [1] D. R. Hopkins, *The Greatest Killer: Smallpox in History, with a New Introduction*, University of Chicago Press, Chicago, Ill, USA, 2002.
- [2] F. Fenner, R. Wittek, and K. Dumbell, *The Orthopoxviruses*, Academic Press, San Diego, Calif, USA, 1998.
- [3] R. M. L. Buller and G. J. Palumbo, "Poxvirus pathogenesis," *Microbiological Reviews*, vol. 55, no. 1, pp. 80–122, 1991.
- [4] D. Baxby, "Poxvirus hosts and reservoirs. Brief review," *Archives of Virology*, vol. 55, no. 3, pp. 169–179, 1977.
- [5] B. Moss, "Poxviridae. The virus and their replication," in *Fields Virology*, B. N. Fields, D. M. Knipe, and P. M. Howley, Eds., pp. 2849–2883, Lippincott-Raven Publishers, Hagerstown, Md, USA, 2001.
- [6] K. L. Roberts and G. L. Smith, "Vaccinia virus morphogenesis and dissemination," *Trends in Microbiology*, vol. 16, no. 10, pp. 472–479, 2008.
- [7] I. V. Babkin and I. N. Babkina, "The origin of the variola virus," *Viruses*, vol. 7, no. 3, pp. 1100–1112, 2015.
- [8] S. N. Shchelkunov, S. S. Marennikova, and R. W. Moyer, *Orthopoxviruses Pathogenic for Humans*, Springer, Berlin, Germany, 2005.
- [9] S. Parker, A. Nuara, R. M. L. Buller, and D. A. Schultz, "Human monkeypox: an emerging zoonotic disease," *Future Microbiology*, vol. 2, no. 1, pp. 17–34, 2007.
- [10] G. McFadden, "Poxvirus tropism," *Nature Reviews Microbiology*, vol. 3, no. 3, pp. 201–213, 2005.
- [11] D. A. Henderson and B. Moss, "Smallpox and vaccinia," in *Vaccines*, S. A. Plotkin and W. A. Orenstein, Eds., W.B. Saunders Company, Philadelphia, Pa, USA, 1999.
- [12] L. Sánchez-Sampedro, B. Perdiguero, E. Mejías-Pérez, J. García-Arriaza, M. Di Pilato, and M. Esteban, "The evolution of poxvirus vaccines," *Viruses*, vol. 7, no. 4, pp. 1726–1803, 2015.
- [13] E. R. Tulman, G. Delhon, C. L. Afonso et al., "Genome of horsepox virus," *Journal of Virology*, vol. 80, no. 18, pp. 9244–9258, 2006.
- [14] B. L. Jacobs, J. O. Langland, K. V. Kibler et al., "Vaccinia virus vaccines: past, present and future," *Antiviral Research*, vol. 84, no. 1, pp. 1–13, 2009.
- [15] E. Jenner, *An Inquiry into the Causes and Effects of the Variolae Vaccinae: A Disease Discovered in Some of the Western Counties of England, Particularly Gloucestershire, and Known by the Name of the Cow Pox*, Sampson Low, London, UK, 1798.
- [16] H. Nishiura and M. Eichner, "Estimation of the duration of vaccine-induced residual protection against severe and fatal smallpox based on secondary vaccination failure," *Infection*, vol. 34, no. 5, pp. 241–246, 2006.
- [17] D. A. Henderson, T. V. Inglesby, J. G. Bartlett et al., "Smallpox as a biological weapon: medical and public health management. Working Group on Civilian Biodefense," *The Journal of the American Medical Association*, vol. 281, no. 22, pp. 2127–2137, 1999.
- [18] E. A. Fenn, "Biological warfare in eighteenth-century North America: beyond Jeffery Amherst," *Journal of American History*, vol. 86, no. 4, pp. 1552–1580, 2000.
- [19] D. M. Maurer, B. Harrington, and J. M. Lane, "Smallpox vaccine: contraindications, administration, and adverse reactions," *American Family Physician*, vol. 68, no. 5, pp. 889–896, 2003.
- [20] R. B. Kennedy, I. G. Ovsyannikova, R. M. Jacobson, and G. A. Poland, "The immunology of smallpox vaccines," *Current Opinion in Immunology*, vol. 21, no. 3, pp. 314–320, 2009.
- [21] G. Chaudhri, V. Tahiliani, P. Eldi, and G. Karupiah, "Vaccine-induced protection against orthopoxvirus infection is mediated through the combined functions of CD4 T cell-dependent antibody and CD8 T cell responses," *Journal of Virology*, vol. 89, no. 3, pp. 1889–1899, 2015.
- [22] M. Bray and M. E. Wright, "Progressive vaccinia," *Clinical Infectious Diseases*, vol. 36, no. 6, pp. 766–774, 2003.
- [23] J. E. Stajich, D. Block, K. Boulez et al., "The Bioperl toolkit: perl modules for the life sciences," *Genome Research*, vol. 12, no. 10, pp. 1611–1618, 2002.
- [24] R. Vita, J. A. Overton, J. A. Greenbaum et al., "The immune epitope database (IEDB) 3.0," *Nucleic Acids Research*, vol. 43, no. 1, pp. D405–D412, 2015.
- [25] P. A. Reche, H. Zhang, J.-P. Glutting, and E. L. Reinherz, "EPIMHC: a curated database of MHC-binding peptides for customized computational vaccinology," *Bioinformatics*, vol. 21, no. 9, pp. 2140–2141, 2005.
- [26] J. D. Bendtsen, H. Nielsen, G. von Heijne, and S. Brunak, "Improved prediction of signal peptides: SignalP 3.0," *Journal of Molecular Biology*, vol. 340, no. 4, pp. 783–795, 2004.
- [27] E. L. Sonnhammer, G. von Heijne, and A. Krogh, "A hidden Markov model for predicting transmembrane helices in protein sequences," in *Proceedings of the International Conference on Intelligent Systems for Molecular Biology (ISMB '98)*, vol. 6, pp. 175–182, Montreal, Canada, June-July 1998.
- [28] E. J. Lefkowitz, C. Upton, S. S. Changayil, C. Buck, P. Traktman, and R. M. L. Buller, "Poxvirus Bioinformatics Resource Center: a comprehensive Poxviridae informational and analytical resource," *Nucleic Acids Research*, vol. 33, pp. D311–D316, 2005.
- [29] P. A. Reche, J.-P. Glutting, and E. L. Reinherz, "Prediction of MHC class I binding peptides using profile motifs," *Human Immunology*, vol. 63, no. 9, pp. 701–709, 2002.
- [30] P. A. Reche, J.-P. Glutting, H. Zhang, and E. L. Reinherz, "Enhancement to the RANKPEP resource for the prediction of peptide binding to MHC molecules using profiles," *Immunogenetics*, vol. 56, no. 6, pp. 405–419, 2004.
- [31] P. A. Reche and E. L. Reinherz, "Prediction of peptide-MHC binding using profiles," *Methods in Molecular Biology*, vol. 409, pp. 185–200, 2007.
- [32] P. A. Reche and E. L. Reinherz, "Sequence variability analysis of human class I and class II MHC molecules: functional and structural correlates of amino acid polymorphisms," *Journal of Molecular Biology*, vol. 331, no. 3, pp. 623–641, 2003.
- [33] C. M. Diez-Rivero, E. M. Lafuente, and P. A. Reche, "Computational analysis and modeling of cleavage by the immunoproteasome and the constitutive proteasome," *BMC Bioinformatics*, vol. 11, article 479, 2010.
- [34] J. S. Blum, P. A. Wearsch, and P. Cresswell, "Pathways of antigen processing," *Annual Review of Immunology*, vol. 31, no. 1, pp. 443–473, 2013.
- [35] W. E. Demkowicz Jr., R. A. Littaua, J. Wang, and F. A. Ennis, "Human cytotoxic T-cell memory: long-lived responses to vaccinia virus," *Journal of Virology*, vol. 70, no. 4, pp. 2627–2631, 1996.

- [36] B. Moss, "Smallpox vaccines: targets of protective immunity," *Immunological Reviews*, vol. 239, no. 1, pp. 8–26, 2011.
- [37] M. Moutaftsi, S. Salek-Ardakani, M. Croft et al., "Correlates of protection efficacy induced by vaccinia virus-specific CD8⁺ T-cell epitopes in the murine intranasal challenge model," *European Journal of Immunology*, vol. 39, no. 3, pp. 717–722, 2009.
- [38] W. Kastenmuller, I. Drexler, H. Ludwig et al., "Infection of human dendritic cells with recombinant vaccinia virus MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity," *Virology*, vol. 350, no. 2, pp. 276–288, 2006.
- [39] C. M. Diez-Rivero, M. Garcia-Boronat, and P. A. Reche, "Integrating T-cell epitope annotations with sequence and structural information using DAS," *Bioinformatics*, vol. 3, no. 4, pp. 156–158, 2008.
- [40] C. P. Toseland, D. J. Clayton, H. McSparron et al., "AntiJen: a quantitative immunology database integrating functional, thermodynamic, kinetic, biophysical, and cellular data," *Immunome Research*, vol. 1, no. 1, article 4, 2005.
- [41] C. Oseroff, F. Kos, H.-H. Bui et al., "HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13980–13985, 2005.
- [42] C. L. Smith, F. Mirza, V. Pasquetto et al., "Immunodominance of poxviral-specific CTL in a human trial of recombinant-modified vaccinia Ankara," *The Journal of Immunology*, vol. 175, no. 12, pp. 8431–8437, 2005.
- [43] L. Jing, D. H. Davies, T. M. Chong et al., "An extremely diverse CD4 response to vaccinia virus in humans is revealed by proteome-wide T-cell profiling," *Journal of Virology*, vol. 82, no. 14, pp. 7120–7134, 2008.
- [44] H. Meyer, G. Sutter, and A. Mayr, "Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence," *Journal of General Virology*, vol. 72, no. 5, pp. 1031–1038, 1991.
- [45] J. W. Golden and J. W. Hooper, "The strategic use of novel smallpox vaccines in the post-eradication world," *Expert Review of Vaccines*, vol. 10, no. 7, pp. 1021–1035, 2011.
- [46] M. Kim, H. Yang, S.-K. Kim et al., "Biochemical and functional analysis of smallpox growth factor (SPGF) and anti-SPGF monoclonal antibodies," *The Journal of Biological Chemistry*, vol. 279, no. 24, pp. 25838–25848, 2004.
- [47] H. Yang, S.-K. Kim, M. Kim et al., "Antiviral chemotherapy facilitates control of poxvirus infections through inhibition of cellular signal transduction," *Journal of Clinical Investigation*, vol. 115, no. 2, pp. 379–387, 2005.
- [48] E. M. Lafuente and P. A. Reche, "Prediction of MHC-peptide binding: a systematic and comprehensive overview," *Current Pharmaceutical Design*, vol. 15, no. 28, pp. 3209–3220, 2009.
- [49] P. A. Reche and E. L. Reinherz, "PEPVAC: a web server for multi-epitope vaccine development based on the prediction of supertypic MHC ligands," *Nucleic Acids Research*, vol. 33, no. 2, pp. W138–W142, 2005.
- [50] A. Sette and J. Sidney, "HLA supertypes and supermotifs: a functional perspective on HLA polymorphism," *Current Opinion in Immunology*, vol. 10, no. 4, pp. 478–482, 1998.
- [51] M. Molero-Abraham, E. M. Lafuente, D. R. Flower, and P. A. Reche, "Selection of conserved epitopes from hepatitis c virus for pan-populational stimulation of T-cell responses," *Clinical and Developmental Immunology*, vol. 2013, Article ID 601943, 10 pages, 2013.
- [52] W. Zhong, P. A. Reche, C.-C. Lai, B. Reinhold, and E. L. Reinherz, "Genome-wide characterization of a viral cytotoxic T lymphocyte epitope repertoire," *Journal of Biological Chemistry*, vol. 278, no. 46, pp. 45135–45144, 2003.
- [53] W. Kastenmuller, G. Gasteiger, J. H. Gronau et al., "Cross-competition of CD8⁺ T cells shapes the immunodominance hierarchy during boost vaccination," *Journal of Experimental Medicine*, vol. 204, no. 9, pp. 2187–2198, 2007.
- [54] C. M. Diez-Rivero and P. A. Reche, "CD8 T cell epitope distribution in viruses reveals patterns of protein biosynthesis," *PLoS ONE*, vol. 7, no. 8, Article ID e43674, 2012.
- [55] P. A. Reche and E. L. Reinherz, "Definition of MHC supertypes through clustering of MHC peptide-binding repertoires," *Methods in Molecular Biology*, vol. 409, pp. 163–173, 2007.
- [56] B. E. Pickett, E. L. Sadat, Y. Zhang et al., "ViPR: an open bioinformatics database and analysis resource for virology research," *Nucleic Acids Research*, vol. 40, no. 1, pp. D593–D598, 2012.
- [57] C. M. Diez-Rivero, B. Chenlo, P. Zuluaga, and P. A. Reche, "Quantitative modeling of peptide binding to TAP using support vector machine," *Proteins: Structure, Function, and Bioinformatics*, vol. 78, no. 1, pp. 63–72, 2010.
- [58] L. Saveanu, O. Carroll, V. Lindo et al., "Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum," *Nature Immunology*, vol. 6, no. 7, pp. 689–697, 2005.