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RESEARCH PAPER

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iVAX: An integrated toolkit for the selection and optimization of antigens and the design of epitope-driven vaccines

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Abbreviations: CIV, Conventional Influenza Vaccine, CTL, Cytotoxic T Lymphocytes, DC, Dendritic Cells, ICS, Immunogenic Consensus sequence, IFN-γ, Interferon-gamma, *H. pylori*, Helicobacter pylori, HA, Hemagglutinin, HCV, Hepatitis C Virus, HIV, Human Immunodeficiency Virus, HLA, Human Leucocyte Antigen, MHC, Major Histocompatibility Complex, ORF, Open Reading Frame, PBMC, Peripheral Blood Mononuclear Cells, S-OIV, Swine-Origin Influenza Virus, TB, Tuberculosis, TCR, T-Cell Receptor, Treg, T regulatory cells

Computational vaccine design, also known as computational vaccinology, encompasses epitope mapping, antigen selection and immunogen design using computational tools. The iVAX toolkit is an integrated set of tools that has been in development since 1998 by De Groot and Martin. It comprises a suite of immunoinformatics algorithms for triaging candidate antigens, selecting immunogenic and conserved T cell epitopes, eliminating regulatory T cell epitopes, and optimizing antigens for immunogenicity and protection against disease. iVAX has been applied to vaccine development programs for emerging infectious diseases, cancer antigens and biodefense targets. Several iVAX vaccine design projects have had success in pre-clinical studies in animal models and are progressing toward clinical studies. The toolkit now incorporates a range of immunoinformatics tools for infectious disease and cancer immunotherapy vaccine design. This article will provide a guide to the iVAX approach to computational vaccinology.

Introduction

After two decades of testing and validation, methods for selecting and optimizing antigens using computational vaccinology tools have been integrated into the vaccine development process in numerous commercial and academic vaccine discovery programs. Even more recently, vaccinologists have recognized the role that *in silico* immunogenicity assessments can play in terms of understanding the efficacy of licensed vaccines. As much for immunopathogenesis as for antigen selection or prediction of vaccine efficacy, immunoinformatics tools have proven to be essential components of the complete vaccine toolkit. The number of tools available has expanded dramatically over the last few years, as well as the number of published studies citing "immunoinformatics" or "computational vaccinology" (**Fig. 1**). Individual computational vaccinology tools, primarily for epitope prediction, are available from a range of sources, such as Jenner-predict[1] and VaxiJen[2] and IEDB.[3]

While a selection of computational vaccinology tools are available, most of these tools are 'stand-alone' algorithms that have not been integrated into a single, continuous workflow. Beginning in 2002, Martin and De Groot at EpiVax have developed an integrated approach to computational vaccine design that was first described in 2003 at a seminal meeting organized by the Novartis Foundation.[4] This set of tools has been further developed and expanded into a single integrated online website, the iVAX toolkit. The toolkit uniquely offers immunoinformatics-driven antigen selection and epitope-based vaccine design as well as the potential for vaccine enhancement using epitope-based immune modulation and removal of Treg epitopes that are discovered using the toolkit. A high-level summary of recent work performed with the iVAX toolkit is provided here, including brief examples of vaccine projects that illustrate the individual tools.

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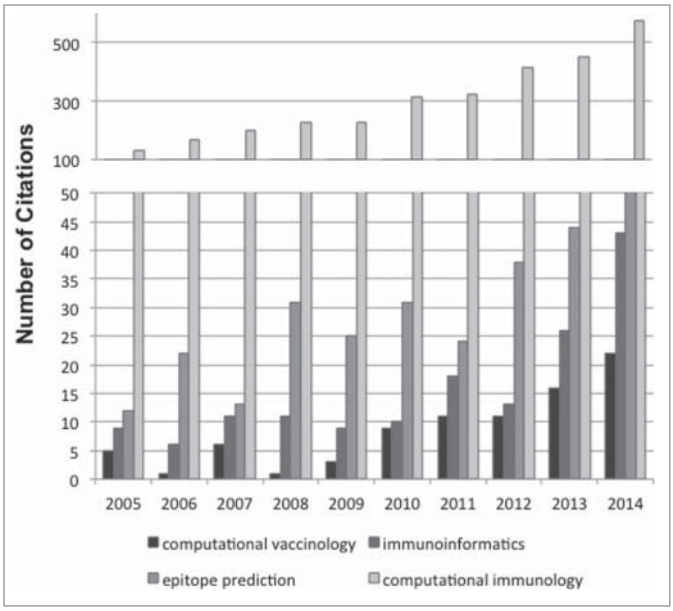

Figure 1. Increasing number of immunology studies involving *in silico* approaches over the last decade. Metrics were extracted from NCBI PubMed for search terms indicated. In the case of epitope prediction, keywords were linked together within quotations in order to return relevant results.

Computational vaccinology

Genome- and 'immunome'-mining tools have played a major role in the design and development of subunit and epitope-based vaccines for infectious diseases in the last decade. The process was at first called "vaccinomics" by Brusic and Petrovsky in 2002.[5] Alternative terms include "reverse vaccinology," a term coined by Rappuoli in 2003[6] and "immunome-derived or genome-derived vaccine design" by Pederson;[7] De Groot and Martin;[4] and Doytchinova, Taylor, and Flower (**Fig. 2**).[8] This method uses computational algorithms to identify a minimal set of immunogens, which may be whole proteins or individual epitopes, which will induce a competent immune response to a pathogen or neoplasm. Enhancement of existing antigens by integrating conserved and immunogenic epitopes, and administration of these immunogens in the right delivery vehicle and with the correct adjuvant has been shown to drive immune responses against infection the pathogen. In short, the computational approach to developing vaccines is based on the concept that selection and design of the epitope or antigen 'payload' is critically important for vaccine development.

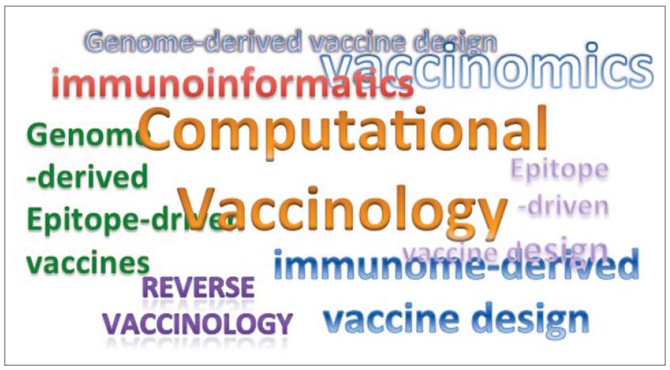

Figure 2. Computational immunology word cloud. Computational immunology Google search term results were input into the Wordle word cloud generator (http://www.wordle.net/) that sizes terms by frequency. The Google search for computational vaccinology terms was performed in March 2015.

Immunoinformatics, as applied to computational vaccinology, allows selection of the payload with significant advantages over conventional vaccines for infectious diseases. For instance, although some viruses exhibit a high degree of strain-to-strain variation at the protein level, highly conserved, immunogenic T cell epitopes can be identified using computational methods; examples can be found in preclinical studies related to tuberculosis (TB), Human Immunodeficiency Virus (HIV), smallpox and *H. pylori* published by our group[9-16] and in those published by other gene-to-vaccine researchers (Sette and Newman,[17] Brusic,[18] Petrovsky,[19] Reche,[20] and He,[21] for example). Vaccines that include multiple epitopes in a single delivery vehicle have been shown to elicit epitope-specific, broad-based immune response, conferring protection against several strains. These vaccines performed at least equivalent to existing whole-protein vaccines, and may show improved efficacy when compared to the whole organism or lysate in animal challenge studies.[22,23] Furthermore, epitope-based vaccines limit the antigenic load, diminishing the need to manufacture and administer large quantities of immunogen, much of which will be immunologically irrelevant or potentially tolerogenic. New immunoinformatics tools also make it possible to select potent T effector epitopes and to remove epitopes that may drive immunopathogenic or immune modulating responses by virtue of cross-reactivity with (human) host sequences.[24-27]

Only two comprehensive vaccine design toolkits have been developed to date. Dr. Yonqun He of University of Michigan has concatenated previously developed "public" tools with other tools developed by his group to construct a toolkit that can be used to select vaccine components; limited validation of this toolkit has been published.[28] In contrast, the set of tools comprised in the iVAX toolkit was developed entirely by the team of De Groot and Martin, with the support of the NIH (NIAID/DAIT), and these tools are currently in use by EpiVax, its commercial clients and academic collaborators. Use by vaccine researchers for collaborative research programs is provided through EpiVax' academic partner, the Institute for Immunology and Informatics (iCubed) at the University of Rhode Island. External collaborators are actively using these tools for a wide range of vaccine programs that are in progress, including research on vaccines against neglected tropical diseases such as Chagas disease, malaria, and HIV.

Significant advancements in immunome-derived, epitope-driven vaccines designed using the iVAX toolkit have been made in recent years; in particular, basic research studies have demonstrated the ability of the tools to select epitopes for Burkholderia species,[29] *H. pylori*[30] and HCV,[31-33] and validated the importance of the presence of T cell epitopes in subunit vaccines. Recently, the collaborating groups at EpiVax and iCubed discovered epitopes that activate T regulatory cells (Tregs) in viral pathogens using

the JanusMatrix tool.[24,34-36] Epitope-driven vaccines designed using the iVAX toolkit have been tested using a number of new delivery methods that will also be reviewed below.

Computational vaccine design tools, the iVAX toolkit

Epitope mapping and design tools

iVAX is an immunoinformatics toolkit consisting of integrated algorithms that facilitate the generation of epitope-based vaccines from whole genome sequences, comprising EpiMatrix, ClustiMer, Conservatrix, EpiAssembler, VaccineCAD, and JanusMatrix (**Fig. 3**). These algorithms are described in detail in the following sections, followed by a brief series of case studies that illustrate their use in vaccine design.

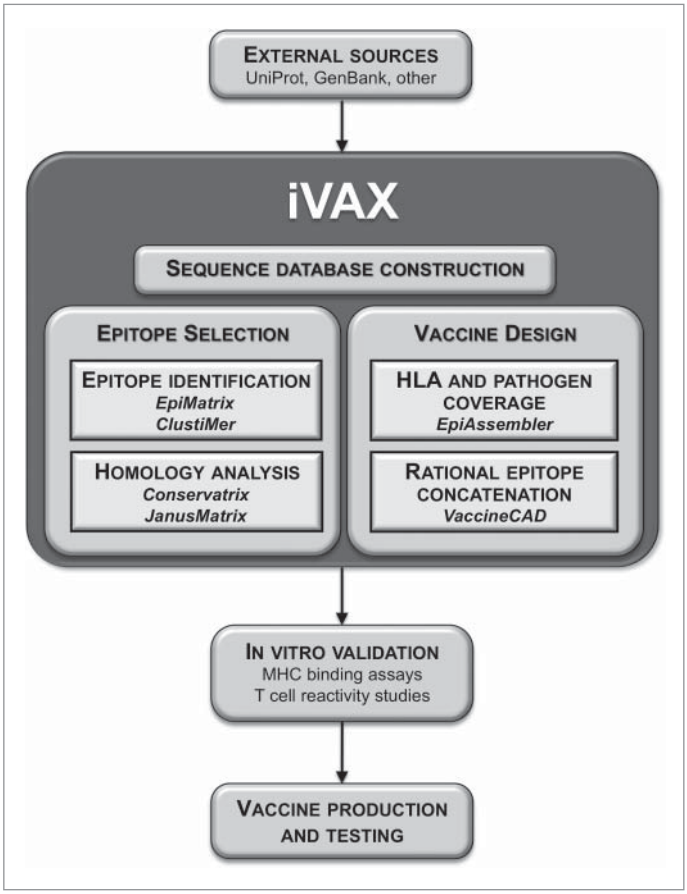

Figure 3. Integration of iVAX into the epitope-driven vaccine development pipeline. A development pathway from genome to vaccine is shown. Pathogen-derived genetic sequences obtained from various sources are initially built into a database in iVAX. T cell epitope vaccine candidates are selected using epitope identification and homology algorithms according to user-defined preferences for HLA coverage, pathogen strain coverage and T cell cross-reactivity. Vaccine construction algorithms assemble epitope ensembles into multi-epitope sequences containing the necessary and sufficient epitope content to activate strong T cell responses specific to the target pathogen. iVAX accelerates epitope identification in comparison with experimental methods using overlapping peptide libraries, for example. *In silico* predictions are confirmed using *in vitro* methods and vaccines are produced and tested for immunogenicity and efficacy.

EpiMatrix

At the heart of the iVAX toolkit is EpiMatrix, an algorithm that models the interaction between a linear peptide and a molecule of the Major Histocompatibility Complex (MHC, or HLA in humans).[37] This binding event initiates the cascade of cellular signals that are necessary for a protective immune response. Advances in the development of the tool and careful curation of the training data (sets of T cell epitopes known to bind to the target HLA) have improved the accuracy of the tool over time.[38]

EpiMatrix parses input protein sequences into overlapping 'frames' of 9 amino acids, corresponding to the minimal length of an HLA-binding peptide. As each frame overlaps the previous one by 8 residues, all possible frames are considered. Using a position-specific scoring matrix, each 9-mer is assigned a score for its predicted likelihood to bind a panel of class I (A*0101, A*0201, A*0302, A*2402, B*0702, and B*4403) and class II (DRB1*0101, 0301, 0401, 0701, 0801, 1101, 1301, and 1501) HLA molecules whose amino acid preferences cover the genetic diversity of >95% of human populations worldwide.[39,40] While it may appear that this selection of alleles is limited compared to other epitope-prediction websites, EpiMatrix exploits the observation that families of HLA alleles share specific pocket preferences,[41] mitigating the problem of HLA polymorphism and diminishing the epitope-mapping redundancy that is problematic for other prediction tools. By tuning its predictive algorithms based upon representative members of these families, called "archetypes," EpiMatrix achieves broad HLA coverage without requiring predictions for a large number of individual haplotypes.

Furthermore, EpiMatrix allele-specific scores are normalized to a standard scale that is based on the observed frequency of T cell epitopes occurring in a set of random protein sequences (over 10,000 such sequences were used to develop the standard), allowing predictions to be compared across multiple alleles. Standardization allows epitopes to be scored based on their deviation from expectation, a z-score of at least 1.64 indicates that for a given HLA allele, the 9-mer belongs to the top 5% of predicted binders and is thus worthy of further consideration. Of course, a number of alternative predictors of HLA binding also exist and are available online; the last head-to-head comparison between EpiMatrix and other available epitope-mapping tools was published in 2009 [38] and new comparison is due in 2015.

However, HLA binding properties, though critical, are not sufficient to drive significant protection against challenge. Other factors that may not be identified by HLA binding predictors govern whether an epitope should be included in a vaccine. The presence of proteolytic cleavage motifs,[42] probability of transport into the appropriate pathway,[43] epitope flanking residues required for T cell recognition[44,45] or cross-conservation with the human proteome[24] may all impact immunogen selection. New tools contained in the iVAX toolkit described below address some of these considerations.

JanusMatrix

Over the past several years, networks of cross-reactive T cell epitopes have been discovered.[46-48] At the level of an HLA-bound peptide, certain amino acids are in contact with the HLA

molecule itself while others are accessible to the T-cell receptor (TCR). If TCR-facing residues from a pathogenic epitope are conserved among multiple HLA-binding sequences from the human genome, the pathogenic epitope may activate T cells specific to these human proteins. This may lead to a lack of response due to low precursor T cell frequency,[49] a regulatory response generated by natural Tregs, or a harmful autoimmune response, all of which are unwanted effects in a vaccine. To address these possibilities, we developed JanusMatrix to identify EpiMatrix-predicted epitopes that may activate tolerogenic or auto-reactive effector T cells.

JanusMatrix searches a large database of human proteins for 9-mers with the same TCR-facing amino acids as those of the input pathogen epitopes (**Fig. 4**).[24,34] The HLA-facing residues of the human 9-mer may vary as long as binding to at least one of the same alleles as the input 9-mer is preserved. In this way, epitopes that are potentially cross-reactive with the human host may be located and removed from consideration for a vaccine. Pathogen sequences can also be screened against other sequence databases built into the tool, such as human microbiome human pathogen databases. The role of JanusMatrix in vaccine design has been highlighted in several recent publications, which describe the discovery of putative Treg-inducing epitopes in HCV,[25] HIV[50] and influenza A .[34,35]

By the same token, JanusMatrix may be used to identify relationships between an input peptide and published epitopes that have been submitted to the Immune Epitope Database,[3] or indeed, any peptide or protein sequence set uploaded by the user. This TCR-focused approach provides a novel alternative to traditional methods of cross-reactivity prediction that rely on whole sequence alignment such as BLAST.[51] In a recent analysis, we found that epitopes deposited in IEDB that had a higher degree of cross-reactivity with the human genome were significantly more likely to be associated with an IL-10 or null (binding but no immune response) and a lower IL-4 response than epitopes that had a lower level of cross-reactivity (**Fig. 5**).

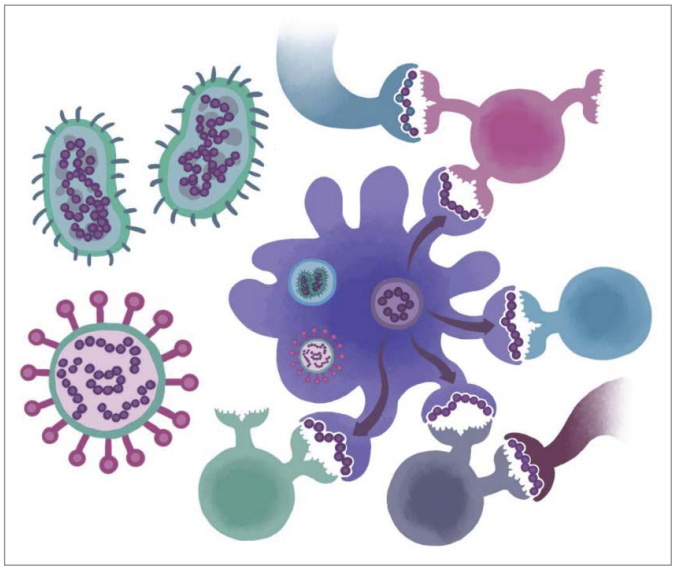

Figure 4. Immune Camouflage. Bacteria and viruses have evolved a number of subterfuges to escape immune response. A recently discovered strategy is to disguise T cell epitopes that may be presented by antigen presenting cells as "self proteins" that are recognized by Tregs (pink and gray) rather than T effector epitopes (blue).

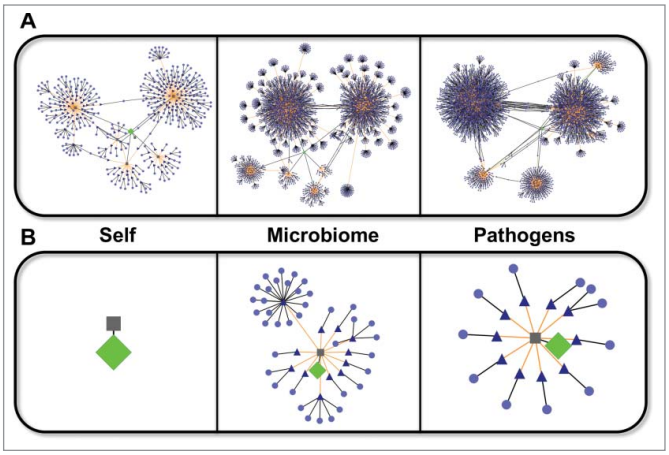

Figure 4. (**B**) JanusMatrix, Epitope Networks for a Regulatory and Effector T cell epitopes. T cell epitope networks were produced for a regulatory T cell epitope from hepatitis C virus (**A**) [24] and an effector T cell epitope from influenza (**B**) [47], using JanusMatrix and Cytoscape. Potentially cross-reactive T cell epitopes were identified by JanusMatrix, which searches proteins in human genome (left), human microbiome (center), and human pathogen genome (right) databases for HLA ligands with TCR-facing residues identical to each of the query epitopes. Green diamonds represent source peptides; gray squares are predicted 9-mer epitopes derived from the source peptide; blue triangles are 9-mers that are 100% identical to the TCR face of the source epitope and that are predicted to bind to the identical HLA; and light purple circles (on the outer rim of the array) are proteins containing the cross-reactive epitope.

Conservatrix

For pathogens that exhibit significant diversity, adequate representation at the epitope level can be challenging. Thus,

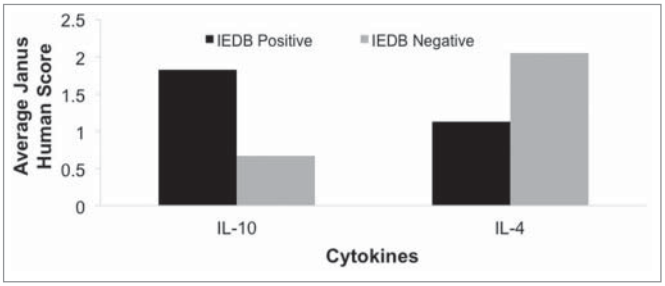

Figure 5. JanusMatrix prediction of epitope cytokine profiles. A retrospective survey of epitope-specific cytokine production data documented in IEDB shows that JanusMatrix analysis of epitope humanness differentiates between cytokine-positive and –negative data of regulatory and effector T cell cytokines. Peptides documented as IL-10-positive have significantly higher potential for human T cell cross-reactivity. Peptides documented as IL-4-positive have significantly lower potential for human T cell cross-reactivity.

prior to identifying putative HLA ligands in the protein sequence of a pathogen, the search space may be efficiently reduced by only considering sequences that are shared among many strains of the vaccine target. Conservatrix facilitates the process of selecting conserved 9-mers by parsing input sequences from multiple strains, clades, or organisms into component strings and searching for matches.[52] The output table includes information on the frequency of each string in the dataset and the identified conserved sequences can be used as an input sequence for EpiMatrix analysis. This tool has been used for defining conserved epitopes in HIV, HCV, influenza, and more recently, Burkholderia.[10,12,14,31]

ClustiMer

EpiMatrix output is formatted for clear visual interpretation of 9-mer frames predicted to bind multiple HLA alleles. When a whole protein sequence is viewed in this way, it becomes apparent that regions spanning multiple 9-mers containing high HLA Class II binding potential are not randomly distributed throughout, but tend to cluster. The minimal cluster, called an "EpiBar," is observed when a single 9-mer frame is capable of binding at least 4 common HLA Class II alleles. To take advantage of this clustering phenomenon and allow down-selection of these regions for epitope-driven vaccine development (and as input into the tools described next), we developed ClustiMer, an algorithm that identifies segments of 15–30 amino acids with elevated class II HLA binding promiscuity, called epitope cluster.[53] Correlation with existing immunodominant and promiscuous epitopes is very high, as was described in a previous publication by our group.[53]

Immunogenicity scale

Considering potential T cell immunogenicity at the protein level can help identify promising whole antigen vaccine candidates. Immunogenic potential over the length of a protein depends on epitope density. The greater the density, the greater the potential to stimulate humoral and cell-mediated immune mechanisms required for protective immunity. EpiMatrix computes whole protein scores by summing top 5% binder scores across all common HLA alleles, normalizing for a 1000-amino acid protein length and determining the deviation from random expectation. On a scale ranging from −50 to +50 or greater, where random expectation is zero, EpiMatrix protein scores above zero indicate the presence of more MHC ligands than expected due to chance and denote a higher potential for immunogenicity (**Fig. 6**). Scores below zero indicate the presence of fewer potential MHC ligands than expected and a lower potential for immunogenicity. Proteins scoring above +20 on this scale are considered to have significant immunogenic potential.

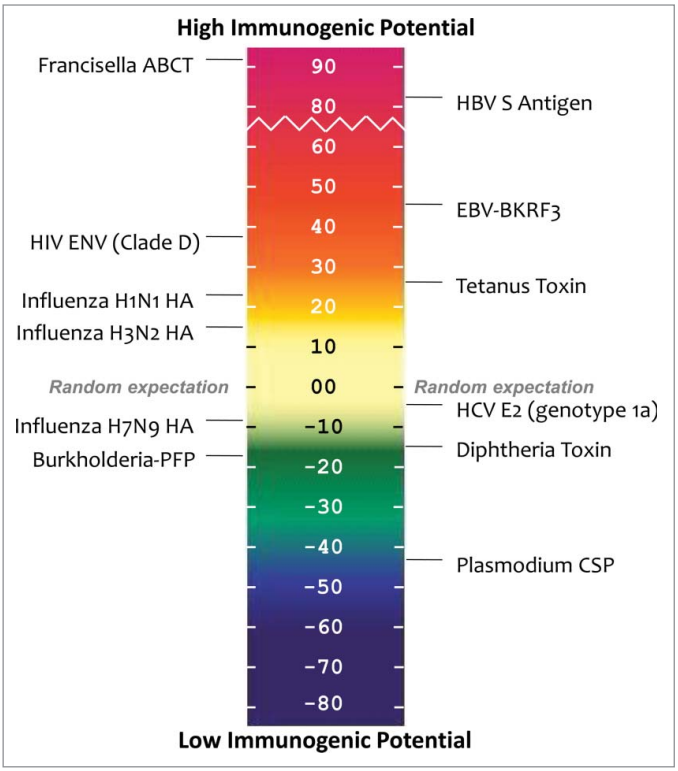

Figure 6. EpiMatrix protein immunogenicity scale. EpiMatrix protein immunogenicity scores higher than +20 are considered to be potentially immunogenic. On the left of the scale are well-known proteins for comparison. Low-scoring proteins near the bottom of the scale are known to engender little to no immunogenicity while higher scoring proteins near the top of the scale are known immunogens.

Vaccine design tools

EpiAssembler

Although including epitopes and/or clusters with broad HLA coverage and selecting peptides that are represented in multiple strains of the target pathogen are addressed by Conservatrix and ClustiMer, sometimes the most conserved linear sequences have very few T cell epitopes, while the least conserved are epitope-rich. Starting with an EpiBar 9-mer frame, EpiAssembler searches other input sequences (from other strains of the same pathogen, for example) for overlapping frames with the optimal combination of HLA promiscuity and sequence conservation, and integrates them into a chain of amino acids called an Immunogenic Consensus Sequence (ICS).[12] Because the construction of an ICS is a goal-directed process, the final ICS may not be identical to any peptide originally present in any of the input sequences or strains; however, each overlapping 9-mer frame is both highly conserved and putatively immunogenic. It is therefore possible to capture the breadth of HLA polymorphism and the depth of pathogenic variation with a relatively small number of ICS peptides. ICS epitopes have been used in epitope-based vaccines by our group with significant success.[22,23,54]

VaccineCAD

Using the aforementioned tools, a trained user can rapidly narrow the range of possible vaccine candidates to a collection of epitope-rich, highly conserved, non-autologous peptides, which may be delivered as a concatemer by linking the peptides together in a "string-of-beads." One potential pitfall is the emergence of new epitopes at the junction between peptides. To overcome

potential non-specific immunogenicity stimulated by these sequences, VaccineCAD shuffles the order of the peptides until it finds an arrangement in which junctional immunogenicity is minimized between adjacent peptides.[23] In some cases, spacers that disrupt HLA binding (e.g. GPGPG for class II) may be inserted if junctional epitopes remain. The end result is a chain of peptides containing a minimum of extraneous HLA binding potential. This construct may be reverse-translated into a nucleotide sequence for DNA vaccination if desired, or for recombinant production of a protein vaccine. Alternative formats for incorporating epitope strings into vaccines include fusions to whole antigens that assemble into virus-like particles[55] and biotinylated peptide strings that self-assemble with peptide antigen carriers.[56]

Case Studies

The iVAX toolkit has been extensively validated in pre-clinical studies. Here, we describe a number of studies in which some or all of the iVAX tools played a central role in designing vaccines with demonstrated antigenicity and/or immunogenicity.

Vaccines for T cell-mediated immune defense against pathogens

Tularemia

Tularemia is a highly infectious disease that is caused by *Francisella tularensis*. No vaccine is currently approved for public use. Class I and class II peptides from *F. tularensis* were predicted from whole genome sequences using EpiMatrix, ClustiMer and EpiAssembler, and synthesized for analysis. The peptides bound soluble HLA *in vitro* and stimulated interferon gamma (IFN-γ) secretion *ex vivo* using peripheral blood mononuclear cells (PBMCs) from infected patients.[57] In "humanized" transgenic mice expressing human HLA class II DRB1*0101, challenge with *F. tularensis* ($5\times LD_{50}$) led to the death of all control mice, while nearly 60% of vaccinated mice survived. Additionally, the epitope-based vaccine was associated with a significant reduction of bacterial burden in the lungs (27,352 CFUs/mg tissue in control mice *vs* 13,561 CFUs/mg tissue in vaccinated mice).[54]

Smallpox

It is well known that the vaccinia immunization protects against variola infection that causes smallpox. In order to develop a safer smallpox vaccine, class II T cell epitopes conserved between the sequences of vaccinia and variola major were predicted by EpiMatrix, selected using Conservatrix and concatenated using EpiAssembler and VaccineCAD.[58] The epitope-based "VennVax" concatemer was constructed, and a plasmid encoding the epitope string was delivered in combination with peptides in liposomes in a DNA-prime/peptide-boost vaccination of HLA-DR3 transgenic mice. All immunized mice (100%) survived challenge with 10X LD_{50} of vaccinia WR, as compared to 19% of the control mice. Robust T cell responses were detected pre-challenge via IFNγ ELISpot performed on splenic leukocytes, but no antibodies were found in the serum, suggesting that the protection was completely driven by T cells.[59] This case study provides evidence that complete protection against a viral challenge may be achieved using T cell epitopes alone.

H.pylori

Infection by *H. pylori* is the most common cause of gastric cancer, and recent antibiotic resistance has renewed interest in developing a vaccine. To cover genetically diverse *H. pylori* strains, 7 publicly available genomes were computationally screened to identify genes that encode proteins with promising antigenic properties. Among conserved 9-mer sequences derived from the core genome and identified with Conservatrix, potential class II HLA binders were predicted by EpiMatrix for a panel of 8 "supertype" alleles. EpiAssembler was used to a maximize immunogenicity and conservation of 9-mers by constructing ICS of 20–25 amino acids in length. The multistep computational selection process greatly narrowed down the *H. pylori* antigenic T cell epitope sequences from 1,241,153 9-mers with binding potential to a total of 1,805 ICS encoded by 676 sequences common to all 7 genomes. The top 100 ICS (~6%), by EpiMatrix Cluster score were selected for peptide synthesis. Overall, 76% of synthesized peptides bound strongly to a panel of 6 classical HLA-DR class II alleles. Human T cell responses to these 90 peptides were generally increased in actively *H. pylori*-infected compared with *H. pylori*-naïve subjects (by IFNγ ELISpot and cytokine ELISA).[29] This program has not yet progressed to *in vivo* studies, but a previous therapeutic vaccine containing a concatenated string of *H. pylori* epitopes, predicted and delivered in the same manner as described for the VennVax vaccine above, was shown to clear infection.[23]

Burkholderia

Burkholderia pseudomallei (BPM) and *Burkholderia mallei* (BM) are Category B pathogens. *Burkholderia cepaciae* (BC) causes chronic lung infections in cystic fibrosis patients. No licensed vaccines are available for these pathogens. We are developing an epitope-driven multi-pathogen vaccine against these 3 Burkholderia species.

The iVAX toolkit was used to develop ICS containing class II T cell epitopes that were conserved in at least 2 Burkholderia species.[12] As epitope selection for this project occurred prior to the development of the JanusMatrix tool, BLAST was used to confirm that none of these ICS clusters were significantly homologous to the human genome. All the ICS bound to at least 2 HLA class II alleles for which they were predicted. Significant human IFN-γ response was induced using the Wullner et al. protocol with modification[60] in at least one donor by all (100%) 44 peptides tested and in at least 3 donors by 35 peptides.

Immunogenicity of the ICS was evaluated in BALB/c and HLA-DR3 transgenic mice by peptide and DNA-prime/peptide-boost immunization. Overall, 29% and 11% of the ICS peptides elicited significant IFN-γ responses in splenocytes from BALB/c and DR3 transgenic mice, respectively, as measured by ELISpot assay. Future studies will be carried out in alternative models as this vaccine progresses in the pre-clinical pathway.

Hepatitis C Virus (HCV)

HCV is an etiologic agent of chronic liver disease. Although new therapies have shown tremendous response rates, they remain expensive, and vector-mediated vaccination with multi-epitope expressing DNA constructs alone or in combination with chemotherapy offers an additional treatment approach. HLA-A2 epitopes and HLA-DR ICS were designed using the iVAX toolkit from HCV genotypes 1a and 1b. In HLA binding assays, 90% and 84% of binding predictions were met for HLA-A2 epitopes and HLA-DR ICS, respectively. All peptides induced IFN-γ responses *ex vivo* in whole PBMC cultures and in cultures of naïve human $CD4^+$ T lymphocytes co-cultured with $CD14^+$ monocyte-derived dendritic cells (DC) pulsed with ICS. These results were confirmed in a HLA-A2/DR1 transgenic murine model vaccinated with pulsed DCs, in which peptide-specific cytokine production was induced to 33/38 (87%) of the vaccine-encoded HLA class I- and class II restricted T cell epitopes.[31]

In addition to confirming the antigenicity of the epitopes, we evaluated whether several epitopes that were highly conserved with the human genome could induce responses in PBMC cultures obtained from chronically infected patients and spontaneous clearers. A highly conserved viral epitope (HCV_G1_p7_794) from HCV non-structural protein p7 induced a marked increase in $CD3^+CD4^+FoxP3^+$ T cells in PBMC donated by chronically infected patients, but not from uninfected individuals or patients who spontaneously resolved infection.[25]

These $CD3^+CD4^+FoxP3^+$ T cells exhibited Treg activity, suppressing the mitogenic response of PBMCs obtained from the same infected patients. HCV_G1_p7_794 alone did not stimulate Treg cell proliferation, but promoted conversion of conventional $CD3^+CD4^+FoxP3^-$ T effector cells to an induced Treg cell population. JanusMatrix analysis indicated that HCV_G1_p7_794 contained sequences whose TCR-facing residues are shared with epitopes associated with hundreds of human proteins, strengthening the evidence that the presence of this epitope should be avoided in HCV vaccine design (**Fig. 3b**).

Accelerated response to bioterror threats

In the case of an outbreak or bioterror attack, response time is a crucial factor in effectively containing a potential epidemic. In order to test the epidemic-preparedness of the iVAX technology for developing 'vaccines 'on demand', the "VaxCelerate" consortium was developed.[61] In a 'live fire' Lassa virus vaccine program, a vaccine composed of a high quality set of 12 putative HLA class I-restricted epitopes and 6 putative HLA class II-restricted T cell epitope clusters was constructed in under 30 days. Epitopes were optimized to contain minimal human cross-reactive epitope content, maximal validation in the literature and sufficient epitope content to observe immune responses in HLA-DR3 transgenic mice. A vaccine was formulated with 6 biotinylated peptides, each composed of a string of 2 class I epitopes and one class II epitope that self-assembled with MTB-HSP70-avidin. Preliminary immunization results demonstrated increased $CD4^+$ IFN-γ^+ T cell frequencies in immunized mice.[56]

Other applications of the iVAX toolkit

Predicting the need for a new vaccine, Pandemic H1N1

H1N1, a novel swine-origin influenza virus (S-OIV) emerged in Mexico and the Western United States in March 2009. In advance of the development of a new H1N1 vaccine, our team used EpiMatrix and Conservatrix to identify T helper and cytotoxic T lymphocyte (CTL) epitopes conserved between S-OIV and 2008–9 CIV in the hemagglutinin (HA) antigen.[14] Based on the analysis, we predicted that there would be moderate to high cross-reactivity in the HA epitopes between the pandemic virus and the 2008–9 seasonal flu vaccine, and that some protection against morbidity may be conferred by prior influenza exposure or seasonal influenza vaccination. The predicted cross-reactivity of these epitopes was later confirmed using blood samples from S-OIV-exposed humans and H1N1-naïve, CIV-immunized subjects.[13]

Prior to 2013, H7N9 influenza virus was known to infect poultry only, giving humans little to no immunity against it.[62] The ongoing circulation of H7N9 in poultry and potential for the virus to be efficiently transmitted from human to human poses a serious threat to public health, raising the development of an effective prophylactic vaccine to a high priority. Unfortunately, attempts at designing an effective vaccine have been largely unsuccessful. For example, whole-inactivated H7N9 vaccines failed to protect ferrets from the infection, and subunit vaccines developed using H7 HA were poorly immunogenic in humans.[63-66]

Predicting vaccine efficacy, H7N9

H7N9 provides an opportunity to showcase 2 iVAX tools. In addition to the low number of $CD4^+$ T cell epitopes predicted by EpiMatrix in H7 HA (as shown in **Fig. 7**, on the EpiMatrix immunogenicity scale), JanusMatrix predicted that several existing epitopes were highly cross-conserved with the human genome

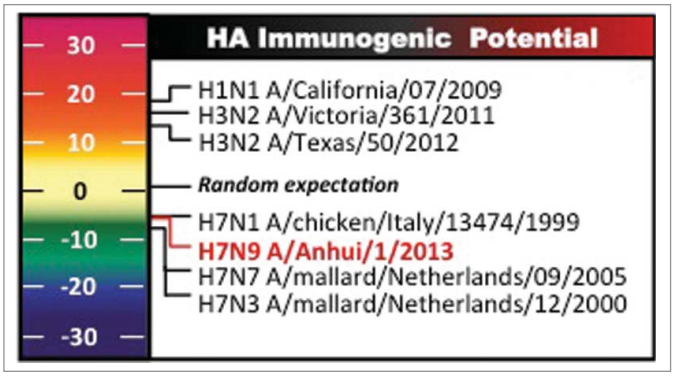

Figure 7. Potential immunogenicity of avian H7 and seasonal influenza HA. Avian H7 HA proteins possess low immunogenic potential based on EpiMatrix-predicted $CD4^+$ T cell epitope content. In contrast recent circulating seasonal HAs bear higher potential and fall within the positive range of the scale. Lower epitope content in H7N9 HA may explain, in part, lower H7N9 immunogenicity observed in infection and vaccination. Re-published from Hum Vaccin Immunother. 2014;10(2),256-62 with permission.

on the TCR side, suggesting that the H7N9 may 'camouflage' itself to appear human [34]. Consistent with this prediction, immunoreactivity screening results using naïve human PBMCs revealed that the degree of cross-conservation of predicted epitopes with the human genome was inversely correlated with their immunogenicity.[67] Immunophenotyping data demonstrate a strong link between these 'human-like' epitopes and Treg expansion.[35] Overall, our observations provide a possible alternative explanation why whole inactivated H7 HA vaccines were poorly immunogenic.

Conclusion

The lack of clinical efficacy of some previous epitope-driven vaccines against microbial pathogens may have been due to their (1) limited number of epitopes, (2) limited sequence conservation with clinical strains, (3) limited HLA population coverage, and/or (4) suboptimal delivery. Recent discoveries add to this list, (4) epitopes that trigger regulatory T cell responses due to cross-conservation with regulatory T cell epitopes present in the human genome appear to be one means by which human pathogens 'camouflage' immune response. Re-designing immunogens to remove these epitopes may improve both humoral and cellular immune responses to vaccines.[50]

The iVAX toolkit addresses each of these issues, enabling the development of computationally designed, epitope-driven vaccines for bio-warfare agents, highly variable pathogens and chronic infectious diseases. The number of epitopes to be included is not a significant obstacle, evidence from animal studies suggests that the number of epitopes required for full protection is a small and definable subset (less than 50);[68,69] thus, achieving a payload of 50–100 epitopes that provide broad coverage of human genetic backgrounds may be sufficient to provide protection. Improving payload quality by choosing epitopes that demonstrate antigenicity in human PBMCs as well as protection in established murine models of disease is now possible. Furthermore, the combination of promiscuous class II epitopes and class I supertype epitopes can provide nearly 100% coverage of human populations. Optimization of payload, delivery, formulation, and adjuvanting can accelerate these vaccines to the clinic.

The use of immunoinformatics tools is not limited to vaccines for infectious diseases. For example, T cell-driven vaccines are becoming an adjunct immunotherapy to treat cancer, more than 250 T-cell driven vaccines are in clinical trials.[70,71] Computational vaccinology should accelerate the identification of immunogenic tumor antigens and improve safety of these new treatments. In the near future, we expect computationally engineered vaccines incorporating recent advances in epitope discovery, vaccine design and delivery and adjuvanting to provide effective protection against infectious diseases as well.

In conclusion, the investigators using computational vaccinology tools have advanced many of the immunoinformatics-designed vaccines described above to the point of formulation and delivery studies over the past 5 years. Sufficient proof of principle has been gathered to suggest that the iVAX vaccine toolkit can be used to design effective vaccines. The toolkit is currently being used in studies that propose to accelerate production of vaccines in response to a bioterror event or to outbreaks of disease that can rapidly spread, such as Ebola in West Africa. The need for new vaccines for protecting against bioterror pathogens and emerging infectious disease is great, and we would argue for the reasons cited above, that the time to integrate computational vaccinology into all existing vaccine pipelines and to advance these vaccines to the clinic is *now*.

Disclosure of Potential Conflicts of Interest

Several of the coauthors on this manuscript are employees of EpiVax (ADG, WM, FT, LM), and ADG and WM are stockholders. RT is employed at the Institute for Immunology and Informatics; he is the primary contact for iVAX collaborations (rtassone.uri@gmail.com). AG is a graduate student in the Department of Cellular and Molecular Biology at the University of Rhode Island (URI). These authors recognize the presence of a potential conflict of interest and affirm that the information represented in this paper is original and unbiased observations. The office of the Vice President of Research at URI manages the relationship between EpiVax and the University of Rhode Island; inquiries can be directed to Attorney Brian Nath (briannath@uri.edu).

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