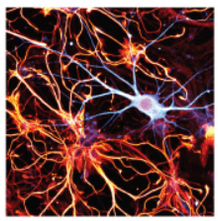
mAbs



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In silico methods for immunogenicity risk assessment and human homology screening for therapeutic antibodies

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ABSTRACT

In silico immunogenicity risk assessment has been an important step in the development path for many biologic therapeutics, including monoclonal antibodies. Even if the source of a given biologic is 'fully human', T cell epitopes that are contained in the sequences of the biologic may activate the immune system, enabling the development of anti-drug antibodies that can reduce drug efficacy and may contribute to adverse events. Computational tools that identify T cell epitopes from primary amino acid sequences have been used to assess the immunogenic potential of therapeutic candidates for several decades. To facilitate larger scale analyses and accelerate preclinical immunogenicity risk assessment, our group developed an integrated web-based platform called ISPRI, (Immunogenicity Screening and Protein Re-engineering Interface) that provides hands-on access through a secure web-based interface for scientists working in large and mid-sized biotech companies in the US, Europe, and Japan. This toolkit has evolved and now contains an array of algorithms that can be used individually and/or consecutively for immunogenicity assessment and protein engineering. Most analyses start with the advanced epitope mapping tool (EpiMatrix), then proceed to identify epitope clusters using ClustiMer, and then use a tool called JanusMatrix to define whether any of the T cell epitope clusters may generate a regulatory T cell response which may diminish or eliminate anti-drug antibody formation. Candidates can be compared to similar products on a normalized immunogenicity scale. Should modifications to the biologic sequence be an option, a tool for moderating putative immunogenicity by editing T cell epitopes out of the sequence is available (OptiMatrix). Although this perspective discusses the in-silico immunogenicity risk assessment for monoclonal antibodies, bi-specifics, multi-specifics, and antibody-drug conjugates, the analysis of additional therapeutic modalities such as enzyme replacement proteins, blood factor proteins, CAR-T, gene therapy products, and peptide drugs is also made available on the ISPRI platform.

GLOSSARY

ISPRI (Interactive Screening and Protein Reengineering Interface): Integrated, cloud-based, comprehensive toolkit for Immunogenicity Risk Assessment.
EpiMatrix Immunogenicity Score: Combined T effector and Treg Epitope Content per unit protein.
Tregitopes: Treg Epitopes found in IgG Framework that have been shown to modulate antigen-specific effector T cell responses.
ClustiMer: Tool for identifying epitope rich polypeptides from within a given protein sequence.
JanusMatrix: Tool for Predicting Tolerance, Putative Treg Epitopes, and Anti-self-immune responses.
OptiMatrix: Tool for modifying T cell epitope sequences to reduce (or enhance) MHC binding.

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Introduction

Immunogenicity is a term that is used in the biopharmaceutical industry to describe undesired immune responses targeting protein and peptide therapeutics, as well as other therapeutic modalities. Immunogenicity is generally defined by the identification of anti-drug antibodies (ADA) in patients who have been treated with the drug. The development of treatment-emergent ADA may be driven by components that are intrinsic to the product, such as T cell epitopes, as well as residual host cell proteins, product and process-related impurities. While the immunogenicity outcome is measured in terms of ADA found in serum samples, the driver for development of these antibodies is a T cell dependent immune response.[1,2] T cells that have T cell receptors (TCR) specific for linear epitopes contained in the sequence of the therapeutic biologic are critical to the development of ADA, which may have consequences for drug safety and efficacy according to the Food and Drug Administration (FDA).[3]

T cell epitope-driven immunogenicity is dependent on engagement of T cells through their specific TCRs with linear T cell epitopes derived from protein sequences and presented on human leukocyte antigens (HLA). The number of studies

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that have validated the link between peptides presented by HLA,[4,5] T cell epitopes,[6] T-regulatory epitopes,[7,8] and the development of ADA, as well as local and systemic immune reactions to biologics,[9] is substantial and continues to increase with time.

Numerous approaches have been developed to assess the immunogenicity risk of biologics using in silico and in vitro methods that explore HLA, T cells, and immunogenicity. One strategy involves the a priori design of monoclonal antibodies (mAbs) with reduced immunogenicity using a variety of methods, including in silico assessment and checking for epitopes in the IEDB database.[10] Cohen has used ISPRI/EpiMatrix to identify critical T cell epitopes for in vitro validation studies[11] and advocates for in vitro immunogenicity methods in her publication with Chung.[12] Additionally, Karle[13] proposes the use of MHC-associated peptide proteomics (MAPPs) assays combined with T cell assays to assess mAb immunogenicity.

Due to the link between T cell epitopes and ADA, in silico T cell epitope prediction methods are increasingly used by protein therapeutics developers to prospectively identify potentially immunogenic protein drug candidates.[14–16] There are many commercially or publicly available tools that have been applied to immunogenicity risk assessment. Most of these tools, including NetMHC, Tepitope, SYFPEITHI, and EpiMatrix, are used to search the amino acid sequences of proteins for putative T cell epitopes that can drive T cell-dependent adaptive responses. Additional outcome-specific in silico tools are also available, such as TcPRo,[17] a risk assessment tool that is specifically tailored for assessing biotherapeutic protein immunogenicity by key HLA alleles.

In contrast with many of the above-cited tools, the *Interactive Screening and Protein Re-engineering Interface* (ISPRI) includes EpiMatrix along with additional tools (ClustiMer, OptiMatrix, JanusMatrix, and ADA prediction) that can aide in reviewing and interpreting the raw data supplied by EpiMatrix. All the tools on the site were developed by EpiVax team members. Access to the ISPRI toolkit is available via a secure, password-protected website. A free-standing, secure copy of the ISPRI website is created for each scientific group that wishes to use the tools: sequences can be stored on the site and data downloaded with archiving capabilities. An Application Programming Interface (API, also known as a "machine-to-machine connection") facilitates high throughput analyses of large numbers of sequences. Several large biotech companies have reported that they are using this tool to assess preclinical immunogenicity risk[16,18–25] and validating studies for the algorithms contained in the ISPRI toolkit have been published.[16,24–34] Due to the use of API connections, more than 2 million sequences are analyzed annually by ISPRI for biotech pipelines.

The ISPRI toolkit has a prolonged track record and a history of validation that is both prospective and retrospective, which has contributed to its adoption by a wide range of experts in the field for the preclinical assessment of immunogenicity. The initial set of tools (the EpiMatrix system) was developed by De Groot and colleagues at Brown University and licensed to EpiVax in 1998. A number of validating case studies are reviewed here. EpiMatrix and JanusMatrix have been applied and validated in the field of vaccine development, most recently for personalized cancer vaccine development.[35] Substantial improvements to the EpiMatrix algorithm have resulted in a high degree of accuracy for class II epitopes (77–100%) and higher than 95% for most class I epitopes.[36,37]

Here, we provide a high-level view of ISPRI algorithms and other aspects of the toolkit and describe how the tools can be used to assess the immunogenicity risk of antibody-like therapeutics including bispecific and multi-specific constructs. Case studies are used to illustrate the application of the toolkit to drug candidates. A schematic of the individual "silos" that are useful for antibody risk assessments is found in Figure 1.

In silico immunogenicity risk assessments

Global analysis: predicting & quantifying T cell epitope content

EpiMatrix.

First developed in 1996,[26,38] the EpiMatrix algorithm is a position-specific scoring matrix (PSSM) epitope prediction tool that has since been extensively used, validated, and refined. EpiMatrix is based on a set of position and allele-specific matrices (20-by-9) of coefficient values. The coefficient matrix represents the 20 naturally occurring amino acids by the nine positions inside of the binding groove of an HLA molecule. Each coefficient represents a single amino acid affinity for a specific position (known as a pocket) within the binding groove of a specific HLA allele. An updated description of the original pocket-profile method[38–40] used to develop and expand EpiMatrix's epitope-prediction matrices was recently published as part of a report describing the application of the method to swine MHC (SLA).[41]

To assess the global immunogenic potential of a biologic protein or mAb, input sequences are parsed into overlapping 9-mer frames, and each frame is evaluated by the EpiMatrix algorithm for binding potential to a panel of nine common Class II HLA-DR supertype alleles selected for broad (>95%) global population coverage[42] (Figure 2). The generation of ADA in clinical studies is positively correlated with the number of HLA-DR-restricted T cell epitopes that are present in the primary sequence of the biologic or peptide drug.[4,43] EpiMatrix assessments are normalized against the mean score of a random set of peptides, and deviations from that mean are reported as Z-scores. The top 5% of assessments (Z-score >1.64) are considered statistically significant. These EpiMatrix "hits" are the most likely peptides from any input sequence to bind to HLA and be presented on the surface of antigen-presenting cells, where they may be interrogated by passing T cells.

Immunogenicity Scale.

The predicted epitope content for a given protein is generated by adding the identified epitopes together and standardizing it by length, making it possible to directly compare input proteins of different lengths. The EpiMatrix Immunogenicity Scale was developed to visualize these comparisons and to compare candidate therapeutics to proteins for which the immunogenicity is well understood. The scale plots epitope content as deviation from an expectation based on randomly generated

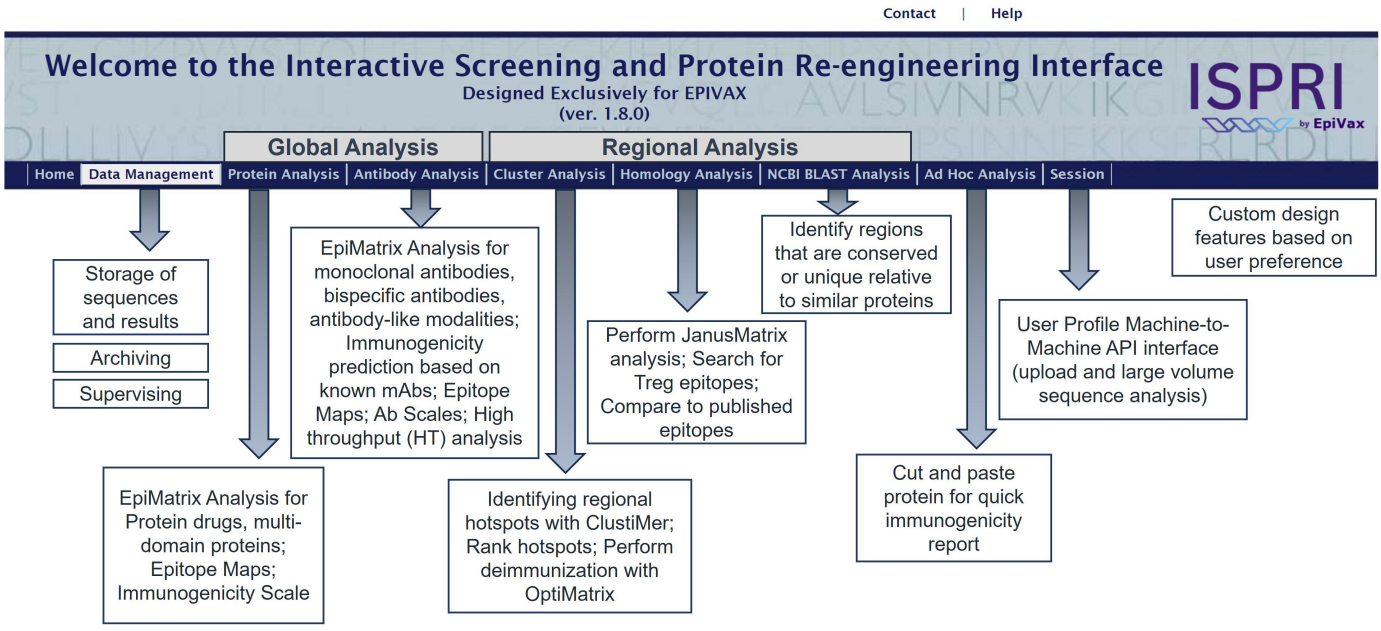


Figure 1. Screenshot of the ISPRI homepage, with arrows connecting ISPRI silos and tools that enable immunogenicity risk assessment. Analysis usually proceeds by starting with a global and regional assessment of the protein sequence for T cell epitopes, followed by an assessment of regulatory T cell epitope (tregitope) content and then by comparisons to known antigens and the human genome. Once global and regional analysis is performed, additional tools included in the ISPRI platform (OptiMatrix) can be used to support deimmunization.

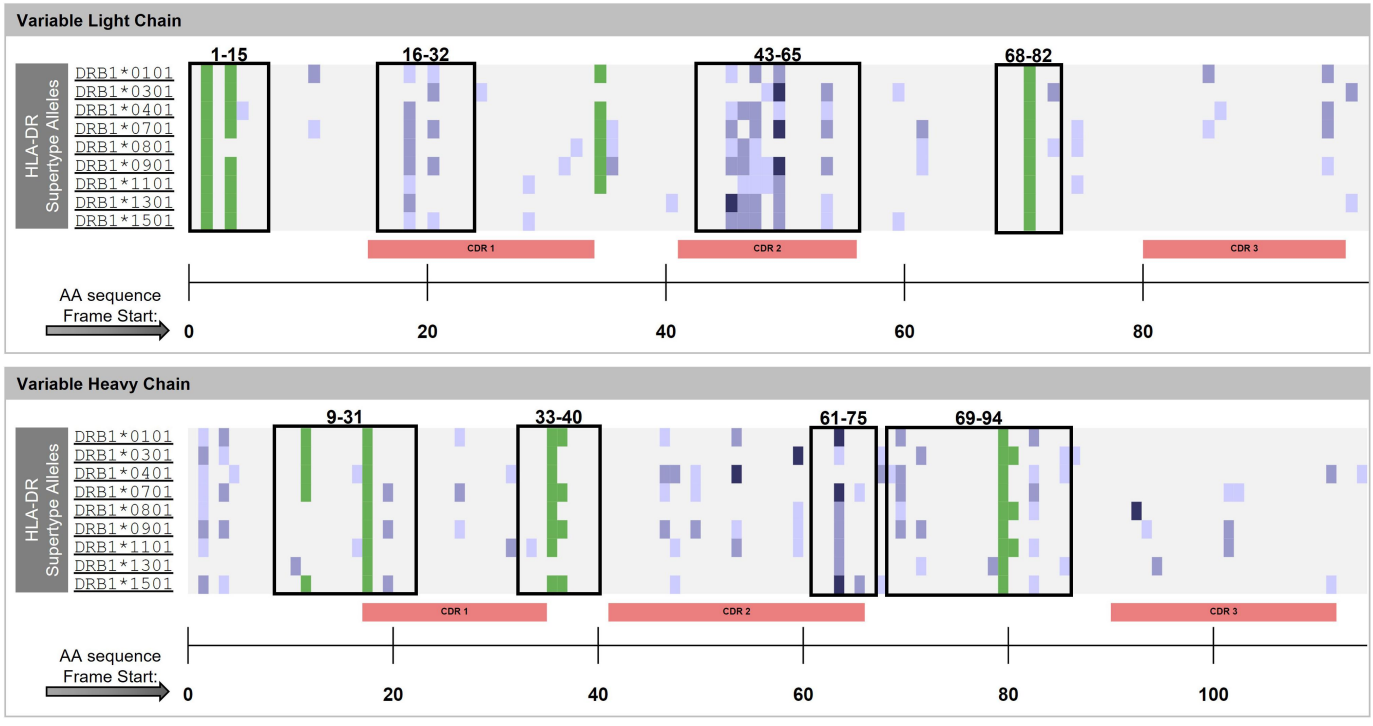


Figure 2. Epitope Map. Input sequences are parsed into overlapping 9-mer frames and each frame is assessed for likelihood to bind to the nine HLA-DR supertype alleles. Blue fill indicates putative class II T cell epitopes for the given frame-by-allele assessment; the strength of the score is indicated by the blue shading. Dark blue indicates assessments in the top 1% of the normal distribution, and medium blue fill indicates scores in the top 5% of the normal distribution. The lightest blue shading indicates scores in the top 10% of the normal distribution and are considered 'near-misses'. Black outlined boxes indicate the relative positions of T cell epitope clusters, or regions of high epitope density, defined by the ClustiMer algorithm. Green fill indicates T cell epitopes for 9-mer frames that are exactly matched to known regulatory T cell epitopes (tregitopes).

proteins as a means of describing the potential for immunogenicity.

The EpiMatrix Immunogenicity Score for candidate biologic proteins is calculated by taking the difference between the number of putative epitopes predicted by the EpiMatrix System for the candidate and the number of predicted T cell epitopes expected to be found in a protein of similar length, where the expectation is based on an analysis of a large dataset

of randomly generated protein sequences with amino acid frequencies observed in human proteins.

The median EpiMatrix score of a set of random proteins is set at zero on the immunogenicity scale. Proteins scoring higher than zero contain more putative T cell epitope content than expected by random chance and have higher immunogenic potential than proteins that score below zero. This scoring system was originally used to differentiate candidates for vaccine development, as higher scores tended to be associated with antigens used for effective vaccines (for more details see the review of the iVAX toolkit).[37] Benchmarks on this scale include protein or mAb sequences for which immunogenicity is known.

Case study 1: benchmarking sets of human proteins on the immunogenicity scale. While the EpiMatrix Score for random proteins is zero, human proteins tend to score lower, and selected human proteins that are critical for survival, such as follicle-stimulating hormone, have remarkably low T cell epitope content, which suggests that lower T cell epitope content may be a feature of critically important human proteins.

As shown in Figure 3(a), well-known and effective vaccine antigens score in the upper range on the Immunogenicity Scale, while well-tolerated therapeutic proteins score in the lower range. The EpiMatrix score is correlated with observed immunogenicity, as has been demonstrated in retrospective and prospective studies.[16,25,44]

This observation, i.e., the low T cell epitope content in critical proteins, lead to an investigation of the T cell epitope content of different classes of human proteins. For example, secreted proteins, which are more likely to encounter the human immune system as they circulate through the lymphatic system and the vascular system, were found to have fewer class II restricted T cell epitopes than non-circulating proteins.

To determine whether low immunogenicity was a universal feature of the human proteome, EpiMatrix was used to score 20,401 human proteins (UniProt Accession UP000005640) for potential immunogenicity. More than 1,000 unique parsed subcellular protein location terms were manually assigned to one of three subcellular locations for analysis: nucleus, cytoplasm, and cell-membrane. The nuclear protein subset contained the largest number of sequences: 2,979 proteins. The "multi-pass" cell membrane protein subset (protein sequences with two or more transmembrane domains) was the smallest subset, containing 1,193 protein sequences. These defined subsets, containing approximately 56% of the human proteome, had different distributions of scores compared to the dataset of random proteins used to construct the EpiMatrix Immunogenicity Scale. While the compiled scores of random proteins closely resemble a normal distribution (Figure 3b), the EpiMatrix Scores for proteins in the human proteome and several human proteome subsets were not normally distributed on the scale. The median score for the human proteome (all subsets) (−9.05) is below the median established using the random protein sequence dataset (0.00).

More specifically, the median EpiMatrix Scores of the non-membrane Cytoplasm (−11.35), Intracellular (−16.80), Nuclear (−21.57), and Secreted (−23.08) protein subsets were all found to be below the median for both random proteins and below the median score for all human sequences, indicating that the number of T cell epitopes contained in human proteins tends to skew toward a lower level of putative T cell epitope content. In contrast, the median EpiMatrix Scores of cytoplasmic membrane-associated proteins (15.32) and multi-pass cell membrane-associated proteins (79.70) were much higher than all human and random protein sets. (Figure 3b). This can be explained by the presence of a high number of hydrophobic

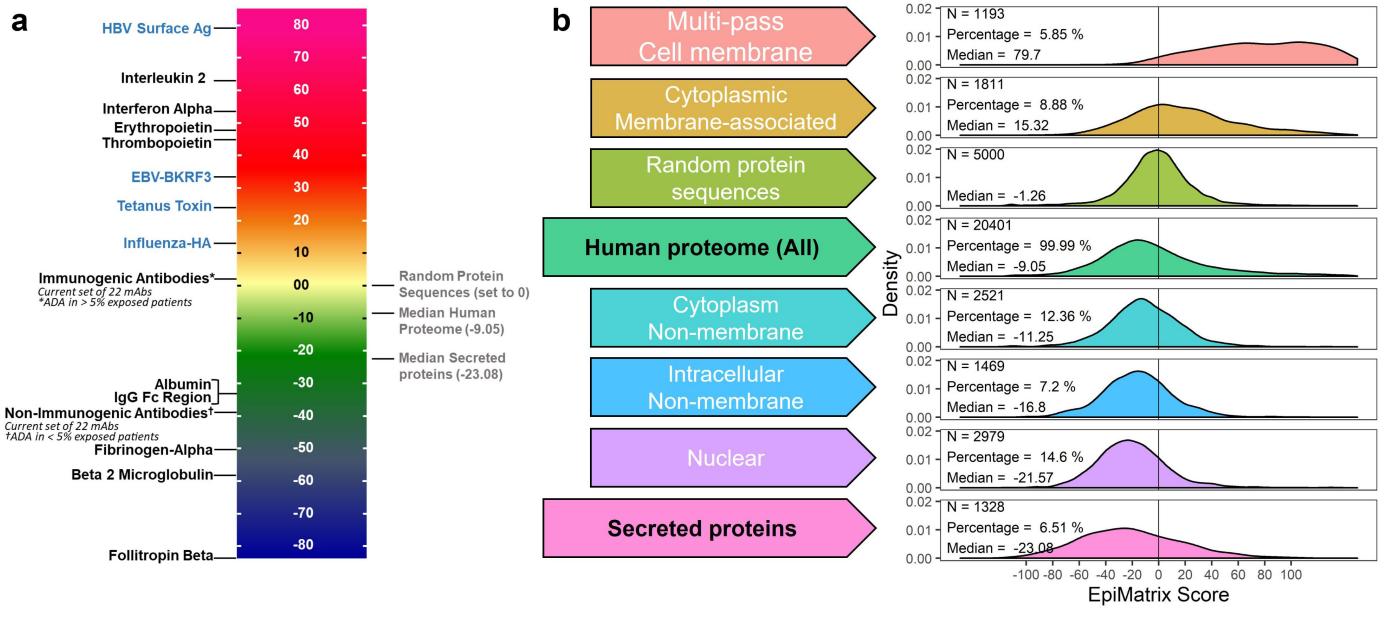


Figure 3. (a)EpiMatrix predicted excess of shortfall in predicted aggregate immunogenicity relative to a Random Protein Standard (per 1,000 9-mer frames analyzed). All scores are adjusted for the presence of tregitopes. *Average of 10 antibodies (VH/VL pairs) known to Induce Anti-Therapeutic Responses in more than 5% of patients, †Average of 10 antibodies (VH/VL pairs) known to Induce Anti-Therapeutic Responses in less than 5% of Patients.(b)Tregitope-adjusted EpiMatrix scores for 20,401 proteins derived from the human proteome (UniProt accession UP000005640) were generated.

residues in trans-membrane proteins. Hydrophobic residues can also act as anchor residues for HLA-DR binding peptides.

Assessing regional immunogenic potential

Assessment of total immunogenic risk can be improved by supplementing the global immunogenicity assessment with the identification of any potential regional hot spots containing MHC binding motifs for multiple HLA alleles. The ClustiMer algorithm is used to identify these high-density regions of immunogenic potential (clusters) within larger protein sequences. The homology analysis tools on ISPRI can then be used to determine the relative humanness or foreignness of each cluster, as well as their relationship to any previously published epitopes.

ClustiMer: finding T cell epitope clusters

The ClustiMer algorithm was designed to identify protein segments with statistically elevated densities of predicted epitopes.[45] Potential T cell epitopes are only rarely randomly distributed throughout peptide or protein sequences, but instead tend to cluster in specific regions (see black outlined boxes in Figure 2). These T cell epitope "clusters" can range from nine to roughly 25 amino acids in length and can contain anywhere from at least four to as many as 40 binding motifs, considering their affinity to bind multiple alleles across multiple frames. The presence of one or more T cell epitope clusters can drive anti-therapeutic immune responses even in otherwise low-scoring peptides or proteins. In our experience, many of the most reactive peptides, in terms of induced T cell responses, contain clusters with several promiscuous 9-mer frames that are predicted to bind to at least four different HLA alleles. These features, which are highlighted in the computer-generated report, are referred to as EpiBars (see detailed description of EpiBars in the special considerations for antibodies section below).

Peptides derived from protein sequences that contain EpiBars bind very well to a range of HLA Class II molecules and tend to be very immunogenic in T cell assays of blood samples drawn from human subjects.[45] Promiscuous T cell epitopes that have been defined for influenza hemagglutinin (amino acids 306–318), tetanus toxin (825–850), and GAD65 (557–567) have EpiBars. Other well-known "positive control" peptides and proteins used in T cell assays also contain this feature.[45] In one study, 100% of subjects exposed to either Tularemia or Vaccinia responded to pools of T cell epitope clusters that score higher than 20 on the EpiMatrix immunogenicity scale.[46,47] In a recent head-to-head comparison, the ClustiMer approach outperformed the standard overlapping peptide approach (usually 15mer peptides overlapping by five amino acids) used by many biologics' researchers.[36] In this comparison, T cell responses to the 15mer overlapping peptides were lower, on average, than the maximal responses induced by the pools predicted using immunoinformatic tools.

JanusMatrix: adjusting immunogenicity assessment for humanness

It is important to determine whether the T cell epitopes identified using in silico tools are T effector epitopes or tolerogenic epitopes (epitopes that invoke a tolerogenic response that dampens effector responses), as the risk of immunogenicity is decreased when Treg epitopes are present. T cell epitopes that are homologues to common human proteins may be tolerated or even tolerogenic in healthy human subjects. To identify specific homologies that may reduce immunogenic potentials, EpiVax developed the JanusMatrix algorithm.[48] This tool, which was first developed in 2013, uncovered new relationships between pathogen and cancer epitopes and other epitopes in the human genome.[35,36,48–50] Specifically, the tool identifies homologies between TCR-facing residues in epitopes that bind to a specific HLA and other epitopes that bind to the same HLA from additional self-proteins. Epitopes with extensive homology to other epitopes in the human genome are postulated to be more likely to be tolerated, as the T cells that recognize the epitopes in the human genome would have been energized or deleted in the course of thymic development.

JanusMatrix begins an analysis by separating the amino acids in each 9-mer that make up a T cell epitope into those that contact and anchor the epitope in the binding groove of the HLA molecule and those that can reasonably be expected to make contact and bind with the TCR of responding T cells (for more details, see Moise et al.).[48] For HLA-DR-restricted 9-mer epitopes, relative positions 1, 4, 6, and 9 make contact in the binding groove of the HLA, and positions 2, 3, 5, 7, and 8 are available to the TCR. Peptide epitope pairs with compatible, but not necessarily exactly matched, HLA binding anchors and exactly matched TCR-facing contours may be cross-reactive. In other words, CD4+ T cells engaged and activated by a given peptide epitope may also be engaged and activated by a TCR contour-matched homologue.

The JanusMatrix tool searches a reference database for proteins with a compatible agretope (i.e., one that is predicted by EpiMatrix to bind the same HLA as the input peptide) and exactly matching the TCR contacts of the input peptide. The JanusMatrix Homology score of a given peptide or protein indicates the average depth of coverage within the reference database (in this case, the human proteome). When comparing peptide epitopes to the human proteome, JanusMatrix Human Homology Scores above two indicate an elevated level of conservation between the TCR-facing features of the input peptide or protein, and the TCR-facing features of proteins resident within the human proteome. Scores above four are considered significant for the generation of a tolerogenic cytokine response. For human proteins, we can extend those thresholds to three and five, because the human proteome reference database will always contain a copy of the input sequence. For a given EpiMatrix Score, a high JanusMatrix Human Homology Score suggests a bias toward immune tolerance. In other words, high JanusMatrix Human Homology Scores tend to offset high EpiMatrix Scores.

The availability of detailed data on large numbers of T cell epitopes in the Immune Epitope Database (IEDB) made it possible to perform a retrospective review of 3,756 HLA Class II-restricted

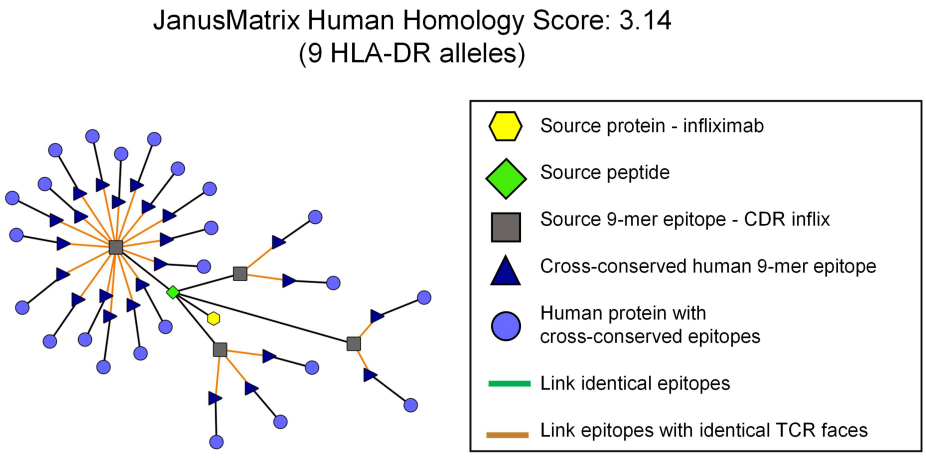


Figure 4. Cytoscape figure of human-like epitope."Non-IgG Tregitope" that was identified in the CDR region of infliximab. This peptide contains several HLA binding motifs (dark grey squares) including one that is extensively conserved with multiple self-peptides that share the same TCR face (dark blue triangles). The JanusMatrix score for this peptide is 3.14. None of the cross-conserved peptides are found in IgG in this instance. This peptide was shown to be associated with IL2 secretion in studies of human T cell responses to infliximab peptides carried out by Vultaggio et al.[6]

T cell epitopes. A statistically significant inverse relationship between high conservation in the human proteome and observed production of IL-4 was identified.[51] IL-4 is commonly associated with effector T cell response.[51] In contrast, a statistically significant relationship was found between HLA Class II-restricted T cell epitopes that had high levels of cross-conservation with the human genome and IL-10 response, suggesting that some of these epitopes may induce regulatory T cell responses.

JanusMatrix has been applied to the evaluation of mAbs for immunogenic potentials, resulting in the discovery of putative Treg epitopes in the highly variable complementarity-determining region (CDR) of some antibodies. Figure 4 shows an example of the significant network of human proteome matches identified by JanusMatrix in an epitope derived from the CDR of the mAb infliximab that was reported to stimulate IL-10 release from infliximab-exposed human subjects.[52]

In parallel studies, JanusMatrix has been used to identify similarly conserved sequences in human pathogens and identified epitopes from human pathogens that had extensive cross-conservation at the TCR face with human T cell epitopes.[49,53,54] Further in vitro and in vivo studies demonstrated that these pathogen epitopes activated and expanded T cells that had a regulatory T cell phenotype, thereby conferring a "stealth" advantage to such pathogens. For example, using JanusMatrix, Losikoff et al. identified a highly cross-conserved Treg epitope in the hepatitis C virus (HCV) genome[55] and another Treg epitope was identified in the hemagglutinin protein of H7N9 avian influenza.[49]

Published "tolerizing" epitopes such as Edratide from IgG[56] and B29 from Heat Shock Protein 70 (HSP 70)[57] have high JanusMatrix homology scores, further corroborating the hypothesis that TCR homology is relevant to tolerance. Additional non-IgG Tregitopes have been found in other autologous protein sequences,[58] and many more are likely to be discovered and validated.

Comparing to previously reported HLA ligands & T cell epitopes

To take advantage of any information regarding the phenotype of response to predicted ligands or their close relatives that may exist in the literature, predicted T cell epitope clusters can be screened against the Immune Epitope Database of previously published HLA ligands and T cell epitopes. This feature is especially useful for reviewing reported MAPPs data. MAPPs assays are used to identify peptides (epitopes) within a therapeutic protein that are processed and presented by antigen-presenting cells (APCs) to T cells. Users can quickly assess the T cell epitope clusters identified by EpiMatrix for their homology to publicly reported epitopes discovered by MAPPs assay evaluation, often saving valuable resources. The IEDB homology analysis on ISPRI is also useful for evaluating the immunogenicity of related epitopes by aligning the T cell epitope clusters to any homologous reported epitopes that have been tested in T cell assays.

We recently performed an analysis of "MAPPs" eluted peptides that have been compiled in the IEDB database to EpiMatrix predictions as of March 2024. We identified 70,594 peptides in the IEDB that were reported to have been eluted from human HLA-DR molecules. Using our usual threshold for binding (EpiMatrix Z-score of 1.64), 58,335 (83%) of these peptides contain at least one HLA allele-specific epitope that is also identified by EpiMatrix. At a slightly lower cutoff that includes "likely HLA-binding 9-mers" (1.28), 64,064 or 91% of the reported eluted peptides contain at least one HLA-allele-specific EpiMatrix ligand. Thus EpiMatrix HLA-binding assessments are highly correlated with MAPPs and, likely, less expensive to perform, especially when assessing multiple mAb candidates.

BLAST (basic local alignment search tool) analysis to reveal other relevant homologies

To identify potential homologies to known proteins, including proteins derived from common bacteria and viruses that may play a role in the education of a healthy human immune system, users can BLAST T cell epitope clusters against the non-redundant protein database at GenBank on the ISPRI platform.

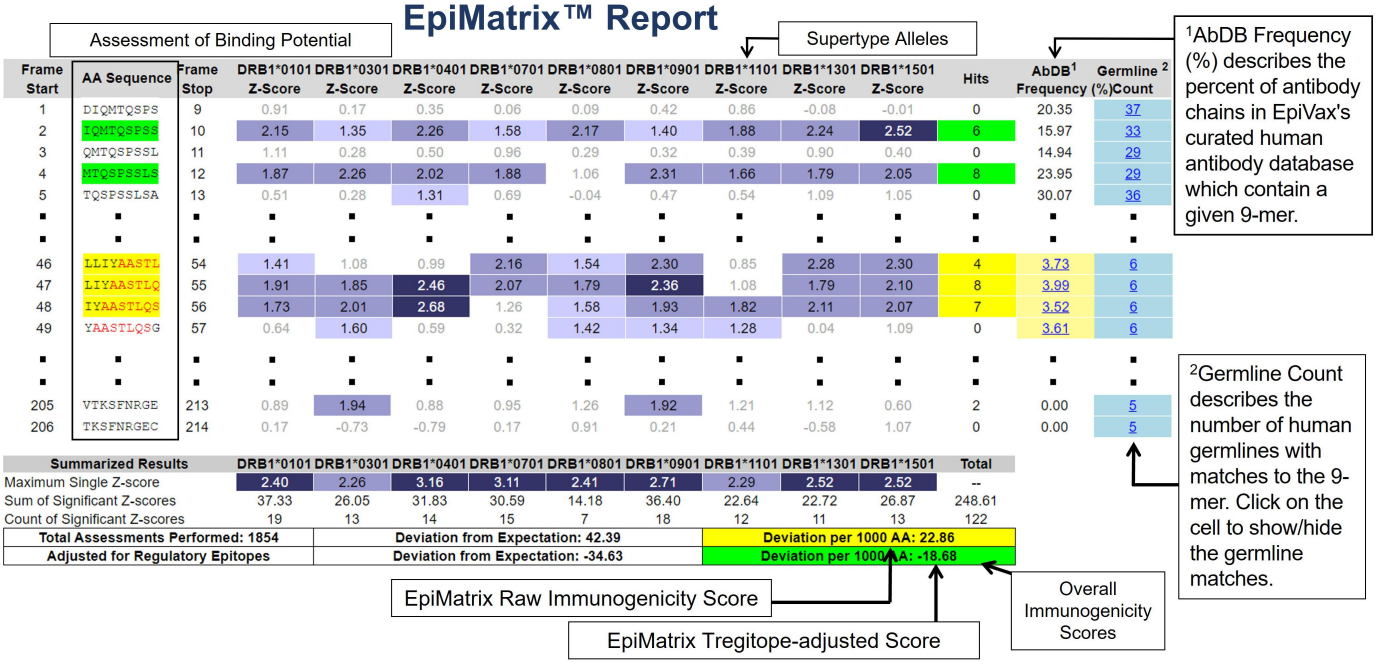
Special considerations for antibodies

Adjusting for validated tregitope content in antibodies

As illustrated above, not all T cell epitopes are drivers of inflammatory immune responses. While some T cell epitopes drive "helper" responses and help mature B cells that produce anti-drug antibodies, other regulatory T cell epitopes may counteract those signals. We previously identified several T cell epitopes that are present in the framework sequences of IgG that regulate immune responses and reduce immunogenicity through activation of regulatory T cells. These peptides, now known as Tregitopes, have been shown to activate regulatory T cells and promote tolerance induction to co-administered antigens. Tregitopes[59] have been the focus of numerous publications by our group,[32] by our collaborators,[60] and others who have used murine versions of the Tregitopes to modulate inflammation or autoimmunity in animal models.[59,61–64] Many of the tolerogenic Tregitopes were re-discovered in studies that examined the phenotype of T cells responding to IgG epitopes obtained from pediatric patients with Kawasaki disease that had been treated with intravenous IgG (IVIG) therapy.[65]

As viewed in the ISPRI toolkit, Tregitopes are promiscuous IgG T cell epitopes that are highly conserved across human immunoglobulins (see column marked AbDB for internal curated antibody database, Figure 5) and within human germline sequences (see column marked Germline Count, Figure 5). In their natural context (in the IgG framework sequence), the function of Tregitopes is not clear: they may regulate the development of antigen-binding antibodies, and particularly, they may reduce the likelihood of anti-idiotypic antibodies to the highly variable antigen-binding CDRs. The role of Tregitopes in the modulation of T follicular helper responses in the maturation of antibody affinity in B cell follicles is currently under investigation.[66] While the Tregitope hypothesis of immune modulation has taken some time to be integrated into the current understanding of immune responses to mAbs and other immunoglobulin therapies, recent publications by Sette and Franco,[65,67,68] among others,[69–72] indicate that the concept has broad support at present.

For the purposes of mAb immunogenicity risk assessment, we developed a Tregitope-adjusted EpiMatrix Score. To calculate the Tregitope-adjusted EpiMatrix Score, the EpiMatrix hits contained in Tregitopes are subtracted from the EpiMatrix Protein score calculation. A regression model that uses this Tregitope-adjusted EpiMatrix Score to predict the potential immunogenic risk of new mAbs before clinical studies of them are started was published in 2013.[73] This model was validated in studies conducted at Amgen.[16,73] Specifically, the Tregitope-adjusted EpiMatrix immunogenicity scores of several Fc-peptide fusion drugs were shown to be highly correlated with clinical immunogenicity.[73]



Frame Start	AA Sequence	Frame Stop	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*0901 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	AbDB[1] Frequency (%)	Germline [2] Count
1	DIQMTQSPS	9	0.91	0.17	0.35	0.06	0.09	0.42	0.86	-0.08	-0.01	0	20.35	37
2	IQMTQSPSS	10	2.15	1.35	2.26	1.58	2.17	1.40	1.88	2.24	2.52	6	15.97	33
3	QMTQSPSSL	11	1.11	0.28	0.50	0.96	0.29	0.32	0.39	0.90	0.40	0	14.94	29
4	MTQSPSSLS	12	1.87	2.26	2.02	1.88	1.06	2.31	1.66	1.79	2.05	8	23.95	29
5	TQSPSSLSA	13	0.51	0.28	1.31	0.69	-0.04	0.47	0.54	1.09	1.05	0	30.07	36
▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪
▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪
46	LLIYAASTL	54	1.41	1.08	0.99	2.16	1.54	2.30	0.85	2.28	2.30	4	3.73	6
47	LIYAASTLQ	55	1.91	1.85	2.46	2.07	1.79	2.36	1.08	1.79	2.10	8	3.99	6
48	IYAASTLQS	56	1.73	2.01	2.68	1.26	1.58	1.93	1.82	2.11	2.07	7	3.52	6
49	YAASTLQSG	57	0.64	1.60	0.59	0.32	1.42	1.34	1.28	0.04	1.09	0	3.61	6
▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪
▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪
205	VTKSFNRGE	213	0.89	1.94	0.88	0.95	1.26	1.92	1.21	1.12	0.60	2	0.00	5
206	TKSFNRGEC	214	0.17	-0.73	-0.79	0.17	0.91	0.21	0.44	-0.58	1.07	0	0.00	5

Summarized Results	DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*0901	DRB1*1101	DRB1*1301	DRB1*1501	Total
Maximum Single Z-score	2.40	2.26	3.16	3.11	2.41	2.71	2.29	2.52	2.52	--
Sum of Significant Z-scores	37.33	26.05	31.83	30.59	14.18	36.40	22.64	22.72	26.87	248.61
Count of Significant Z-scores	19	13	14	15	7	18	12	11	13	122

Total Assessments Performed: 1854	Deviation from Expectation: 42.39	Deviation per 1000 AA: 22.86
Adjusted for Regulatory Epitopes	Deviation from Expectation: -34.63	Deviation per 1000 AA: -18.68

Figure 5. Regional assessment for Immunogenicity. EpiMatrix assessment of an example antibody in the ISPRI toolkit. Each overlapping 9 mer is scored for predicted affinity to one of nine HLA class II alleles. All scores in the top 5% (Z-Score ≥ 1.64) are considered "hits". Scores in the top 10% are considered elevated, other scores are grayed out for simplicity. Frames containing four or more alleles scoring above 1.64 are referred to as EpiBars and are highlighted in yellow. These frames have an increased likelihood of binding to HLA. Frames conserved in IgG antibodies and believed to be either passively tolerated or actively regulatory are highlighted in green.

In the retrospective studies,[73] Tregitope-adjusted scores were well correlated with observed clinical immune responses for 22 antibodies granted marketing approvals.[14] In this study, the average Tregitope-adjusted EpiMatrix Score of 10 mAbs known to induce immune responses in more than 5% of exposed subjects was −1.84. The average Tregitope-adjusted EpiMatrix Score of 10 mAbs that were known to induce immune responses in fewer than 5% of exposed subjects was −32.01. Therefore, using the original Tregitope-adjusted model of ADA prediction (Version 1.0) for similar antibodies and antibody-derived products for the same indications, calculation of T cell epitope content and adjustment for Tregitope content is likely to provide a more accurate forecast of immunogenic potential. It is important to note, however, that these antibodies were not checkpoint inhibitors, which directly modulate immune responses, as discussed below.

Antibody immunogenicity: new regression analysis

In previous retrospective studies, the Tregitope-adjusted T cell epitope score was significantly better correlated to immunogenicity in the clinic than the overall (unadjusted) T cell epitope content per unit length.[73] Additional studies were performed with a separate set of 43 antibodies for which clinical data from various sources, including FDA labels and publications, are available (see Supplementary Table 1) to determine if the Tregitope-adjusted EpiMatrix score could be used to prospectively evaluate immunogenicity risk, keeping in mind that published ADA incidence is highly dependent on the sensitivity of the assay, patient co-medication and, potentially, the mechanism of action of the therapeutic and thus may vary among studies. This retrospective study also enabled a re-assessment and update of our existing regression model.

The revised Tregitope-adjusted model of ADA prediction (Version 2.0) is based on an exponential equation. Evaluating this updated dataset with our revised model, we found a significant correlation between Tregitope-adjusted EpiMatrix Scores and predicted ADA responses (Pearson correlation coefficient = 0.75, p-value <0.001). The predicted ADA based on the Tregitope-adjusted EpiMatrix Score also had a low root mean squared error (9.53), suggesting that predicting immunogenicity (as measured by ADA) using the Tregitope-adjusted EpiMatrix Score in an exponential model was close to the observed immunogenicity. Further, the regression line established by the revised model is highly correlated with the regression line established by our original model (Figure 6).

Additional studies incorporating more recently approved antibodies are ongoing. These results using 43 antibodies (Supplementary Table 1) and the new preliminary results with additional mAbs suggest that the relationship between the Tregitope-adjusted EpiMatrix score and observed immunogenicity is maintained.

Case study 2: Bococizumab

We analyzed the amino acid sequences of a set of mAbs with the same cardiovascular molecular target, PCSK9: evolucumab, alirocumab, bococizumab, and determined their potential immunogenicity risk by calculating the Tregitope-adjusted

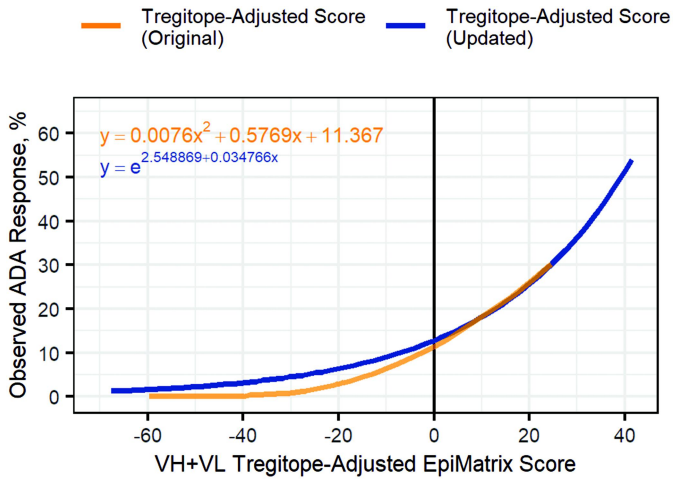


Figure 6. Twenty-two licensed antibodies make up the polynomial regression used for predicting T-dependent ADA responses in ISPRI (orange line). The updated regression model adds 21 new mAb examples with clinical immunogenicity data. Observed immunogenicity indicates the percent of exposed patients with a positive immunogenic response as defined by a positive ADA titer and reported from clinical trials identified in the FDA-approved drug product labels.

Immunogenicity Score. The predicted immunogenicity of these antibodies was calculated using their Raw (unadjusted) and Tregitope-adjusted EpiMatrix Score and the established (2013) ADA regression model. Their Tregitope-adjusted EpiMatrix Scores were: evolucumab (−45.06), Alirocumab (−35.05), and bococizumab (−20.44). While the scores for evolucumab and alirocumab are lower than the average score for non-immunogenic antibodies (−32.01), the score of bococizumab is higher than this benchmark and suggests a greater potential for immunogenicity. These scores were corroborated in the clinic: the highest ADA rate was observed for bococizumab as compared to evolucumab and alirocumab (Figure 7, Box B).

In addition to its Tregitope-Adjusted EpiMatrix Score, the identification of a high-risk cluster in the variable light chain contributes to the overall high estimated immunogenicity risk for bococizumab.[74] Further, others have confirmed the high numbers of potential CD4 T cell epitopes in bococizumab and other immunogenic mAbs using alternative methods, such as MAPPs, and in vitro peripheral blood mononuclear cell assays[75] also align with the in silico observations made with ISPRI. Development of bococizumab was terminated due to its higher overall immunogenicity in the clinic.

Considering clinical context: antibody target and mechanism of action

While Tregitope-adjusted EpiMatrix Score is generally well-correlated with the observed clinical incidence of ADA, the clinical indication for the antibody is important to consider. For example, if the target of the antibody is an immune-modulating protein, or the mechanism of action of the therapeutic modifies the T cell (e.g., anti-CD3 antibodies) or B cell response (e.g., anti-CD19 antibodies), the target and mechanism of action may play a role in the overall immunogenic risk.

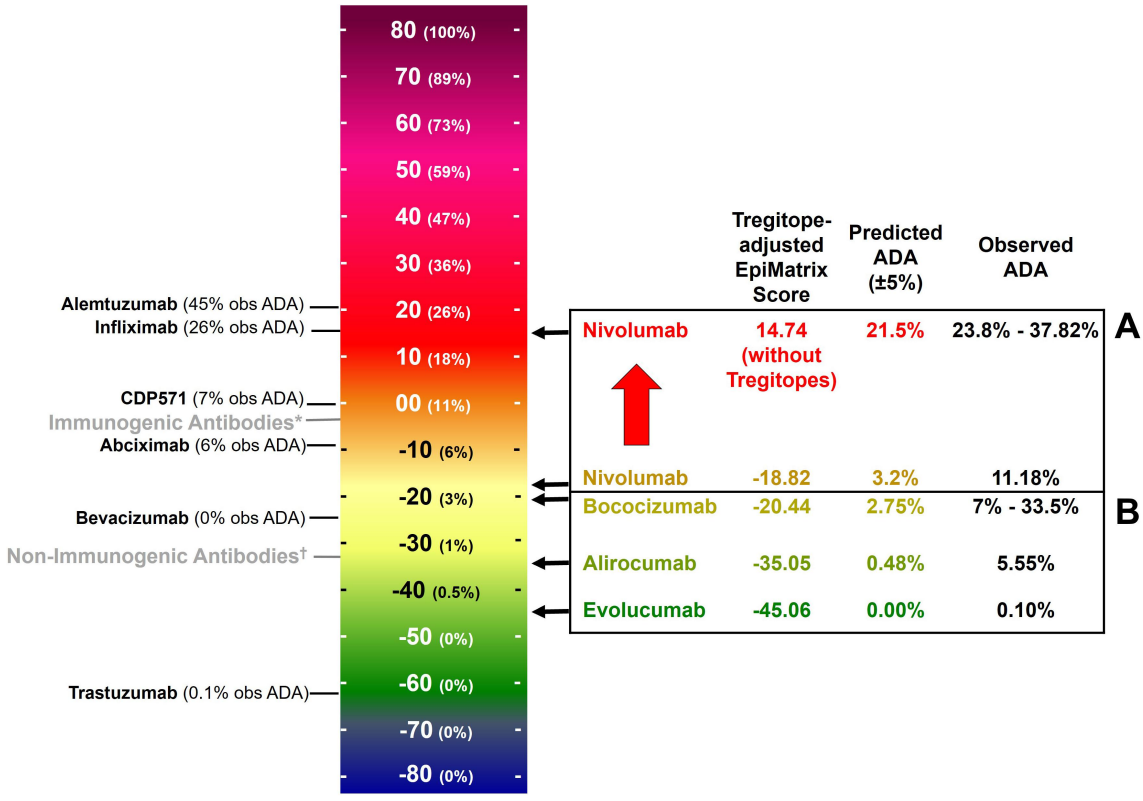


Figure 7. EpiMatrix predicted excess of shortfall in predicted aggregate immunogenicity relative to a Random Protein Standard (per 1,000 9-mer frames analyzed). All scores are adjusted for the presence of tregitopes. Predicted ADA responses are indicated in parentheses. Predicted response = 11.367 + 0.5769 * tregitope-adjusted score + 0.0076 * tregitope-adjusted Score^2 (R^2=0.6722). Scores shown represent the combined protein score for the VH/VL sequences. Observed ADA responses for benchmark antibodies are indicated in parentheses on the lefthand side of the scale. *Average of 10 antibodies (VH/VL pairs) known to Induce Anti-Therapeutic responses in more than 5% of patients. †Average of 10 antibodies (VH/VL pairs) known to Induce Anti-Therapeutic responses in less than 5% of patients.

As another example, the effect of regulatory T cell epitopes such as Tregitopes may be reduced in the context of checkpoint inhibitor therapy, which is specifically designed to activate quiescent T cells expressing checkpoint inhibitory molecules, including PD-1, PDL-1, and CTLA-4.

Case study 3: nivolumab and ipilimumab

We evaluated the immunogenicity of two checkpoint inhibitors, anti-PD-1 nivolumab and anti-CTLA-4 ipilimumab. Nivolumab was reported to induce an ADA response in 11.18% of subjects receiving monotherapy, but in nearly 38% of subjects receiving a combination of nivolumab and ipilimumab,[76–78] which may be related to the synergistic effect of ipilimumab on regulatory T cells. In this case, considering the clinical context and the expectation that Treg function will be impeded, the Tregitope-adjusted EpiMatrix Score may underestimate the immunogenic potential of the checkpoint inhibitor. The Tregitope-adjusted EpiMatrix Score of nivolumab (−18.82) predicts ADA response in 3.2% of exposed subjects. However, if the Raw EpiMatrix Score for nivolumab (14.72) is used instead of the Tregitope-adjusted score, the regression model predicts ADA response in 21.5% of exposed subjects, which is more consistent with the clinical observations (Figure 7, Box A). Thus, for mAbs in which the mechanism of action is immunostimulatory, and where the impact of Tregitopes may be limited, the raw EpiMatrix score may be preferentially used over the Tregitope-adjusted score for ADA prediction.

Managing immunogenicity risk (OptiMatrix)

When combined, the observations related to JanusMatrix and Tregitope and those related to clinical immunogenicity suggest that foreign T cell epitopes play a significant role in the development of ADA.[27] Efforts to humanize mAbs have rendered these biologics less foreign to the human immune system and successfully reduced the potential for development of the ADA.[79] Building on humanization of murine or chimeric mAb sequences, another technique for reducing the potential development of ADA is deimmunization, which decreases the overall HLA binding affinity of defined sequences based on the identification of high-ranking T cell epitopes within even fully human sequences. OptiMatrix is a specialized tool that can be used to reduce the predicted binding of clustered, or "promiscuous' epitopes in silico, while preserving regulatory T cell epitopes if they are present in the same sequence.

Tolerization is another concept that we have proposed.[80] Options for modification include making amino acid substitutions that increase homology to the human germline, or match known Tregitopes, or eliminate predicted T-helper epitopes. First, an in-silico analysis is performed to identify key amino acid residues, and then modifications to the sequence are iteratively analyzed in silico, producing a list of potential modified peptides and proteins. Of course, in silico analysis does not permit a comprehensive assessment of the impact of changes on the overall structure and stability of the mAb. Other methods, such as in vitro binding assays and three-dimensional modeling, are

required to estimate the impact of OptiMatrix changes on stability and affinity. The modified peptides can then be synthesized and compared in HLA binding assays and T cell assays to validate the deimmunization of the modified peptides. Immunogenicity mitigation approaches can be used in these cases to create a less immunogenic therapeutic protein.[80,81] As described in a number of publications, OptiMatrix has been successfully applied to a range of proteins, including Factor VIII, alpha-interferon, and Complement Factor 3 (C3d) peptides.[81–83]

Discussion

As shown by the case studies described above, the tools included in the ISPRI toolkit can be used to analyze protein and peptide sequences for immunogenicity liabilities and to mitigate immunogenicity risk prior to the start of clinical studies. Over the course of time, the EpiMatrix epitope prediction algorithms have been refined, ancillary analysis tools have been expanded, users have been trained, and case studies supporting the utility of the tool have been published. The availability of in silico tools such as those provided in ISPRI has re-shaped the pharmaceutical industry's approach to immunogenicity screening, moving it earlier in the pipeline, which has likely contributed to the development of safer, more effective products[5,25,84–87] and saved organizations from having to terminate costly clinical studies of therapeutics due to immunogenicity and related adverse events.

As was shown for fully human therapeutics such as human erythropoietin and thrombopoietin, the presence of significant CD4 epitope content is a potential risk[88] that, when realized, can lead to unwanted immunogenicity given the right circumstances.[89,90] Judicious use of the ISPRI platform in the future could guide drug developers to make informed decisions at critical junctures. By eliminating high-risk candidates early, drug developers can save valuable time and resources that would be required to perform in vitro or in vivo screening of the myriad leads that must be triaged on the path from discovery to market.

In addition to defining methods that contribute to the successful development of biologic products, ISPRI tools have been used to define new concepts in immunology. Consider, for example, the preliminary evidence provided in this perspective that the human proteome has been shaped by the CD4 T cell response over evolutionary time.[91] Human proteins contain fewer putative HLA-DR ligands than we would expect by random chance, and that effect is even more pronounced in the subset of secreted proteins, those most likely to be presented by the class II degradation pathway. This finding suggests the need for a more conservative filter when screening candidate therapeutic proteins. The phrase "no greater than observed for a random protein sequence" does not necessarily imply safety from an immunogenicity perspective, since lower T cell epitope content overall appears to be linked to better clinical outcomes.

Also consider the discovery of Tregitopes in 2008,[59] which significantly affected the immunogenicity risk assessments of mAb drugs going forward, highlighting the importance of conserving specific sequences in IgG frameworks to counterbalance immunogenic regions in CDRs. Discovery of Tregitopes may have also elucidated the immunomodulatory impact of IVIg therapy. JanusMatrix is contributing to the discovery of new Treg epitopes in pathogens and human proteins.

We have shown that immunogenicity risk assessment for mAbs and antibody-modality product candidates requires compensation for the presence of regulatory T cell epitopes, as well as consideration for the clinical setting in which these products will be used. Furthermore, a clear demarcation between immunogenic mAb sequences, which carry elevated putative T cell epitope content, and non-immunogenic mAbs has been defined. Integrating data from hundreds of clinical studies for more than 40 approved mAbs enabled the definition of a threshold that differentiates potentially immunogenic (>5% ADA) and non-immunogenic (<5% ADA) mAbs based on analysis of their sequence for T cell epitopes.

As clinical indications change over time, it may be necessary to adjust our risk assessments. As shown above, for mAbs that are being applied to checkpoint blockade, where Treg function is impeded, the Tregitope-adjusted score may overestimate the benefit of regulatory T cell epitopes on dampening immunogenic potential. On the contrary, if Treg epitopes are present but regulatory T cells are not functional or are temporarily impeded from functioning normally by an inflammatory milieu, T cell responses may be biased away from tolerance. In these cases, the Raw EpiMatrix Score may provide the best assessment of immunogenic risk. In the context of indications and respective therapeutics where Treg function is not affected, the Tregitope-adjusted EpiMatrix Score may provide a more accurate risk assessment.

Validation studies

As described above, a number of retrospective and prospective studies have provided additional validation of the ISPRI platform for monospecific and bispecific mAbs.[21,23,24,34,74,92] Recent publications on immunogenicity risk assessment can provide additional detail.[30,93] Other clinically relevant factors such as dose and frequency of administration, route of administration, concomitant medications, and the mechanism of action, and other product-related considerations, including formulation (for example, the presence of innate immune response modifiers in the formulated drug product that may act as immunostimulatory adjuvants), aggregation, oxidation, deamidation, and other chemical degradative processes, may modify the immunogenic potential of biologic therapeutics. Clinical context factors may also bear on immunogenicity, such as the extent of inflammatory and ongoing immune responses at the time of product administration, which may contribute to immunogenicity vs. ongoing treatment with immunomodulatory agents, which may diminish the immune response to introduced biological agents. ISPRI users are always encouraged to use additional methods for orthogonal validation of the computed immunogenicity risk assessments provided by ISPRI.

Additional studies are underway to prospectively identify new, potentially tolerogenic epitopes using JanusMatrix. While

previously published studies of epitopes from viruses have confirmed the association between high JanusMatrix Scores (epitopes containing TCR-facing motifs that are highly conserved within the human proteome) and the induction of regulatory T cell responses,[51,94] additional studies are likely to confirm additional Treg epitopes in biologic therapeutics. For example, it is likely that high JanusMatrix-scoring T cell epitopes may randomly occur in the CDRs of mAbs in the process of affinity maturation; thus, it is important to check for human homology at the TCR face of these sequences before considering any engineering modifications to the T cell epitopes in the CDR regions. Further in vitro studies will be performed so as to better define thresholds for tolerance using JanusMatrix.

Meanwhile, our group has accumulated over 15 years of experience working with a relatively small set of Ig-derived regulatory T cell epitopes in our laboratory and the laboratories of our collaborators.[32,58,62,63] New Tregitopes are being identified over time; these are added to the ISPRI analysis and immunogenicity scoring calculation as they are validated. Regulatory epitopes are likely to exist in many other autologous proteins, and these epitopes may also be restricted by individual HLA. In the process, we have developed an in vitro assay for validating new Treg epitopes and re-validating existing Tregitope peptides by demonstrating their ability to suppress the inflammatory response to a memory T cell response.[58] This assay will be used to test new candidate Tregitopes, both promiscuous and personalized, and will align the in silico and in vitro data we collect to establish a better-supported threshold for future prospective studies.

Conclusions

The strong correlation between T cell epitope content, HLA-binding peptides, tolerogenic epitopes and immunogenicity is likely to be reconfirmed again and again, as newer antibodies enter the clinical pipeline. Access to rapid immunoinformatics tools such as the ISPRI toolkit is likely to reduce drug development costs while improving safety and efficacy. As data become available for newer antibody modalities and indications, we will continue to add to our regression model for antibody ADA prediction and will be able to perform high throughput analyses of large antibody datasets. Updated models for immunogenicity risk assessment will support deimmunization, humanization, and other approaches to addressing the potential immunogenicity of antibody therapeutics within the ISPRI toolkit.

Future directions

High throughput/big data/artificial intelligence

As newer and even larger datasets emerge, the ISPRI toolkit can be put to work to uncover patterns and improve immunogenicity risk assessment accuracy. For example, MAPPs data is now published on publicly accessible databases such as the Immune Epitope Database.[95] Nielsen et al. have suggested that T cell epitope prediction can be improved using this data source,[96] and artificial intelligence interventions can be expected. Additional information that is available in large datasets of antibody sequences could also be leveraged for application to risk assessment. One example is high throughput B-cell receptor sequencing and cloning. Recent academic collaborations that implement the ISPRI toolkit have included a high throughput analysis of more than 200,000 B cell-derived heavy-light chain sequence pairs for HLA-DR-restricted T effector and Treg epitopes, matched to the HLA-DR type of five individual donors.[66] These studies will provide valuable insight into the personalized assessment of immunogenic potential in the context of antibody somatic hypermutation.

Automated upload options to ISPRI are possible to facilitate the screening of hundreds of light chain and heavy chain pairs that are produced in the process of optimizing antibody candidates. The epitope mapping data generated by ISPRI, including Tregitope-adjusted immunogenicity scores, can be automatically provided via machine-to-machine interface to developers, who can screen hundreds of sequences using the API connection to the ISPRI platform every month. The need for high throughput immunogenicity screening analysis capacity is evident as biologics developers pioneer advanced technologies to discover new molecules with therapeutic potential and construct them in novel modalities to improve their function and efficacy.

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Disclosure statement

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Abbreviations

ADA	Anti-drug Antibodies
API	Application Programming Interface
CDR	Complementarity Determining Region
HLA	Human Leukocyte Antigen
MAPPs	MHC-associated peptide proteomics
MHC	Major Histocompatibility Complex
PSSM	Position-Specific Scoring Matrix
TCR	T cell receptors
Teff	T Effector Epitope
Treg	Regulatory T cell

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