Prediction of immunogenicity for therapeutic proteins: State of the art

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Immunogenicity is a significant problem associated with protein therapeutics, but can be predicted in advance by in silico, in vitro, and in vivo tools, which can identify sequences within the therapeutic protein that, when processed by T-cells, elicit an immune response. Recent developments in T-cell-dependent immunology relating to the immunogenicity of therapeutic products include the description of toll-like receptor ligands and the identification and classification of regulatory T-cells. A limitation in determining the relative immunogenicity of potential therapeutic proteins is the variance in the immunogenicity determined by in vitro or in vivo techniques in animal and human models. However, given the sophistication and high-throughput capacity of existing in silico tools and the availability of precise in vitro validation assays, accurate prediction of immunogenicity for therapeutic protein products, and more rapid translation of research discoveries into clinical success, may be within reach.

Keywords Immunogenicity, immunoinformatics, protein therapeutic, T-cell epitope prediction

Abbreviations

ADA anti-drug antibodies, **ANN** artificial neural network, **APC** antigen-presenting cell, **B1** B-cell signal 1, **B2** B-cell signal 2, **B3** B-cell signal 3, **B4** B-cell signal 4, **CD** cluster of differentiation, **DC** dendritic cell, **ELISA** enzyme-linked immunosorbent assay, **ELISpot** enzyme-linked immunosorbent spot, **EMEA** European Agency for the Evaluation of Medicinal Products, **FDA** Food and Drugs Administration, **FVIII** clotting factor VIII, **HLA** human leukocyte antigen, **IFN** interferon, **IEDB** Immune Epitope Database and Analysis Resource, **Ig** immunoglobulin, **IL** interleukin, **iTEM** individualized T-cell epitope measure, **mAb** monoclonal antibody, **MHC** major histocompatibility complex, **PAMP** pathogen-associated molecular pattern, **T1** T-cell signal 1, **T2** T-cell signal 2, **TCR** T-cell receptor, **Td** T-cell-dependent, **TGF** transforming growth factor, **TLR** toll-like receptor, **TNF** tumor necrosis factor, **Treg** regulatory T-cells

Introduction

Why should drug developers worry about immunogenicity? All exogenous proteins, including therapeutic proteins, have the potential to cause antibody formation. Therapeutic proteins have revolutionized the treatment of many diseases and, because many more therapeutic proteins are likely to become available for an increasingly wide range of indications, scientists and clinicians are becoming increasingly aware of the importance of assessing the immunogenicity of new molecules to avoid the development of treatment resistance and potentially life-threatening immune responses (exemplified by the responses of some patients to a recombinant human erythropoietin used in the European market [1-3]). Although the production of therapeutic proteins has improved with advanced recombinant expression, purification and formulation technologies, the use of recombinant, purely human or humanized proteins has not completely eliminated immunogenicity problems associated with therapeutic proteins. Anti-drug antibodies (ADA) develop following repeated administrations of a number of clinically approved antibodies in immunocompetent patients; thus, the FDA and European Agency for the Evaluation of Medicinal Products (EMEA) are beginning to develop standards for immunogenicity assays to clarify for drug developers which tests must be performed prior to, and following, the drug approval process.

Until recently, most of the publications and position papers addressing immunogenicity focused on methods for measuring ADA [4]. However, drug developers are now beginning to view immunogenicity from the perspective of T-cells. This is because antibodies that develop following administration of a protein therapeutic are not generated independently of a T-helper cell immune response. For example, when immunoglobulin (Ig)G isotype ADAs are detected, it is probable that T-cells contributed to the observed response, as they are usually required for isotype switching within from IgM to IgG. Furthermore, B-cells that do not receive T-cell help are not able to persist as B-memory cells (see later for a fuller description of the role of the T- and B-cell in the antibody response). Standards for measuring ADA have been formulated, but standards for measuring T-cell responses to therapeutic proteins have yet to be defined. How well do the current tools measure T-cell epitope content? Are the tools accurate? Which tools are the most useful? Efforts to develop a consensus on methods for measuring T-cell-dependent immunogenicity are now underway.

In addition to measuring immune responses, drug developers need rapid, low-cost means for predicting which protein-based drugs will be least likely to induce ADAs in clinical trials. Most developers are painfully aware of the number of new protein-based drugs that fail in the clinic because of immunogenicity, even though few such reports are published. Fortunately, the ability to discriminate between non-immunogenic protein therapeutics and those that will generate immune responses in the clinic is now possible using rapid *in silico* approaches. Scientists are now able to prospectively identify monoclonal antibodies

(mAbs), protein therapeutics and other protein-based drugs (eg, enzymes, fusion proteins, peptibodies and protein scaffolds) that are likely to generate an ADA response. However, as with any informatics tool, it is necessary to validate immunogenicity predictions by *in vitro* and *in vivo* assays. The best approach to measure and/or predict a T-cell response to a protein therapeutic is to couple rapid *in silico* mapping of the protein sequence to find potential epitopes with *in vitro* confirmation (using human leukocyte antigen [HLA] binding assays or T-cell assays) as described later. *In vivo* immunogenicity analysis in suitable animal models is an additional important validation step prior to moving a potential protein therapeutic into clinical trials.

This review will discuss the measurement and prediction of T-cell related peptide immunogenicity. This type of immunogenicity is also known as T-cell-dependent (Td) immunogenicity. A brief review of the biogenesis of T-cell responses, the different types of T-cell epitope prediction tools, and the validation of *in silico* predictions using *in vitro* and *in vivo* assays are presented. The review will also highlight the improved understanding of new immunogenicity screening technologies that are currently available and the principles behind immunogenicity, which will hopefully lead to the development of safer protein therapeutics.

General immunology principles

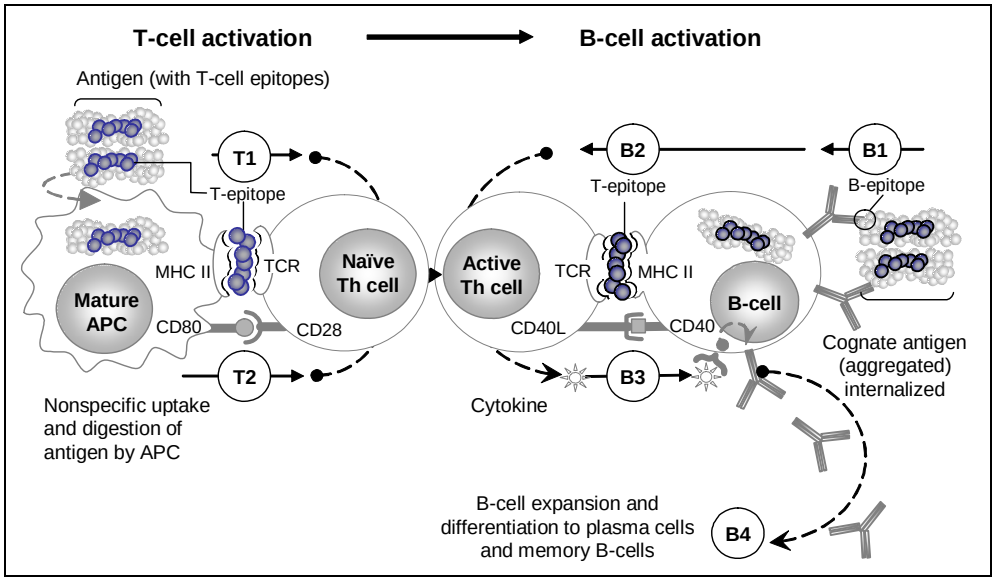
T-cell-dependent antibody responses

Figure 1 provides an overview of the role of the T-cell in the antibody response.

In order to induce a Td antibody response to a protein antigen (such as a therapeutic protein), several events must be coordinated, usually within specialized regions of secondary lymphoid organs (eg, lymph nodes and spleen). The first step in this process is internalization of antigen by professional antigen-presenting cells (APCs) such as dendritic cells (DCs; left side of Figure 1). The mature APC processes the antigen into peptides (epitopes) that are then presented to naïve T-cells by major histocompatibility complex (MHC) class II molecules on the APC surface. The interaction of a T-cell receptor (TCR) with this MHC II-epitope complex is identified as T-cell signal 1 (T1). In order to fully activate the T-cell, which is required for B-cell activation, T1 must be accompanied by additional signals from costimulatory molecules such as cluster of differentiation (CD)80 and CD86, which are provided by the APC; these additional signals are termed T-cell signal 2 (T2).

In the absence of T2, T-cells vital to an immune response may become inactive. However, once fully activated, these T-cells divide and produce an array of cytokines. Activation of the naïve B-cell is initiated by an interaction between IgM and IgD receptors on the surface of the B-cell and their cognate antigen; this is termed B-cell signal 1 (B1). Upon encountering a B-cell that has recognized cognate antigen bound via a specific T-cell epitope/MHC:TCR interaction, the T-cell delivers cytokines that stimulate the B-cell to proliferate and mature toward a plasma cell (B-cell signal 2 [B2]). This interaction results in the engagement of CD40 and CD40 ligands, which communicate a further signal to the presenting B-cell – B cell signal 3 (B3) – leading to antibody production via B-cell clonal expansion and differentiation into antibody-secreting plasma cells and memory B-cells (which persist after an initial immune response to combat re-infection); this is termed B-cell signal 4 (B4).

Figure 1. Activation of CD4 T cells and the T-cell dependent antibody response.



APC antigen-presenting cell, **B1** B-cell signal 1, **B2** B-cell signal 2, **B3** B-cell signal 3, **B4** B-cell signal 4, **CD** cluster of differentiation, **MHC** major histocompatibility complex, **T1** T-cell signal 1, **T2** T-cell signal 2, **TCR** T-cell receptor, **Th** T-helper cell

Central tolerance

T-cells mature in the thymus to express newly recombined TCR sequences. T-cells bearing TCRs that bind with high affinity to self-peptides presented on MHC class I or class II molecules in the thymus undergo negative selection, whereby these cells are either deleted or enter a state of inactivity in which cells are unable to produce an effective immune response. This process generates central tolerance, an important checkpoint for immune system development. Autoreactive B-cells are also deleted in the course of immune system development (possibly in the bone marrow), although a few may escape. Autoreactive B- and T-cells are controlled by regulatory T-cells (Tregs), as described in the next paragraph.

Peripheral tolerance

Peripheral tolerance mechanisms also exist as a safety net for tolerance against self-antigens expressed in peripheral tissues, but not primary lymphoid organs, and for control of autoreactive B- and T- cells that escape regulation by central tolerance mechanisms. Tregs secrete anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)β, which directly downregulate autoreactive T-cells. Similarly, circulating autoreactive B-cells may undergo functional inactivation. For example, in the absence of T-cell help, these autoreactive B-cells are not stimulated to produce antibodies. Because Tregs serve as a means of inducing peripheral tolerance, they may be exploited to modify existing, unwanted immune responses, and therefore represent an important target for improving protein therapeutics [5•,6•].

The nature of immune responses to therapeutic proteins

T-cell responses to protein therapeutics can be classified in one of two categories, depending on whether the protein is foreign or autologous. In the former case, the T-cell responses are described as 'vaccine-like' and in the latter, 'autoimmune-like'.

Vaccine-like reactions to protein drugs

The outcome of repeat administration of a non-autologous protein therapeutic will be governed by the classic principles of immune responses to foreign proteins. Factors that influence this outcome include inherent protein antigenicity, as measured by the T-cell epitope content of the protein's amino acid sequence, the inadvertent presence of contaminants, such as bacterial DNA, in the therapeutic formulations, and other factors that induce an innate immune response, such as dose size, route of administration and frequency of dosing.

If T-cell epitopes are present in the foreign protein, T-cell responses can usually be detected *in vitro*. T-cell epitopes are expected to induce the cytokines IL-2, IL-4, IL-5 and interferon (IFN)γ production in an epitope-specific manner. When exposure to the foreign antigen is recent, T-cell responses are easily detectable. For T-cell populations that are expected to be reduced in number because of no recent exposure to antigen, expansion of these antigen-specific populations prior to performing the T-cell assay would elicit such a cytokine response. For further information, De Groot & Rappuoli provide a more complete picture of T-cell immunogenicity related to vaccine-like reactions [7•].

Immune responses to autologous protein drugs

The principle of self-tolerance suggests that human-derived autologous proteins, such as fully human or humanized antibodies, should not elicit an immune response. However, T- and B-cell responses to autologous proteins are frequently observed. For example, T-cell responses have been observed for a number of autologous proteins in the treatment of diabetes [8,9•] and multiple sclerosis [10,11]. Furthermore, T-cell epitopes have been clearly defined in the response to clotting Factor VIII, to which some hemophiliacs may be partially tolerant [5•]. Immunogenicity induced by autologous proteins is probably the result of perturbations of B- and T-cell tolerance. In this setting, a complicated series of immunological events occur, which are not yet fully understood.

It is likely that the anti-self or autoimmune response to some therapeutic proteins involves aspects of the innate immune system and the regulation of adaptive immune responses by Tregs. T-cells responding to epitopes derived from autologous proteins may respond as either effector T-cells (inducing IL-2, IL-4 or IFNγ proliferation) or as Tregs (inducing TGFβ, tumor necrosis factor (TNF)α and chemokines such as macrophage inflammatory protein [MIP]1α/β). To distinguish between these two possibilities, it is important to measure cytokine profiles that can differentiate between the two cellular phenotypes, or to directly identify the nature of the T-cells by their distinct cell-surface or intracellular markers (such as CD25 or Foxp3, respectively), rather than evaluate only cytokine response or cellular proliferation. Thus, identification of T-cell epitopes within the sequence of therapeutic proteins must be coupled with phenotypic studies to determine whether the T-cell response will be inflammatory (effector), or suppressive (regulatory).

Immunoinformatics – a guide

Immunoinformatics facilitates the understanding of immune function by modeling the interactions among immunological components, and in the context of this review, immunoinformatics can be used to determine the likely immunogenicity of a therapeutic protein. Despite the potential of bioinformatics to accelerate immune system research, the field of immunoinformatics is still in its infancy. Yet the complexity of immune interactions and the data-intensive nature of immune research makes immunology a prime area for computational methodologies. Current immunoinformatics resources include searchable databases of epitopes and immune-related molecules, and analysis tools for T- and B-cell epitope prediction, vaccine design, and protein structure comparisons.

What can be predicted using immunoinformatics?

T-cell epitopes

The T-cell response to a protein therapeutic antigen is dependent on the binding of T-cell epitopes to MHC, presentation of the MHC:epitope complex at the cell surface,

and recognition of that complex by either an effector or Treg, an interaction that can be evaluated in MHC or HLA binding assays. An additional means of determining whether a protein therapeutic contains sequences that have a propensity to stimulate a Td antibody response is to evaluate whether T-cells respond to the peptide epitopes derived from the therapeutic protein in *in vitro* T-cell assays. Because the synthesis of hundreds or thousands of short overlapping peptides for testing in such assays is cost- and labor-prohibitive, selective *in silico* methods are practical alternatives. Indeed, the critical determinant of T-cell immunogenicity is related to the strength of epitope binding to MHC molecules [12]. Therefore, bioinformatics tools that can accurately model the MHC:epitope interface and accurately predict immunogenic peptide sequences have become essential tools for evaluating the potential immunogenicity of protein therapeutics.

Protein-derived T-cell epitopes are linear, and their binding to MHC is determined by the interaction of their side chains with binding pockets in the floor of the MHC epitope-binding groove. The presence or absence of specific amino acid side chains determines how tightly an epitope binds to MHC, if at all. A number of MHC-binding motif-based tools that permit the scanning of protein sequences for potential T-cell epitopes have been developed. 'Matrix-based' T-cell epitope selection algorithms were developed by Davenport & Ho Shon [13] for one MHC class II allele in 1995, and by Jesdale & De Groot for an array of MHC class I and class II alleles in 1997 [14]. More than 20 T-cell epitope mapping tools currently exist, but not all are equivalent.

A list of T-cell epitope mapping tools is provided in Table 1. For reviews of T-cell epitope mapping tools, see De Groot & Berzofsky [15•,16•] and the 2005 web server issue of *Nucleic Acids Research* [17•]. Furthermore, Sturniolo *et al* [18•] and Zhang *et al* [19] developed the pocket profile method, one of the more important advances in epitope mapping. According to this method, similarities in MHC binding constraints are reflected in commonalities in the composition of MHC binding pockets.

T-cell epitope content and coverage

Highly immunogenic proteins contain many T-cell epitopes or concentrated clusters of T-cell epitopes within their amino acid sequences, whereas non-immunogenic proteins tend to contain fewer epitopes. One concept, developed by Martin & De Groot, is that T-helper cell epitope content per unit

Table 1. An overview of validated T-cell epitope-mapping tools.

Tool	Developer	HLA class I (number)	HLA class II (number)	Description	Web site
EpiMatrix	EpiVax Inc	24	74	Matrix-based, pocket profile methods	*http://www.epivax.com*
Class I/class II binding prediction	IEDB	34	13	ANN, average relative binding method, stabilized matrix method	*http://tools.immuneepitope.org/analyze/html/mhc_binding.html/http://tools.immuneepitope.org/tools/matrix/iedb_input?matrixClass=II*
SYFPEITHI	University of Tübingen	19	6	Extended anchor method	*http://www.syfpeithi.de/*
MHC Thread	Hebrew University	4	–	Protein threading/fold recognition	*http://margalit.huji.ac.il*
MHCPred	The Edward Jenner Institute for Vaccine Research	11	3	Partial least squares-based multivariate statistical method	*http://www.jenner.ac.uk/MHCPred*
EpiJen	The Edward Jenner Institute for Vaccine Research	18	–	Additive approach based on Free-Wilson	*http://www.jenner.ac.uk/EpiJen*
NetMHC	Institute of Medical Microbiology and Immunology	120	–	ANN, weighted matrices	*http://www.cbs.dtu.dk/services/NetMHC*
NetCTL	Institute of Medical Microbiology and Immunology	12	–	ANN	*http://www.cbs.dtu.dk/services/NetCTL/*
nHLAPred	Institute of Microbial Technology	91	–	ANN, matrices	*http://www.imtech.res.in/raghava/nhlapred/*
SVMHC	University of Tübingen	20	+[a]	Support vector machine for MHC class I	*http://www-bs.informatik.uni-tuebingen.de/Services/SVMHC*
Bimas	NIH	32	–	Matrix-based	*http://thr.cit.nih.gov/molbio/hla_bind/*

[a]Published by Sturniolo *et al* [18•], **-** Human leukocyte antigen (**HLA**) class molecule is not available, **+** HLA class molecule is available.
ANN artificial neural network, **IEDB** Immune Epitope Database and Analysis Resource, **MHC** major histocompatibility complex, **NIH** National Institutes of Health

protein count may explain the differences in observed antibody responses to slightly different versions of the same recombinant human protein [20]. These authors developed the Td immunogenicity scale, which enables the evaluation and comparison of protein sequences for T-cell epitopes using EpiMatrix, a matrix-based algorithm for T-cell epitope mapping (Figure 2). EpiMatrix is standardized so that comparisons can be made across predictions for different HLA MHC alleles.

To create Figure 2, the EpiMatrix prediction matrices were used for eight MHC class II alleles that are representative of more than 98% of human populations. First, the number of potential T-helper epitopes that would occur in random-sequence pseudo proteins composed of amino acids at their naturally occurring frequencies were measured, which computed a mean 'epitope score' per 1000 assessments of 0.5, with a standard deviation of ± 7.9. The variable light and heavy chains of the antibodies specific to the relevant tested protein identified were compared by summing the total number of EpiMatrix scores for each protein that was above an accepted cutoff for immunogenicity (epitope score > 1.67), and the difference between the scores for random proteins and the scores for the light and heavy chain variable regions in aggregate were then measured.

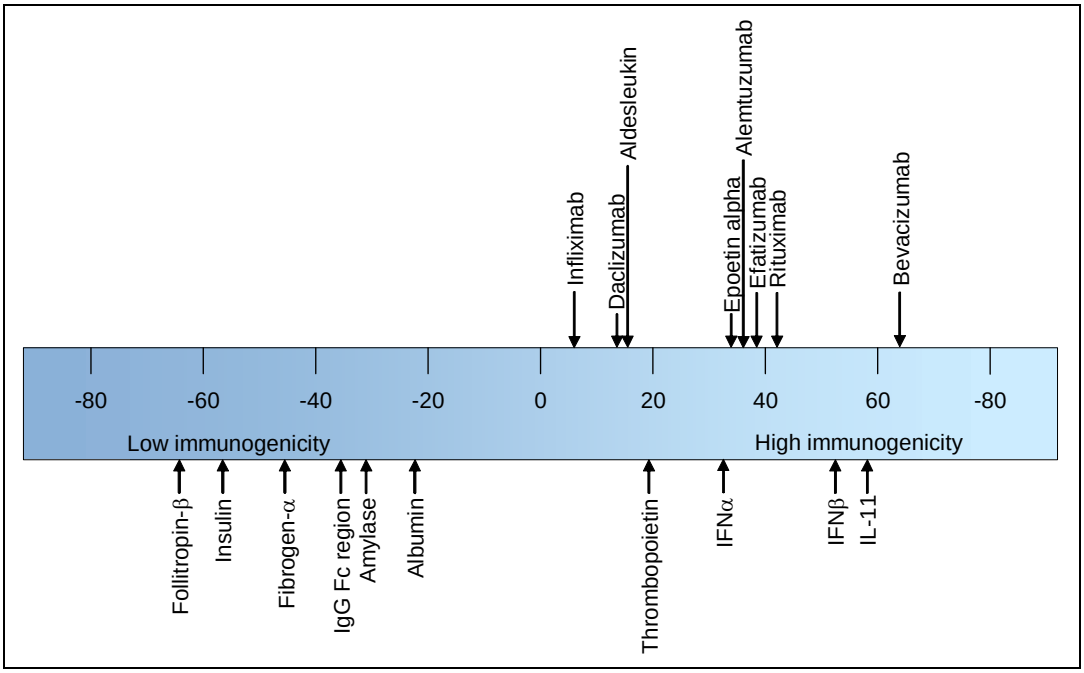
Caution has to be taken to avoid inaccurate predictions when using the described tools to predict T-cell epitopes in therapeutic proteins. The main reasons that these tools fail to predict T-cell epitopes accurately include (i) overtraining; that is, the tool is trained on the epitopes that it is then asked to predict; (ii) limited or flawed sources of information for the MHC ligand datasets from which most tools are created; and (iii) flawed interpretation of results, such as erroneous summing across MHC types when the MHC pocket profiles that define MHC binding are similar. An example of this third reason occurs in the approach used by EpiBase, which evaluates each protein sequence for T-cell epitopes. EpiBase is restricted by all human MHC class II HLAs or by a subset of HLA (such as Japanese MHC HLA or Caucasian MHC HLA), which may result in overweighting of selected HLA pockets, unnecessarily skewing the epitope selections [21]. In contrast, the approach developed by De Groot *et al*, uses only eight EpiMatrix matrices that predict 99% of possible HLA MHC class II binding pockets and also predict 98% of human class II alleles to calculate an overall immunogenicity score [22].

New tools for the prediction of T-cell epitopes continue to be developed on a regular basis, making the decision about which tool to use for immunogenicity prediction more difficult. Useful tools provide a standardized score, so predictions can be compared across alleles. An important new feature of the immune epitope database (*http://www.immuneepitope.org*) is the ability to compare public T-cell epitope prediction tools against a gold standard set of epitopes defined by the La Jolla Institute for Allergy and Immunology. Additional means of comparing and contrasting T-cell epitope and immunogenicity predictions are likely to become available in the future.

Human leukocyte antigen (HLA) association with immune response

While there are known links between autoimmunity and the genetic determinants of immune response (class II MHC), the number of papers describing an association between

Figure 2. An EpiMatrix-generated protein immunogenicity report for common protein-based therapeutics.



IFN interferon, **IL** interleukin

ADA responses and MHC haplotypes are few [23,24]. Nonetheless, because Td antibody development is determined by epitope specificity, MHC-restriction suggests that it may be possible, with improved algorithms, to relate Td ADA responses to MHC type. Martin *et al* have developed a parameter – the individualized T-cell epitope measure (iTEM) – which describes this relationship and may be useful in clinical trials [24]. T-helper cell epitope content, as measured by iTEM, takes into account individual HLA differences and may also explain differences between subjects with regard to observed antibody responses to slightly different versions of the same recombinant human protein. Combining epitope mapping with the iTEM method may allow researchers to screen clinical cohorts for subjects that are at higher risk for developing ADAs.

What cannot be predicted by immunoinformatics

B-cell epitopes and antibody-antigen interactions

B-cell epitope mapping algorithms are not as developed as their T-cell epitope counterparts. Recent examples of B-cell epitope mapping algorithms include 3DEX [25] and CEP [26]. Many such algorithms have been created and used to analyze existing datasets *in silico*, but only a handful of these tools have been used in prospective research studies and validated using *in vitro* or *in vivo* methods [27]. As a result, few B-cell epitope mapping algorithms are in current use and more research is needed [28]. In general, the best method for predicting the potential of a therapeutic protein to produce B-cell immune responses is to immunize HLA transgenic mice with the protein (see later section).

T-regulatory versus T-effector epitopes

Both effector T-cells and Tregs recognize T-cell epitopes. Thus, while prediction of a T-cell response is the first step in the evaluation of potential immunogenicity of an exogenous or endogenous protein, the nature of the immune response, be it inflammatory or suppressive, is likely to be determined by the presence or absence of Tregs. *In vitro* evaluation of the Treg phenotype (via cytokine profiling or by cell-surface markers identification) is therefore required when mapping T-cell epitopes.

Effect of PAMPs and protein aggregates

The stimulation of APCs and T-cells by pathogen-associated molecular patterns (PAMPs), such as those recognized by toll-like receptors (TLRs), results in quantitative and qualitative changes in T-cell epitope presentation and cellular activation [29]. Therefore, any contamination of protein drug products by leachates, cell-wall components or bacterial DNA that act as TLR ligands may increase adaptive immune responses, resulting in more potent ADA responses.

Another complication is that high-molecular-weight aggregates of a therapeutic protein are more likely to elicit Td and T-cell independent ADA responses by stimulating DCs or by cross-linking of B-cell receptors, than non-aggregated proteins [30]. The formation of these aggregates depends on drug solubility characteristics and handling of the drug during the production process. Methods for predicting aggregation are not currently available.

In vitro validation of immunogenicity prediction

HLA binding assays

The primary parameter that dictates the ultimate hierarchy of T-cell responses appears to be the half-life of class II MHC:epitope complexes [12]. Therefore, the authors recommend that class II MHC binding assays be used to confirm epitope predictions and the probability of the epitope being immunogenic. Two types of MHC binding assays can be used: cell-based binding assays and soluble HLA binding assays. The *in vitro* evaluation of class II MHC:epitope binding can be performed by measuring the ability of exogenously added peptides to bind to the surface of lymphoblastoid cell line B-cells expressing class II MHC alleles, as described by Kessler *et al* [31]. Alternatively, a competition-based HLA binding assay can be adapted for high throughput [32]. Such an assay is dependent on the availability of soluble HLA MHC class II proteins.

T-cell assays

Monitoring antigen-specific T-cell responses *in vitro* may involve measurement of their proliferation by radiolabeled thymidine incorporation (an *in vitro* method) or release of cytokines (measured by the ELISpot or ELISA methods) such as IL-2 or IFNγ. Whichever method is used, T-cell responses to the therapeutic protein antigen must be compared in order to control measurements taken in the absence of antigen, as there is high variability in baseline immune responses from person to person and animal to animal [33•]. The use of cytokine assays to monitor antigen-specific T-cell responses has advantages over T-cell proliferation assays by providing information about the nature of the T-cell response. The ELISpot technique is one of the most sensitive methods for detecting T-cell responses to therapeutic proteins, as it enables the detection of individual T-cells directly from splenocytes or peripheral blood, and permits the determination of their antigen-specific cytokine release. ELISpot assays are regularly used to identify low-frequency autoreactive T-cells. Both IL-2- and IL-4-based ELISpot assays can be performed using commercial kits. Flow-based assays can also be used to measure T-cell responses. For example, T-cells that respond to a particular epitope can be directly labeled using tetramers (MHC class II:epitope complexes), or the number and phenotype of T-cells that respond to the antigen can be determined by staining for cell-surface markers and intracellular cytokine expression [34].

In general, the best source of T-cells for immunogenicity studies in a protein therapeutic clinical trial would be from blood obtained from exposed subjects. When previous exposure to the protein therapeutic is remote and antigen-specific T-cells are low in number, re-stimulation of the T-cells can be performed *in vitro* in order to activate and expand relevant memory T-cells. Alternatively, naïve donor T-cell assays can be performed. In both cases, if an effector response to autologous proteins is to be measured, depletion of suppressive Tregs may be necessary prior to measurement of T-cell response *in vitro*, although markers for these cells are not yet definitive in humans.

The use of the whole therapeutic protein antigen in T-cell assays may not correlate with *in vivo* observations because of differences in how the protein is processed into its peptide components. During assays, most T-cell immunologists substitute overlapping peptides that represent the whole antigen, either in pools or in single-well assays. However, there are several inherent problems in the design of overlapping peptides sets for use in T-cell assays. These problems include the artificial truncation of T-cell epitopes and the lack of stabilizing flanking residues. Selecting peptides for immunogenicity assays based on epitope prediction tools is an alternative approach.

Measuring immunogenicity *in vivo*

Another means of evaluating the potential immunogenicity of a protein therapeutic is to expose HLA-transgenic mice to the protein, either as the whole protein or as epitope peptides. Outbred murine models, protein transgenic murine models and other non-humanized animal models have been used to evaluate immunogenicity; however, animal MHC molecules are not equivalent to human MHC molecules as they can have different amino acid side chain restrictions in the epitope binding groove. Therefore, the results of these types of *in vivo* assays, when performed with the goal of predicting Td immunogenicity in humans, should be interpreted with caution.

Fortunately, a number of transgenic mouse strains that express the common HLA gene products (HLA-A, HLA-B and HLA-DR molecules) have been developed [35,36]. A direct correlation has been found between T-cell responses in infected humans and T-cell responses induced in immunized HLA-transgenic mice [37,38]. Hence, HLA-transgenic mice are now routinely used to assay and optimize human epitope-driven vaccines in preclinical studies [39,40]. It is possible to measure both T-cell response to the whole wild-type therapeutic protein and T-cell response to epitopes derived from the wild-type therapeutic protein; furthermore, it is also possible to compare T-cell response to modified versions of immunogenic proteins. In addition to measuring T-cell responses, antibodies induced by exposure to the therapeutic protein may be measured by ELISA and neutralizing antibody assays; the results of these studies can be compared with T-cell responses to the same proteins.

In vivo methods for modeling immunogenicity are also dependent on the ability of the animal model to recognize the antigen in the proper context. While HLA-transgenic mice are better models of human MHC presentation of processed protein than wild-type mice, HLA transgenics are not tolerized to human autologous proteins in the course of development. For example, many mice have previously been exposed to autologous (murine) clotting Factor VIII (FVIII); hence the ability of mice to develop antibodies to a given formulation of FVIII depends on the differences in the sequences between their autologous FVIII and the FVIII being evaluated. Thus evaluations of autologous proteins such as human FVIII in murine or non-human models may not reflect the phenotype of T-cell response as accurately as studies performed in FVIII knockout or protein-transgenic mice. A number of different protein-transgenic mouse strains have been developed for this purpose.

Tools for reducing immunogenicity

The observation that protein therapeutics elicit Td antibody responses has not only led to the development of innovative immunoinformatics tools that measure immunogenicity, but these same tools are also being applied to aid the removal of T-cell epitopes within protein therapeutics to reduce immunogenicity [20,41]. The immunogenicity of therapeutic proteins is probably linked both to (i) the presence of T-helper epitopes generated from the therapeutic protein; and (ii) an event (signal) that triggers an immune response. Therefore, the removal of T-cell epitopes from any potential protein therapeutic may reduce the protein's overall potential to stimulate an illicit T-cell immune response. This process may be thought of as an artificially directed version of the process that occurs naturally when tumor cells [42] and pathogens [43,44] evolve to escape immune pressure by accumulating mutations that reduce the binding of their constituent epitopes to host HLAs, rendering the host cell unable to signal the presence of the tumor or pathogen to T-cells [45]. The existence of these viable 'immune escape mutants' in tumor and pathogen proteins demonstrates that proteins, and indeed whole organisms, can tolerate certain de-immunizing mutations. Thus, it is reasonable to expect that an iterative approach to modifying protein sequences that reduces their epitope content, will also lead to successfully de-immunized and functional therapeutic proteins. Successful de-immunization of therapeutic proteins has been demonstrated in studies performed by researchers at BioVation Ltd [46], Epimmune Inc [41], Genencor International [47], and EpiVax Inc [20] using a range of different approaches.

In silico tools

EpiVax has developed a tool called OptiMatrix that iteratively substitutes all 20 amino acids in any given position of a protein sequence and then re-analyzes the predicted immunogenicity of the sequence, following the change. OptiMatrix identifies critical residues that contribute most to MHC binding affinity across multiple nonomer peptide frames and multiple HLA MHC alleles, and averages the contribution of each amino acid to binding across nonomer peptide binding frames and HLA MHC alleles.

An alternative approach to predicting reduced immunogenicity has been developed for humanization of mAbs by Xencor Inc [48]. This algorithm measures the extent of 'human-ness' of strings of nonomer peptide frames, and aims to maximize the human-like sequence content by substitution of amino acids derived from human germline sequences of antibody framework and complementarity-determining regions. The method operates on the assumption that substituted human sequences bear lower T-cell epitope potential.

In vitro and in vivo tools

In vitro tools for modifying immunogenicity are essentially the same as described in the section on measuring Td

immunogenicity. In general, when *in vitro* evaluations are performed, a direct comparison is made between T-cell proliferation or cytokine production levels induced by control, wild-type and modified sequences. Another means of evaluating the impact of epitope modifications on *de novo* T-cell response to therapeutic proteins is to measure the immunogenicity of modified T-cell epitopes in HLA-transgenic mice, either as whole protein or as epitope peptides. Using this approach it is possible to measure (i) T-cell response to the whole wild-type protein; (ii) T-cell response to epitopes derived from the wild-type protein; and (iii) abrogation of T-cell response to modified versions of immunogenic proteins, and furthermore to compare and contrast humoral immune responses in the same model.

Conclusion

In silico T-cell epitope mapping tools provide drug developers with a means of rapidly screening and contrasting therapeutic proteins for potential immunogenicity. As these tools become standardized and validated *in vitro* and *in vivo*, it may be possible to use *in silico* analysis without performing additional *in vitro* and *in vivo* studies. However, presently, most *in silico* tools need to be used in conjunction with *in vitro* and *in vivo* validation.

In vitro methods for measuring Td immunogenicity, including T-cell proliferation and cytokine assays, are well-established. In addition, T-cell phenotyping (the identification of Tregs versus effector T-cells) is an important aspect of measuring T-cell response. Caution should be exercised when using these methods, as they can be misinterpreted. For example, problems relating to the processing of the therapeutic protein can lead to misinterpretations of immunogenic responses (eg, the classification of a nonreactive therapeutic protein *in vitro* despite an *in vivo* response).

Given the right combination of immunogenicity screening tools, drug developers can screen large numbers of proteins, limiting the cost and effort associated with bringing a potential protein therapeutic to trial. However, much remains to be learned about immunogenicity, and *in silico* methods are not yet stand-alone. Presently the best approach, whether measuring potential immunogenicity or modifying immunogenicity of therapeutic proteins, is to combine *in silico* prediction with *in vitro* and *in vivo* validation.

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