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Prediction of immunogenicity: *in silico* paradigms, *ex vivo* and *in vivo* correlates

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Immunogenicity can be a major obstacle to successful protein drug therapy. Antidrug antibodies may neutralize therapeutic function, influence pharmacokinetics and, in some cases, lead to severe adverse effects. These effects depend on factors including dose, regimen, delivery route and contaminants, among others. Importantly, immunogenicity is a consideration that is better addressed during preclinical development before complications arise in clinical trials or following licensure. This article will address the development and application of computational tools for immunogenicity assessment of protein therapeutics, and validation of those predictions using peripheral blood from exposed subjects or alternative *in vivo* methods.

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Introduction

As anticipated, molecular biology has revolutionized medicine and the treatment of human diseases. The revolution is ongoing: researchers continue to identify new approaches for modulating cellular processes and means of delivering these new therapies to their targets with heretofore-unimaginable precision. On the basis of these findings, armies of molecular biologists are developing novel therapeutic proteins, monoclonal antibodies (mAbs) and antibody-like protein scaffolds, intent on improving human health. In the rush to deliver the promise of molecular medicine, biologists have, on occasion, overlooked the well-known implications of protein immunogenicity. In addition, the determinants of immunogenicity of autologous or human-like proteins have not been fully determined, meaning that assumptions about immune tolerance, too, require a second look in protein therapy design.

Fortunately, immune response to *foreign* proteins is relatively well understood, owing to years of thorough study of parameters influencing vaccine efficacy. Factors including delivery route delivery vehicle, dose regimen, aggregation, innate immune system activation and the ability of the protein to interface with the humoral (B cell) and cellular (T cell) immune systems, all impact the potential immunogenicity of *vaccine* immunogens when delivered to humans (for a review of immunogenicity determinants, see De Groot and Scott [1•]).

Similarly, protein therapeutics, when administered in an immunostimulatory milieu, engender both cellular and humoral immune responses. Development of antidrug antibodies (ADA) is considered an adverse immune response, as ADA may neutralize the therapeutic effects of the drug and/or alter its pharmacokinetics. T cells are certainly involved in this immune response when IgG class ADA are observed because antibody isotype switching is a hallmark of T-dependent antigens [2•].

More serious adverse events can be provoked if ADA crossreact with a crucial autologous protein. Examples of adverse ADA responses include autoimmune thrombocytopenia (ITP) following exposure to recombinant thrombopoietin [3•], and pure red cell aplasia, which was associated with a particular formulation of erythropoietin (Eprex) [4•]. Since the impact of immunogenicity can be quite severe, regulatory agencies are developing risk-based guidelines for immunogenicity screening [5•].

In silico paradigms

Immunoinformatics algorithms for identifying T-cell epitopes have improved dramatically since they were first developed by Berzofsky, Margalit, and DeLisi in the 1980s [6]. It is now possible to measure the T-cell epitope content of a protein relatively accurately using *in silico* tools, and also to evaluate the regional and overall immune potential of a protein therapeutic. Given the resulting 'immunogenicity score' of a protein [7•], and taking into consideration other determinants as described above, it is possible to make an informed decision about the likelihood that a protein will provoke an immune response. For example, the EpiMatrix suite of computational tools, together with *ex vivo* immunogenicity testing, has been applied to evaluate protein therapeutics in the preclinical phase and correctly predicted clinical

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immunogenicity in at least two cases which were subsequently published [8•,9•]. Recognizing the value of preclinical immunogenicity screening, a number of therapeutic protein developers have incorporated *in silico*, *ex vivo*, and *in vivo* preclinical immunogenicity screening protocols into their product development strategy.

T-cell epitope prediction

'*In silico*' predictions of T-helper epitopes have been available for more than two decades, and consequently, their application to vaccine design [10–12] and to selection of autoimmunity epitopes [13] is well documented. During this time, the number of T-cell epitope prediction tools has steadily increased (for reviews, see Petrovsky and Brusic [14] and De Groot and Berzofsky [15]).

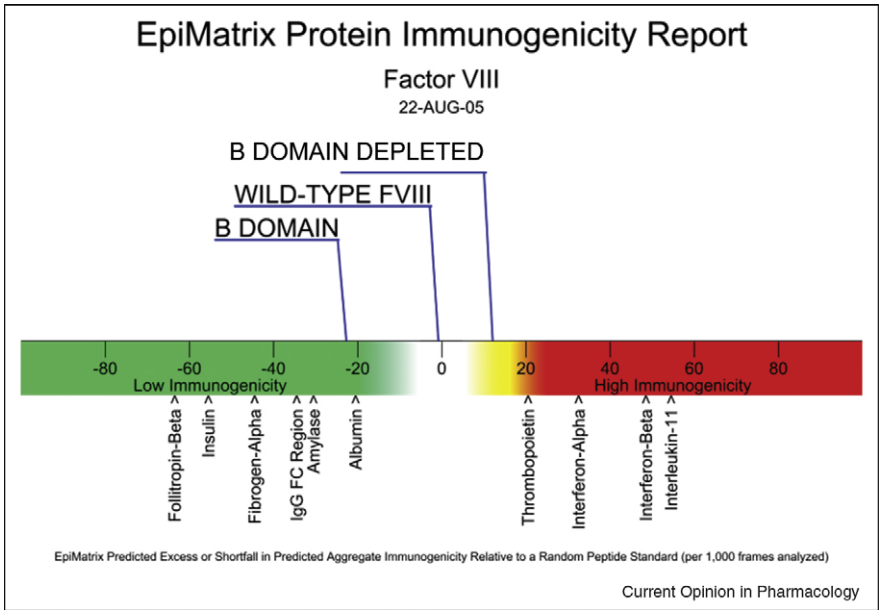
The EpiMatrix approach is described here to illustrate the basic process of screening a protein therapeutic for immunogenicity. A protein sequence is first parsed into overlapping 9-mer peptide frames, each of which is then evaluated for binding potential to each of eight common class II HLA alleles that 'cover' the genetic backgrounds of most humans worldwide [16]. Normalization of allele-specific scores makes it possible to compare scores of any 9-mer across multiple HLA alleles and enables immunogenicity prediction on a global scale [17].

By calculating the density of high-scoring frames within a protein, it is possible to estimate a protein's overall 'immunogenicity score'. In Figure 1 several types of FVIII and the B domain of FVIII provide an illustration of the concept. In addition, subregions of densely packed high-scoring frames can be identified, and regional or 'cluster' scores can be calculated (Figure 2) [6,7•,8•,18•]. We and others have observed that potential immunogenicity is not randomly distributed throughout protein sequences but instead tends to reside in regions (which are often also immunodominant) [19–21]. A T-cell epitope 'cluster' usually ranges from 9 to about 25 amino acids and can contain anywhere from 4 to 40 binding motifs. Regions of proteins where HLA binding potentials cluster, reaching a cumulative EpiMatrix sum of scores that is greater than 10, are associated with significant T-cell immunogenic potential [22,23].

Epitopes: tolerance and ignorance

Not all clusters of immunogenic potential can be considered to be potentially immunostimulatory. Would-be epitopes in autologous proteins might have triggered T cells that are absent from the peripheral circulation, since T cells that are auto-reactive are said to be deleted in thymic development. However, some T cells specific for autologous proteins escape thymic deletion and become natural regulatory T cells (Tregs); they appear to serve as regulators or suppressors of autoimmune, auto-reactive immune responses [24•]. Just as the inadvertent addition of stimulatory T-cell (T-effector) epitopes to proteins may lead to increased immunogenicity, removal or alteration of

Figure 1



EpiMatrix immunogenicity scale analysis of human FVIII sequence (from Genbank). This graph shows the potential immunogenicity based on T-cell epitope content per 10 000 amino acids, of wild-type FVIII, B domain depleted FVIII, and B domain itself. The analysis suggests that B domain depleted FVIII might be as immunogenic, if not more immunogenic than wild-type FVIII. Of course, the effect of FVIII is also determined by an individual's HLA type, his/her exposure to autologous FVIII (whether entirely absent or mutated but still expressed), route of administration, dose, frequency of dosing, FVIII source (recombinant or natural), and whether the FVIII is contaminated with innate immune system triggers such as CpG DNA or leachates from the components included in the final formulated drug.

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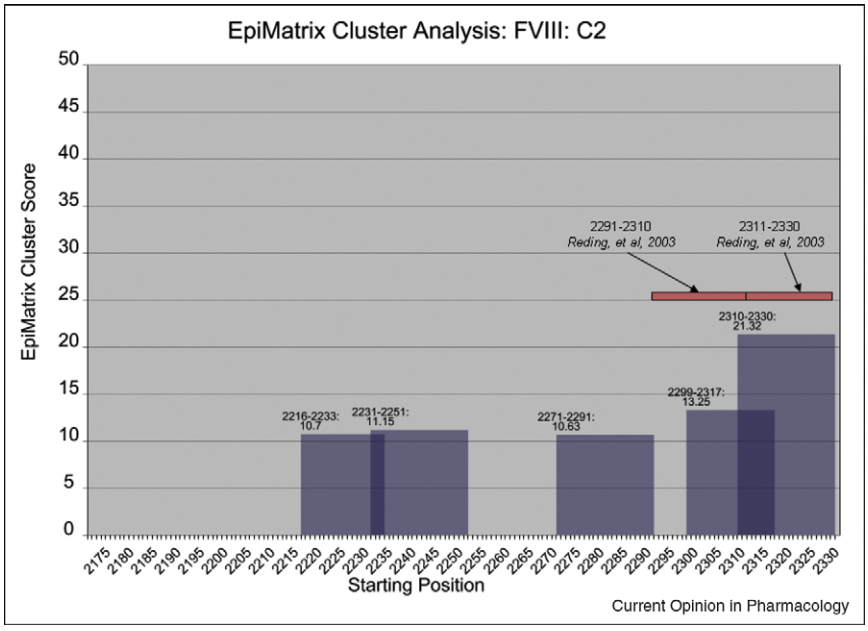
Figure 2



FVIII C2 ClustiMer analysis. A cluster map for domain C2 charts the location and potential immunogenicity of each cluster. The location of the cluster is indicated by amino acid number on the *x*-axis and the width of the bar correlates with the length of the cluster. EpiMatrix Cluster Score is shown on the *y*-axis; a score above 10 indicates high likelihood of immunogenicity. The red bar at right indicates the span of two published T-cell epitopes [57].

regulatory T-cell epitopes in the drug development process may alter the natural T-regulatory immune response to recombinant autologous proteins. The link between T-cell (and HLA-restricted) immune response and the development of autoantibodies is still being defined; early evidence points to the reduction of Treg immune responses and to the induction of T-effector responses as significant contributors in the context of immune responses [25,26].

The conceptual basis of tolerance induction for protein therapeutics is the observation that immunoglobulin therapies induce expansion of Tregs *in vitro* and *in vivo* [27]. We have shown that coincubation of donor PBMC with T-cell epitopes derived from autologous proteins can lead to suppression of immune response to bystander antigens in human PBMCs, and that the corresponding murine epitopes suppress *in vivo* immune response in HLA DR4 transgenic mice [58]. Building on these observations, we are now evaluating whether modification of autologous proteins to include such Treg epitopes will lead to the development of less immunogenic antibodies and improved replacement proteins for protein deficiency diseases.

The dynamic balance between regulatory T-cell and T-effector (T-helper or cytotoxic T cell) immune responses to autologous proteins is best understood in the context of a mutated or partially deleted protein and recombinant protein replacement therapy. For example, FVIII may be expressed in a truncated or mutated nonfunctional form in some individuals. Although Figure 1 provides an illustration of the potential immunogenicity of FVIII (based on total T-cell epitope count), it is important to note that the presence of regulatory T-cell epitopes in FVIII (because of recognition of some of these epitopes by Treg cells) may affect the expression of replacement FVIII immunogenicity in the individual patient. Thus, the immunogenicity of replacement FVIII might be better represented by summing the net effect of T-effector epitopes (positive signals for immunogenicity), regulatory T-cell epitopes (suppressors) and epitopes to which T cells have been deleted in the course of thymic development (neutral).

B-cell epitope prediction

It would be advantageous to predict B-cell antigenicity to identify neutralizing antibody targets. Computational tools that accurately predict B-cell epitopes remain elusive because of the conformational dependence of antibody:antigen interactions. B-cell epitope prediction tools such as 3DEX and CEP [28–30] do not, as far as can currently be determined, accurately predict B-cell epitopes on a high-throughput basis. Notably, in some cases, defining a T-cell epitope may lead to identification of a B-cell epitope since B-cell epitopes have been shown to colocalize with T-helper epitopes [31,32].

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De-immunization

De-immunization by epitope modification is an approach based on the disruption of HLA binding, an underlying requirement for T-cell stimulation. The idea of rational epitope modification is rooted in the natural process that occurs when tumor cells and pathogens evolve to escape immune pressure by accumulating mutations that reduce the binding of their constituent epitopes to host HLA, rendering the host cell unable to 'signal' to T cells the presence of the tumor or pathogen [33].

Ex vivo and *in vivo* correlates

Human T-cell assays

Immunogenicity screening using immunoinformatics tools requires validation *in vitro* and *in vivo*. If blood from antigen-exposed individuals is available, predicted peptide epitopes can be tested for their reactivity with T cells. The type of immune response (effector or regulatory T cell) can be determined by evaluating cell surface or intracellular markers corresponding to the different classes of T cells such as CD4/CD25, FoxP3, and CD127. Class II (T-helper) epitopes can be 'promiscuous'; that is to say that a single sequence can fit MHC of various haplotypes because of the open-ended configuration of the peptide-binding groove.

ELISA and ELISpot are related methods for detecting antigen-specific T-cell responses by measurement of cytokines secretion (e.g. interferon-gamma, IL-2, and IL-4). T-cell proliferation can be measured by the dilution of a fluorescent dye or by radioactive thymidine incorporation. T cells that respond to a particular epitope can be directly labeled using tetramers (comprised of HLA class II:peptide complexes), or the number and phenotype of T cells that respond to the antigen can be determined using cell surface markers and intracellular cytokine staining [34]. The pros and cons of the different types of T-cell assays have been evaluated in side-by-side studies [35•].

HLA transgenic mouse models

Most proteins intended for therapeutic use in humans are relatively foreign in mice and therefore immunogenic [36]. Although protein therapeutics are evaluated in many different animal models, these studies typically do not consider the effect of T-cell recognition of peptides in the context of HLA. Therefore, immunogenicity analyses in non-HLA transgenic models should be interpreted with caution as murine, rat and nonhuman primate MHC differ from human HLA at the amino acid level, and their responses do not necessarily reflect those of humans. Indeed, different strains of mice also possess different MHC types, which is one explanation for differential immune responses to pathogens, vaccines and therapeutic mAbs, in C57Bl/6 and Balb/C mice [37].

Accurate preclinical evaluation of protein therapeutics on *de novo* T-cell responses can be done in mice that are transgenic for human MHC (HLA Tg mice) [38]. Those HLA transgenic mice express that have human HLA genes and have a MHC class II deficient background [39] are preferred, for this purpose, since CD4 T-cell-mediated immune response is completely restricted by human HLA molecules, and not by mouse MHC.

T-cell responses in infected humans correlate directly with T-cell responses in immunized HLA transgenic mice [40,41]; thus, HLA transgenic mice are now routinely used to assay and optimize (human) epitope-driven vaccines in preclinical studies [42–44]. Fortunately, several transgenic mouse strains expressing the most common HLA DR molecules are available (HLA DRB1*0101, *0201, *0301, *0401, *1501) [39,45] enabling immunogenicity measurements in models that represent a large proportion of the human population.

Sequence differences between human and murine proteins add an additional layer of complexity to the evaluation of human protein immunogenicity in mice. These differences may result in dramatic immune responses in mice where none would have been encountered in humans or *vice versa*; this phenomenon has been exploited for the development of cancer vaccines [46]. The degree of foreignness may depend on the number of amino acids that are different among peptides that are processed and presented by the animal MHC molecule. One means of addressing this issue is to develop mice that are transgenic for the human protein of interest, however, the issue of T-cell epitope presentation in the context of class II murine MHC remains problematic in these models [47].

A number of groups are pioneering studies of 'immune-system-humanized' mice as a translational model for studying immunogenicity [48–52]. New mouse strains such as NOD-SCID IL2rgamma(null) mice that lack the IL-2 receptor common gamma chain make the development of such mice possible. These novel chimeric mice lack adaptive immune function, display multiple defects in innate immunity, and support heightened levels of human immune cell engraftment. The models are created by engraftment of hematopoietic stem cells or peripheral blood mononuclear cells into immunodeficient mice; one drawback of these mice is that each mouse represents only one single patient's immune phenotype; thus multiple immune-system-humanized mice would be required for adequate representation of human MHC diversity [53•].

When *in vivo* studies are not possible

In the absence of animal models and access to exposed human blood, *in vitro* assays can be used to determine the potential for predicted epitopes to engender immune responses. In particular, HLA binding assays can be used to assess the affinities of therapeutic-derived epitope

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sequences for multiple HLA alleles. *In vitro* evaluation of MHC binding can be performed by quantifying the ability of exogenously added peptides to compete with a fluorescently labeled known MHC ligand [54]. Competition-based HLA binding assays can be adapted for high-throughput *in vitro* [55•]. A correlation between HLA binding and immunogenicity is often observed [56].

Conclusion

In the context of studies of therapeutic proteins, *in vivo* confirmation validates the accuracy of immunogenicity screening using *in silico* methods. Because T-cell epitopes are necessary for a robust humoral response, accurate T-cell epitope predictions will correlate to the actual response *in vivo*. The effect of regulatory T-cell epitopes and their counterparts, the effector T-cell epitope, need to be taken into consideration when measuring the immune potential of a therapeutic protein. The implementation of regular protocols for screening therapeutic proteins in preclinical stages, using epitope mapping in combination with *in vitro* and *in vivo* studies, may allow researchers to avoid the development of ADA and may also reduce the costs of recombinant protein drug development by eliminating candidates that are determined to be too immunogenic.

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