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REVIEW

# Reducing risk, improving outcomes: Bioengineering less immunogenic protein therapeutics

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**Abstract** One of the great surprises of the biologics revolution has been the discovery that recombinant human proteins, including monoclonals of human origin, can cause immune responses when administered to immune-competent subjects. Preclinical and clinical evaluations of the potential immunogenicity of biologics have been primarily focused on humoral immune responses and as a result, the critical contribution of T cells to the development of anti-monoclonal antibodies (also known as anti-drug antibodies or ADA) has been somewhat overlooked. Recent publications have confirmed the role of effector T cells and begun to explore the role of regulatory T cells in the development of anti-drug antibodies. This review will focus on the role of T-cell-dependent (Td) immunogenicity assessment in the preclinical and clinical phases of drug development and summarize new data on regulatory T-cell epitopes contained in the Fc and CH1 domains of IgG. Recommendations for Td immunogenicity screening and assessment provided in this article may contribute to the development of safer protein-based drugs for human use.

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## Introduction

A number of industry-based and regulatory guidelines for immunogenicity screening and assessment of protein therapeutics have been published. Most of these publications have focused on methods for measuring anti-drug antibodies (ADA) [1,2] and have not directly addressed the role of T cell immunogenicity. The association between the presence of T-cell epitopes in a protein sequence and the titer and longevity of ADA has now been documented for several therapeutic proteins [3–7] and methods for rapid T-cell epitope screening (in silico) and T-cell epitope validation (in vitro and in vivo) are being applied in the therapeutic development pipeline. Standards for the preclinical assessment of Td immunogenicity have yet to be established and are urgently needed. Regulatory agencies and drug developers need rapid, low-cost means for predicting, in the preclinical setting, which protein drugs will be more likely to induce anti-drug antibodies (ADA) in clinical trials. This paper describes the available in silico, in vitro, and in vivo methods of immunogenicity assessment as they relate to Td immune response, and discusses how they might best be put to use.

## Methods for Td immunogenicity assessment

### What methods are available to measure T-cell epitope content?

T cells carry out their roles following binding of their T-cell receptor (TCR) to T-cell epitopes, small linear fragments derived from protein antigens, displayed on the surface of antigen presenting cells (APC) by various alleles of the major histocompatibility complex (MHC; HLA refers to human MHC). While B cells and antibodies generally recognize conformational epitopes from surface proteins, T cells recognize epitopes derived from all types of proteins that are processed by APCs.

Once taken up by the antigen presenting cell, antigenic proteins are broken down by proteolytic enzymes (Fig. 1). During this process very large numbers of peptide fragments are released. Any one of these fragments could be a T-cell epitope, but only about 2% of all the fragments generated

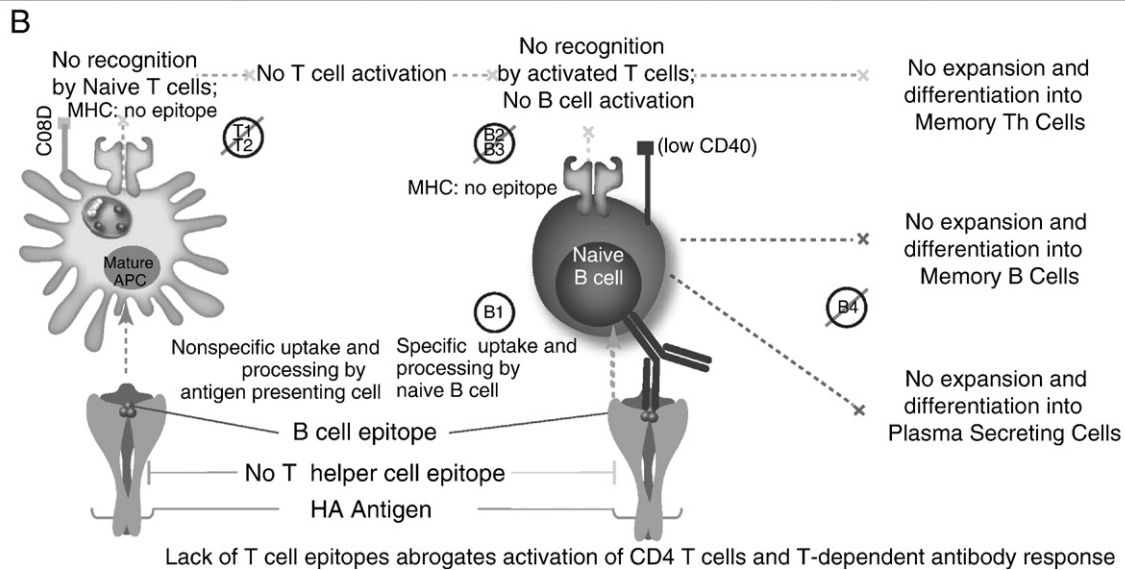
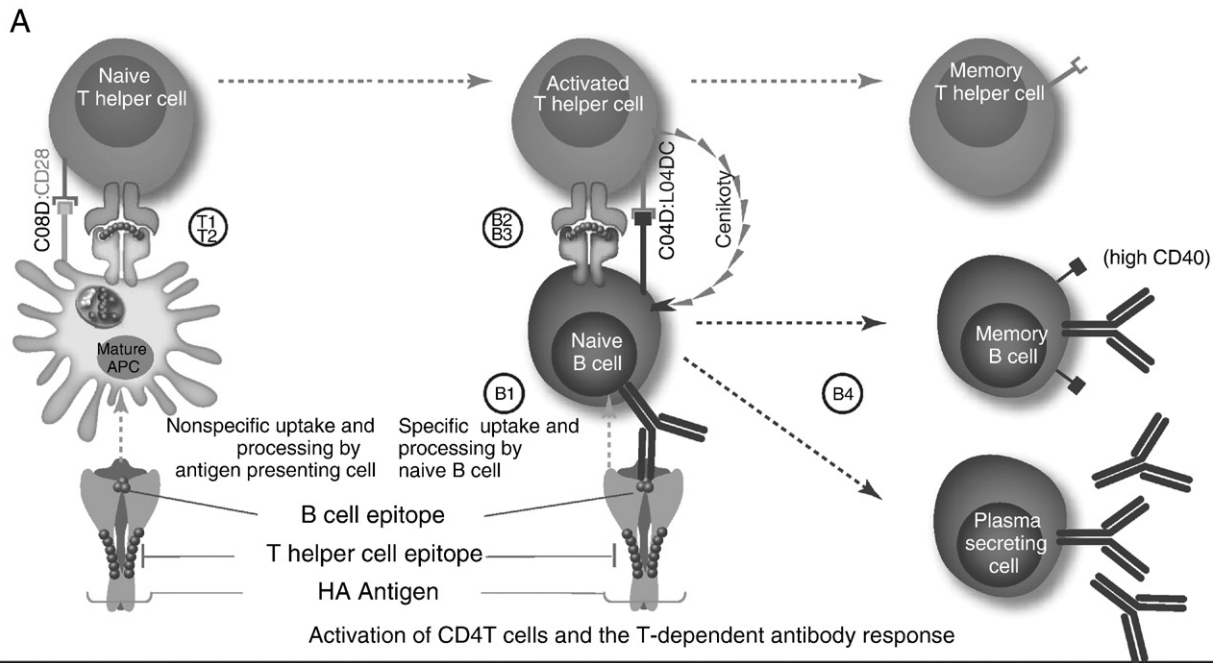
have the right amino acid side chains (“R groups”) that allow them to bind in the binding groove of a MHC molecule and be presented on the surface of the APC. One of the critical determinants of T-cell epitope immunogenicity is the strength of T-cell epitope binding to MHC molecules [8]. Peptides binding with higher affinity to MHC are more likely to be displayed on the cell surface where they can be recognized by T lymphocytes.

### In silico tools for predicting T-cell epitopes

Over the last 10 years, researchers have developed a number of computer algorithms that map the locations of MHC class I- and class II-restricted T-cell epitopes within proteins of various origins. The in silico methods include frequency analysis, support-vector machines, hidden Markov models, and neural networks. A number of algorithms for finding T-cell epitopes have been developed; for recent reviews see publications by Sette [9], Brusci [10] and De Groot [11] among others. Several of these algorithms are currently in use by drug developers. What these tools have in common is an ability to quickly screen large volumes of genomic sequences for putative epitopes; this preliminary screen reduces the search space dramatically, typically by at least 20-fold. MHC class II prediction methods are more directly related to Td immunogenicity assessment, as class II-restricted T cells provide help to B cells, leading to the development of ADA.

Such “in silico” predictions of T-helper epitopes have already been successfully applied to the design of vaccines [12,13] and to the selection of epitopes in studies of autoimmunity [14]. For example the EpiMatrix system, a suite of epitope-mapping tools, has been validated following more than a decade of use for in vitro and in vivo studies (see references [15–19]). The EpiMatrix algorithm uses Class I and Class II HLA “pocket profiles” that describe HLA pocket binding coefficients, and applies these coefficients to the prediction of overlapping 9- and 10-mer peptide epitopes.

Some epitope-mapping algorithms allow researchers to measure the potential immunogenicity of whole proteins such as bioengineered protein therapeutics, monoclonal antibodies (mAbs) and their sub-regions. For example, EpiMatrix assesses the epitope density of a given protein and compares that density to those of a set randomly-generated pseudo-



**Figure 1** T-cell-dependent Td antibody response. Stimulation of T helper (TH) cells by antigens involves first activation by interleukin 1 (IL-1) (T1) and then presentation of the antigen at the surface of antigen-presenting cells (usually macrophages, dendritic cells or B cells) (T2) in association with class II major histocompatibility complex molecules (MHC II); for extrinsic (foreign) proteins this requires initial capture of the protein, followed by denaturation and/or degradation so as to associate the molecule or fragments with MHC II. T cells so stimulated express receptors for interleukin 2 (IL-2), and secrete various molecules, including factors which stimulate B cells to divide and/or secrete Ig, interferon-gamma and IL-2. (B1) In turn, IL-2 causes proliferation of TH and cytotoxic/suppressor T cells. Encounter of the same antigen by a B cell results in processing and presentation of the epitope at the B cell surface in the context of MHC (B1). Co-stimulatory factors such as B7 interact with the memory B cell (B2), resulting in secretion of cytokines by the T cell (B3). These cytokines allow the B cell to mature and become a dedicated, IgG producing (isotype switched), memory B cell (B4).

protein sequences of similar size. Setting aside for a moment the well-established concept of tolerance (see below), EpiMatrix has been used to evaluate a subset of self-proteins (specifically those that are abundant in serum) and found that these proteins have lower inherent class II epitope-restricted epitope densities, as compared to random proteins

and to known antigens [20]. This epitope-density approach is gaining acceptance as a means of comparing one protein therapeutic (or MAb) to another, so as to select the best lead to carry forward from preclinical to clinical development, reducing the risk of failure due to immunogenicity in the clinical setting.

### In vitro assays

Peptides are used to measure T-cell responses *in vitro*; they can be of variable lengths (9 to 25). Peptides presented in the context of class I MHC are generally limited to 9 or 10 amino acids in length, although some processing is believed to occur during the T-cell assay and so 15-mers are also used for class I assays. Class I epitopes are short and fit tightly in the bounded MHC molecule; by contrast, Class II (T helper) epitopes lie within an open-ended groove in the MHC II. As such, a class II epitope can shift within the groove thereby accommodating MHC of various haplotypes. The only limit on the size of the peptide is its ability to remain in a linear conformation in the open-ended groove. HLA binding assays can be used to assess whether peptides derived from protein sequences (usually following a first-pass immunoinformatics analysis) can bind to either MHC class I or class II. HLA binding assays use a range of techniques to measure the affinities of predicted epitope sequences for the HLA alleles *in vitro*. *In vitro* evaluation of MHC binding can be performed by quantifying the ability of exogenously added peptides to compete with a fluorescently-labeled MHC ligand [21] and can be adapted for high throughput [22]. HLA binding assays are a robust method for confirming epitope predictions *in vitro*. A concordance between HLA binding and immunogenicity is often observed [23]; however longer peptides (over 20 AA) sometimes fold (in solution) and thus do not bind *in vitro*.

### In vitro assays using human blood cells

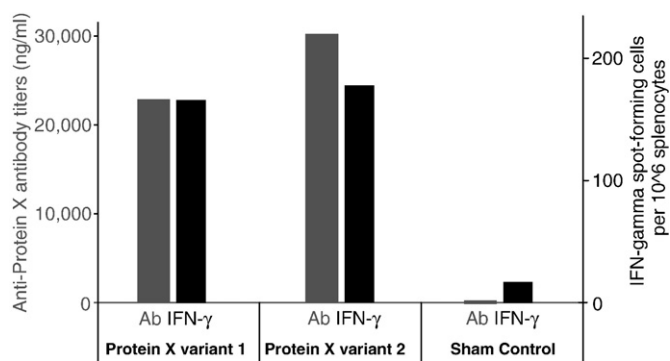
ELISA and ELISpot are related methods for detecting T-cell responses by measurement of cytokines secreted by the T cells (gamma interferon, IL-2, and IL-4 are examples). ELISpot is generally considered to be the standard in immunogenicity screening because of its greater level of sensitivity and specificity, compared to ELISA. T cell response can also be measured by (1) the dilution of a fluorescent dye in subsequent generations of cells (CFSE) and (2) the incorporation of a radioactive label in the proliferating cell's DNA (tritiated thymidine incorporation assay). Fluorescent labeling (FACS) and intracellular cytokine staining (ICS) are the most precise methodologies available for measuring and defining T cell response. For example, T cells that respond to a particular epitope can be

directly labeled using tetramers (comprised of MHC class II: peptide complexes). Labeled cells can then be sorted and counted, and the phenotype of T cells that respond to the antigen can be determined using cell surface markers and intracellular cytokine staining [24]. Both peptides and whole antigens can be used in T-cell assays. The recognition of whole antigens requires the presence of an antigen-presenting cell that is capable of processing and presenting peptides derived from the antigen; proteins that have multiple intra-molecular bonds may not be fully processed and therefore immune responses to such proteins, as measured *in vitro*, may be more variable.

### HLA transgenic mice

Even though a range of animal models are used for the evaluation of vaccines, immunogenicity studies in these models should be interpreted with caution. Although their functions may be similar, the MHC of mice, rodents, and non-human primates differ from human MHC (known as HLA in the context of human immune response) at the amino acid level and these differences affect which T-cell epitopes can be presented. These differences explain why different strains of mice (Balb/C, C57Bl/6) have different immune responses to pathogens as well as vaccines for those pathogens [25]. In particular, predictions that are developed using human T-cell epitope mapping tools can only be tested in murine models that are transgenic for (express) Human MHC.

Fortunately, a number of transgenic mouse strains that express the most common HLA A, HLA B and HLA DR molecules have been developed. T-cell responses in these mice correlate directly with T-cell responses observed in infected/vaccinated humans [26,27]. HLA transgenic mice are now routinely used to assay and optimize (human) epitope-driven vaccines in preclinical studies [28–30]. Despite the limited number of HLA class II alleles for which Tg mice have been developed (HLA DR 0101, 0301, 0401, 1501), comparisons of immunogenicity can be done to a high degree of accuracy in the mouse model. A side-by-side comparison of immune responses to two biosimilar proteins is shown in the figure below. Both Td immune response (gamma interferon by ELISpot) and antibody response (ELISA) response to protein therapeutics can be assessed in HLA transgenic mice (Fig. 2).



**Figure 2** In vivo evaluation of protein immunogenicity. In this scenario, two variants of the same protein are compared for potential immunogenicity. Antibody responses (red bars) and T cell responses (blue bars) are correlated. Both T cell response and B cell response (Ab) to the variants are slightly different; variant 2 appears to be marginally more immunogenic than version 1.

## Autologous proteins and tolerance

Anti-drug antibodies can also develop to recombinant autologous proteins such as erythropoietin [31], products that, in theory, should not breach tolerance [32,33]. Some researchers believe that the development of auto-antibodies to autologous proteins may be due to denaturation or aggregation of the protein drug, and others believe that the protein's inherent potential for immunogenicity may be uncovered by the addition of a "danger signal" such as a Toll-receptor ligand or another type of contaminant. Human immune response to recombinant autologous proteins probably also involves T regulatory cells. The peptide sequence of T-cell epitopes does not intrinsically define the type of T cell response that is observed, rather, T cell phenotype is dependent on the immune context in which the epitope is presented to the T cell. An epitope may be associated with tolerance in one setting (regulatory T cell recognition), and an effector immune response in another (effector T cell recognition).

When considering factors that contribute to immunogenicity of autologous or homologous proteins, a brief review of the mechanism of tolerance is in order. There are two types of tolerance: central and peripheral. Central T-cell tolerance occurs in the thymus wherein anti-self T cells are deleted. B cells that can recognize self-proteins may similarly be deleted, most likely in the bone marrow. In addition, peripheral tolerance mechanisms exist to control each of these repertoires and prevent auto-reactivity after lymphocytes/B Cells have emigrated from the thymus/bone marrow.

Tolerance can be broken, and antibodies do develop to autologous proteins. Consider, for example, Graves' disease in which antibodies to thyroid stimulating hormone receptor develop, and myasthenia gravis, in which antibodies develop to the acetylcholine receptor. The link between the development of auto-antibodies and T cell (and HLA-restricted) immune response is well established. Auto-immune diseases are characterized by auto-antibody subsets that are associated with MHC genes, most specifically HLA class II (HLAII or MHCII), which are involved in the presentation of the autoantigen to T helper cells (for more details see reference [34]).

Tolerance can be overcome in a number of different contexts that are directly relevant to the therapeutic administration of autologous proteins. As is true for auto-immune disease, T-cell responses to epitopes within autologous proteins are a necessary component of anti-therapeutic antibody responses. Where peripheral tolerance plays a role, immunogenicity may represent a perturbation of the natural balance between effector T cells and regulatory T cells that recognize epitopes in the autologous protein. Regulatory T cells can be detected by cell-surface markers such as CD25 and CD127 and by intracellular markers such as FoxP3. In the evaluation of immune response to autologous proteins, it is important to bear in mind the effect of these cells when analyzing results. This implies that Td immunogenicity assessment should be coupled to T cell immunophenotyping – as the presence of a T-cell epitope, detected by cell-based assays and in silico screening, is not sufficient to determine what type of immune response may ensue in a clinical setting.

## Comparison of in silico tools

### Selection of tools used for immunogenicity screening

Efforts to "bench mark" in silico tools have only recently been made possible, thanks to the Immune Epitope Database's (IEDB) development of a "gold standard list" of T-cell epitopes. Using the list as a benchmark, a number of tools have been compared by the team at the IEDB [35]. Available online epitope-mapping algorithms were compared for their ability to correctly predict a set of validated class I and class II HLA-restricted epitopes published in the IEDB as of June 2008; Standard Receiver Operated Curve (ROC) and Area Under the Curve (AUC) were used [47]. As competitors, an area under the ROC (Receiver Operating Characteristic) Curve score of 0.5 is equivalent to a random prediction and a score of 1.0 is equivalent to a perfect prediction. This measure can also be interpreted as the probability that when one positive example and one negative example are randomly chosen, the classifier will assign a higher score to the positive example than to the negative. Therefore, scores above 0.6 demonstrate positive predictive power, and scores above 0.75 indicate significant predictive power.

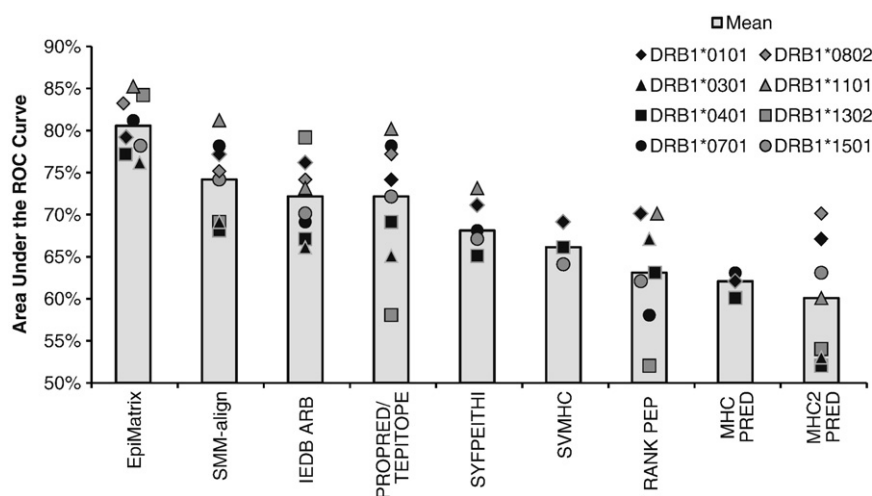
A summary of the results of the published comparison is shown in Figure 3; the IEDB tools appear to be the strongest predictors of T cell epitopes among tools that are available online according to the published comparison.

A similar epitope prediction algorithm, EpiMatrix, was not evaluated in the Wang and Sette study because use of the tool is limited to commercial users and selected academic collaborators. The EpiMatrix set of tools is currently in use by a number of biotech companies for immunogenicity screening (and by the department of defense, among other non-commercial users). The algorithm has been validated extensively over the past 10 years; EpiMatrix predictions have been published for a number of in vitro and in vivo studies (see for examples references [15] De Groot et al.; Bond et al. 2001 [16]; Dong et al. 2004 [17]; McMurphy et al. 2005 [18]; and Koita et al. 2006 [19]).

We recently evaluated the accuracy of the EpiMatrix algorithm using the same dataset of epitopes as published by Wang and Sette (see Fig. 3 for unpublished data provided by Matt Ardito, Bill Martin, presented in poster format at the 2nd annual Vaccine Conference, December 2–4, 2008, Boston MA USA [36]). For class II predictions, which are more relevant to ADA development than class I predictions, EpiMatrix was at least 5% better, and at times 15% better, than available online tools.

### ClustiMer: finding epitope clusters

Several additional tools are used by the authors when analyzing proteins for potential immunogenicity. Using EpiMatrix it is possible to compare potential epitopes across multiple HLA alleles as EpiMatrix raw scores are converted to a normalized "Z" scale. Peptides scoring above 1.64 on the EpiMatrix "Z" scale (typically the top 5% of any given sample), are likely to be MHC ligands. Since class II epitopes can be promiscuous, such epitopes can be discovered by estimating the binding potential of each frame with respect to each of a panel of eight common class II alleles (DRB1\*0101, \*0301, \*0401, \*0701, \*0801, \*1101, \*1301, and \*1501). Taken



**Figure 3** A comparison of online tools. Comparison data taken from Wang P, Sidney J, Dow C, Mothé B, Sette A, et al. [see reference [47]. For class II predictions (which are more relevant to ADA development than class I predictions), EpiMatrix was 5% better than the IEDB ARB tool, 17% better than MHC2PRED, 15% better than MHC PRED, 9% better than SYFPEITHI, and 5% better than TEPTOPE (PROFPRED), based on comparisons published by Wang and Sette [49]. The sensitivity rating, on average, of Class II prediction was 77%, which gives EpiMatrix an advantage in correctly predicting binders that are actually binders and increasing the ratio of true positives to false positives.

together these alleles represent the predominant types of “pockets” for the most common MHC and they “cover” the genetic backgrounds of most humans worldwide [37]. A number of different tools are available for the purpose of finding clusters of HLA binding motifs (promiscuous epitopes); the authors use a tool called ClustiMer.

Scanning the output produced by the EpiMatrix algorithm, the ClustiMer algorithm identifies polypeptides predicted to bind to an unusually large number of HLA alleles; these sequences are then extended at the n- and c-terminal flanks until the predicted epitope density of the candidate epitope cluster falls below a given threshold value. In general, T-cell epitope clusters identified by the ClustiMer algorithm tend to be promiscuous MHC binders and are frequently T-cell epitopes [32].

#### EpiBar: a signature feature of a highly promiscuous T-cell epitope

Further, we have noticed that many of the most reactive T-cell epitope clusters present a feature we refer to as an “EpiBar”. An EpiBar is a single 9-mer frame that is predicted to be reactive to at least four different HLA alleles (Fig. 4B). EpiBars may be a signature feature of highly immunogenic, promiscuous class II epitopes. Sequences that contain EpiBars include Influenza Hemagglutinin 307–319 (Cluster score of 18), Tetanus Toxin 825–850 (Cluster score of 16), and GAD65 557–567 (Cluster score of 19) [unpublished data, Bill Martin, EpiVax]. An example of an EpiBar is shown below. Class II-associated invariant chain peptide (CLIP) which is a highly conserved protein that stabilizes MHC class II molecules in the proteolytic vesicles; it is cleaved from invariant protein (IP). Note the horizontal bar of high Z scores in this peptide which is known to bind promiscuously across HLA class II alleles (Fig. 4B).

#### Immunogenicity scale

Using the concept of epitope density, it is possible to scale proteins for epitope content. Highly immunogenic proteins

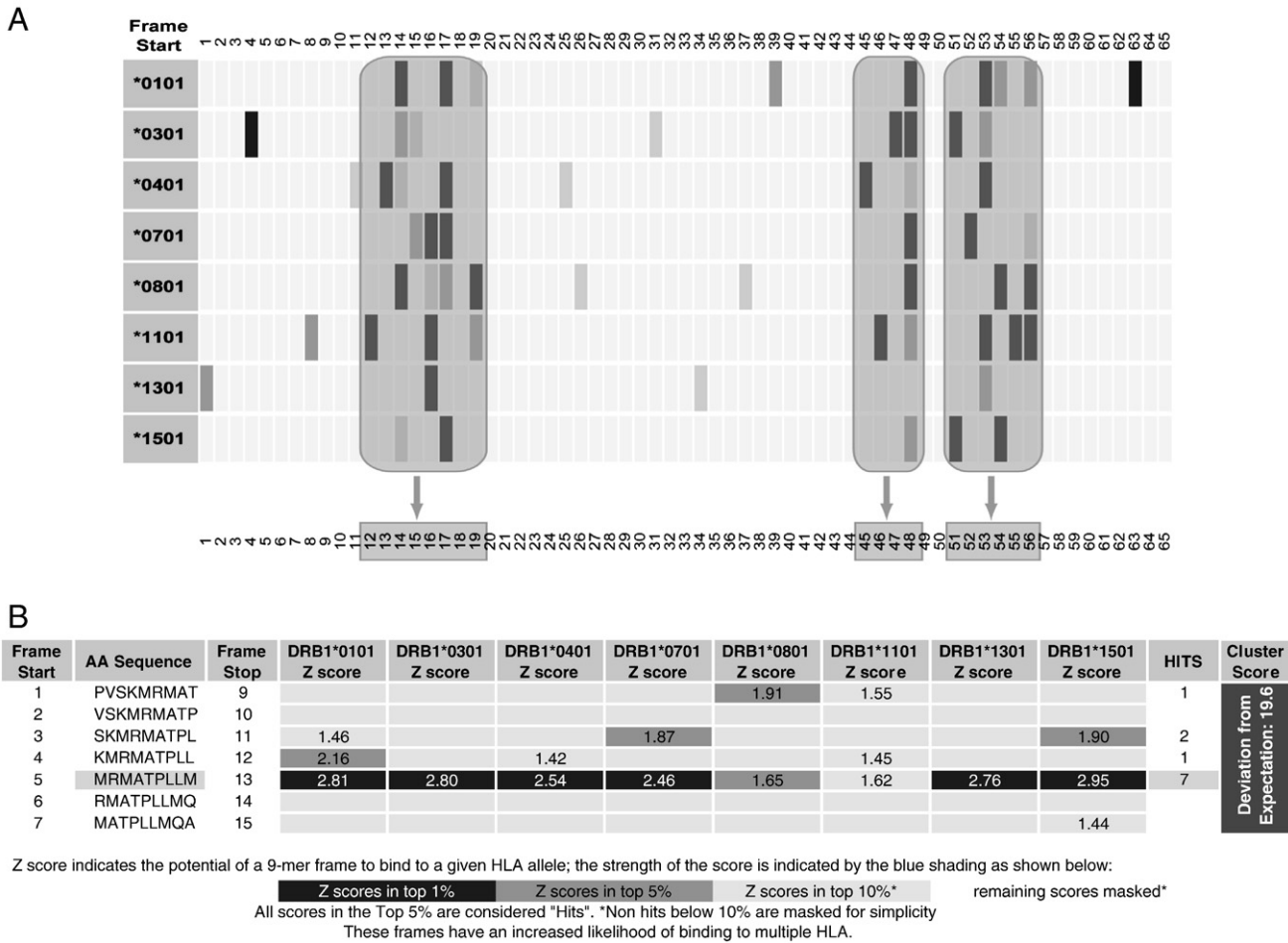
may contain many T-cell epitopes or concentrated clusters of T-cell epitopes, whereas non-immunogenic proteins would tend to contain fewer epitopes.

EpiMatrix and the immunogenicity scale have been utilized to measure the potential immunogenicity of whole proteins. In this context, EpiMatrix assesses the aggregate epitope density of a given protein with respect to the aggregate epitope density of a set of randomly-generated pseudo-protein sequences of similar size [32]. By correcting for size and expected epitope density, the potential immunogenicity candidate vaccine antigens and protein therapeutics can be directly compared. In general, proteins having higher epitope densities tend to be more immunogenic while low-density proteins tend to be immunologically inert.

The density of T-helper epitopes may explain differences in observed antibody responses to slightly different versions of the same recombinant human protein, such as that observed to chimeric and humanized monoclonal antibodies [32]. The authors have therefore developed a “Td immunogenicity scale” that allows the evaluation and comparison of protein sequences for immunogenicity based on T-cell epitope content. Given the “immunogenicity score” of a protein, and taking into consideration other determinants as described above, it is possible to make an informed decision about the likelihood that a protein may provoke an immune response in the clinic (Fig. 5).

#### What is the evidence for T-cell epitope content being predictive of immunogenicity?

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 immunogenicity in at least two cases (GDNF, FPX) which were subsequently published [17,38]. Recognizing the value of preclinical immunogenicity screening, a number of therapeutic protein developers have incorporated in silico, ex vivo and in vivo preclinical

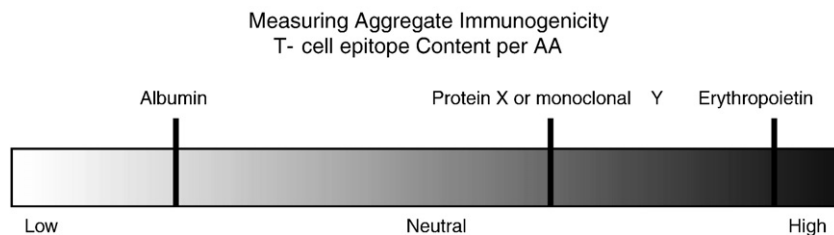


**Figure 4** (A) ClustiMer Analysis. Clustering of HLA binding potential illustrated for a hypothetical protein sequence: as shown above, HLA binding motifs can occur both within and outside of “cluster” regions. In both retrospective [50] and prospective studies [19], we have determined that synthetic peptides representing these regions of dense HLA binding potential are promiscuous binders to HLA molecules and are highly immunogenic in vitro and in PBMC obtained from patients exposed to such peptides in the context of natural infections [22]). A similar cluster, found in an invariant chain (an autologous protein) is shown in panel b. (B) “CLIP” EpiBar. Class II-associated invariant chain peptide (CLIP) region of an invariant chain (Ii) is involved in the assembly and transport of MHC class II  $\alpha\beta$  Ii complexes through its interaction with the class II peptide-binding site. CLIP sequences acts as a promiscuous binder with moderate affinity for all class II molecules. See Thayer et al. [51]. The EpiMatrix analysis for CLIP is shown above: high scores are clustered in the one region previously known to associate with MHC class II molecules. An “EpiBar” is located at invariant chain residues 91–99.

immunogenicity screening protocols into their product development strategy.

The FPX protein therapeutic illustrates the usefulness of in silico screening for prospective prediction of immunogeni-

city. FPX is a recombinant fusion protein consisting of two identical, biologically active, peptides attached to human Fc fragment. EpiMatrix predicted a strong signal for immunogenicity within the 14-amino acid carboxy-terminal region of



**Figure 5** Proteins ranked by T-epitope content per Amino Acid. The immunogenicity scale illustrated here, is used as to compare proteins one to another. The concept of ranking proteins by epitope content was developed by De Groot and Martin at EpiVax and applied in Koren et al. Clinical Immunology, 2007 [4].

the peptide portion of FPX. On administration of FPX in 76 healthy human subjects, 37% developed antibodies after a single injection. A memory T cell response against the carboxy-terminus of the peptide was observed in antibody positive but not in antibody negative subjects. Promiscuity of the predicted T-cell epitope(s) was confirmed by representation of all common HLA-alleles in antibody positive subjects. As predicted by EpiMatrix, HLA-haplotype DRB1\*0701/1501 was associated with the highest T cell and antibody response.

Several other publications have also linked T cell response and immunogenicity. For example, Barbosa et al. [6] confirmed the role of T cells in the immune response to Betaseron by linking ADA to HLA DR type. Tatarewicz and Moxness used EpiMatrix to screen GDNF, a protein therapeutic that was subsequently proven to be immunogenic in clinical trials [7]. In addition to the two published studies [4,7], *in silico* evaluations have been predictive of clinical immunogenicity in at least two unpublished studies (Vibha Jawa, unpublished data presented in San Diego, September 2008 [39]). To date, there is no published documentation of preclinical studies using naive blood from human donors that were predictive of clinical immunogenicity.

### Role of regulatory T-cell epitopes

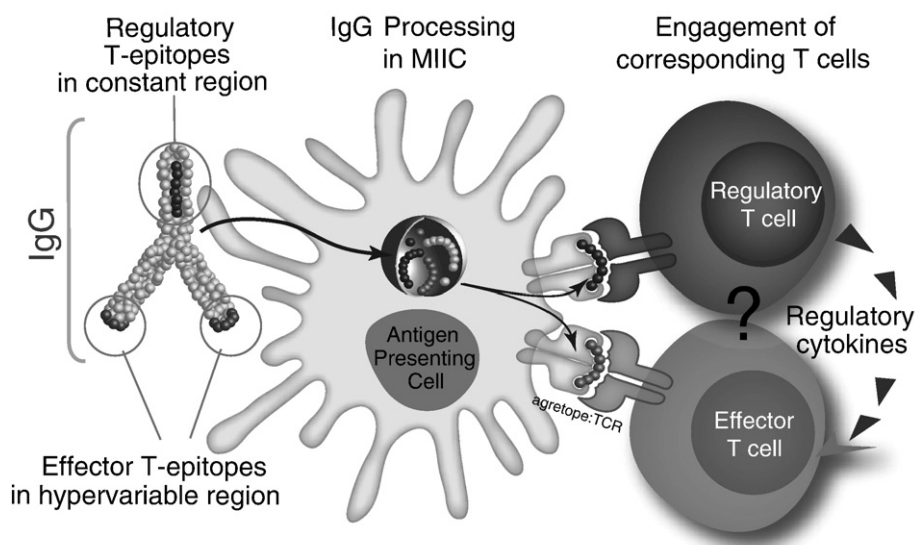
Not all clusters of immunogenic potential can be considered to be potentially immunostimulatory. Would-be epitopes in autologous proteins may relate to T cells that are absent from the peripheral circulation, since T cells that are auto-reactive are said to be deleted in thymic development. However, 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 [40]. According to the most widely accepted theory regarding

T-cell tolerance, T cells with high affinity for self antigens are deleted whereas those with moderate affinity for self antigens may escape deletion and may be re-programmed to function as "natural" regulatory T cells (Treg) cells [51]. Anti-self immune responses may be ongoing and controlled by circulating regulatory T cells (Tregs).

Peripheral tolerance can be overcome in a number of different contexts that most likely relate to the milieu in which the immune response is taking place [41]. For example, inflammation caused by minute amounts of contaminants such as LPS, or bacterial DNA, both of which can act as toll-like receptor (TLR) agonists, may stimulate antigen presenting cells to release sufficient amounts of cytokines and chemokines to overcome Treg immune responses in the periphery [42].

The heavy chain of antibodies has both a constant and a variable region. As the name implies, the constant region is conserved in all antibodies of the same isotype while the variable region differs in each B cell. The light chain also has one constant and one variable domain. The variable domains of each chain are most crucial since they bind to antigen and determine the specificity of the antibody. The resulting novel heterogeneity within the V segments becomes the common theme for a small subset of B cells that out-compete other clonally-related B cells by virtue of their superior antigen-binding capacity. There have been many explanations for the apparent lack of induction of T-cell responses to antibody variable regions, including T regulatory cells and apparent T-cell tolerance to B cell receptor variable regions [43].

The link between HLA-restricted/T cell immune response and the development of auto-antibodies is still being defined; early evidence points to the reduction of Treg immune responses and to induction of T effector responses as significant contributors to HAHA [44,47,48] (Fig. 6). Tolerance to immunoglobulin is perhaps explained by the

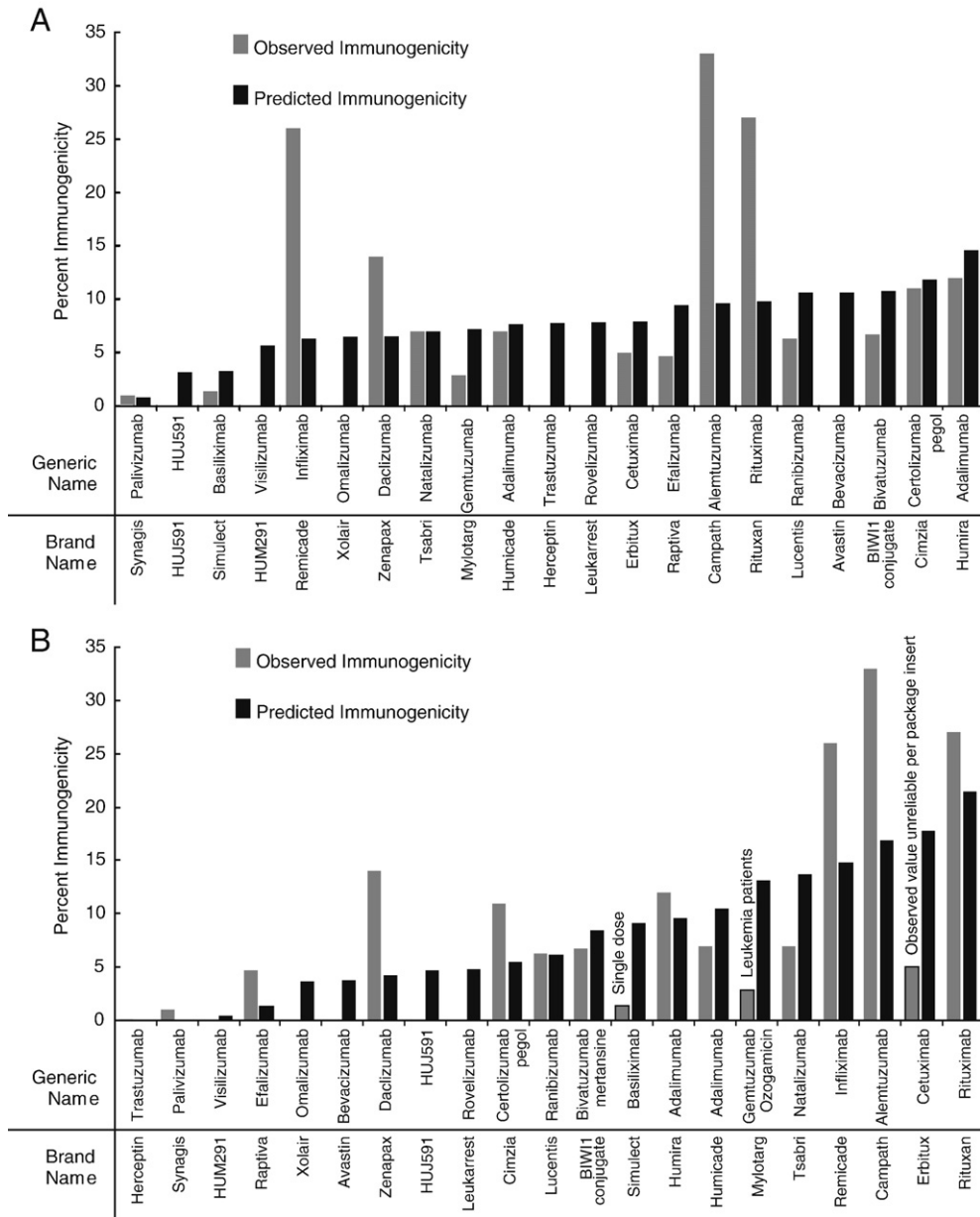


**Figure 6** Theoretical role of regulatory T cell epitopes in IgG. Adapted with permission from De Groot et al. [45]. We have discovered conserved T-cell epitopes in IgG that engage natural regulatory T cells. We hypothesize that antibody-derived Treg epitopes (dark blue epitope) activate regulatory T cells, which leads to suppression of effector T cells that recognize effector epitopes (red epitope), like those of IgG hypervariable regions to which central tolerance does not exist. Whether this suppression is mediated by regulatory cytokines alone or by contact-dependent signaling, or both, has yet to be determined.

observation that immunoglobulin therapies induce expansion of Tregs in vitro and in vivo [45]. In recently published work, we identified regulatory T-cell epitopes within the heavy and light chains of Ig. Termed “Tregitopes”, these regulatory epitopes synthesized as peptides, were able to suppress effector immune responses to coadministered antigen, both ex vivo and in vivo. In mouse models, the level of suppression

was greater than that achieved by Fc, perhaps explaining also the mechanism of IVIG suppression. The Tregitopes were found to upregulate CD25 and Foxp3 expression leading to the secretion of IL-10 and TGF- $\beta$  [46].

The discovery of regulatory T-cell epitopes in the Fc and CH1 domains of IgG has important implications for the design of mAbs and indeed for therapeutic proteins in general.



**Figure 7** (A and B) Td immunogenicity of antibodies: comparison of scoring with and without removal of Tregitope scores. Twenty monoclonal antibody sequences were obtained from the literature or the US Patent and Trademark office (USPTO). Each sequence was then scanned both for epitopes (any epitope restricted by eight common HLA alleles). Positive EpiMatrix scores for each of the antibodies were summed; scores are shown in panel A without the Tregitope correction, and in panel B, epitopes contained in putative T regulatory epitope (as defined above) were set aside (not included in the final sum). These putative Treg epitopes (IgG “Tregitopes”) were defined as 9-mers conserved in more than 1% of observed antibodies (>4000) and scoring at least 5 on the EpiMatrix cluster scale. The final regressions are shown. No Fc regions were scored for this study as all are highly conserved; the immunogenicity of Cimzia is notable in this context, as it lacks any Fc and therefore does not have the Fc-region Tregitope described in De Groot et al. [45]).

Hypothetically, removal or alteration of regulatory T-cell epitopes by mAb developers in the “humanization” process, may also adversely affect the immune response to the mAbs. The implied role of natural Tregs in the outcome of immune response to mAbs is particularly interesting to consider in the case of CIMZIA, a Fab fragment anti-TNF drug. This drug is more immunogenic than was expected (when compared to other antibodies, which do not lack the Fc region), perhaps due to the absence of the critical Fc fragment Tregitope.

Just as the inadvertent addition of stimulatory T cell (T effector) epitopes to proteins may lead to increased immunogenicity, removal or alteration of regulatory T-cell epitopes in the drug development process may alter the natural T-regulatory immune response to recombinant autologous proteins.

### Scaling monoclonal antibodies for immunogenicity

Building on these observations, we have evaluated monoclonal antibodies for the presence or absence of T-effector and regulatory T-cell epitopes. The immunogenicity of monoclonals can be 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) (Fig. 7).

To perform this analysis, we selected 21 human and chimeric antibodies that had been studied for immunogenicity were selected from the literature (see Table 1). Where multiple studies were available, the scores were “averaged”. Amino acid sequences for the variable regions of the heavy and light chains were obtained from GenBank and the USPTO. All sequences were scored with EpiMatrix and rated on the EpiMatrix immunogenicity scale. Each sequence was then scanned for the presence of putative Tregitopes. This included only the most common variants on the EpiBars

found in the regulatory T-cell epitopes previously identified as potentially tolerogenic. Observed immunogenicity was then regressed against the immunogenicity (epitope per AA) score of the combined light and heavy chains. When Tregitopes were removed from the scoring, the immunogenicity score was found to be a significant predictor of immunogenicity (correlation coefficient=0.67).

A number of caveats apply to the interpretation of the data as provided in Fig. 7. The data set used for this pilot analysis is very small ( $n=21$ ). In some cases the data was only provided for subjects who were clinically immunosuppressed prior to having received the antibody therapy. In most cases, the number of study subjects is small (20–30). Furthermore, some of the antibodies target antibodies target receptors involved in human immune response. And finally, as stated previously, the presence or absence of MHC class II ligands is not the only factor necessary to the formation of robust humoral response.

### Recommendations

ADA can be minimized by improving product purity and reducing aggregates, however screening methods such as antibody immunogenicity assays, T-cell epitope mapping, HLA typing, T cell phenotyping, and animal studies (particularly HLA transgenic) are important additions to the battery of tests that might be required in preclinical and clinical evaluations of immunogenicity.

The authors believe that a combination of in silico assessment and in vitro/in vivo validation should become the standard for the biotherapeutics industry for both prospective (preclinical) and ongoing (clinical) assessment of immunogenicity. Given the association between T-cell responses and immunogenicity, the vast array of new therapeutic proteins that are being developed, and the enormous expense associated with clinical trials, researchers

**Table 1** List of online tools for T-cell epitope prediction

Name	Developers/institution	Type	Website
EpiMatrix	A.S. De Groot, W.D. Martin EpiVax, Inc.	Commercial	<a href="http://www.epivax.com">www.epivax.com</a>
IEDB ARB/ SMM-align	Public	Public	<a href="http://www.immuneepitope.org">www.immuneepitope.org</a>
MHC2PRED	G.P.S Raghava Bioinformatics Center, Institute of Microbial Technology, Chandigarh India Bioinformatics Center, Institute of Microbial Technology, Chandigarh, India	Public	<a href="http://www.imtech.res.in/raghava/mhc2pred">www.imtech.res.in/raghava/mhc2pred</a>
MHCPRED	D.R. Flower The Jenner Institute	Public	<a href="http://www.jenner.ac.uk/mhcpred">www.jenner.ac.uk/mhcpred</a>
PROPRED/ TEPITOPE	G.P.S. Raghava, H. Singh	Public	<a href="http://www.imtech.res.in/raghava/propred">www.imtech.res.in/raghava/propred</a>
RANKPEP	P.A. Reche Harvard Medical School	Public	<a href="http://bio.dfci.harvard.edu/RANKPEP">http://bio.dfci.harvard.edu/RANKPEP</a>
SVRMHC	P. Donnes, A. Elofsson Division for Simulation of Biological Systems, University of Tübingen, Germany Division for Simulation of Biological Systems, University of Tübingen, Germany	Public	<a href="http://www-bs.informatik.uni-tuebingen.de/SVMHC">http://www-bs.informatik.uni-tuebingen.de/SVMHC</a>
SYFPEITHI	H-G Rammensee Dept of Immunology, Tübingen, Germany Dept. of Immunology, Tübingen, Germany	Public	<a href="http://www.syfpeithi.de/home.htm">http://www.syfpeithi.de/home.htm</a>

and developers would do well to consider the potential for their protein product to induce a Td immune response prior to testing the proteins in the clinic. In silico, in vitro, and in vivo tools are available for this purpose. In silico tools represent a high-throughput, low-cost, and accurate means of assessing immunogenicity.

It may be useful for regulatory agencies to encourage companies to incorporate immunogenicity screening early in the development pipeline by requesting this information in pre-IND conferences. Regarding immunogenicity monitoring in the clinical phase of development, the authors are of the opinion that both T cell responses and ADA should be carefully monitored. Furthermore, although it can be said, that proteins that have fewer T-cell epitopes are generally less immunogenic; the balance between Treg epitopes and T effector epitopes may also be critical to the outcome. Thus, additional in vitro studies that examine the role of regulatory T cells in the response to autologous proteins should be considered in the biologics development process. Should it prove impossible to remove T cell epitopes in the preclinical development process, in silico and in vitro epitope mapping may provide a means for monitoring Td immunogenicity in the clinical development phase.

In silico epitope mapping using the tools reviewed in this article may permit developers to triage protein drugs to higher and lower risk categories. At the preclinical phase, in silico analysis would enable developers to identify proteins to be assigned to higher risk categories; additional studies might be performed for protein drugs that contain many T cell epitopes. At the clinical trial phase, in silico analysis might be used to identify which peptide sequences from the protein drug should be synthesized and used for monitoring for Td immunogenicity ex vivo.

### What efforts to develop a consensus on in silico, in vitro, and in vivo methods for measuring T cell dependent immunogenicity are underway?

A number of open "consensus building" meetings between experts and drug developers whose goal is to formulate a white paper on the role of cellular immunity in the development of anti-drug antibodies to biologics were held in 2007–2008. Methods for predicting and confirming T cell immunogenicity (in silico, in vitro and in vivo) are currently being compared and contrasted. An attempt will be made to clarify "what is known" about the contribution of cellular immune response to protein therapeutics and "what is not known" including such topics as the role of T regulatory cells and cytotoxic T cells. A consensus "white paper" paper is currently in development and can be expected to be submitted for publication in 2009.

### The role of regulatory agencies

Regulatory agencies such as the EMEA and the FDA have an opportunity to declare their stance regarding the observed association between T-cell epitope content and immunogenicity; by doing so they may encourage pharma companies to use available tools for to develop safer biologics. In the absence of this acknowledgement, drug developers will continue to measure antibody, only. Lack of information may lead to missed opportunities to understand the mechanisms

of immunogenicity (see Epo and PRCA for example) and to improve drug safety. As has been done for the vaccine field, the agencies can set "standards" for Td immunogenicity assays. Such methods can be standardized now. When drug developers pay attention to T-cell epitopes, which are one of the root causes of immunogenicity, safer biologics are likely to be developed.

### Disclosure

Two of the contributing authors, William Martin and Anne S. De Groot, are senior officers and majority shareholders at EpiVax, a privately-owned immunotherapeutics company located in Providence RI. These authors acknowledge that there is a potential conflict of interest related to their relationship with EpiVax and attest that the work contained in this research report is free of any bias that might be associated with the commercial goals of the company.

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