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Minimizing the immunogenicity of protein therapeutics

Arthur J. Chirino, Marie L. Ary and Shannon A. Marshall

As is cautioned in many package inserts, 'with all therapeutic proteins, there is a potential for immunogenicity'. Immunogenicity problems in humans, which currently can be detected only in clinical trials or after product launch, pose a significant barrier to the development and acceptance of protein drugs. Recent and ongoing research, presented in this review, seeks to address the challenge of protein therapeutic immunogenicity by elucidating the mechanisms underlying immune recognition of protein therapeutics, establishing preclinical methods for assessing immunogenicity and developing strategies for minimizing immune responses.

Arthur J. Chirino*
Marie L. Ary
Shannon A. Marshall
Xencor
111 W. Lemon Avenue
Monrovia
CA 91016, USA
*e-mail: art@xencor.com

▼ Many therapeutic proteins, including products isolated from human blood, recombinant human cytokines, and recombinant growth factors, have elicited immune responses when administered to patients [1–3]. In some cases, the formation of neutralizing and non-neutralizing antibodies reduces drug efficacy and potency. For example, several studies have found a correlation between anti-interferon-α antibodies and the failure of interferon treatment in hepatitis C patients [4]. Other immune responses, such as those seen with megakaryocyte growth and development factor (MGDF) and Johnson & Johnson's (http://www.jnj.com) erythropoietin (EPO) product Eprex® (epoetin alfa), pose a far greater danger: formation of cross-reactive neutralizing antibodies to the corresponding endogenous protein resulted in thrombocytopenia and pure red cell aplasia, respectively [5,6]. These more serious cases, although relatively rare, have brought the issue of unwanted clinical immunogenicity to the forefront. Although even native human proteins can elicit an immune response, the perceived risk of clinical immunogenicity in response to non-native protein sequences is especially high. Non-native protein sequences, including fusion proteins, bacterially derived biotherapeutics, engineered variants, and most monoclonal antibodies (mAbs), can possess novel functions and superior clinical performance; therefore, the development of safe and effective engineered and non-human protein therapeutics is crucial to the continued expansion of the biotherapeutics industry. To address the immunogenicity of native and engineered protein therapeutics, several companies and academic groups are developing better assays to quantify immune responses in patients, new methods to detect potential immunogenicity problems before clinical trials, and sophisticated tools to limit protein immunogenicity while maintaining biotherapeutic function.

Immune recognition of therapeutic proteins

To elicit an antibody response, a protein therapeutic must interact with several classes of immune cells, including antigen presenting cells (APCs), T cells and B cells. Each cell type recognizes distinct antigen features, and blocking any of these recognition events can significantly reduce the incidence of clinical immunogenicity (Figure 1).

Antigen uptake

One of the first steps in raising an immune response to a protein therapeutic is APC uptake via pinocytosis, receptor-mediated endocytosis or phagocytosis. The efficiency of antigen uptake, a major determinant of immunogenicity, varies significantly with the route of administration, the aggregation state of the protein, and its receptor-binding specificity. Protein aggregates are typically far more immunogenic than soluble proteins. The presence of aggregates is linked to the immunogenicity of therapeutics including human growth hormone and interferons [7,8], and a

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connection between erythropoietin immunogenicity and aggregation has been proposed [9]. Increased uptake by APCs and prolonged exposure to APCs and T cells are thought to be key factors in the enhanced immunogenicity of particulate antigens, such as protein aggregates, although it is likely that other mechanisms are also important [10,11].

Recently, Schellekens suggested that aggregation-dependent antibody formation toward Eprex® might involve direct B-cell activation and differentiation rather than a T-cell mediated response [9]. T-cell-independent antibody production has been demonstrated for polyvalent antigens of bacterial and viral origin, such as lipopolysaccharides and structurally well-ordered viral capsids. Antibodies produced by direct B-cell activation are mostly low affinity, high avidity IgM and limited low affinity IgG isotypes, as affinity maturation and class switching require T-cell help [12]. However, clinical administration of Eprex® resulted in sustained production of high affinity IgG1 and IgG4 antibodies (K_d ~110 pM) [9], strongly suggesting a T-cell dependent process.

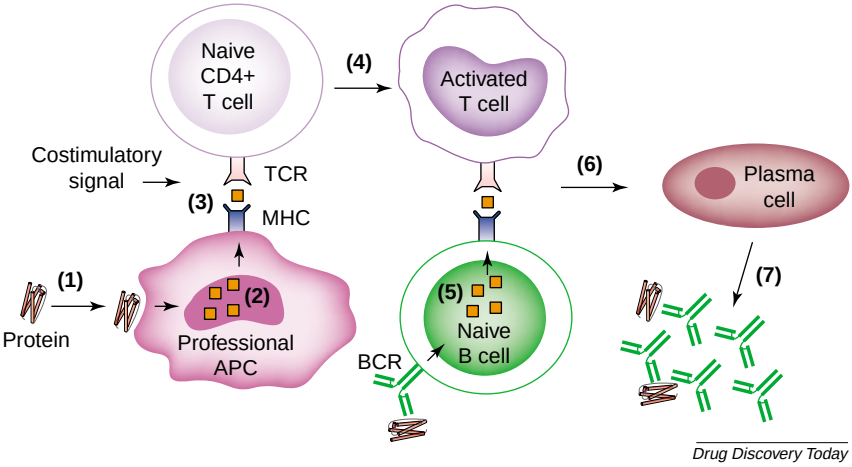

Figure 1. The process of raising an immune response to a protein drug involves several recognition events and signals: (1) protein uptake by antigen presenting cells (APCs), (2) protein antigen processing, (3) interaction between APCs and T cells, including formation of peptide–major histocompatibility complex (MHC)–T-cell receptor (TCR) complexes in the presence of costimulatory signals such as those from infection or inflammation, (4) T-cell maturation, (5) interaction between T cells and B cells, including protein binding to B-cell receptors and formation of peptide–MHC–TCR complexes, (6) B-cell maturation, including isotype switching and affinity maturation, and (7) antibody binding to the protein drug. Blocking any of the above steps can reduce the immunogenicity of protein therapeutics.

APCs express a tremendous diversity of cell-surface receptors, including antibody Fc receptors and a wide range of cytokine receptors. Binding enables receptor-mediated endocytosis and, for immunomodulatory therapeutics, can stimulate signaling pathways that promote or inhibit APC maturation and activation. Receptor-mediated endocytosis is a far more efficient means of antigen uptake than non-specific endocytosis, enabling presentation of antigens that are present in much smaller concentrations. For example, the therapeutic efficacy of AltaRex's OvaRex® (oregovomab), a monoclonal antibody directed against the ovarian cancer antigen CA125, is directly attributable to increased antigen presentation of CA125 as a result of Fc receptor-mediated internalization [13].

Binding determinants of class II MHC, T-cell receptors and B-cell receptors

In T-cell-dependent immune responses, specific portions of an antigen are recognized by class II major histocompatibility complex (MHC) molecules, T-cell receptors (TCR) and B-cell receptors (BCR); the MHC-binding determinants are referred to as agretopes, while TCR and BCR binding determinants are called epitopes. Following antigen uptake and processing, antigen-derived peptides bind to class II MHC molecules. TCRs recognize these peptide-MHC complexes, so T-cell epitopes and MHC agretopes must be overlapping. BCRs and antibodies, by contrast, recognize linear or conformational motifs that are present on the surface of the intact protein, and might include residues that are far apart in the protein sequence. Consequently, T-cell epitopes are usually distinct in sequence and location from antibody epitopes.

The immune system generates tremendous molecular diversity. Each individual can produce an antibody repertoire of ~10^8, which is increased further during affinity maturation, and an even greater number of T-cell receptors. Due to this tremendous diversity, a wide variety of sequences and structures can be recognized by the adaptive immune system. As a result, while physiologically relevant antibody and T-cell epitopes in protein drugs can be identified, it would be nearly impossible to remove all of the potential T-cell or antibody epitopes on a protein therapeutic. MHC molecules, by contrast, are static but highly polymorphic proteins, meaning that while each person only expresses a handful of MHC molecules, >1000 class II MHC alleles have been identified in the human population (http://www.ebi.ac.uk/imgt/hla/) [14]. The class II human leukocyte antigen (HLA) gene complex includes three loci (DP, DQ, and DR), each containing genes encoding the α and β subunits of an MHC molecule. Among the class II

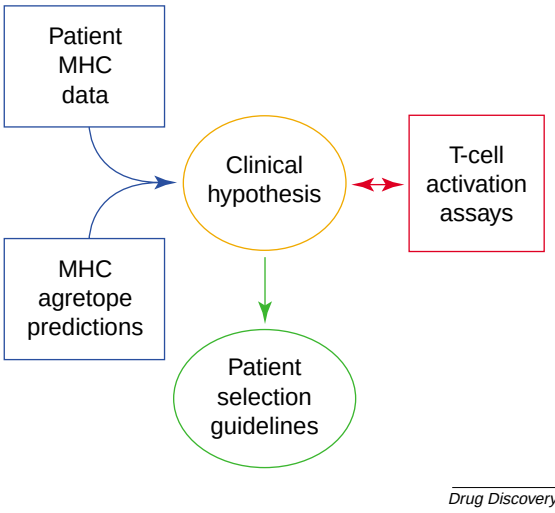

Figure 2. The major histocompatibility complex (MHC) genotype of patients is known to affect susceptibility to autoimmune diseases and vaccine-induced immune responses. It is also likely that specific MHC genotypes are associated with immune responses to various protein therapeutics. Analysis of the patient MHC genotype data and allele-specific agretope prediction algorithms can be used to generate hypotheses relating specific alleles to clinical outcomes; these hypotheses can be validated using *ex vivo* T-cell activation assays. The results of such studies could be used to identify patients who are especially likely or unlikely to raise a harmful immune response to a given protein therapeutic.

loci, DRB1 is especially polymorphic, DRB3/4/5, DQA1, DQB1, DPA1 and DPB1 are moderately polymorphic, and DRA is essentially monomorphic [15]. However, the complete class II MHC genotype of >90% of the relatively diverse US population can be accounted for with ~100 of the most prevalent heterodimer combinations (data obtained from The High-Resolution Donor-Recipient Pair Project, an ongoing study of the National Marrow Donor Program®; see http://www.nmdpresearch.org/).

MHC alleles differ with respect to their peptide binding specificity [16]; therefore, depending on HLA genotype, individuals will vary in their ability to mount an immune response to a given protein sequence. On a population level, MHC diversity helps to ensure effective pathogen recognition, enabling at least a fraction of the human population to combat infection. Among individuals, allelic differences in class II MHC molecules are manifest as variation in susceptibility to specific infectious diseases [17] and autoimmune diseases [18], as well as variation in the efficacy of some vaccines [19,20]. Similarly, individual allelic differences in peptide binding specificity are expected to affect the immunogenicity of protein therapeutics. The immunogenicity of interferon-α in mouse models is affected by MHC alleles [21] and an early study hints that antibody response to insulin might be associated with DR7 alleles [22], but further studies with larger sample sizes will be required to confirm a link between clinical immunogenicity and patient MHC haplotype. Understanding the dependence of immune responses on MHC haplotype could aid in the design and monitoring of clinical trials (Figure 2).

Preclinical and clinical methods for assessing immunogenicity

Historically, preclinical immunogenicity testing has been limited to monitoring antibody formation in rodents and non-human primates. However, immune responses to therapeutic proteins in conventional animal models have poorly predicted immunogenicity problems in humans [1,23]. One limitation of traditional animal models is that tolerance, a key aspect of the immune response, is highly species-specific. Many endogenous human proteins are used as therapeutics. For a human to raise an immune response to such a protein, a patient must break tolerance, while in animal models such proteins are foreign. Furthermore, MHC sequences, and hence MHC agretopes vary among species, resulting in differences in antigen recognition and T-cell repertoire. As a result, immunogenicity in animal models does not necessarily predict immunogenicity in humans. Several alternative cell-based and animal models for predicting clinical immunogenicity have been discussed recently [1], which might provide greater predictive power: *in vitro* T-cell activation assays and studies in genetically engineered animal models are especially promising. However, no model fully recapitulates the human immune response, so long-term monitoring of immunogenicity in patients during clinical trials and post-marketing is essential.

Antibody detection

Robust, selective, sensitive and well-validated antibody detection methods are crucial for monitoring clinical immunogenicity in both preclinical and clinical studies. The sensitivity, and hence the incidence of false negatives, varies appreciably among the commonly used ELISA and radioimmunoprecipitation antibody-detection assays. In addition, assay protocols must correct for interference caused by the presence of drug in clinical samples [24]. Surface plasmon resonance detection, which more sensitively detects extremely low antibody concentrations and low affinity antibodies, is emerging as a superior assay for antibody formation [25]. Highly sensitive methods can be critical, as recent studies in Eprex® patients demonstrate that low neutralizing antibody titers can cause clinically relevant sequelae [26].

Traditional animal models

To assess the predictive power of traditional animal models, we compared the clinical immunogenicity for several approved protein therapeutics with the animal immunogenicity data described in FDA reviews (http://www.fda.gov/cder/biologics/products/etanimm091202.htm) and found a poor correlation. Immunogenicity was detected in many animal studies, even if the protein was minimally immunogenic in humans. For example, in studies with Amgen's (http://www.amgen.com) anti-TNF fusion protein Enbrel® (etanercept), anti-drug antibodies were detected in <5% of patients with rheumatoid arthritis or psoriatic arthritis, while the incidence of immunogenicity was much higher (83–100%) in toxicology studies performed in cynomolgus monkeys, rats, rabbits and mice.

Animal studies can nonetheless provide useful information. For example, studies in non-human primates accurately predicted the relative immunogenicity of different forms of human growth hormone [27], and mouse models demonstrated that interferon-α aggregates are more immunogenic than soluble protein [8]. Animal studies have also been useful in revealing clinical sequelae that might develop when neutralizing antibodies are produced [28,29]. For example, preclinical safety studies in monkeys demonstrated that MGDF elicits neutralizing antibodies that are associated with decreased platelet counts; a similar response was later observed in the clinic [29]. Animal studies can be improved by performing specialized immunogenicity experiments in addition to general toxicology studies. Although expensive, non-human primates are considered the best species for immunogenicity testing. When the sequence of a therapeutic based on a human protein is highly conserved between humans and a non-human primate, immune tolerance might be similar in both species. Furthermore, many of the polymorphic differences in class II MHC molecules arose before humans and non-human primates diverged, meaning that orthologous MHC sequences share significantly greater similarity than paralogous sequences [30]. As a result, it is likely that class II MHC peptide binding specificities of humans have more in common with those of non-human primates than with those of other species. Monkey MHC alleles are now being genotyped [31], and macaques with defined MHC haplotypes are being bred for vaccine studies [32]. As such efforts continue, it might be possible to breed non-human primates with MHC alleles that share peptide binding specificities with the most prevalent human alleles, thus providing an animal model with improved predictive power.

Genetically engineered mouse models

Genetically engineered mice are also promising models for immunogenicity. Wild-type mice will typically raise an immune response to therapeutics based on human proteins, while tolerance limits immunogenicity to these therapeutics in humans. To better capture the effects of tolerance on the immune responses to engineered variants of human proteins, transgenic mice that express the endogenous human protein can be treated with that protein or a variant and monitored for immune responses. For example, the relative immunogenicities of native human insulin, insulins from other species, and engineered variants, as well as native versus variant tissue plasminogen activator, were accurately determined using transgenic mouse models [33,34]. Furthermore, transgenic mouse models can be superior models for preclinical testing, because efficacy disruption by clearing, sustaining and neutralizing antibodies is minimized. However, as mentioned previously, inter-species differences in MHC alleles and T-cell repertoire limit predictability. Autoimmunity to human antigens can also be studied in mouse models that are engineered to express human rather than murine MHC molecules [35,36]. Although it would be quite laborious to produce and maintain sufficient mouse models to consider all relevant human MHC alleles, a more tractable number of mouse lines expressing human MHC molecules could provide reasonable predictions for a fraction of the human population. In addition, models in which the mouse homolog of a protein therapeutic is knocked out can be used to assess the potential clinical consequences of cross-reactive neutralizing antibodies, as knockouts can indicate whether a protein is required for an essential physiological function.

Ex vivo *T-cell activation assays*

Stimulation of CD4+ T cells is required for sustained antibody production (Figure 1); therefore, assays that measure T-cell activation using human APCs and T cells can be used to test the immunogenicity of a peptide or protein. Several experimental techniques have been developed to measure antigen binding by class II MHC molecules and subsequent T-cell activation; thorough comparisons of these methods have been published previously [37,38]. Typically T-cell activation assays have been used for vaccine development or to study autoimmune disease, so this discussion focuses on issues that specifically relate to testing the immunogenicity of protein therapeutics. Sensitivity is a key concern in measuring the immunogenicity of therapeutics derived from native human proteins, because most self-reactive T cells are deleted during negative selection. One promising solution (Figure 3) is to invoke multiple rounds of T-cell priming to stimulate clonal expansion and detect T-cell activation using EliSpot assays [13,39,40], which monitor cytokine production with single-cell sensitivity [41].

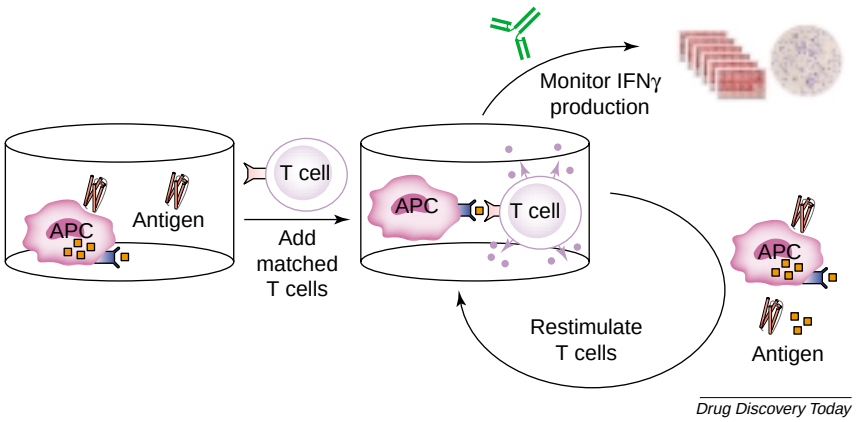

Figure 3. *Ex vivo* T-cell activation assays are a promising method to assay the immunogenicity of protein therapeutics. T cells and antigen presenting cells (APCs) are isolated from human blood and exposed to a peptide or protein antigen. Potential immunogens will induce T-cell activation, which can be detected by measuring the production of cytokines, such as interferon-γ (IFNγ) production in an EliSpot assay. Only a small number of naive T cells will be reactive to most protein therapeutics (especially those derived from human sequences); therefore, repeated stimulation with antigen-pulsed APCs can be used to enhance the sensitivity of the assay.

Ideally, initial T-cell stimulation should be performed with protein rather than peptide antigens so that the assay can capture antigen uptake and processing events as well as presentation. However, peptide assays can still be useful for identifying MHC agretopes. Finally, to accurately understand the potential immunogenicity of a protein, T-cell activation assays should be conducted using a panel of donors expressing all of the prevalent MHC alleles in the relevant patient population. Although T-cell activation assays do not account for certain crucial factors, such as peripheral tolerance and antibody binding, they use fully human APCs and T cells and are therefore the best available model for antigen processing, presentation and recognition by MHC and T-helper cells.

Current strategies to reduce immunogenicity

Initial successes in reducing immunogenicity were obtained by replacing drugs obtained from non-human sources with human sequences (e.g. insulin, monoclonal antibodies) or by improving manufacturing processes to minimize impurities (e.g. human growth hormone) [42]. Such precautions, although necessary, are often insufficient, so additional approaches for immunogenicity reduction have been proposed. Several companies, shown in Table 1, now use a variety of immunogenicity reduction strategies to ensure the quality of their internal pipeline and to create partnering opportunities. A crucial requirement is that modifications introduced to minimize immune responses must not destroy the structure, function and stability of the therapeutic. Retaining activity while reducing immunogenicity is especially challenging for therapeutics that must bind to one or more protein receptors to function, as some of the motifs that form functional binding interfaces might also be recognized by the molecules of the immune system.

Increasing human sequence content

Human anti-murine antibody (HAMA) responses limited the efficacy of early murine mAbs [43,44]. To reduce HAMA responses, several approaches to minimize or eliminate murine sequence content were developed: chimeric antibodies comprising mouse variable regions and human constant regions, humanized antibodies in which murine CDRs were grafted onto a human framework, and fully human antibodies produced by phage display or in transgenic mice. These approaches often dramatically reduced immunogenicity; for instance, the murine anti-CD3 antibody OKT®3 (muromonab) elicited neutralizing antibodies after a single dose in all patients tested, while a humanized version was immunogenic in only 25% of patients following multiple injections [45]. Nonetheless, antibody production affects several chimeric, humanized, and fully human mAb therapies and, in at least one case, the incidence of immunogenicity exceeded 60% [46]. Even Abbott's (http://www.abbott.com) Humira® (adalimumab), the first FDA-approved fully human antibody, elicits a detectable immune response in 12% of patients when administered without an immunosuppressant (http://www.fda.gov/cder/foi/label/2002/adalabb123102LB.pdf). As the experiences with recombinant human Eprex® demonstrates, even fully human proteins can be immunogenic, as immune tolerance can be broken under some circumstances. The following immunogenicity reduction strategies should prove useful in addressing immunogenicity problems even in native human proteins.

Improving solution properties

As discussed previously, protein aggregates are typically significantly more immunogenic than soluble proteins. Historically, poor protein solubility has been considered an obstacle that could be overcome with sufficient effort by optimizing expression, purification, formulation and solution conditions. However, aggregation-linked immunogenicity continues to plague important protein therapeutics, such as interferons, despite decades of research on these

Table 1. Companies supplying immunogenicity reduction services and technologies

Company[a]	Immunogenicity reduction strategy	Epitope/agretope identification and validation strategy	Epitope/agretope modification and removal strategy
Biovation	MHC II agretope removal	Threading[b]/*in vitro* MHC binding/*Ex vivo* T-cell proliferation	Substitutions in single and combined agretope variants by iterative site-directed mutagenesis
Epimmune	MHC II agretope removal	*In vitro* MHC binding	Iterative site-directed mutagenesis
EpiVax	MHC II agretope removal	Quantitative matrix[c]	Substitution suggestions based on quantitative matrix results
Genencor	MHC II agretope removal	Multi-donor T-cell proliferation	Iterative site-directed mutagenesis/ alanine scanning
Nektar	Antibody epitope blocking (PEG)	Not applicable	Advanced PEGylation
Novozymes	Antibody epitope removal	Search of proprietary epitope database	Directed evolution
Xencor	MHC II agretope removal/Antibody epitope removal/antibody epitope blocking (PEG)/solubility improvement	Quantitative matrix/ multi-donor, *in vitro* protein-primed T-cell activation	Rational, structure-based design (PDA® technology)

[a]Biovation, http://www.biovation.co.uk; Epimmune, http://www.epimmune.com; EpiVax, http://www.epivax.com; Genencor, http://www.genencor.com; Nektar, http://www.nektar.com; Novozymes, http://www.novozymes.com; Xencor, http://www.xencor.com
[b]A computational method that models the structure of peptide–MHC complexes and calculates binding affinities using a force field.
[c]A computational method that assigns scores to peptide–MHC interactions based on experimental peptide binding data. The matrix comprises scores for each amino acid, at each 'pocket' in the MHC peptide-binding site, for each MHC allele considered.
Abbreviations: MHC, major histocompatibility complex; PEG, polyethylene glycol; PDA®, Protein Design Automation®.

molecules. An alternate approach is to use rational solubility engineering, discussed in a recent review [47], to identify mutations that will minimize aggregation. As with any mutagenesis-based strategy for immunogenicity reduction, it is crucial to confirm, using the computational and experimental approaches described here, that any modifications introduced to improve solubility do not increase immunogenicity by other mechanisms.

Removing antibody epitopes

Identification of antibody epitopes has typically been arduous, although recent developments in mass spectrometry now enable more rapid detection [48]. Antibody epitopes are often located at a small number of discrete sites on the surface of a protein and antibody-binding affinity can be dominated by interactions with only a handful of residues. Modifications of the crucial residues in an antibody epitope can therefore reduce binding by existing antibodies. For example, modified variants of Factor VIII have been produced that are not neutralized by anti-Factor VIII antibodies [49,50]. Similarly, a significant reduction in the antigenicity of staphylokinase was achieved by site-directed mutagenesis of antibody epitopes: neutralizing antibodies were observed following a single treatment in 81% of patients administered wild-type staphylokinase but in only 47% of those treated with the best variant [51,52]. The reduction in antigenicity was linked to replacement of hydrophobic and charged residues with polar neutral residues. For therapeutics such as staphylokinase that are not intended for protracted use, B-cell epitope removal via residue replacement could be beneficial. However, repeated treatment with such a variant protein will likely elicit a new antibody response against a different set of epitopes, rather than sustained immunogenicity reduction.

PEGylation

An alternate approach for immunogenicity reduction is to sterically block antibody binding by derivatizing the protein with polyethylene glycol (PEGylation). PEGylation can also decrease immunogenicity by promoting solubility and permitting less frequent dosing [53]. PEGylation has been used successfully to minimize the immunogenicity of therapeutic enzymes such as arginase, asparaginase and purine nucleoside phosphorylase, because PEGylation blocks antibody binding but does not prevent the diffusion of small molecule substrates [54,55]. However, for other classes of protein therapeutics, there is often a direct trade-off between blocking antibody-binding sites and leaving

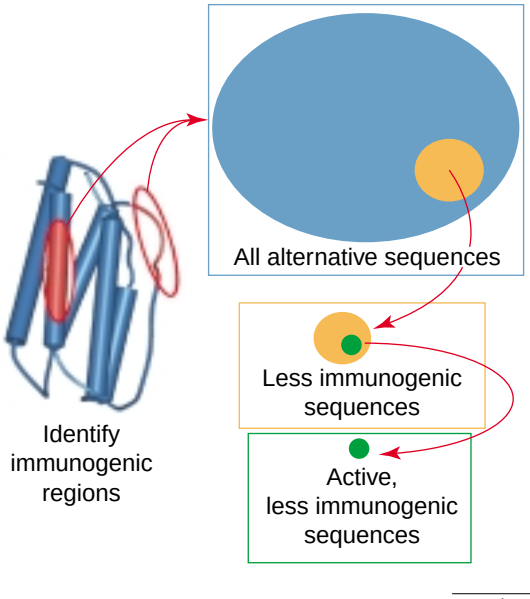

Figure 4. Once the regions of a protein that confer immunogenicity are identified, sequence modifications can be introduced to reduce immunogenicity while retaining activity. A key challenge is that, although a large number of alternate sequences (blue oval) could be tested, only a small fraction (orange oval) will be less immunogenic. Furthermore, only a small fraction of these less immunogenic sequences will be stable and active (green circle). Experimental assays for immunogenicity are at most medium-throughput; therefore, computational methods that pre-screen possible variants to identify those that are most likely to be active and less immunogenic will greatly aid in future efforts to discover effective, safer protein therapeutics.

receptor binding sites unoccluded. For example, although PEGylation of interferon-α_{2a} decreased immunogenicity and increased serum half-life, it also decreased *in vitro* antiviral activity by 93% [56]. In other cases, serious immunogenicity issues arise despite PEGylation; for instance, the MGDF molecule that elicited cross-reactive neutralizing antibodies was N-terminally PEGylated. In the future, rational protein design approaches could be used to select the PEG attachment sites that provide the best balance between improving pharmacokinetics, reducing immunogenicity and maintaining activity [47].

Identifying and removing class II MHC agretopes

The production of the high-affinity IgG antibodies responsible for clinically relevant immunogenicity [5,9] can be evaded by identifying and removing class II MHC agretopes from the biotherapeutic. The factors that determine peptide binding affinity and specificity have been thoroughly characterized using biochemical and structural methods. Antigen-derived peptides bind in an extended conformation along a groove in the class II MHC molecule. Although the peptides are typically 13–18 residues long, a 9-residue region is most critical for binding [16]. Removal of MHC agretopes offers a more tractable approach to immunogenicity reduction over antibody epitope removal because the factors affecting binding are better defined, the diversity of binding sites is much smaller, and MHC molecules and their binding specificities are static throughout an individual's lifetime.

Several computational methods can be used to predict MHC agretopes; these have been reviewed recently [1]. Similarly, several qualitative and quantitative experimental methods for analysis of peptide-MHC class II interactions have been reviewed elsewhere [37,57]. It is worth noting that methods for detecting MHC-binding agretopes were initially developed to identify cytotoxic T-lymphocyte (CD8+) and T-helper (CD4+) epitopes for vaccine development [58], because peptides that promiscuously bind MHC molecules make effective vaccines. Minimizing immunogenicity, however, requires removing all of the agretopes that are recognized by prevalent class II alleles, including DP, DQ and DR alleles, a much more difficult task. Most current computational and experimental methods consider binding to a limited subset of alleles. In the future, allele coverage can be efficiently maximized by judiciously selecting donors expressing prevalent MHC alleles and by experimentally characterizing the peptide binding propensities of additional MHC alleles; such work is currently in progress at Xencor (http://www.xencor.com).

Following computational or experimental identification of MHC agretopes, mutagenesis approaches can be used to produce variant sequences that do not interact with MHC. Typically, alanine scanning or random mutagenesis has been used [59]. However, assays to experimentally confirm immunogenicity reduction are relatively low-throughput, limiting the number of variants that can be screened. An alternate approach is to identify active, less immunogenic sequences using rational protein design methods (Figure 4). Computational protein design approaches, such as Xencor's Protein Design Automation® (PDA®) technology, have a demonstrated record of success in engineering stable, active proteins with desired biophysical and clinical properties [60–62]. We have incorporated computational methods for predicting MHC agretopes, as well as solubility engineering, rational PEGylation and B-cell epitope removal approaches, into the computational component of PDA® technology, enabling the discovery of stable, functional and less immunogenic protein therapeutics. *Ex vivo* T-cell activation assays and other preclinical immunogenicity studies are then used to confirm immunogenicity reduction in a

tractable number of variants; we refer to this combination of computational and experimental immunogenicity reduction platforms as ImmunoPDA™ technology.

Conclusions

The immunogenicity of protein therapeutics presents a challenge to the biotherapeutics industry. Several mechanisms play a role in eliciting an immune response; therefore, multiple strategies have been developed to reduce interactions between the therapeutic and immune effector molecules. A key requirement is to minimize immunogenicity while maintaining desired structural and functional properties. The combination of rational protein design technologies, recently reviewed in *Drug Discovery Today* [47], and the immunogenicity reduction strategies described here, is uniquely suited to aid in the development of active, less immunogenic protein therapeutics. *Ex vivo* T-cell activation assays are a promising medium-throughput screen for confirming immunogenicity reduction and predicting immune responses in humans. Specialized animal models including genetically engineered mice and MHC-defined primates closely mimic critical aspects of the human immune response, such as tolerance and T-cell repertoire, and might therefore justify their high costs of development. Continuing progress in the prediction, detection and prevention of harmful immune responses brings the promise of safer and more effective biotherapeutics for the future.

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