

Review

Evaluating and Mitigating the Immunogenicity of Therapeutic Proteins

Zuben E. Sauna,^{1,*} Daniel Lagassé,¹ Joao Pedras-Vasconcelos,² Basil Golding,³ and Amy S. Rosenberg²

Therapeutic proteins provide interventions for some of the most complex and intractable diseases and are an essential part of modern medicine. Immunogenicity is the development of immune responses, usually measured by antibodies, to therapeutic proteins. These responses can adversely affect the safety and efficacy of the therapeutic agent and may have the following consequences: neutralization of a life-saving biotherapeutic, crossreactivity to non-redundant endogenous proteins, and hypersensitivity responses. These concerns have been underscored by the discontinuation of development of several drugs in recent years owing to immunogenicity issues. We review here recent progress in technological approaches that are useful for the clinical and non-clinical risk assessment of immunogenicity as well as mitigation strategies including deimmunizing protein molecules and inducing immune tolerance to the therapeutic protein.

Therapeutic Proteins and Immunogenicity

Once representing a scientific frontier, therapeutic protein products are now part of routine medical practice. These drugs treat diverse diseases, make up >20% of the pharma market, and have a growth rate double that of small-molecule drugs [1]. Therapeutic proteins have succeeded in clinical practice and the marketplace despite key technological challenges. A small molecule, for example ibuprofen, is made up of 33 atoms, while a therapeutic protein such as factor VIII (FVIII) consists of ~37 000 atoms, not including glycoforms. Reliably and reproducibly producing these large molecules, with their complex structures, requires sophisticated manufacturing facilities, large capital outlays, and recombinant engineering. Nonetheless, therapeutic proteins perform remarkably well and, in general, provide a high degree of targeted efficacy with limited side effects. Consequently, therapeutic proteins have provided medical interventions which were either not possible with small molecules or have resulted in clinical outcomes that are considerably superior.

In addition to the operational and manufacturing issues, the clinical use of therapeutic proteins also presents unique challenges. **Immunogenicity** (see [Glossary](#)) is one such issue that is of considerable concern during drug development, licensure, and in the post-licensure phase. While an immune response to a vaccine is the desired clinical outcome, immune responses to therapeutic proteins are undesirable. When **anti-drug antibodies** (ADAs) develop, these may or may not affect the activity of the drug depending on numerous factors including titer, duration, and neutralizing activity. Those that affect drug activity by binding to active protein domains are called **neutralizing ADAs** (NABs). Non-neutralizing antibodies (called binding antibodies) are not necessarily benign because they can result in more rapid clearance of the drug, affecting the pharmacokinetic/pharmacodynamic (PK/PD; i.e., activity) profile and

Highlights

Immunogenicity compromises the safety and/or efficacy of therapeutic proteins, is a priority for regulatory agencies, and adds to the risks and costs associated with drug development.

In the clinical setting, the risks and consequences of anti-drug antibodies translate into complex decision-making, and reliable immunogenicity testing strategies are therefore needed to inform clinical decisions.

A multitude of non-clinical approaches (computational, *in vitro*, *ex vivo*, and animal models) are currently used to assess immunogenicity risk.

Newly emerging technologies are expected to contribute to the rapid advancement of non-clinical assessments and have the potential to reduce the burden of immunogenicity.

Novel strategies applied to protein therapeutic deimmunization or immune tolerance induction have the potential to reduce the incidence of immunogenicity and aid in better clinical management.

¹Hemostasis Branch, Division of Plasma Protein Therapeutics, Office of Tissues and Advanced Therapeutics, Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, MD 20993, USA

²Laboratory of Immunology, Division of Biotechnology Product Review and Research 3, Office of Biotechnology Products, Center for Drugs Evaluation and Research, US Food and Drug Administration, Silver Spring, MD 20993, USA

³Plasma Derivatives Branch, Division

causing loss of tissue targeting. In addition, ADAs can crossreact with endogenous proteins or elicit **anaphylactic reactions** which can be life-threatening. Immune responses to drugs can result in a drug not being approved [2], withdrawn, or discontinued from further development owing to adverse events and their impact on clinical outcomes [3–5].

In this review we address immunogenicity only in the context of therapeutic proteins. There are several detailed white papers [6–10], guidance documents [11–13], and research articles [14–16] that detail the development and use of assays for detecting ADAs and NABs in clinical samples which will not be reviewed here. This review focuses on recently developed (or evaluated) non-clinical methods to predict immunogenicity as well as advances in deimmunizing **epitopes** for both **T cells** and **B cells** and in modulating immune responses to mitigate the risks and effects of immunogenicity.

Evaluating Immunogenicity

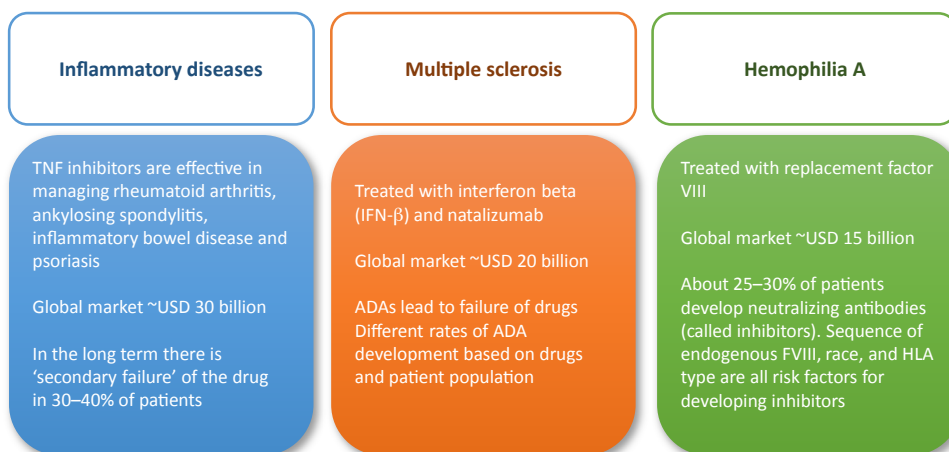
Immunogenicity in the Clinic

In real-world experience, immunogenicity risks and consequences translate into complex decision making based on numerous factors. The influence of immunogenicity can be illustrated by the effects of immunogenicity on tumor necrosis factor (TNF) inhibitors and on therapeutic proteins used to treat multiple sclerosis (MS) and hemophilia (HA) (Figure 1). Together, these therapies have the potential to affect the lives of almost 30 million people.

TNF inhibitors can be extremely effective in treating inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, and psoriasis. However, over time (from 6 months to 4 years), only 60–70% of patients continue to respond to these medications [17,18]. It is recognized that ADAs to monoclonal antibody (mAb) TNF inhibitors can bind to the idiotope and thus cause loss of efficacy [19]. While clinical laboratory test-guided strategies [20] have been proposed to reduce the costs of therapeutic non-responsiveness and provide an evidence-based and personalized approach to minimize treatment delays, the use of immunogenicity testing to guide therapy is controversial [17] despite the fact that real-world evidence suggests that immunogenicity limits efficacy in a substantive fraction of patients. Because an

of Plasma Protein Therapeutics, Office of Tissues and Advanced Therapeutics, Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, MD 20993, USA

*Correspondence:
zuben.sauna@fda.hhs.gov (Z.E. Sauna).



Trends in Biotechnology

Figure 1. Examples of Real-World Consequences of Immunogenicity. For each therapeutic area, the most common treatment options, the size of the global market, and the impact of immunogenicity on therapy are highlighted. Abbreviation: ADA, anti-drug antibody.

evidence-based consensus regarding how immunogenicity assays may be used to inform clinical decisions [21] is lacking, prospective randomized studies to establish testing parameters to tailor therapy are warranted.

In contrast to anti-TNF agents, the treatment of MS using interferon β (IFN- β) and natalizumab include well-established norms for ADA testing (at least in most of Western Europe) [22–24]. However, there are key issues that have not been fully resolved for assaying ADAs and NABs to such agents, including assays which differ in sensitivity and consistency disparities between laboratories [25]. Therefore, efforts are underway to harmonize data and compare assay results across different clinical laboratories for binding and neutralizing ADAs against IFN- β and natalizumab as well as to develop novel assays [25,26]. While appealing as a concept, the feasibility of operationalizing standardized ADA assays is fraught with challenges including the following: requiring the agreement of multiple stakeholders (i.e., drug manufacturers, clinical laboratories, and physicians); the appropriate clinical context for usage of ADA testing strategies included in post-marketing commitments and the availability of such assays for physician referral; regulations pertaining to *in vitro* diagnostic testing by the Clinical Laboratory Improvement Amendments (CLIA) and the availability of National Institute for Biological Standards and Control (NIBSC)/World Health Organization (WHO) endorsed 'Reference Standards' for ADAs; and, most crucially, issues of insurance coverage.

Immune responses to therapeutic proteins that have endogenous counterparts present additional safety concerns. An example is the development of pure red cell aplasia in patients treated with erythropoietin that had undergone manufacturing, container-closure, and administration changes, and which induced NABs to the therapeutic that cross-reacted to endogenous erythropoietin [3]. Another example is the treatment of hemophilia A patients with replacement FVIII. Even though the frequency of developing ADAs is relatively high (25–30%), these drugs are marketed because the consequences of immunogenicity are clinically manageable. On the other hand, bypass therapy with factor VIIIa (FVIIIa) is used for FVIII- or factor IX (FIX)-deficient patients who develop NABs. Because these patients are not deficient in factor VII (FVII), crossreactive antibodies to endogenous FVII could cause a life-threatening bleeding state, such that any incidence of ADAs is considered to be too high a risk. Similarly, bovine thrombin products purified from bovine plasma have bovine factor V (FV) as a contaminant which was shown to induce crossreactive ADAs to human FV (1 in several million treatments) [27]. However, such crossreactivity is clinically a potentially life-threatening adverse event. Consequently, the product label for bovine thrombin carries a Boxed Warning [28] and human thrombin products have been developed [29].

Individual Methods Used To Assess Immunogenic Peptides in Therapeutic Proteins

The multitude of preclinical approaches (computational, *in vitro*, *ex vivo*, and animal models) currently in use to assess the immunogenicity risk of therapeutic proteins have been reviewed extensively [30–32]. These methods (Table 1) for the most part rely on the premise that the immune responses of concern elicited against therapeutic proteins are T cell-dependent. The rationale for studying the T cell response is provided below.

A substantial body of work has established that T helper cells (Th cells: **CD4⁺** T cells) responding to protein epitopes are crucial for the development of significant ADA responses. These epitopes are presented to Th cells by **human leukocyte antigen** (HLA) molecules (major histocompatibility complex class II, MHC-II) on the surface of **antigen-presenting cells** (APCs). Therefore, it is essential to identify potential HLA-restricted immunogenic epitopes

Glossary

Affinity maturation: a process that increases antibody affinity that occurs during adaptive, thymus-dependent immune responses.

Anaphylactic reaction: a rapid-onset allergic reaction which is an immediate response to antigenic challenge, leading to the release of inflammatory mediators. Anaphylaxis can result in sharp drops in blood pressure, difficulty in breathing, and death if left untreated.

Antigen: a foreign substance that is recognized by the immune system and binds to T and B cell receptors.

Antigen-presenting cell (APC): any cell that can process and present antigen on MHC class II molecules. The deliver costimulatory signals necessary for T cell activation. Professional APCs include macrophages, DCs, and B cells.

Anti-drug antibodies (ADAs): any antibody that binds to a protein that is used as a therapeutic (drug).

B cell: a lymphocyte that expresses B cell receptor. These differentiate into memory B cells or antibody-secreting plasma cells after encounter with antigen.

CD4: a receptor found on helper T cells and monocytes. The CD4 receptor on helper T cells binds to HLA class II, and the interaction causes CD4 helper T cell activation.

Cytokine: a group of secreted, low-molecular weight molecules that regulate immune responses. The actions of cytokines are mediated through specific cytokine receptors on the surfaces of target cells.

Dendritic cells (DCs): professional APCs; DCs express MHC class II molecules and are efficient presenters of antigen to T cells.

Epitope: the part of an antigen that is recognized by an antibody or T cell receptor.

Human leukocyte antigen (HLA): HLA class I molecules comprise cell-surface receptors, composed of an α chain and a non-covalently associated protein called β 2-microglobulin, that are expressed on all nucleated cells in the body and are used to identify non-self protein antigens derived from the cell cytoplasm. The presence of the foreign peptide antigen binding to the HLA class I molecule activates CD8⁺ CTLs (cytotoxic T lymphocytes)

Table 1. Methods Used To Assess Immunogenic Peptides in Therapeutic Proteins

Class	Method	Immune process probed	Rationale	Refs
Computational/ <i>in silico</i>	HLA-binding algorithms	Antigen presentation	Identifies potential T helper cell epitopes (algorithms are designed around experimental observations and machine learning of HLA DRB1 binding)	[95,96]
<i>In vitro</i>	Peptide/HLA-binding assay	Antigen presentation	Measures potential T helper cell epitope binding affinity (can assess the impact of variables such as non-natural amino acids and post-translational modifications)	[97,98]
<i>Ex vivo</i>	LC/MS ^a -based MHC-associated peptide proteomics (MAPPs) assay	Antigen processing and presentation	Identifies naturally processed and presented peptide antigens (T helper cell epitopes); can identify dominant T helper cell epitopes (helps to address the overprediction of <i>in silico</i> algorithms because many fewer peptides are processed and presented than are theoretically possible)	[99,100]
	MHC-II tetramer-guided epitope mapping (TGEM)	Antigen recognition	Mapping of HLA-restricted T cell epitopes	[101,102]
	Protein-specific T cell amplification	Antigen processing, presentation, and recognition	Generation of antigen-specific T cell lines from naïve PBMC ^b donors	[103,104]
	Human blood-derived cell-based assays (PBMCs/DCs as APCs; T cells as effector cells)	Depends on assay design	T cell activation measured by proliferation (thymidine/BrdU ^c incorporation; CFSE ^d dilution) or cytokine production (ELISPOT) Most beneficial when powered with diverse donor pool and HLA genotypes representative of the world population Can measure immunogenicity risk due to foreign epitope content and immune activation danger signals (due to product attributes)	[105–107]
Animal model/ <i>in vivo</i>	HLA transgenic mice (humanized immune system)	All	Assessment of risk for ADA development in context of human HLA	[108–110]

^aLC/MS, liquid chromatography combined with mass spectrometry.

^bPBMCs, peripheral blood mononuclear cells.

^cBrdU, bromodeoxyuridine.

^dCFSE, carboxyfluorescein succinimidyl ester.

because these constitute a necessary condition for eliciting an immune response to therapeutic proteins (see Box 1 for examples).

The methods currently in use for predicting immunogenicity risk (Table 1) thus rely on measurement of one or more events that eventually lead to the proliferation of **antigen**-specific T cells [32]. However, these T cell assays do not directly correlate with clinical outcomes. ADA responses are ultimately the product of (i) the generation of the relevant peptide–HLA complex, (ii) antigen presentation to and activation of cognate T cells, and (iii) presentation and interaction

specific for that antigen. Receptors are encoded by the major histocompatibility complex (MHC) genes. HLA class II molecules comprise cell-surface receptors composed of an α and a β chain that are expressed specialized cell types, including DCs, macrophages, and B cells (all of which are professional APCs), and are encoded by the MHC genes located on chromosome 6. There are three sets of genes that encode functional receptors: HLA-DP, –DQ, and –DR. APCs process soluble antigens such as toxins or other proteins made by bacteria (for example) and then display them on their surface as peptide antigens in combination with HLA class II molecules. CD4⁺ helper T cells specific for these antigens are then able to be activated and respond to the presence of the presented peptide derived from the invading bacteria or microbe.

Immunogenicity: in the context of protein therapeutics, refers to an immune response to the drug.

Neutralizing ADAs (NABs): antibodies against protein therapeutics that neutralize the functional activity of the drug.

Peptidome: the complete set of peptides encoded by a particular genome, or that is present within a particular cell type or organism.

Plasma cell: antibody-secreting cell derived from B cells.

T cell: a lymphocyte that matures in the thymus and expresses a T cell receptor.

T regulatory cells (Tregs): a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease.

Box 1. Examples of Immunogenic Peptide Assessments

Multiple studies have identified FVIII HLA-restricted peptide epitopes (typically a core of 6–18 distinct epitopes per individual) that predominantly span the A2, A3, and C2 domains of FVIII [111–113]. Similarly, T cell epitopes in the variable regions of chimeric therapeutic mAbs (rituximab, anti-CD20; and infliximab, anti-TNF- α) have been identified using the reactivity to these mAb peptides of peripheral blood mononuclear cells (PBMCs) isolated from healthy donors [114]. The Th peptide epitopes identified using healthy donors were subsequently used to stimulate cytokine production by T cells isolated from ADA-positive patients [114]. Such T cell assays have been used to identify immunogenic T cell epitopes, while calculating their overall abundance has allowed ranking of therapeutic antibodies by immunogenicity risk, which is consistent with rates of clinical ADA [115]. Studies such as these are almost always retrospective, and thus unfortunately do not demonstrate the utility of the non-clinical methods as predictive tools. With respect to mAbs, immunogenicity in the complementarity-determining regions (CDRs) of the variable regions has been shown to be related to the degree of humanization [116].

Obviously, the identification of Th epitopes is not a sufficient predictive biomarker of immunogenicity because the relevant peptide–HLA complexes first must be generated via antigen processing and presentation as well as downstream recognition by antigen-specific cognate CD4⁺ T cells. As such, antigen-specific T cell clones isolated from mild HA patients with missense F8 mutations (R2150H, A2201P, R593C) with NABs have been identified [117]. Indirect evidence that T helper cells are key players in the development of FVIII inhibitors was provided by the observation that HA patients in the advanced stages of HIV1 infection (absolute CD4⁺ counts: <200/mm³) as well as mild to moderate CD4⁺ T cell depletion due to HIV1 infection (absolute CD4⁺ counts: 262–935/mm³) were not able to develop anamnestic responses upon FVIII rechallenge, despite a history of high-responder NABs [61]. Moreover, HIV1 seropositivity has also been associated with lower rates (odds ratio, 0.32) of developing new FVIII inhibitors [118].

of antigen-specific B cells with T helper cells. This leads to driving naive B cells to the generation of antibody-secreting **plasma cells** as well as memory B cells. In ADA-positive patients, these assays reflect the recall/memory of T cell responses.

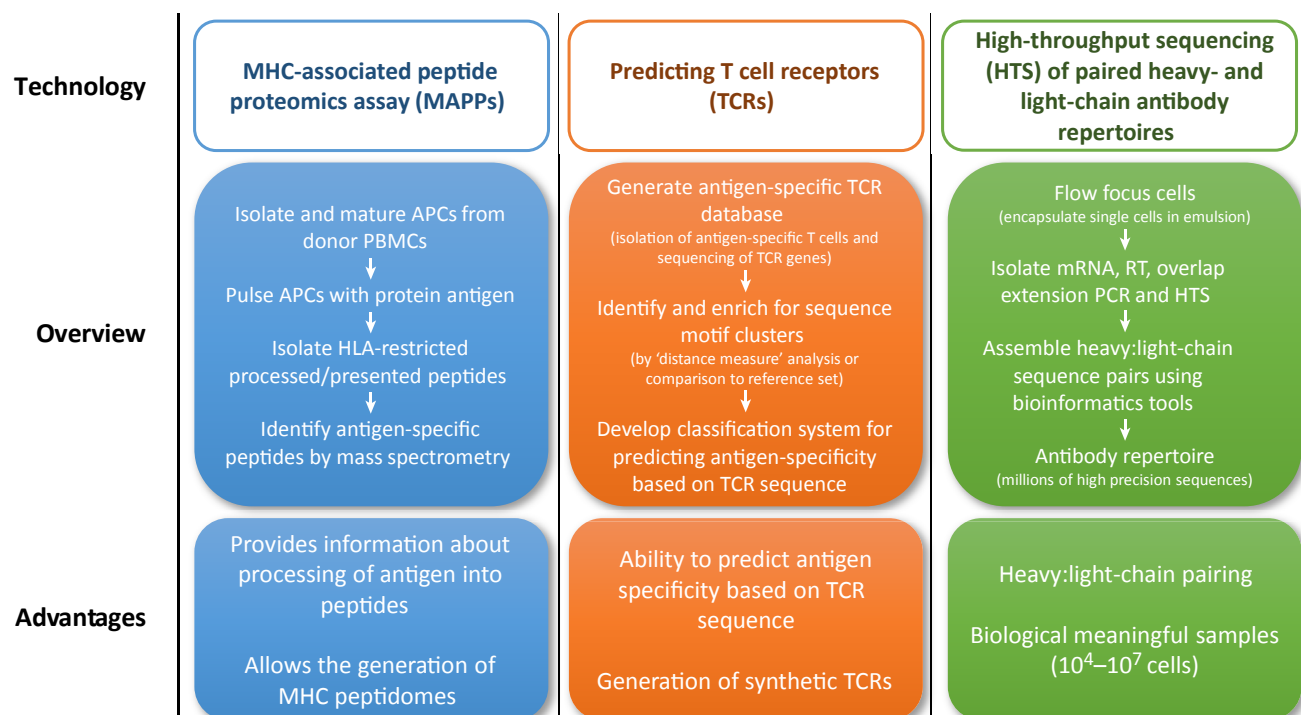
Emerging Technologies for the Non-Clinical Assessments of Immunogenicity

The unprecedented advances in analytical methods, computational power, and high-throughput sequencing have impacted on almost all biological disciplines. Applications of these advances to immunology are given below (Figure 2).

Mass Spectrometric Profiling of the HLA-Associated Peptidome

Antigen processing and presentation are key to eliciting specific T cell responses. Therefore, a variety of APC-based non-clinical immunogenicity assessments have been employed to evaluate **dendritic cell** (DC) binding, activation (via surface markers or **cytokine** production), as well as protein internalization and intracellular (endosomal) trafficking of therapeutic proteins [33,34]. Many of the traditional methods for identifying T cell epitopes (Table 1) focus on measuring the response to individual peptides, or pools of peptides. These approaches, while useful, have several disadvantages [35]. Most importantly, they provide little or no information about cellular processing of protein into peptides, or about which peptides are presented in the context of HLA molecules. Moreover, even if the intact protein is used in the assay it is not possible to determine which peptides elicited the T cell response. The use of individual overlapping peptides that span the entire protein to identify specific T cell epitopes is expensive, tedious, time-consuming, and provides information about peptide presentation but not about antigen processing (i.e., under physiological conditions it is not known whether the peptide of interest is generated). Moreover, the assays have considerable variability and are not suitable for large-scale proteomic surveys of antigens.

First developed in the early 1990s, mass spectrometry-based strategies to identify peptides bound to MHC-II proteins have been used to understand the molecular and cellular mechanisms underlying protein processing and peptide presentation (reviewed in [36,37]). This technology has subsequently been used to understand processing of autoantigens in autoimmune diseases [38].



Trends in Biotechnology

Figure 2. Emerging Technologies for the Non-Clinical Assessments of Immunogenicity. For each emerging technology – MAPPs, predicting TCRs, and HTS of paired heavy- and light-chain antibody repertoires – a general overview including flow of major steps and the advantages are described. Abbreviations: APC, antigen-presenting cell; PBMCs, peripheral blood mononuclear cells; RT, reverse transcription; TCR, T cell receptor.

In the context of immune responses to therapeutic proteins, the MHC-associated peptide proteomics (MAPPs) assay has been used to characterize a neo-sequence in an engineered protein as a neo-epitope in some patients [39]. In this study, an analog of FVIIa with three amino acid substitutions induced ADAs in 11 % of the study population, terminating drug development [2,39]. A *post hoc* analysis showed that: (i) mutant peptides bound with high affinity to at least one of the HLA-DRB1 variants found in patients, which could be considered to be high risk; (ii) the mutant peptides could elicit T cell proliferation when APCs carried one or more high-risk HLA-DRB1 variants; and (iii) when DCs from donors with high-risk HLA-DRB1 variants were incubated with full-length mutant protein, the mutant peptides were identified in the MAPPs assay. In this comprehensive characterization, the MAPPs assay was the only analytical tool that could demonstrate that the foreign antigen (i.e., peptides with the neo-sequence) was both processed and presented by the immune system.

The patient-related genetic sequence of endogenous FVIII as a risk factor for immunogenicity has also been the subject of considerable interest. Similarly, the protection of T cell epitopes by von Willebrand factor (vWF) [40] and differences in the cellular processing of plasma-derived and recombinant FVIII have also been the subject of numerous hypotheses [40,41]. These hypotheses are being tested using MAPPs assays, which permit the comparison of peptide repertoires when cells are treated with the different products and/or combinations of proteins [42–44].

The approaches described above are technically demanding and very expensive. Currently the technology does better at addressing relatively straightforward questions related to which

protein-derived peptides are detected in a specific donor or patient. Of great importance in advancing more precise assessments, new high-throughput workflows have facilitated the reproducible and often quantitative identification of MHC-II-peptide complexes [45]. These methods allow the generation of MHC **peptidomes** [46] and the initiation of studies to elucidate how such peptidomes change in response to clinical interventions.

Predicting Antigen Specificity Based on T Cell Receptor (TCR) Sequence

During T cell development, genes encoding the variable regions of the TCR undergo rearrangement, resulting in a unique TCR for each T cell clone. The number of total possible TCRs has been estimated at 10^{18} . Consequently, determining the antigen-specificity of any TCR based solely on its amino acid sequence was considered almost impossible until 2017 when two studies reported methods to address this issue. Both involved the use of fluorescently labeled complexes of antigens (T cell epitope plus MHC) to isolate antigen-specific T cells. A large database of antigen-specific TCR sequences was then generated using high-throughput, single-cell DNA sequencing of the TCR genes, following which computational strategies were employed to identify the sequence motifs that correlated with antigen specificity [47,48].

One approach [47] was to exploit a 'distance measure' to compute the similarity of any two TCR sequences and assign highly similar sequences to clusters. The TCR repertoires selected for binding to a specific antigen were enriched for clusters with specific sequence motifs. No such enrichment could be found in unselected TCR repertoires. These enriched sequence motifs were used to develop a classification system for predicting antigen-specificity based on TCR sequence and achieved a success rate of ~80% in correctly identifying the antigen-binding specificity for 10 different human and mouse TCRs [47].

An alternative approach [48] analyzed TCRs from humans that bound to specific antigens, again leading to the identification of the relevant TCR motifs. The enriched sequence motifs specific for a *Mycobacterium tuberculosis* antigen were used to design a set of 10 synthetic TCRs not found in the biological samples. This approach proved successful because eight of the 10 synthetic TCRs specifically bound the antigen [48].

There is potential for the complementarity of datasets generated by the aforementioned technologies (MAPPs assays and predicting TCR antigen-specificity) because the prediction of TCR antigen-specificity for therapeutic proteins will likely rely upon potential T cell epitopes identified by the MAPPs assay.

High-Throughput Sequencing (HTS) of Paired Heavy- and Light-Chain Antibody Repertoires

There are relatively few methods to obtain detailed information about the antibody repertoire associated with an immune response to therapeutic proteins. In most instances it is not possible to develop a protein drug that is non-immunogenic in all patients. Consequently, the management of immunogenicity is part of the clinical care of patients and could benefit from a more detailed understanding and/or characterization of the antibody repertoire.

High-throughput sequencing of the variable domains of antibodies can provide a more granular understanding of adaptive immune responses at the molecular level, and methodologies to perform high-throughput next-generation sequencing of antibody repertoires have existed for some time [49–51]. However, a key challenge has been the sequencing of both the heavy and light chains at the single-cell level in large and biologically meaningful samples (i.e., 10^4 – 10^7 lymphocytes). Obtaining sequence information for correctly paired heavy and light chains is difficult because these are encoded by separate mRNAs. This problem has recently been

surmounted by using an experimental process called flow focusing. Flow focusing involves encapsulation of single cells in an emulsion containing magnetic beads for mRNA capture. The mRNA transcripts are then reverse-transcribed and physically linked to their partners by overlap extension PCR. Subsequent HTS and bioinformatics permit obtaining sequences of the paired heavy and light chains of the antibody repertoire (millions of sequences) with high precision (>97% has been reported [52,53]).

In the past decade a familiar trajectory with the application of HTS methods has been that, following the initial proof-of-principle demonstration, there have been dramatic decreases in cost, increases in efficiency, and widespread adoption of the technology, resulting in large datasets. Thus, large datasets of antibody repertoires of patients treated with different therapeutic proteins may soon be available for statistical analyses and data mining. Moreover, the technology also permits the tracking of the immune response over time, providing unprecedented insight into millions of B cell clones as they undergo somatic hypermutation and **affinity maturation** to improve antigen binding. Again, the feasibility of operationalizing such clinical studies may prove difficult because there is currently sparse sampling and a lack of engagement by clinical teams to implement such approaches, as well as cost considerations. Moreover, such efforts would require engagement with statisticians for data analysis and the development of mechanistic models. Nonetheless, these data promise a better molecular understanding of the dynamics of the humoral immune response to therapeutic proteins.

Immunogenicity-Mitigating Approaches

The Deimmunization of Therapeutic Proteins

The earliest deimmunization efforts to ‘humanize’ murine mAbs did not specifically alter individual T cell epitopes but focused on removing non-self amino acid sequences to avoid eliciting unwanted immune responses. In recent years, mAb deimmunization strategies have become more tailored toward potentially immunogenic regions of the primary amino acid sequence, and include antibody optimization via reducing non-germline amino acid sequences. Such efforts have resulted in minimizing predicted human T cell epitope content [54]. Some deimmunization strategies applied to other protein classes have been reported in recent years and are described below.

Immunotoxins

A novel class of genetically engineered proteins known as recombinant immunotoxins (RITs) have shown promise in treating cancers. These fusion proteins rely upon specific immune targeting of toxins that are enzymatic inhibitors of protein synthesis, such as *Diphtheria* toxin, *Pseudomonas* exotoxin A, and ricin toxin [55,56]. In these cases, the protein toxin fusion partner is typically of bacterial or plant origin, and therefore elicits NABs, making immunogenicity a major hurdle to recurrent treatment, particularly in immunocompetent patients.

Pastan and colleagues have implemented a deimmunization strategy that, for the first time, incorporates the elimination of both B cell and T cell epitopes from an immunogenic protein (*Pseudomonas* exotoxin A, PE38) [57] (Box 2). However, these PE38 deimmunization approaches have not eliminated the immunogenicity because subdominant epitopes remained. Therefore, immune tolerance approaches (see below) have been included to mitigate PE38 immunogenicity [58,59].

Currently, the only FDA-approved immunotoxin, denileukin diftitox (Ontak[®]), was approved in 1999 and indicated for the treatment of cutaneous T cell lymphomas which express the CD25 component of the IL-2 receptor [60]. This drug thus targets and kills IL-2R α (CD25) activated T

Box 2. Examples of Protein Deimmunization

Immunotoxins

The Pastan group removed the B cell [119] and T cell epitopes [120] on the assumption that both are necessary to reliably silence immunogenic regions within a bacterial protein. However, as with all mutational approaches, this strategy has the potential to unintentionally introduce neo-epitopes which must be evaluated. This group has also incorporated protein folding and structural considerations into their approach by implementing the Rosetta protein design algorithm package to assess changes to free energy ($\Delta\Delta G_0$), that could indicate an effect on stability, that may result from the deimmunization of T and B cell epitopes [121].

Factor VIII

A study by Moise and colleagues focused on previously identified T cell epitopes in FVIII [63]. Several proprietary software packages were used to design *in silico* strategies to first identify T cell epitope clusters and mutants to disrupt the binding of these epitopes to MHC-II molecules. The EpiMatrix and ClustiMer algorithms were used to identify promiscuous epitopes that bound to eight MHC-II variants that occur with high frequency in the human population. Another proprietary algorithm, OptiMatrix, was then used to identify and evaluate the contribution of the individual FVIII amino acid residues in the T cell epitope clusters. Based on these analyses, amino acid substitutions most likely to decrease peptide–MHC-II affinity while preserving protein structure and function were identified by (i) minimizing the number of substitutions, and (ii) designing alternative mutants for each peptide, which could then be evaluated in functional assays.

Enterobacter cloacae P99 β -Lactamase Used in Antibody-Directed Enzyme Prodrug Therapy (ADEPT)

ADEPT is a good model system in need of deimmunization [122]. A typical ADEPT therapy involves the following: (i) a patient is treated with an antibody–enzyme fusion protein to localize the drug to cancer cells, (ii) a relatively non-toxic prodrug is then administered systemically, and (iii) the enzyme targeted to cancer cells converts the prodrug to a toxic agent (only at the targeted site). This specificity is achieved by using enzymes of non-human origin. Bacterial enzymes have activities orthogonal to the human proteome, and thus there is minimal conversion of the prodrug by the endogenous (human) enzymes of the patient. Unfortunately, the bacterial enzymes are highly immunogenic, and this has limited the success of this approach [123,124]. The DP2 algorithm was applied to *Enterobacter cloacae* P99 β -lactamase (P99 β L) [66]. This deimmunization approach provided several sequences that were predicted to have limited engagement with MHC-II proteins, and which were also likely to be stable and exhibit enzymatic activity.

lymphocytes as well as **T regulatory cells** (Tregs) that are known to express the target moiety. Thus, the immunogenicity of the fusion protein is not independent of the mechanism of action of the drug in eliminating T cells with antithetical activities on immune responses.

Factor VIII

FVIII has been used as a replacement therapy for HA patients for several decades, resulting in considerable clinical experience. The development of NABs to FVIII has been recognized as an important obstacle to the management of these patients: ~30% of previously untreated HA patients and 50% of patients with severe disease (who have major deletions in the *F8* gene) develop NABs. Thus, this protein is an ideal candidate as a model system for developing deimmunization strategies. Animal studies and clinical evidence both indicate that the development of NABs to FVIII is a T cell-dependent process [61,62]. A study by Moise and colleagues focused on deimmunizing previously identified FVIII T cell epitopes [63] (Box 2). The mutant ‘deimmunized’ peptide sequences did show reduced binding to the MHC-II variants used in the algorithms. Furthermore, animal studies were performed in two models: (i) a FVIII knockout mouse that expresses no C2 domain at any point in development and normally expresses mouse MHC-II, and (ii) HLA-DR transgenic models, which express whole FVIII protein throughout development, a single human HLA-DR allele, and no mouse MHC-II. Although the results of this study are promising, the design and development of a fully functional FVIII molecule that shows reduced immunogenicity to a more diverse set of HLA/MHC-II variants remains a challenge.

Enterobacter Cloacae P99 β -Lactamase Used in Antibody-Directed Enzyme Prodrug Therapies

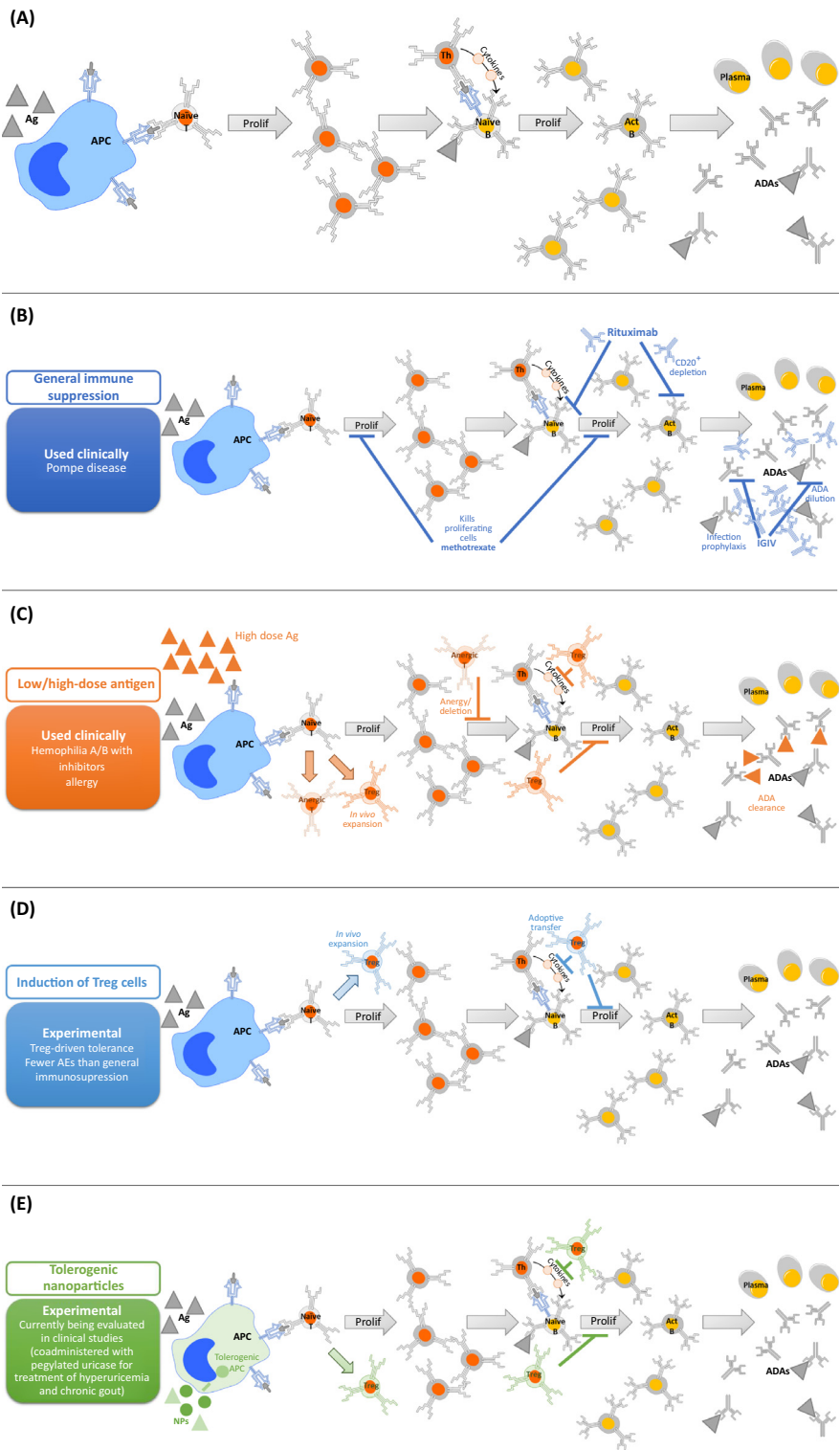
Griswold and coworkers have proposed a comprehensive computational stratagem for protein deimmunization termed 'dynamic programming for deimmunizing proteins' (DP2) [64] (Box 2). This approach is aimed at identifying and then disrupting T cell epitopes [65] using peptide–MHC-II affinity as a surrogate biomarker for a T cell epitope. This approach, moreover, has integrated computational approaches for maintaining protein stability and activity during the process of deimmunization. Protein stability is determined by estimating the $\Delta\Delta G_0$ values for the deimmunized protein. Amino acid conservation, on the other hand, is used to assess the effect of deimmunization on protein function. An example of the application of the DP2 algorithm and characterization of the deimmunized molecule has recently been published [66]. Two of the deimmunized protein variants were expressed, purified, and characterized. This is a good preliminary example of computational redesign of protein molecules for deimmunization. However, in the experimental studies only four MHC-II molecules were used, and although some of the engineered variants showed substantive (17–48-fold) decreases in peptide–MHC-II affinities, others did not. It is important to note that the heterozygosity of the MHC-II molecules and contribution of each allele to peptide binding is not reflected in single human MHC allele transgenic mice. Nevertheless, the clinical significance of these deimmunized variants should be ascertained.

Immune Tolerance Induction (ITI)

ITI regimens are appropriate to consider when the immune response of a patient abrogates the efficacy of a lifesaving therapeutic and no other therapeutic options, such as bypass therapy, are available (Figure 3). Moreover, it may be applied when deimmunization strategies fail [58,59].

ITI Strategies Used in the Treatment of Pompe Disease

The best example is that of immune responses to enzyme replacement therapy (ERT) in Pompe disease, a lysosomal storage disease (LSD) predominantly affecting muscle [67]. Abrogation of ERT efficacy in infant-onset Pompe disease leads to loss of previously gained motor milestones, precipitous clinical decline, and death due to cardiorespiratory failure [68,69]. In other, more slowly progressive LSDs, the effect of the immune response to ERT (e.g., rh α -galactosidase in Fabry disease; rh α -L-iduronidase for mucopolysaccharidosis type 1, MPS1) is less clear in patient studies, although a preponderance of evidence from animal models and spontaneous animal disease strongly support the benefits of immune tolerance to the ERT in its ability to penetrate crucial target tissues. In Pompe disease, patients who have severe genetic mutations in the α -glucosidase gene such that they are a 'knockout' phenotype [crossreactive immunologic material (CRIM)-negative] almost uniformly generate robust immune responses to ERT, leading to rapid clinical decline and death [69], while most CRIM-positive patients generate lower-level, transient, and non-neutralizing ERT antibody responses and enjoy sustained benefit from ERT. However, a subset of CRIM-positive patients generate ADA responses similarly to CRIM-negative patients and do as poorly. Because patients with Pompe disease can now be identified from newborn screening [70], CRIM-negative patients are treated with an ITI protocol at the onset of ERT. A short course of combination therapeutics including rituximab (to eliminate CD20⁺ B cells), methotrexate (kills T and B cells proliferating to the ERT antigens), and immune globulin intravenous (IGIV) (immunomodulation and infection prophylaxis) is highly effective in most patients without incurring serious adverse events [71]. A subset of patients required a second course of treatment. This treatment has spectacularly prolonged ventilator-free survival in these patients, although disease severity and the extent of disease before initiation of therapy significantly modifies



Trends in Biotechnology

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clinical course. At the other extreme, for patients in whom a high-titer sustained antibody response has gone unchecked, despite administration of the prophylactic immune suppressive agents, bortezomib, a proteasome inhibitor that targets the long-lived (CD20⁺) plasma cells mediating such responses, has proven dramatically successful and, combined with additional courses of rituximab and methotrexate, led to durable immune tolerance [72]. For CRIM-positive patients in whom ERT elicits high-titer antibody responses, ITI is initiated following a rise in antibody titer to 1/25 000 that is sustained. Such patients may require more prolonged tolerance-inducing therapies and/or treatment with bortezomib.

Tolerogenic nanoparticles

Because Pompe disease is life-threatening, the risk of combination therapy with immunosuppressive drugs is acceptable; however, this may not be the case for other non-fatal diseases. Recently, alternative strategies involving immunomodulatory agents have been devised to mitigate the effects of therapeutic protein immunogenicity through antigen-specific or antigen-targeted tolerance induction. Synthetic virus particles (SVPs), also known as tolerogenic nanoparticles (tNPs), are self-assembling particles that consist of a biodegradable polymer, poly(lactide-co-glycolide) (PLGA), that have encapsulated a small-molecule immunomodulator, rapamycin, or in an alternative presentation the antigen is incorporated directly into the particles [73]. In the rapamycin nanoparticle approach, SVPs containing rapamycin are concomitantly administered with a free protein antigen, resulting in the induction of tolerogenic DCs and antigen-specific Tregs *in vivo* [74]. If validated in clinical studies, this and other promising nanoparticulate regimens may have broad-ranging applications because they may elicit durable antigen-specific immunotolerance without altering the protein therapeutic, and therefore could be paired with a variety of immunogenic products. The use of SVPs would have few side effects such as increased risk of infection because the encapsulated rapamycin does not lead to global immunosuppression [75].

ITI Approaches in HA and Hemophilia B (HB)

First reported over 40 years ago, the clinical knowledge and understanding of ITI for hemophilia patients is derived from small cohort studies, and retrospective national and international registries. In the case of hemophilia patients with inhibitors, the most common clinical immunomodulation via antigen desensitization or a tolerance-induction protocol involves daily high-dose (up to 200 IU/kg/day) FVIII infusions to eliminate FVIII inhibitors and induce tolerance. While the mechanisms by which high-dose treatments induce tolerance are unknown, it is hypothesized that repeated dosing leads to anergy and deletion of antigen-specific T cells [76] or that these high-dosing regimens can lead to the induction of Tregs. However, in the case of HA patients, successful outcomes were obtained in only 50–70% of patients. In addition, the cost of a completed ITI protocol, measured by inhibitor titer falling below 0.6 BU/ml and FVIII

Figure 3. Immune-Tolerance Induction (ITI) Strategies. (A) Immune processes, including different cell types and cell-cell interactions, leading to the development of ADAs in response to a therapeutic protein are shown. (B–E) The impacts of different ITI strategies on the development of ADAs are highlighted with color. The ITI strategies and potential mechanisms illustrated are: (B) general immune suppression (killing of proliferating cells by methotrexate, depletion of CD20⁺ cells by rituximab, infection prophylaxis and ADA dilution by IGIV; yellow), (C) low/high-dose antigen (antigen dose leading to anergic T cells/T cell deletion, *in vivo* expansion of Tregs, clearance of ADAs; orange), (D) induction of Treg cells (via *in vivo* expansion or *ex vivo* expansion and adoptive transfer; blue), and (E) tolerogenic nanoparticles (induction of Tregs; green). Current clinical/experimental applications for each ITI strategy are also included. Abbreviations: act B, activated CD20⁺ B cell; ADAs, anti-drug antibodies; AE, adverse event; Ag, therapeutic protein antigen; anergic T, anergic CD4⁺ T cell; APC, antigen-presenting cell; IGIV, immune globulin intravenous; naïve B, naïve CD20⁺ B cell; naïve T, naïve CD4⁺ T cell; NPs, nanoparticles; plasma, antibody-secreting plasma cell; proliferate, cellular proliferation; Th, helper CD4⁺ T cell; Treg, regulatory CD4⁺ T cell.

functional normalization, can exceed \$1 million. In the case of HB patients, ITI regimens are typically met with less favorable outcomes and poor success rates. Many of the HB patients with FIX inhibitors that undergo ITI present with an allergic phenotype and are prone to complications such as anaphylaxis and nephrotic syndrome owing to formation of circulating immune complexes upon high FIX dosing [77,78].

Given the high cost and variable success of standard ITI regimens for hemophilia patients, there is a need for alternative approaches for inducing immune tolerance to FVIII or FIX products. One such approach to mitigate the effects of FVIII immunogenicity is through the deliberate expansion of antigen-specific Tregs that suppress the proliferation and cytokine release of antigen-specific effector T cells. Several groups have been working toward generating cellular therapy protocols that rely upon either (i) the adoptive transfer of Tregs [79] or (ii) the expansion of Tregs [80,81]. So far these protocols have been successful in animal models. However, their utility in hemophilia patients has yet to be determined. The ability to induce tolerance through such a mechanism would be advantageous in the prevention or reversal of inhibitors to FVIII products. Again, these Treg-centric approaches would be more selective than more globally immunosuppressive protocols that involve the blockade of costimulatory pathways or the depletion of T or B cells. Other approaches to immune tolerance in the hemophilia space include (i) oral tolerance regimens [82,83], (ii) generation of tolerogenic APCs [84], and (iii) ectopic cellular expression of FVIII [85,86]. Given the abundance of novel experimental ITI treatment strategies that have the potential to be less expensive and more efficacious than the current high-dose hemophilia ITI regimens, novel tolerizing therapies are likely to emerge soon.

Concluding Remarks

The manufacture and licensure of therapeutic proteins almost always involves risk-based approaches to reduce/mitigate immunogenicity. Many of the recently developed non-clinical methods to predict immunogenicity together with concurrent advances in the field of immunology and in novel technologies will further our ability to accurately assess therapeutic protein immunogenicity, deimmunize proteins, and modulate anti-drug immune responses. A key bottleneck to accurately predicting immunogenicity has been the large numbers of molecules and/or interactions involved: the MHC proteins are among the most diverse in the human genome; the binding of each MHC protein needs to be evaluated for potential binding to all peptides generated from the drug of interest; the vastness of the TCR repertoire in an individual [87]; and the similarly vast numbers of possible antibodies in a human ($\sim 10^{11}$) [88–90]. The emerging technologies described in this review make it plausible for us to obtain data that capture the salient epitopes and MHC interactions that drive potential immune responses to therapeutic proteins. It has become apparent in other aspects of biology that high-throughput technologies that rely on proteomics and genomics mature rapidly and are accompanied by exponentially reducing costs and a resulting deluge of data [91]. We anticipate that the application of these technologies to immune responses to proteins used as therapeutics will follow a similar trajectory. The crucial challenge then will be the collection of samples from well-designed clinical studies and appropriate cohorts (see Outstanding Questions). It will also be necessary to generate mathematical models to help in appropriate clinical study design, leading to iterative cycles of hypothesis generation, testing, and model refinement in addition to using these data to predict immune outcomes at the individual and population levels. Although the basic formulations of such models have begun to emerge [92–94], forthcoming datasets will be necessary to validate them.

The availability of a diverse set of *in silico*, *in vitro*, and *ex vivo* methods to measure different steps in the human immune response to therapeutic proteins also provides an important

Outstanding Questions

Can clinical studies be designed to demonstrate correlation (not only association) between results of non-clinical assays and the development of ADAs?

Can ‘big data’ (e.g., genomic and proteomic data) collected in clinical trials of drugs be interrogated to identify biomarkers of immunogenicity?

If sufficient biochemical, biophysical, and cellular information is collected from subjects, can mathematical models be developed to integrate this information and make good predictions of immunogenicity? What is the minimal dataset that would be required to accomplish this?

If biomarkers for predicting immunogenicity are validated, can these be used to design protein drugs that have optimal activity and reduced immunogenicity?

The past few years have seen the application of a new generation of genomic and proteomic tools to interrogate the immune response; how can these advances be leveraged to develop better predictive algorithms for immunogenicity to protein therapeutics?

Can protein therapeutics and tolerizing regimens be codeveloped?

advantage in advancing new approaches for the development of immune-tolerizing regimens. It is increasingly being recognized that clinical studies should not only be focused on outcomes but should also address mechanistic hypotheses. This, for example, is the model adopted by the Immune Tolerance Network (www.immunetolerance.org/) whose stated mission is 'To advance the clinical application of immune tolerance by performing high quality clinical trials of emerging therapeutics integrated with mechanism-based research.' Integrated mechanism-based clinical research can only be successful when a critical mass of underlying tools, assays, and technologies are available.

Taken together, the rapid advances, increasing accessibility, and decreasing costs for methods used in the non-clinical assessment of immunogenicity should, within the next decade, significantly reduce the burden due to immunogenicity. The result will be medications with a reduced incidence of immunogenicity or, in cases where this is not possible, better management of immunogenicity.

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Disclaimer Statement

The findings and conclusions in this article have not been formally disseminated by the US FDA and should not be construed to represent any Agency determination or policy.

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