

# Immunogenicity assessment during the development of protein therapeutics

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## Abstract

**Objective** Here we provide a critical review of the state of the art with respect to non-clinical assessments of immunogenicity for therapeutic proteins.

**Key findings** The number of studies on immunogenicity published annually has more than doubled in the last 5 years. The science and technology, which have reached a critical mass, provide multiple of non-clinical approaches (computational, in vitro, ex vivo and animal models) to first predict and then to modify or eliminate T-cell or B-cell epitopes via de-immunization strategies. We discuss how these may be used in the context of drug development in assigning the immunogenicity risk of new and marketed therapeutic proteins.

**Summary** Protein therapeutics represents a large share of the pharma market and provide medical interventions for some of the most complex and intractable diseases. Immunogenicity (the development of antibodies to therapeutic proteins) is an important concern for both the safety and efficacy of protein therapeutics as immune responses may neutralize the activity of life-saving and highly effective protein therapeutics and induce hypersensitivity responses including anaphylaxis. The non-clinical computational tools and experimental technologies that offer a comprehensive and increasingly accurate estimation of immunogenic potential are surveyed here. This critical review also discusses technologies which are promising but are not as yet ready for routine use.

## Introduction

The first recombinant therapeutic protein, insulin, was approved in 1982. This class of drugs now represents an essential and rapidly growing sector of clinical therapeutics used to treat a wide variety of clinical indications including autoimmune disease, cancer, infectious diseases and genetic disorders.<sup>[1]</sup> Over the years, advances in protein engineering have permitted alterations in the structure of protein therapeutics to enhance desirable functional characteristics and thus, product efficacy and safety.<sup>[2]</sup> Consequently, protein therapeutics, such as monoclonal antibodies (mAbs) and enzyme replacement therapies (ERTs), is increasingly being developed with 'fit for purpose' modifications to treat diseases with unmet medical needs. However, an important factor that still limits therapeutic efficacy and impacts

product safety of therapeutic proteins is immunogenicity, that is immune responses directed to the therapeutic.<sup>[3–5]</sup>

Immune responses to therapeutic proteins generate anti-drug antibodies (ADAs) which may cause serious adverse events including abrogation of the efficacy of life-saving agents, either by antibody-mediated neutralization of such factors or by mediating hypersensitivity responses, forcing discontinuation of product administration.<sup>[6–9]</sup> ADAs may also greatly impact the efficacy of agents which, though not life-saving, are highly effective in mitigating disease manifestations such as the TNF inhibitors for rheumatologic diseases.<sup>[10]</sup>

Immune responses to biotherapeutics have been recognized as an important safety concern by regulators and the industry, prompting guidance on clinical risk assessment and assay performance (see section on resources, i.e. guidance documents and white papers, below). The large surge

in therapeutic proteins entering the drug development pipeline<sup>[11–13]</sup> and the added complexity of proteins drugs which are bioengineered and that engage the immune system (such as the IgG constant region, Fc) have all made immunogenicity an even more critical issue in the last few years.<sup>[2,14]</sup> The validity of these concerns is exemplified by the engineered protein drugs that have been discontinued during phase 3 clinical trials and postlicensure due to serious immune reactions.<sup>[6,7,15]</sup>

Here, we review the recent literature on the underlying immunology of the immune response to therapeutic proteins and technological advances for prediction, prevention and abrogation of immunogenicity. We also identify the key gaps in our knowledge.

## The Immune Response to Therapeutic Proteins

T-cell-dependent antibody responses<sup>[16]</sup> to therapeutic proteins are of primary concern in that they engender class switching, affinity maturation and the induction of long-lived memory B and plasma cells<sup>[17]</sup> which produce high titre, sustained, high affinity, and often neutralizing antibody responses that mediate adverse events.<sup>[18]</sup> The early events in this immunologic train involve uptake of the therapeutic by dendritic cells or product-specific B cells which process the protein and present a subset of its peptides on HLA-class II molecules to CD4+ T cells.<sup>[19]</sup> Below and in Figure 1 are illustrated the process of antigen processing and presentation culminating in the activation (proliferation, cytokine secretion and upregulation of costimulatory molecules) of T cells specific for the therapeutic protein:

- 1 Uptake and internalization of a therapeutic protein by antigen presenting cells (APCs) including dendritic cells and antigen-specific B cells.
- 2 Proteolytic processing of the therapeutic protein into peptides.
- 3 Binding of peptides derived from the therapeutic protein to HLA-II molecules.
- 4 Peptide-HLA-II presentation on the cell surface.
- 5 Engagement by the APC of CD4+ T-cells bearing TCRs specific for the peptide-HLA-class II complex.
- 6 Upregulated expression of costimulatory molecules on the T cell and APC and production of cytokines leading to activation, proliferation and expression of effector function

To generate an ADA response, T cells must collaborate with antigen-specific B cells (Figure 1) which predominantly takes place in germinal centres of lymphoid tissues. Importantly, while T cells are specific for linear peptides within the context of HLA-II, B cells are generally specific for 3-D epitopes of the native protein consisting of discontinuous peptide segments

within a protein, and thus, B-cell epitopes are much more difficult to predict. Epitope specificity of antibodies generated to the therapeutic can be evaluated through epitope mapping studies performed with animal or patient generated antibodies post-treatment; such studies may shed light on the amino acid sequences that compile such highly dimensional epitopes and potentially allow modification of the protein to delete such targets. As B-cell epitopes are more complex than T-cell epitopes, modification of a therapeutic protein to remove amino acid sequences predicted to be a source of immunogenicity (de-immunization) using predictive algorithms or more direct *in-vitro* studies have focused on T-cell epitopes.<sup>[18]</sup> De-immunizing strategies that modify B-cell epitopes are complex, time-consuming and expensive. One of the best examples of the use of such a strategy is one developed to identify and engineer B-cell epitopes on recombinant immunotoxins.<sup>[20]</sup> The process involved as follows: (a) RNA extraction from blood samples obtained from patients who had been treated with recombinant immunotoxins. (b) Synthesis and amplification of cDNAs encoding Fvs. (c) Phage display of the Fvs. (d) Isolation of the phage that contained Fvs reacting with the immunotoxins. (e) Sequencing of individual phage that bound to the immunotoxin domains. (f) Design, synthesis and evaluation of recombinant immunotoxins-based information obtained above. While the process to design a molecule with reduced reactivity with human antisera is complex, it is even more challenging to ensure that the functional (activity of cytotoxic and antitumor) properties are retained. This may be even more difficult to achieve with therapeutic proteins that require complex functions such as the catalytic activity of enzymes.

## Immunogenicity Assessments During Drug Development

Therapeutic proteins are known to elicit immune responses whether they are therapeutic counterparts of endogenous proteins or foreign proteins with little human homology.<sup>[21]</sup> Thus, immunogenicity assessments are now an integral part of the development, licensure and postmarketing evaluations of therapeutic proteins (see guidance documents listed in Table 1). However, the likelihood of the development of such immune responses and the risks posed to patients by the immune response to such diverse therapeutics differs dramatically indicating, per guidance, that a risk-based strategy should be adopted to assess immunogenicity.<sup>[22]</sup> Factors influencing immunogenicity of therapeutic proteins are well described and are both patient- and product-related with the latter often associated with the manufacturing process.<sup>[23]</sup> Indeed, there are examples in which manufacturing changes that had no apparent effect on the biophysical and biochemical properties of the

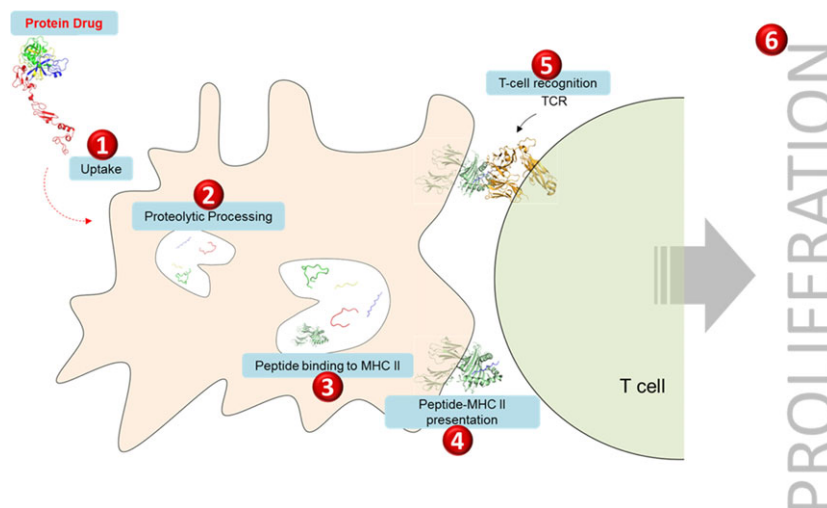


**Figure 1** A simplified schematic showing series of (mostly linear) events that lead to a T-cell-mediated immune response to a therapeutic protein. The important steps which can be monitored using experimental or computational tools are illustrated in numbered red circles. The protein drug is first be internalized into professional antigen presenting cells such as dendritic cells (Step 1). Internalized proteins are enclosed in endosomes (Step 2) where short peptides are generated through the action of proteasomes and other enzymes (Step 2). MHC-II molecules are transported from the endoplasmic reticulum ER to the endosomes where the peptide antigens are exchanged with the CLIP (Class II-associated invariant chain peptide, that binds MHC class II groove during MHC assembly) protein (Step 3). It should be noted that whether processing occurs prior to, concurrently with or following binding has not been conclusively resolved and is the subject of active investigations. Peptide-MHC-II complexes are transported to the plasma membrane and the processed antigen is presented to CD4+ T cells in the lymph nodes (Step 4). Recognition of the peptide-MHC-II complex by the appropriate T-cell receptor (Step 5) results in the proliferation of those T-cells (Step 6).

molecule nonetheless resulted in significant increases in immunogenicity.<sup>[24]</sup>

To illustrate some of the nuances in immunogenicity risk assessments consider the following examples. New variants of FVIII products that result in neutralizing antidrug antibodies in 20–30% of the patient population continue to be approved. This is because this is the prevalence of nADAs observed in all FVIII products<sup>[25]</sup> and a salvage therapy, using FVIIa, can be used to bypass the FVIII step in the coagulation cascade in nADA-positive patients, thus maintaining a life-saving therapeutic for these patients.<sup>[26]</sup> On the other hand, the development of a new mutant variant of the FVIIa product, vatreptacog alfa, was discontinued when about 10% of patients in the phase 3 clinical trial developed ADAs as the parent product (wild type FVIIa) generates ADAs in less than 1% of treated patients.<sup>[6]</sup> Thus, the critical salvage product with its much lower incidence of immunogenicity makes the risk of the new product with its higher ADA responses too high. Omontys (peginesatide) was approved for the treatment of anaemia in dialysis patients.<sup>[27]</sup> Postmarketing it was determined that a small proportion of patients (0.2%) developed allergic reactions to the drug and fatal reactions were found to occur in about 0.02% of the patients.<sup>[28]</sup> Although the proportion of patients was very small, given the large number of potential patients, the well-known safety profile of erythropoietin

agents, and the fatalities that were associated with the adverse reaction,<sup>[29]</sup> the drug was withdrawn.<sup>[30]</sup> These examples illustrate that critical aspects of an immunogenicity risk assessment may vary and that decisions are made on a case-by-case basis taking into account many factors.

The examples given above relate to safety risks associated with immunogenicity. In addition antidrug antibodies can result in clinically significant effects on the efficacy of drugs. A recently published study provides a well-documented and quantified example.<sup>[31]</sup> Bococizumab is a humanized monoclonal antibody targeting that was being developed to reduce levels of low-density lipoprotein cholesterol. An exhaustive, multinational, study was recently carried out on 4300 patients enrolled in six clinical trials. The study demonstrated antidrug antibodies developed in a large proportion of the patients and significantly attenuated the lowering of LDL cholesterol levels.

Given that experience with the clinical use of therapeutic proteins has clearly shown that any protein drug is potentially immunogenic, including therapeutic counterparts of endogenous proteins to a variable extent, and that adverse events may arise from such responses, immunogenicity assessments are required for all protein therapeutics during product development.<sup>[3]</sup> Currently, clinical trials in which patients are evaluated for clinical responses consistent with ADA, as well as assays for ADA and extensive

**Table 1** Resources for developing immunogenicity assessment strategies during clinical studies

|                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                           |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>EMA guidelines</b>                                                                                                                                                                                                                                     |                                                                                                                                                                                                                           |
| Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins, 2007                                                                                                                                                                | <a href="http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003946.pdf">http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003946.pdf</a>         |
| Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use has been released for consultation, 2012                                                                                                                | <a href="http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf">http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128688.pdf</a>         |
| Guideline on the clinical investigation of recombinant and human plasma-derived factor VIII products                                                                                                                                                      | <a href="http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109692.pdf">http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109692.pdf</a>         |
| Guideline on the clinical investigation of recombinant and human plasma-derived factor IX products                                                                                                                                                        | <a href="http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109691.pdf">http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109691.pdf</a>         |
| <b>FDA guidelines</b>                                                                                                                                                                                                                                     |                                                                                                                                                                                                                           |
| Assay development for immunogenicity testing of therapeutic proteins, 2009                                                                                                                                                                                | <a href="http://www.fda.gov/downloads/Drugs/%20GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf">http://www.fda.gov/downloads/Drugs/%20GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf</a> |
| Immunogenicity Assessment for Therapeutic Protein Products, 2014                                                                                                                                                                                          | <a href="http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338856.pdf">http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM338856.pdf</a>       |
| <b>World Health Organization guidelines</b>                                                                                                                                                                                                               |                                                                                                                                                                                                                           |
| Guidelines on the quality, safety, and efficacy of biotherapeutic products prepared by recombinant DNA technology. Annex 4, WHO Technical Report Series No 987, 2013                                                                                      | <a href="http://apps.who.int/iris/bitstream/10665/129494/1/TRS_987_eng.pdf?ua¼1&amp;ua¼1">http://apps.who.int/iris/bitstream/10665/129494/1/TRS_987_eng.pdf?ua¼1&amp;ua¼1</a>                                             |
| Guidelines on evaluation of similar biotherapeutic products (SBPS). Annex 2, WHO Technical Report Series No 977, 2009                                                                                                                                     | <a href="http://www.who.int/biologicals/areas/biological_therapeutics/BIOTHERAPEUTICS_FOR_WEB_22APRIL2010.pdf">http://www.who.int/biologicals/areas/biological_therapeutics/BIOTHERAPEUTICS_FOR_WEB_22APRIL2010.pdf</a>   |
| <b>White papers</b>                                                                                                                                                                                                                                       |                                                                                                                                                                                                                           |
| Koren E, Smith HW, Shores E, Shankar G, Finco-Kent D, Rup B. Recommendations on risk-based strategies for detection and characterization of anti- bodies against biotechnology products. <i>J Immunol Methods</i> 2008; 333: 1e9                          | <a href="http://www.sciencedirect.com/science/article/pii/S0022175908000197">http://www.sciencedirect.com/science/article/pii/S0022175908000197</a>                                                                       |
| Gorovits B, Wakshull E, Pillutla R, Xu Y, Manning MS, Goyal J. Recommendations for the characterization of immunogenicity response to multiple domain biotherapeutics. <i>J Immunol Methods</i> 2014; 408: 1e12                                           | <a href="http://www.sciencedirect.com/science/article/pii/S0022175914001550">http://www.sciencedirect.com/science/article/pii/S0022175914001550</a>                                                                       |
| Mire-Sluis A, Barrett YC, Devanarayan V, Koren E, Liu H, Maia M, et al. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. <i>J Immunol Methods</i> 2004; 289: 1e16 | <a href="http://www.sciencedirect.com/science/article/pii/S0022175904001978">http://www.sciencedirect.com/science/article/pii/S0022175904001978</a>                                                                       |

pharmacovigilance programmes remain the most reliable way to assess immunogenicity. Sensitive and specific assays are absolutely critical to such evaluations, and their degree of validation is clinical phase dependent (see guidance documents listed in Table 1). Thus, FDA requires qualified assays to screen for antidrug antibodies before commencement of clinical trials and, depending on risk, additional bioassays to monitor for neutralizing antidrug antibodies.<sup>[32]</sup> Such assays are expected to undergo validation by phase III trials. The resources to develop assays for detecting antidrug antibodies in a risk-based manner include guidance documents from regulatory agencies and white papers from industry groups as discussed below.

It should also be noted that ADA assays are also critical for evaluation of preclinical toxicology studies. ADA that neutralizes the activity of the product will indeed mask the toxicology profile related to the activity of the agent, thus conferring a false picture of potential toxicity. Moreover, although animals do not often predict the incidence of ADA in humans to therapeutic counterparts of endogenous human proteins, they may indeed predict the consequences of such responses depending on the extent of evolutionary conservation of the endogenous protein. Recent advances in technologies (see below), resulting in improved predictive performance, have also contributed to greater confidence in the incorporation of these tools in decision-making.

## Resources for Developing Strategies for Immunogenicity Assessments During Drug Development

The current consensus is that immunogenicity can only be accurately assessed in the patient population because *in silico*, *in vitro*, *ex vivo* and animal models have not been convincingly demonstrated to be predictive. This view is reflected, for example, in the World Health Organization's, 'Guidelines on the quality, safety, and efficacy of biotherapeutic products prepared by recombinant DNA technology'.<sup>[21]</sup> Consequently, the immunogenicity assessments of protein therapeutics for licensure predominantly rely on the use of validated assays to accurately measure antidrug antibodies during clinical trials.<sup>[33]</sup> The European and US regulatory agencies have therefore published detailed strategies and agency expectations for the identification and characterization of product-specific antibodies during clinical trials (Table 1). While endeavouring to delineate broad principles and regulatory expectations, most experts are cognizant of the complexities and recognize that no single assay or strategy may be used for all products. This is apparent, for example, by the release of specific guidance documents for specific product classes which show unique characteristics [FVIII/FIX].

Notwithstanding the complexity, the overarching goals of immunogenicity assessments are straightforward and consistent across all product classes: does the therapeutic protein elicit antibodies and, if so, what are the clinical consequences? Consequently, although assay formats may vary, the general strategy for the immunogenicity assessment of most therapeutic proteins pursues the following sequence:

- 1 A screening assay is used for the identification of so-called 'binding' antibodies. This initial screening is typically performed using an assay that allows for a false-positive rate of ~5%.<sup>[32]</sup>
- 2 Samples which test positive in the screening assay are then subjected to a confirmatory assay to eliminate the false-positive samples. Pre-incubation of clinical samples with an excess of the therapeutic protein is the most commonly applied strategy in competitive assays. Determining the confirmatory cut-point is a critical component of the qualification and validation of these assays.
- 3 Depending on risk and stage of development, samples that test positive in both screening and confirmatory assays are titrated to determine antibody levels and then subjected to a suitably designed bioassay to determine whether the antidrug antibodies are neutralizing (i.e. able to inhibit the function of the protein).
- 4 Isotype identification, at least as regards IgG, IgM, and in the face of hypersensitivity responses, IgE, is required.

Sub isotype analyses (IgG1, IgG4 etc.) may be required if such information is considered to be of value based on the clinical consequences of the immune response.

## Recent Advances in Technologies to Make Preclinical Predictions of Immunogenicity

As emphasized above, *ex-vivo* assessments of immunogenicity are not considered reliable enough to make regulatory decisions. However, there has been considerable progress in the development of *in silico*, *in-vitro* and *ex-vivo* tools for preclinical assessments of immunogenicity risk and quantitative assessments have shown that many of these tools have improved predictive performance. This has led to increased use of such assessments in the early stages of drug development principally at the stage in which candidate molecules are designed and selected.

### *In silico* algorithms for determining peptide-HLA affinity

*In silico* prediction algorithms are among the most commonly utilized tools for preclinical assessments of candidate molecules. A large number of these tools are available in the public domain (see<sup>[34]</sup> for a comparative evaluation of algorithms in the public domain) while others are proprietary<sup>[35]</sup> and require user fees. For the most part, these algorithms seek to predict HLA-class II-peptide epitopes to which CD4+ T-cells respond. The underlying premise is that the development of antidrug antibodies requires presentation of peptides derived from the protein therapeutic by HLA-II proteins.<sup>[18]</sup> Also, given the exogenous administration of these proteins, the HLA-II repertoire, and not HLA-I, which generally obtains peptides from an intracellular source, for example viral peptides<sup>[36]</sup> are associated with the immune response to infused therapeutic proteins.

Scientific studies have suggested that strong peptide-HLA-II affinity can increase the probability of an immune response to the protein from which the peptide was derived.<sup>[19,37]</sup> This has been the underlying motivation for the development of predictive algorithms. Unfortunately, developing computational methods that accurately estimate peptide-HLA-II binding affinities is more challenging than development of those for peptide-HLA-I affinities.<sup>[38]</sup> Nonetheless, the performance of these algorithms has steadily improved,<sup>[34]</sup> and predictive performances when measured as AUC for ROC curves are now in the range of 0.7–0.9 (see<sup>[39]</sup> for a comprehensive discussion of the use of ROC curves for evaluating diagnostics and performance of predictive tools). Despite these encouraging signs of progress, some caveats are in order. Foremost, the algorithms

are designed to estimate peptide-MHC-II affinity, a prerequisite for which is that the peptide(s) of interest are generated by the antigen processing machinery. Computationally this is a very difficult problem and algorithms to predict the generation of peptides during antigen processing have so far performed poorly.<sup>[40]</sup> In addition when the predictive performance of a specific tool is reported, the sole parameter assessed is peptide-MHC-II affinity and not the consequences of that affinity value. The software is also either programmed to provide cut-off values or the users can apply their own judgement in assigning cut-offs to identify potential T-cell epitopes. Although there is some rationale based on experience and the reading of scientific literature, for the selection of such values it is important to be cognizant that the cut-off values are essentially arbitrary.

Despite the limitations discussed above, there are many scenarios where *in silico* tools for estimating peptide-HLA-II binding affinities can be profitably used in immunogenicity assessments, and some examples are provided below. In computational assessments comparing the affinities of neo-sequences inserted in a candidate drug, the selection of the variants with lower affinities for HLA-II molecules is likely to be advantageous from an immunogenicity perspective, as they are expected to form less stable complexes and thus, be less capable of T-cell activation. Similarly, the computational assessment could identify some HLA-II molecules that bind the neo-sequence(s) with particularly high affinity. This would indicate the need for additional *in-vitro* and *ex-vivo* studies and even incorporating this information into the clinical studies such as a requirement to HLA type patients and closely monitor those potentially most at risk.

### In-vitro measurements of peptide-MHC-II affinity

Compared to *in silico* tools for measuring peptide-MHC-II affinity, *in-vitro* peptide-HLA-II binding assays are time-consuming and expensive, but give a better predictive read out of T-cell activation. Thus, the best path may be to use the computational tools as an initial screen and confirm the affinities of the candidate drug peptides so identified to purified recombinant HLA-II molecules before making critical decisions related to molecule selection. Such a hybrid approach that combines *in silico* tools with binding assays may be of use in reducing the number of peptide-HLA-II combinations that require testing in an *in-vitro* experimental setting. (see<sup>[41]</sup> for such an approach). In addition, the wide range of analytical tools that are now available allows the measurement of on- and off-rates as well as the half-life of peptide-HLA-II complexes in addition to the  $K_d$  (affinity) (e.g. Surface Plasmon Resonance).<sup>[42]</sup> Studies have suggested that biophysical

measurements that are surrogates for the stability of the peptide-HLA-II complex (such as half-life, and off-rates) may better predict overall immunogenicity.<sup>[37]</sup> Thus using *in silico* tools to screen large numbers of peptide-HLA-II binding affinities followed by exhaustive experimental characterization of those of concern may provide data leading to better decision-making. Such a strategy can keep the size of the experimental sets manageable, screening a broad, meaningful cross-section of peptides and HLA-II variants and comprehensively characterize the peptide-HLA-II interactions using direct binding assays,<sup>[43]</sup> completion binding assays<sup>[44]</sup> and kinetic measurements.<sup>[42]</sup>

### In-vitro assays for antigen presentation

A consistent criticism of using *in silico* algorithms to estimate peptide-HLA binding is that the estimates are made on short peptide sequences (15–25 mers). Similarly, assays that measure T-cell responses (see below) are carried out using either the whole therapeutic protein or the relevant peptides that define the T-cell epitopes. As shown in Figure 1, therapeutic proteins must be internalized by antigen presenting cells (APCs) and enzymatically processed into peptides before presentation by HLA-II molecules. Depending on the manner in which a protein is folded and the number and position of enzyme cleavage sites only a small fraction of all potential peptides may actually be generated.<sup>[45]</sup> Thus, the identification of T-cell epitopes based on peptide-HLA binding affinities may be moot if the peptides are not generated in the first place. In this regard, mass spectrometry has become the tool of choice for T-cell epitope identification.<sup>[46]</sup> Although the so-called MHC-associated Peptide Proteomics, or MAPPs, assays<sup>[45]</sup> require specialized skills, are quite expensive and not universally accessible, considerable progress has been made in their implementation. The technique is becoming increasingly simpler to perform, more affordable than in the past and the associated software tools are getting more user friendly. These assays involve the *in-vitro* profiling of protein-derived peptides displayed by HLA-II molecules after uptake of the full-length protein by monocyte-derived dendritic cell APCs. This is accomplished by elution of peptides isolated from HLA proteins followed by high resolution mass spectrometry analysis. HLA-II-binding peptides have been found to range from 12 to 25 amino acids, often sharing a common core but differing in the length of the flanking regions.<sup>[47]</sup> The *in-vitro* profiling of peptides displayed by HLA-II molecules obviates the need for methods to determine peptide processing because peptides of importance to generating immune responses will be identified only if they are first generated by proteolysis and loaded into HLA molecules. Despite the progress noted above, and compared to the other assays described in this section,

MAPPs assays are complex, expensive and need specialized equipment and skills. They are also limited by the HLA variants found on donors.

### T-cell proliferation assays

T-cell proliferation assays pertain to step 5 in Figure 1. If these assays are conducted with the full-length therapeutic protein, the assay measures the cumulative effect of processing of the therapeutic protein by antigen presenting cells, formation of peptide-HLA-II complexes expressed on the APC surface and productive engagement of a T-cell bearing a relevant TcR for individual peptide-HLA complexes (Figure 1). Proliferation and activation of T-cell effector function require additional costimulatory signals (e.g. CD80/86 on the APC and ligand CD28 on the T cell; CD40-APC and ligand CD40L-T cell) and cytokines (e.g. IL-2).<sup>[48]</sup> Thus, of all the *in-vitro* and *ex-vivo* assessments of immunogenicity, these assays provide a readout that is more likely predictive of T-cell responses *in vivo*.<sup>[49]</sup> Although these assays are most informative when carried out using the full-length intact protein, they are unfortunately most often performed using individual peptides or peptide pools. This is because preliminary preclinical assessments are performed using naïve cells from donors who have not been exposed to the therapeutic as at this stage of drug development, no patients have been treated with the therapeutic. If peptide sequences in the therapeutic-protein constitute T-cell epitopes, TCRs that recognize the neo-epitope-HLA-II complex may be present in the population of cells derived from naïve donors, albeit at very low frequencies (approximately 1 per million T cells).<sup>[50]</sup> Thus, 'overloading' of the APCs with one (or a few) peptides of interest (i.e. those that are identified as potential T-cell epitopes) will likely be necessary to get meaningful signals. If this is not done, the low density of both the required peptide-MHC-II complex and the TCR that can recognize it result in very poor signal-to-noise ratios.

A wide variety of methods are used to measure T-cell proliferation.<sup>[51]</sup> These include uptake of <sup>3</sup>H-tritium, dilution of fluorescent dyes loaded into live cells and the use of the ELISPOT assay to measure cytokines associated with proliferation.<sup>[48]</sup> These methods have all been well-established and are reasonably reproducible. Additionally a standardized more sensitive assay to monitor CD4+ T-cell response has been recently developed under the auspices of the ABIRISK consortium.<sup>[52]</sup> These assays should be considered in the non-clinical drug development programme to better assess the potential risks and benefits of moving a molecule into clinical trials. The pros and cons of the various methods have been discussed in noteworthy reviews.<sup>[18]</sup> Although T-cell proliferation measures a step in the immune response that is further advanced and thus may be

more physiologically relevant, there are nonetheless several limitations. The assays all rely on human donors and thus the HLA-II variants present in the donors. Consequently it can be difficult to evaluate such activation for patients whose HLA-II variants occur infrequently in the donor population. In addition, unless they are homozygous, each donor presents two HLA-II variants for each HLA-II class (DP, DQ and DR) and this may complicate the interpretation of the data with respect to HLA-restriction. Finally, T cells can perform both effector and regulatory functions and a more accurate prediction of the outcome of the immune response would require additional characterization of the proliferating cells.

### Identification of T-cell epitopes

T-cell proliferation assays alone can provide epitope identification, but the format for these assays requires the synthesis of overlapping peptides that cover the entire protein and the identification of donors whose HLA-II alleles are representative of the population. The peptides are then pooled into groups: peptides recurring in the pools that induce proliferation are confirmed as T-cell epitopes. Given the large number of possible peptide-HLA-II combinations, the experimental identification of promiscuous T-cell epitopes is time-consuming and may be expensive. Therefore, first collecting peptide-HLA-II binding data can substantively limit the number of peptide-HLA-II combinations tested in proliferation assays and use of mass spectrometric analyses to identify the relevant peptides may further increase efficiency. This may make the identification of potential T-cell epitopes more rapid, efficient, and less expensive but further proof regarding this strategy is needed. When identifying T-cell epitopes for large peptides the cost and time associated with all overlapping peptides can be prohibitive, and thus, it is a common practice to offset the peptides by a few amino acids. This, however, risks some regions of the protein being missed.

### Limitations of Non-Clinical (ex vivo, in silico) Assessments of Immunogenicity and Gaps in Knowledge

It is thus clear that a number of tools and assays can be used in the *ex-vivo* evaluation of immunogenicity. However, the key limitation to the ability to make accurate predictions of immunogenicity is the development of tools to incorporate information from the diverse set of assays and computational assessments to generate a risk ranking of potential outcomes. Consider a hypothetical example where several mutant variants of a candidate drug are available and the different neo-sequences at the sites of the

mutations are being evaluated for their potential immunogenicity. Let us assume that an *in silico* tool with a very good predictive performance (Area Under a ROC Curve > 0.9) was used to estimate the affinity of peptide-HLA-II (step 3 in Figure 1). Let us also assume that peptide-HLA-II estimates were performed selecting a range of HLA-II alleles that adequately represent the potential patient population. Although the evaluation was carefully designed and the tool used for assessment provided accurate results, it is likely that with respect to peptide-HLA-II combinations classified as 'high risk', some false positives will be identified. These false positives are not due to deficiencies in the experimental tool or design but simply because the step in the progression of the immune response that is being evaluated is necessary but not sufficient for immunogenicity. Thus, further improvements in the individual tools alone are unlikely to resolve important impediments to accurate predictions of immunogenicity in a clinical setting. A high-level mathematical model capable of synthesizing this non-clinical data and giving appropriate weights to individual data points/data sets is also needed. For such a model to be able to make good predictions, it should be able to provide quantitative weights to the data obtained from different assays and then synthesize this information based on known immunological mechanisms.

Although our understanding of basic immunology has grown considerably, it is by no means complete. Thus, a good mathematical model should perform two functions: (a) As new immunological mechanisms emerge or old hypotheses are rejected, it should be possible to integrate these changes into the model, even with the extant data sets. (b) As new analytical or computational assays and programmes generate new data sets, it should be possible to integrate these into the model. There have been many attempts, going back several decades, to construct mathematical models to describe immune responses.<sup>[53–55]</sup> In recent and more sophisticated approaches, each entity (such as an immune cell) is modelled; the entities are referred to as agents and the models as agent-based. In these models, the agents (e.g. T cells) adapt their behaviour (e.g. differentiation) over time based on set rules as well as interactions with other agents. Examples of multiagent-based models in immunology include the following: those that allow simulations of the human immune system (ImmunoGrid<sup>[56]</sup>); immune responses during peptide vaccination therapy for solid tumours [VaccImm<sup>[57]</sup>]; interactions between the cells of the innate and adaptive immune systems (Basic Immune Simulator<sup>[58]</sup>).

The models described above share an important goal with those that aim to simulate the immune response to protein therapeutics, namely, that the model should reflect both the diversity and relative proportions of molecules and cells of which it is comprised in a human-scale model.

Thus, a neo-sequence in a protein therapeutic may be identified as a potential T-cell epitope in non-clinical studies, but a model can help make better predictions of immune responses of significance. Such a model would use estimates of the proportion of APCs, the number and frequency of the specific T-cell epitopes presented, affinity and half-life of the peptide-HLA-II complex in patients with different HLA-II variants and the number of naïve T cells with receptors specific for this neo-sequence. Simulations would include the available non-clinical data (hopefully reflecting the diversity of cells and molecules involved in the immune response) but also the relative frequencies (see above) to link data obtained from individual assays/tools to a prediction of clinically relevant antidrug antibody generation.

The basic formulations of these models have begun to emerge in recent years.<sup>[59]</sup> The theoretical basis for<sup>[60]</sup> and partial validation of<sup>[61]</sup> one such model has been published. This model incorporates the key immunological events (e.g. antigen presentation, activation, proliferation and differentiation) that occur at the molecular/subcellular, and cellular scales. These reports represent a noteworthy start but require comprehensive validation and possibly modifications of the mathematical algorithms. This in turn requires a systematic collection of *ex vivo* and clinical data.

At least until an overarching framework for a model for immunogenicity is validated, it is important that all parameters are well defined, weighted and evaluated in an *ex-vivo* setting to be correlated with data from the clinical trial. It is also important that a comprehensive strategy for validation of the model is planned by computational scientists and immunologists involved in the formulation of the model. This would include the choice of the assays, validation of individual assays (as distinct from the validation of the model), appropriate biological samples/donors, samples to be collected and frequencies of the assays. This risky and expensive proposition may best be undertaken by public-private or industry wide consortia such as the ABIRISK project. The collection and biobanking of appropriate cells/tissues from patients during clinical trials could also be useful in a post hoc evaluation of clinical samples. Thus, the mathematical models themselves could aid in the design of appropriate prospective studies for validating immunogenicity assessments.

We would like to emphasize that even with resources and commitment from the different stakeholders, these validation studies will be extremely challenging. The computational tools and *in-vitro* and *ex-vivo* assays are not difficult to run. However, the interpretation of results, the underlying statistical analysis and cut-points used to distinguish responders from non-responders will all pose significant challenges. In addition, the low numbers of antigen-specific cells in a naïve population can strongly impact experimental design (e.g. use of peptides vs full-length protein in a T-

cell proliferation assay) because the alternative design will have a low signal-to-noise ratio and thus, make it more difficult to identify a true positive response.

## Conclusion

Immunogenicity is a significant impediment to development and licensure of therapeutic proteins and limits the clinical utility of many approved products. Moreover, as a new generation of therapeutic proteins which have been engineered to improve product attributes or to enhance process characteristics, are rapidly entering the drug development pipeline, the challenge becomes even greater. Such engineered proteins inevitably require novel amino acid sequences which are likely to generate potentially immunogenic neo-epitopes. Decisions about progressing with drug development and licensure when immune responses are detected generally follow a risk-based strategy. As many therapeutic proteins provide the only medical option in otherwise life-threatening illnesses, mitigation strategies for immunogenicity are critical in the management of these patients. Here, we have discussed the current consensus on the evaluation of immunogenicity in clinical studies. We have also discussed the emerging repertoire of computational and experimental tools that can be used in the non-clinical assessment of immunogenicity, currently mostly of use in identification of new drug candidates. These technologies offer a comprehensive and increasingly accurate estimation of immunogenic potential. In addition, there are numerous technologies which are not as yet ready for routine use in non-clinical studies of immunogenicity but hold immense promise.

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These include systems to more accurately replicate the clinical use of the drug such as humanized mice and artificial human lymph node systems. Concurrently the development of mathematical models of immunogenicity offers a potential platform for using data from a diverse set of assays for better immunogenicity predictions. The utility of non-clinical studies and the willingness of the wider community to further investigate these will depend in large part on establishing clear associations with clinical outcomes.

## Declarations

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