PEER REVIEWED

Early Interventions to Reduce the Impact of Immunogenicity on the Development of Biopharmaceuticals

The development of biological therapeutics such as humanised antibodies has led to remarkable clinical benefits in many indications. Despite this success, the induction of anti-drug antibodies (ADAs), due to the potential immunogenicity of these therapeutics, remains a significant challenge to the successful development of biotherapeutics. This article looks at the impact of immunogenicity and approaches that can be taken in pre-clinical development to reduce the impact on product success and patient experience.

The decades that followed the approval of the first biotherapeutic (insulin, 1982), have seen a dramatic increase in both the number of approvals and the rate at which they are being approved. Biologics now account for over 30% of licensed pharmaceutical products and accounted for over 25% of the new drug approved by the CDER in 2015. For monoclonal antibodies alone, since the commercialisation of the first mAb in 1986, this class of product has grown significantly. By 2014, forty-seven mAb products had been approved in the US or Europe. At the current approval rate, it can be anticipated that approximately 70 monoclonal antibody products will be on the market by 2020, with combined worldwide sales of nearly $125 billion[1].

Despite this success, biologics can elicit immunological responses when administered to patients, which can affect their safety and efficacy. These side-effects fall into two categories; pharmacological (largely predictable adverse reactions resulting from the interaction between the biologic and its target) and non-pharmacological. Non-pharmacological adverse events include immunotoxicity, which includes both immune response-mediated and non-immune response-mediated reactions.

This article focusses on the impact of immunogenicity, and the resultant anti-drug antibodies (ADAs), that can result in neutralisation and hypersensitivity reactions to a range of biotherapeutics. We will also comment on the clinical consequences of immune responses to biopharmaceuticals, why this should be avoided and approaches that can be taken in the pre-clinical development phase to provide an approach to reduce the immunogenicity risk of biopharmaceuticals.

The Whys and Wherefores of Immunogenicity

Immune responses to biopharmaceutical agents are wide-ranging, and can be directed against agents that are both human and non-human in origin. The mechanism by which immunogenicity is triggered remains unclear, although the tolerance to self-proteins may be broken by a number of factors linked to both the product and the patient[2,3]. Product factors include dose, route and frequency of administration, immunomodulatory capacity of the protein therapeutic, and formulation4. Patient factors that influence immunogenicity include immune competence (e.g. whether the patient is receiving immunosuppressive treatment), patient's MHC haplotype and intrinsic tolerance to the protein therapeutic. Other factors influencing immunogenicity are listed in Table 1.

Product-related
- Molecular structure – primary amino acid sequence or variants
- Aggregates, degradation products, oxidised or deaminated forms
- Host cell DNA/proteins
- Duration of treatment
- Previous exposure
- Cellular or soluble target
- Biological properties of the therapeutic product

Patient-related
- Genetic profile
- Immune status
- Disease state

Other medication

Table 1: Examples of factors that influence unwanted immunogenicity of therapeutic proteins

Antigen processing and presentation is central to the development of an immunogenic response. Patient and biologic-specific factors mentioned above, exert their influences on immunogenicity by modulating antigen processing and presentation events such as antigen uptake, epitope presentation and maturation of antigen-presenting cells (Figure 1)[5].

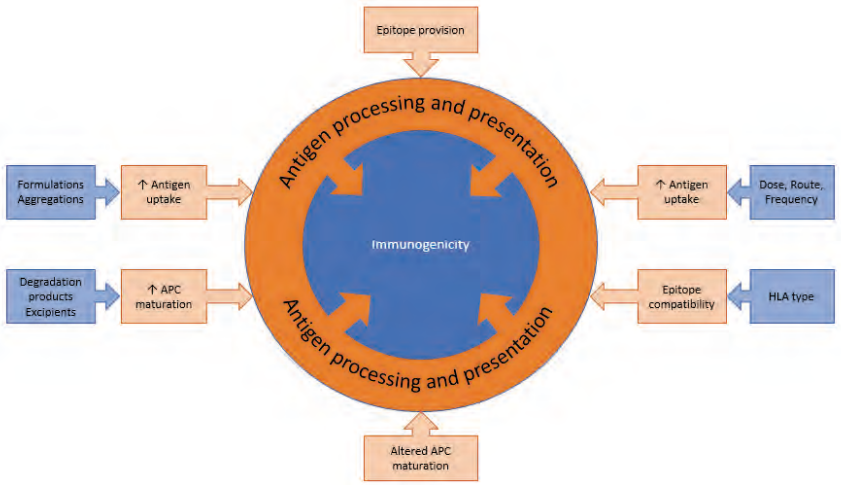

Figure 1: Antigen processing and presentation is central to immunogenic responses to biotherapeutics (Adapted from Sethu et al,2011[5])

Immunogenicity – Why do we Care?
So why do we care about immunogenicity? What are the consequences of anti-drug antibodies? The impact of ADAs can have a wide range of consequences, ranging from clinical consequences and reduced efficacy, to safety and cost implications.

Clinical Consequences of Immunogenicity
Unintended immune responses to biopharmaceutical products (as opposed to desired immunogenicity with vaccine products) can have a severe impact on the safety and efficacy of a biologic. Consequences can range from no discernible or weak effects, to efficacy-limiting effects, or at worst, can include significant morbidity and even mortality in patients.

Neutralising ADAs binding to or near the active site of the biologic can prohibit the drug from carrying out its therapeutic function, thus affecting efficacy. Non-neutralising ADAs bind outside the active site of the biotherapeutic, forming immune complexes that can lead to increased clearance of the drug, affecting the pharmaco-kinetics (PK) of the drug.

Immune complexes can also lead to increased toxicity and hypersensitivity by accumulating in tissues, causing inflammation and damage. The most serious complications can arise with the production of neutralising antibodies, especially when they target non-redundant recombinant self-proteins and therefore have the potential to cross-react with the patient's own endogenous protein[6].

Impact of Immunogenicity Exemplified
In 1998, there was an upsurge in the numbers of cases of erythropoietin-induced pure red cell aplasia (epo-PRCA) in patients with chronic kidney disease treated with Eprex, an approved erythropoietin (EPO) product. Incidence peaked at approximately 250 newly-reported cases in 2001/2002; surprisingly, many of the cases reported during this period were in patients who had been receiving EPO-α for up to a decade without an issue with PRCA[7].

Investigations revealed that most of the cases occurred in patients treated with subcutaneous EPO-α (ortho-biotech) following a formulation change requested by the European Medicines Agency (EMEA). EMEA had requested that human serum albumin in the product be replaced by polysorbate 80. Subsequent studies suggested the cause of PRCA was due to either changes in product stability or the formation of micelles caused by high polysorbate 80 concentration. The latter explanation postulated that the EPO molecules were presented on the surface of the micelles at regular intervals exposing them to the immune system[8]. Later studies identified soluble tungsten in the syringes (from pins used to manufacture the syringes), which may have mediated unfolding and aggregation of the epoetin, causing increased immunogenicity[9]. This is just one example of how multiple factors can influence the immunogenicity of a therapeutic protein.

The Cost of Immunogenicity
Companies involved in biological drug development care about the risks of immunogenicity, not only from a desire to deliver safe and efficacious products to the patients, but also from a cost perspective. There are serious development and manufacturing costs associated with managing overt immunogenicity to a product should it occur after launch, to say nothing of the cost of late-stage termination of product development due to overt immunogenicity[10]. Estimates of the average costs of research and development of a successful new medicine have increased over the last decade from an estimated US$802M at 2000 prices (DiMasi *et al*. 2003[11]) to US$1,867M at 2000 prices (Paul *et al*. 2010[12]).

In 2012, Novo Nordisk discontinued the development of vatreptacog alfa following analysis of Phase III results. The adept™2 trial was one of the largest of its kind for assessing bypassing agents. Vatreptacog alfa, a fast-acting FVIIa analogue with only three amino acid substitutions compared to native FVIIa was found to induce anti-drug antibodies in a few patients. Although not all antibodies were neutralising, some of them exhibited cross-binding to Novoseven® (recombinant coagulation FVII, eptacog). ADAs had not previously been reported to Novoseven®, so this raised concern for the treatment of haemophilia patients with these agents. Subsequent post hoc assessment of the immunogenicity of vatreptacog alfa using multiple pre-clinical immunogenicity assessment approaches, including *in silico*, MHC-associated peptide proteomics (MAPPs) and *ex vivo* T cell assays identified an HLA-restricted neoepitope that likely resulted in the observed ADAs[13]. These findings suggest that use of such tools in pre-clinical screening and development of biologics could aid candidate selection and reduce the chances of failure at late-stage development due to immunogenicity. Even manageable immunogenicity can have cost implications for companies, as payers may be less inclined to reimburse a drug which requires costly immunogenicity management, when better alternatives are available.

When and How to Tackle Immunogenicity
The example of Novo Nordisk's vatreptacog alfa illustrates how costly it can be to find out about a product's immunogenicity late in the development process, and why most biopharmaceutical companies are incorporating immunogenicity analysis in general, and T cell epitope analysis specifically into their pre-clinical or even discovery processes. Immunogenicity assessment is also one of the regulatory requirements for biotherapeutic approval, with guidelines advocating the use of immunogenicity testing as early as pre-clinical development[14,15]. To this end, Abzena has developed a suite of tools to evaluate the immunogenicity of a protein or antibody at the pre-clinical stage.

The evidence clearly supports the need for early assessment of immunogenicity, but how do you identify which candidates have issues and what they are? Just as there are multiple elements that contribute to the immunogenic impact of a biotherapeutic, there

are also multiple points for the assessment of immunogenicity. Figure 2 details the steps involved in the development of an immunogenic response, and the points which can be evaluated to assess this response. Specific tools and assays exploiting these different parameters of the immune response can be utilised to inform and evaluate the immunogenic potential of biopharmaceutical candidates.

Immunogenicity – What Can We Do?

Problems associated with immunogenicity to biopharmaceuticals, especially monoclonal antibodies, have been significantly reduced due to advances in molecular biology. There are, however, many recombinant protein biologics that are identical to endogenously expressed human sequences that still elicit potent neutralising immune responses in patients[8,16,17]. Regardless of how immunogenicity is triggered, one of the single most important factors in the development of an immune response is the presence of epitopes that stimulate a potent CD4+ T cell response.

T Cell Epitopes Drive Immunogenicity

CD4+ T cell epitopes are critical in driving T cell dependent immune responses to antigens. During the initiation of a T cell dependent immune response, antigen-presenting cells such as dendritic cells (DCs), capture and process antigens, including therapeutic antibodies as shown in Figure 1a, and present them in the form of short, linear peptides bound in the groove of the MHC class II (b). Binding of the T cell receptor to these MHC class II/peptide/complexes by CD4+ cells (c) triggers an activation cascade in which T cells proliferate, differentiate and provide help to B cells by producing costimulatory cytokines (e.g. IL-2 & IL-4) and by cell-cell contact (d).

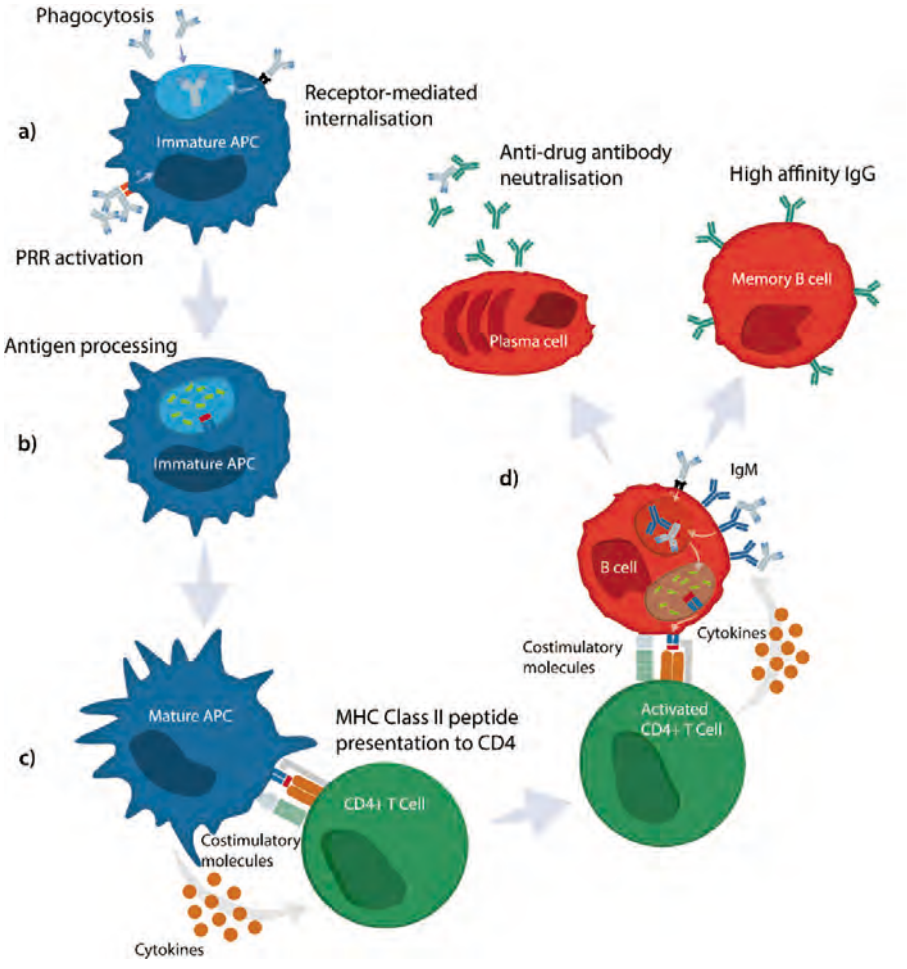

Figure 2: Schematic View of T Cell and B Cell Mediated Immune Response to a Therapeutic Antibody

The immune cascade, from antigen processing and presentation through to secretion of ADAs from plasma cells, provides multiple points to assess the immunogenic response to a biotherapeutic. Whilst in silico tools provide a rapid method for the evaluation of peptide MHC class II interactions and can be used for assessing large numbers of candidates, it is the *ex vivo* T cell assays that provide a qualitative and quantitative measurement of T cell epitopes. Here we focus on a sub-set of Abena's EpiScreen™ capabilities to exemplify how immunogenicity to a biopharmaceutical can be understood and mitigated.

We will discuss the role of four different assays from Abzena's EpiScreen™ suite; Figure 3 shows how the tests we will discuss fit into the flow of early biotherapeutic development. The EpiScreen™ suite includes three *ex vivo* immunogenicity assessment assays that utilise a panel of peripheral blood mononuclear cells (PBMCs) from 50 individuals. The panel is selected to ensure the distribution of HLA-allotypes is representative of the European/North American population and can be optimised to be representative of the intended population of the candidate drug, consistent with FDA and EMEA recommendations[14,15]. The three assays are the time course T cell assay, the DC:T cell assay and the T cell epitope mapping assays.

EpiScreen™ Time Course and DC:T Cell Assay.

The EpiScreen time course and DC:T cell assays are used for immunogenicity risk assessment of whole protein biologics. The time course assay is a PBMC-based assay whilst the DC:T cell assay loads DCs with whole protein for processing and presentation to autologous CD4 T cells. The DC:T cell assay is typically used for biologics that exhibit direct T cell pharmacology. Both assays assess the frequency and magnitude of responses (proliferation and IL-2 secretion) against the whole protein in the study cohort. Assessment of relative risk of clinical stage immunogenicity is achieved by benchmarking against clinically relevant controls included within the assay.

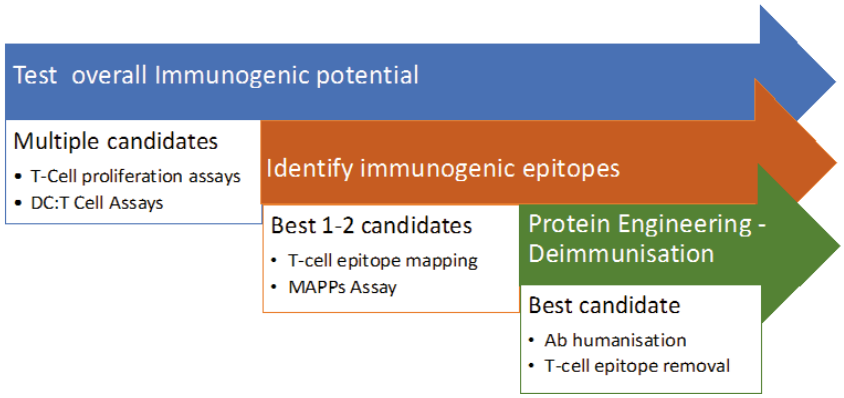

Figure 3: The application of Immonugenicity Testing and Protein Engineering in Early Biotherapeutic Development

Whilst these two assays provide an assessment of the immunogenic potential of the biotherapeutic, it is also important to understand which specific elements of the biotherapeutic elicit this T cell response for the purposes of protein engineering with the aim of reducing immunogenicity. Understanding which individual elements drive the immunogenicity in a biologic can be achieved by carrying out T cell epitope mapping assays, enabling the selection of better lead candidates and facilitating "deimmunisation" of the product to reduce the risk of clinical immunogenicity. EpiScreen™ provides two assays to address this question; the T cell epitope mapping and MHC class II associated peptide proteomics (MAPPs) assays respectively.

EpiScreen™ T cell Epitope Mapping

If identification and removal of the elements that drive immunogenicity is desired, then T cell epitope mapping studies can pinpoint the exact region of the biotherapeutic to engineer around. EpiScreen™ T cell epitope mapping assays identify CD4+ T cell epitopes within protein sequences by utilising 15mer peptides which span the test sample sequence. These assays provide quantitative (frequency of response to each peptide) and qualitative (magnitude of response to each peptide to compare their relative potency) measurements to map the location of T cell epitopes. From here a core 9mer peptide within the stretch of 15 amino acids can be identified and designated a T cell epitope. Once identified, the epitope itself can be deimmunised by including amino acid substitutions at key residues that reduce or abrogate binding to MHC class II molecules.

These assays allow for pre-clinical assessment of the immunogenic potential of whole molecules or by identifying T cell epitopes. Whole protein assays allow for natural processing and presentation of T cell epitopes in the context of MHC class II, however the data generated by these whole protein assays differs from that of assays using synthetic 15mer peptides, which can introduce cryptic epitopes – i.e. presentation of epitopes that are not naturally presented by APCs resulting in false positives and potential for over-engineering of a molecule if taken on their own. The value of these peptide-based MAPPs assays lies in their ability to generate a shortlist of potential T cell epitopes for testing in T cell mapping assays.

EpiScreen™ MAPPs Assay

The EpiScreen™ T cell epitope mapping assay is a comprehensive means of identifying putative T cell epitopes but relies on extracellular binding of peptides to MHC class II molecules on the surface of DCs. In contrast, MAPPs allow the identification of putative T cell epitopes captured on MHC class II molecules as a result of uptake and intracellular processing of whole protein – more reflective of the natural antigen presentation process.

For MAPPs, DCs are loaded with test proteins for processing and presentation of linear peptides in the groove of MHC class II. Following immunoprecipitation of the MHC class II/peptide complexes from the surface of the DC, the peptides are eluted and analysed by nano-LC-MS/MS. The peptides identified are typically different length variants that cluster together and share the same core HLA-DR binding motif. The peptides can then be synthesised and further characterised using T cell assays to determine which of them can elicit T cell proliferation for the purpose of protein re-engineering of the original biotherapeutic by de-immunisation to reduce the risk of clinical immunogenicity, with lower risk of over-engineering due to falsely identified T cell epitopes.

Appropriate Assay Format

Biopharmaceutical companies continue to develop novel biologics against new targets with new modes of action (MOA). For example, new immuno oncology drugs that target T cells directly and induce T cell proliferation may confound the proliferation observed through immunogenicity in a PBMC-based assay. In these instances, the DC:T cell assay is a more appropriate assay, potentially avoiding direct T cell pharmacology and only presenting linear peptides derived from the biologic to T cells. However, bi-specific antibodies that target T cells on one arm can target or tether to the surface of DC cells depending upon the specificity of the second arm, and this can confound this [DC:T] approach too. Such molecules can be present in the *in vitro* culture as intact molecules for the duration of the assay and exert pharmacology when the autologous T cells are re-introduced into the assay. In these instances, consideration of testing each arm individually or T cell epitope mapping the variable domains are options to consider. A final alternative strategy for such molecules would be to assess the naturally processed and presented peptides identified by MAPPs in a T cell peptide assay. Immunogenicity risk assessment assay formats will need to adapt and evolve as the biologics they are intended to assess evolve. Only by understanding the MOA of the biologic will it be possible to elect an appropriate assay format from the existing range of assays or

indeed develop new assays going forward.

Conclusion

The increase in the number of biotherapeutics being developed and approved across a range of therapeutic areas, has thrown a spotlight on the importance of understanding the potential immunogenicity profile of these drug types and the risk this might cause to patients.

Early immunological testing can provide data about the overarching immunogenicity of a candidate as well as identifying specific immunogenic sequences and epitopes to inform the design and selection of the best clinical candidate. Immunogenicity testing in early candidate selection and lead optimisation is a well-established strategy supported by the FDA and other regulatory bodies. The EpiScreen™ suite of assays provides data that can facilitate lead selection based on the immunogenicity of biotherapeutics, including mAbs and other human and non-human protein therapeutics. It can also confirm reduction in the immunogenicity risk following antibody humanisation and protein deimmunisation.

This article demonstrates that early intervention not only helps identify these risks, but opens the opportunity to mitigate those risks through a combination of improved candidate selection and the use of protein engineering techniques to deimmunise the lead candidate.

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