Sustained immune responses may affect efficacy and safety of protein therapeutics and share common determinants with tolerance mechanisms.

Immunogenicity of protein therapeutics and the interplay between tolerance and antibody responses

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Patients can mount sustained immune responses to protein therapeutics with the production of neutralizing antibodies (NAbs) that can compromise efficacy or safety of these drugs. Dendritic cells (DCs) are required for immunoglobulin (Ig) isotype switching and the production of IgG, a process involving presentation of MHC class II binding epitopes to helper T cells (CD4+ T cells) and subsequent B cell activation. DCs, CD4+ T cells and MHC class II binding epitopes are also involved in self-tolerance. While many assay formats are available for reliable antibody detection, the complex *in vivo* interplay between immunogenicity and tolerance hinders accurate pre-clinical predictions of protein drug immunogenicity to humans.

Introduction

Protein therapeutics generate billions of dollars in annual revenues for the pharmaceutical industry, can provide treatments for diverse diseases ranging from microbial infections to metastatic cancer and in some instances represent the only available therapy option. Therefore there is much interest in avoiding unwanted side effects derived from the unexpected immunogenicity of these drugs, which may decrease their efficacy and safety. Although immunogenicity may involve production of several immunoglobulin (Ig) isotypes, causing allergic reactions or even anaphylactic shock [1–3], our discussions here will focus on neutralizing antibodies (NAbs) of sustained immune responses, typically of the IgG class. NAbs to protein drugs that have an endogenous counterpart may have severe consequences. Such has been the case for recombinant human thrombopoietin (TPO) [4] and erythropoietin (EPO) [5]. Healthy volunteers mounting anti-TPO NAb responses during clinical trials developed thrombocytopenia [4,6], and anti-EPO NAbs caused pure red cell aplasia [7] because those Abs neutralized activity of the endogenous protein. The presence of anti-IFN-β NAb in multiple sclerosis patients affects the efficacy of this drug [8]. Besides, the development of Axokine® (a modified form of ciliary neurotrophic factor) for weight loss was discontinued after approximately two thirds of the patients developed anti-Axokine NAbs during clinical trials [9].

There are some indications that helper T cells expressing the CD4 marker (hereafter referred as CD4+ T cells) are necessary for class switching and affinity maturation, leading to strong antibody responses with the production of NAbs of the IgG class [10–12]. Antigen presenting cells (APCs), such as dendritic cells (DCs) and B cells, uptake and process proteins and present antigenic peptides (epitopes) bound to MHC class II proteins to CD4+ T cells. Epitope recognition by the T cell receptors (TCR) in the presence of co-stimulatory molecules triggers activation of CD4+ T cells and leads to activation of B cells and their subsequent differentiation into Ab-producing plasma cells. In normal circumstances, the antigenic determinants of the endogenous proteins are involved in self tolerance, keeping an exquisite balance *in vivo* which prevents individuals from mounting sustained antibody responses to their own proteins, despite possessing epitopes that can bind to MHC molecules [10,13].

Much progress has been made in developing and validating assays to detect Abs against protein drugs [14,15]. However, advances in predicting and eliminating immunogenicity in the absence of human data lag behind due to several factors, including difficulties in consistently standardizing assays with T cells from human blood, lack of extensive clinical data correlating with

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predictive models and the interplay between immunogenicity and tolerance. Significant progress in this area would involve not only the availability of robust assays with enough sensitivity and reproducibility, but also further unraveling the complexities of human pathways leading to antibody production. Here we provide brief summaries of the science underlying essential aspects of sustained immune responses against therapeutic proteins and review the latest information in the field, including currently available techniques for immunogenicity detection and prediction. We also scrutinize clinical data available for patients with autoimmune diseases or mounting antibody responses to protein therapeutics and how it relates to tolerance or immunogenicity.

Association between MHC class II binding epitopes and immunogenicity of therapeutic proteins

Several cell types expressing MHC molecules can present peptides to T cells, including macrophages [16], γδ-T-cells [17], dendritic cells (DCs) [12] and B cells [18]. Among the known APCs, DCs and B cells have a well-established role in antigen presentation to CD4+ T-cells [19], leading to sustained immune responses. DCs have a dual role: they initiate adaptive immune responses and also induce tolerance and T cell anergy, preventing autoimmune disorders [12,20]. DCs induce the activation of resting CD4+ T cells, a process known as priming. Although DCs alone can prime T cells, B cells can subsequently enhance T cell expansion [21]. Following uptake and processing of antigens by APCs, antigenic peptides are displayed on the cell surface bound to MHC class II proteins [22]. T cell activation requires interaction of the T cell receptor (TCR) with the peptide-MHC complex, and also interactions between co-stimulatory molecules such as CD28 and CD80/CD86 [23,24]. Activated CD4+ T cells induce B-cell growth and antibody production [12]. B cells produce five major classes of antibodies: IgA, IgD, IgE, IgG and IgM. The B-cell antibody response can be T cell-independent – with the production of IgM, the early Ab [25] – or T cell mediated, with isotype switching and the production of IgG, the major immunoglobulin in serum [11,26].

Self-protein immunogenicity: Is there more to the MHC class II binding epitope history?

Immune responses to human protein therapeutics often include the formation of binding Abs and NAbs (Table 1). Based mainly on clinical data available for autoimmune diseases and vaccines, it was postulated that MHC class II binding epitopes are essential components for the production of Abs against therapeutic proteins and an array of methods for identifying and removing those epitopes were developed [27,28]. More recently a clinical study with multiple sclerosis (MS) patients identified an association between anti-IFN-β Ab responses and the MHC haplotype DRB1*0701-DQA1*0201; a lack of association was observed between IFN-β immunogenicity and DRB1*1501 [13]. By contrast, previous work using an *in vitro* T cell assay and an animal model concluded that the HLA-DR2 haplotype (HLA-DR15+/HLA-DQ6+) was associated with IFN-β immunogenicity and identified a prominent IFN-β epitope that binds to DRB1*1501 [29,30]. IFN-β epitopes binding to the MHC DRB1*1501 molecule were also identified with a binding assay [31], and various epitopes binding to this MHC protein can be predicted with one of several *in silico* computer-based algorithms. The discrepancy between the IFN-β clinical data and results from *in vitro* assays or predictive tools may arise from the complex interplay of immunogenicity and tolerance that takes place *in vivo*. It is interesting to notice that the IFN-β epitope which activated *in vivo* primed T cells from multiple sclerosis patients binds weakly to DRB1*0701 in an *in vitro* assay [13] suggesting that high affinity DRB1*0701 binding epitopes were not involved in the anti-IFN-β antibody response (Figure 1). This rationale would be consistent with conclusions obtained for Graves disease (GD), which is associated with development of antibodies against the thyrotropin receptor – TSHR [32]. HLA-DR3 and HLA-DQA1*0501 predispose to GD, while HLA-DR7 has a protective effect. *In silico* and *in vitro* studies using purified HLA proteins and *in vitro* primed cells from GD patients indicate that TSHR epitopes bind to HLA-DR3 and HLA-DR5 with moderate affinity and to HLA-DR7 with high affinity [32]. Also in support for a role of high affinity of binding epitopes in tolerance, intravenous administration of a myelin basic protein (MBP) immunodominant epitope ($_{85}$VVHFFKNIVTP$_{96}$) induced tolerance in multiple sclerosis (MS) patients [33]. MS is an inflammatory autoimmune disease of the central nervous system (CNS) in which patient CD4+ T cells recognize MBP peptides in an MHC class II restricted manner, mounting an immune response to MBP [34,35]. In addition, HLA-DQ0602 (HLA-DQA1*0102/DQB1*0602) is associated with dominant protection in insulin-dependent diabetes mellitus (IDDM) [36].

Immune responses against protein therapeutics are well documented but in most instances genetic data is not available and patients were not reportedly HLA typed during clinical trials or in the normal course of treatment (Table 1). The scarcity of clinical data makes it difficult to assess the validity of pre-clinical immunogenicity prediction tools for risk assessment and de-immunization strategies.

Non-self protein therapeutics, antibodies and antimicrobial vaccines

The human body recognizes non-self protein therapeutics as foreign and therefore immune responses to them may resemble those produced during infections or against antimicrobial vaccines. Adaptive immune responses are the basis for vaccination, a process first utilized approximately two hundred years ago, although the science behind it was not understood at the time. Both B cells and CD4+ T cells are central to the adaptive immune responses that include the production of antibodies by activated B cells. Some of the most effective antimicrobial vaccines, including anti-tetanus and anti-diphtheria, are based on inactivated exotoxins. In addition, high immunogenicity has also been observed with non-self protein therapeutics, such as bacterial Streptokinase, one of the therapeutic agents currently used to treat myocardial infarction [37]. Some first generation murine therapeutic monoclonal Abs were also very immunogenic. The subsequent use of human–mouse chimeric, humanized or human antibodies generally decreased, but not completely eliminated, the incidence of immunogenicity. For example, the murine anti-cancer antibody 17-1A (Panorex; Edrecolomab), which targets EpCam, elicited antibody responses in 41/43 patients at doses varying from 0.2 to 2 g [38]. In another clinical trial, 13/14 patients receiving a combination of interferon gamma and 0.4 g of 17-1A developed anti-17-1A antibodies [39]. By contrast, MT201, a fully human

TABLE 1

Implication of MHC class II binding epitopes on self protein immunogenicity and autoimmune diseases

		Clinical				Non-clinical		
		Antibodies	**MHC class II association**	**T cell epitopes**	**Refs**	**T cell assays (epitopes)**	**Binding assays (epitopes)**	**Refs**
Proteins therapeutics	IFN-β	Anti- IFN-β NAbs	DRB1*0701 and IgG	nd	[13,62]	aa118-132/DRB1*1501 aa121-165/DRB1*0701	Several BE	[13,29–31]
	IFN-α	Anti-IFN-α NAbs	nd	nd	[77]	BE regions	nd	[78]
	EPO	Anti-EPO NAbs	nd	nd	[79]	Several BE	Several BE	[31]
	TPO	Anti-TPO NAbs	nd	nd	[4,6]	nd	nd	na
	FVIII	Anti-FVIII NAbs	DRB1*1501/DRB5*01 haplotype and NAB	nd	[80,81]	BE aa2098-2112(C1) aa2144-2161(C1) aa2191-2220(C2)	BE aa2098-2112(C1) aa2144-2161(C1)	[80,82,83]
	AXO	Anti-AXO NAbs	nd	nd	[9]	nd	nd	na
Autoimmune diseases	MS	Anti-MBP	DRB1*1501/DQA1*0102/ /DQB1*0602 haplotype and MS	aa83-102 Epitope Spreading	[34,84–86]	aa81-99/DR2a/DR2b aa131-159/DR2a	Several BE	[44,86–89]
	IDDM	Ant-GAD65 Anti-IA-2 Anti-insulin	HLA-Q, DR and IDDM	nd	[90–95]	Pro-insulin-BE Insulin peptide B:9-23 (DOB mice) Insulin A peptide 1-15 (DR4 restricted)	BE from GAD65, IA-2 and insulin	[31,36,96–100]
	RA	Antivarthritogenic Ag	DRB1*0401, haplotype c1 and RA	nd	[101–104]	BE Citruline-containing peptides	BE Citruline-containing peptides	[104–106]
	GD	Anti-TSHR	DQA1*0501, DR3 and GD	nd	[107–109]	Several BE	Several BE	[32]

Abbreviations: IFN-β, interferon beta; IFN-α, interferon alpha; EPO, erythropoietin; TPO, thrombopoietin; FVIII, factor VIII; AXO, Axokine®; MS, multiple sclerosis; IDDM, insulin dependent diabetes mellitus; RA, rheumatoid arthritis; GD, graves disease; nd, not determined (to our knowledge); aa, amino acid; C1 or C2, factor VIII domains; TSHR, thyrotropin receptor; BE, MHC class II binding epitope; MBP, myelin basic protein; GAD65, glutamic acid decarboxilase65; IA-2, islet associated-2; DQ0602, HLA-DQA1*0102/DQB1*0602; haplotype c1, DQB1*0201-DRB1*0301-D6S1014*143-D6S273*139-D6STNFa*99-MIB*350-C1-2-5*196; Epitope, amino acid sequence binding to HLA molecules; Ag, antigen; na, not available (to our knowledge).

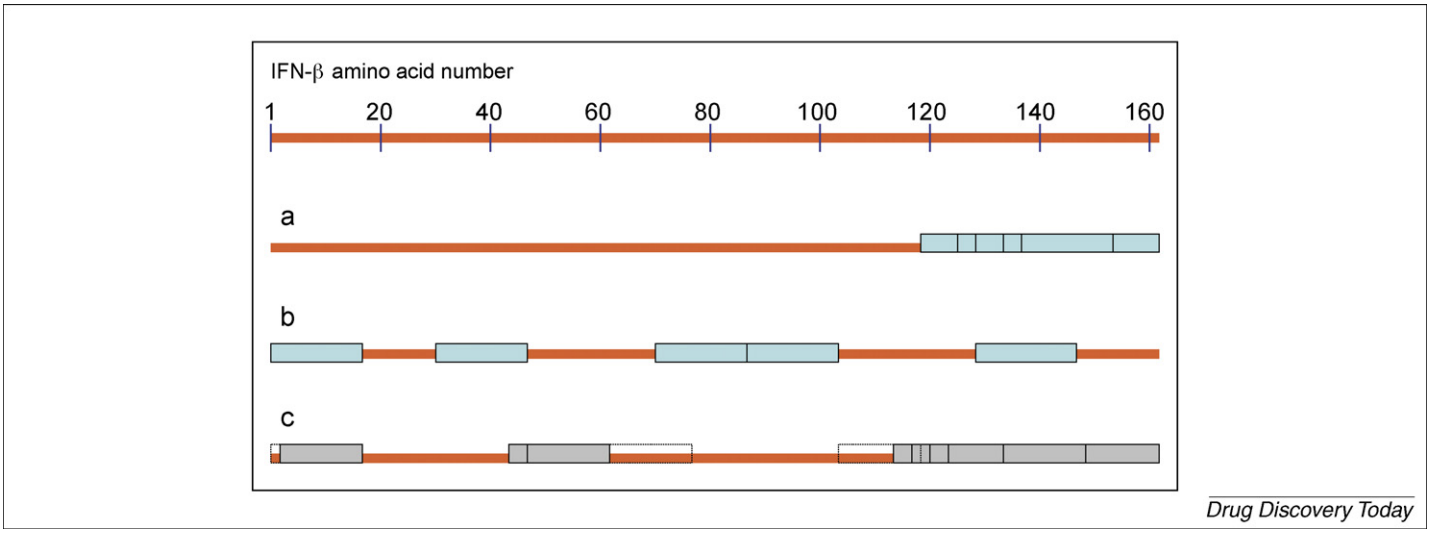

FIGURE 1

IFN-β epitopes that bind to DRB1*0701 determined with different techniques. **(a)** Elispot assay with *in vivo* primed T-cells from anti-IFN-β antibody positive patients [13]; **(b)** *In vitro* binding assay using a B cell line expressing DRB1*0701 [13]; **(c)** Peptide binding assay using purified HLA protein [31]. The boxes represent amino acid regions containing IFN-β epitopes.

monoclonal Ab that also targets EpCam, displayed lower levels of immunogenicity to humans [40]. Several clinically tested approaches are available to engineer therapeutic antibodies with decreased immunogenicity. Human antibodies have been rescued from immune donors and can also be obtained from phage display libraries or using transgenic mice. However, even human antibodies can elicit Ab responses in patients [41].

Epitope spreading

The process of epitope spreading, in which immunogenicity is triggered by antigenic determinants distinct from the epitope involved in the initial immune response, has been reviewed in detail elsewhere [42]. With regards to the topic discussed here, epitope spreading has been observed in several instances of autoimmune disease progression. It has been involved with the progression of MS [34] and has been observed during disease advance in two demyelinating animal models of MS [experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus-induced demyelinating disease-TMED-IDD] [35,43,44]. Epitope spreading was also documented with cancer vaccines [45] and during the induction of experimental autoimmune glomerulonephritis in rats immunized with a peptide from the alpha3(IV)NC1 domain of type IV collagen [46]. It is conceivable that a similar process might occur in the course of antibody responses to protein drugs, although it has not been investigated. Epitope spreading in the context of antibody responses against protein drugs may have major implications for our capability to effectively use clinical data to evaluate the accuracy of pre-clinical immunogenicity predictions.

Imunogenicity versus tolerance induction

Tolerance mechanisms allow distinction between self and foreign elements, eliciting protection and yet avoiding autoimmune reactions. Central tolerance in the thymus is responsible for eliminating self-reactive T cells; the ones escaping negative selection in the thymus are subject to peripheral tolerance, including anergy [47,48]. DCs can elicit immunogenicity or tolerance during the process of antigen presentation [10,20,49,50]. To avoid immune reactions, protein therapeutic regimens leading to tolerance have been utilized. Administration of high doses of Factor VIII to haemophilia may lead to tolerance to this, otherwise very immunogenic, protein drug [51,52]. Tolerance induction regimens have also be used for therapeutic antibodies [53,54].

Product and host-related factors involved in the immunogenicity of protein therapeutics

As discussed previously, the amino acid sequence of a protein drug, that is, the presence of epitopes capable of binding to MHC class II proteins is necessary – although not alone sufficient – for a sustained immune response and the production of IgG. Other factors that may affect the *in vivo* balance between immunogenicity and tolerance given the same protein sequence include (a) drug manufacturing specifications (e.g., formulation, aggregation), (b) drug administration protocols (e.g., dose, route and frequency of administration), (c) immunocompromised patients and (d) genetic predisposition of the individuals receiving the drug. The human gene complex coding for MHC class II proteins includes three loci (HLA-DR, -DQ and -DP), each containing genes for the alpha and beta subunits of an MHC molecule. MHC proteins vary in their antigen binding specificities and, therefore, individuals may respond differently to a given antigen, depending on their HLA type. As noted above, a clinical study with multiple sclerosis (MS) patients undergoing IFN-β therapy identified a strong association between the development of anti-IFN-β antibodies and the haplotype DRB1*0701-DQA1*0201 [13]. That same study also observed a positive anti-IFN-β antibody response with other alleles. Four out of five MS patients with the allele DRB1*1301 were antibody positive in a direct capture Elisa assay, and five out of five were positive in an indirect capture ELISA [13]. However, because those alleles were rare among the MS study population, a larger number of samples would be required to perform analysis reaching statistical significance. A study with

haemophilia A patients treated with Factor VIII identified a weak association between anti-Factor VIII antibodies and DQA1*0102 [55]. Besides, haemophilia patients with certain mutations of the Factor VIII gene are prone to mounting anti-Factor VIII Nab responses [56]. The inclusion of HLA typing tests during clinical trials of protein drugs or post-marketing may unravel other associations between HLA types and immunogenicity. Subjects genetically predisposed to developing anti-drug antibodies should be more closely monitored and perhaps considered for alternative treatments. Moreover, a broader knowledge about cellular mechanisms involved in protein immunogenicity may aid the development of pre-clinical tools for risk assessment.

There is scarce information about other determinants of protein immunogenicity, such as the production system and glycosylation (mammalian cells or bacteria used for production of recombinant proteins), formulation, denaturation, aggregation, dose and length of treatment and route of administration [3,57–60]. Process specifications, impurities, formulation, route and frequency of administration and dosage represent extrinsic risk factors that may influence immunogenicity but are not a direct consequence of epitope removal approaches [27,61]. Propensity of the protein to aggregate may represent an intrinsic risk factor eliciting NAb responses, which in some cases can be minimized by the formulation. Different preparations of the same IFN-β amino acid sequence – Avonex and Rebif – present differential immunogenicity to humans [62]. Because of ethical issues regarding tests in humans, animal models are likely to be utilized further to study the effect of aggregation in protein immunogenicity. Antibody responses to aggregated recombinant human Factor VIII (rFVIII) were investigated in haemophilia A mice, and the results suggested that the aggregated protein acted as a distinct antigen [59]. Immuno tolerant mice expressing human IFN-α2b have been used to test the effect of aggregation and other modifications on IFN-α2b immunogenicity. [57,58,63]. Clinical and pre-clinical assessments of a role for aggregation in therapeutic protein immunogenicity have been reviewed elsewhere [3].

Pre-clinical immunogenicity predictions and protein de-immunization

Some fundamental questions that need to be addressed before undertaking a project to de-immunize a therapeutic protein for use in humans are (a) how will immunogenicity be assessed during clinical trials? (b) If a naturally occurring protein has already been tested in the clinic, was its immunogenicity high enough that changes could be detected with the limited number of patients used for clinical trials? (c) Are there alternative treatments for the disease and do the possible benefits to patients outweigh the risks involved? (c) Is there clinical data for the therapeutic protein in question to guide pre-clinical de-immunization efforts? When testing an association between an HLA type and antibody responses, the total number of patients needed to perform analysis reaching statistical significance depends on (a) the percentage of patients that have the allele being tested and (b) the percentage of the study population developing antibodies against the test drug [13]. What follows is that for biotherapeutics such as EPO, which elicited antibody responses in a small percentage of patients, a very large study population would be necessary to perform analysis reaching statistical significance. This undermines the practicality of developing less immunogenic EPO variant proteins [31], as immunogenicity may not be detected with a limited number of patients used for clinical trials. Moreover, modification of the native protein sequence may lead to increased immunogenicity, which in turn may be ameliorated by production specifications including formulation [61]. For proteins such as Axokine®, which elicited antibody responses in about two thirds of the patients [9], genetic data from clinical trials might be a more useful tool to guide either de-immunization efforts or patient selection. Some considerations about the intrinsic nature of the protein may be used for assessing risk to patients in the event of a NAb response, either to the native protein or to a modified version.

Epitope identification and removal

Once basic issues have been addressed and a decision made to de-immunize a protein by epitope removal, techniques that can be used to identify epitopes include *in silico* prediction algorithms [27,28,64], *in vitro* peptide binding assays [13], T cell activation assays [13,29], tetramer analyses [65], artificial antigen presenting chips [66] or peptide elution and identification following protein uptake, processing and presentation by antigen presenting cells [67]. Several epitope prediction tools are available in public databases or can be purchased from vendors [27,28,68,69]. *In vitro* peptide binding assays can use MHC proteins-purified from B cells or produced as recombinant proteins – or whole cells expressing MHC proteins on their surface [13,70,71]. Assays measuring T cell responses typically monitor cytokine production – ELISPOT [72], intracellular cytokine staining, ELISA – or T cell proliferation by any of several methods, including [^{3}H]-thymidine incorporation [29], flow cytometry [73], CFSE labeling [74] and BrdU incorporation[75]. Choices are made according to specific situations. T cells can be primed *in vitro* or *in vivo* and in the latter case the assay is performed with cells from patients treated with the drug.

Typically, algorithms or binding assays overpredict MHC binding epitopes without accounting for interactions with TCRs. Moreover, epitopes that bind to a TCR leading to T cell activation *in vitro* do not necessarily trigger antibody responses *in vivo*, as related mechanisms are involved in tolerance. Techniques identifying epitopes that are presented by DCs *in vitro* following uptake and processing of proteins might identify high affinity binding epitopes, which, as reviewed above, may be involved in tolerance rather than immunogenicity of the protein. Considering all the uncertainties of currently available techniques in face of the complexities of the immune system, clinical data from patients – particularly associations between HLA types and Nab responses – that are not immunocompromised might represent the most reliable alternative to guide pre-clinical de-immunization attempts (Figure 2). This would be specially relevant in cases when Nab responses stop development of otherwise promising drugs [9]. Besides, performing HLA typing during clinical trials would expand our knowledge of the subject. Obtaining valid associations would involve performing the analysis with a fraction of the patients treated with the drug that are not immunocompromised. In cases when a very large percentage of patients develop antibodies against the protein drug it may be feasible early during clinical trials to identify HLA associations reaching statistical significance [13].

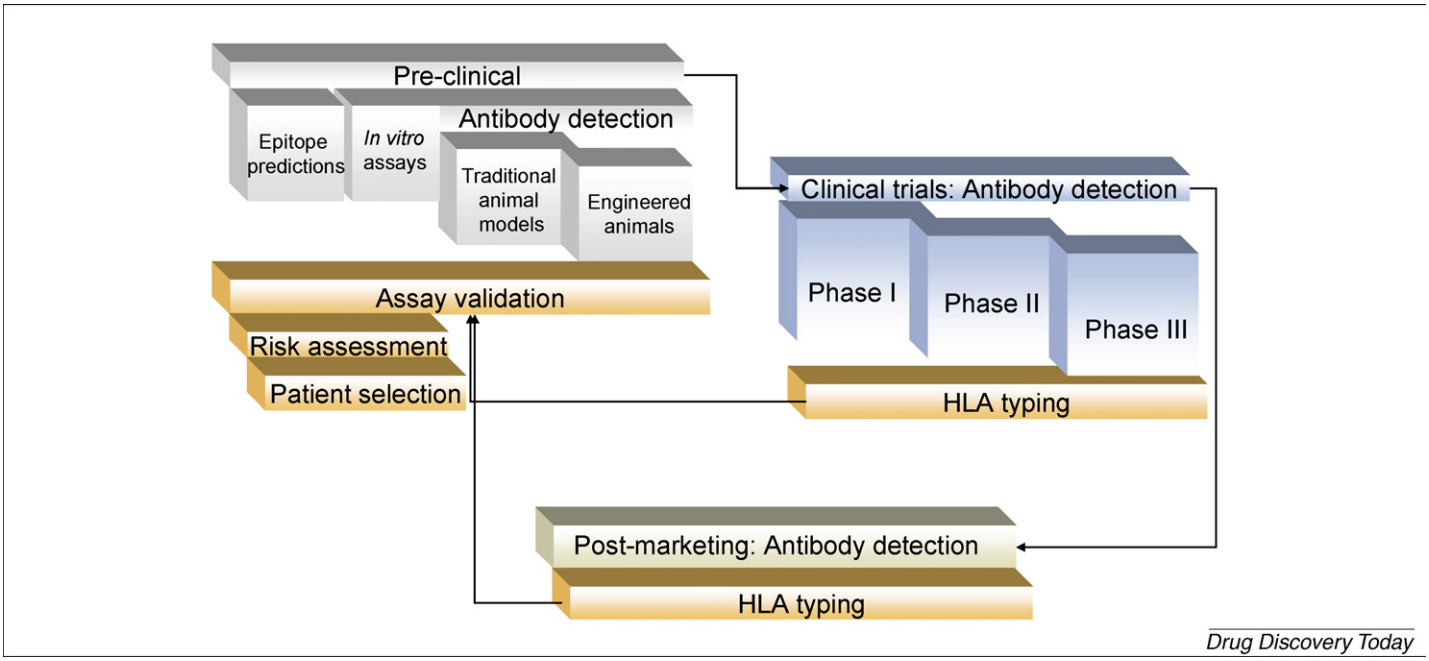

FIGURE 2

Assessing immunogenicity of protein therapeutics: validation of pre-clinical immunogenicity predictions requires clinical data. HLA typing of patients during clinical trials and post-marketing of protein drugs allows testing associations between antibody responses and MHC class II alleles. HLA associations might lead to rational selection of patients for therapy and guide de-immunization attempts.

Detecting antibody responses to protein therapeutics

While we are only now starting to gain insights into humoral immunogenicity of protein biologics that might lead to effective pre-clinical immunogenicity predictions and de-immunization strategies, much progress has been made in accurately detecting antibody responses in humans. Recommendations for the design, optimization and qualification of assays used to detect both non-neutralizing Ab and NAb responses against biotherapeutics are available [14,15]. Often, immunoassays are used for primary screens and samples yielding positive results are subsequently tested for the presence of neutralizing antibody using an *in vitro* cell-based assay or a non-cell-based competitive ligand-binding assay. Drug interference is one of the hurdles requiring careful consideration during the choice of assay [13,14,76].

Conclusions

Significant advances have been made regarding development and standardization of assays to measure antibody responses to protein therapeutics and also understanding the cellular pathways involved in the immunogenicity of these drugs. While associations between MHC class II alleles and sustained antibody responses to protein drugs have been documented, antigen presentation by DCs to CD4+ T cells is involved in both immunogenicity and tolerance. The co-existence of these two mechanisms utilizing the same pathway adds to the difficulties associated with pre-clinically predicting immunogenicity of protein therapeutics. Clinical data and HLA typing of patients treated with protein drugs may provide important information guiding patient selection and de-immunization attempts.

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