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## REVIEW

# T-cell dependent immunogenicity of protein therapeutics: Preclinical assessment and mitigation



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**KEYWORDS**

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**Abstract** Protein therapeutics hold a prominent and rapidly expanding place among medicinal products. Purified blood products, recombinant cytokines, growth factors, enzyme replacement factors, monoclonal antibodies, fusion proteins, and chimeric fusion proteins are all examples of therapeutic proteins that have been developed in the past few decades and approved for use in the treatment of human disease. Despite early belief that the fully human nature of these proteins would represent a significant advantage, adverse effects associated with immune responses to some biologic therapies have become a topic of some concern. As a result, drug developers are devising strategies to assess immune responses to protein therapeutics during both the preclinical and the clinical phases of development. While there are many factors that contribute to protein immunogenicity, T cell- (thymus-) dependent (Td) responses appear to play a critical role in the development of antibody responses to biologic therapeutics. A range of methodologies to predict and measure Td immune responses to protein drugs has been developed. This review will focus on the Td contribution to immunogenicity, summarizing

**Abbreviations:** Td, T-cell dependent, thymus dependent; T, thymus; ADA, anti-drug antibodies; Ti, T-cell independent; APC, antigen-presenting cells; HLA, human leukocyte antigen; MHC, major histocompatibility complex; TCR, T cell receptor; Treg, regulatory T cells; FVIII, factor VIII; nTregs, natural regulatory T cells; aTreg, adaptive regulatory T cells; iTreg, induced regulatory T cells; IEDB, Immune Epitope Database Analysis Resource; IC<sub>50</sub>, 50% inhibitory concentration; ELISpot, enzyme-linked immunosorbent spot-forming; ELISA, enzyme-linked immunosorbent assay; CFSE, carboxyfluorescein succinimidyl ester; PBMC, peripheral blood mononuclear cells; ALN, artificial lymph node; ORG, unmodified original epitopes; FPX, recombinant Fc fusion protein; SFC, spot-forming cells.

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current approaches for the prediction and measurement of T cell-dependent immune responses to protein biologics, discussing the advantages and limitations of these technologies, and suggesting a practical approach for assessing and mitigating Td immunogenicity.

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## 1. Introduction

### 1.1. The immunogenicity of protein therapeutics

Since the approval of the first recombinant biological therapeutic, insulin, in October 1982, more than 165 biotherapeutic

agents have entered the marketplace and have generated an estimated \$99 billion in sales worldwide [1–4]. Therapeutic biologics offer the advantages of increased specificity and reduced toxicity compared to small molecules. However, when administered to patients, these protein-based drugs have the potential to elicit immune responses that may directly impact drug safety, efficacy, and potency. For example, anti-drug

antibodies (ADA) that develop in response to a therapeutic protein may alter the drug's pharmacokinetic profile and abrogate its pharmacodynamic effect (neutralizing activity) [5–8].

Immune responses to proteins are characterized by the generation of antibodies (humoral immune response) that could be T cell dependent or independent. T-independent antibody responses may be generated when B cells recognize a repeated pattern (motif) in the therapeutic protein and respond by transiently producing low-affinity, predominately IgM antibodies [9]. Antibodies that are generated in conjunction with T cell help are referred to as T cell-dependent or thymus-dependent (Td) antibodies. This process, described in the next section, involves a complex interplay among antigen presenting cells, T cells, secreted cytokines, and B cells, emphasizing the importance of genetic factors such as HLA haplotype expression and T cell/B cell repertoire in immune responses to administered proteins. Thus, measurement of ADA IgG responses usually indicates that T cells are involved in the immune response to the protein. Moreover, a number of clinical studies now suggest that high levels of T cell-driven IgG ADA have the potential to cross-react with the endogenous counterpart, an adverse effect that can have serious consequences [10–12].

T cell responses contribute to the generation of ADA. Proteins with therapeutic potential are produced in cell lines that are derived from a variety of sources, including mammals (both human and non-human), insects, bacteria, plants, yeast, and viruses. Small differences in the protein sequence and/or post-translational modifications (e.g., glycosylation, oxidation, deamidation, acylation, and alkylation) can contribute to the immunogenic potential of the therapeutic protein. Furthermore, the manufacturing process may involve multiple steps (e.g., regulation of gene expression, purification, concentration, formulation, and long-term stabilization) to produce recombinant proteins of sufficient quantity and quality to meet clinical release criteria. At each of these steps, there is potential for the introduction of biochemical or biophysical modifications into the molecules that may influence the immunogenicity profile of the biologic product. While these aspects of protein production may not impact function of the therapeutic protein, modifications inherent in the manufacturing process can have a major impact on host immune responses. For example, consider the case of Erbitux, a protein with non-human type glycosylations that caused anaphylaxis in selected patients who had pre-existing antibodies targeting these carbohydrate structures [13,14]. Host cell proteins derived from recombinant protein-producing cell lines may co-purify with a therapeutic product to become part of the final formulation [15,16]. These impurities, even in small quantities, have the potential to stimulate an unwanted immune response. In at least one recent case, they induced anti-host cell protein antibodies and contributed to the suspension of a clinical trial [15,16].

So as to provide biologics developers with a structured approach to measuring immunogenicity risk, the European Medicines Agency (EMA) has published a "Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins" [17], in which factors influencing the immunogenicity of therapeutic proteins were classified

into patient-, disease-, or product-related categories. The patient- and disease-related categories describe factors that may pre-dispose a particular individual to an immune response. In contrast, product-related factors, i.e. factors intrinsic to the final drug product itself that contribute to immunogenicity, may include modifications in the glycosylation profile [18–20], biophysical and biochemical attributes [21–24], or factors introduced during formulation [14,20,25,26]. Table 1 summarizes some of these patient-, disease-, and product-related factors that have the potential to influence immune responses to a biologic therapeutic. Many of the factors described in Table 1 that might predispose a therapeutic protein to be immunogenic have also been identified as "critical quality attributes" in the FDA-sponsored Quality-by-Design initiative [27] focused on manufacturing "process development". For protein-based drugs, the primary amino acid sequence itself can be a strong determinant of immunogenic potential. Unfortunately, primary sequence is difficult, if not impossible, to modify in the manufacturing process.

Although many protein drugs are associated with ADA, as mentioned above, only a few have been associated with severe adverse events such as profound anemia or thrombocytopenia when the antibodies cross-reacted with the drug's endogenous counterparts [10,11,28]. These types of serious outcomes resulting from cross-reactive ADA have inspired the development of a multitude of in vitro methods for measuring the presence of ADA, which have been carefully reviewed in several white papers and regulatory guidance documents [17,22,29–33]. In addition, methods for identifying drivers of Td immune responses have also expanded and have been described in a number of publications [34–42].

The goal of this paper is to review recent developments in the understanding and identification of Td immune response

**Table 1** Factors that can influence immune responses to a therapeutic protein.

FACTORS	IMPLICATIONS
Intrinsic immunogenic dominant epitopes	Higher risk of immunogenicity based on reactivity to HLA (DR, DP, DQ) alleles
Sequence differences between therapeutic protein and endogenous protein	Cross-reactive neutralizing antibodies to endogenous proteins
Differences in glycosylation patterns. Structural alterations induced by storage condition, production/purification and formulation	Breaking of tolerance due to <ul style="list-style-type: none"> <li>- Aggregation</li> <li>- Oxidation</li> <li>- Deamidation and degradation</li> <li>- Conformational changes</li> </ul>
Route of delivery <ul style="list-style-type: none"> <li>- Route/frequency of administration</li> <li>- Syringe environment and associated reconstitution</li> <li>- Non-physiologic concentrations</li> </ul>	<ul style="list-style-type: none"> <li>- Differences in antigen presentation following intravenous and subcutaneous delivery</li> <li>- Presence of leachates from syringe (i.e. tungsten or silicone) can facilitate immune responses</li> <li>- Exposure to high dose proteins in formulations can lead to an immune response</li> </ul>
Patient-related factors <ul style="list-style-type: none"> <li>• Immune status <ul style="list-style-type: none"> <li>- Immunosuppression due to supportive therapies</li> <li>- Autoimmune-diseased state</li> <li>- Genetic features like HLA haplotypes</li> </ul> </li> <li>• Intensity of treatment at the first dose</li> <li>• Age</li> </ul>	<ul style="list-style-type: none"> <li>- Immune status can enhance or reduce the likelihood of an immune response</li> <li>- Presence of HLA alleles that have high potential to bind to immune dominant epitopes</li> </ul>
Host Cell Proteins (HCP) <ul style="list-style-type: none"> <li>• Recombinant protein therapeutics produced in mammalian cell lines may contain process-related contaminants.</li> </ul>	The homology of HCP with human proteins raises concern over the inherent risk of anti-self immune responses.

drivers, and to organize these into a framework to approach the prediction and measurement of Td immune responses as one component of a risk assessment strategy. Case studies will be presented to illustrate Td immunogenicity and the practical application of these methods. An evidence-based roadmap is proposed for identifying Td responses in protein therapeutics and developing criteria for assessing immunogenicity by (i) sequence-driven assessment using *in silico* algorithms, (ii) *in vitro* assays, and (iii) *in vivo* models. Lastly, we touch on the emergence of methods for mitigating Td immunogenicity, such as de-immunization and tolerance induction.

## 2. The central role of T cells in immunogenicity

### 2.1. The T cell contribution to antibody responses

Immune responses to therapeutic proteins can be broadly divided into two general categories. The first involves activation of the classic adaptive immune system by what are considered to be “foreign” proteins, like the response elicited against pathogens, vaccines, or allotypic antigens. Replacement proteins would be viewed as foreign to the immune system of an individual who is lacking, in whole or in part, the endogenous counterpart. The second involves a breach of B and/or T cell tolerance, like the response elicited to autologous self-proteins in certain autoimmune diseases. The mechanisms for the breach of immune tolerance are not as well defined as the mechanisms for immune induction to microbial infections, but may include epitope mimicry, cross-reactivity of T cells, presence of trace levels of innate immune activators such as toll-like receptor agonists [25,43,44], and/or multivalency as might be presented by aggregated proteins [45].

For the classic immune pathway, production of anti-therapeutic protein responses is the culmination of a series of events that eventually leads to B cell activation and antibody secretion. This antibody production can be thymus independent [T-cell independent, (Ti)] or Td in origin [46,47]. B cells are activated in a Ti manner when particular structural patterns, such as polymeric repeats or carbohydrate molecules, directly induce activation of B cells. Ti activation of B cells can be distinguished from Td activation, as the antibodies resulting from Ti activation are limited in both isotype and affinity and if memory B cells are generated they are not long-lived [48,49]. In contrast, Td activation of B cells is characterized by class switching (IgM to IgG) and development of memory B cells that produce higher-affinity, more robust, and longer-lived antibody responses. The development of IgG-class antibodies following administration of a biological protein generally indicates that the therapeutic is driving a Td immune response.

Td responses, by definition, are contingent upon T cell recognition of therapeutic protein-derived epitopes through the basic processes of protein antigen processing and presentation. The therapeutic protein is taken up by antigen-presenting cells (APC) and proteolytically processed into small peptides. Certain of these peptides will bind to human leukocyte antigen (HLA)/major histocompatibility complex (MHC) class II molecules, where interactions of

sufficiently high stability with MHC will facilitate presentation of the peptide/MHC complex on the APC cell surface<sup>3</sup> [50]. Because human populations express a number of different HLA class II alleles, the interaction between antigenic epitope and HLA may exhibit a full spectrum of binding stabilities. HLA polymorphism and its impact on the binding of specific peptides (HLA restriction) are primary mechanisms by which patient genetics contributes to immune responses to particular protein therapeutics. Examples of HLA-associated immune responses to therapeutic proteins are provided below.

CD4<sup>+</sup> T cell recognition of a specific antigen-MHC class II complex, combined with co-stimulatory signals delivered by the APC, culminates in robust T cell activation that in turn facilitates a mature antibody response. In the absence of activated T helper cells, naïve B cells do not fully mature, and activated antigen-specific B cells are rendered anergic or undergo apoptosis. Therefore, T cell recognition of peptide epitopes derived from a protein therapeutic is the key determinant of Td antibody formation.

### 2.2. T cell epitope stability and immunogenicity

Immunogenicity prediction is based on well-defined interactions between the amino acids comprising a protein sequence and an individual's HLA molecules. Setting aside the influence of T cell receptor (TCR) affinity, the greater the stability of a given peptide within the binding groove of a particular HLA class II molecule, the greater the likelihood that this peptide will elicit a T cell response [50]. Thus, the identification of peptide sequences in a biologic drug that bind to HLA is highly relevant for achieving the goal of screening for immunogenic potential.

The presence of HLA-binding sequences in a protein therapeutic is part of the larger biological process of antigen presentation, T cell receptor (TCR) recognition, and T cell activation, and thus is not an absolute guarantee that T cell responses will occur. As elegantly described by others, peptide processing and abundance, intracellular editing and MHC loading by the DM protein, peptide-MHC stability, TCR specificity and abundance, phenotype of the responding T cell, and presence or absence of secondary signals all contribute to initiating and shaping the quality and quantity of a T cell response [18,20,25,26,50]. While the detection of a T cell response to a putative epitope is an important predictor of immunogenic potential, it has been much more difficult to reliably model the intricate yet critical steps that occur between the HLA binding event and the activation of T cell response. Furthermore, the important contribution of regulatory T cells (Treg), which may proliferate and produce inhibitory signals in response to particular T cell epitopes, has gained appreciation in recent years. Methods for detecting and discriminating between regulatory and helper T cell responses (Treg and T helper, respectively) are discussed in greater detail below. Quite a few clinical trials of protein therapeutics have now linked the presence of T helper responses to immunogenicity, confirming the direct

<sup>3</sup> The terms HLA (human) and MHC (encompasses both human and mouse) are used interchangeably in this manuscript.



relationship between T cell epitopes and the immunogenicity of biologics [6,39,51–54].

### 2.3. Td immunity to therapeutic proteins

A classic T cell and Td antibody response to a therapeutic protein antigen can occur after one or two administrations of a particular protein therapeutic, persist for prolonged periods of time, and ultimately inhibit the efficacy of the therapeutic product. A classic Td immune response to a protein therapeutic is illustrated by the induction of ADA “inhibitors” in response to blood replacement factor VIII (FVIII) in hemophilia patients. Although the incidence and intensity of the immune response to FVIII can vary depending on the extent to which endogenous FVIII is expressed in the individual patient, immune responses to FVIII are driven by T cell epitopes [55]. The differential between effector and regulatory T cell immune responses to FVIII epitopes, on the individual level, may be a factor that determines whether that individual develops anti-FVIII ADA [56].

### 2.4. The role of central and peripheral tolerance to biologics

The absence of an immune response to autologous proteins is attributed to central (thymus-derived) immune tolerance to proteins present in the respective individual's proteome. According to the theory of central tolerance, T cells that respond to epitopes derived from autologous proteins expressed in the thymus undergo deletion or are rendered anergic during thymic development [57]. Nonetheless, tolerance to autologous proteins is now known to be incomplete, since autoreactive T cells to self-antigens have been uncovered in peripheral circulation in the context of autoimmunity and are also present in the circulation of healthy individuals. Natural regulatory T cells (nTregs) are T cells generated in the thymus that circulate in the periphery. Upon antigen-specific activation through their TCR, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> nTregs are able to suppress bystander effector T cell responses to unrelated antigens by contact-dependent and -independent mechanisms [58,59]. nTregs may hold immune responses to some autologous proteins (such as erythropoietin) in check.

Adaptive Tregs, developed in reaction to foreign antigens to which central tolerance may not exist, are an additional source of control over immune responses [60]. Sustained tolerance (to exogenous proteins) may require the existence of these ‘adaptive’ or ‘induced’ Treg cells (iTreg, also known as iTreg, [58]) with the same antigen specificity as the self-reactive effector T cells [58,61–63]. Administration of antigen in the absence of an innate-immune stimulator (danger signal) can lead to tolerance; this approach has been used for the induction of tolerance to allergens. A strong link connecting HLA, presentation of T cell epitopes (both regulatory and effector) in the context of HLA, and the maintenance of peripheral tolerance has been described [64]. The implications of immune control by nTregs and the induction of tolerance via aTregs will be discussed in greater detail below.

## 3. Methods for predicting Td immune responses

Detailed knowledge of the regions of localized immunogenicity within the protein sequence may facilitate immunogenicity mitigation efforts. Here we begin with a review of immunogenicity prediction methods, followed by a brief description of strategies that may mitigate Td immunogenicity including tolerization, deimmunization, and the coadministration of the drug with immunosuppressive therapies such as methotrexate, prednisone, cyclophosphamide, or Rituxan [65–68].

Predictive immunogenicity screening often involves more than one approach, as each method has strengths and weaknesses. A first step in the process may be to screen a protein for the presence of T cell epitopes by sequence analysis *in silico*. This step can be followed by *in vitro* studies using a variety of methodologies, including HLA binding assays, naïve blood assays, or humanized mouse models (see below for descriptions of these assays).

### 3.1. *In silico* T cell epitope-screening methods

The core residues of the T cell epitope sequence that mainly define the affinity and stability of binding to HLA pockets are limited in length to 9–10 amino acids; thus prediction of T cell epitopes based on the amino acid sequence of a peptide is computationally feasible when sufficient information on a set of peptides that are known to bind to a particular MHC is available. Databases such as the Immune Epitope Database Analysis Resource (IEDB; [www.tools.immunoepitope.org](http://www.tools.immunoepitope.org)) [69] provide the raw material for developing T cell epitope prediction tools.

Presentation of epitopes by both MHC I and MHC II contributes to the initiation of an immune response. MHC II is more relevant to anti-drug antibody responses, as MHC Class II-restricted T helper cells are responsible for driving humoral immunity. HLA DR, DQ, and DP are the three loci of peptide-carrying HLA class II molecules. *In vitro* and *in vivo* observations indicate that HLA DR binding peptides are generally 12 amino acids in length, as the flanking sequences serve to stabilize the peptide (by hydrogen bonds) in the HLA binding groove [70]. Systematic assessments of MHC class II peptide binding domains of proteins have been developed using machine learning methods, software algorithms, and data transformations [71–73]. For a brief overview of T cell epitope mapping tools, see Table 2 [69,74–80]. A common denominator among these tools is the ability to quickly screen large datasets, including whole genomes or proteomes, for putative T cell epitopes. Several common HLA-DR types share HLA binding pocket repertoires [81], meaning their ability to bind peptides is more promiscuous than for class I alleles. Moreover, analysis focused on as few as eight representatives of the 875 known HLA-DR alleles can “cover” the genetic backgrounds of most humans worldwide [82]. Additionally, MHC class II-binding T cell epitopes have been observed to occur in clusters of up to 25 amino acids in length [72,83]. Thus identifying MHC class II-binding T cell epitope clusters can be a strong indication for T cell responses because they represent regions of the protein in which sequences that have high affinity across multiple HLA alleles and multiple frames are located [79].

It is notable that there are few examples in the literature of an association between HLA-DP or -DQ and immunogenicity. This may be due to the fact that binding motifs of HLA-DP and HLA-DQ alleles are less well defined than HLA-DR alleles, and/or that HLA-DP and HLA-DQ may contribute very little to drug-related immunogenicity. A recent retrospective analysis of previously published immunogenicity prediction data [72] found no association between the presence of DP or DQ motifs (as predicted using online tools accessible through the IEDB) and immunogenicity of 23 monoclonal antibodies that have been evaluated in human clinical trials [199].

The computational approach to MHC class II T helper cell epitope prediction and its application to Td immunogenicity assessment has been described in previous publications [84–90] and is now well accepted in vaccine discovery efforts [85,91–94] and for the identification of key epitopes triggering autoimmunity [95]. In the context of biologics, several studies of therapeutic proteins mentioned above [6,51] have demonstrated the prospective predictive accuracy of these algorithms by showing a positive correlation between the presence of an MHC binding sequence and observed Td immunogenicity in the clinic (demonstrated for a recombinant fusion protein, FPX, in Table 3). A specific example is provided in detail in the case studies section of this article. Some groups have also developed tools that are expressly designed to rank therapeutic proteins by T cell epitope content [72] and to define means of modifying epitope content in silico [35,96,97]. Application of in silico tools reduces downstream in vitro testing dramatically,

typically by at least 20-fold [98], and provides an opportunity in early development to modify a protein to decrease its immunogenic potential.

### 3.2. Strengths and limitations of in silico analysis

#### 3.2.1. Antigen processing

In vivo, immunologically relevant epitopes are produced by the APC, which processes the protein into discrete peptide fragments, assembles the peptides in complexes with MHC molecules, and displays these complexes on its cell surface [99,100]. The non-classical MHC class II molecule HLA-DM acts as a T cell epitope editor, reducing the number of epitopes that are eventually presented at the APC surface through kinetic proofreading [101]. Methods that will predict products of antigen processing and contribute to the accuracy of Td immunogenicity assessments are under development [50,102,103]. One means to qualitatively identify the peptide sequence of epitopes that are presented following HLA-binding and DM-editing is to directly elute naturally processed HLA-associated peptides, followed by peptide sequencing [103–105].

#### 3.2.2. MHC affinity

A prerequisite for T cell epitope immunogenicity is the stable binding of a linear epitope in the groove of the MHC molecule; the higher the kinetic stability (slow peptide off-rate), the longer it will reside within the MHC cleft, and the greater the likelihood that an epitope will be recognized by a T cell [106]. Improved prediction of peptide–MHC

Table 2 Summary of T cell epitope mapping tools, in alphabetical order.

NAME	DEVELOPERS/INSTITUTION	TYPE	WEBSITE
Epibase	I. Lasters and P. Stas Algonomics NV/Lonza, Inc.	Commercial	www.lonza.com
EpiMatrix	A.S. De Groot and W.D. Martin EpiVax, Inc.	Collaborative/Commercial	www.epivax.com
IEDB	Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, Damle R, Sette A, Peters B. The immune epitope database 2.0. <i>Nucleic Acids Res.</i> 2010 Jan;38:D854-62.	Mixed collection of tools of assorted derivation	www.iedb.com
MHC2PRED	G.P.S. Raghava Bioinformatics Center, Institute of Microbial Technology, Chandigarh, India	Public	www.imtech.res.in/raghava/mhc2pred/
MHCPRED	D.R. Flower The Jenner Institute	Public	www.ddg-pharmfac.net/mhcpred/MHCPred/
PROPRED/ TEPITOPE	G.P.S. Raghava and H. Singh Bioinformatics Center, Institute of Microbial Technology, Chandigarh, India	Public	www.imtech.res.in/raghava/propred/
RANKPEP	P.A. Reche Harvard Medical School	Public	http://bio.dfci.harvard.edu/RANKPEP/
SVRMHC	P. Donnes, A. Elofsson Division for Simulation of Biological Systems, University of Tübingen, Germany	Public	http://svrmhc.biolead.org/
SYFPEITHI	H.G. Rammensee Department of Immunology, Tübingen, Germany	Public	http://www.syfpeithi.de
SMM-Align/ NetMHCII-2.2	M. Nielsen, C. Lundegaard, and O. Lund Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark	Public	www.cbs.dtu.dk/services/NetMHCII-2.2/
TCED/iTope	M. Baker and F. Carr Antitope, Ltd.	Commercial	www.antitope.co.uk/

**Table 3** Association of algorithm-predicted immunogenicity to clinical immunogenicity rates. EpiMatrix-generated scores associated with each FPX protein and their respective rates of antibody incidence (binding and neutralizing) are shown. An assessment of Tregitope content in each molecule was also performed and scores were adjusted accordingly. FPX 1, for example, had a high rate of clinical immunogenicity and was associated with elevated T-cell epitope content and low Tregitope content, as reflected by its high Z score. FPX 2, 3, and 4 were associated with a low EpiMatrix score, and Tregitope adjustment further reduced the predicted potential for binding. Predictably, FPX 2, 3, and 4 exhibited only minor clinical immunogenicity.

Protein Therapeutics	FPX 1	FPX 2	FPX 3	FPX 4
EpiMatrix Score	21.97	1.76	-0.76	1.63
Tregitope – adjusted EpiMatrix score	21.97	1.62	-1.76	-111.25
Binding Antibodies	37%	7.80%	5.60%	4.50%
Neutralizing Antibodies	40%	0.50%	Not Analyzed	0%

stability might improve the ability of *in silico* methods to predict activation of CD4<sup>+</sup> T cells *in vivo* [106]. While *in silico* tools have dramatically improved over time, and consideration of epitope “off-rate” or peptide “residence time” on the MHC molecule is probably within reach of future generations of algorithms, in contrast, TCR affinity is unlikely to be amenable to *in silico* prediction, as the TCR is pleiomorphic and exhibits three-dimensional complexity akin to B cell epitopes.

### 3.2.3. T cell phenotype

Identification of T cell epitopes in a protein may indicate that the protein could induce an immune response, though the nature of the response remains heavily dependent on the phenotype of the T cell that recognizes this epitope in the context of MHC class II and on the local milieu (inflammatory or regulatory). The prediction of the type of T cell (T helper, T regulatory, etc.) responding to a potential T cell epitope remains beyond the capabilities of the *in silico* approaches described here: phenotyping requires *in vitro* characterization, except in cases where known regulatory T cell epitopes have been previously described.

### 3.2.4. Individual versus population-level predictions

Caution is advised when applying T cell epitope prediction tools that have been developed for the purpose of predicting immune responses in large populations to an individual subject. As HLA binding is intimately associated with T cell response, individual responses to protein therapeutics will vary depending on multiple factors, such as an individual's HLA haplotype [53,89] and immune status. Individual T cell repertoire variation further complicates the interpretation of individual subject responses; current thinking is that a T cell repertoire is defined by previous exposures to related epitopes, vaccination, and the internal (gut) microbiome [107,108]. Such individual specificity, in addition to antigen processing (see above), may explain why some MHC binding

peptide sequences do not induce T cell activation [109]. Thus, Td immunogenicity on the population level, as predicted by tools that measure the potential for immunogenicity across HLA alleles, can be quite different from immunogenicity at the individual level. *In silico* tools can be used to predict the former in the pre-clinical phase of development; the same tools may be useful to analyze subject-by-subject immune responses to a protein therapeutic in the clinical phase of development.

### 3.2.5. Post-translational factors

Additional determinants of Td immunogenicity that are not considered in the *in silico* prediction process may be revealed *in vitro*. For example, effects of non-sequence-derived post-translational modifications including those of a biophysical or biochemical nature such as deimination (or citrullination), deamidation, oxidation, dimerization, and protein folding-induced conformational changes that are all widely accepted as determinants of immunogenicity [21,110].

Despite these caveats, T cell epitope prediction has advanced greatly over the last decade. *In silico* tools provide the advantages of high throughput, low cost, and the ability to reduce the search space for further *in vitro* testing. *In vivo* immunogenicity, however, is also influenced by a number of factors that cannot yet be accurately predicted *in silico* [71,80]. The results of *in silico* analyses can then be carried to the next step of pre-clinical analysis, such as HLA binding, antigen processing and presentation assays, and T cell assays, in which other factors that contribute to generating an immune response can be examined.

## 3.3. HLA binding assays

Having defined the regions of interest *in silico*, one means of validating these predictions is to perform HLA/MHC binding assays. A number of different HLA class II binding assays can be used to measure the affinity of predicted epitope sequences to HLA alleles *in vitro*; several are described in this section.

### 3.3.1. Competition binding assays

One method that is employed is a competition-based HLA binding assay, described previously [111–113] that can be adapted for high throughput [114]. The method is described in detail in other publications, but briefly, experimental peptides are tested for their ability to compete against a labeled high-affinity peptide for binding to purified class II molecules. The reaction is carried out at several peptide concentrations; a non-linear regression analysis of the resulting dose–response curve is then used to calculate the IC<sub>50</sub> (concentration of test peptide required to compete off 50% of the target peptide). Binding assays can be performed for a broad representation of class II alleles [82]. One drawback of this method is that measurements of peptide affinity are always relative to this standard reference peptide, as the appropriate reference peptide may differ from HLA allele to allele. A value that describes the 50% inhibitory concentration (IC<sub>50</sub>) can be derived by fine-tuning the protocol for each allele through repeated experiments, and performing the assays at multiple concentrations. IC<sub>50</sub> values

can be converted to method-independent  $K_i$  values via the Cheng–Prusoff equation for comparison of peptide affinities across platforms [115].

### 3.3.2. Direct binding assays

It is also possible to measure peptide affinity directly, rather than observing its ability to displace a known ligand. In such experiments, a variety of methods may be used to separate peptide bound to HLA molecules from free peptide in solution, thus allowing for assessment of the peptide's affinity. Spin-column filtration and gel electrophoresis are ways to accomplish this distinction by molecular weight, since peptide–HLA complexes will weigh more than unbound peptides. These methods, however, are tedious and can suffer from complex dissociation during the separation process. Other novel methods have been proposed, such as proximity-based detection utilizing a Luminescent Oxygen Channeling Immunoassay [116]. All of these techniques allow more direct binding affinity determination than competition studies, but have methodological limitations and require costly equipment.

### 3.3.3. Real-time kinetic measurements

Apart from determining the quantity of displaced reference peptide (competition assay) or bound vs. free test peptide (direct assay), the rate at which peptides interact with HLA molecules is a dimension of epitope strength that can be measured *in vitro*. Protocols based on fluorescence polarization are suitable for kinetic studies due to the absence of interference from ELISA wash steps, allowing multiple readings to be taken throughout the binding reaction [117]. Additionally, surface plasmon resonance methods have also been employed to measure peptide binding to MHC II [118].

## 3.4. Strengths and limitations of HLA binding assays

*In vitro* HLA binding assays are relatively straightforward and easier to perform than live-cell experiments. However, each of the assay formats can be affected by peptide purity and length as described here. Peptides that are synthesized for HLA binding assays must include carboxy- and amino-terminal flanks that stabilize the peptide in the MHC groove [119]. Selection of peptides based on artificially defined overlapping sequences (e.g., 15mers overlapping by ten amino acids) can lead to the truncation of MHC binding motifs and elimination of critical flanking regions, which limits the accuracy of the binding assay. Furthermore, artificially synthesized peptides may contain impurities, truncations, and errors in the sequence that can lower or alter the binding of the pure peptides; thus high-quality peptides (greater than 90% purity) are required to obtain more accurate results in binding assays. Long peptides can fold or peptides can aggregate in solution, leading to underperformance in binding assays. Solvents used in the experiment may interact negatively with certain amino acids, causing oxidation or the formation of unwanted disulfide bonds. Quality-controlled peptide manufacturing and storage, along with proper reagent selection, can minimize the impact of these problems [113,114].

While epitopes can be predicted (*in silico*) and validated in binding assays (*in vitro*), the final impact of the T cell epitope is through its activation of a T cell. Activation is related to TCR specificity, avidity and T cell phenotype (none of which are measured in HLA binding assays), which makes straightforward interpretation of immunogenicity based upon HLA binding assays impossible. Thus, HLA binding assays may improve the accuracy of immunogenicity predictions when applied in a step-wise process after *in silico* epitope prediction and before the employment of a biological assay such as enzyme-linked immunosorbent spot-forming (ELISpot) assays or HLA transgenic mouse studies, but additional assays that assess T cell responses may need to be performed [120].

## 3.5. *In vitro* T cell assay methods for Td immunogenicity analysis

For many years, *in vitro* assays based on HLA haplotype have been used in transplantation research to assess the risk of engrafted T cells responding to host tissue (graft-versus-host disease). Adaptation of these assays to protein therapeutics may improve the pre-clinical assessment of the potential for Td immunogenicity. The presence of T cells that actively suppress immune responses to autologous proteins is a significant confounding factor in their development and evaluation. In addition, these assays are dependent on the selection of a culture milieu that accurately accounts for the *in vivo* conditions of human immune stimulation [40,54,121]. In this respect, bulk cultures of PBMC, either with or without co-stimulatory signals (anti-CD28 antibody, IL-2, IL-7, etc.), have been utilized to assess immunogenicity of therapeutic proteins.

A number of biological outcomes for T cell activation can be measured in these *in vitro* assays, including cytokine secretion (IFN- $\gamma$ , IL-2, IL-4, etc.), regulation of cell surface markers of activation, signal transduction events, and proliferation [122,123]. Supported by such evidence, *in silico*-identified peptides that stimulate multifunctional T cell responses *in vitro* can be considered *bona fide* T cell epitopes with the potential to contribute to an ADA response.

### 3.5.1. Measurement of T cell cytokine responses

ELISAs and ELISpots are two related methods of measuring cytokines secreted by T cells (i.e., IFN- $\gamma$ , IL-2, IL-4, and IL-10). The enzyme-linked immunosorbent assay (ELISA) can measure cytokine levels in culture supernatants generated under conditions of T cell stimulation. Cytokine levels measured in an ELISA can provide information about the magnitude of the response (how much cytokine is secreted into the supernatant) as well as the type and quality of the response (which cytokines are or are not secreted into the supernatant). Multi-analyte, high throughput cytokine testing can also be performed in cell-derived supernatants in bead-based assays [124–127]. Enzyme-linked immunosorbent spot-forming (ELISpot) assays provide information regarding the number of cytokine-producing cells (down to 1 cell per million) within a cell population stimulated *ex vivo*; these assays are considered to be more sensitive and



quantitative whereas ELISA and bead-based assays are slightly less sensitive and more qualitative.

Intracellular cytokine staining measured by flow cytometry is another method for detecting cytokines and linking their expression to the phenotype of individual cells. These assays can be used to accurately measure T cell polyfunctionality relative to the phenotypic classifications of CD4<sup>+</sup> T cells based on cell surface markers.

### 3.5.2. T cell proliferation

T cell proliferation in response to stimulation by a peptide–MHC complex can be measured by (1) the incorporation of the radioactive nucleotide tritiated thymidine into the DNA of dividing but not resting cells or (2) the dilution of a fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), that decreases in fluorescence intensity by half with each round of cell division and can be measured by flow cytometry. Thymidine incorporation assays are gradually being replaced by CFSE staining, which presents significant advantages in terms of ease-of-use. In addition to CFSE labeling, cells can be co-stained for expression of other cell surface markers, transcription factors, and/or intracellular cytokines that distinguish between regulatory T cells and effector T-helper cell phenotypes, including Th1, Th2, Th17, Th22, etc. as reviewed in detail elsewhere [128–130].

### 3.5.3. Tetramers

Fluorescently labeled tetrameric complexes of MHC class II molecules loaded with the peptide of interest (i.e., “tetramers”) can also be used to enumerate T cells recognizing a particular epitope. However, relative to MHC I tetramers, MHC II tetramer staining has proven problematic and so their utility has been restricted. The technical limitations experienced specifically with MHC II tetramers may be due to weaker TCR–peptide–MHC II interactions or the fact that CD4 does not participate in the stability of tetramer binding as does CD8 [131]; either one or both of these features may contribute to increased variability and poor quality of the experimental output. Efforts continue to improve MHC II tetramer–TCR interactions towards the goal of increasing the utility of MHC II tetramers so that they may be more widely used for the identification of specific CD4 T cell responses, including those against protein therapeutics.

### 3.5.4. Naïve T cell in vitro assays

Naïve peripheral blood mononuclear cells (PBMC) have been used to describe Td immunogenicity of therapeutic proteins [34,40,132,133]. Compared to a recall response, the precursor frequency of antigen-specific cells in a naïve population is quite low; it was postulated that the higher the precursor frequency reactive with a certain peptide or protein, the higher the potential of the respective peptide or protein to induce an immune response [134]. Multiple rounds of antigen stimulation, sometimes over several weeks, are required to expand sufficient numbers of antigen-specific T cells for reliable measurement. By conditioning naïve blood samples ex vivo through prolonged and/or repeated exposure to experimental antigen, immune responses can be expanded to the point where they can be measured. What is not known is how expansion affects the phenotype of the T cell response; either regulatory or effector responders may eventually dominate in in vitro cultures.

### 3.5.5. T cell assays using whole antigens

T cell assays using whole antigens also can be used to measure T cell responses to protein therapeutics in vitro. The recognition of these antigens requires the presence of an APC that is capable of processing and presenting peptides derived from the antigen. Advantages of whole PBMC assays include the ability to set up several assays and/or assay conditions with a limited blood sample volume and the ease of assay performance, features which lend themselves to high-throughput assay development [40]. Human monocyte-derived dendritic cells can be manipulated in vitro to model antigen processing by professional APC in vivo (H. Kropshofer, unpublished data). However, as applied to evaluating immunogenicity of biologics, optimization of variables such as the ratio of DCs to autologous T cells are important factors for ensuring that the in vitro results are relevant to the clinical scenario.

### 3.5.6. T cell re-stimulation assays using “exposed” donors

T cell re-stimulation assays are generally used to identify and measure a recall or memory response in PBMC derived from subjects who have been exposed at a distant time point to a protein or a given biologic product. Whereas ‘exposed blood’ assays cannot be performed for novel therapeutics, this type of assay can be used to evaluate the impact of pre-existing immune responses to a new version of an existing biologic in use such as a re-engineered FVIII. Epitope mapping of the recall response can be performed using specific peptides from the whole protein; however, this approach may be over-predictive. Therefore, studies using whole therapeutic protein should ideally be performed in parallel with studies using sets of predicted peptides. Antigen-specific T cell responses can be assessed after re-stimulation ex vivo by ELISA, ELISpot, proliferation, and flow cytometric analysis of activation markers and intracellular cytokine expression.

### 3.5.7. Reconstitution of T cell immune responses in vitro

New methods for “reconstituting T cell immune responses” in artificial media may permit improved in vitro assessments of the interactions between the professional APC and the T cells. To this end, several artificial lymph node (ALN) systems have been developed [135–137]. These methods attempt to replicate, in three dimensional structures and in APC:PBMC ratios, the natural immune environment [138]. In at least one approach, human blood-derived dendritic cells are cultured in transwells partitioned by human vascular endothelial cells. Addition of autologous CD4<sup>+</sup> T cells to the co-culture allows activated APC expressing chemokine receptors to respond to inflammatory chemokines and migrate through the transwells, mimicking the migration of APC from the periphery to the lymph nodes. The antigen-specific CD4<sup>+</sup> T cell response is monitored by CD154, IFN- $\gamma$ , IL-2, IL-5, IL-17, and IL-21 expression. A good correlation between previously established immune responses in vivo and ALN immunogenicity has been observed, at least for protein-based vaccines [139]. Even though the human ALN model is primarily being used for in vitro evaluation of vaccine efficacy [140], the application of this technology for the prediction of therapeutic protein immunogenicity is feasible.

Alternatively, some groups have used small flow-through systems that induce PBMC to self-assemble into lymph

node-like structures [141]. Pre-activation of the T cells improves the readouts; however, the introduction of specialized (non-self) APC may generate false positives for some biologic proteins. In other cases, these ALN systems have given results that correlate with predicted immune responses [135,142].

### 3.6. Strengths and limitations of in vitro T cell assays for Td immunogenicity analysis

The benefit of in vitro T cell assays utilizing easily accessible human peripheral blood is that the assays may provide a preview of the immunogenicity of a therapeutic protein without the risks typically associated with first-in-human use. Careful consideration of the composition of the cells in culture, such as monocyte-derived macrophages, immature/mature dendritic cells, and T and B cells, may improve the ability of a protein to initiate and propagate an immune response. Furthermore, these assays incorporate antigen processing and presentation pathways for whole proteins as well as for discrete peptides.

A limitation of ELISpot and ELISA assays is that PBMC contain several cell types capable of secreting particular cytokines, e.g., IFN- $\gamma$  (NK cells, NKT cells, CD4<sup>+</sup> or CD8<sup>+</sup> T cells). Thus PBMC-based assays to measure cytokine production by a specific lymphocyte subset may need to be fine-tuned such as by the use of subset-depleted or subset-enriched PBMC preparations. Another potential issue related to T cell assays is that the concentration of whole protein or peptide in the cell culture may need to be titrated relative to the number of T cells. The optimal concentration of protein required for proper evaluation of immune responses in vitro may be non-physiologic due to a limited antigen-presenting population and/or co-stimulation. T cell assays also require support with homeostatic cytokines (IL-2, IL-7, IL-15, etc.) to reduce bystander effects [40]. In addition, optimization of T cell concentrations may be required [143].

While “irrelevant” cells secreting cytokines in vitro may contribute to overestimation of the frequency of activated T cells, their presence also contributes to the overall level of activation of cells in the assay. Cells such as NK cells, CD8<sup>+</sup> T cells, and basophils may play a supportive role in these cultures [144], thus their removal can modify the outcome of a truly representative immune response. Finding the right balance between minimization of irrelevant immune responses and support for the in vitro immune response is one of the major obstacles to widespread adaptation of in vitro T cell assays for pre-clinical screening of protein therapeutics.

Viable and functional T cells are also required for the assay; standardized and optimized procedures for handling and storing whole blood are needed to ensure the accuracy of subsequent assays. Furthermore, blood from naïve and drug-exposed individuals can differ in the content of antigen-specific T effector and T regulatory cells. Based on the nature of response (in vivo primed vs. stimulated and recalled), stimulation methods and the amount of antigen required for challenge can also differ. Naïve cells will require multiple in vitro stimulations to amplify detection, while antigen-specific recall responses can be elicited even with a single challenge.

Similarly, stimulation can be performed with peptides or whole proteins alone, peptides in the context of tetramers, or APC pulsed with whole proteins. New advances in tetramer/multimer technology can enhance detection of epitope-specific T cells and thus should allow more sensitive and standardized approaches to evaluate individual responses to T cell epitopes identified in protein therapeutics [145].

Finally, the number of individual blood (PBMC) donors that would normally be required to address the HLA diversity of a patient population is quite large (more than forty), and the volume of blood required ranges from 15 mL to more than 50 mL. Maintenance of a large supply of blood samples from pre-qualified donors that is sufficient to reduce assay-to-assay variation can be done, but is cost prohibitive for most preclinical laboratories.

Much remains to be done to improve the accuracy of in vitro T cell assays in predicting clinical immunogenicity. Future considerations for improving in vitro T cell assays include: better linkage between the spectrum of immune responses to a therapeutic protein and predictive power in clinical trials using statistically derived criteria, such as fold-increase or stimulation-index; improved means to distinguish responders from non-responders; evaluation of T cell responses from diseased states associated with inflammation or immune suppression; selection of the optimal set of markers for the identification of activated T cells; and improved ability to differentiate Treg and CD4<sup>+</sup> Teff responses (one schema has been offered by the HIPC consortium [146]). Additional improvements require establishing clear parameters that define memory versus naïve T cell populations, influence due to bystander cells, and standard methods for PBMC harvest, preparation, and storage. Concurrent with efforts to standardize, in vitro immunogenicity screening assays are being incorporated into the preclinical pipeline by a number of drug developers.

### 3.7. Mouse models of in vivo Td immunogenicity of human therapeutic proteins

Important advances in understanding MHC restriction, mapping of epitope recognition for murine epitopes presented by murine MHC, and T cell function have been achieved with in vivo mouse studies. However, when we turn to in vivo mouse studies for prediction and validation of Td immunogenicity for clinically relevant proteins, there are two major limitations that must be taken into consideration. The first is that human and murine proteins are not identical, thus administration of protein therapeutics to mice may result in responses to components of the protein that are foreign to mice; and second, murine MHC will present mouse, not human, T cell epitopes. Since murine models provide a means of evaluating immunogenicity and an important bridge to the clinic, a number of enhanced models have been developed.

#### 3.7.1. HLA transgenic mice

The HLA transgenic lines are generated by incorporation of specific human HLA genes into murine MHC class II-deficient mice, producing a mouse strain that expresses human class II HLA in the absence of mouse class II MHC [147,148]. Thus, these mice process and present epitopes in the context of

human HLA, and their T cells recognize epitopes presented by human HLA. They are most useful when directly comparing two proteins that are very similar (such as FVIII and versions of FVIII that have fewer epitopes or new glycosylations) [97]. A direct correlation has been found between epitopes that elicit T cell responses in infected humans and those that induce T cell responses in immunized HLA transgenic mice [149–152]. HLA transgenic mice are now routinely used to test and optimize (human) epitope-driven vaccines in preclinical studies [153–155]. For example, Hanke et al. mapped HIV epitopes in transgenic mice, and then moved their DNA vaccine through abbreviated primate studies after proving that the one non-human primate epitope engineered into the vaccine stimulated T cells [156].

The formation of anti-FVIII antibodies, also known as inhibitors, is a major obstacle to FVIII gene replacement therapy in hemophilia A patients. After intravenous administration of FVIII, the immune response mounted is dependent on CD4<sup>+</sup> T helper cells, as has been demonstrated by numerous studies in mice and humans [157–162]. More currently, interference with T–B cell interactions in hemophilic mice was shown to reduce inhibitor formation [159, 160, 162].

An example of the use of HLA transgenic mice for evaluating the application of immunogenicity prediction tools towards the goal of deimmunizing a therapeutic protein was recently published in *Clinical Immunology* [97]. In silico tools were utilized to predict immunogenic peptides within the C2 domain of FVIII. Changes to amino acids in positions predicted to be important for binding to the HLA DR3 MHC class II pocket were modified with the intent of disrupting peptide–MHC binding. The same predictive tools were reapplied to assess the binding potential of the modified peptides. This process was reiterated until the predicted binding to HLA DR3 was reduced. The de-novo immunogenicity of these modified peptides was tested in hemophilic E16 mice (H-2b; [163]) and in HLA-DR3 transgenic mice [164]. The initial immunogenicity study results, in which mice were immunized with the unmodified original (ORG) epitopes predicted by in silico analysis of the C2 domain, were consistent with predicted responses for either H-2b- or HLA-DR3-expressing mice. These two mouse strains were crossed to produce E16xDR3 mice, in which an immune response of intermediate magnitude was observed. Specifically, the immunogenicity of epitopes derived from FVIII in the E16xDR3 mice was consistent with the absence of tolerance induction to this sequence (the E16 mice do not express full-length FVIII) and the presence of MHC (I–Ab) binding motifs in the sequence [97]. In proliferation assays, modified epitope peptides were less antigenic than ORG peptides. In general, lower antigenicity was observed for those peptides that had two rather than just one amino acid substitution. Similarly, in terms of de novo immunogenicity, the more mutations, the lower the observed proliferative response in general.

In addition to providing an example of how in silico tools can be applied early in the development process to mitigate immunogenicity risk, this study also highlights certain limitations of available mouse models for risk assessment. While certain epitopes are predicted to bind promiscuously to both human HLA DR3 and I–Ab MHC, others demonstrate greater restriction by either the human or the mouse MHC. Thus the lack of observed immunogenicity may be attributed to the absence of a relevant MHC expressed in the mouse model.

With regard to the HLA DR transgenic models, those peptides predicted to be HLA ligands were only immunogenic when the sequence contained mismatches between the human FVIII sequence used in the immunization and the corresponding sequence in the native mouse FVIII protein [97]. To be immunogenic, the mismatches had to occur within 9-mer sequences that also contained HLA DR3 or DR4 binding motifs. When presented to the mouse T cell, these 9-mers would appear “foreign” and thus stimulate T cell proliferation. Peptides that were predicted to bind HLA DR3 and/or DR4 but did not stimulate immune responses were found to contain no mouse/human sequence mismatches.

In summary, identification of T cell epitopes and development of de-immunized versions by targeted sequence modification can lower HLA binding and proliferation responses, but the process has the potential to impact protein function

### 3.7.2. Humanized mouse models

“Humanized” mice engrafted with a functional human immune system are now being used to study human hematopoiesis, immunity, regeneration, stem cell function, cancer, and human-specific infectious agents. Immunocompromised SCID/NOD/ $\gamma$  chain<sup>−/−</sup> or RAG2<sup>−/−</sup>/ $\gamma$  chain<sup>−/−</sup> mice, utilized as recipients to facilitate acceptance of human tissue, are engrafted with functional human hematopoietic stem cells (CD34<sup>+</sup>), liver, and thymus [165]. The result is a cohort of mice in which human myeloid and lymphoid lineages are reconstituted from a single human donor, and the interactions of these cells in a complex biological environment can be studied. XenoMouse® (described below), in addition to the humanized mice such as NOD/Shi-*scid*/IL-2R $\gamma^{\text{null}}$  (NOG), NOD *scid* IL2 receptor gamma chain knockout mice (NSG), bone marrow, liver, thymus transplanted mouse (BLT), and bone marrow transplanted mouse (BMT) have all been used as animal models to evaluate human immune responses [165–168].

### 3.8. Strengths and limitations of mouse models of in vivo Td immunogenicity

These evolving mouse models could provide functional and testable elements of the innate and adaptive human immune system without putting patients at risk [169]. The species specificity of a number of cells and molecules critical for a fully functional immune system remains a limitation in these models. For example, in HLA transgenic mice, the T cell repertoire will be shaped by epitopes derived from mouse proteins presented by a single human HLA allele to mouse TCR. This confounds the application of HLA transgenic mice for determination of immunogenicity of a human protein therapeutic. And while humanized (SCID/Hu) mouse models are improving, certain aspects of a fully functional immune response relevant to immunogenicity prediction and mitigation are lacking, such as the ability to elicit the complete spectrum of B cell antibody responses or the ability to proteolytically process antigens in a way that recapitulates what has been observed for human endosomal/lysosomal proteases.

The XenoMouse® model has been of particular interest, as it is transgenic for nearly the complete human immunoglobulin

locus, thus is tolerant for human IgG2/k $\lambda$  antibodies, but is deficient for mouse IgH and Igk chains. The human-like humoral immune response in XenoMouse® is restricted by mouse MHC and T cell help but is not as robust as in wild type mice, potentially due to inefficient signal transduction and isotype switching mediated by accessory factors that are necessary for B cell maturation. Hence the utility of such a model to study immune responses to human proteins remains somewhat limited.

Clearly, more work is required to develop these mouse models to accurately reflect human immune responses to protein therapeutics before they can become accurate, useful, and routine components of a Td immunogenicity screening program.

## 4. Applied Td immunogenicity prediction

### 4.1. In silico prediction supported by subsequent clinical data

Koren et al. [54] demonstrated a correlation between the in silico evaluation of T helper epitope content of a protein therapeutic and its observed immunogenicity when administered to human subjects in a clinical trial. The therapeutic protein of interest was a recombinant Fc fusion protein (FPX) consisting of human germ line Fc  $\gamma$  fragment with two identical, biologically active, 24-amino-acid peptides attached to the amino terminal end of the Fc fragment. In the in silico analysis, the carboxy terminal region of the peptide scored high for binding to five of eight common HLA molecules, suggesting that this peptide had the potential to be presented by five different HLA molecules to T cells. Moreover, the C-terminus peptides were associated with a cluster of overlapping 9-mers that could bind across several HLA DR alleles.

The antibody response to FPX was consistent with the high immunogenic potential predicted in silico. A single subcutaneous or intravenous administration of the protein resulted in high-affinity binding antibodies in 40% and 33% of total individuals, respectively (Table 3). T cell-mediated recall responses to the therapeutic protein were also assessed in vitro for donors exhibiting a strong humoral response in vivo. In vitro PBMC activation by the FPX peptide, and the amino-terminal and carboxy-terminal fragments thereof, was measured as a function of the number of IFN- $\gamma$  and IL-4 spot-forming cells (SFC) in a standard ELISpot assay. The antibody data suggested a strong T cell-driven response, which was corroborated by the in vitro cytokine responses observed in PBMC culture. Thus, the in silico prediction of immunogenic T helper cell epitope(s) within the carboxy-terminal region of the FPX peptide correlated with the in vitro T cell assays and the in vivo antibody responses.

HLA typing confirmed the predicted binding promiscuity of the carboxy-terminal epitope(s), as antibody-positive subjects possessed all of the eight most common HLA alleles. The magnitude of the immune response also appeared to correlate with the HLA haplotype and with the best carboxy-terminal peptide binding scores. One subject who possessed the DRB1\*0701 allele had the highest antibody concentration as well as the highest number of IFN- $\gamma$  and IL-4 SFCs, as was predicted based on the in silico EpiMatrix results. Another subject who had DRB1\*0701 allele also

showed relatively high antibody concentration with lower, but measurable, SFCs in vitro. Immune responses (both in vivo antibody and in vitro T cell) to different regions of the protein correlated with in silico predictions. The carboxy-terminal region of the FPX peptide showed the highest MHC binding score in the context of the DRB1\*0701 allele; T cell and antibody responses to this fragment were observed in vitro and in vivo, respectively, for individuals possessing that allele. In contrast, the DRB1\*0301 allele had very low MHC binding scores, and patients who possessed DRB1\*0301 but not any of the other higher binding alleles demonstrated low responses in ELISpot and no evidence of an antibody response to the protein therapeutic. The immunogenicity of the FPX fragments and the association between clinical results and the HLA class II alleles were supported in the naïve blood T cell assays in further studies performed by Jawa et al. [40,54] the reactivity of naïve (pre-exposure) PBMC to FPX1 was associated with therapeutic-induced antibody responses observed in the clinic as well as with expression of specific HLA class II alleles that were predicted in silico to present FPX1-derived epitopes.

This case study illustrates several important principles regarding the immunogenicity assessment of protein therapeutics:

- (i) *Clinical correlation*: Clinical incidence of high immunogenicity to FPX1 from exposed donors was retrospectively associated with the in silico immunogenicity predictions.
- (ii) *Promiscuous epitopes*: This study demonstrated that immunodominance was associated with clusters of epitopes within the sequence of the FPX1 peptide. The clustering of epitopes was associated with greater immunogenicity as measured by a high incidence of binding and neutralizing antibodies to FPX fusion protein. Moreover, due to the clustering, the peptide was more promiscuous and was able to bind across several HLA DR alleles. This was validated when ADA positive subjects were observed to express high-binding alleles.
- (iii) *Antibody–HLA correlation*: The responders with high antibody titers expressed HLA-DR alleles that had been predicted by the in silico algorithm to be the best epitope binders.
- (iv) *In silico–T cell assay correlation*: The C-terminal region of the FPX1 peptide elicited T cell responses in PBMC from FPX1-exposed, antibody-positive donors, supporting a correlation between in silico prediction and observed clinical immunogenicity.
- (v) *Correlation to in vitro naïve response*: The predicted immunodominant regions of the peptide were able to elicit response from naïve PBMC with the HLA DR predicted in silico.

### 4.2. Clinical link between MHC class II haplotype and IFN- $\beta$ immunogenicity [53]

A similar association between the HLA DRB1\*0701 allele and a strong antibody response to recombinant beta-interferon was observed by Barbosa et al. The IFN- $\beta$  epitopes were



identified using a peptide library and a peptide binding assay with B cell lines expressing this allele. Peptides were synthesized as overlapping 17-mers covering the entire sequence of IFN- $\beta$  and combined into 10-peptide pools. Recall responses from subjects with multiple sclerosis receiving Type I IFN- $\beta$  therapy were assessed in vitro by measuring T cell IFN- $\gamma$  ELISpots (Fig. 1). The PBMC from antibody-positive subjects (haplotype DRB1\*0701/DQA1\*0201) had high levels of IFN- $\gamma$ -secreting T cells in the presence of two of the 10 peptide pools tested, while antibody-negative subjects showed no response. Depending on the availability of cells, peptide pools could be de-convoluted to identify the minimal epitope and the restricting HLA class II allele. One limitation of such an approach is the difficulty of defining the true immunogenic epitope, and consequently the most relevant HLA-DR motifs, among the overlapping peptides, as is illustrated in Fig. 1.

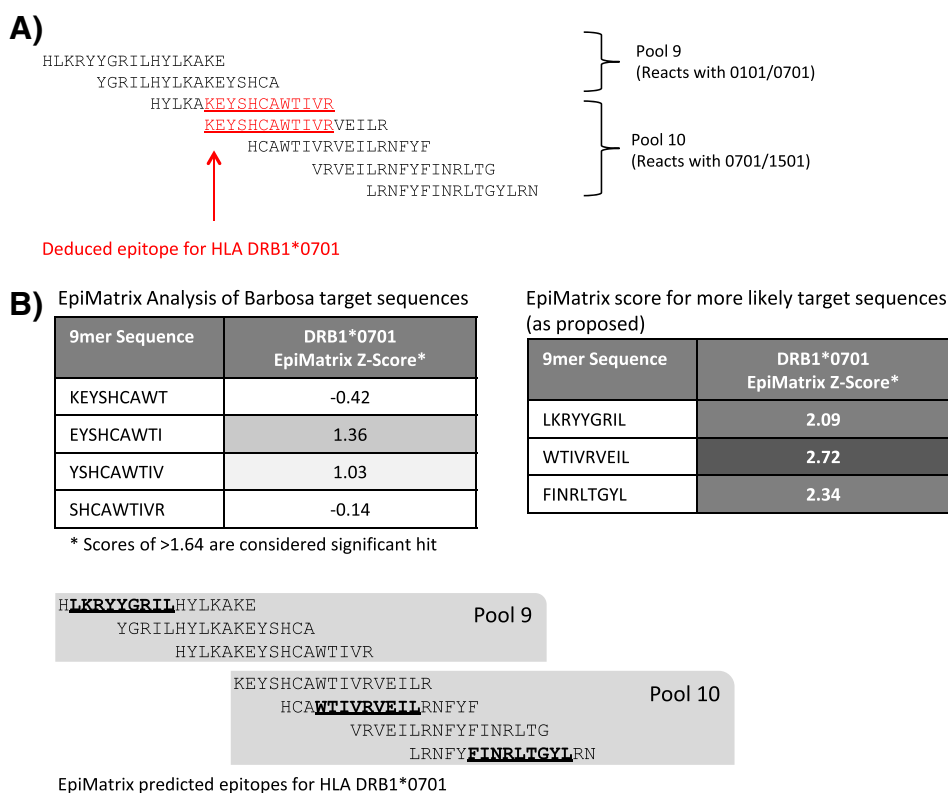
Stickler et al. [170] observed that DRB1\*1501/DQB1\*0602 haplotypes are associated with a high rate of reactivity towards human IFN- $\beta$  in the naïve T cell assay format. Accordingly, the C-terminal IFN- $\beta$  peptide 147–161 was eluted from HLA-DR molecules after IFN- $\beta$  exposure of human DC expressing the DRB1\*1501 allele (H. Kropshofer, personal communication). Due to limitations in the number of subjects tested, a clinical correlation with the HLA-DRB1\*1501 allele could not be detected in the Barbosa et al. study [53]. Thus the Barbosa et al. publication nicely

illustrates some of the limitations and lessons learned in the interpretation of in vitro studies:

- (i) *The overlapping peptide approach may lead to motif truncation:* The approach of using overlapping peptides that are then pooled for in vitro analysis may make it difficult to define a specific epitope or epitope cluster, particularly if limited PBMC samples do not accommodate de-convolution of these peptide pools (T cell assays).
- (ii) *HLA skewing:* Natural limitations on the number of subjects that are exposed to the drug or HLA-association of the disease itself may lead to skewed representation of HLA alleles, thus some associations between HLA and clinical immunogenicity may be overlooked.
- (iii) *Combining methods may lead to new insights:* Careful comparison of the HLA-binding and T cell assay outcomes may uncover linkages that can better explain the results; limiting immunogenicity screening to a single approach may lead to misinterpretations.

## 5. Mitigation of T cell-dependent immunogenicity

Computer algorithms, epitope databases, and improved statistical methods have given researchers superior tools



**Figure 1** Association of HLA with patient immune responses. Pools of overlapping 17mers derived from IFN- $\beta$  were tested in ELISpot assays using PBMC from multiple sclerosis patients with antibody response to beta-interferon therapy (Barbosa et al., Clinical Immunology 2006). Pools 9 and 10 stimulated significant IFN- $\gamma$  responses in patients sharing a single HLA allele, DRB1\*0701, thus the minimal epitope for this allele was determined to comprise the residues shared between the two stimulatory pools. However, immunoinformatic analysis using the EpiMatrix algorithm reveals three distinct 9-mer binding motifs for DRB1\*0701 in these peptides, none of which are fully represented in the region of overlap between the two peptide pools.

for assessing the potential of T cell immunogenicity of therapeutic proteins [42]. As a result, a number of different approaches to mitigate Td immunogenicity of therapeutic proteins are now under consideration. These approaches include: direct modification of the therapeutic protein by pegylation and/or glycosylation to mask “immunogenic epitopes”, thereby reducing recognition by the immune system; modification of HLA class II anchor residues of immunodominant epitopes to disrupt presentation; and application of strategies that tolerize the immune system to the therapeutic protein. The primary focus of this review is on methods for predicting and measuring Td immunogenicity; hence, a thorough discussion of methods for mitigating immunogenicity is beyond its scope. However, it is important here to link our evolving ability to identify contributors to a therapeutic protein's immunogenicity with our ability to modify that feature to mitigate unwanted immune responses. Currently, tools to predict T cell epitopes can be applied to remove T cell epitopes. Indeed, a method for deimmunization (protein/sequence re-engineering) has been introduced, which involves the elimination of predicted T cell epitopes or a reduction in the total number of T cell epitopes. The approach has been described in detail in a number of publications [41,171–175], and readers are referred to those articles for details on the methodology. Efforts to render effector T cells non-responsive through the actions of immunosuppressive drugs or induction of Treg cells are evolving. Finally, the ability to identify drug-induced or drug-responsive T cells may in the future bring opportunities to specifically deplete them.

### 5.1. Deimmunization

The first published attempt to deimmunize a protein involved the introduction of alanine substitutions at the MHC anchoring residues Y73, K74, R77, E80, and D82 of staphylokinase, alone or in combination. These modifications to the native protein were subsequently shown to reduce or eliminate a T cell response and clinical immunogenicity [176]; this was followed by a number of additional deimmunization studies [29,41,171–175,177,178]. Ongoing efforts include deimmunization of (1) botulinum neurotoxin type A, (2) lysostaphin, and (3) Factor VIII [97], as well as (4) removing T regulatory epitopes from a monoclonal antibody (anti-DEC205) for vaccine applications. Epitope modification has also been applied to several other therapeutic proteins in studies performed by researchers at Biovation [179], Epimmune [52], Genencor [180], and elsewhere, using a range of methodologies.

Clearly, modification of the sequence of a protein can have an impact on the secondary or tertiary structure. Thus the utility of this approach depends on the location and extent of amino acid changes and the impact of those changes on the pharmacological activity of the modified protein. On a case-by-case basis, an immunoinformatics-driven and cell-based approach to modifying protein sequences may eventually lead to less immunogenic proteins while preserving their therapeutic potential. Several de-immunized therapeutic proteins are in clinical use [176]; however, in the majority of instances, the immunogenic potential of the parent molecule was determined by the presence of T cell epitopes and not through clinical testing. This is the key limitation of all predictive methods, and

of course, clinical testing is the only way to “validate” any of the methods discussed in this review. Retrospective studies may be the only approach to testing the concept, but without a head-to-head comparison of the original and deimmunized proteins (FVIII, for example) in a clinical setting, all else will be correlative.

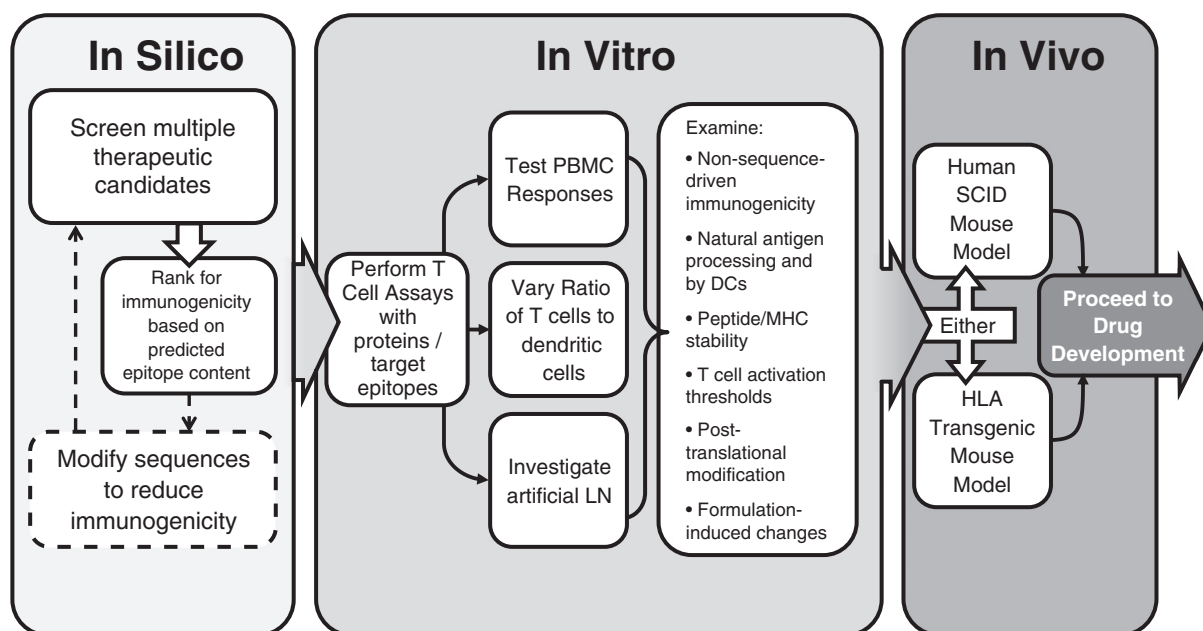
### 5.2. Tolerization

Active interference with T cell responses to protein therapeutics by inducing tolerance to the drug is an approach that has garnered significant interest in the last few years. Tolerance can be induced with non-depleting anti-CD4 antibodies [181,182]. IVIG has also been used to induce tolerance in solid organ transplant [183], to reduce FVIII inhibitors [184–189], and to inhibit ADA in Pompe patients undergoing Myozyme treatment [190]. Tolerance-inducing protocols combining Rituxan, methotrexate and IVIG that have been effectively applied to mitigate existing anti-therapeutic responses in the context of life-saving enzyme replacement therapy may provide an immediate solution for these patients [68,190,191]. However, there are new opportunities opening up along the developmental pipeline to build next-generation protein therapeutics to which patients are more tolerant. Towards this goal, De Groot et al. have identified a set of natural, human regulatory T cell epitopes (“Tregitopes”) present in the Fc and Fab domains of IgG that have also been shown to induce tolerance to co-administered proteins; these Tregitopes may be responsible for tolerance to idiotypic epitopes. When incubated with PBMC in vitro, Tregitopes specifically activate CD4<sup>+</sup> T cells, increase CD25/Foxp3 expression, and increase expression of regulatory cytokines and chemokines [41]. Administration of Tregitopes with protein antigens in vivo inhibits T cell proliferation and effector cytokine expression, and induces antigen-specific adaptive tolerance induction. Methods for co-administering Tregitopes with protein therapeutics are currently under development [192–195].

## 6. Considerations: assessing immunogenicity of therapeutic proteins

Given the potential impact of immunogenicity on the safety, efficacy, and utility of a final therapeutic protein drug product, there is increasing interest in developing approaches that will help to establish an accurate immunogenicity profile for a protein therapeutic. Immunogenicity predictions provide the greatest opportunity, and have the greatest benefit, early during selection of a lead molecule. Introduction of changes to the structure of a lead molecule could be prohibitively expensive with respect to time and resources necessary to repeat prior development and safety studies. We suggest consideration of a multi-step approach such as the following (Fig. 2) to evaluate the Td immunogenicity potential of a therapeutic protein:

Step 1 *HLA binding prediction in silico and confirmatory assessment in vitro*. A high-throughput in silico epitope-mapping algorithm applicable across common HLA class II haplotypes could identify areas of



**Figure 2** Example roadmap for immunogenicity prediction. A potential step-wise approach to pre-clinical immunogenicity testing is depicted here. *Step 1* would consist of the in silico screening of linear sequences from multiple therapeutic candidates for T cell epitopes and clusters. At the conclusion of Step 1, therapeutic candidates would be rated for immunogenic risk. *Step 2* would be an in vitro evaluation of immunogenicity in a series of T cell assays. This phase could include examination of antigen processing and presentation, post-translational modifications, and different formulations. Subsequent *Step 3* immunogenicity testing of therapeutic candidates may be carried out in vivo in established “humanized” animal models such as the Hu-SCID and HLA-transgenic mice. The culmination of this immunogenicity prediction strategy would be advancement into the drug development pipeline.

putative epitope clusters within a larger protein and allow drug candidates to be ranked in order of their potential for immunogenicity. However, in silico screening should never be viewed as a stand-alone technology. The in silico results could be carried forward by having the epitopes identified synthesized for testing in an in vitro peptide-binding assay. From this analysis, epitopes could be prioritized by strength of binding ( $IC_{50}$ ) and by the breadth of HLA to which they bind. To strengthen the predictive value of in silico tools and HLA binding assays that do not take into account antigen processing, editing, or presentation, cell-based approaches as described above [103] may also be applied.

**Step 2 Stimulation of T cell activity in vitro.** The focused set of epitopes or portions of a larger protein identified in Step 1 could be further assessed by in vitro human T cell assays to study immune responsiveness to this protein in Step 2. Knowledge gained from the in silico analysis can be applied here to demonstrate specificity of the response. Proliferation assays are sensitive, low in cost, and can be designed to assess phenotype of the responder cells. ELISpot assays and multiplex cytokine bead arrays are moderate in cost and more sensitive; intracellular cytokine staining methods have the highest sensitivity but can be technically challenging and higher in cost. It is recommended to utilize at least two independent readouts of T cell activity to ensure a balance between specificity and sensitivity. Results from Step 2 should strengthen the findings of the in silico prediction from Step 1. In addition, in vitro

screening may enable the evaluation of processing-associated changes, such as posttranslational modifications and misfolding. Where target-mediated, agonist effects, or formulation changes of an immunomodulatory therapeutic are a concern, in vitro studies can be performed in tandem with in silico studies.

Immunogenicity assessment is an important adjunct to preclinical studies that may facilitate the identification and selection of the best candidates to bring towards the clinic. The stepwise approach to preclinical Td immunogenicity assessment proposed here progresses from higher-throughput, lower-cost methods to narrow the search space, to lower-throughput, higher-cost methods to screen for T cell reactivity. At this point, a combination of these experimental results, experience with the protein of interest, and the stage of the development process may together prompt an effort to either proceed into in vivo animal experiments and further development (Fig. 2, Step 3), or perhaps to reengineer the protein to lower immunogenicity.

## 7. Conclusion

In response to concerns about the potential side effects of anti-drug immune responses, regulatory bodies such as the FDA and the EMA have begun to request that each protein therapeutic be accompanied by an immunogenicity risk assessment [196,197]. For example, the recent EMA guidance mentions “predictive immunogenicity” as an approach sponsors could consider in their preclinical studies [17]. In

this context, the historical focus has been on measurement of antibody responses as the read-out for immunogenicity, supported by the obvious consequences of ADA responses on protein therapeutic pharmacokinetics and efficacy. Td immunogenicity assessment has been considered by many drug developers to be an “upstream” activity, associated with the lead candidate optimization/selection process. Given the contribution of T cell responses to the development of a detrimental ADA response and the emerging suite of tools for predicting Td immunogenicity, this focus is shifting in some instances. For example, mitigating immunogenicity by modification of T cell responses, with careful attention to potential Treg epitopes, is now mentioned in the recently released FDA draft guidance entitled “Immunogenicity Assessment for Therapeutic Protein Products” [120].

The drug discovery and early development phases present prime opportunities to extract the greatest value from immunogenicity prediction tools. The cost of modifying or, in extreme cases, abandoning drugs that are near or have reached clinical trial stage can be quite significant [6]. Thus applying Td immunogenicity screening earlier in the drug-development pathway may be an important adjunct to preclinical studies in some circumstances.

At present, drug developers are applying a range of strategies and assays to assess and modulate humoral and/or cell-mediated immune responses to protein therapeutic drugs at both the preclinical and clinical phases of development. Guidelines for standardizing immunogenicity testing of protein therapeutics across the industry are emerging from these activities and parallel assay standardization and validation efforts in the vaccine discovery field. Approaches for reducing biologic protein immunogenicity are also proceeding apace; a number of drugs that have been de-immunized or tolerized are now being introduced into the clinic. Additional methods, such as tolerization using cytotoxic and immunosuppressive drugs, are currently in use in high-risk enzyme replacement therapy patients [66,68,191,198].

A wide range of Td immunogenicity screening methods examining different aspects of the process by which a protein therapeutic may trigger an immune response has been presented here. No single method has emerged as a definitive tool for determining whether or not a protein therapeutic will elicit a detrimental immune response in patients, and given the complexity of the immune system, a singular solution may not be realistic. Rather, the field is evolving to apply strategic combinations of multiple methods to most closely predict and mitigate immunogenicity risk. Td immunogenicity screening is a rapidly advancing science that could eventually impact drug development, reducing risks to patients and costs to industry. As more preclinical immunogenicity testing is performed and clinical correlations become available, the accuracy of preclinical immunogenicity screening methods and their utility to industry are bound to improve.

## Conflict of interest statement

Anne S. De Groot is a senior officer/majority shareholder and Leslie Cousens is an employee at EpiVax, Inc., a privately-owned immunotherapeutics company located in Providence RI. These authors acknowledge that there is a potential

conflict of interest related to their relationship with EpiVax and attest that the work contained in this report is free of any bias that might be associated with the commercial goals of the company.

## References

- [1] D.S. Dimitrov, Therapeutic proteins, *Methods Mol. Biol.* 899 (2012) 1–26.
- [2] W.R. Strohl, D.M. Knight, Discovery and development of biopharmaceuticals: current issues, *Curr. Opin. Biotechnol.* 20 (2009) 668–672.
- [3] G. Walsh, Biopharmaceutical benchmarks 2010, *Nat. Biotechnol.* 28 (2010) 917–924.
- [4] M.D. Barbosa, Immunogenicity of biotherapeutics in the context of developing biosimilars and biobetters, *Drug Discov. Today* 16 (2011) 345–353.
- [5] K.U. Eckardt, N. Casadevall, Pure red-cell aplasia due to anti-erythropoietin antibodies, *Nephrol. Dial. Transplant.* 18 (2003) 865–869.
- [6] S.M. Tatarewicz, X. Wei, S. Gupta, D. Masterman, S.J. Swanson, M.S. Moxness, Development of a maturing T-cell-mediated immune response in patients with idiopathic Parkinson's disease receiving r-metHuGDNF via continuous intraputaminial infusion, *J. Clin. Immunol.* 27 (2007) 620–627.
- [7] V. Jawa, M. Hokom, Z. Hu, N. El-Abaadi, Y. Zhuang, D. Berger, S. Gupta, S.J. Swanson, N. Chirmule, Assessment of immunogenicity of romiplostim in clinical studies with ITP subjects, *Ann. Hematol.* 89 (2010) 575–585.
- [8] G. Shankar, C. Pendley, K.E. Stein, A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs, *Nat. Biotechnol.* 25 (2007) 555–561.
- [9] M. Sauerborn, E. van de Vosse, D. Delawi, J.T. van Dissel, V. Brinks, H. Schellekens, Natural antibodies against bone morphogenic proteins and interferons in healthy donors and in patients with infections linked to type-1 cytokine responses, *J. Interferon Cytokine Res.* 31 (2011) 661–669.
- [10] N. Casadevall, J. Nataf, B. Viron, A. Kolta, J.J. Kiladjian, P. Martin-Dupont, P. Michaud, T. Papo, V. Ugo, I. Teyssandier, B. Varet, P. Mayeux, Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin, *N. Engl. J. Med.* 346 (2002) 469–475.
- [11] J. Li, C. Yang, Y. Xia, A. Bertino, J. Glaspy, M. Roberts, D.J. Kuter, Thrombocytopenia caused by the development of antibodies to thrombopoietin, *Blood* 98 (2001) 3241–3248.
- [12] F. Baert, M. Noman, S. Vermeire, G. Van Assche, D.H.G., A. Carbonez, P. Rutgeerts, Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease, *N. Engl. J. Med.* 348 (2003) 601–608.
- [13] C.H. Chung, B. Mirakhur, E. Chan, Q.T. Le, J. Berlin, M. Morse, B.A. Murphy, S.M. Satinover, J. Hosen, D. Mauro, R.J. Slebos, Q. Zhou, D. Gold, T. Hatley, D.J. Hicklin, T.A. Platts-Mills, Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose, *N. Engl. J. Med.* 358 (2008) 1109–1117.
- [14] S.K. Singh, Impact of product-related factors on immunogenicity of biotherapeutics, *J. Pharm. Sci.* 100 (2011) 354–387.
- [15] A.H. Gutierrez, L. Moise, A.S. De Groot, Of [hamsters] and men: a new perspective on host cell proteins, *Hum. Vaccin. Immunother.* 8 (2012).
- [16] K. Lewis, Inspiration Biopharmaceuticals Announces Clinical Hold of Clinical Trials Evaluating IB1001 for the Treatment and Prevention of Bleeding in Hemophilia B, Inspiration Biopharmaceuticals, 2012.
- [17] In: E.M. Agency (Ed.), Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins EMEA/CHMP/BMWP/14327/2007, 2007.



- [18] B. Monzavi-Karbassi, S. Shamloo, M. Kieber-Emmons, F. Jousheghany, P. Luo, K.Y. Lin, G. Cunto-Amesty, D.B. Weiner, T. Kieber-Emmons, Priming characteristics of peptide mimotopes of carbohydrate antigens, *Vaccine* 21 (2003) 753–760.
- [19] A. Beck, E. Wagner-Rousset, M.C. Bussat, M. Lokteff, C. Klinguer-Hamour, J.F. Haeuw, L. Goetsch, T. Wurch, A. Van Dorselaer, N. Corvaia, Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins, *Curr. Pharm. Biotechnol.* 9 (2008) 482–501.
- [20] R. Mueller, A. Karle, A. Vogt, H. Kropshofer, A. Ross, K. Maeder, H.C. Mahler, Evaluation of the immuno-stimulatory potential of stopper extractables and leachables by using dendritic cells as readout, *J. Pharm. Sci.* 98 (2009) 3548–3561.
- [21] A.S. Rosenberg, Effects of protein aggregates: an immunologic perspective, *AAPS J.* 8 (2006) E501–E507.
- [22] I.C. Buttel, P. Chamberlain, Y. Chowes, F. Ehmann, A. Greinacher, R. Jefferis, D. Kramer, H. Kropshofer, P. Lloyd, A. Lubiniecki, R. Krause, A. Mire-Sluis, T. Platts-Mills, J.A. Ragheb, B.M. Reipert, H. Schellekens, R. Seitz, P. Stas, M. Subramanyam, R. Thorpe, J.H. Trouvin, M. Weise, J. Windisch, C.K. Schneider, Taking immunogenicity assessment of therapeutic proteins to the next level, *Biologicals* 39 (2011) 100–109.
- [23] M.K. Joubert, M. Hokom, C. Eakin, L. Zhou, M. Deshpande, M.P. Baker, T.J. Goletz, B.A. Kerwin, N. Chirmule, L.O. Narhi, V. Jawa, Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses, *J. Biol. Chem.* 287 (2012) 25266–25279.
- [24] M.K. Joubert, Q. Luo, Y. Nashed-Samuel, J. Wypych, L.O. Narhi, Classification and characterization of therapeutic antibody aggregates, *J. Biol. Chem.* 286 (2011) 25118–25133.
- [25] D. Verthelyi, V. Wang, Trace levels of innate immune response modulating impurities (IIRMs) synergize to break tolerance to therapeutic proteins, *PLoS One* 5 (2010) e15252.
- [26] M.M. van Beers, W. Jiskoot, H. Schellekens, On the role of aggregates in the immunogenicity of recombinant human interferon beta in patients with multiple sclerosis, *J. Interferon Cytokine Res.* 30 (2010) 767–775.
- [27] A.S. Rathore, H. Winkle, Quality by design for biopharmaceuticals, *Nat. Biotechnol.* 27 (2009) 26–34.
- [28] K. Praditpornsilpa, P. Kupatawintu, W. Mongkonsritagoon, O. Supasynh, S. Jootar, T. Intarakumthornchai, C. Pongskul, W. Prasithsirikul, B. Achavanuntakul, P. Ruangarnchanasetr, S. Laohaviniij, S. Eiam-Ong, The association of anti-r-HuEpo-associated pure red cell aplasia with HLA-DRB1\*09-DQB1\*0309, *Nephrol. Dial. Transplant.* 24 (2009) 1545–1549.
- [29] A.R. Mire-Sluis, Y.C. Barrett, V. Devanarayan, E. Koren, H. Liu, M. Maia, T. Parish, G. Scott, G. Shankar, E. Shores, S.J. Swanson, G. Taniguchi, D. Wierda, L.A. Zuckerman, Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products, *J. Immunol. Methods* 289 (2004) 1–16.
- [30] S. Gupta, S.R. Indelicato, V. Jethwa, T. Kawabata, M. Kelley, A.R. Mire-Sluis, S.M. Richards, B. Rup, E. Shores, S.J. Swanson, E. Wakshull, Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics, *J. Immunol. Methods* 321 (2007) 1–18.
- [31] E. Koren, H.W. Smith, E. Shores, G. Shankar, D. Finco-Kent, B. Rup, Y.C. Barrett, V. Devanarayan, B. Gorovits, S. Gupta, T. Parish, V. Quarmby, M. Moxness, S.J. Swanson, G. Taniguchi, L.A. Zuckerman, C.C. Stebbins, A. Mire-Sluis, Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products, *J. Immunol. Methods* 333 (2008) 1–9.
- [32] G. Shankar, V. Devanarayan, L. Amaravadi, Y.C. Barrett, R. Bowsher, D. Finco-Kent, M. Fiscella, B. Gorovits, S. Kirschner, M. Moxness, T. Parish, V. Quarmby, H. Smith, W. Smith, L.A. Zuckerman, E. Koren, Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products, *J. Pharm. Biomed. Anal.* 48 (2008) 1267–1281.
- [33] In: U.F.C. CBER (Ed.), *Assay development for immunogenicity testing of therapeutic proteins*, 2009.
- [34] M. Stickler, N. Rochanayon, O.J. Razo, J. Mucha, W. Gebel, N. Faravashi, R. Chin, S. Holmes, F.A. Harding, An in vitro human cell-based assay to rank the relative immunogenicity of proteins, *Toxicol. Sci.* 77 (2004) 280–289.
- [35] A.S. De Groot, D.W. Scott, Immunogenicity of protein therapeutics, *Trends Immunol.* 28 (2007) 482–490.
- [36] A.S. De Groot, J. McMurry, L. Moise, Prediction of immunogenicity: in silico paradigms, ex vivo and in vivo correlates, *Curr. Opin. Pharmacol.* 8 (2008) 620–626.
- [37] L.C. Perry, T.D. Jones, M.P. Baker, New approaches to prediction of immune responses to therapeutic proteins during preclinical development, *Drugs R D* 9 (2008) 385–396.
- [38] P. Stas, I. Lasters, Strategies for preclinical immunogenicity assessment of protein therapeutics, *IDrugs* 12 (2009) 169–173.
- [39] F.A. Harding, M.M. Stickler, J. Razo, R.B. DuBridge, The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions, *MAbs* 2 (2010) 256–265.
- [40] D. Wullner, L. Zhou, E. Bramhall, A. Kuck, T.J. Goletz, S. Swanson, N. Chirmule, V. Jawa, Considerations for optimization and validation of an in vitro PBMC derived T cell assay for immunogenicity prediction of biotherapeutics, *Clin. Immunol.* 137 (2010) 5–14.
- [41] A.S. De Groot, L. Moise, J.A. McMurry, E. Wambre, L. Van Overtvelt, P. Moingeon, D.W. Scott, W. Martin, Activation of natural regulatory T cells by IgG Fc-derived peptide “Tregitopes”, *Blood* 112 (2008) 3303–3311.
- [42] C.J. Bryson, T.D. Jones, M.P. Baker, Prediction of immunogenicity of therapeutic proteins: validity of computational tools, *BioDrugs* 24 (2010) 1–8.
- [43] M.C. Honeyman, N.L. Stone, B.A. Falk, G. Nepom, L.C. Harrison, Evidence for molecular mimicry between human T cell epitopes in rotavirus and pancreatic islet autoantigens, *J. Immunol.* 184 (2010) 2204–2210.
- [44] M.F. Cusick, J.E. Libbey, R.S. Fujinami, Molecular mimicry as a mechanism of autoimmune disease, *Clin. Rev. Allergy Immunol.* 42 (1) (2012) 102–111.
- [45] R.F. Siliciano, A.D. Keegan, R.Z. Dintzis, H.M. Dintzis, H.S. Shin, The interaction of nominal antigen with T cell antigen receptors. I. Specific binding of multivalent nominal antigen to cytolytic T cell clones, *J. Immunol.* 135 (1985) 906–914.
- [46] R.H. Zubler, Naive and memory B cells in T-cell-dependent and T-independent responses, *Springer Semin. Immunopathol.* 23 (2001) 405–419.
- [47] C.C. Goodnow, C.G. Vinuesa, K.L. Randall, F. Mackay, R. Brink, Control systems and decision making for antibody production, *Nat. Immunol.* 11 (2010) 681–688.
- [48] M. Onda, Reducing the immunogenicity of protein therapeutics, *Curr. Drug Targets* 10 (2009) 131–139.
- [49] T. Defrance, M. Taillardet, L. Genestier, T cell-independent B cell memory, *Curr. Opin. Immunol.* 23 (2011) 330–336.
- [50] J.M. Weaver, C.A. Lazarski, K.A. Richards, F.A. Chaves, S.A. Jenks, P.R. Menges, A.J. Sant, Immunodominance of CD4 T cells to foreign antigens is peptide intrinsic and independent of molecular context: implications for vaccine design, *J. Immunol.* 181 (2008) 3039–3048.
- [51] M. Moxness, S. Tatarewicz, D. Weeraratne, N. Murakami, D. Wullner, D. Mytych, V. Jawa, E. Koren, S.J. Swanson, Immunogenicity testing by electrochemiluminescent detection for antibodies directed against therapeutic human monoclonal antibodies, *Clin. Chem.* 51 (2005) 1983–1985.

- [52] S. Tangri, B.R. Mothe, J. Eisenbraun, J. Sidney, S. Southwood, K. Briggs, J. Zinckgraf, P. Bilsel, M. Newman, R. Chesnut, C. Licalsi, A. Sette, Rationally engineered therapeutic proteins with reduced immunogenicity, *J. Immunol.* 174 (2005) 3187–3196.
- [53] M.D. Barbosa, J. Vielmetter, S. Chu, D.D. Smith, J. Jacinto, Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity, *Clin. Immunol.* 118 (2006) 42–50.
- [54] E. Koren, A.S. De Groot, V. Jawa, K.D. Beck, T. Boone, D. Rivera, L. Li, D. Mytych, M. Koscec, D. Weeraratne, S. Swanson, W. Martin, Clinical validation of the “in silico” prediction of immunogenicity of a human recombinant therapeutic protein, *Clin. Immunol.* 124 (2007) 26–32.
- [55] K.P. Pratt, A.R. Thompson, B-cell and T-cell epitopes in anti-factor VIII immune responses, *Clin. Rev. Allergy Immunol.* 37 (2009) 80–95.
- [56] C. Kamate, P.J. Lenting, H.M. van den Berg, T. Mutis, Depletion of CD4+/CD25high regulatory T cells may enhance or uncover factor VIII-specific T-cell responses in healthy individuals, *J. Thromb. Haemost.* 5 (2007) 611–613.
- [57] S. Yarkoni, A. Kaminitz, Y. Sagiv, I. Yaniv, N. Askenasy, Involvement of IL-2 in homeostasis of regulatory T cells: the IL-2 cycle, *Bioessays* 30 (2008) 875–888.
- [58] J.A. Bluestone, A.K. Abbas, Natural versus adaptive regulatory T cells, *Nat. Rev. Immunol.* 3 (2003) 253–257.
- [59] N.M. Tsuji, A. Kosaka, Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells, *Trends Immunol.* 29 (2008) 532–540.
- [60] M.C. Eyerman, X. Zhang, L.J. Wsocki, T cell recognition and tolerance of antibody diversity, *J. Immunol.* 157 (1996) 1037–1046.
- [61] D.T. Umetsu, R.H. DeKruyff, The regulation of allergy and asthma, *Immunol. Rev.* 212 (2006) 238–255.
- [62] M.R. Karlsson, J. Rugtveit, P. Brandtzaeg, Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy, *J. Exp. Med.* 199 (2004) 1679–1688.
- [63] A.R. Kendal, Y. Chen, F.S. Regateiro, J. Ma, E. Adams, S.P. Cobbold, S. Hori, H. Waldmann, Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance, *J. Exp. Med.* 208 (2011) 2043–2053.
- [64] M. Congia, S. Patel, A.P. Cope, S. De Virgiliis, G. Sonderstrup, T cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 3833–3838.
- [65] R.J. Farrell, M. Alsahli, Y.T. Jeen, K.R. Falchuk, M.A. Peppercorn, P. Michetti, Intravenous hydrocortisone premedication reduces antibodies to infliximab in Crohn's disease: a randomized controlled trial, *Gastroenterology* 124 (2003) 917–924.
- [66] M. Rohrbach, A. Klein, A. Kohli-Wiesner, D. Veraguth, I. Scheer, C. Balmer, R. Lauener, M.R. Baumgartner, CRIM-negative infantile Pompe disease: 42-month treatment outcome, *J. Inherit. Metab. Dis.* 33 (2010) 751–757.
- [67] E. Moran, F. Carbone, V. Augusti, F. Patrone, A. Ballestrero, A. Nencioni, Proteasome inhibitors as immunosuppressants: biological rationale and clinical experience, *Semin. Hematol.* 49 (2012) 270–276.
- [68] A. Joseph, K. Neff, J. Richard, L. Gao, D. Bangari, M. Joly, K. Culm-Merdek, R. Garman, J. Williams, S. Richards, M. Ruzek, Transient low-dose methotrexate induces tolerance to murine anti-thymocyte globulin and together they promote long-term allograft survival, *J. Immunol.* 189 (2012) 732–743.
- [69] Y. Kim, A. Sette, B. Peters, Applications for T-cell epitope queries and tools in the Immune Epitope Database and Analysis Resource, *J. Immunol. Methods* 374 (1–2) (2011) 62–69.
- [70] A.J. Godkin, K.J. Smith, A. Willis, M.V. Tejada-Simon, J. Zhang, T. Elliott, A.V. Hill, Naturally processed HLA class II peptides reveal highly conserved immunogenic flanking region sequence preferences that reflect antigen processing rather than peptide–MHC interactions, *J. Immunol.* 166 (2001) 6720–6727.
- [71] I.A. Doytchinova, V.A. Walshe, N.A. Jones, S.E. Gloster, P. Borrow, D.R. Flower, Coupling in silico and in vitro analysis of peptide–MHC binding: a bioinformatic approach enabling prediction of superbinding peptides and anchorless epitopes, *J. Immunol.* 172 (2004) 7495–7502.
- [72] A.S. De Groot, W. Martin, Reducing risk, improving outcomes: bioengineering less immunogenic protein therapeutics, *Clin. Immunol.* 131 (2009) 189–201.
- [73] Y. El-Manzalawy, D. Dobbs, V. Honavar, Predicting MHC-II binding affinity using multiple instance regression, *IEEE/ACM Trans. Comput. Biol. Bioinform.* 8 (4) (2011) 1067–1079.
- [74] A. Sette, S. Buus, E. Appella, J.A. Smith, R. Chesnut, C. Miles, S.M. Colon, H.M. Grey, Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis, *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 3296–3300.
- [75] A.S. De Groot, J. Rayner, W. Martin, Modelling the immunogenicity of therapeutic proteins using T cell epitope mapping, *Dev. Biol. (Basel)* 112 (2003) 71–80.
- [76] A.S. De Groot, J.A. Berzofsky, From genome to vaccine—new immunoinformatics tools for vaccine design, *Methods* 34 (2004) 425–428.
- [77] G.L. Zhang, N. Petrovsky, C.K. Kwok, J.T. August, V. Brusic, PRED(TAP): a system for prediction of peptide binding to the human transporter associated with antigen processing, *Immunome Res.* 2 (2006) 3.
- [78] J.C. Tong, Z.H. Zhang, J.T. August, V. Brusic, T.W. Tan, S. Ranganathan, In silico characterization of immunogenic epitopes presented by HLA-Cw\*0401, *Immunome Res.* 3 (2007) 7.
- [79] G.L. Zhang, A.M. Khan, K.N. Srinivasan, A. Heiny, K. Lee, C.K. Kwok, J.T. August, V. Brusic, Hotspot Hunter: a computational system for large-scale screening and selection of candidate immunological hotspots in pathogen proteomes, *BMC Bioinforma.* 9 (Suppl. 1) (2008) S19.
- [80] D.R. Flower, K. Phadwal, I.K. Macdonald, P.V. Coveney, M.N. Davies, S. Wan, T-cell epitope prediction and immune complex simulation using molecular dynamics: state of the art and persisting challenges, *Immunome Res.* 6 (Suppl. 2) (2010) S4.
- [81] T. Sturniolo, E. Bono, J. Ding, L. Raddrizzani, O. Tuercii, U. Sahin, M. Braxenthaler, F. Gallazzi, M.P. Protti, F. Sinigaglia, J. Hammer, Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices, *Nat. Biotechnol.* 17 (1999) 555–561.
- [82] S. Southwood, J. Sidney, A. Kondo, M.F. del Guercio, E. Appella, S. Hoffman, R.T. Kubo, R.W. Chesnut, H.M. Grey, A. Sette, Several common HLA-DR types share largely overlapping peptide binding repertoires, *J. Immunol.* 160 (1998) 3363–3373.
- [83] D. Valmori, F. Levy, E. Godefroy, L. Scotto, N.E. Souleimanian, J. Karbach, V. Tosello, C.S. Hesdorffer, L.J. Old, E. Jager, M. Ayyoub, Epitope clustering in regions undergoing efficient proteasomal processing defines immunodominant CTL regions of a tumor antigen, *Clin. Immunol.* 122 (2007) 163–172.
- [84] K.B. Bond, B. Sriwanthana, T.W. Hodge, A.S. De Groot, T.D. Mastro, N.L. Young, N. Promadej, J.D. Altman, K. Limpakarnjanarat, J.M. McNicholl, An HLA-directed molecular and bioinformatics approach identifies new HLA-A11 HIV-1 subtype E cytotoxic T lymphocyte epitopes in HIV-1-infected Thais, *AIDS Res. Hum. Retroviruses* 17 (2001) 703–717.
- [85] A.S. De Groot, A. Bosma, N. Chinai, J. Frost, B.M. Jesdale, M.A. Gonzalez, W. Martin, C. Saint-Aubin, From genome to vaccine: in silico predictions, ex vivo verification, *Vaccine* 19 (2001) 4385–4395.

- [86] Y. Dong, S. Demaria, X. Sun, F.R. Santori, B.M. Jesdale, A.S. De Groot, W.N. Rom, Y. Bushkin, HLA-A2-restricted CD8<sup>+</sup>-cytotoxic-T-cell responses to novel epitopes in *Mycobacterium tuberculosis* superoxide dismutase, alanine dehydrogenase, and glutamine synthetase, *Infect. Immun.* 72 (2004) 2412–2415.
- [87] J. McMurry, H. Sbai, M.L. Gennaro, E.J. Carter, W. Martin, A.S. De Groot, Analyzing *Mycobacterium tuberculosis* proteomes for candidate vaccine epitopes, *Tuberculosis (Edinb.)* 85 (2005) 95–105.
- [88] O.A. Koita, D. Dabito, I. Mahamadou, M. Tall, S. Dao, A. Tounkara, H. Guiteye, C. Noumsi, O. Thiero, M. Kone, D. Rivera, J.A. McMurry, W. Martin, A.S. De Groot, Confirmation of immunogenic consensus sequence HIV-1 T-cell epitopes in Bamako, Mali and Providence, Rhode Island, *Hum. Vaccin.* 2 (2006) 119–128.
- [89] T. Cohen, L. Moise, M. Ardito, W. Martin, A.S. De Groot, A method for individualizing the prediction of immunogenicity of protein vaccines and biologic therapeutics: individualized T cell epitope measure (iTEM), *J. Biomed. Biotechnol.* 2010 (2010).
- [90] L. Moise, R.M. Buller, J. Schriewer, J. Lee, S.E. Frey, D.B. Weiner, W. Martin, A.S. De Groot, VennVax, a DNA-prime, peptide-boost multi-T-cell epitope poxvirus vaccine, induces protective immunity against vaccinia infection by T cell response alone, *Vaccine* 29 (3) (2011) 501–511.
- [91] J.D. Ahlers, I.M. Belyakov, E.K. Thomas, J.A. Berzofsky, High-affinity T helper epitope induces complementary helper and APC polarization, increased CTL, and protection against viral infection, *J. Clin. Invest.* 108 (2001) 1677–1685.
- [92] A.S. De Groot, W. Martin, From immunome to vaccine: epitope mapping and vaccine design tools, *Novartis Found. Symp.* 254 (2003) 57–72 (discussion 72–56, 98–101, 250–102).
- [93] A.S. De Groot, D.S. Rivera, J.A. McMurry, S. Buus, W. Martin, Identification of immunogenic HLA-B7 “Achilles’ heel” epitopes within highly conserved regions of HIV, *Vaccine* 26 (2008) 3059–3071.
- [94] A.S. De Groot, M. Ardito, E.M. McClaine, L. Moise, W.D. Martin, Immunoinformatic comparison of T-cell epitopes contained in novel swine-origin influenza A (H1N1) virus with epitopes in 2008–2009 conventional influenza vaccine, *Vaccine* 27 (2009) 5740–5747.
- [95] H. Inaba, W. Martin, A.S. De Groot, S. Qin, L.J. De Groot, Thyrotropin receptor epitopes and their relation to histocompatibility leukocyte antigen-DR molecules in Graves’ disease, *J. Clin. Endocrinol. Metab.* 91 (2006) 2286–2294.
- [96] C.A. Weber, P.J. Mehta, M. Ardito, L. Moise, B. Martin, A.S. De Groot, T cell epitope: friend or foe? Immunogenicity of biologics in context, *Adv. Drug Deliv. Rev.* 61 (2009) 965–976.
- [97] L. Moise, C. Song, W.D. Martin, R. Tassone, A.S. De Groot, D.W. Scott, Effect of HLA DR epitope de-immunization of Factor VIII in vitro and in vivo, *Clin. Immunol.* 142 (2012) 320–331.
- [98] M. Nielsen, O. Lund, NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction, *BMC Bioinforma.* 10 (2009) 296.
- [99] H.G. Rammensee, Antigen presentation—recent developments, *Int. Arch. Allergy Immunol.* 110 (1996) 299–307.
- [100] D.R. Fooksman, G.K. Gronvall, Q. Tang, M. Edidin, Clustering class I MHC modulates sensitivity of T cell recognition, *J. Immunol.* 176 (2006) 6673–6680.
- [101] A.J. Sant, F.A. Chaves, S.A. Jenks, K.A. Richards, P. Menges, J.M. Weaver, C.A. Lazarski, The relationship between immunodominance, DM editing, and the kinetic stability of MHC class II:peptide complexes, *Immunol. Rev.* 207 (2005) 261–278.
- [102] C. Kuttler, A.K. Nussbaum, T.P. Dick, H.G. Rammensee, H. Schild, K.P. Hadeler, An algorithm for the prediction of proteasomal cleavages, *J. Mol. Biol.* 298 (2000) 417–429.
- [103] H. Kropshofer, T. Singer, Overview of cell-based tools for pre-clinical assessment of immunogenicity of biotherapeutics, *J. Immunotoxicol.* 3 (2006) 131–136.
- [104] T.A. Rohn, A. Reitz, A. Paschen, X.D. Nguyen, D. Schadendorf, A.B. Vogt, H. Kropshofer, A novel strategy for the discovery of MHC class II-restricted tumor antigens: identification of a melanotransferrin helper T-cell epitope, *Cancer Res.* 65 (2005) 10068–10078.
- [105] R.M. Chiczy, R.G. Urban, Analysis of MHC-presented peptides: applications in autoimmunity and vaccine development, *Immunol. Today* 15 (1994) 155–160.
- [106] C.A. Lazarski, F.A. Chaves, S.A. Jenks, S. Wu, K.A. Richards, J.M. Weaver, A.J. Sant, The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance, *Immunity* 23 (2005) 29–40.
- [107] K. Berer, G. Krishnamoorthy, Commensal gut flora and brain autoimmunity: a love or hate affair? *Acta Neuropathol.* 123 (2012) 639–651.
- [108] R.M. Welsh, J.W. Che, M.A. Brehm, L.K. Selin, Heterologous immunity between viruses, *Immunol. Rev.* 235 (2010) 244–266.
- [109] P. Wang, J. Sidney, C. Dow, B. Mothe, A. Sette, B. Peters, A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach, *PLoS Comput. Biol.* 4 (2008) e1000048.
- [110] L.A. Trouw, T.W. Huizinga, R.E. Toes, Autoimmunity in rheumatoid arthritis: different antigens—common principles, *Ann. Rheum. Dis.* 72 (Suppl. 2) (2013) ii132–ii136.
- [111] J.H. Kessler, W.E. Benckhuijsen, T. Mutis, C.J. Melief, S.H. van der Burg, J.W. Drijfhout, Competition-based cellular peptide binding assay for HLA class I, *Curr. Protoc. Immunol.* (2004)(Chapter 18, Unit 18 12).
- [112] J.H. Kessler, B. Mommaas, T. Mutis, I. Huijbers, D. Vissers, W.E. Benckhuijsen, G.M. Schreuder, R. Offringa, E. Goulmy, C.J. Melief, S.H. van der Burg, J.W. Drijfhout, Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides, *Hum. Immunol.* 64 (2003) 245–255.
- [113] A.C. Steere, W. Klitz, E.E. Drouin, B.A. Falk, W.W. Kwok, G.T. Nepom, L.A. Baxter-Lowe, Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a *Borrelia burgdorferi* peptide, *J. Exp. Med.* 203 (2006) 961–971.
- [114] J.A. McMurry, S.H. Gregory, L. Moise, D. Rivera, S. Buus, A.S. De Groot, Diversity of *Francisella tularensis* Schu4 antigens recognized by T lymphocytes after natural infections in humans: identification of candidate epitopes for inclusion in a rationally designed tularemia vaccine, *Vaccine* 25 (2007) 3179–3191.
- [115] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K<sub>1</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction, *Biochem. Pharmacol.* 22 (1973) 3099–3108.
- [116] S. Justesen, M. Harndahl, K. Lamberth, L.L. Nielsen, S. Buus, Functional recombinant MHC class II molecules and high-throughput peptide-binding assays, *Immunome Res.* 5 (2009) 2.
- [117] R. Buchli, R.S. VanGundy, H.D. Hickman-Miller, C.F. Giberson, W. Bardet, W.H. Hildebrand, Development and validation of a fluorescence polarization-based competitive peptide-binding assay for HLA-A\*0201—a new tool for epitope discovery, *Biochemistry* 44 (2005) 12491–12507.
- [118] L.C. Wu, D.S. Tuot, D.S. Lyons, K.C. Garcia, M.M. Davis, Two-step binding mechanism for T-cell receptor recognition of peptide MHC, *Nature* 418 (2002) 552–556.
- [119] S.T. Chang, D. Ghosh, D.E. Kirschner, J.J. Linderman, Peptide length-based prediction of peptide–MHC class II binding, *Bioinformatics* 22 (2006) 2761–2767.
- [120] Guidance for industry: immunogenicity assessment for therapeutic protein products, DHHS FDA Docket No. FDA-2013-D-0092,



- Document No. 2013-03019, 2013, (<https://federalregister.gov/a/2013-03019>).
- [121] M.P. Baker, T.D. Jones, Identification and removal of immunogenicity in therapeutic proteins, *Curr. Opin. Drug Discov. Devel.* 10 (2007) 219–227.
- [122] L. Findlay, D. Eastwood, C. Ball, C.J. Robinson, C. Bird, M. Wadhwa, S.J. Thorpe, R. Thorpe, R. Stebbings, S. Poole, Comparison of novel methods for predicting the risk of pro-inflammatory clinical infusion reactions during monoclonal antibody therapy, *J. Immunol. Methods* 371 (2011) 134–142.
- [123] G. Suntharalingam, M.R. Perry, S. Ward, S.J. Brett, A. Castello-Cortes, M.D. Brunner, N. Panoskaltis, Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412, *N. Engl. J. Med.* 355 (2006) 1018–1028.
- [124] C. Czerkinsky, G. Andersson, H.P. Ekre, L.A. Nilsson, L. Klareskog, O. Ouchterlony, Reverse ELISpot assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells, *J. Immunol. Methods* 110 (1988) 29–36.
- [125] C.C. Czerkinsky, L.A. Nilsson, H. Nygren, O. Ouchterlony, A. Tarkowski, A solid-phase enzyme-linked immunospot (ELISpot) assay for enumeration of specific antibody-secreting cells, *J. Immunol. Methods* 65 (1983) 109–121.
- [126] R.T. Carson, D.A. Vignali, Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay, *J. Immunol. Methods* 227 (1999) 41–52.
- [127] B. Makitalo, M. Andersson, I. Arestrom, K. Karlen, F. Villinger, A. Ansari, S. Paulie, R. Thorstensson, N. Ahlborg, ELISpot and ELISA analysis of spontaneous, mitogen-induced and antigen-specific cytokine production in cynomolgus and rhesus macaques, *J. Immunol. Methods* 270 (2002) 85–97.
- [128] R. Basu, R.D. Hatton, C.T. Weaver, The Th17 family: flexibility follows function, *Immunol. Rev.* 252 (2013) 89–103.
- [129] B. Foster, C. Prussin, F. Liu, J.K. Whitmire, J.L. Whitton, Detection of intracellular cytokines by flow cytometry, *Curr. Protoc. Immunol.* John Wiley and Sons, 2007, (Chapter 6; Unit 6.24).
- [130] P. Pala, T. Hussell, P.J. Openshaw, Flow cytometric measurement of intracellular cytokines, *J. Immunol. Methods* 243 (2000) 107–124.
- [131] L. Wooldridge, A. Lissina, D.K. Cole, H.A. van den Berg, D.A. Price, A.K. Sewell, Tricks with tetramers: how to get the most from multimeric peptide–MHC, *Immunology* 126 (2009) 147–164.
- [132] A. Jaber, M. Baker, Assessment of the immunogenicity of different interferon beta-1a formulations using ex vivo T-cell assays, *J. Pharm. Biomed. Anal.* 43 (2007) 1256–1261.
- [133] M.M. Stickler, D.A. Estell, F.A. Harding, CD4+ T-cell epitope determination using unexposed human donor peripheral blood mononuclear cells, *J. Immunother.* 23 (2000) 654–660.
- [134] S. Delluc, G. Ravot, B. Maillere, Quantification of the preexisting CD4 T-cell repertoire specific for human erythropoietin reveals its immunogenicity potential, *Blood* 116 (2010) 4542–4545.
- [135] R.G. Higbee, A.M. Byers, V. Dhir, D. Drake, H.G. Fahlenkamp, J. Gangur, A. Kachurin, O. Kachurina, D. Leistritz, Y. Ma, R. Mehta, E. Mishkin, J. Moser, L. Mosquera, M. Nguyen, R. Parkhill, S. Pawar, L. Poisson, G. Sanchez-Schmitz, B. Schanen, I. Singh, H. Song, T. Tapia, W. Warren, V. Wittman, An immunologic model for rapid vaccine assessment – a clinical trial in a test tube, *Altern. Lab. Anim.* 37 (Suppl. 1) (2009) 19–27.
- [136] C. Giese, A. Lubitz, C.D. Demmler, J. Reuschel, K. Bergner, U. Marx, Immunological substance testing on human lymphatic micro-organoids in vitro, *J. Biotechnol.* 148 (2010) 38–45.
- [137] Y. Ma, L. Poisson, G. Sanchez-Schmitz, S. Pawar, C. Qu, G.J. Randolph, W.L. Warren, E.M. Mishkin, R.G. Higbee, Assessing the immunopotency of Toll-like receptor agonists in an in vitro tissue-engineered immunological model, *Immunology* 130 (2010) 374–387.
- [138] C. Qu, V.A. Nguyen, M. Merad, G.J. Randolph, MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells, *J. Immunol.* 182 (2009) 3650–3659.
- [139] B.C. Schanen, A.S. De Groot, L. Moise, M. Ardito, E. McClaine, W. Martin, V. Wittman, W.L. Warren, D.R. Drake III, Coupling sensitive in vitro and in silico techniques to assess cross-reactive CD4(+) T cells against the swine-origin H1N1 influenza virus, *Vaccine* 29 (2011) 3299–3309.
- [140] V. Dhir, M. Fort, A. Mahmood, R. Higbee, W. Warren, P. Narayanan, V. Wittman, A predictive biomimetic model of cytokine release induced by TGN1412 and other therapeutic monoclonal antibodies, *J. Immunotoxicol.* 9 (1) (2012) 34–42.
- [141] C. Giese, C.D. Demmler, R. Ammer, S. Hartmann, A. Lubitz, L. Miller, R. Muller, U. Marx, A human lymph node in vitro–challenges and progress, *Artif. Organs* 30 (2006) 803–808.
- [142] J.M. Moser, E.R. Sassano, C. Leistritz del, J.M. Eatrises, S. Phogat, W. Koff, D.R. Drake III, Optimization of a dendritic cell-based assay for the in vitro priming of naive human CD4+ T cells, *J. Immunol. Methods* 353 (2010) 8–19.
- [143] R.A. Subramanian, S. Basha, R.C. Brady, S. Hazenfeld, M.T. Shata, D.I. Bernstein, Age-related changes in magnitude and diversity of cross-reactive CD4+ T-cell responses to the novel pandemic H1N1 influenza hemagglutinin, *Hum. Immunol.* 71 (2010) 957–963.
- [144] H. Karasuyama, K. Mukai, Y. Tsujimura, K. Obata, Newly discovered roles for basophils: a neglected minority gains new respect, *Nat. Rev. Immunol.* 9 (2009) 9–13.
- [145] M.M. Davis, J.D. Altman, E.W. Newell, Interrogating the repertoire: broadening the scope of peptide–MHC multimer analysis, *Nat. Rev. Immunol.* 11 (2011) 551–558.
- [146] H.T. Maecker, J.P. McCoy, R. Nussenblatt, Standardizing immunophenotyping for the Human Immunology Project, *Nat. Rev. Immunol.* 12 (2012) 191–200.
- [147] S. Pan, T. Trejo, J. Hansen, M. Smart, C.S. David, HLA-DR4 (DRB1\*0401) transgenic mice expressing an altered CD4-binding site: specificity and magnitude of DR4-restricted T cell response, *J. Immunol.* 161 (1998) 2925–2929.
- [148] Y.C. Kong, L.C. Lomo, R.W. Motte, A.A. Giraldo, J. Baisch, G. Strauss, G.J. Hammerling, C.S. David, HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1\*0301 (DR3) gene, *J. Exp. Med.* 184 (1996) 1167–1172.
- [149] M. Shirai, T. Arichi, M. Nishioka, T. Nomura, K. Ikeda, K. Kawanishi, V.H. Engelhard, S.M. Feinstone, J.A. Berzofsky, CTL responses of HLA-A2.1-transgenic mice specific for hepatitis C viral peptides predict epitopes for CTL of humans carrying HLA-A2.1, *J. Immunol.* 154 (1995) 2733–2742.
- [150] A.X. Le, E.J. Bernhard, M.J. Holterman, S. Strub, P. Parham, E. Lacy, V.H. Engelhard, Cytotoxic T cell responses in HLA-A2.1 transgenic mice. Recognition of HLA alloantigens and utilization of HLA-A2.1 as a restriction element, *J. Immunol.* 142 (1989) 1366–1371.
- [151] A. Didierlaurent, J.C. Ramirez, M. Gherardi, S.C. Zimmerli, M. Graf, H.A. Orbea, G. Pantaleo, R. Wagner, M. Esteban, J.P. Kraehenbuhl, J.C. Sirard, Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-A2-restricted cytotoxic T-cell responses, *Vaccine* 22 (2004) 3395–3403.
- [152] S. Man, M.H. Newberg, V.L. Crotzer, C.J. Luckey, N.S. Williams, Y. Chen, E.L. Huczko, J.P. Ridge, V.H. Engelhard, Definition of a human T cell epitope from influenza A non-structural protein 1 using HLA-A2.1 transgenic mice, *Int. Immunol.* 7 (1995) 597–605.
- [153] J. Charo, M. Sundback, A. Geluk, T. Ottenhoff, R. Kiessling, DNA immunization of HLA transgenic mice with a plasmid expressing mycobacterial heat shock protein 65 results in HLA class I- and II-restricted T cell responses that can be augmented by cytokines, *Hum. Gene Ther.* 12 (2001) 1797–1804.



- [154] G.Y. Ishioka, J. Fikes, G. Hermanson, B. Livingston, C. Crimi, M. Qin, M.F. del Guercio, C. Oseroff, C. Dahlberg, J. Alexander, R.W. Chesnut, A. Sette, Utilization of MHC class II transgenic mice for development of minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes, *J. Immunol.* 162 (1999) 3915–3925.
- [155] B.D. Livingston, M. Newman, C. Crimi, D. McKinney, R. Chesnut, A. Sette, Optimization of epitope processing enhances immunogenicity of multi-epitope DNA vaccines, *Vaccine* 19 (2001) 4652–4660.
- [156] T. Hanke, A.J. McMichael, M. Mwau, E.G. Wee, I. Ceberej, S. Patel, J. Sutton, M. Tomlinson, R.V. Samuel, Development of a DNA-MVA/HIVA vaccine for Kenya, *Vaccine* 20 (2002) 1995–1998.
- [157] M.V. Ragni, F.A. Bontempo, J.H. Lewis, Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC, *Transfusion* 29 (1989) 447–449.
- [158] E.A. Clark, J.A. Ledbetter, How B and T cells talk to each other, *Nature* 367 (1994) 425–428.
- [159] B.M. Reipert, M. Sasgary, R.U. Ahmad, W. Auer, P.L. Turecek, H.P. Schwarz, Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance, *Thromb. Haemost.* 86 (2001) 1345–1352.
- [160] M.J. Holder, H. Wang, A.E. Milner, M. Casamayor, R. Armitage, M.K. Spriggs, W.C. Fanslow, I.C. MacLennan, C.D. Gregory, J. Gordon, et al., Suppression of apoptosis in normal and neoplastic human B lymphocytes by CD40 ligand is independent of Bcl-2 induction, *Eur. J. Immunol.* 23 (1993) 2368–2371.
- [161] G.G. Klaus, M.S. Choi, E.W. Lam, C. Johnson-Leger, J. Cliff, CD40: a pivotal receptor in the determination of life/death decisions in B lymphocytes, *Int. Rev. Immunol.* 15 (1997) 5–31.
- [162] J. Qian, L.C. Burkly, E.P. Smith, J.L. Ferrant, L.W. Hoyer, D.W. Scott, C.C. Haudenschild, Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer, *Eur. J. Immunol.* 30 (2000) 2548–2554.
- [163] L. Bi, A.M. Lawler, S.E. Antonarakis, K.A. High, J.D. Gearhart, H.H. Kazazian Jr., Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A, *Nat. Genet.* 10 (1995) 119–121.
- [164] A. Mangalam, M. Rodriguez, C. David, A new humanized HLA transgenic mouse model of multiple sclerosis expressing class II on mouse CD4 T cells, *Ann. N. Y. Acad. Sci.* 1103 (2007) 112–117.
- [165] M.A. Brehm, A. Cuthbert, C. Yang, D.M. Miller, P. Dilorio, J. Laning, L. Burzenski, B. Gott, O. Foreman, A. Kavirayani, M. Herlihy, A.A. Rossini, L.D. Shultz, D.L. Greiner, Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rgamma(null) mutation, *Clin. Immunol.* 135 (2010) 84–98.
- [166] F. Ishikawa, Y. Saito, S. Yoshida, M. Harada, L.D. Shultz, The differentiative and regenerative properties of human hematopoietic stem/progenitor cells in NOD-SCID/IL2rgamma (null) mice, *Curr. Top. Microbiol. Immunol.* 324 (2008) 87–94.
- [167] A.K. Wege, M.W. Melkus, P.W. Denton, J.D. Estes, J.V. Garcia, Functional and phenotypic characterization of the humanized BLT mouse model, *Curr. Top. Microbiol. Immunol.* 324 (2008) 149–165.
- [168] P. Reddy, R. Negrin, G.R. Hill, Mouse models of bone marrow transplantation, *Biol. Blood Marrow Transplant.* 14 (2008) 129–135.
- [169] T. Willinger, A. Rongvaux, T. Strowig, M.G. Manz, R.A. Flavell, Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement, *Trends Immunol.* 32 (2011) 321–327.
- [170] M. Stickler, A.M. Valdes, W. Gebel, O.J. Razo, N. Faravashi, R. Chin, N. Rochanayon, F.A. Harding, The HLA-DR2 haplotype is associated with an increased proliferative response to the immunodominant CD4(+) T-cell epitope in human interferon-beta, *Genes Immun.* 5 (2004) 1–7.
- [171] A.S. Parker, W. Zheng, K.E. Griswold, C. Bailey-Kellogg, Optimization algorithms for functional deimmunization of therapeutic proteins, *BMC Bioinforma.* 11 (2010) 180.
- [172] J.R. Cantor, T.H. Yoo, A. Dixit, B.L. Iverson, T.G. Forsthuber, G. Georgiou, Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 1272–1277.
- [173] R.G. Holgate, M.P. Baker, Circumventing immunogenicity in the development of therapeutic antibodies, *IDrugs* 12 (2009) 233–237.
- [174] T.D. Jones, L.J. Crompton, F.J. Carr, M.P. Baker, Deimmunization of monoclonal antibodies, *Methods Mol. Biol.* 525 (2009) 405–423(xiv).
- [175] A.S. De Groot, M. Goldberg, L. Moise, W. Martin, Evolutionary deimmunization: an ancillary mechanism for self-tolerance? *Cell. Immunol.* 244 (2006) 148–153.
- [176] P.A. Warmerdam, S. Plaisance, K. Vanderlick, P. Vandervoort, K. Brepoels, D. Collen, M. De Maeyer, Elimination of a human T-cell region in staphylokinase by T-cell screening and computer modeling, *Thromb. Haemost.* 87 (2002) 666–673.
- [177] A.S. De Groot, P.M. Knopp, W. Martin, De-immunization of therapeutic proteins by T-cell epitope modification, *Dev. Biol. (Basel)* 122 (2005) 171–194.
- [178] D.W. Scott, A.S. De Groot, Can we prevent immunogenicity of human protein drugs? *Ann. Rheum. Dis.* 69 (Suppl. 1) (2010) i72–i76.
- [179] K. Hellendoorn, T. Jones, J. Watkins, M. Baker, A. Hamilton, F. Carr, Limiting the risk of immunogenicity by identification and removal of T-cell epitopes (DeImmunisationTM), *Cancer Cell Int.* 4 (2004) S20.
- [180] V.P. Yeung, J. Chang, J. Miller, C. Barnett, M. Stickler, F.A. Harding, Elimination of an immunodominant CD4+ T cell epitope in human IFN-beta does not result in an in vivo response directed at the subdominant epitope, *J. Immunol.* 172 (2004) 6658–6665.
- [181] S.P. Cobbold, S.X. Qin, H. Waldmann, Reprogramming the immune system for tolerance with monoclonal antibodies, *Semin. Immunol.* 2 (1990) 377–387.
- [182] D. Winsor-Hines, C. Merrill, M. O'Mahony, P.E. Rao, S.P. Cobbold, H. Waldmann, D.J. Ringler, P.D. Ponath, Induction of immunological tolerance/hyporesponsiveness in baboons with a nondepleting CD4 antibody, *J. Immunol.* 173 (2004) 4715–4723.
- [183] S.C. Jordan, M. Toyoda, A.A. Vo, Regulation of immunity and inflammation by intravenous immunoglobulin: relevance to solid organ transplantation, *Expert. Rev. Clin. Immunol.* 7 (2011) 341–348.
- [184] G. Zandman-Goddard, A. Krauthammer, Y. Levy, P. Langevitz, Y. Shoenfeld, Long-term therapy with intravenous immunoglobulin is beneficial in patients with autoimmune diseases, *Clin. Rev. Allergy Immunol.* 42 (2) (2012) 247–255.
- [185] A.C. Dykes, I.D. Walker, G.D. Lowe, R.C. Tait, Combined prednisolone and intravenous immunoglobulin treatment for acquired factor VIII inhibitors: a 2-year review, *Haemophilia* 7 (2001) 160–163.
- [186] D. Provan, Characteristics of immune thrombocytopenic purpura: a guide for clinical practice, *Eur. J. Haematol. Suppl.* (2009) 8–12.
- [187] D.B. Cines, H. Liebman, R. Stasi, Pathobiology of secondary immune thrombocytopenia, *Semin. Hematol.* 46 (2009) S2–S14.

- [188] P. Lozeron, D. Adams, Advances in the treatment of chronic inflammatory demyelinating neuropathies in 2010, *J. Neurol.* 258 (2011) 1737–1741.
- [189] M. Mahdi-Rogers, Y.A. Rajabally, Overview of the pathogenesis and treatment of chronic inflammatory demyelinating polyneuropathy with intravenous immunoglobulins, *Biologics* 4 (2010) 45–49.
- [190] N.J. Mendelsohn, Y.H. Messinger, A.S. Rosenberg, P.S. Kishnani, Elimination of antibodies to recombinant enzyme in Pompe's disease, *N. Engl. J. Med.* 360 (2009) 194–195.
- [191] R.D. Garman, K. Munroe, S.M. Richards, Methotrexate reduces antibody responses to recombinant human alpha-galactosidase A therapy in a mouse model of Fabry disease, *Clin. Exp. Immunol.* 137 (2004) 496–502.
- [192] Y. Su, R. Rossi, A.S. De Groot, D.W. Scott, Regulatory T cell epitopes (Tregitopes) in IgG induce tolerance in vivo and lack immunogenicity per se, *J. Leukoc. Biol.* 94 (2) (2013) 377–383.
- [193] L.P. Cousens, Y. Su, E. McClaine, X. Li, F. Terry, R. Smith, J. Lee, W. Martin, D.W. Scott, A.S. De Groot, Application of IgG-derived natural Treg epitopes (IgG tregitopes) to antigen-specific tolerance induction in a murine model of type 1 diabetes, *J. Diabetes Res.* 2013 (2013) 621693.
- [194] F. Mingozzi, K.A. High, Immune responses to AAV vectors: overcoming barriers to successful gene therapy, *Blood* 122 (1) (2013) 23–26.
- [195] S. van der Marel, A. Majowicz, K. Kwikkers, R. van Logtenstein, A.A. te Velde, A.S. De Groot, S.L. Meijer, S.J. van Deventer, H. Petry, D.W. Hommes, V. Ferreira, Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis, *World J. Gastroenterol.* 18 (2012) 4288–4299.
- [196] D.T. Mytych, S. La, T. Barger, J. Ferbas, S.J. Swanson, The development and validation of a sensitive, dual-flow cell, SPR-based biosensor immunoassay for the detection, semi-quantitation, and characterization of antibodies to darbepoetin alfa and epoetin alfa in human serum, *J. Pharm. Biomed. Anal.* 49 (2009) 415–426.
- [197] M. Wadhwa, R. Thorpe, Unwanted immunogenicity: lessons learned and future challenges, *Bioanalysis* 2 (2010) 1073–1084.
- [198] J.M. Cruzado, O. Bestard, J.M. Grinyo, New immunosuppressive protocols with the advent of novel biological drugs, *Transplantation* 88 (2009) S20–S23.
- [199] A.S. De Groot, R. Tassone, L.P. Cousens, F. Terry, R. Martin, M. Ardito, W. Martin, In silico prediction of HLA-DP and ?DQ epitope content is poorly correlated with clinical immunogenicity of therapeutic proteins, manuscript in preparation (2013), Abstract presented at the American Association of Pharmaceutical Scientists (AAPS) National Biotechnology Conference (NBC), San Diego, CA, USA. May 20-22, 2013.