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# Immunogenicity of Therapeutic Fusion proteins: Contributory Factors and Clinical Experience

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## **Immunogenicity of Therapeutic Fusion proteins: Contributory Factors and Clinical Experience**

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## Introduction

The past two decades have been remarkable for advances in the field of protein engineering. The impact of these advances on human health is now becoming evident. A wide variety of biologically engineered products are already in clinical use or under pre-clinical development by the biotechnology industry. Developers are engineering novel therapeutic proteins, monoclonal antibodies (mAbs), fusion proteins and antibody-like protein scaffolds, intent on expanding the number of clinical applications for these products. The caveat is that virtually all therapeutic proteins elicit some level of immune response that can lead to loss of efficacy or other adverse events, some of them serious; these effects have led to costly failures in clinical trials. Thus, while there is a greater emphasis on the development of biologics as a pathway to developing new drugs as compared to small molecules, drug developers and regulatory agencies are turning their attention to factors that contribute to protein immunogenicity as a means of improving clinical success. Fortunately, years of thorough study of the parameters influencing vaccine efficacy allow parallels to be drawn for protein therapeutics. Factors including delivery route, delivery vehicle, dose regimen, aggregation, innate immune system activation and the ability of the protein to interface with the humoral (B cell) and cellular (T cell) components of the immune system all impact the intended immunogenicity of vaccine immunogens when delivered to humans. The influence of these factors on the unintended immunogenicity of therapeutic proteins will be discussed here, with a particular focus on Fc fusion proteins, together with the use of state-of-the-art technology

in predicting and mitigating this risk. Notwithstanding the recent advances discussed here, the current state of science is such that clinical studies are required to evaluate immunogenicity of protein therapeutics.

### **Basis of Therapeutic Protein Immunogenicity**

Therapeutic protein products encompass diverse proteins like human cytokines, cellular growth factors, hormones, clotting factors, enzymes, fusion proteins and mAbs. Therapeutic proteins are attractive drug products, as they are generally considered safe, specific, and non-toxic. However, their efficacy can be dramatically compromised by the development of anti-therapeutic protein responses [1, 2]. Anti-therapeutic antibodies have the potential to neutralize clinical effects of this class of therapeutics. Moreover, anti-therapeutic protein responses can be associated with serious adverse events when they cross-react with autologous protein antigens.

Anti-therapeutic protein responses are not unexpected when the protein is foreign, either as the result of a different species of origin or a recipient in whom the natural analog of the therapeutic protein is deleted or modified. In these cases, immune responses to foreign proteins are fairly well delineated, owing largely to factors such as B and T cell epitopes that have been extensively characterized in the field of vaccinology. However, anti-therapeutic protein responses can also develop against recombinant autologous proteins to which, theoretically, the recipient should be tolerant. Biopharmaceuticals such as antibodies, “humanized” proteins and fusion proteins fall

somewhere between these two poles, as they are homologous with autologous protein sequences, and yet they frequently incorporate point mutations that confer therapeutic activity. Even such small changes may present the risk of introducing new epitopes never before encountered by the host.

For better understanding of immune responses to therapeutic proteins, it is helpful to categorize these responses into one of two general types: (i) activation of the classical immune system by foreign proteins, such as that elicited against pathogens or vaccines involving T cells, B cells and the innate immune system and (ii) breach of B and T cell tolerance to autologous proteins, a series of immunological events otherwise modulated by T regulatory cells (Treg) that has yet to be fully understood. In the case of classical immune induction, production of anti-therapeutic protein responses is the end result of a sequence of events that lead to B cell activation, and can be divided into T cell-independent (Ti) or T cell-dependent (Td) antibody production [3, 4]. Ti activation of B cells occurs when particular structural patterns, such as polymeric repeats, are able to directly induce stimulation and activation of a B cell subset. Although Ti activation is often credited as the source of anti-therapeutic protein responses, Ti activation of B cells is easily distinguished, as it generally does not lead to affinity maturation or to the generation of memory B cells [5]. In contrast, Td activation of B cells is generally characterized by isotype switching, development of B cell memory and overall more robust antibody responses. Thus, the induction of IgG-class antibodies (measured by standard immunogenicity assays) through isotype-switching indicates that the therapeutic protein contains a Td antigen. Td responses, by definition, are contingent upon T cell recognition of therapeutic protein-derived epitopes through the following basic process. The therapeutic protein is processed by antigen-presenting cells (APCs)

and presented on the APC surface by HLA (human leukocyte antigen) MHC class II molecules. This complex of epitope–HLA is recognized by antigen-specific helper T cells that facilitate B cell antibody production and isotype class switching. In the absence of signals provided by the cytokines released downstream of T cell-APC interactions, naïve B cells do not mature, and activated antigen-specific B cells can be rendered anergic or undergo apoptosis. Therefore, T cell recognition of peptide epitopes derived from a protein therapeutic is a key determinant of Td antibody formation and subsequent anti-therapeutic protein responses. Moreover, a number of clinical studies now suggest that serious adverse events are associated with high levels of T cell-dependent IgG antibodies [6-8].

The measurement of discrete peptide binding potential to the MHC molecules can be an important and quantitative tool for immunogenicity assessment. Indeed, the greater the affinity of a given peptide for the binding groove of a particular MHC molecule, the greater the opportunity to elicit a T cell response. However, this piece of the immunogenicity puzzle should be viewed in a broader context. For instance, rather than being randomly distributed throughout a protein, potential T cell epitopes tend to occur in clusters ranging from 9 to 25 amino acids in length [9, 10]. When considered in total, T cell epitope clusters predict larger sets of binding motifs with affinity across multiple alleles and multiple frames [11]. In addition, it is important to remember that in vivo, immunologically relevant epitopes are created by the antigen presenting cell that processes the protein into discrete peptide fragments, assembles the peptides in complexes with MHC molecules, and displays these complexes on its cell surface [12, 13]. Thus, while the accurate assessment of peptide binding potential to MHC molecules is an important tool for honing in on T cell epitopes and clusters thereof, it is only one

facet of the process by which functional T cell epitopes are generated.

Tolerance to self-proteins is a fundamental feature of the immune system [14, 15]. Thus, the development of immune responses to recombinant human or humanized proteins can be considered a breaking of this tolerance. Indeed, although self-reactive B cells are often observed, they are typically not stimulated to produce antibodies in response to natural levels of endogenous proteins [16]. Current efforts are underway to understand the role of T regulatory cells (Treg) in establishing and maintaining tolerance [17, 18]. The reduction or the overwhelming of Treg immune responses in the application of therapeutic proteins are potential contributors to anti-therapy immune responses that need to be considered. Moreover, the role of Treg in the development of autoimmune disease may be instructive for better understanding their contributions to the immunogenicity of therapeutic proteins.

In addition to the basic principles of immunology that govern T and B cell responses to foreign and self proteins [19], the implications of extrinsic factors that are particular to the therapeutic protein or its administration should also be considered in this context. These include protein modifications, aggregation and innate immune system activation. Modifications in recombinant proteins that occur in the in vitro expression and purification processes, including post-translational modifications and denaturation, could render them sufficiently different from their natural “self” analogs to be immunogenic. Aggregates of a therapeutic protein can be recognized by the immune system in a number of ways. One way that aggregates may trigger immune responses is through the recurrence of B-cell epitopes on the surfaces of the aggregate that could simulate the presence of highly repetitive arrays, like those seen on the surfaces of bacteria and viruses, facilitating cross-linking of the B cell receptor to activate proliferation.

Alternatively, aggregates are, like the VLP that so effectively induce immune responses in the vaccine context, more likely to be taken up by APCs, where degradation and presentation of T cell epitopes is then able to drive a Td response [20, 21]. Finally, engagement of Toll-like receptor (TLR) family members by products of microbial degradation has been shown to stimulate B cell responses and antibody production in the absence of T cell help [22]. Thus even low-level contaminants or impurities like endotoxins, microbial agents or host cell proteins present along with the therapeutic proteins could contribute to their immunogenicity.

### **Relevance to fusion proteins**

Full-length Fc fusion proteins are normally generated by fusing the N-terminus of the protein of interest containing the biologically active site to murine or human Fc (hinge-CH2-CH3). The fusion of proteins with Fc allows for large-scale manufacturing (milligram to gram quantities), easy purification and an increased in vivo half-life that proves useful in immunotherapeutic applications [23, 24]. Taking what we know about general protein immunogenicity, we can envision a number of Fc fusion protein attributes that should be given special consideration in preclinical development. For example, the joining of two non-immunogenic or self-proteins together can form junctions never before “seen” by the immune system that could comprise new T and B cell epitopes. Likewise, Fc fusion proteins may be taken up and processed differently than either of their native counterparts, contributing to higher immunogenicity than predicted for either component. Immunogenicity is enhanced by binding to Fc receptors on the surface of antigen presenting cells thereby targeting the antigen to these cells [25]. If this new combination

increases uptake or differentially stimulates antigen presenting cells, then the host immune system may respond differently. Also, as these novel protein sequences could be processed and presented in a way that is sufficiently distinct from the native protein components, resulting in greater immunogenicity. Deimmunization of the new immunogenic epitopes generated by fusion at the junctional region can be used to reduce their interaction with a T-cell receptor [26]. Due to these potential concerns of immunogenicity due to the fusion of two or more protein components, a robust immunogenicity evaluation strategy is often required as a part of risk assessment to support the drug development at preclinical and clinical stages [27]. Swanson et al. have reviewed the various assay platforms and their suitability for immunogenicity assessment [28]. From this comparative analysis, surface plasmon resonance-based Biacore assays emerge as ideal for testing the immunogenicity of fusion proteins. With the Biacore approach, each component of the fusion protein, such as the peptide portion, Fc portion or any linker regions, can be independently assessed for antibody reactivity simultaneously on parallel immobilized surfaces [28].

## **Tools for Immunogenicity Screening**

### **In Silico Analysis Tools**

As discussed above, immunogenicity of a therapeutic protein depends largely on its ability to trigger either a cellular or humoral immune response. In the case of cellular responses, T cells recognize small linear peptides derived from protein antigens upon their uptake and proteolytic processing by an antigen-presenting cell. These peptides are then presented to T cells as a complex of epitope in the binding groove of the major

histocompatibility complex (MHC) on the APC surface. One of the critical determinants of T cell epitope immunogenicity is the affinity of that epitope for the groove of the MHC; the higher the affinity the greater the likelihood that an epitope will be recognized by a T cell [29]. In contrast to T cell epitope recognition, which is restricted by presentation on MHC molecules, B cells and the antibodies that they produce generally recognize conformational epitopes directly on the surface of native proteins. Compared to MHC-T cell epitope interactions, those between the B cell receptor and B cell epitope are much more difficult to describe mathematically for use in prediction algorithms [30, 31]. Thus the groundbreaking work towards establishing reliable methods for immunogenicity prediction appears somewhat biased towards the definition of T cell epitopes; accessible, high throughput, and accurate prediction of B cell epitopes may be beyond the reach of current computational algorithms.

There are a number of factors inherent in the binding between a T cell epitope and an MHC binding groove that make this relationship amenable to computer-based prediction algorithms. Because several common HLA-DR types share largely overlapping peptide binding repertoires, analysis focused on as few as 8 MHC molecules can “cover” the genetic backgrounds of most humans worldwide [32]. Likewise, epitopes are generally limited to 9-21 amino acids in length, making their analysis manageable. Databases are available, such as the Immune Epitope Database Analysis Resource (IEDB; [www.tools.immunoepitope.org](http://www.tools.immunoepitope.org)), that manually curate experimentally characterized immune epitopes [33]. Taken together, amino acid preferences for interacting with different parts of the MHC binding groove can be observed and then extrapolated to predict interactions between unknown epitopes and certain MHC molecules.

Many computer-based algorithms created to identify MHC-restricted T cell epitopes have emerged from the research community over the last 10 or more years (Table 1 Epitope Prediction Tools). These *in silico* methods include frequency analysis, support-vector machines, Markov models and neural networks [34-36]. The common ground between these tools is the ability to quickly screen large datasets, including whole genomes, for prediction of T cell epitopes. These types of predictions have successfully been applied both to vaccine development [37-41] and to the identification of key epitopes triggering autoimmunity [42].

Additional tools for immunogenicity screening that go beyond these standard prediction methods have been developed by De Groot et al. [9, 36, 43]. Using their EpiMatrix algorithm, protein sequences are parsed into overlapping 9-mer peptide frames, each of which is then evaluated for binding potential to each of the 8 common MHC class II HLA alleles that generally represent the natural breadth of genetic diversity in human HLA [32]. This algorithm uses MHC “pocket profiles” that describe HLA binding groove coefficients, and applies these coefficients to predict whether a given 9-mer peptide will bind to a given MHC allele. The method initially described by Sturniolo and Hammer [44] has been adapted by De Groot et al. to generate Class II prediction tools [45]. One of the unique features of this algorithm is that allele-specific scores are normalized, making it possible to compare scores of any 9-mer across multiple HLA alleles and enabling a more broad assessment of predicted immunogenicity [36]. The predictive value of this algorithm has been extensively tested and is supported by published *in vitro* and *in vivo* studies [40, 45-50].

Mapping putative epitopes within a candidate protein therapeutic is a starting point for assessing the potential immunogenicity of a whole protein. It provides

information about the number of epitopes in a given protein as well as the tendency of these epitopes to cluster within particular regions of a protein. Sub-regions of densely packed high-scoring frames or “clusters” of potential immunogenicity can be identified, and cluster scores calculated and compiled, a process that has been well-established by the team of De Groot and Martin [9]. The frequency and density of these T cell epitope clusters can then be compared to a set of randomly generated pseudoprotein sequences of similar sizes. Proteins containing many, or concentrated clusters of, T cell epitopes are predicted to be highly immunogenic, while those containing few sparse epitopes are more likely to be less immunogenic. The relationship between the density of epitope clusters and protein immunogenicity has been validated extensively *in vitro* and *in vivo*. Moreover, these characteristics can be factored into the estimate of a whole protein’s overall “immunogenicity score”, a useful tool for consideration in the bioengineering of protein therapeutics. The ability to assign this standardized measure of immunogenicity to a protein facilitates informed decisions about the likelihood that a protein will provoke an immune response [43].

To aid in the direct comparison of immunogenicity predictions between proteins, an “immunogenicity scale” that maps immunogenicity predictions of analyzed proteins onto a scale of known proteins likewise analyzed and validated for immunogenicity can be developed (Figure 1 Immunogenicity Scale). Using this approach, the De Groot and Martin group has correctly predicted the clinical immunogenicity of a novel “peptibody”, a bioengineered autologous protein, and antibodies in clinical use [9, 51, 52] among others. Consequently, a number of biotech companies have integrated this approach to immunogenicity screening into their drug development pipeline. Beyond its use in evaluating a single therapeutic candidate, future applications of the immunogenicity

scale might include its utility in the early development process by aiding in the selection a lead low-immunogenicity candidate from among a panel of protein variants with a range of predicted immunogenicities.

### **In Vitro Analysis Tools**

For many years, in vitro assays have been used in transplantation research to predict the responsiveness of T cells to a target tissue based on HLA haplotype matching. Likewise, given the inherent risks posed by unwanted immunogenicity elicited by therapeutic proteins, there is an urgent need to develop specific and sensitive in vitro methods to analyze the potential immunogenicity of new therapeutic proteins. The challenge in developing these types of assays is to provide an appropriate milieu in culture that accurately represents in vivo conditions of immune stimulation [52-54]. In this respect, bulk cultures of peripheral blood lymphocytes, 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. If blood from individuals participating in clinical trials is available, peptides predicted as T cell epitopes and validated in MHC binding assays can be tested for their reactivity with T cells in vitro. A positive immune response, measured by peptide-specific cytokine production, proliferation, etc., is a strong indicator that the parent protein is immunogenic. Supported by this type of evidence, these

peptides can be considered validated T cell epitopes with the potential to contribute to an anti-drug Ab response.

ELISA and ELISpot are two related methods, both of which measure cytokines secreted by T cells (i.e. IFN $\gamma$ , IL-2, and IL-4). ELISAs are a means to 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 quality of the response (which cytokines are or are not secreted into the supernatant). ELISpot assays provide information regarding the numbers of cytokine-producing cells within a cell population stimulated *ex vivo*. ELISpots are sensitive because they are able to identify cells that produce relatively low quantities of the cytokine of interest.

The proliferation of T cells in response to stimulation by a peptide-MHC complex can be measured by (1) the dilution of a fluorescent dye, CFSE, that will decrease in fluorescence intensity by half with each round of cell division, a property measured by flow cytometry, and (2) the incorporation of a radioactive nucleotide, tritiated thymidine, into the DNA of dividing but not resting cells. Some of the most precise methodologies available for measuring and defining T cell responses are measured by flow cytometry. For example, T cells bearing T cell receptors specific for a particular epitope can be directly enumerated using fluorescently labeled tetrameric complexes of MHC class II molecules loaded with the peptide of interest. Antigen-specific T cells detected in this manner can be further examined for phenotypic and activation markers as well as for intracellular cytokine expression.

*HLA binding assays* can be used to measure the affinity of peptides derived from immunoinformatics analyses for binding to soluble MHC Class II molecules *in vitro*.

Evaluation of MHC binding can be performed as a competition assay, where a “test” peptide added over a range of concentrations is measured for its ability to inhibit the binding of a labeled “reference” peptide with a well-characterized affinity for a particular MHC allele [55]. This standardized method is cell-free and can be adapted for high throughput [56]. The advantage to using in vitro HLA binding assays is that detailed information about a peptide’s HLA affinity may be obtained prior to performing experiments on live cells. Data acquired from these assays may be indicative of immune responses in later experiments, such as ELISpots or HLA transgenic mouse studies, providing a reliable framework in which to interpret results. It is important to note, however, that the conditions under which candidate epitopes are tested in vitro are different from the cellular environment in ways that make a 1:1 comparison difficult. Artificially synthesized peptides may contain impurities or sequence errors. Solvents used in the experiment may interact negatively with certain amino acids, causing oxidation or forming unwanted disulfide bonds. Quality-controlled peptide manufacturing and storage along with proper reagent selection can minimize the impact of these problems.

Because competition binding assays yield indirect measures of affinity based on how effective a peptide inhibits the binding of a labeled reference peptide, only relative measurements proportionate to specific assay parameters can be made. A set of peptides can be “ranked” for binding to a given HLA allele provided they have been tested under the same conditions, but comparisons across alleles are not as straightforward. By fine-tuning the protocol for each allele through repeated experiments, a consensus among data points can be approached.

*T cell re-stimulation assays* can be utilized to identify and measure a recall or memory response in PBMC derived from subjects who have been exposed to a given biologic product within the context of a clinical trial. As the precursor frequency of the antigen-specific cells in a recall response is relatively high, whole PBMC assays are generally both rapid and sensitive with no pre-treatment or enrichment required. Epitope-mapping of the recall response can be performed using specific peptides from the whole protein at the time of challenge. T cell responses after re-stimulation *ex vivo* can include ELISA, ELISpot, proliferation, and flow cytometric analysis of activation markers of intracellular cytokine expression.

*Naïve PBMC assays* have been used to predict immunogenicity of therapeutic proteins [54, 57]. Compared to a recall response, the precursor frequency of antigen-specific cells in a naïve population is quite low. Thus, 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 accurately characterized. A kinetic analysis of responses following stimulation with the protein antigens of interest can be useful for detecting both low- and high- frequency antigen specific effector cells.

Both whole proteins and peptides can be used to measure T-cell responses *in vitro*; peptides can be of variable length (9 to 25 residues). Peptides presented in the context of class I MHC are generally limited to 9 or 10 amino acids in length, although some processing is believed to occur during the T cell assay and so 15-mers are also used for class I assays. In contrast with Class I epitopes which are short and fit tightly in the MHC molecule to which they bind, Class II (T helper) epitopes are displayed on an

open-ended groove in the MHC II. As such, a class II epitope can shift within this groove, thereby increasing the number of discrete 9-mer sequences able to interact with the MHC molecule. The only limit on the size of the peptide is its ability to remain in a linear conformation in the groove. Like peptides, whole antigens too can be used to measure T-cell responses in vitro. The recognition of these antigens requires the presence of an antigen-presenting cell 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. However, since PBMC contain several cell types capable of secreting IFN- $\gamma$  (NK cells, NKT cells, CD4 or CD8 T cells), assays employing PBMC to measure this cytokine may encounter obstacles. As not all IFN $\gamma$ -producing cells utilize the HLA class II - TCR activation pathway, the variety of cell types can contribute to a non-specific signal. This bystander effect can be minimized by use of CD8+ T cell-depleted PBMCs. The optimized in vitro concentration of protein required for challenge may be non-physiologic, potentially due to a limited antigen presenting population and/or co-stimulation. Moreover, there is a limitation in the number of antigen presenting cells when either whole PBMCs or enriched PBMCs are used in a long-term assay. The response rate of T cells could be further improved by co-culture of optimal antigen presenting cell: T cell ratios.

While the practice of inducing T cell responses in vitro through MHC-bound peptides on antigen presenting cells can generate valuable information, the complete nature of the immune response cannot be fully replicated in these experiments.

Future considerations for developing effective in vitro assays include the ability to:

- (a) Characterize responders by setting statistically derived criteria like fold increase or stimulation index,
- (b) Distinguish responders from non-responders,
- (c) Select the optimal set of markers for the identification of activated T cells and distinguishing between Treg and Teff subsets.
- (d) Identify parameters that define memory versus naïve T cell populations,
- (e) Standardize methods for cell harvest and preparation from whole blood.

Naïve and pre-sensitized blood can differ in the content of antigen-specific T effector and T regulatory cells. Hence, immediate handling of whole blood with a stringent freezing process is essential to ensure viability and functionality. Based on the nature of response (primed vs. stimulated and recalled), stimulation methods and the amount of antigen required for challenge can also differ. The naïve cells will require multiple challenges 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 context of tetramers or APC pulsed with whole proteins.

## **Approaches for Risk Assessment and Minimization**

### **Importance to product development**

The extent to which one characterizes immunogenicity of a new protein therapeutic is determined in part by the potential risk of immune-mediated sequelae in the human population targeted for its use. Factors to consider include, but are not limited to,

inherent potential immunogenicity of the protein, its biological role, indication of use, route of administration, duration of treatment as well as health status of the patient (e.g. immune competence).

The immunogenicity of therapeutic protein products is an important issue in product development as it can pose a serious safety risk to the health of patients if not thoroughly addressed during the drug development process. Indeed, serious and unexpected immune responses observed in clinical trial patients can derail a promising therapeutic otherwise on the path to successful licensing and marketing. To preclude these events, strategies for assessing immunological risk of a potential protein therapeutic should be incorporated into the earliest stages of the drug development process. Even before the clinical trial stage of development, immunogenicity should be considered for its impact on the ability to accurately measure pharmacokinetics, pharmacodynamics, bioavailability and efficacy during pre-clinical testing. Implementation strategies might include the use of current methods for immunogenicity prediction, the development of in vitro techniques to test the immunogenicity of predicted epitopes as well as validated and sensitive in vivo assays for detecting immune responses in pre-clinical animal models and clinical trial patients. Because of the diverse nature of therapeutic proteins, testing strategies should be considered on an individual basis. An aspect of risk-based analysis concerns the design of the molecule, encompassing in silico identification of any immunological “hot spots” and their reengineering so as to reduce or eliminate these hot spots. Performing binding, in vivo and cell-based assays to understand the functional role of drug attributes in a physiological context can be an additional component of the risk assessment. Clinical evaluation to confirm acceptable immunogenicity is always required because the in silico

and in vitro methods cannot account for all sources of variability. The types of tools for risk assessment and mitigation described herein can increase the stringency of selecting which therapeutics to move forward into clinical trials, making the process safer and more efficient.

### **Approaches for Risk Minimization**

Prediction of immunogenic epitopes has led to the strategy of deimmunization by epitope modification to disrupt HLA binding, an underlying requirement for T cell stimulation. Indeed, rational epitope modification is based on the natural process that occurs when tumor cells and pathogens escape immune pressure by accumulating mutations to reduce binding of their constituent epitopes to host HLA, rendering the host cell unable to alert T cells to the presence of the tumor or pathogen [58, 59]. As deimmunized protein therapeutics make their way into the clinic, the initial results appear to support this approach to reducing immunogenicity risk.

### **Tolerance induction**

Clusters of epitopes with MHC binding potential can stimulate an immune response through T<sub>H</sub> cell induction, but the ability of a peptide to bind to MHC does not necessarily imply an effector immune response. Alternatively, a peptide with MHC binding potential may interact with regulatory T cells to modulate an immune response. For example, potential epitopes in autologous proteins may trigger T cells that are absent from the peripheral circulation. While the majority of auto-reactive T cells are thought to be deleted during thymic development, it is now well accepted that some T

cells specific for autologous proteins escape thymic deletion to become natural regulatory T cells (nTregs). This subset of T cells serves as a suppressor of autoimmune and auto-reactive immune responses [18]. Just as the inadvertent addition of stimulatory T effector epitopes to proteins may lead to increased immunogenicity, removal or alteration of regulatory T cell epitopes in the drug development process may alter the natural T-regulatory immune response to recombinant autologous proteins. The link between HLA-restricted T cell immune response and the development of autoantibodies is still being defined, but early evidence points to the reduction of Treg immune responses and to induction of T effector responses as significant contributors in the context of unwanted immunogenicity [16].

During the last several decades, a variety of approaches have been developed for the induction of tolerance in experimental animals. Some of these approaches have progressed to clinical trials. Our goal herein is not to cover this area of research, which has been extensively reviewed elsewhere [15, 60]. Rather, the fundamental principles favoring tolerance will be outlined, following which we will focus on approaches developed in our lab and those of our collaborators.

As noted in the introductory section, the route of immunization with a protein significantly influences its immunogenicity and tolerogenicity. An intravenous injection favors tolerance whereas a subcutaneous or intramuscular injection leads to direct uptake by dendritic cells. These cells travel to the lymphoid follicles, where antigens are processed, and immune responses follow. In contrast, intravenous antigen is taken up by APCs in the spleen, primarily B cells. Oral administration of antigens can lead to unresponsiveness due to processing of antigens in the gut immune system. This route

may also deviate the response toward mucosal immune responses like IgA formation [61].

Alternatively, if co-stimulation is blocked (e.g. by CTLA-4-Ig or antibodies to CD80/CD86), anergy may ensue. The strategy is to provide “signal 1” (T cell epitopes bound to MHC) to the T cell in the absence of “signal 2” (co-stimulation). This approach has been widely employed in transplant models but has not been successful for other applications. Additional methods are based on treatments to block or subvert T cell signaling using either antibodies to the CD3 co-receptor or drugs like rapamycin that inhibit downstream signaling pathways. Clinical trials of drugs employing these approaches also are in progress [62, 63].

Recently, the use of so-called “tolerogenic” APC, which are tolerogenic immature dendritic cells (iDC) pulsed with target antigens, has gained popularity. Such iDC express low amounts of CD80/CD86, and thus provide little of the co-stimulation necessary to generate a robust adaptive response [64]. However, maintaining this immature phenotype *in vivo* can be challenging. In a related approach, B cells as tolerogenic APC in combination with gene therapy are being explored. Like iDC, naïve B cells are low in co-stimulatory molecules [65]. Even mature or activated B cells can still be tolerogenic under certain circumstances. This approach has been successful in a variety of mouse and rat models for autoimmune diseases and hemophilia; it is now moving forward to proof of concept in non-human primates.

Over two decades ago, B cells with immunoglobulin (Ig) fusion proteins began to be used as tolerogens. Immunoglobulins, especially IgG subclasses, had long been known to be excellent tolerogenic carriers by virtue of long half-life, favorable

pharmacodynamics and their ability to cross-link inhibitory Fc receptors [66]. The fusion protein *per se* was shown to be tolerogenic [67], a point to which we shall return. Animal studies demonstrated that retroviral expression of this Ig fusion in stem cells and B cells induced tolerance [68] and that Treg cells were induced by tolerogenic B cell presentation of epitopes, an effect that was enhanced by the presence of the IgG heavy chain scaffold [69]. Thus, Fc fusion proteins are one option to consider for accessing tolerogenic mechanisms and potentially decreasing the immunogenicity of a biologic drug.

#### **Regulatory T cell epitopes in IgG framework regions: Tregitopes**

“Tregitopes” are one potential explanation for the tolerogenicity of Fc fusion proteins as described in 2008 [70]. Tregitopes are highly conserved, tolerogenic peptides, discovered during epitope mapping and deimmunization programs with human protein (especially IgG) therapeutics. It was observed that all IgG subclasses contained epitopes in constant regions that were predicted to have strong binding to MHC class II, that were promiscuous and conserved in multiple mammalian species. It was predicted that these epitopes may be recognized by Treg cells. Further experimental data provided evidence that Tregitopes not only activate FoxP3-expressing T cells, they also suppress effector T cell reactions *in vitro* and *in vivo* [70]. In their natural state, Tregitopes are integrated into the arms and stem of human antibodies, and may serve to balance the inflammatory triggers that are present in the re-arranged segments (variable loops) of the antibody arms. Tregitopes are therefore present in intravenous immunoglobulins (IVIg), a blood-derived product that is used clinically to control autoimmune conditions [71]. Further studies may provide some insight into the mechanisms by which IVIg acts as an

immunosuppressive agent in the clinical setting [71-74]. Immunoglobulin therapies have previously been shown to induce expansion of Tregs in vitro and in vivo [72, 74]. Consistent with this observation, co-incubation of donor PBMC with Tregitopes derived from IgG has been shown to lead to suppression of immune response to bystander antigens. The corresponding murine epitopes suppress in vivo immune response in HLA DR4 transgenic mice [70-72]. De Groot and collaborators have now shown that the Tregitopes are immunosuppressive, dampen inflammation and induce tolerance in animal models of T1D, gene therapy, and organ transplants [75-81]. These observations have been corroborated by other researchers who have identified similar peptides in IgG, but have not recognized them as being Treg epitopes [82-85]

## **Case Study and Clinical Experience**

### **Observed vs. Predicted Immunogenicity of 21 antibodies in clinical use**

The accuracy of in silico methods for immunogenicity prediction has been tested on more than 20 antibodies in clinical use, in vitro or in a retrospective study. Protein sequence information for the variable regions of heavy and light chains was obtained from GenBank and the United States Patent and Trademark Office. All sequences were analyzed and scored for immunogenicity according to an immunogenicity scale. Observed immunogenicity from multiple studies was obtained, averaged, and regressed against the predicted overall immunogenicity score of the combined heavy and light chains (Figure 2 Predicted versus Observed Immunogenicity). The results of this regression analysis showed that the immunogenicity score was a statistically significant

predictor of observed immunogenicity (correlation coefficient = 0.67). Notable in this analysis is the relatively small data set, limited by the numbers of therapeutic antibodies in clinical trials, with only 20-30 participants on average per study. With recent evidence suggesting that observed immunogenicity for some therapeutics has been under-reported due to inadequate assays [86], this retrospective study supports the use of in silico immunogenicity assessment as part of a comprehensive preclinical development strategy [9].

### **Correlation of Clinical Immunogenicity of fusion proteins with In Silico Risk Estimates**

In a prospective study, the sequences from four different peptibody-based fusion proteins were assessed in silico for binding to HLA-DR alleles using the EpiMatrix algorithm, which assigns a Z score to overlapping 9-mer sequences based on their predicted affinity for 8 Class II MHC alleles [52, 54]. All four molecules were also administered in clinical trials, where an independent immunogenicity assessment was conducted. Table 2, Correlation of Clinical Immunogenicity with In Silico Risk Estimates, summarizes the EpiMatrix-generated scores associated with each protein and their respective rates of antibody incidence (binding and neutralizing). An assessment of Tregitope content in each molecule was also performed and the Z scores adjusted accordingly. FPX1, which had a high rate of clinical immunogenicity, was associated with elevated T cell epitope content reflected by its high Z score. Low Tregitope content was also associated with FPX1. FPX2-4 was associated with a low EpiMatrix score, and Tregitope adjustment reduced the predicted potential for binding further. Accordingly, FPX2-4 showed only minor clinical immunogenicity. Hence an inverse relationship

between Tregitope content and immunogenicity rate could be noted for these fusion proteins.

This retrospective study linking high T cell epitope content and low Tregitope content with observed clinical immunogenicity of FPX1 was followed by an evaluation of FPX1 immunogenicity in vitro by Koren et al. [52]. Whole blood-derived PBMC were obtained from healthy individuals dosed with FPX1. Cells were collected from both antibody-positive and antibody-negative subjects. Peptides, derived from either the N-terminus (predicted as non-immunogenic by in silico algorithm) or the C-terminus (predicted to be immunogenic by in silico algorithm) from FPX1, were used for challenge in the recall response. Antigen-specific responses from PBMC were assessed using IFN $\gamma$  and IL-4 ELISpots as readouts. A significant induction of IL-4 and IFN $\gamma$  spot forming cells was observed in PBMC derived from FPX1 antibody-positive subjects when the in vitro cultures were stimulated with the C-terminus peptide as well as the whole protein FPX1 (Table 3 High PBMC Response to FPX Peptides among Donors with High anti-FPX titers). Neither IL-4 nor IFN $\gamma$  spot forming cells were observed in PBMC derived from antibody-negative subjects and N-terminus peptide stimulation in vitro. There was an excellent correlation between the HLA restriction of the peptides, as predicted by EpiMatrix algorithms, and the HLA of the patients who responded to the epitopes [49].

The FPX1 fusion protein was further evaluated in a naïve human PBMC-derived in vitro sensitization assay [54]. Whole PBMC were stimulated with FPX1-derived N-terminus and C-terminus peptides multiple times to amplify the frequency of antigen-specific cells. Immune responses were measured by enumerating IFN $\gamma$ -secreting cells by ELISpot. A significant induction of IFN $\gamma$ -secreting cells was evident for the PBMC challenged with the immunogenic C-terminus of FPX1 peptide. No reactive cells were

observed when challenged with non-immunogenic N terminus peptide and a clinically proven and EpiMatrix-predicted non-immunogenic monoclonal antibody therapeutic.

The above retrospective study validates the hypothesis that immunogenicity of a biotherapeutic can be predicted through the systematic application of in silico and in vitro tools such as those described here (Figure 3 In Silico Prediction of Immunogenicity - Proof of Concept Study in Humans with FPX 1). Based on the in silico, in vitro and clinical findings related to FPX1 and their concordance to each other, a similar risk assessment strategy can be proposed for biotherapeutics under early stage development.

### **Preclinical and Clinical Immunogenicity Assessment Strategy**

The assessment of immunogenicity of a biotherapeutic is under considerable scrutiny by regulatory agencies. Hence, as a part of risk minimization and mitigation, drug manufacturers should have a strategy to detect and characterize the potential for immunogenicity. An evaluation of all samples for binding antibodies in a screening assay is one such strategy. The reactive samples are confirmed to contain antibody using a secondary species-specific antibody. Additionally, a drug depletion step confirms the specificity of the sample. The optimal platform for binding antibody assessment is chosen based on the nature and modality of the biotherapeutic. Lastly, a biological assay should be used to test if these antibodies are capable of neutralizing the biological effect of the drug [87, 88]. The appropriate assays when developed should be sensitive and specific enough to eliminate false positive results. A fully characterized antibody

response to a biotherapeutic enables risk assessment and clinical relevance for the patient [89].

### Strategy and Recommendation

A comprehensive approach to pre-clinical immunogenicity testing could begin with a high-throughput in silico screening followed by an in vitro evaluation and end with testing in vivo in transgenic animal models (Figure 4 Immunogenicity Prediction Strategy). Immunogenicity screening could follow a tiered approach where Tier 1 would entail screening of linear sequences from multiple therapeutic candidates for T cell epitopes and clusters therein. Candidates could then be ranked based on the quantity and quality of immunogenic epitopes, adjusted for Tregitope content.

Once the field of candidates has been prioritized and/or narrowed, Tier 2 screening would test the immunogenicity of these molecules in one or more in vitro assays. At this point the in vitro assays would, in addition to validating in silico predictions, bring forward any non-sequence-related immunogenicity concerns like processing-associated changes, post-translational modifications and alterations due to misfolding. In vitro assays can also be utilized to test therapeutics, which may have target-mediated, or agonist effects, and to overcome hurdles during formulation such as interference from aggregates. Such variables can enhance the immunogenicity of the drug product in ways that cannot be captured by in silico modeling, which only attends to the amino acid sequence. Even in vitro tests cannot be fully indicative of immunogenicity, and hence clinical evaluation of immunogenicity is always required to confirm the development predictions.

## Conclusions

The ability to analyze and prospectively predict the immunogenicity of a potential protein therapeutic has tremendous benefits at every stage of the drug development process. Fusion protein based biotherapeutics can pose an immunogenicity risk due to their structural differences from conventional fully human monoclonal or recombinant protein therapeutics. Reliable in silico immunogenicity screening makes it possible to rank lead candidates at the preclinical stage of development and/or reengineer proteins to make them less immunogenic. Moving forward in the preclinical development process, in vitro methods and in vivo animal models are important for validating the in silico findings. The in vitro methods can also address any non-sequence post translational and manufacturing-associated changes such as aggregation or contaminants. Due to limitations of the in silico and in vitro approaches, these assessments cannot substitute for clinical studies during drug development. Further downstream, the measurement of immunogenicity in patients enrolled in Phase I/II clinical trials of protein therapeutics should reflect the considerations taken in the preclinical process and provide additional opportunities to refine a lead candidate should anti-drug immunity arise. Finally, rigorous data collection in clinical trials may confer the ability to identify prospectively individuals at a higher risk of developing an anti-drug response, such as by HLA typing, where alternative therapies would be indicated.

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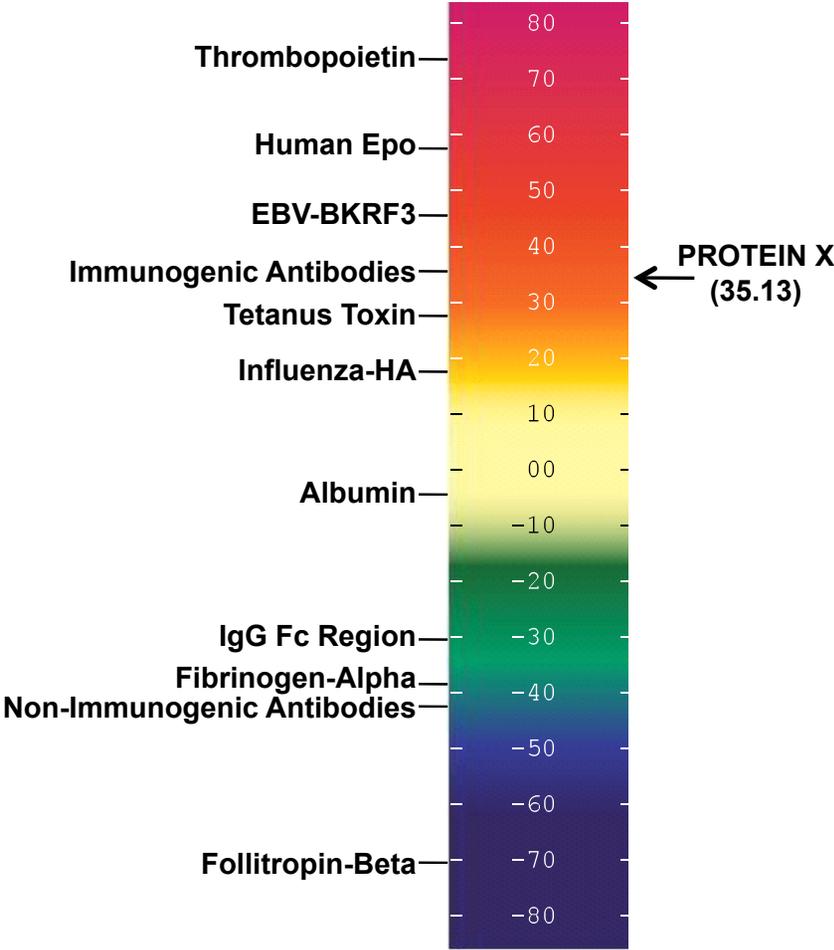
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**Table 1. Epitope Prediction Tools**

<b>NAME</b>	<b>DEVELOPERS/INSTITUTION</b>	<b>TYPE</b>	<b>WEBSITE</b>
EpiScreen	M. Baker and F. Carr Antitope, Ltd.	Commercial	<a href="http://www.antitope.co.uk/">www.antitope.co.uk/</a>
Epibase	I. Lasters and P. Stas Algonomics NV/Lonza, Inc.	Commercial	<a href="http://www.algonomics.com">www.algonomics.com</a>
EpiMatrix	A.S. De Groot and W.D. Martin EpiVax, Inc.	Collaborative/Commercial	<a href="http://www.epivax.com">www.epivax.com</a>
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. Nucleic Acids Res. 2010 Jan;38:D854-62.	Public	<a href="http://www.immuneepitope.com">www.immuneepitope.com</a>
MHC2PRED	G.P.S. Raghava Bioinformatics Center, Institute of Microbial Technology, Chandigarh, India	Public	<a href="http://www.imtech.res.in/raghava/mhc2pred/">www.imtech.res.in/raghava/mhc2pred/</a>
MHCPRED	D.R. Flower The Jenner Institute	Public	<a href="http://www.darrenflower.info/MHCPRED/">www.darrenflower.info/MHCPRED/</a>
PROPRED/ TEPITOPE	G.P.S. Raghava and H. Singh Bioinformatics Center, Institute of Microbial Technology, Chandigarh, India	Public	<a href="http://www.imtech.res.in/raghava/propred/">www.imtech.res.in/raghava/propred/</a>
RANKPEP	P.A. Reche Harvard Medical School	Public	<a href="http://bio.dfci.harvard.edu/RANKPEP/">http://bio.dfci.harvard.edu/RANKPEP/</a>
SVRMHC	P. Donnes, A. Elofsson Division for Simulation of Biological Systems, University of Tubingen, Germany	Public	<a href="http://svrmhc.biolead.org/">http://svrmhc.biolead.org/</a>
SYFPEITHI	H.G. Rammensee Department of Immunology, Tubingen, Germany	Public	<a href="http://www.syfpeithi.de/home.htm">www.syfpeithi.de/home.htm</a>
SMM-Align/ NetMHCII-2.2	M. Nielsen, C.Lundegaard, and O. Lund Center for Biological Sequence Analysis, Department of Systems Biology, Technical Univeristy of Denmark	Public	<a href="http://www.cbs.dtu.dk/services/NetMHCII-2.2/">www.cbs.dtu.dk/services/NetMHCII-2.2/</a>

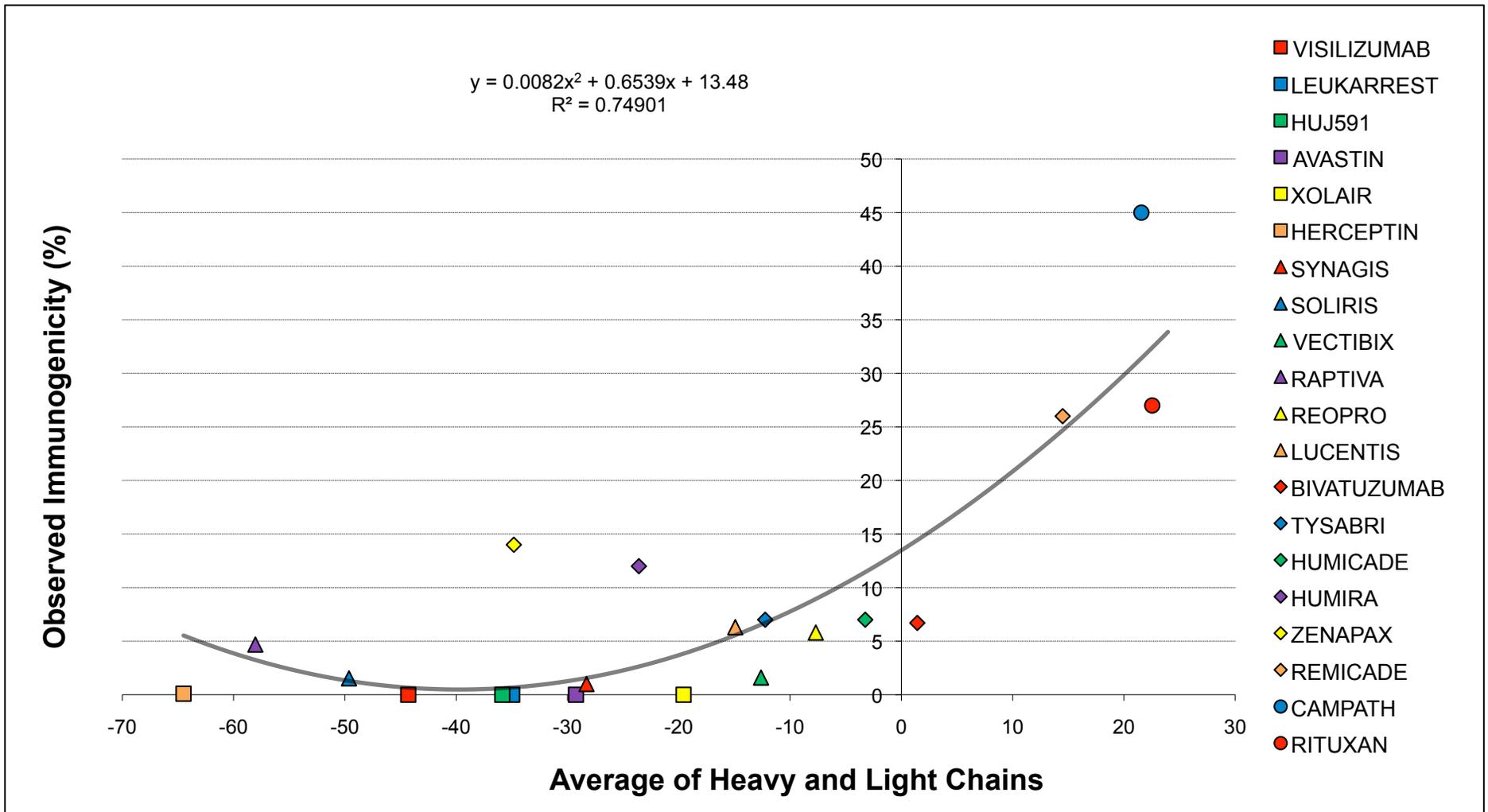
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Figure 1. Immunogenicity Scale



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Figure 2. Predicted versus Observed Immunogenicity



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**Table 2. Correlation of Clinical Immunogenicity with In Silico Risk Estimates**

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

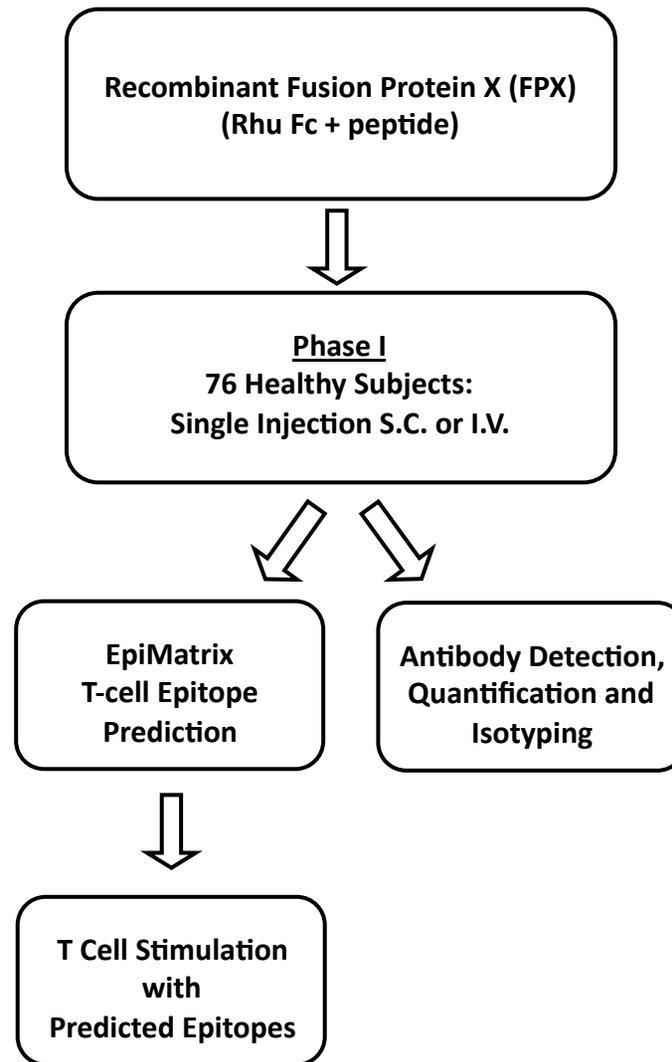
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Table 3. High PBMC Response to FPX Peptides among Donors with High anti-FPX Titers

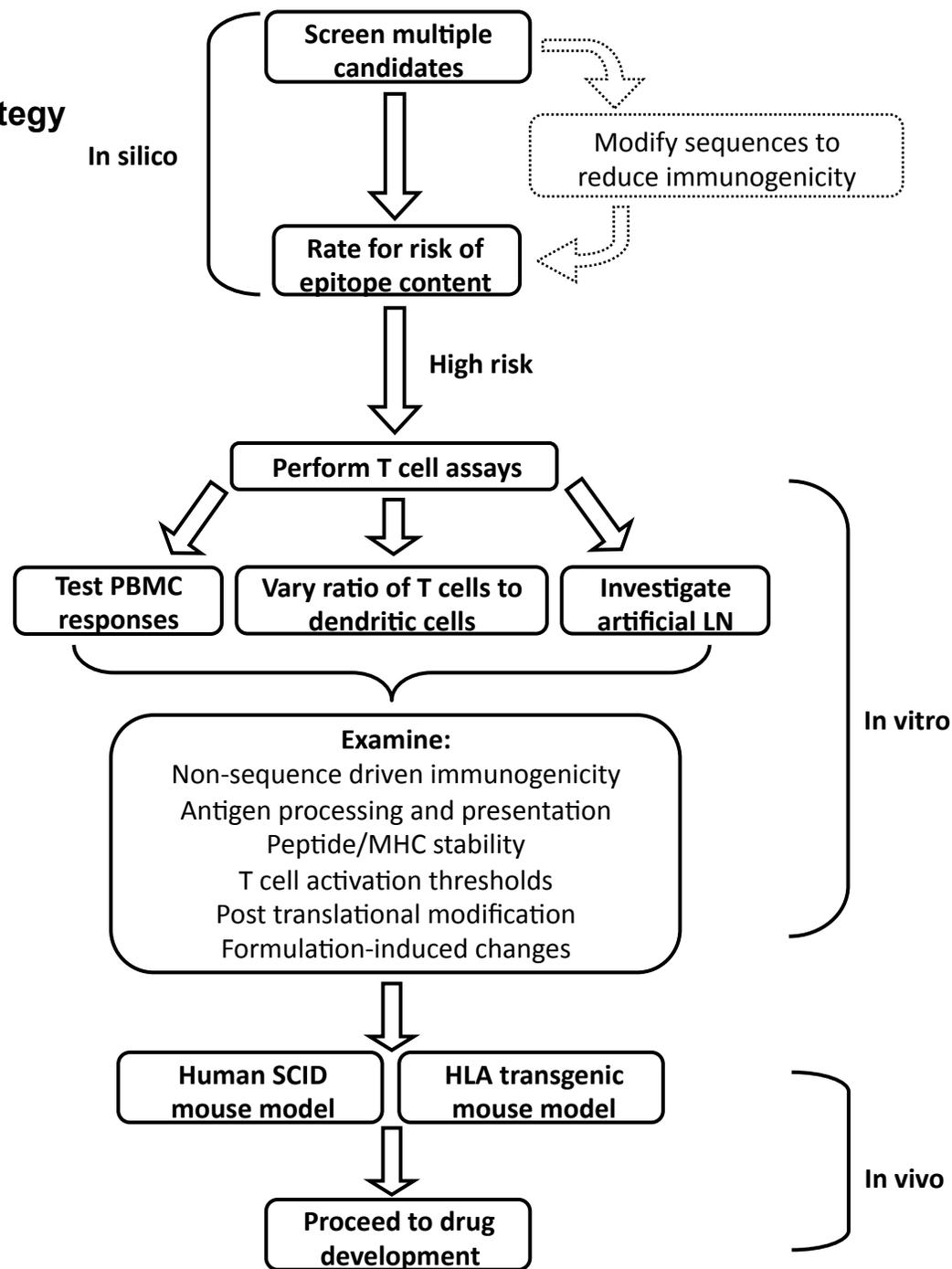
Subject ID	HLA-DRB1*	Antibody Concentration (mg/ml)	aa 1-10		aa 11-20		aa 1-24	
			IFN $\gamma$	IL-4	IFN $\gamma$	IL-4	IFN $\gamma$	IL-4
1	0701/1501	20.2	1.8	0.8	26	89	34	92
2	0101/1301	1.5	1.5	1.8	9.9	4.7	26.6	30.1
3	0101/1701	1	0.6	1.4	14.6	6.8	16.9	14.5
4	0405/0101	1.1	1.4	1.2	6	4.7	9.4	7.1

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Figure 3. In Silico Prediction of Immunogenicity: Proof of Concept Study in Humans with FPX1



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Figure 4. Immunogenicity Prediction Strategy



**Figure 1. Immunogenicity Scale.** Protein Immunogenicity Scale. Protein “X” is mapped on to the Protein Immunogenicity Scale according to its EpiMatrix score. Proteins scoring above +20 are considered to be potentially immunogenic. On the left of the scale are included some well-known proteins for comparison representing a range of immunogenicities, arranged by EpiMatrix score, from highest (red) to lowest (purple).

**Figure 2. Predicted versus Observed Immunogenicity.** Twenty human and chimeric antibodies were selected from the literature based on the availability of in vivo immunogenicity data and averaged where multiple studies of observed immunogenicity was available. Protein sequence information for the variable regions of heavy and light chains was obtained (GenBank and the United States Patent and Trademark Office). These sequences were analyzed with EpiMatrix and scored according to the EpiMatrix Immunogenicity Scale. Observed immunogenicity was regressed against predicted immunogenicity score of the combined heavy and light chains.

**Figure 3. In Silico Prediction of Immunogenicity - Proof of Concept Study in Humans with FPX 1.** In a prospective study, sequences from four different fusion proteins were assessed in silico for binding to HLA-DR alleles using the EpiMatrix algorithm. High T cell epitope content and low Tregitope content were retrospectively linked with observed clinical immunogenicity of FPX1, and followed by an evaluation of FPX1 immunogenicity in vitro. Based on the in silico, in vitro and clinical findings related to FPX1 and their concordance to each other, a similar risk assessment strategy may be proposed for biotherapeutics under early stage development.

**Figure 4. Immunogenicity Prediction Strategy.** A potential tiered approach to pre-clinical immunogenicity testing is depicted here. Tier 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 Tier 1, therapeutic candidates would be rated for immunogenic risk. Tier 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. Tier 3 would focus on immunogenicity testing of therapeutic candidates in vivo, in established “humanized” animal models such as the Hu-SCID and HLA-transgenic

mouse models. The culmination of this immunogenicity prediction strategy would be advancement into the drug development pipeline.