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## Immunogenicity of Protein Pharmaceuticals

Robert Dingman, Sathy V Balu-Iyer\*

Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY 14214

### Abstract

Protein therapeutics have drastically changed the landscape of treatment for many diseases by providing a regimen that is highly specific and lacks many off target toxicities. The clinical utility of many therapeutic proteins has been undermined by the potential development of unwanted immune responses against the protein, limiting their efficacy and negatively impacting its safety profile. This review attempts to provide an overview of immunogenicity of therapeutic proteins, including immune mechanisms and factors influencing immunogenicity, impact of immunogenicity, pre-clinical screening methods, and strategies to mitigate immunogenicity.

### Introduction:

Protein pharmaceuticals are one of the fastest growing class of drug molecules, including over 250 proteins used clinically for various indications. Advances in recombinant technology not only paved the way for unlimited supply of proteins, but also improved safety profiles by eliminating viral transmission that can occur with proteins isolated from natural sources. In addition, understanding the disease at the molecular level contributed to the growth of protein-based therapies by identifying key proteins that impact the severity or progression of the disease and harnessing their therapeutic potential to provide treatment. These examples include life-saving replacement therapies to treat Pompe Disease and Hemophilia A with recombinant acid alpha-glucosidase (rhGAA)<sup>1,2</sup> and Factor VIII (FVIII)<sup>3</sup>, respectively, and cytokine based therapies such as interferon beta for Multiple Sclerosis<sup>4,5</sup>. In addition, monoclonal antibody based drugs such as adalimumab (Humira), Fc-Fusion proteins such as Etanercept (Enbrel) for rheumatoid arthritis and Eloctate (Fc-FVIII) and immune checkpoint inhibitors such as pembrolizumab (Keytruda) and atezolizumab (Tecentriq), among several other antibody products have been developed.

Unfortunately, the clinical utility of many therapeutic proteins has been undermined by the potential development of unwanted immune responses against the protein. The incidence of immunogenicity of several clinically approved products has been well documented<sup>6–8</sup>

\*To whom correspondence should be addressed: Dr. S.V. Balu Iyer (formerly known as Sathyamangalam V. Balusubramanian), 359 Kapoor Hall, Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY 14214, Telephone: +1(716) 645-4836; Fax: +1 (716) 645-3693; svb@buffalo.edu.

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(Tables 1 and 2), and some have failed before even reaching clinical trials. The development of antibodies can limit efficacy and negatively impact safety, hampering the clinical utility of the protein.

## 1. Mechanism of immunogenicity:

**i. The Immune Response**—Immune responses against therapeutic proteins can arise due to two different mechanisms; a classical immune response, or by breaking tolerance<sup>9–11</sup>. The immunological discrimination of self and non-self-proteins is key to determining the mechanism of the immune response. In healthy individuals, the immune system maintains homeostasis between self and non-self-proteins by negative selection of self-reactive immune cells in thymus<sup>12</sup>, and proteins that are recognized as foreign will initiate a classical immune response in patients. This response is characterized by the formation of antibodies and is typically first seen within days to weeks after administration, and is often triggered after a single injection. These types of responses are long lasting and very difficult to reverse once memory B-cells have been generated<sup>13–15</sup>. Upon subsequent exposure to the protein, the immune system mounts a ‘secondary response,’<sup>13,16,17</sup>, primarily characterized by significant IgG release that greatly impacts therapy. The most well-known examples of therapeutic proteins that evoke classical immune responses are replacement therapies such as rhGAA<sup>18</sup>, and FVIII<sup>19,20</sup>. It also affects Mab therapeutics, as the CDR region is highly immunogenic and results in the generation of anti-idiotypic alloantibodies due to a lack of central tolerance to this region<sup>21,22</sup>. Therapeutic proteins that are homologous to endogenous self-proteins generally do not mount a response due to established immunological tolerance, but can become immunogenic by breaking B-cell tolerance<sup>23,24</sup>. B cell tolerance is maintained in the bone marrow mediated by negative selection of auto-reactive B cells and high BCR affinity cells are forced to undergo apoptosis<sup>25</sup>. Through repetitive administration, tolerance can be broken to therapeutic proteins<sup>23</sup>, as in IFN- $\gamma$ , IFN- $\beta$ , and Erythropoietin (Epo)<sup>26</sup>.

**ii. Cellular and Molecular Mechanisms of Immunogenicity:** The cellular mechanism leading to anti-drug antibody (ADA) formation to therapeutic proteins involves two major cell types, antigen presenting cells (APC), including dendritic cells and macrophages, and T and B lymphocytes<sup>9</sup>. Due to the high phagocytic capacity of APCs such as immature dendritic cells, administered proteins are engulfed, processed and presented in the context of major histocompatibility complex (MHC-II) to T-lymphocytes in lymph nodes<sup>10,27</sup>. This process is also accompanied by maturation of APC by upregulation of co-stimulatory markers such as CD40, CD80 and CD86, and migration of APCs to local lymph nodes<sup>28</sup>. With the help of secreted cytokines, APCs stimulate antigen specific T-lymphocytes that prompt the activation of B-lymphocytes and promotes their differentiation into memory B-cells and plasma cells (Figure 1 in Reference 31)<sup>29–31</sup>. Memory B-cells reside dormant until subsequent exposures to the therapeutic protein and plasma cells secrete antibodies that recognizes specific epitopes on the protein presented by APC MHC receptors<sup>17,32</sup>. Immunogenicity is also possible through T cell independent processes in which the antigen engages B cells directly<sup>33</sup>.

### iii. Immunological response of Therapeutic Proteins and

**Antibodies:** Immunogenicity of therapeutic proteins manifests as the development of ADA as well as hypersensitivity reactions<sup>34–36</sup>. Hypersensitivity reactions can be distinguished into four types depending on the mechanism of response and effector molecule generated<sup>37–39</sup>. Type I hypersensitivity is mediated by IgE antibodies that cross link proteins and mast cells with release of vasoactive mediators, most notably histamine, and typically manifests as anaphylaxis, rhinitis, hives and asthma<sup>37</sup>. Type I reactions are observed within minutes to hours after administration of proteins and can typically be prevented by prophylactic administration of anti-histamines. Type I reactions can also occur independent of IgE release, involving T cells, more commonly seen with food proteins<sup>34</sup>. In type II reactions, IgG and IgM antibodies directed against cell-surface antigens mediate cell destruction and complement activation<sup>40</sup>. A common example of Type II hypersensitivity is blood cell incompatibility. Due to cell surface markers, blood cells from different types (A, B, O) are perceived differently upon blood transfusion and the body reacts by attacking the “invading” cells. Type II hypersensitivity is also observed with Mab products that target cell surface antigens. Upon binding of the administered Mab to cell surface antigens expressed on target or non-target tissues, the immune system can elicit antibody dependent cellular toxicity (ADCC) causing cell lysis and death<sup>41</sup> (Fig 1). For example, cardiotoxicity observed with trastuzumab (Anti HER-2) has been at least partially attributed to the expression of HER-2 on cardiac muscle in addition to cancerous tissue due to ADCC<sup>42–44</sup>. Administered antibody bound to antigen can also evoke complement activation by binding to C1q and eventual deposition at the surface of the target cell, leading to complement-dependent cytotoxicity (CDC)<sup>45,46</sup> (Fig. 2). The design of the Fc portion of the antibody and antibody subtypes can also influence cellular cytotoxicity, as IgG2 and IgG4 show lower ADCC compared to IgG1 or IgG3, while IgG4 show no CDC effects<sup>44,47</sup>. Type III hypersensitivity is predominantly characterized as immune-complex mediated hypersensitivity. If the amount of protein is significantly higher than ADA present, small immune complexes can form that are not effectively cleared by phagocytic cells, leading to Type III reactions due to immune complex or protein-antibody complex deposits in various tissues. Complex deposition can lead to complement activation and inflammatory responses that cause serum sickness<sup>48</sup>. The size of the immune complexes of ADA and protein are primary determinants of type III reactions. Type IV hypersensitivity (delayed type hypersensitivity) is cell mediated, and is characterized by sensitized T helper cells that release cytokines and activate macrophages and T-cells<sup>48</sup>.

Based on clinical observation, about 63% of hypersensitivity reactions generated against Mabs are type I reactions, 13% experience cytokine release, while about 3% are delayed type IV reactions<sup>34</sup>. Immune related toxicities against Mabs, particularly immune modulating Mabs, arise from two mechanisms: first, target binding in healthy tissues as described and secondly, enhanced pharmacology, attenuating the activity of target molecules on the cells<sup>35</sup>. Recently, immune related adverse reactions have been documented for immune checkpoint inhibitors (anti CTLA-4, anti PDL –1 and anti PD1)<sup>49,50</sup>, which manifests as inflammation involving multiple organ systems, including the gastrointestinal tract, endocrine system, skin and liver and to a lesser degree the, central nervous system, cardiovascular and pulmonary systems. These immune related adverse reactions can present

at any time within first few weeks after treatment initiation up to months after therapy has been stopped. The pathophysiology underlying the toxicity is not fully understood, however it is likely to be related to the role that immune checkpoints play in maintaining immunologic homeostasis. Currently, steroid and immunosuppressive treatment is recommended for patients who have developed these adverse immune related reactions.

## 2. Impact of Immunogenicity on Efficacy

ADA responses against therapeutic proteins can be separated into two distinct classes; neutralizing and binding antibodies. Neutralizing antibodies recognize regions within therapeutic proteins that are critical for its biological activity, abrogating its activity directly. For example, about 30% of severe Hemophilia A patients who take recombinant FVIII as replacement, develop neutralizing antibodies, referred as “inhibitors” and in these inhibitor positive patients, hemostatic efficacy is lost<sup>19</sup>. Similarly, 89-100% of Pompe Disease patients who are treated with recombinant human acid alpha-glucosidase (rhGAA), develop antibodies against rhGAA<sup>51,52</sup>. Once a sustained immune response is established, the efficacy of rhGAA therapy is lost and no safe clinical options are available. In contrast, binding antibodies interact with the protein and can alter the pharmacokinetics of the protein, indirectly impacting its efficacy by reducing overall systemic exposure. During dose escalation clinical trials of emicizumab, a bi-specific FVIII mimetic, one out of six patients developed ADA that resulted in rapid clearance of the protein, indirectly impacting hemostatic efficacy<sup>53</sup>. Similarly, long-term use of adalimumab resulted in development of ADA in about 28% of patients and it was associated with lower adalimumab concentration and poorer clinical outcomes<sup>54,55</sup>.

**i. PK/PD Modeling of Immunogenicity:** The use of pharmacokinetic and pharmacodynamic (PK/PD) approaches to assess the impact of immunogenicity on efficacy has recently gained considerable interest. As discussed, binding antibodies can impact PD indirectly by altering the PK of the protein, often by increasing the clearance<sup>56,57</sup>. An increasing number of studies have incorporated the effect of immunogenicity to develop PK models that describes the impact of antibodies on absorption, disposition, metabolism and elimination (ADME) of therapeutic proteins<sup>58</sup>. A few model designs have been used; models that include immunogenicity as a covariate, models that include ADA concentration as a continuous variable, and models that include immunogenicity as a supplementary clearance (Fig 3). Zhou et al. modeled immunogenicity as a covariate and found that it has a significant impact on golimumab clearance<sup>58</sup>. Alternatively, the effect of ADA can be modeled as a separate clearance pathway, leading to direct elimination from the circulation as opposed to proteolytic degradation in tissue. Deng et al. developed a PK model for adalimumab, incorporating ADA as a factor increasing clearance<sup>59</sup>. Castele et al developed a non-linear mixed effects model to describe certolizumab PK in a Crohn’s disease patient and modeled ADA concentration as a continuous variable<sup>60</sup>. ADA can also be modeled as a categorical variable, as done by Rosario et al<sup>61</sup>, in which ADA is modeled based on if it is present or absent. This type of model however does not account varying levels of ADA in patients. Another consideration is the incorporation of a lag component in the model to account for the time it takes for ADA to develop. Vicini et al. modeled immunogenicity as a clearance pathway, and included a series of delay compartments through which the antibody

must pass before entering the central compartment<sup>62</sup>. Gomez-Mantilla has published an excellent review surveying the field of incorporating ADA in PK models<sup>63</sup>.

Very few studies have been published that integrate ADA response using PK/PD approaches, which is critical to completely delineate the impact of immunogenicity on efficacy. Such PK/PD integration also requires consideration of the direct effect ADA have on the PD of therapeutic proteins. We recently proposed an integrated model to define the impact of inhibitor development against FVIII in Hemophilia A where the fraction unbound of FVIII was elucidated from PK analysis and integrated with a direct response model to determine inhibitor effects on PD<sup>64</sup>.

**ii. Measurement of Anti-drug Antibodies**—Measurement of ADA in patients is important in clinical trials as a requirement for new drug approvals. Different assays can have an impact on the perceived immunogenicity rates and severity, as assays must be sensitive enough to detect low levels of ADA and be able to differentiate between the therapeutic protein and the ADA. Additionally, characterizing and comparing methods is often difficult in most cases because human ADA standards are not available to determine exact concentrations. Challenges of ADA measurement include the selectivity of the assay for ADA and not drug product, the presence of false positive and negatives of several assays, and a lack of standardization across experiments makes it difficult to compare immunogenicity of different products tested differently. It is likely that early phase 3 clinical trials under or overestimated immunogenicity in protein products due to assay limitations in detection and differentiation between drug and ADA. Assay selection is key to determining the patients' clinical outcome and this is no better shown than with adalimumab and TNF blockers. Bartelds showed that ADA against adalimumab directly correlates with patient outcomes and estimated immunogenicity rate using a radioimmunoassay<sup>65</sup>. Schouwenburg et al compared an antigen binding test and a pH shift anti-idiotypic binding test (PIA) and showed that the drug tolerant PIA assay better correlated with clinical outcomes of adalimumab treatment<sup>66</sup>. Bartelds, Schouwenburg, and Hart published a novel assay to detect anti-adalimumab antibodies which showed better detection of immunogenicity than previous assays<sup>67</sup>.

**a) Bridging Assays:** Bridging assays are one of the most commonly used assays in industry to detect ADA. In bridging immunoassays, the drug is labeled and ADA will form a bridge between two labeled drug molecules that can be detected by the label. These assays are useful in detecting all antibody classes, and can be used in any sample, as the ADA will always bind to the labeled drug. Recently, the Gyrolab platform has been used to develop binding assays for ADA detection<sup>68</sup>. It offers advantages such as automation and smaller volume sizes that can eliminate the use of costly resources and human error.

Unfortunately, there are some limitations to bridging assays. First, soluble antigen in plasma can cause drug molecules to bridge, resulting in false positives<sup>69</sup>. Secondly, drug interference is a major issue that can result in the determination of false negatives due to drug tolerance, seemingly decreasing the rate of immunogenicity. Drug tolerance is the amount of free drug that can be present in a sample that doesn't interfere with the detection of ADA. If too much free drug is present in a sample, the drug can conceal the presence of

low level ADA in the sample. Finally, in select cases, pre-existing antibodies can interfere with bridging immunoassays<sup>70</sup>. One example is rheumatoid factor, which can bind to the Fc region on antibodies, and result in false positives<sup>71</sup>.

**b) Ligand Binding Assays:** Ligand binding assays (LBA) are commonly used methods that are used to detect binding of a target. Shibata et al. compared three types of LBA to detect anti-EPO antibodies according to World Health Organization guidelines<sup>72</sup>. In their paper, they compared surface plasmon resonance, electrochemiluminescence, and biolayer interferometry. Electrochemiluminescence is a method in which light is measured as a result of chemical and electrical reactions. Biolayer interferometry is an optical method that measures light interference on a protein surface. As was hypothesized, each assay differed in their ability to detect ADA, confirming that assay selection is highly important in detecting ADA.

**c) Protein Specific Assays:** In some cases, protein specific assays can be used to measure ADA concentrations. In the case of FVIII, the Bethesda Assay can be used to specifically measure neutralizing anti-FVIII antibodies in concentration of BU/mL, which allows for better comparison of inhibitory ADA between studies<sup>73</sup>. In addition, FVIII is one of the few proteins where a standard is available to measure ADA by ELISA. ESH8 is an anti-FVIII antibody that can be used to generate a standard curve and is used to measure total FVIII ADA. Dong et al. developed an assay specific for anti-PEG antibodies in which biotin-PEG is conjugated to magnetic beads and anti-PEG antibodies bound to the bead is measured using a sensor that detects changes in size of the complex<sup>74</sup>. Previous assays for anti-PEG antibodies generally had high background, which is eliminated in this specific method because only the complex is detected by the sensor.

**d) Drug Tolerant Assays:** The presence of the drug in the sample can cause issues when detecting ADA, so drug-tolerant assays have been developed to overcome these problems and allow for better quantification of ADA. Several labs have conducted studies comparing drug tolerant assays in the detection of adalimumab ADA, looking at pH shift idiotypic antigen binding, acid dissociation assays, temperature shift assays, and electrochemoluminescence assays<sup>75,76</sup>. They concluded that all assays correlated well with the amount of ADA, however the rate of immunogenicity ranged from 51-66%, with many of the issues coming from low ADA patients. Increasing the sensitivity of these assays will best identify ADA-positive samples. Mikulskis et al. developed a solution ELISA drug tolerant assay to detect ADA against monoclonal antibodies after comparing it to several other methods<sup>77</sup>.

**e) ELISA:** ELISAs can also be used to detect ADA by coating the plate with the protein and incubating samples to measure ADA bound to the plate. This method has been used in our lab in conjunction with the Frey method to measure anti-rhGAA antibodies<sup>78,79</sup>. The utility of ELISAs can be limited because standards are not available for all ADA. Modified methods such as reciprocal dilutions, or optical density may be used in the place of actual concentration, however it makes it difficult to compare results between studies and between labs.

**f) Other:** In addition to the discussed methods, other less common methods are also used. Immune-PCR is an extension of bridging assays, used in special cases with high drug interference, in which the complex is labeled with biotin that is detected using an anti-biotin antibody conjugated to DNA<sup>80</sup>. Using PCR, the DNA can be quantified to assess ADA levels. Immuno-LC/MS can also be used to detect ADA in plasma<sup>81</sup>. Using a bridging assay, human ADA were identified and specific proteolytic cleavage patterns were identified to segregate Ig into subclasses. Before using immune-LC/MS, samples must be enriched, either by tagging the drug with biotin, or spiking excess drug into the sample to saturate ADA binding. Qu et al have also investigated methods of using LC/MS to detect antibody therapeutics, which could also be used to detect ADA<sup>82</sup>.

**g) Limitations of ADA detection:** There are two major complications in measuring ADA in patient samples. The first is interference from the drug and the second is assay sensitivity. Interference from circulating drug has the ability to complicate detection and removing free drug can improve these assays. The most common method to remove free drug is acid dissociation, which enriches the ADA and removes drug from the sample so that only the ADA is being detected. Secondly, some studies incorporate a wash-out period to allow circulating drug to leave the system, however this is limited in multi-dose studies. An easy, efficient method to reduce drug interference is by diluting the sample to reduce overall drug concentration so it doesn't interfere. Sensitivity issues have been well documented and can be present in any assay. It is important to improve sensitivity to prevent the presence of false negatives that may bring down the overall immunogenicity rate.

Further, most labs have developed their own methods for ADA detection, and a reliable standardized method is not available. Standard curve generation is difficult without a standard ADA for each drug, making it difficult to compare results between studies. In addition, with many different assays available, it is not uncommon that studies may use different methods to quantify ADA of the same protein. These issues may be present in older phase 3 clinical studies in which assay sensitivity was not high, resulting in numerous false negatives and making the detection of ADA difficult, lowering overall immunogenicity.

The selection of an appropriate assay is also critical when discussing PK studies. When measuring the impact on immunogenicity on PK, it is important to accurately quantify ADA and the concentration of protein in the sample. Using an appropriate assay can influence parameters, especially in the terminal phase where sensitivity of the assay is key when determining concentration<sup>83</sup>. It can also make it difficult to compare PK profiles between studies if different assays are used.

**iii Clinical Management and Monitoring ADA**—The clinical management of patients with immunogenicity is very challenging and depends on the clinical condition of the patient and the disease. In patients with Hemophilia A, after inhibitors develop, bypass therapies are available, such as NovoSeven, an activated FVIIa product, emicizumab, a bi-specific antibody that mimics FVIII by binding to FIX and FX, or activated prothrombin complex concentrate. To reverse inhibitor development, immune tolerance induction (ITI) strategies using high dose FVIII over a period of time desensitize the immune system to FVIII and induce antigen specific tolerance. The exact mechanism is still the subject of

research, however it has been used successfully for decades, and various dosing regimens have been used with success to induce ITI<sup>84,85</sup>. Unfortunately, ITI approaches are very expensive, costing nearly a million dollars and is not effective in all patients. Patients who develop sustained ADA against rhGAA in the treatment of Pompe Disease have no alternative clinical options. Due to the dire situation, immunosuppressive regimens of rituximab, methotrexate and IVIG (to be discussed later) are attempted that are very costly and globally suppress the immune system, leaving the patient susceptible to infection<sup>86</sup>. The use of immune checkpoint inhibitors is complicated by immune related adverse events, as discussed (IRAE). A team approach is necessary to manage IRAE as it is a complicated process because patients must receive supportive care for management of these incidences, and careful monitoring is necessary to reduce toxicity. Management of IRAE is summarized by Amin et al<sup>87</sup>. Based on these experiences, it is clear that clinical management of ADA is challenging as most treatments are complicated, prohibitively expensive, can be ineffective in some patients, and may expose the patient to significant risk. Prophylactic prevention of ADA development is desirable, and may be accomplished using low dose immunosuppressants or oral nanoparticles that will be discussed in detail below.

For many protein therapeutics, monitoring ADA can provide life and cost saving benefits. Routine monitoring of ADA development can be effective at improving clinical outcomes, as well as reducing the cost of therapy. Laine et al. used data collected in Finland of adalimumab and infliximab drug trough concentrations and ADA development to develop a model that shows testing patients for ADA is cost-saving in real life clinical practice<sup>88</sup>. Vincent et al. also proposed a model where drug levels and ADA measurements can be used to predict the best biologics to use in chronic inflammatory diseases<sup>89</sup>. The ABIRISK Consortium did a retrospective analysis of ADA in patients treated with IFN- $\beta$  and natalizumab and found that immunogenicity testing in patients would reduce the potential number of ADA positive patients and reduce the amount of money lost treating patients that are not receiving benefit<sup>90</sup>.

### 3. Factors influencing immunogenicity:

Several factors can influence the immunogenicity of therapeutic proteins and can be classified as patient, product, or treatment related factors. The duration of treatment, route, and frequency of administration impact the immunogenicity of the protein<sup>91</sup>. It is commonly accepted that subcutaneous administration of therapeutic proteins, while more user friendly, is more immunogenic than intravenous administration, likely due to migratory potential of cutaneous dendritic cells<sup>92,93</sup>. As discussed, prolonged, chronic therapies are often more immunogenic and frequent administration has also been shown to increase immunogenicity<sup>94</sup>.

Patient related factors that can influence immunogenicity include the immune status of the patient and polymorphisms of the MHC receptor. The MHC (or HLA in humans) is highly polymorphic<sup>95</sup> and several different alleles have been identified for MHC II with many different subunits, such as DP, DM, DOA, DOB, DQ and DR<sup>96-98</sup>. These receptor subtypes differ in binding affinity for epitopes and therefore, inter-patient differences in MHC subtypes can influence the generation of an immune response. Immune status can also



greatly influence a patient's immune response to the protein, as autoimmune patients often respond more strongly to therapeutic proteins than immunocompromised patients do.

Alterations in the actual protein product can have a significant effect on their immunogenic potential. The presence of immunogenic epitopes recognized by MHC-II<sup>99</sup>, aggregates in the final product<sup>100</sup>, and post translation modification such as glycosylation all influence the immunogenicity of protein products. The link between aggregates and immunogenicity is currently the subject of intense research<sup>101,102</sup>. The nature of aggregates that evoke an immune response are not clear, as there are several aggregates with distinct molecular and biophysical characteristics that have been identified that differ in their ability to engage the immune system. To get a better understanding of the link between aggregation and immunogenicity, a classification system has been formulated to organize aggregates based on size, reversibility, structure, covalent modification and morphology<sup>103,104</sup>. For example, the presence of sub visible particles and native like aggregates has been shown to evoke a strong immune response and may be partially complicit in breaking tolerance due to the repeated exposure to immunogenic epitopes<sup>105,106</sup>. Additionally, it has been shown that native-like aggregates of IFN- $\alpha$  are much more immunogenic than non-native aggregates and aggregates that lack a native epitope<sup>107,108</sup>. Studies performed by our group using Factor VIII also showed that native-like aggregates of FVIII are more highly immunogenic when administered in a murine model of Hemophilia A<sup>107</sup>. In addition to aggregation, oxidation of proteins and aggregates in formulations have also been shown to influence immunogenicity. Jiskoot et al. showed that oxidation-mediated aggregation resulted in increased immunogenicity of IFN- $\beta$ <sup>109</sup>. Hermeling et al also showed that oxidized aggregates are more immunogenic than native protein<sup>108</sup>.

Recombinant proteins can be produced in bacterial cells, such as *E. Coli*, or mammalian cells, such as CHO cells. Proteins expressed in bacteria do not undergo post translational modifications such as glycosylation, while proteins from mammalian cells do, which can result in different immunogenic profiles. A classic example of the influence of glycosylation can be seen in Interferon- $\beta$  products. The first product developed, Betaseron, is produced in *E. Coli* cells and contained no glycosylation. Avonex was developed after, and is produced in CHO cells with recombinant DNA technology. Between the two products, Betaseron has much higher immunogenicity than Avonex, at 35% versus 5%, respectively<sup>110</sup>. The difference in immunogenicity can be partially attributed to the changes in glycosylation patterns of these products that can lead to aggregation. Glycosylation patterns on Mabs can also influence their toxicity profile in the body. Most Mabs in the clinic are IgG class and contain a glycosylation site at amino acid 297, and occasionally in the Fab region as well. Fucose structures on the Mab reduce IgG binding to the Fc receptor, reducing ADCC, while less terminally bound galactose reduced CDC activity<sup>111</sup>. Several very informative review articles are available regarding the impact of aggregation and glycosylation on immunogenicity<sup>6,100-102,112</sup>

#### 4. Prediction of immunogenicity using preclinical studies:

Prediction of clinical immunogenicity using preclinical approaches can be useful when assessing new biologic products. Bococizumab, a Mab in development to lower LDL levels

in the body by inhibiting PCSK9 was recently discontinued after phase III clinical trials, citing higher immunogenicity incidence rates causing reduced efficacy of the Mab over time as compared to similar products in its class.<sup>113,114</sup> A bioengineered version of Factor VIIa (rFVIIa) was discontinued from further clinical development after phase III clinical trials, due to the detection of unwanted immunogenicity. A post hoc analysis of the bioengineered rFVIIa proposed to modify neo-epitopes not present on rFVIIa, demonstrating the value of rigorous preclinical screening<sup>115</sup>. The ability to predict such immune responses during preclinical screening could reduce drug attrition and also lead to selection of better protein candidates that have been specifically engineered to lower immunogenicity<sup>115</sup>. Unfortunately, most pre-clinical immunogenicity models are limited in their ability to predict the incidence rate of immunogenicity, but have indispensable value in assessing relative immunogenicity between similar products, rank ordering the immunogenicity of multiple molecules and formulations, and elucidating the mechanism of immune response to further our ability to design less immunogenic proteins. Preclinical studies *in vivo* are commonly used to screen immunogenicity of therapeutic proteins and the relative immunogenicity between different products. *In vitro* methods such as epitope mapping, HLA affinity, and T cell proliferation assays are used to assess the immunogenic potential of proteins and identify immunogenic regions. *In silico* programs have been developed that focus on epitope mapping of the proteins three dimensional structure.

**a) In Vivo Immunogenicity Assessment:** Generally speaking, any human or humanized protein for therapeutic use is going to be immunogenic when administered to an animal, thus results should be interpreted with caution. However, mouse models have made significant contributions to biomedical research, including our understanding of the immune response against antigens and foreign proteins. The ease of breeding and housing, and manipulation of their genome system for specific applications are key reasons for their utility in biomedical research.

**i. Mouse Models:** Mouse models have been used to predict immunogenicity, compare relative immunogenicity between products and elucidating immune mechanisms<sup>116–120</sup>. In an experiment by Jiang et al, they compared the relative immunogenicity of several types of FVIII products to determine if their new FVIII fc fusion was less immunogenic than the currently used products<sup>121</sup>. It was found that mice treated with rFVIII<sub>fc</sub> had significantly lower antibody response as compared to B-Domain deleted FVIII or full length FVIII, which is confirmed by clinical observation of Eloctate (rFVIII-Fc) having much lower immunogenicity<sup>122–124</sup>. In a study by Lu et al, BALC/c mice were used to compare the relative immunogenicity of DNA vaccines by different routes of administration and showed that intramuscular administration was the least immunogenic<sup>125</sup>. Studies from our lab have used mouse models to show that administration of FVIII or rhGAA complexed with phosphatidylserine based liposomes is significantly less immunogenic than administration of the free protein alone<sup>31,78,126</sup>. B6-129 mice with the *GAA* gene knocked out<sup>127</sup> have been used as a model to assess immunogenicity of rhGAA<sup>78,128</sup>.

With increasing interest regarding factors influencing immunogenicity, mouse models have also been used to compare the immunogenicity of aggregated products. Using a mouse

model of Hemophilia A, our lab showed native aggregates are relatively more immunogenic than monomeric protein and non-native aggregates<sup>129</sup>. Jiskoot et al. showed that the aggregated forms of insulin were more immunogenic than the commercial products<sup>130</sup>. Braun et al. performed a study with IFN- $\alpha$  which showed the formulation became significantly more immunogenic after the formation of aggregates, which occurs in nearly all marketed IFN- $\alpha$  products<sup>131</sup>.

Another important use of mouse models is to provide mechanistic insight into immune system activity. Adoptive transfer and T cell proliferation studies can be performed to assess the role of T and B cells in protein immunogenicity. Adoptive transfer studies performed by our group suggest a role for CD4+CD25+ T cells in suppressing an immune response against FVIII. T cells were transferred from mice that were tolerized to FVIII into recipient mice, which then exhibited significantly lower ADA development<sup>31</sup>. A recent study by van Beers et al. showed that immunological memory did not develop after administration of IFN- $\beta$ , despite the development of anti-IFN- $\beta$  antibodies<sup>132</sup>. This has also been seen clinically in patients that have switched interferon products due to immunogenicity. Anti-IFN antibodies have been known to be cross reactive, however when switching products, patients do not see an increase in antibody level<sup>5,133</sup>.

**ii. HLA Transgenic Mice:** Due to physiological differences, animal MHC receptors don't directly mimic human HLA receptors<sup>134</sup>. HLA and MHC genes are some of the most polymorphic genes, making it difficult to assess protein immunogenicity due to high inter-subject variability in HLA/MHC expression.<sup>96,97,135</sup> To combat this, transgenic (Tg) mouse models have been developed that mimic a human subject and are tolerized to a specific protein.<sup>136,137</sup> The resulting mouse will tolerate the therapeutic protein of interest, and the development of immunogenicity will result from the breaking of self-tolerance, as opposed to a classical immune response to foreign antigens. Transgenic mouse models have been developed for a wide range of therapeutic proteins, such as IFN- $\beta$ <sup>136,138</sup>, human growth hormone<sup>139</sup>, insulin<sup>140,141</sup>, and FVIII<sup>137</sup>.

One of the earliest uses of transgenic tolerant mice investigated the immunogenicity of human insulin and insulin analogues. Ottesen et al showed that transgenic mice are tolerant of human insulin and did not produce antibodies<sup>141</sup>. Incidentally, they also found that increasing the number of protein modifications correlated with an increase in ADA. Beers et al utilized a transgenic mouse model tolerant to human IFN- $\beta$  to investigate immunogenicity towards IFN- $\beta$  products. They showed that Betaferon was the only product tested that broke immune tolerance in transgenic mice. In addition, it had the highest immunogenicity amongst all products tested<sup>138</sup>, which agrees with other reports of IFN- $\beta$  immunogenicity<sup>142</sup>. Transgenic murine models are useful, however development of the model is a labor intensive process, and new models must be developed for each therapeutic protein of interest. Some transgenic mouse models are provided in Table 3.

NOD *scid* gamma (NSG) mice are highly immunocompromised and can be transfected to investigate the human immune system in an *in vivo* model. NSG mice are deficient in most immune cells; T and B cells, dendritic cells, macrophages and natural killer cells, as well as complement and cytokine signaling. The Jackson Laboratories pioneered the development of

the NSG model, and have developed humanized NSG mouse models<sup>143–145</sup>. CD34+ humanized mice are engrafted with cord blood derived hematopoietic stem cells to develop a functional immune system in the mouse, displaying normal T cell and inflammatory function. To this point, NSG mice have primarily been used to investigate the pathology of infectious diseases in a humanized mouse model, but used properly, these mice could provide insight into the mechanism of therapeutic protein immunogenicity.

**iii. Higher Order Animal Models:** Higher order animal species may have better clinical utility for predicting immunogenicity due to a higher degree of homology between human and non-human primate proteins and more similarities in immune mechanism. Rhesus monkeys and chimpanzees have been used to examine the relative immunogenicity of human growth hormone (HGH) and tissue plasminogen activator (tPA). Zwickl et al. investigated immunogenicity of HGH in rhesus monkeys and found that their results correlated well with clinical observation in regards to relative immunogenicity, however the predictive power was lessened when trying to assess incidence rate of immunogenicity<sup>146</sup>. In a separate study, this group also investigated the impact of immunogenicity of a marketed tPA analog compared to native monkey tPA. They concluded that antibody development is not more significant against the tPA analog and these low antibody levels do not impact its enzymatic behavior, suggesting immunogenicity does not pose a great risk to clinical treatment<sup>147</sup>.

In addition to relative immunogenicity, primate models can also be used to investigate the impact neo-epitopes may have on protein immunogenicity. Zwickl again used rhesus monkeys to investigate the impact of neo-epitopes on the immunogenicity of insulin. Monkeys were administered human insulin, LysPro insulin and porcine insulin and the development of antibodies was assessed. Their results showed that alterations present in modified human insulin (LysPro insulin) does not give rise to neo-epitopes in monkeys, as only 1 monkey developed low levels of anti-insulin antibodies<sup>148</sup>. These results are corroborated by clinical observation showing human and LysPro insulin have similar incidence rates of immunogenicity<sup>149</sup>.

Canine models have also occasionally been utilized to assess immunogenicity. In studies performed by Finn et al, they assessed the immunogenicity of modified Factor IX in a dog model of Hemophilia B. They showed there was no antibody formation against FIX-R338L even after treatment with wild type FIX<sup>150,151</sup>. In a separate study by Randolph et al, they investigated whether administration of recombinant canine erythropoietin was immunogenic in dogs and found there were no immunogenicity risks<sup>152</sup>.

**iv. Limitations of Animal Models:** Despite their uses, animal models still possess significant hurdles impacting their usefulness in predicting immunogenicity due to the lack of genetic diversity in mice and basic differences in immune mechanisms. Factors influencing immunogenicity even in humans are not fully understood, and variability between human and animal systems makes prediction difficult. The strain of mouse used to develop a model, or investigate immunogenicity can also have a large impact on their usefulness. C57BL/6 mice and Balb/c mice differ greatly in their immunologic systems and can respond very differently to stimulation by proteins<sup>153–155</sup>. Generally, Balb/c mice have

higher cytokine release and T cell function as compared to C57BL/6, while C57BL/6 mice generally have higher natural killer cell activity<sup>155,156</sup>. In addition, both strains of mice respond differently to pro- and anti-inflammatory cytokines<sup>156</sup>. Differences between humans and mice exist in Toll-like receptors, antibody subsets and the balance of leukocyte subsets that make it difficult to directly correlate human and murine immune responses.

As with murine models, higher order animal species are still ineffective at predicting immunogenicity incidence rates. Gunn et al performed a study looking at the immunogenicity of interleukin-3 (IL-3) and found that all primates formed antibodies, while clinically, few patients form antibodies against IL-3<sup>157</sup>. Additionally, primates are not an effective model for assessing immunogenicity of monoclonal antibodies. In a study by van Meer et al. they attempted to assess the incidence rate and impact of immunogenicity of several approved mAb products in monkeys and compared it to available clinical data<sup>158</sup>. They found that only 59% of the mAbs tested had comparable immunogenicity between monkeys and humans, while 30% over-predicted and 11% under-predicted.

Laboratory animals commonly used for pre-clinical immunogenicity testing are bred in hyper hygienic conditions and lack exposure to pathogens that strengthen the basal activity of the immune system<sup>159</sup>. In contrast, humans are constantly exposed to various pathogens in everyday life that keep the immune system well maintained. Therefore, the physiological microbiome may not be representative of a natural human immune system. Nevertheless, the use of pre-clinical models is indispensable in terms of comparing relative immunogenicity and elucidating the mechanism of immune responses in studies that are not ethically possible or practical in humans.

**b. In vitro Immunogenicity Assessment:** Compared to *in vivo* testing, *in vitro* systems are typically less time consuming and more cost effective. *In vitro* assays can be used to investigate the cellular mechanism of the immune response, identify immunogenic epitopes on the proteins, and evaluate MHC affinity, T cell proliferation, and immunogenic effects of the whole protein. In theory, *in vitro* scaling can be used effectively with screening tools to investigate the mechanistic basis by which the immune system responds to foreign antigens, as well as identifying key immunogenic epitopes present on the protein.

**i. Epitope Mapping:** Epitope mapping is a robust method that can identify immunogenic epitopes of a protein by individually analyzing peptide fragments of the protein<sup>160,161</sup>. Therapeutic proteins are broken down into overlapping amino acid chains, and peptides are exposed to immune cells. After exposure, immunogenicity can be assessed by measuring cytokines and surface markers that indicate an inflammatory immune response has occurred. This exhaustive method was used by Herzog and colleagues to map the immunogenic T-cell epitopes of rhGAA<sup>162</sup>. Mice were immunized with free GAA protein after which the splenocytes were extracted and exposed to amino acid chains developed from the full length protein. Using an ELISpot assay, peptides were assessed for IFN- $\gamma$  secretion and immunogenic epitopes were identified<sup>162</sup>. Hamze et al mapped infliximab and rituximab to identify CD4 T cell epitopes. Using lymphocytes from healthy donors, they identified nine T cell epitopes present in the variable region of both antibodies that were able to stimulate PBMC to secrete a variety of cytokines<sup>163</sup>. Epitope mapping has been performed for

additional proteins, such as FVIII<sup>164</sup> and AAV2<sup>165</sup>. Epitope mapping from the full protein is highly labor intensive, and *in silico* programs are often used to identify regions that may be immunogenic to narrow down epitope candidates.

Epitope spreading can complicate the prediction of immunogenic epitopes on a protein. During a typical immune response, T cell activation is dependent on recognition of immunodominant epitopes, however epitope spreading results in the recognition of sub-dominant or cryptic epitopes<sup>166</sup>. Epitope spreading can occur as a result of altered endocytic process, variable T cell interaction, or mutations in B cell processing, which causes alterations in epitope recognition<sup>167</sup>. Epitope spreading has been implicated in autoimmune conditions, as it can result in continual tissue damage as the immune system recognizes different epitopes. In a study by Bernard et al, they showed that after immunization with MOG protein, the ADA target changed and new immunogenic MOG epitopes were identified<sup>168</sup>. As a result of epitope spreading, continual tissue damage occurs as the next wave of autoreactive T cells invades with a different epitope target. The unpredictability of epitope spreading is extremely difficult to identify in pre-clinical models, and while immunodominant epitopes can be predicted, it may be more difficult to predict sub-dominant epitopes that arise later on.

**ii. MHC Binding Assays:** MHC binding assays can be used to detect high affinity peptides and are often used in conjunction with epitope mapping to identify protein regions that are at a high risk of immunogenicity<sup>169–171</sup>. Griswold et al<sup>172</sup> describe a high throughput method to perform a peptide MHC binding assay that allows for the rapid analysis of up to 90 peptides on a single plate using a mechanized protocol. Peptides of interest are incubated at various doses with control peptides and soluble MHC proteins to assess affinity. Higher affinity peptides bind more strongly to MHC, resulting in an increased likelihood that a T cell will recognize the epitope<sup>170,173–175</sup>. ProImmune has developed a peptide binding assay that can be used to provide MHC binding data and identify potentially immunogenic epitopes<sup>176</sup>.

**iii. T-Cell Dependent Assays:** Upon stimulation by an immunogenic protein, T cells proliferate and release cytokines to exert their effect. Typically, T cell proliferation is measured through the use of radiolabeling with thymidine<sup>177</sup> or fluorescent labeling dyes such as carboxyfluorescein succinimidyl ester (CFSE)<sup>178,179</sup>. CFSE is a fluorescent dye that can be used to assess repeated T-cell divisions<sup>179</sup> and is rapidly replacing radiolabeling cells due to less hazardous waste and simplicity. CFSE staining can also be accompanied with phenotypic staining to distinguish different subtypes of proliferating T cells. Karle et al. and Rubic-Schneider et al assessed T cell proliferation using thymidine to determine the immunogenicity of secukinumab and EPO aggregates, respectively<sup>180,181</sup>

In addition to proliferation, cytokine release can also be measured to determine immunogenicity. Assaying the type and degree of cytokine activity can give general information about the immune response that is occurring in these T cells. Commonly, ELISA and ELISpot methods are used to measure cytokines such as IL-2 and I FN- $\gamma$  released by T cells *in vitro*<sup>174,182</sup>. ELISpot assays are highly sensitive methods that can be used to determine the number of cells that secrete specific cytokines. Developed by

Czerkinsky et al in 1983, ELISpot assays are more sensitive than ELISA assays and are typically more effective in quantifying the cytokine response<sup>183</sup>. Cytokines can also be detected by flow cytometry and can be performed in conjunction with T cell phenotyping to classify the type of T cell response that is occurring<sup>184,185</sup>. Peripheral Blood Mononuclear Cell (PBMC) assays can provide insight into the potential immune response without testing in humans using preparations that contain several types of immune cells<sup>169,186</sup>, better mimicking *in vivo* immune systems. PBMC preparations contain several different cell types, including macrophages, monocytes, and lymphocytes, and can include cells from both the adaptive and innate immune systems. PBMC from healthy human donors were used to assess the immunogenicity of aggregated antibodies by assessing cytokine secretion and T cell proliferation in a study by Joubert et al<sup>187</sup>.

**iv. Innate Immune Screening:** Given the increasing evidence of interplay between the innate and adaptive arms of the immune response, using innate cell systems for *in vitro* screening may improve the predictive power of pre-clinical screening. Ahmadi et al used PBMC preparations in which CD8 reactive T cells were removed to model the innate response, and showed excellent correlation between *in vitro* testing and clinical immunogenicity seen with Mabs<sup>188</sup>. Innate lymphoid cells (ILCs) may be useful as an *in vitro* system but has not yet been explored in the context of immunogenicity of therapeutic proteins<sup>189</sup>. *In vitro* innate system cell lines transfected with toll-like receptors in combination with human and mouse macrophages have been successfully used to screen for impurities in therapeutic protein preparations<sup>190</sup>.

**v. B cell Epitope Mapping:** B cells recognize discontinuous epitopes composed of amino acid sequences that are in close proximity in the protein's three dimensional conformation that more closely mimic the native protein<sup>191,192</sup>. As a result, the prediction of B cell epitopes is much more difficult than two dimensional epitopes recognized by T cells<sup>192,193</sup>. In B cell epitope mapping, immunogenic regions of the protein are identified and modifications can be made to the protein to remove these sections. There are two types of epitopes that can be recognized by antibody-producing B cells; structural and functional epitopes<sup>192</sup>. Structural epitopes are generally larger, around 16-22 amino acids, and comprise the amino acids on the protein that come in contact with the antibody. Functional epitopes are shorter 3-5 amino acid chains that influence the affinity of the therapeutic protein and the antibody<sup>192,193</sup>.

Currently, the most accurate method of predicting structural epitopes is through the use of X-ray crystallography to identify epitopes on the protein that bind to the antibody. X-ray crystallography allows for the identification of precise sequences that bind to the antibody and can provide information on strength of binding as well<sup>194,195</sup>. Nuclear Magnetic Resonance (NMR) can also be used to identify structural epitopes without forming crystals, however it is limited to use with small proteins and peptides<sup>194,196</sup>. In addition to the preferred methods listed above, electron microscopy can be used to examine structural epitopes on larger antigens such as viral capsids. For example, electron microscopy was used to identify structural epitopes on the surface of the Human Papilloma Virus 16 capsid and showed that antibody binding results in conformational changes<sup>197,198</sup>.

Functional epitopes are commonly determined by antigen fragmentation, competitive binding or modification testing. Fragmentation assays are performed to assess whether or not an antibody binds to the epitope fragment of interest<sup>199,200</sup>. Primarily a qualitative approach, western blots or ELISAs are commonly used to assess whether the antibody will bind to the epitope fragment. Positive binding interactions indicate the peptide fragment may be immunogenic. Competitive binding assays can be used to determine if multiple antibodies can bind to epitopes on a protein at the same time<sup>201</sup>, providing information on the number of potentially immunogenic epitopes on a therapeutic protein. An easy way to determine if a specific sequence is immunogenic is to modify the amino acid chain and assess its binding to ADA. Modification testing relies on mutagenesis of amino acid residues to assess their impact on binding of the antibody to the immunogenic sequence<sup>202</sup>. By mutating individual amino acids, one can identify detrimental residues, and can replace them if it does not abrogate activity of the protein.

**vi. Limitations of in vitro immunogenicity assessment:** One of the main issues predicting immunogenicity *in vitro* is attempting to replicate the immune system *ex vivo*. The immune system is a complex system of T-cells, B-cells dendritic cells, and macrophages, and this cannot be easily replicated in *in vitro* systems. It is difficult to predict the interplay between two dimensional epitopes and the three dimensional protein structure and how APC will digest the protein to present epitopes via MHC. It is likely there is variability in the way the proteins are processed by immune cells *in vivo* versus *in vitro* that is undetected by *in vitro* assays. Synthesized peptide sequences may not contain post-translational modifications that are essential to the protein<sup>203,204</sup> which would lead to altered signaling, potentially resulting in false negatives or false positives<sup>204</sup>. Even if immunogenic epitopes are detected, they can only be modified if they do not interfere with protein activity. As with many systems, genetic diversity is an issue that is difficult to replicate. Patient-derived PBMC from various genetic backgrounds are a possibility, however a very large pool of donors would be required to adequately capture HLA polymorphisms to investigate.

Several well-known factors influencing immunogenicity such as route of administration, frequency, and dose are very difficult to predict *in vitro*<sup>7,91</sup>. Unpredictable treatment factors also can emerge that influence immunogenicity and are impossible to predict. One example is Eprex, an Epo analog that was used to increase red blood cell growth in anemic patients. Initial treatment with Eprex was successful, however some patients being treated with Eprex started showing higher incidence of red cell aplasia<sup>205,206</sup>. It was determined that the issue was antibody mediated and treatment with Eprex was becoming less effective. Initial investigations suggested that organic compounds leached from the rubber stopper of the pre-filled syringes was the most probable cause of immunogenicity<sup>205,206</sup>, however further testing has concluded that tungsten present during the manufacturing process may be the root cause of Eprex aggregation<sup>181,207</sup>. Small factors like these are likely to go undetected during preclinical testing and there is little chance of predicting such occurrences.

**c. In Silico Predictions:** Due to technological advances in proteomics and sequence mapping, epitope databases have been constructed that provide a library of existing epitopes



and methods of prediction. Several databases exist such as IEDB, Epiteome, and SEDB<sup>208–210</sup>, that provide information on two dimensional epitopes that are recognized by T cells. The linear binding interface between epitopes and MHC make it easier to predict as opposed to B cell epitopes that are influenced by protein folding. Table 4 highlights several key databases that have been developed.

Several programs and webpages have also been developed that can be used to analyze molecules and predict immunogenic epitopes on potential therapeutic proteins. Soria-Guerra et al reviewed several programs that can be used for *in silico* epitope prediction<sup>211</sup>. These various programs range in MHC subtype predictions and methods, and some programs have the ability to simulate amino acid substitutions to assess changes in immunogenicity. MHCpred is an algorithm developed by Doytchinova that can be used to predict immunogenicity of epitope sequences<sup>212,213</sup>. Users can input the amino acid sequence and assess its MHC binding potential to various alleles. EpiMatrix was developed by EpiVax to predict allele specific binding of therapeutic proteins to MHCII receptors, and has been improved to also assess binding at the T-cell receptor interface<sup>169,214</sup>. Table 5 summarizes some of the algorithms that have been developed for peptide-MHC binding.

Epitope prediction can be applied to most therapeutic proteins, however predictions must be assessed in conjunction with *in vitro* methods. Predictions should also be combined with activity assessment to ensure altering immunogenic epitopes does not impact protein activity. *In silico* methods could be improved with greater ability to predict peptide-MHC affinity, which drives epitope immunogenicity. It is also difficult to predict peptide affinity for the T-cell receptor, as the TCR recognizes three dimensional structure. As described above, HLA polymorphisms make it extremely difficult to predict population-wide immunogenic epitopes. Algorithms may be able to identify epitopes that are most immunogenic, but patients may still experience immunogenicity due to alternative epitopes that are presented by their individual APC. Post translational modifications are also difficult to simulate *in silico*.

The use of quantitative structure activity relationship (QSAR) models to improve safety and reduce immunogenicity of protein pharmaceuticals is gaining momentum to predict and reduce immunogenic regions of proteins. This approach attempts to employ structural, statistical, and machine based learning to connect structural features to immunogenicity and activity. Generating an integrated data set of protein structures from databanks, pre-clinical and clinical outcomes could be a first step in designing less immunogenic proteins and formulations. Similar QSAR models have been used to predict skin sensitivity to several compounds<sup>215</sup>. This approach could also be utilized to predict immunogenicity of protein pharmaceuticals. For example, Kumar et al utilized computational tools to delineate the relationship between epitopes and aggregates using a structure based approach<sup>216,217</sup>.

## 5. Strategies to mitigate immunogenicity of therapeutic proteins:

Strategies are being developed that are aimed to prevent as well as reverse an established ADA response by (i) inducing immunological tolerance, (ii) modifying the product or (iii) formulation, (iv) modulation of the immune system using small molecule drugs, and (v) manipulating T and B cells to reverse established immune responses. The reversal of an

established immune response is very difficult, often requiring lymphocyte deletion, and have been met with limited success, thus prophylactic regimens to induce tolerance to therapeutic proteins are preferred.

**i. Immune Tolerance Induction:** Immune tolerance induction is a method of presenting an antigen in a way that will prevent or reverse the development of ADA in an antigen specific manner. Immunologically,  $T_{\text{regs}}$  have shown to have a critical role in immune tolerance induction through TGF- $\beta$  mediated pathways<sup>218</sup>. Our group is developing a method to induce tolerance to various therapeutic proteins by co-administering the antigen with phosphatidylserine (PS) based liposomes. PS is a naturally occurring phospholipid present on the inner leaflet of cells. Upon cellular apoptosis, PS flips to the outer membrane and acts as an “eat me” signal to phagocytic cells that engulf and digest cellular debris without the initiation of an immune response. We have shown that co-administration of PS with various therapeutic proteins and antigens (including rhGAA, FVIII and ovalbumin) can induce durable tolerance and prevent the development of antibodies<sup>78,126</sup> (unpublished data). Additionally, we have shown this method to be antigen specific, as rechallenge with an unrelated antigen showed a robust immune response<sup>31</sup>. Sule et al. showed that dendritic cells conditioned with anti-inflammatory cytokines IL-10 and TGF- $\beta$  were able to suppress antibody development in Hemophilia A (HA) mice<sup>219</sup>. In their study, they transferred DC that were treated with IL-10 and TGF- $\beta$  into HA mice and then challenged the mice with free protein. They also showed that this method is antigen specific, as challenge with human serum albumin resulted in a normal immune response. Zhang and Scott have developed a method to induce immunologic tolerance to FVIII, rhGAA, and AAV8 using Poly lactic-co-glycolic acid (PLGA) based nanoparticles containing rapamycin<sup>128,220,221</sup>. These studies showed that administration of antigen with their nanoparticle can significantly reduce antibody development in mouse models.

Another method of inducing immunological tolerance is hepatic gene therapy. In this method, viral vectors containing the gene sequence of the protein of interest are administered and incorporated into hepatocytes, which produce the protein of interest. Work done by Herzog first showed that immune tolerance can be induced to Factor IX by adeno-associated virus (AAV-FIX) gene delivery to the liver<sup>222</sup>. Mice that were administered AAV-FIX showed no antibody development and substantially lower T cell responses *in vitro*. They showed this method was antigen specific and induced the proliferation of regulatory CD4+ T cells. Gene therapy for the treatment of Hemophilia A and B is quickly gaining interest in the industry and the FDA.

**ii. Protein Modifications:** Directly modifying the protein may lead to a decrease in immunogenicity by modifying immunogenic regions identified on the protein. Reducing impurities, aggregates and host cell associated proteins have further improved the safety of therapeutic proteins. Using protein engineering approaches and *in silico* tools, deimmunization and epitope removal strategies have been employed to design less immunogenic therapeutic proteins, which were employed to engineer a less immunogenic form of FVIII. Pratt et al. identified key immunogenic epitopes on the FVIII protein and assess amino acid substitutions that would reduce immunogenicity, while maintaining

procoagulant activity. Using in vitro T cell proliferation assays and MHC binding assays, they determined that substitutions at the 2196 amino acid of full length FVIII reduced T cell proliferation while maintaining activity, illustrating the ability of in vitro methods to develop a less immunogenic FVIII <sup>223</sup>. Additionally, Pastan et al. developed a less immunogenic anti-CD25 immunotoxin by identifying and eliminating immunogenic T cell epitopes. After amino acid modification, they showed that the T cell response was reduced by 74.8%, while maintaining cell cytotoxicity <sup>224</sup>. One strategy that has been shown to reduce clinical immunogenicity for Mabs is to increase the human content in the antibody. One example showed that switching the construct of the antibody OKT3 from murine to chimeric, humanized, or fully human drastically reduced the incidence rate of immunogenicity <sup>225</sup>. Anne de Groot reviewed several potential mechanisms to bioengineer less immunogenic proteins in her review <sup>226</sup>.

**iii. Formulation Related Changes:** Modifying the formulation to decrease aggregation may also help to reduce therapeutic protein immunogenicity. Our lab has shown that the presence of O-Phospho-L-serine (OPLS) prevents the formation of FVIII aggregates by occupying the lipid binding region that is susceptible to aggregation in aqueous formulations <sup>227</sup>. Senga and Honda showed that the inclusion of a small peptide AF.2A1 in Mab formulations will bind to the Fc region of non-native IgG conformations. Once bound, the formulation can be separated to remove Mab bound to AF.2A1 and the resulting formulation will be devoid of aggregates<sup>228</sup>. AF.2A1 peptide beads can be used to rid a formulation of aggregates, while OPLS can be used to prevent the formation of aggregates in FVIII formulations.

**iv. Immune Modulatory Drugs—**Small molecules drugs can also be used to help patients better tolerate therapeutic proteins by modulating the immune response primarily through immunosuppression. Commonly used immunosuppressive drugs are methotrexate, rapamycin, bortezomib, and cyclophosphamide. Methotrexate (MTX) is an anti-cancer drug that is commonly used to treat certain types of cancer and autoimmune diseases, such as breast cancer, leukemia, rheumatoid arthritis, and Crohn's disease. MTX is used to treat autoimmune diseases due to its ability to suppress T cell activation and down-regulate B cell activity. Kishnani et al. and Richards et al. developed low dose MTX regimens for use in Infantile Pompe Disease patients to induce tolerance to administered rhGAA <sup>79,229</sup> and shown clinically to prevent immunogenicity. Maini et al. showed that the administration of MTX with the anti-TNF antibody infliximab reduced antibody development and patient response improved <sup>230</sup>. Rapamycin is a small molecule macrolide that is commonly used to prevent transplant rejection by inhibiting the activation of T and B cells via the mTOR receptor <sup>231,232</sup>. As mentioned, Scott and Zhang used rapamycin in their PLGA nanoparticle to induce tolerance. Herzog et al. used rapamycin in combination with FVIII delivered orally to induce tolerance to FVIII in Hemophilia A mice <sup>233</sup>. Bortezomib is a small molecule drug that inhibits proteasome activity, preventing the degradation of pro-apoptotic signals, leading to cell death. It has been used in Infantile Pompe Disease patients along with MTX and RTX to reverse high sustained ADA and restore efficacy of therapy <sup>234</sup>. Cyclophosphamide is primarily used as a chemotherapeutic agent to kill rapidly proliferating cells but at low doses has been recognized to have immunomodulatory effects through its influence on dendritic

cell homeostasis and cytokine secretion<sup>235</sup>. Historically, cyclophosphamide has been shown to induce tolerance to FVIII when given as a pre-treatment<sup>236,237</sup>. Investigative therapies also include cyclophosphamide/steroid combinations to induce tolerance in FVIII inhibitor positive patients<sup>238</sup> and has also been tested for use in preventing ADA formation in the treatment of Pompe Disease to no avail<sup>239</sup>. Azathioprine is an immunosuppressive drug that can be given in combination with infliximab to improve the treatment of Crohn's Disease<sup>240</sup>. It was suggested by Colombel et al that the improvement may be partially attributed to reducing the immunogenicity of infliximab<sup>241</sup>. Additionally, azathioprine was used in combination with infliximab or adalimumab to reduce ADA frequency by 47%<sup>242</sup>.

**v. Lymphocyte Manipulation to Reverse ADA development**—In addition to tolerance induction strategies, methods have also been investigated which manipulate and eliminate lymphocytes to prevent or reverse an immune response against the therapeutic protein (Fig. 4). These approaches can impact ADA development, but also compromise the immune system of the patient, leaving them susceptible to infections. Kishnani et al. developed an immunosuppressive regimen that showed the ability to stop and reverse the development of antibodies against GAA in infantile Pompe Disease patients<sup>229,243</sup>, comprised of rituximab (RTX), MTX, and gammaglobulins. While this method was able to stop antibody development, it also caused a significant reduction in CD20 positive B cells during the course of RTX and MTX administration that did not recover until after treatment with RTX and MTX was stopped. Anti-CD3 antibodies have been used with success in preventing antibody development against rhGAA<sup>244</sup> and FVIII<sup>245</sup> in mice by altering cytokine production to favor a Th1 response, however they were not able to modulate ADA development long term, likely due to the generation of new lymphocyte cells that are reactive to the therapeutic protein. Active tolerance is not being induced with the use of immunosuppressive drugs, therefore is only maintained for one cell lifespan.

Scott et al. engineered FVIII expressing cytotoxic T cell that could selectively target and kill FVIII-specific B cells. They engineered immunodominant epitopes on the FVIII protein that were expressed as antigen receptors on the surface of cytotoxic T cells, which bound to and specifically killed FVIII reactive B cells *in vivo* and *in vitro*<sup>246</sup>. In the interest of B cell targeting, Brettschneider et al. fused the C2 domain of FVIII to endotoxin A of *pseudomonas aeruginosa* which selectively targeted FVIII specific memory B cells and eliminated them<sup>247</sup>.

There are a variety of methods being assessed to induce immunological tolerance and the clinical translatability of these methods is still being investigated. Antigen specific tolerance is the desired method, as patients would not be susceptible to secondary infection. There is also a degree of risk assessment required in these tolerance induction strategies. In some cases, such as Pompe Disease, antibodies develop in nearly all patients, leaving the patient without relief and no other clinical options. In this case, administering an immunosuppressive regimen may be a more acceptable option, whereas in other diseases, the incidence of immunogenicity is lower, and patients can transition to another product. Mitigation strategies are still under development, however there are several promising methods being investigated.

## Conclusion

Clinical immunogenicity of therapeutic proteins is a growing issue that threatens to undermine the development of potent protein drugs. Understanding the mechanism of the immune response and factors influencing immunogenicity of therapeutic proteins will allow us to design safer drugs and prevent immunogenicity. Engineering proteins that are inherently less immunogenic, or developing methods to prevent or reverse an established immune response to therapeutic proteins can help solve the major issue of immunogenicity. Several methods are available that can attempt to predict immunogenicity of therapeutic proteins, but there is still large debate over the usefulness of these predictive tools. As was described, *in vivo* models are most useful for relative immunogenicity ranking, but the prediction of immunogenicity incidence rate is something that is very difficult to perform in models due to basic differences between human and animal immune systems. *In vitro* models are useful in predicting if the protein will interact with MHC molecules, however these systems do not expose the protein to the complete array of factors that can influence immunogenicity in human patients. *In silico* models are rapidly being developed and can be useful in predicting immunogenic epitopes, but need to be combined with *in vitro* testing to ensure the predictions are useful. Despite their limitations, preclinical methods for predicting immunogenicity are instrumental to drug development. Immunogenicity of therapeutic proteins is a clinical challenge, but success in overcoming it would improve the safety and efficacy of several lifesaving therapies. Several mitigation strategies are being actively pursued to not only prevent, but reverse an established immune response.

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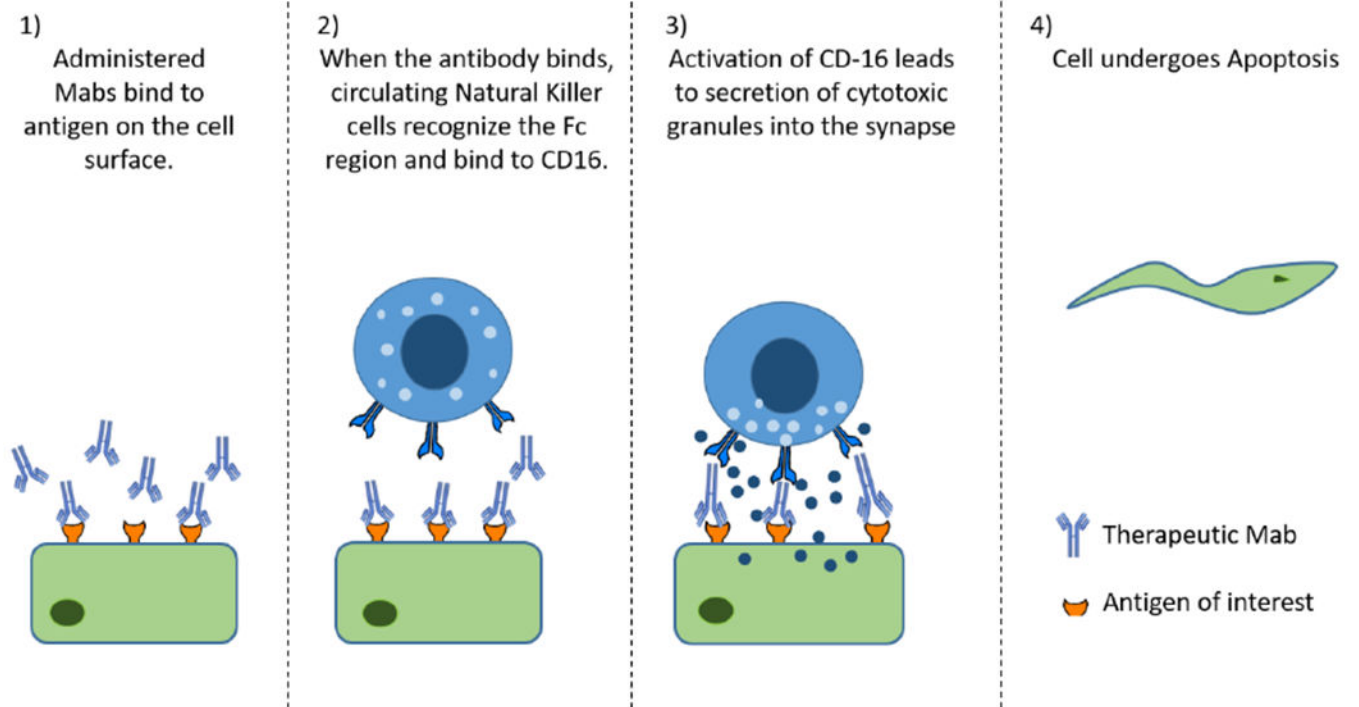


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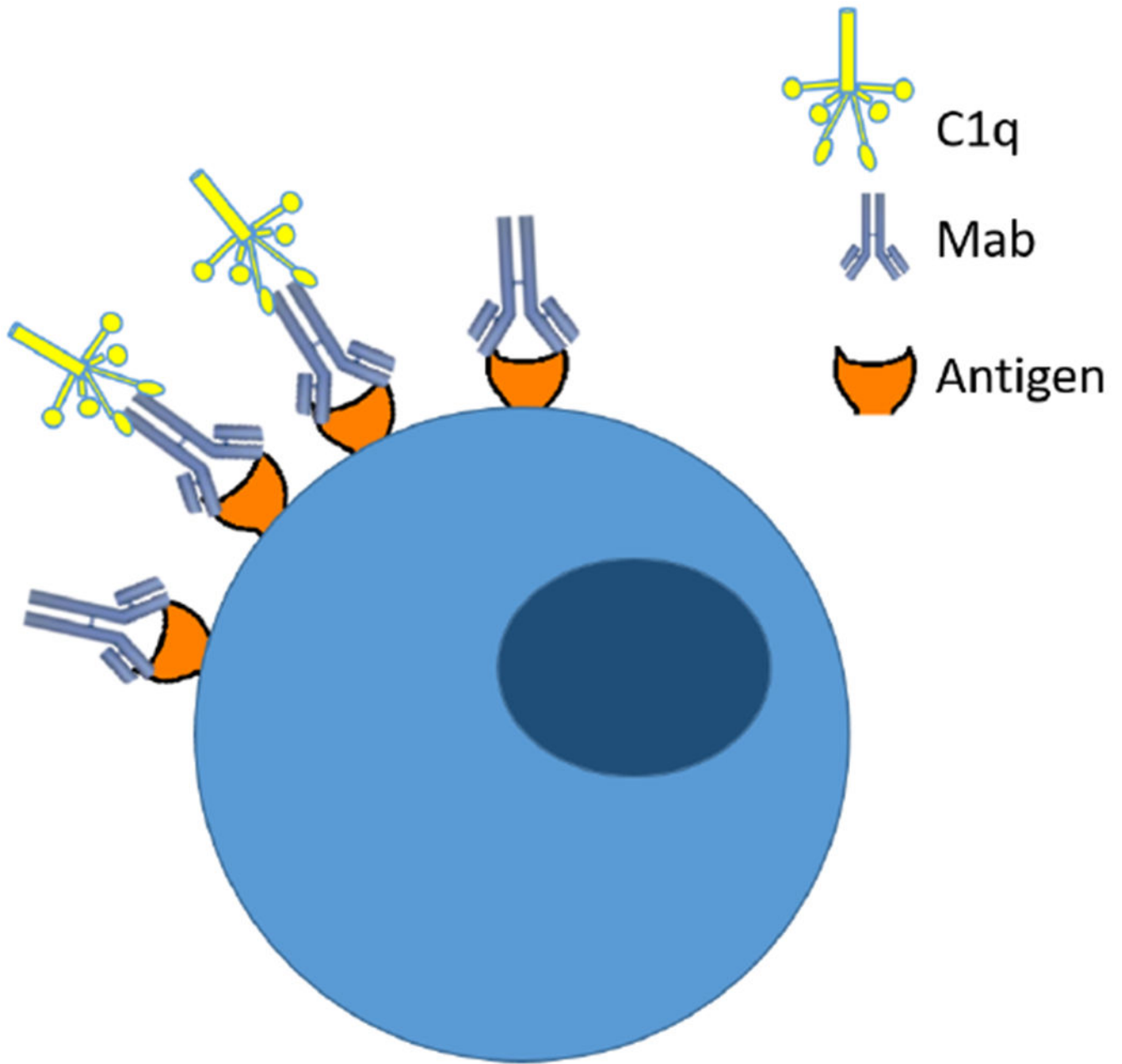
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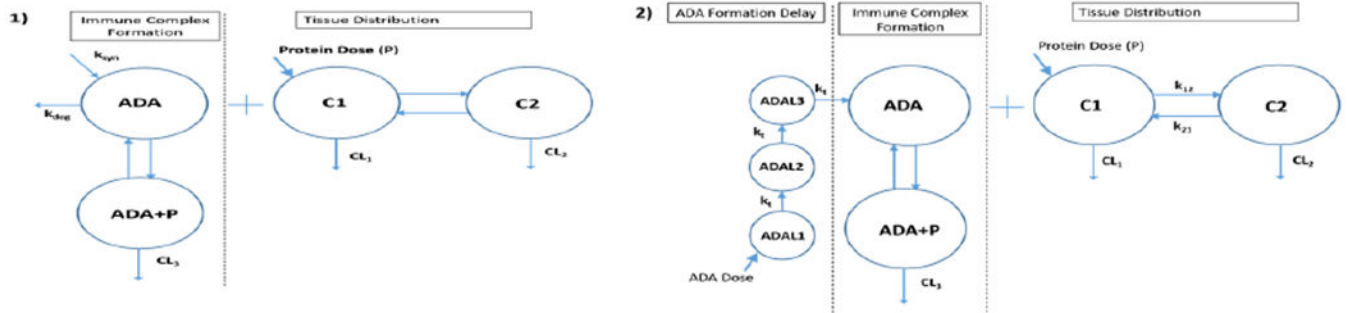
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**Figure 1:** Antibody Dependent Cellular Cytotoxicity Mechanism: When Mabs bind to target antigen on the cell surface, it can lead to natural killer cells binding to the Fc region via CD16. Activation of this receptor leads to secretion of cytotoxic granules that enter the healthy cell, forcing it to undergo apoptosis.

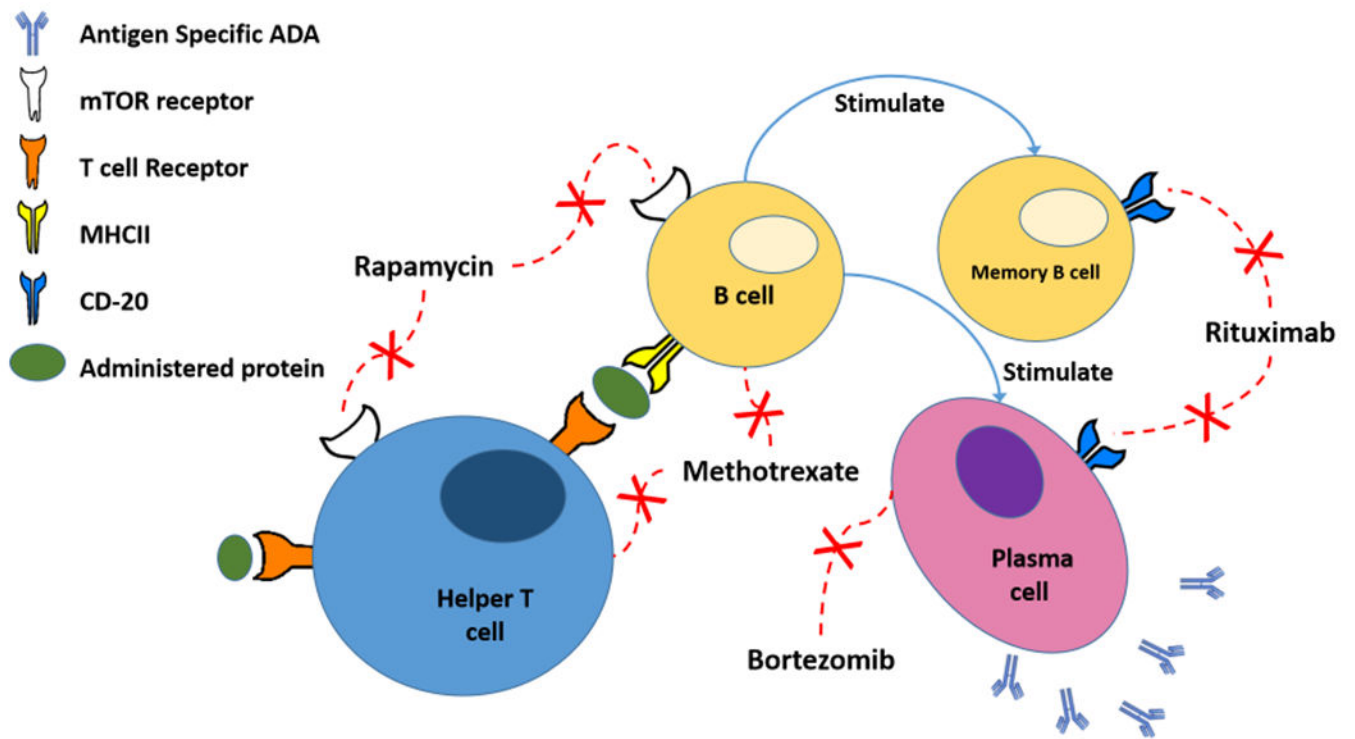


**Figure 2:**  
Complement Dependent Cytotoxicity: When Mabs bind to target antigen, it can deposit in healthy tissue. Circulating complement can bind to the immune complex and trigger complement activation, leading to cell death.



**Figure 3:**

A Two compartment model (1) depicting the impact of ADA on the PK of a protein. Protein is administered into the central compartment (C1) where it can distribute into the tissue compartment (C2).  $CL_1$  and  $CL_2$  depict clearance from the central compartment and tissue compartment, respectively. The left side depicts immune complex formation. ADA in the central compartment (ADA) can bind to protein to form immune complexes (ADA+P) that can be directly eliminated via degradation pathways ( $CL_3$ ), complementing  $CL_1$  and  $CL_2$ . The second model (2) has the same basic structure, with the addition of  $T_{lag}$  component (ADAL), accounting for the time it takes to initiate an immune response. ADA begins as hypothetical bolus dose before passing through a series of compartments before reaching the central compartment.



**Figure 4:** Effect of various immunomodulatory drugs on the immune system. Rapamycin inhibits T and B cell activation through the mTOR pathway. Rituximab blocks CD20, killing CD20 expressing B cells. Methotrexate inhibits DNA synthesis and kills rapidly dividing cells. Bortezomib inhibits proteasomes, promoting apoptosis in rapidly dividing cells.



Table 1:

Extensive list of antibody products and immunogenicity

Trade Name	INN	Company	Target	Type	FDA Approval	IMG Rate <sup>1</sup>
RgeoPro	Abciximab	Eli Lilly	GP1Ib/IIIa	Chimeric IgG1 Fab	1994	6-44%
Humira	Adalimumab	Abbott	TNF- $\alpha$	Human IgG1	2002	5-26%
Lentradra	Alemtuzumab	Sanofi	CD-52	Humanized IgG1	2014	1.9-8.3%
Praluent	Alirocumab	Sanofi	PCSK-9	Human IgG1	2015	1.2-4.8%
Tecentriq	Atezolizumab	Genentech	PD-L1	Human IgG1	2016	30-48%
Bavencio	Avelumab	Merck & Co.	PD-L1	Human IgG1	2017	4.1%
Simulect	Basiliximab	Novartis	CD-25	Chimeric IgG1	1998	1.2%
Benlysta	Belimumab	GSK	BLyS	Human IgG1	2011	0-4.8%
Fasenra	Benralizumab	Astrazeneca	CD-125	Humanized IgG1	2017	13.0%
Avastin	Bevacizumab	Genentech	VEGF	Humanized IgG1	2004	0.6%
Zinplava	Bezlotoxumab	Merck & Co.	C. Difficile Toxin B	Human Monoclonal Antitoxin Antibody	2016	0.0%
Adcetris	Brentuximab	Seattle Genetics	CD-30	Chimeric IgG1 ADC	2011	7-30%
Siliq	Brodalumab	Valeant Pharmaceuticals	IL-17RA	Human IgG2	2017	3.0%
Ilaris	Canakinumab	Novartis	IL-1 $\beta$	Human IgG1	2009	0.0%
Libtayo	Cemiplimab	Regeneron	PD-1	Human IgG	2018	1.3%
Cimzia	Certolizumab pegol	UCB	TNF- $\alpha$	Humanized IgG Fab fragment	2008	6-23%
Erbitux	Cetuximab	Eli Lilly	EGFR	Chimeric IgG1	2004	5.0%
Darzalex	Daratumumab	Janssen	CD-38	Human IgG1	2015	0.0%
Prolia/Xgeva	Denosumab	Amgen	RANKL	Human IgG2	2010	<1%
Dupixent	Dupilumab	Regeneron	IL-4R $\alpha$	Human IgG4	2017	7.0%
Imfinzi	Durvalumab	Astrazeneca	PD-L1	Human IgG1	2017	Unknown
Soliris	Eculizumab	Alexion Pharmaceuticals	Complement C5	Humanized IgG2/4	2007	1-3%
Empliciti	Elotuzumab	BMS	SLAMF7	Human IgG1	2015	18.5%
Hemlibra	Emicizumab	Genentech	Factor VIII	Humanized IgG4	2017	Undetected
Aimovig	Erenumab	Amgen	CGRPR	Human IgG2	2018	2.6-6.2%
Repatha	Evolocumab	Amgen	LDL-C / PCSK9	Human IgG2	2015	0.1%

Trade Name	INN	Company	Target	Type	FDA Approval	IMG Rate <sup>1</sup>
Ajovy	Fremanezumab	Teva Pharmaceuticals	CGRP	Humanized IgG2	2018	0.4-1.6%
Emgality	Galcanezumab	Eli Lilly	CGRP	Humanized IgG4	2018	4.8-12.5%
Mylotarg	Gemtuzumab ozogamicin	Wyeth	CD-33	Humanized IgG4 / toxin conjugate	2000	Undetected
Simponi	Golimumab	Janssen	TNF- $\alpha$	Human IgG1	2009	2-7%
Trogarzo	Ibalizumab	TaiMed Biologics	CD-4	Humanized IgG4	2018	Undetected
Remicade	Infliximab	Janssen	TNF- $\alpha$	Chimeric IgG 1	1998	10-51%
Vervoy	Ipilimumab	BMS	CTLA-4	Human IgG1	2011	1.1-4.9%
Nucala	Mepolizumab	GSK	IL-5	Human IgG1	2015	6.0%
Poteligeo	Mogamulizumab	Kyowa Kirin	CCR4	Humanized IgG 1	2018	3.9%
Lumoxiti	Moxetumomab	Astrazeneca	CD-22	Recombinant Immunotoxin	2018	56.0%
Tysabri	Natalizumab	Biogen	VLA-4	Humanized IgG4	2004	9-10%
Portrazza	Necitumumab	Eli Lilly	EGFR	Human IgG1	2015	4.1%
Opdivo	Nivolumab	BMS	PD-1	Human IgG4	2015	11.2-37.8%
Gazyvaro	Obinutuzumab	Roche	CD-20	Humanized IgG 1	2013	7.0%
Ocrevus	Ocrelizumab	Genentech	CD-20	Humanized IgG1	2017	0.5-1.5%
Atzerra	Ofatumumab	GSK	CD-20	Human IgG1	2009	Undetected
Lartuvo	Olaratumab	Eli Lilly	PDGFR- $\alpha$	Human IgG1	2016	3.5%
Xolair	Omalizumab	Genentech	IgE	Humanized IgG1	2003	<0.1%
Synagis	Palivizumab	MedImmune	F-protein of RS virus	Humanized IgG1	1998	0.7%
Vectibix	Panitumumab	Amgen	EGFR	Human IgG2	2006	0.8-4.6%
Keytruda	Pembrolizumab	Merck & Co.	PD-1	Human IgG4	2014	2.0%
Perjeta	Pertuzumab	Roche	HER-2	Humanized IgG1	2012	2.8%
Cyramza	Ramucirumab	Eli Lilly	VEGF	Human IgG1	2014	6.0%
Lucentis	Ranibizumab	Genentech	VEGF-A	Humanized IgG1 Fab fragment	2006	0-6%
Cinqair	Reslizumab	Teva Pharmaceuticals	IL-5	Human IgG4	2016	4.8-5.4%
Rituxan	Rituximab	Biogen Idec	CD-20	Chimeric IgG1	1997	1.1-11%
Cosentyx	Secukinumab	Novartis	IL-17a	Human IgG1	2015	Undetected
Sylvant	Siltuximab	Janssen	cCLB8	Chimeric IgG1	2014	0.2%
Ilumya	Tildrakizumab	Sun Pharma	IL-23	Humanized IgG1	2018	6.5%

Trade Name	INN	Company	Target	Type	FDA Approval	IMG Rate <sup>1</sup>
RoActemra	Tocilizumab	Chugai	IL-6 receptor	Humanized IgG1	2010	2.0%
Herceptin	Trastuzumab	Genentech	HER-2	Humanized IgG1	1998	0.1%
Stelara	Ustekinumab	Janssen	IL-12/IL-23	Human IgG1	2009	3-6%
Entyvio	Vedolizumab	Takeda	Integrin- $\alpha$ 4 $\beta$ 7	Humanized IgG1	2014	13.0%

<sup>1</sup> Rates of Immunogenicity were derived solely from FDA licensed package inserts. In cases where IMG rates were not reported, the table was labeled as 'Unknown'. IMG rates listed can be impacted by confounding factors such as co-medication, immune status of the patient, and detection assays used.

Table 2:

Extensive list of protein products and immunogenicity

Trade Name	INN	Company	Target	Type	FDA Approval	IMG Rate <sup>2</sup>
Actvase	Alteplase	Genentech	Thrombolytic	Recombinant	1987	Unreported
Advate	Octocog Alfa	Baxter	Antihemophilic Factor VIII	Recombinant in CHO	2003	20.0%
Amevive	Alefacept	Biogen	CD-2 inhibitor	Recombinant in CHO		3.0%
Andexxa		Portola Pharmaceuticals	Coagulation Factor Xa	Recombinant in CHO	2018	17.0%
Avonex	Interferon Beta-1a	Biogen	Interferon- $\beta$ Mimetic	Recombinant in CHO	1996	5.0%
Betaseron	Interferon Beta-1b	Bayer	Interferon- $\beta$ Mimetic	Recombinant in <i>E. Coli</i>	1993	45.0%
Bravelle	Urofollitropin	Ferring	Follicle Stimulating Hormone	Purified from Urine	2002	Unreported
Byetta	Exenatide	Astrazeneca	GLP-1 agonist	Solid Phase Peptide Chemistry	2005	9-38%
Ceredase	Alglucerase	Genzyme	$\beta$ -glucocerebrosidase Mimetic	Purified from Human Plaenta and Modified	1994 (discontinued)	Unreported
Eliprase	Idursulfase	Shire	Iduronate-2-sulfatase mimetic	Recombinant in HT-1080	2006	51-68%
Eloctate		Bioverativ	Antihemophilic Factor VIII	Recombinant in HEK	2014	Unreported
Epogen	Epoetin Alfa	Angen	Erythropoietin Mimetic	Recombinant in CHO	1989	Unreported
Helixate		CSL Behring	Antihemophilic Factor VIII	Recombinant in BHK	1993 (discontinued)	12.5-15%
Kepivance	Palifermin	Sobi	KGF Mimetic	Recombinant in <i>E. Coli</i>	2004	2.0%
Koate		Grifols	Antihemophilic Factor VIII	Purified from Human Plasma	2012	Unreported
Levemir	Insulin Detemir	Novo Nordisk	Insulin Mimetic	Recombinant in <i>Saccharomyces Cervisiae</i>	2005	0.0%
Monarc-M		American Red Cross	Antihemophilic Factor VIII	Purified from Blood	1974	Unreported
Myozyme	Alglucosidase Alfa	Genzyme	Acid Alpha-Glucosidase Mimetic	Recombinant in CHO	2006	89.0%
Neulasta	Pegfilgrastim	Angen	G-CSF mimetic	Recombinant in <i>E. Coli</i>	2002	0.8%
Neumega		Wyeth	IL-11 Mimetic	Recombinant in <i>E. Coli</i>	1997	1.0%
NovoSeven		Novo Nordisk	Antihemophilic Factor VII	Recombinant in BHK	1999	Unreported
Nulojix	Belatacept	BMS	CD-80/86 blocker	Recombinant in CHO	2011	7.8%
Palynziq	Pegvaliase	BioMarin	Metabolizing Enzyme	Recombinant	2018	100.0%
Proleukin	Aldesleukin	Prometheus	IL-2 Mimetic	Recombinant in <i>E. Coli</i>	1992	66-74%
Pulmozyme	Domase Alfa	Genentech	rhdNase	Recombinant in CHO	1993	Unreported
Raplixa		The Medicines Company	Fibrin Sealant of Wounds	Purified from Human Plasma	2015	2-4%

Trade Name	INN	Company	Target	Type	FDA Approval	IMG Rate <sup>2</sup>
ReFacto		Pfizer	Antihemophilic Factor VIII	Recombinant in CHO	2000	Unreported
Refludan	Lepirudin	Bayer	Direct Thrombin Inhibitor	Recombinant in Yeast	2004 (discontinued)	Unreported
Tanzeum	Albuglutide	GSK	GLP-1 agonist	Recombinant in <i>Saccharomyces Cervisiae</i>	2014	5.5%

<sup>2</sup>Rates of Immunogenicity were derived solely from FDA licensed package inserts. In cases where IMG rates were not reported, the table was labeled as 'Unknown'. IMG rates listed can be impacted by confounding factors such as co-medication, immune status of the patient, and detection assays used.

**Table 3:**

## Transgenic Mouse Models

<b>Protein of Interest</b>	<b>Reference</b>
<b>Tissue Plasminogen Activator</b>	Stewart et al. <sup>248</sup>
<b>Insulin</b>	Ottensen et al. <sup>141</sup>
<b>Interferon-<math>\beta</math></b>	Hermeling et al. <sup>136</sup>
<b>Human Growth Hormone</b>	Sweetsen et al. <sup>139</sup>
<b>Factor VIII</b>	Fakharzadeh et al. <sup>137</sup>
<b>Factor VIIa</b>	Lenk et al. <sup>249</sup>
<b>Transforming Growth Factor-<math>\beta</math></b>	Carrier et al. <sup>250</sup>
<b>Acid Alpha-glucosidase</b>	Khanna et al. <sup>251</sup>

**Table 4:***In Silico* Epitope Databases

<b>Database</b>	<b>Developer</b>
<b>SEDB</b>	Pondicherry University, India <sup>210</sup>
<b>Epitome</b>	Schlessinger et al. <sup>209</sup>
<b>IEDB</b>	National Institute of Allergy and Infectious Disease <sup>208</sup>
<b>CED</b>	Huang et al. <sup>252</sup>
<b>AntiJen</b>	Edward Jenner Institute, Toseland et al. <sup>253</sup>
<b>Bcipep</b>	Saha et al. <sup>254</sup>
<b>HLA Epitope Registry</b>	Duquesnoy et al. <sup>255</sup>

**Table 5:**

## Commonly Used Algorithms and Programs for Epitope Prediction

Algorithm	Developer
MHCPred	Guan et al. <sup>213</sup>
MHCPred 2.0	Guan et al. <sup>212</sup>
EpiJen	Edward Jenner Institute <sup>256</sup>
ProPred	CSIR-Institute of Microbial Technology
MMBPred	Bhasin et al. <sup>257</sup>
SVMHC	Dönnies et al. <sup>258</sup>
Protean 3D	DNASTAR <sup>259</sup>
SVMTriP	Yao et al. <sup>260</sup>
EpiMatrix/JanusMatrix	EpiVax <sup>214,261</sup>