

Immunogenicity of protein therapeutics

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Protein therapeutics, such as monoclonal antibodies, enzymes and toxins, hold significant promise for improving human health. However, repeated administration of protein therapeutics, whether natural or recombinant, often leads to the induction of undesirable anti-drug antibodies (ADAs), which interfere with or neutralize the effect of the drug. Although an immune response to foreign proteins can be expected and is well understood, the basis for the development of responses to therapeutic autologous proteins is the subject of some debate. Inflammatory components of the drug delivery vehicle, T cell responses, T and B cell epitopes in the protein drug, and the associated B cell response are all targets for interventions aimed at reducing ADA responses. Here, we review some theories put forward to explain the immunogenicity of therapeutic proteins and describe some emerging protein-engineering approaches that might prevent the development of anti-drug antibodies.

Two broad categories of immune response to protein therapeutics

The number of therapeutic protein products available for use in clinical settings has dramatically increased in recent years. This category of biomedical products, also known as biological therapeutics, includes recombinant human cytokines (e.g. α and β interferon), cellular growth factors (e.g. GM-CSF), hormones (e.g. glucagon), neuromuscular antagonists (e.g. botulinum toxin), blood products (e.g. clotting factor VIII) and monoclonal antibodies. Although therapeutic proteins are generally considered safe and non-toxic, anti-therapeutic protein antibodies can develop during treatment. Anti-therapeutic protein antibodies [known as anti-drug antibodies (ADAs)] might neutralize or otherwise compromise the clinical effect of therapeutics and can also be associated with serious adverse events related to cross-reactivity with autologous proteins. Examples of ADAs include those to botulinum toxin (used to treat dystonia) and to clotting factor VIII (FVIII), a therapeutic for Hemophilia A [1,2]. Botulinum toxin is a foreign antigen, whereas therapeutic FVIII could appear foreign to the recipient because Hemophilia A is usually associated with deletion or modification of the FVIII gene. The generation of ADAs is not particularly surprising in

these instances because it might be considered a vaccine-like reaction to foreign protein. Immune responses to vaccination are related to the number of drug doses administered, delivery route, and adjuvant. ADAs can also develop to recombinant autologous proteins, such as erythropoietin [3], which should not, in theory, breach tolerance [4]. The development of auto-antibodies in such cases might be owing to degradation or aggregation of the protein drug, or to the administration of the protein in the presence of a 'danger signal', such as a toll-like receptor (TLR) ligand. This will be covered in more detail later in the review.

As highlighted previously, immune responses to protein therapeutics might be categorized into one of two types of immune response: (i) activation of the classical immune system by foreign proteins, similar to the immune response against pathogens or vaccines; and (ii) breach of B and T cell tolerance to autologous proteins – a complicated series of immunological events that is not fully understood (Table 1). These two mechanisms overlap but are slightly different. The most easily explained mechanism is a 'vaccine-like' response to foreign proteins, involving T cells, B cells, and the innate immune system. By contrast, human immune responses to autologous proteins might also involve overcoming the regulation of adaptive immune responses by T regulatory cells (Treg).

Immunological mechanisms: B cell activation can be T cell-independent (Ti) or T cell-dependent (Td)

To identify effective interventions that might prevent the development of ADAs, it is worthwhile to review the sequence of immunological events leading to ADA secretion (summarized in Figure 1a).

The sequence of events that lead to B cell activation, and the resultant production of antibodies, can be divided into Ti and Td scenarios. Ti activation of B cells occurs when structural features of certain molecules, such as polymeric repeats, induce the 'signals' required to stimulate activation of a B cell subset, although the complement component C3d might also have a role when a T-dependent immune response cannot be activated, leading to the development of immune memory [5]. Although Ti activation is often cited as a source of antibodies to protein therapeutics, Ti activation of B cells generally does not lead to affinity maturation or to the generation of memory B cells. In general, Td activation of B cells results in a more robust antibody response, isotype switching, and the development

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Table 1. Parallels and differences between classical immune responses to exogenous antigens and autologous (therapeutic) proteins^a

	Classical immune response	Immune response to recombinant autologous (therapeutic) proteins
Antibody	Present	Might be induced if the protein is altered, enabling recognition by B cell receptors (IgM, IgG)
T cell response	Present	Might be present under some circumstances
T regulatory cells	Absent	Might control anti-self response
Inflammation	Not absolutely necessary	Probably contributes to over-riding T regulatory cells and subsequent immunogenicity
Adjuvants	Increase likelihood of immunogenicity by stimulating innate immune response	Probably required to breach tolerance
Usual character of immune response	Effector or inflammatory immune response	Tolerance

^aThe two slightly different but overlapping mechanisms involved in the development of immunogenicity in response to the use of therapeutic proteins: (i) the classical immune response to foreign proteins, and (ii) incomplete tolerance to autologous proteins.

of B cell memory. The induction of IgG class ADAs (measurable in standard immunogenicity assays) generally implies that the therapeutic protein is a Td antigen that has led to isotype switching [6]. Because Td responses require T cell recognition of epitopes contained in the protein drug, binding of peptide epitopes [derived from internal processing by antigen-presenting cells (APCs)] to HLAs (human leukocyte antigens) MHC class II molecules, and recognition of the epitope–HLA complex by activated helper T cells can be assumed to occur. In the absence of signals provided by the cytokines released as a result of T cell interaction with APCs, the naïve B cell does not mature. Indeed, without T cell help, activated antigen-specific B cells might be rendered anergic or undergo apoptosis (Figure 1b). Therefore, T cell recognition of the peptide epitopes derived from the antigen can be considered a key determinant of Td antibody formation.

Tolerance and the regulation of the anti-self response

Tolerance to self-proteins is a basic immune system feature and the development of ADAs to recombinant autologous proteins can be regarded as a 'breach' of tolerance. Although auto-reactive B cells can be found in normal individuals, these cells are not usually stimulated to produce antibodies by the circulating levels of native proteins. There might be many parallels between the development of autoimmune disease and immunogenicity of therapeutic proteins, including the involvement of Treg. Reduction of Treg immune responses and induction of T-effector responses are probably significant contributors to ADAs because the link between T cell (and HLA-restricted) immune response and the development of auto-antibodies is well defined [7].

Extrinsic factors contributing to immunogenicity

Repeated dosing, routes of immunization, effective adjuvants, and other co-factors that are known to contribute to effective vaccination are best avoided with protein therapeutics. For example, reducing the total number of doses, minimizing aggregates, diminishing T cell activation, and avoiding delivery vehicles that have adjuvant effects, are rational strategies for reducing immunogenicity. These factors are discussed in greater detail in the following sections.

'Danger' signals

B cells might begin to produce antibodies in response to an auto-antigen if stimulated by a second signal from T helper

cells, or by direct stimulation via TLR ligands, such as products of microbial degradation. TLR ligands, such as bacterial DNA (CpG) have recently been shown to stimulate B cell responses directly in the absence of T cells [8]. Thus, even low-level contamination of recombinant therapeutic protein with bacterial DNA, lipids or endotoxins might contribute to the development of unanticipated immune reactions to autologous proteins [9].

Arrays

One potent way to induce high levels of IgG independent of T cell help is to present the self antigen arrayed on viruses and virus-like particles (VLP). For example, self antigens conjugated with papilloma VLP evoked a strong antibody response to self-antigens [10,11]. Highly repetitive arrays present in a protein therapeutic might, therefore, induce T_H ADAs.

Aggregates

The role of aggregates in the development of immune responses to proteins is well-defined, particularly with respect to intravenous immunoglobulin (IVIG) and human growth hormone. Aggregates might engender an immune response by cross-linking the B cell receptor, which activates B cells to proliferate, targets protein to the lysosomal pathway and efficiently elicits T cell help [12]. In addition, aggregates of proteins can present epitopes in an array form, evoking T cell-independent antibody induction (see previous section). Furthermore, aggregates are readily taken up by APCs and might drive dendritic cell (DC) maturation, enhancing Td immune response.

Endocytosis of the target

Whether or not a therapeutic protein (or monoclonal antibody) is immunogenic might depend on whether the protein interfaces with the protein-processing machinery of APCs. Proteins that are internalized because their target is endocytosed; for example, anti-tumor necrosis factor (TNF) antibodies against surface TNF might be more immunogenic than proteins that are not endocytosed (for example, anti-TNF antibodies against soluble TNF).

Protein modification

Protein modification by sequence modification, denaturation or introduction of alternative glycosylation sites might render a protein enough unlike 'self' to induce a

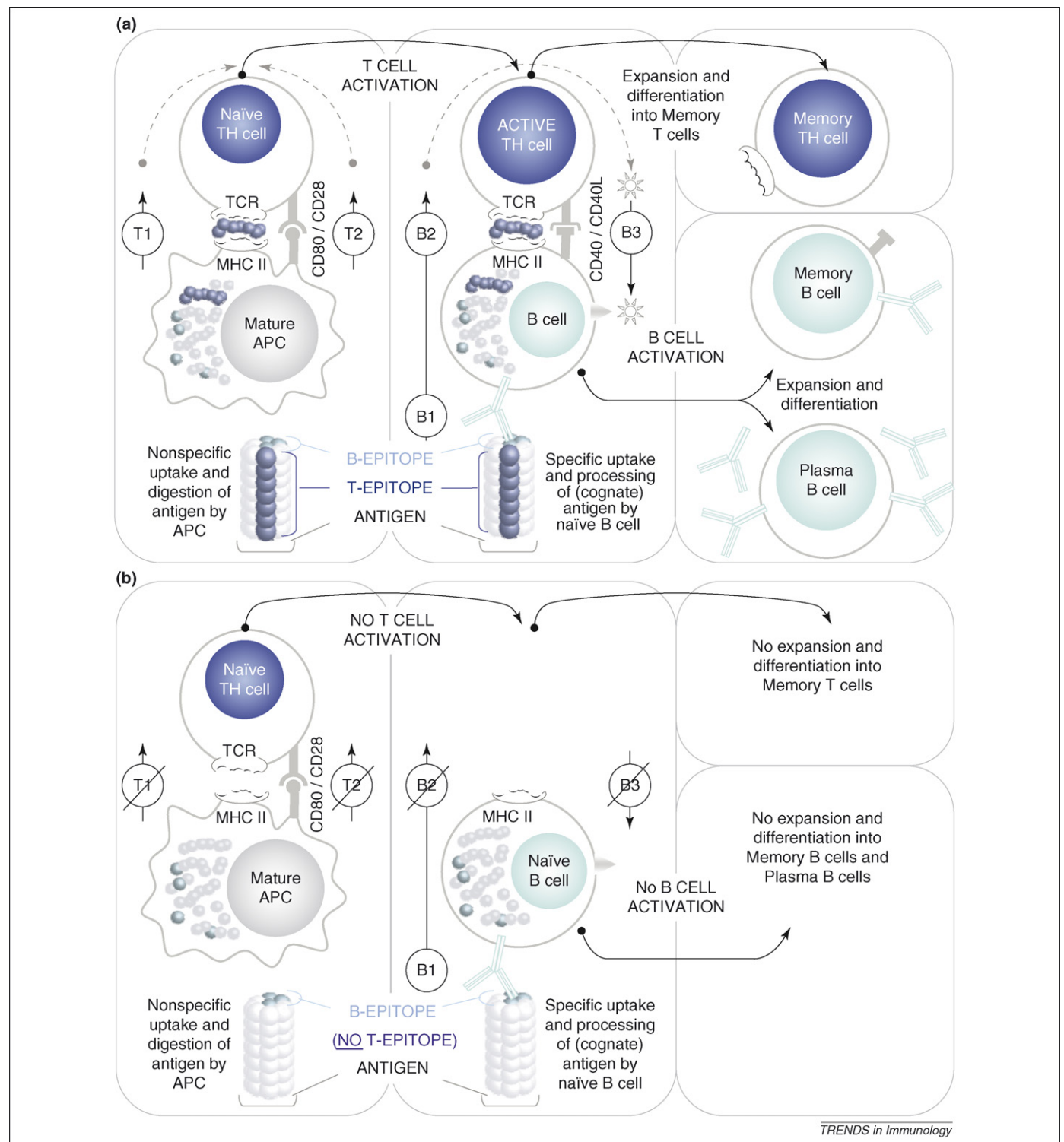


Figure 1. (a) To induce naïve B cells to produce antibodies to a protein antigen and to initiate an adaptive immune response, several events must be coordinated, usually within specialized regions of secondary lymphoid organs (e.g. lymph nodes, spleen, etc.). The first step in this process is the nonspecific internalization of the antigen by professional APC, such as mature DC. The mature DC process the antigen into peptides, which are then presented to naïve T cells in MHC class II molecules on the APC surface. The interaction of the TCR with this MHCII-peptide complex is indicated as T cell Signal 1 (T1). To fully activate the T cell, this must be accompanied by additional signals from what are termed 'co-stimulatory molecules', such as CD80 and CD86, provided by the APC, indicated as T cell Signal 2 (T2). In the absence of such a second signal, T cells might become anergic. Once fully activated, these CD4 T cells divide and can produce an array of cytokines with various activities. An interaction between B cell IgM and IgD receptors and cognate antigen is required to initiate activation of the naïve B cell (middle panel), this is termed B cell Signal 1 (B1). The B cell internalizes and processes the antigen-Ig complex and presents cognate T cell epitopes in the context of MHC class II on its surface. On encountering the cognate epitope-MHC complex, the T helper cell delivers cytokines, B cell Signal 2 (B2), which drive B cells to activate, proliferate, and differentiate into memory B cells and antibody-secreting plasma cells (right panel). Activated B cells express CD40 and bind CD40 ligand, communicating a further signal to the presenting B cell and the engaged T cell; T-B cell Signal 3 (B3). Thus, full activation of a B cell and its corresponding T cell is dependent on (i) proper presentation of the T cell epitope derived from the protein antigen by a professional APC and a B cell, and (ii) recognition of the peptide-MHC complex by the TCR. Cytokine signals from the T cell initiate a cascade of further immuno-stimulatory events that cause B cells to expand, switch isotypes and undergo phenotypic changes resulting in the establishment of memory B cells. (b) A complete lack of T cell epitopes would eliminate autologous T help and lead to B cell apoptosis.

classical immune response, while still resembling the human homolog enough to retain function. A single amino acid change or post-translational modification (or lack thereof) could create a neo-epitope for immune system recognition. Therapeutic proteins looking more like 'self', or with few mutations compared with the wild-type, are considerably less immunogenic than proteins that appear unique to the immune system. This is particularly true of mutated or fusion proteins containing novel antigenic B and T cell epitopes. In addition, proteins denatured during formulation might be more immunogenic than their intact counterparts [13]. These products might present new B cell or even T cell epitopes that were not present in the parent molecule, leading to the stimulation of an immune response [14].

Patient-dependent factors

Patients can show different types of immune response to therapeutic protein products, owing to allelic variation (e.g. allotypes), genetic deficiency, disease state (immunosuppressed or not) or HLA background. Allelic variation might have a more important role in the immunogenicity of autologous proteins than previously suspected and deserves greater study. For example, hemophilia patients with deletions or inversion of the FVIII gene have different immune responses to FVIII replacement therapy. Although the relationship between FVIII mutations and FVIII 'inhibitors' (neutralizing ADAs) needs to be better defined, two recent publications have documented the relationship between immunogenicity of selected proteins and HLA-associated T-dependent immune response [15,16]. Finally, some protein therapeutics might have immunological effects that contribute to, or are independent of, their own inherent immunogenicity (i.e. by perturbing key immune system components, such as anti-IL-2 or even IL-2 itself).

Leachates

Although the specific mechanism of action of soluble factors leached from uncoated rubber stoppers (so-called leachates) is unknown, they have been implicated in the formation of antibodies against recombinant erythropoietin (see following section). The leachates might have had a directly pro-inflammatory effect, similar to TLR ligands, providing the crucial 'danger signal' required for antibody induction.

Extrinsic factors that might diminish immunogenicity

Masking T cell epitopes with sugars (glycosylation), a process that occurs during the biosynthesis of the product, might reduce the immunogenicity of therapeutics. By contrast, addition of unusual sugars might increase immunogenicity [17,18]. Addition of polyethylene glycol (PEG) molecules (PEGylation) might camouflage proteins and interfere with ADA formation in addition to providing a means for prolonging the half-life of the product (see following section).

Consequences of antibodies to therapeutic proteins

Some types of antibodies do not interfere with the protein therapeutic, whereas others have dramatic and sometimes

life-threatening effects. For example, antibodies that neutralize or interfere with the effect of the protein therapeutic and, occasionally, with the function of the endogenous analog, were initially described in the context of treatment with megakaryocyte growth and differentiation factor (MGDF) [4]. By contrast, severe general immune reactions, such as anaphylaxis associated with the use of animal antisera, have become rare, presumably because product purity has increased substantially over the past decades. Delayed-type infusion-like reactions resembling serum sickness are more common, particularly with monoclonal antibodies and other proteins administered in relatively large amounts. These reactions might be owing to immune complex formation. Patients with steadily increasing antibody titers are reported to show more infusion-like reactions than patients with short, temporary antibody responses. In many instances, the presence of ADAs is not associated with direct interference with protein function. Instead, the ADAs might influence the pharmacokinetics of the drug, potentially increasing or decreasing its half-life [19].

Neutralizing and cross-reactive ADAs

By contrast, neutralizing ADAs can contribute to loss of activity of the protein therapeutic. By definition, neutralizing antibodies that interact with a ligand–receptor interaction inhibit the efficacy of all ligands in the same class. For example, antibodies that neutralize recombinant erythropoietin (rEPO) by interfering with the binding of the protein to its receptor might interfere with all forms of erythropoietin, leading to anemia. Neutralizing antibodies are of greatest concern when they develop in conjunction with conditions for which there are no treatment alternatives [20]. Cross-reactive ADAs might also cause particular problems. In general, when neutralizing antibodies cross-react with an endogenous factor that has an essential biological function, there is a higher likelihood of adverse events. For example, ADAs were recently associated with the development of pure red cell aplasia (PRCA) following treatment with rEPO. This severe form of anemia was due to the development of antibodies cross-reactive with endogenous erythropoietin [21]. Cases of PRCA were associated with exposure to just one recombinant EPO product, suggesting that factors extrinsic to the protein itself were associated with the adverse outcome. Current evidence suggests that this effect was associated with a specific reformulation [22–24]. This formulation had replaced human serum albumin with polysorbate-80 and used uncoated stoppers in the product packaging. PRCA was 17 times more common in subjects exposed to this formulation than in those who were not. Cases of rEPO-associated PRCA dropped dramatically after the drug was reformulated and uncoated stoppers were replaced with coated versions [24].

Methods for the precise measurement of ADAs and their effects are essential for the rational design of future therapeutics. These methods have evolved rapidly over the past few years and have become the subject of two industry 'white papers'. Assays for measuring immune responses to therapeutic proteins are discussed in [Boxes 1 and 2](#); see also the review by Sluis *et al.* [25]. Currently, the

Box 1. Pros and cons of assays for measuring immunogenicity: antibody assays

Several excellent reviews have been published regarding the development and validation of different assay formats for antibodies to therapeutic proteins [25]. Here we only discuss some caveats concerning interpretation of these means of measuring antibody response in *in vitro* assays.

Lack of standardization. Given that there are no international antibody assay standards, antibody titers measured in clinical trials are difficult to compare, unless the antibody assays were performed in the same laboratory. There is also wide variation in the reported incidence of immunogenicity associated with a single product. Lack of standardization is one explanation for these results.

Methodologies. Two different methodologies are used to evaluate patient serum for the presence of ADA. Methods evaluating antibody–drug binding include enzyme-linked immunosorbent assays (EIA), radioimmunoassay (RIA), and surface plasmon resonance (SPR). Binding assays and bioassays are often used in combination. For example, a sensitive binding assay might be used initially to screen samples for antibody and bioassays typically follow, because these are usually technically difficult and time-consuming.

Biological assays. Bioassays might be particularly useful for demonstrating that neutralization is caused by antibodies, if the presence of other inhibitory factors, such as a soluble receptor, must be excluded.

Isotype. SPR technology can further characterize a neutralizing antibody response for isotype, affinity and specificity; however neutralizing capability cannot be linked to isotype.

Chronology. The immune response is a dynamic process and, therefore, the course of antibody development (kinetics) must be evaluated as the response evolves over time. Increasing titers of neutralizing antibodies are thought to be associated with epitope spreading and might correlate with some biological effect, however a particular therapeutic product might not be immunogenic enough to achieve such levels.

Measuring response in populations. An additional method for comparing relative immunogenicity in patient groups is to use 'mean population antibody titers' (describing the method by which the titer was determined). This is a more accurate means of describing immune responses to protein therapeutics than describing the percentage of subjects who convert from antibody-negative to antibody-positive.

only means of discriminating between neutralizing and non-neutralizing ADAs is to perform a biological assay using cell lines that express the target and mimic the therapeutic effect of the drug. Measurement of the biological effects of ADAs was also discussed by Gupta *et al.* in a recent white paper [26].

Prediction of immunogenicity

Several approaches can be used to predict the potential immunogenicity of the therapeutic in a patient, and thereby minimize the risk of adverse reactions or neutralization of the product.

Prediction based on physico-chemical characterization

For non-human proteins, sequence and T cell epitope analysis are good predictors of an immune response in the clinic. For biopharmaceuticals with a high degree of homology to native proteins, the main physico-chemical determinants of immunogenicity are impurities, heterogeneity, aggregate formation, and protein degradation, such as oxidation and deamidation. Aggregate formation is one aspect of the production process that can be monitored by physico-chemical characterization [27]. However,

Box 2. Pros and cons of assays for measuring immunogenicity: T cell immunogenicity monitoring

Monitoring T cell response *in vitro* requires measurement of an indicator (usually a cytokine, or a T cell function, such as proliferation), in the presence of antigen.

Methodologies. Several different assays can be used to measure T cell responses. The assays measure proliferation of T cells in response to an antigen (thymidine incorporation) or release of cytokines, such as IL-2 or IFN γ , by T cells responding to antigen (ELISA or ELISpot assays). In every case, T cell responses to antigen must be compared with controls containing medium without antigen because there is significant variability in baseline (background) immune responses from person to person, and from study animal to study animal [69]. The enzyme-based ELISpot technique is one of the most sensitive methods for detecting T cell responses to therapeutic proteins. It enables the detection of individual T cells directly from splenocytes or peripheral blood and permits determination of their antigen-specific cytokine release. ELISpot assays are regularly used to identify low-frequency autoreactive T cells. IL-2 and IL-4 ELISpot assays can be performed using commercial kits.

Flow cytometry. Fluorescent labeling (FACS) and intracellular cytokine staining (ICS) are the most precise methodologies available for measuring and defining T cell responses because they allow gating of specific types of T cells (including Tregs) but they are associated with a higher cost per assay. For example, T cells that respond to a particular epitope can be directly labeled using tetramers (comprising MHC class II-peptide complexes), or the number and phenotype of T cells that respond to the antigen can be determined using FACS-based assays, cell surface markers, and intracellular cytokine staining [70].

Antigen. The use of whole antigen might not correlate with *in vivo* responses owing to differences in the processing of the protein into its peptide components. Most T cell immunologists substitute overlapping peptides representing the whole antigen, either in pools or in single well assays. There are several inherent problems in the design of overlapping peptides sets for use in T cell assays, which include the artificial truncation of T cell epitopes and the lack of stabilizing flanking residues. Selection of peptides for immunogenicity assays based on epitope-prediction tools is an alternative approach.

factors such as the formation of repeating arrays might influence immunogenicity and cannot be identified by current physico-chemical methods [28].

Purity

The relatively high immunogenicity of the earliest therapeutic recombinant DNA-derived growth hormone was blamed on high levels of contaminating bacterial products [29]. The considerable reduction in immunogenicity observed over time in products such as insulin and growth hormone is probably due to heightened stringency in production and increased purity. Based on the recent description of the role of TLR ligands in generating a humoral immune response, these products should be removed in the production process, as far as possible [30].

Prediction based on T cell responses

T cell assays can determine whether peptides derived from the therapeutic protein contain sequences with a propensity to stimulate an antibody response. Generally, for protein administered in the context of clinical trials, the best source of T cells for immunogenicity studies is blood obtained from exposed subjects. T cell assays can be

performed with fresh cells *ex vivo* [31], or memory cells can be expanded and activated *in vitro*, if necessary. In both cases, measurement of the T cell response to autologous proteins might require prior depletion of Tregs to unmask weak responses [32]. T cell proliferation, ELISA, or ELISpot without assessment of concurrent Treg responses might give an inaccurate picture of the potential for immunogenicity of autologous proteins. It has been the experience of the authors that assays using blood from exposed individuals are easier to perform and more reliable than assays that use naïve donor cells (A.D.G. and D.S., unpublished results).

HLA-binding assays

HLA-binding assays can be used to assess whether peptides derived from the therapeutic bind to MHC Class II. These assays measure the affinity of predicted epitope sequences for multiple HLA alleles. *In vitro* evaluation of MHC binding can be performed by measuring the ability of exogenously added peptides to compete with a fluorescently-labeled MHC ligand [33]. Competition-based HLA-binding assays can be adapted for high throughput [34].

In silico T cell epitope prediction and content

Several computerized T cell epitope-mapping tools are now available for use in the identification of T cell epitopes contained within protein sequences [35,36]. Highly immunogenic proteins contain many T cell epitopes or concentrated clusters of T cell epitopes, whereas non-immunogenic proteins tend to contain fewer epitopes. One concept, developed by Martin and De Groot, is that T helper (Th) epitope content might explain differences in observed antibody responses to slightly different versions of the same recombinant human protein [37]. Their 'Td immunogenicity scale' enables the evaluation and comparison of protein sequences for T cell epitopes using EpiMatrix (and the matrices for eight common class II alleles). T cell epitope content, as defined for the immunogenicity scale, relates to the number of T cell epitopes per 1,000 amino acids in a protein sequence. For example, monoclonal antibodies that are slight sequence variants of one another can be compared for epitope content (and potentially for immunogenicity), facilitating the process of selecting the best candidate for clinical trials. Epitope prediction is best performed using a combination of the above techniques (*in silico* and *in vitro*). Because *in silico* tools might vary (see [35] for review of epitope-prediction tools), protein developers are advised to request or perform comparisons of prediction accuracy before selecting one T cell epitope prediction tool over another.

Animal studies

In principle, all proteins intended for therapeutic use in humans are foreign for animals, and therefore immunogenic. Animal and human responses to proteins of non-human origin will be similar because these proteins are comparably foreign for all mammalian species. Although the use of the respective animal proteins might help elucidate basic immunological properties of the molecules studied, animal and human MHC are not equivalent because they can have different amino acid side-chain

restrictions. This is best illustrated by the variability of immune response to chimeric antibodies in different strains of inbred mice [38]. For human homologs, standard animal models are even less predictive because the induction of immunogenicity in humans is based on breaking B and T cell tolerance, although such models do allow comparison of relative immunogenicity of different preparations of the same protein. In non-human primates, there might be a higher degree of sequence homology between the human product and the primate native molecule, to which the animal is immune tolerant. However, one should be aware of significant differences in immunogenicity between monkeys and humans. For example, rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) respond differently to human EPO-GM-CSF hybrids, whereas human FVIII at therapeutic doses can be immunogenic in rhesus monkeys (D.S. *et al.*, unpublished results). Another means of evaluating the impact of epitope modifications on *de novo* T cell response is to measure immunogenicity of the proteins and their derivative peptide epitopes in HLA-transgenic mice [39]. Fortunately, several transgenic mouse strains expressing the most common HLA DR molecules are available [40,41]. To compare immunogenicity of wild-type and modified epitopes, mice are immunized with peptide epitopes in adjuvant. T cell responses in infected humans correlate directly with T cell responses in immunized HLA transgenic mice [42,43]. Hence, HLA transgenic mice are now routinely used to assay and optimize (human) epitope-driven vaccines in pre-clinical studies [44–46].

Protein-transgenic mice for predicting breakage of B cell tolerance

An alternative model for the evaluation of immunogenicity of autologous human proteins is a transgenic animal expressing the human protein in question, which is therefore tolerant to it. Breaking of immune tolerance can be studied in these animals as reported by Ottesen *et al.* [47], Palleroni *et al.* [48] and Stewart *et al.* [49] for insulin, IFN α 2 and human-tissue plasminogen activator (htPA), respectively. Such transgenic mice were used to show that aggregates of human IFN α 2 produced during storage at room temperature of freeze-dried preparations were responsible for enhanced immunogenicity [50].

Reduction of immunogenicity: therapeutic protein modification

Protein therapeutic developers aiming to decrease potential immunogenicity of their products have several options available: careful formulation (to avoid contaminants and aggregates), reduction of immunogenicity by glycosylation and PEGylation, and, in some cases, linkage of the therapeutic protein to a human Ig Fc fragment. Reduction of T cell epitopes by sequence modification also improves immunogenicity, as described below.

PEGylation and glycosylation

PEGylation and glycosylation are the two best known methods for post-translational modification of proteins that impact therapeutic efficacy. PEG is non-toxic, non-immunogenic, non-antigenic, highly soluble in water and

has US FDA approval for use in a range of therapeutics. PEGylated therapeutic protein conjugates have several advantages, including decreased degradation by metabolic enzymes and prolonged residence time in the body. In addition to prolonging protein half-life, PEG might decrease immunogenicity by decreasing proteolysis, interfering with protein processing and presentation, and by physically blocking antibody- or HLA-epitope binding [51]. Glycosylation is also believed to interfere with antibody binding and to have an impact on autoimmunity [52]. The protein-processing machinery of APCs might be impeded by glycosylated amino acid residues and, alternatively, peptide processing might modify (partially or fully deglycosylated) sugars, potentially affecting epitope presentation to T cells [53]. A glycan-containing T cell epitope might also be presented as several variants. This might impair or diversify the immune response by interfering with the binding of the epitope to HLAs or by modifying the interaction with the TCR. Larger glycan structures can lead to loss of MHC class II-restricted T cell recognition.

IgG fusion proteins

Isologous and heterologous immunoglobulins are extremely effective as carriers of therapeutic moieties. The efficacy of these proteins is due, in part, to their long half-life *in vivo*, as well as their ability to bind to Fc receptors. The concept of using IgG as a carrier molecule to induce unresponsiveness in the adult immune system has been exploited for simple haptens, such as nucleosides, as well as for peptides [54]. The most recent example of a successful effector–Fc fusion is etanercept (Enbrel) (Amgen Inc.; <http://www.amgen.com/>), a fusion protein between soluble TNF receptor Type II and the Fc region of human IgG1, which has been used in clinical practice for the control of a wide range of chronic inflammatory diseases, such as rheumatoid arthritis.

Humanization

Much effort has been applied to re-engineering murine and mouse–human chimeric antibodies so that the antibodies have as little non-human structure as possible, to minimize anti-therapeutic effects. In some cases, modification of the therapeutic protein sequence from a foreign protein (a murine monoclonal) to a less foreign protein (a humanized monoclonal) has resulted in reduced immunogenicity. Replacement of mouse immunoglobulin constant regions with human sequences results in the largest immunogenicity reduction. Humanization of variable domains affects a further decrease. Although there is clear evidence that chimeric antibodies are less immunogenic than murine monoclonal antibodies, humanization has not solved the monoclonal antibody immunogenicity problem. For example, the humanization approach reduced, but did not entirely eliminate, the immunogenicity of the Campath antibody [55,56].

Sequence modification

Because the T cell epitope has a crucial role in the development of Td antibody responses, it stands to reason that protein-sequence modifications resulting in removal of potential T cell epitopes from autologous (recombinant

therapeutic) proteins could reduce potential for the induction of immune response to the protein. The literature is replete with evidence for the attenuating effect of epitope-sequence modification on T cell response, particularly with reference to immune escape from class I- and class II-restricted immune response in viral infections. Loss of T cell help removes Signal 2 for specific B cells and, in theory, could lead to B cell tolerance [57]. T cell epitope modification might be thought of as the directed version of the process that occurs naturally when tumor cells [58] and pathogens [59,60] evolve to escape immune pressure by accumulating mutations that reduce the binding of their constituent epitopes to host HLAs [61]. This approach might be relevant to the design of novel replacement proteins, as illustrated by the development of a deimmunized rEPO [62] and recent progress on a novel FVIII [63], and by the development of deimmunized monoclonal antibodies [64–66]. Successful deimmunization of therapeutic proteins has been demonstrated by Genencor Inc. (<http://www.genencor.com>) [67] and EpiVax Inc. (<http://www.epivax.com>) [68] using slightly different approaches. However, such modification could introduce new epitopes that could be presented by other MHC molecules or could create novel B cell epitopes – possibilities that can be assessed in (HLA transgenic) animal studies or by reiterative T cell epitope analysis (*in silico*). Finally, these modifications must maintain the biological function of the product.

Conclusion: is there a bright new future?

Given the vast array of therapeutic proteins that are entering the clinic, all researchers and developers involved in the production of therapeutic proteins should consider the potential for their product to induce an immune response in humans, and should carry out careful immunogenicity studies in the preclinical and clinical phases of the development program. They might also add T cell epitope mapping and screening studies in suitable animal models to their pre-clinical repertoire, before moving a promising candidate into the clinic. In addition to T cell epitope mapping, a careful evaluation of the phenotype of the type of T cells responding to the study protein is warranted because newer data are now demonstrating that not all T cell ‘responses’ are equivalent. This holds true particularly for proteins that are in part, or wholly, recombinant autologous proteins, such as FVIII or any of the cytokines. Although these proteins might contain multiple epitopes, the T cells that respond to the epitopes might be effector or regulatory in nature.

Research on the nature of the immune response to autologous (homologous) therapeutic proteins could improve the selection and screening of candidates. It should build on information about the innate immune system, B cell signaling and tolerance, the importance of Treg, and the regulation of autoimmunity in the periphery. Whatever the ‘final’ mechanism might be, it is important to remember that the goal of this research is to accelerate the development of new compounds that are safe for use in human subjects. The bright new future of therapeutic proteins will arrive when, and if, developers remember to consider immunogenicity.

Conflict of interest statement

Dr. Annie De Groot is a majority stockholder in EpiVax and an employee of EpiVax.

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