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Brief Reviews

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Auditing Protein Therapeutics Management by Professional APCs: Toward Prevention of Immune Responses against Therapeutic Proteins[1]

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Alloimmunization is a crippling concern in the management of patients undergoing administration of protein therapeutics as evidenced in replacement therapy and other treatment procedures. Several issues in the genesis and modulation of such deleterious immune responses have been studied. While authors have focused on the downstream events of the specific immune response and suggested modification of protein therapeutics to eliminate epitopes that interact with B cell receptors, T cell receptors, or MHCII molecules, the mechanisms underlying Ag interaction with APCs, a step upstream of immune effectors, have been grossly neglected. We wish to emphasize that the recent knowledge in understanding the capacities of an APC to handle an Ag and the importance of the surrounding microenvironment in this process are crucial for designing novel protein therapeutics with reduced immunogenicity. ***The Journal of Immunology,*** **2008, 181: 1609–1615.**

Numerous pathologies are treated with drugs that are protein in nature. These therapeutic exogenous proteins can be given either to overcome partial or complete deficiencies of various self-proteins as in replacement therapy or as alien agents independent of the presence of an endogenous counterpart to ameliorate certain pathological conditions. Protein therapeutics are screened rigorously for various biosafety measures like toxicity and viral and bacterial contamination before being applied to the patients. Beyond these measures lies the risk of the immune system responding to the administered protein drug. This immune response attributes another parameter to be checked in a protein drug and is called "immunogenicity". Immune response to a protein drug can be manifested in the form an allergic reaction, an anaphylactic shock, or in the production of immunoglobulins against epitopes on therapeutic proteins (1–3). The first two manifestations have now become less frequent due to the development of modern purification procedures and clinical grade good manufacturing technologies and by using human or humanized proteins (4). However, production of immunoglobulins in patients against a protein drug remains a major problem. This is particularly because, in several instances, patients develop Abs that are specific for and inhibit the functions of administered therapeutic proteins (Table I). Occasionally, administration of human therapeutic proteins may also be associated with anaphylactic shock as has been observed in the case of therapeutic human factors VIII (FVIII) and IX (FIX) in hemophilia patients (5, 6). The development of inhibitory Abs has enormous implications in the day-to-day management of the patients and the clinical outcome and lays a heavy socioeconomic burden; it thus represents the major pitfall in the treatment of the patients. For instance, the average annual cost of care of patients with hemophilia A who develop inhibitory ani-FVIII Abs following FVIII replacement therapy, reached 0.2 million euros per patient in 2003 in developed countries (7).

Initiation of immune response

Understandably, in the case of replacement therapy such immune responses may be due to a lack of T cell and B cell tolerance consequent to the absence of the endogenous protein. However, neutralizing Abs may also develop against protein drugs in recipients who have endogenous counterparts, as seen in healthy individuals treated with thrombopoietin or erythropoietin (8). These observations suggest a role for factors other than the mere lack of T cell tolerance in governing the development of immune responses to protein therapeutics. The incriminated factors include the following: 1) the prevalence of immunodominant epitope(s) in the therapeutic protein that ensures preferential interactions with B cell receptors, T cell receptors, or with MHC II molecules; and 2) patient-related factors such as route of administration, frequency of treatment, age/intensity of treatment at time of first exposure, and various genetic features including HLA haplotypes and polymorphisms in immune genes (9).

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Table I. *Examples of protein therapeutics reported to induce inhibitory immune responses upon administration to patients*

Human Protein Therapeutics	Disease	References
Replacement therapy		
Insulin	Diabetes	85
Growth hormone	Pituitary dwarfism	86
Coagulation factor IX	Hemophilia B	87
Coagulation factor VIII	Hemophilia A	88
von Willebrand factor	von Willebrand disease type 3	89
Erythropoietin	Anemia in patients with chronic renal failure	90
Nonreplacement therapy		
Recombinant streptokinase and staphylokinase	Thrombolysis in acute myocardial infarction	91–93
Cytokines (IFNα, IL-2)	Hepatitis C, some cancers	94–96
Growth factors (GM-CSF)	Some cancers, neutropenia	97, 98
Receptors and antagonists (CD4, TNFR fusion protein)	HIV, multiple sclerosis	99, 100
Targeting MAbs (e.g., anti-IL-10 mAbs)	Systemic lupus erythematosus	101, 102

A simple view of the induction of an immune response to an Ag includes the endocytosis of the Ag by APCs that process the Ag and present the derived peptides onto MHC molecules to Ag-specific T lymphocytes. The T cells in turn activate Ag-specific B lymphocytes that differentiate into Ab-secreting cells or memory B cells. Because the development of neutralizing Abs is central to the failure of replacement therapy, the role of the "effector arm" of the adaptive immune system, i.e., B and T lymphocytes, in the alloimmune response to protein therapeutics has been explored in depth (10). However, the importance of cross-talk between protein therapeutics and APCs, a critical event upstream from the activation of Ag-specific T and B lymphocytes, has been underrated. In this review, we summarize the recent knowledge accumulated on the mechanisms of "Ag management" by APCs that should pave the way to the design of novel protein therapeutics with reduced immunogenicity.

Professional APCs are the first immune-sensitive depots for incoming exogenous protein therapeutics

Endocytosis and the processing of protein therapeutics. The initial step in the development of an immune response is the recognition of Ag by the professional APCs. At present, three major types of APCs have been described: dendritic cells (DCs),[3] macrophages, and B lymphocytes. Discriminatory handling of Ags by APCs occurs at different levels. At the cell surface, APCs are endowed with various endocytic machineries. Macrophages and DCs, in addition to receptor-independent macropinocytosis, have a plethora of surface-expressed receptors through which they capture Ags. In contrast, B lymphocytes are able to endocytose exclusively by Ag-specific, surface-expressed Igs (also known as B cell receptors or BCRs). The fate of the Ag within an APC may vary depending on the cell type. Thus, the content of lysosomal proteases and the amount and distribution of MHC II molecules determine the immunological fate of the endocytosed Ag (11). Indeed, as compared with B lymphocytes and DCs, macrophages have a very high endocytosing capacity, are rich in lysosomal proteolytic enzymes, and are low in MHC II content. Conversely, DCs and Ag-specific B lymphocytes are low in proteolytic capacity, rich in MHC II content, and induce primary immune responses by the activation of naive $CD4^+$ T lymphocytes (12).

Specific peptide motifs as well as carbohydrates of distinct type, linkage, and structure on protein therapeutics may serve as differential ligands for various endocytic receptors on APCs. The physical and functional characteristics of the endocytic receptors, such as their internalization efficiency, ligand-sensitive intracellular routing, associated activation, or inhibitory motifs, together with the type of APC, prove to be essential in governing the magnitude and nature of an immune response (13). Indeed, recent experimental evidence demonstrates that the type of receptor targeted on specific subsets of murine DCs governs the nature of the T cell response, which is generated following Ag administration (14). Although the subsets of DCs in mice and human are different, such an APC-restricted immunological fate of a given protein therapeutics in patients should be meticulously considered. Several opportunities lie in this direction of investigation, because both mouse and human DCs have various subdivisions based on surface marker phenotype that eventually differ in function (15, 16). Mouse DC subtypes are more evident than those in humans. Classically, a murine DC expresses the integrin CD11c along with the costimulatory molecules CD80, CD86, and CD40 and have moderate levels of MHCII. Interestingly, the T cell markers CD4 and CD8 are the differentiating markers of different subtypes of murine DCs. However, CD8 on DCs is in the form of an $\alpha\alpha$-homodimer rather than the $\alpha\beta$-heterodimer that is typical of T cells. The other two markers on murine DCs important for subtypes are the integrin CD11b and the lectin CD205. DCs populate different organs in mice differently. For example, DCs in the thymus are principally of the subtype $CD4^-CD8^{high}CD205^{high}CD11b^-$ while those in the spleen are in the order of $CD4^+CD8^-CD205^-CD11b^+ > CD4^-CD8^{high}CD205^{high}CD11b^- > CD4^-CD8^-CD205^-CD11b^+$ (15, 17, 18). In human beings, the DC subtypes have been less investigated. The major subtypes of human DCs that are identified are Langerhans cells, interstitial dermal DCs, BDCA-1^+ and BDCA3^+ myeloid DC subsets, and plasmacytoid DCs. The major difference with mouse DCs is the absence of CD8α marker, and this poses a major difficulty in comparing the subtypes of DCs found in both species. However, the Langerhans cell (marked by Birbeck granules, CD1a, and langerin) is considered as a subtype in both murine and human systems. Evidences of similarity between mouse and human DCs also came from studies with the human thymus wherein most DCs are $CD11c^+CD11b^-CD45RO^{low}$, express many "myeloid markers," and thus resemble mouse thymic $CD8^+$ DCs (19, 20).

[3] Abbreviations used in this paper: DC, dendritic cell; PEGylation, derivatization of A protein with polyethylene glycol; Treg, regulatory T cell.

Macrophages also are of different subtypes. In the mouse spleen macrophages are principally of three types: the CD169-positive metallophilic macrophages and the MARCO- or SIGN-R1-positive macrophages of the marginal zone and the F4/80-bearing macrophages of the red pulp (21). Another CD169-bearing macrophage present on the floor of the subcapsular sinus and in the medulla of lymph nodes in mice has been recently reported to present Ag in a unique way to migrating B cells (22). Whether such macrophage populations exist in human and are capable of Ag-presenting function remains to be confirmed. In another recent study, normal human dermis has been shown to contain immunostimulatory dendritic cells marked by CD11c and BDCA-1 while the macrophage population identified by CD163 and factor XIIIA showed much less immunostimulatory potency (23).

Importantly, the type of APC that will be involved in "managing" a given protein therapeutics will also vary with the route of administration of the drug. Blood-borne Ags are preferentially internalized and processed by splenic APCs (21), which is also the case for some i.v. administered protein therapeutics such as procoagulant factor VIII (A.-M. Navarrete, S. Dasgupta, S. Delignat, S. André, G. Caligiuri, Y. Meslier, Y. Repessé, B. Wootla, N. van Rooijen, A. Rice, J. Bayry, A. Nicoletti, S. V. Kaveri, and S. Lacroix-Desmazes, unpublished data). Conversely, i.m. or s.c. administered therapeutic proteins should be processed by APCs in peripheral tissues and be transported to draining lymph nodes for presentation to immune effectors. Thus, the role of a specific type of APC in relation to immune response against a particular protein therapeutics remains to be surveyed in a systematic manner, as has been done in case of several pathogenic Ags (24, 25).

Inflammatory signals in mounting an immune response. An APC expresses several surface and intracellular "inflammatory signal-sensing" molecules such as TLRs and Nod-like receptors. Activation of an APC via such sensory molecules and an associated local inflammatory environment has been reported to be necessary for a sustained and effective immune response (14). In particular, DCs in the context of inflammatory stimuli undergo a "maturation and activation program" that includes augmentation of surface costimulatory molecules like CD86 and CD80, enhancement of MHC II surface expression consecutive to a reduction in their ubiquitination, an increase in activation markers like CD40, suppression of endocytosis, secretion of inflammatory cytokines relevant for T cell programming, and enhancement in migratory properties (26, 27). Interestingly, in a recent clinical study by Devaraj et al., type 1 diabetes, which is a well-known proinflammatory state, has been shown to be associated with a significantly increased expression of TLRs on monocytes (28). The type 1 diabetes patients also showed significant augmentation of downstream elements of TLR signaling and release of the proinflammatory cytokines IL-1 β and TNF-α when compared with the control population. The release of proinflammatory cytokines strongly correlated with the increased expression of TLRs in the patient population that in turn strongly correlated with pathological signs like hemoglobin glycosylation (28). Proinflammatory cytokines like GM-CSF have been shown to augment MHCII and costimulatory molecules like CD86 and CD40 on monocytes (29).

Proinflammatory situations leading to the activation of APCs may develop for several reasons in the context of the administration of protein therapeutics. Protein therapeutics of human origin may per se provide signals via TLR triggering. Indeed, while the general notion is that self-proteins are not endowed with a capacity to activate APCs, recent reports demonstrate that various endogenous molecules target TLRs on APCs, leading to their activation (30–32).

Proinflammatory signals may also be provided by the systemic or local environment consequent to the pathological state of the patient. For instance, repeated bleeding in the context of hemorrhagic disorders causes release and accumulation of various TLR ligands and other proinflammatory molecules (33). Similarly, infectious and autoimmune conditions create a proinflammatory state that may strengthen and precipitate the adaptive immune response to a given protein therapeutics. Furthermore, several pathological conditions are associated with defective functions of naturally occurring $CD4^+CD25^+$ regulatory T cells (Tregs) (34). The fact that defective Tregs fail to control the functions of APCs and effector T cells provides an ideal platform for the development of an immune response to protein therapeutics. This is of particular relevance in cases where the endogenous counterpart of the protein therapeutics is absent and where protein-specific effector T cells have not been eliminated during ontogeny. Interestingly, efficient immune responses may also be elicited in the absence of an "adjuvant-like" danger signal. Thus, sustained Ag presentation, as is the case in the repeated administration of protein therapeutics, may lead to effective T cell activation and differentiation (35). Furthermore, the targeting of specific receptors on APCs even in the absence of proinflammatory signals may generate efficient Ab responses (36).

Prediction of immunogenicity

Correct prediction of the immunogenicity of protein therapeutics forms a challenge to the biopharmaceutical industry. Such predictions are approached in various ways involving modern immunological tools. Broadly, these approaches can be classified as described in the following paragraphs.

In silico approach. The field of bioinformatics has given rise to various computation-based approaches to understand immune responses toward any Ag. This field, generally termed "immunoinformatics," can be used to predict various elements in a protein that might precipitate an immune response in a recipient. Specifically, software programs like EpiMatrix developed by EpiVax, NetCTL developed by the Institute of Medical Microbiology and Immunology (Copenhagen, Denmark), and Bimas developed by the National Institutes of Health (Bethesda, MD) use matrix and artificial neural network-based algorithms to map, quantify, and compare T cell epitopes on proteins (37). In many of these algorithms, the strength of the binding of a T cell epitope to a MHC molecule is considered to be greatly important in the prediction of T cell-mediated immunogenicity (38). MHC molecules exhibit several allele-based heterogeneities and because the MHCII molecule is so essential in Ab-mediated immune response, it is judicious to search for the association of a particular haplotype of MHCII for immune response against a given protein therapeutics. However, this association has been found to be scarce or weak as evidenced in clinical literature (39–41). Nonetheless, with improved versions of algorithms that take into account differences in individual MHCs, attempts may be made in the future to link T cell-dependent Ab responses with MHC types, thereby allowing the screening of clinical cohorts for subjects who are at a higher risk of developing Abs against a therapeutic protein.

Clinical validation of an in silico-based prediction has been observed when a recombinant fusion protein was administered to human volunteers and an Ab-mediated immune response was studied (42). EpiMatrix software predicted promiscuous T cell epitope(s) on the protein and 37% of the subjects elicited Ab response. Indeed, memory T cell response was found in Ab-positive subjects only. Promiscuity of the predicted T cell epitope(s) was confirmed by representation of all common HLA alleles in Ab-positive subjects. Also, as predicted by the software, a particular MHC type (DRB1*0701/1501) was associated with the highest T cell and Ab response. In silico approaches thus form a significant tool in the repertoire of rational designs of protein therapeutics (43).

In vitro approach. In earlier times, in vitro testing for immunogenicity included assessment of the physicochemical properties of the protein drug in question as regards, for example, aggregation and degradation of the protein (9). However, due to recent advances in immunological assays, the current emphasis is to understand how the T cell compartment is affected by the protein therapeutics. This is done mainly in one of two ways: 1) fresh T cells from exposed patients or naive individuals are assessed ex vivo (44); or 2) memory T cells are expanded in vitro in the presence or absence of the said Ag or derived peptides. In these assays, parameters such as T cell proliferation capacities and T cell-specific cytokine release are followed (45). Other types of in vitro assays that have been developed to predict immunogenicity include MHC II binding assays (either cell based or soluble) for peptides (46, 47).

Animal models. Preclinical animal models for various diseases are available. In these animal models, effectiveness of the therapeutic proteins are evaluated. However, due to the potential difference between species, a drug that yields good result in an animal model might not necessarily yield the expected benefit clinically. Unexpected side effects like cytokine release syndrome and improper dosing have posed a great threat and necessitated re-evaluation in postregistration studies. As in the case of mAb treatment of chronic human disease, demonstration of the safety and efficacy of the prolonged administration of anti-TNFα mAb in rheumatoid arthritis patients realized the potential for the therapy (48). The species barrier poses a further problem for predicting a parameter like the immunogenicity of a therapeutic protein because, in general, human proteins would generally be immunogenic in an animal system. It is especially vital to consider the immunological differences between mouse and man before making any predictions (49, 50). To shorten the species barrier, drugs are now being tested in a nonhuman primate system (51). Along with the species barrier problem, the need to develop new disease models to understand drug efficacy (in terms of pharmacodynamics, pharmacokinetics, and toxicity) have been felt (52).

To test the immunogenicity of a protein drug in vivo, various transgenic animal models can be used. Conceptually, they can be in the lines of animal models developed and used for vaccine candidates. Such models for vaccine development include transgenic animals with human MHC molecules designed especially to bridge the gap between human and mouse systems (53, 54). Interestingly, direct correlations of T cell responses between those observed in humans and those in immunized transgenic mice have lead to the routine use of such models in preclinical studies (55–58). MHC transgenics are, however, not sufficiently suitable for studying a break in tolerance as observed in the case of therapy with human autologous proteins like insulin, IFNa2, and human tissue plasminogen activator, because they are not tolerized to such proteins in the course of their development. Hence, to study such cases, animals expressing the human proteins are better suited (59–61). To study the immunogenicity of proteins used in replacement therapy, animals genetically stripped of the analogous protein are better suited because they mimic the actual patient situation. These cases have been well documented in the hemophilia model (62, 63).

Although in vitro assays coupled to in silico prediction tools appear to provide ideal platforms for the prediction of immunogenicity in the future, animal models have their distinct advantages. They provide an ideal platform for understanding the effect of the route of administration and the elimination of protein therapeutics. Proteins administered via a s.c. or an intradermal route may be more immunogenic than when administered via other routes, probably by mimicking an active immunization process (64, 65). Although theoretically local administration may be considered least likely to be associated with immunogenicity, Ab production can still occur such as with intranasal synthetic salmon calcitonin for Paget's disease or aerosolized recombinant human DNA for cystic fibrosis (66, 67). The oral route offers an attractive alternative way to deliver protein therapeutics (68). However, the immunogenicity of a protein drug needs/remains to be deciphered when delivered orally.

It has been detailed in the literature how the development of Abs against a therapeutic protein affects its elimination, especially in the case of anti-idiotypic Abs developed against an administered monoclonal (69–71). Bearing in mind the intricate relationship between catabolism and immunogenicity, it is interesting to speculate that elimination mechanisms could provide vital clues for studying protein drug-mediated immune response.

Current approaches for reducing alloimmunization against protein therapeutics

General strategies for reducing the immunogenicity of protein therapeutics include the prediction of epitopes that bind to MHC II, T cell receptors, or BCR via an in vitro assay or in silico modeling, followed by their removal or alteration (72). These approaches may, however, be hampered by the loss of functional activity of the proteins. The success of these approaches may also be limited by the large diversity of the MHC II molecules, by the uncovering of cryptic epitopes, a phenomenon called "epitope spreading" that results in the creation of neoantigens, and by the generation of novel linear or conformational epitopes with unpredicted immunogenicity. An alternate approach for immunogenicity reduction is to sterically block Ab binding by derivatizing the protein with polyethylene glycol (PEGylation). Beyond the steric effect it provides, PEGylation can also decrease immunogenicity by promoting solubility and permitting less frequent dosing (73). PEGylation has been used successfully to minimize the immunogenicity of therapeutic enzymes such as arginase, asparaginase, and purine nucleoside phosphorylase (74, 75). In the case of some therapeutics like IFNα2a, although PEGylation has reduced immunogenicity, the loss in function of ~93% has been a major concern (76). Sialylation of proteins can also reduce immunogenicity. Sialic acid-conjugated T glycopeptides were found to be less immunogenic than the nonsialylated ones (77).

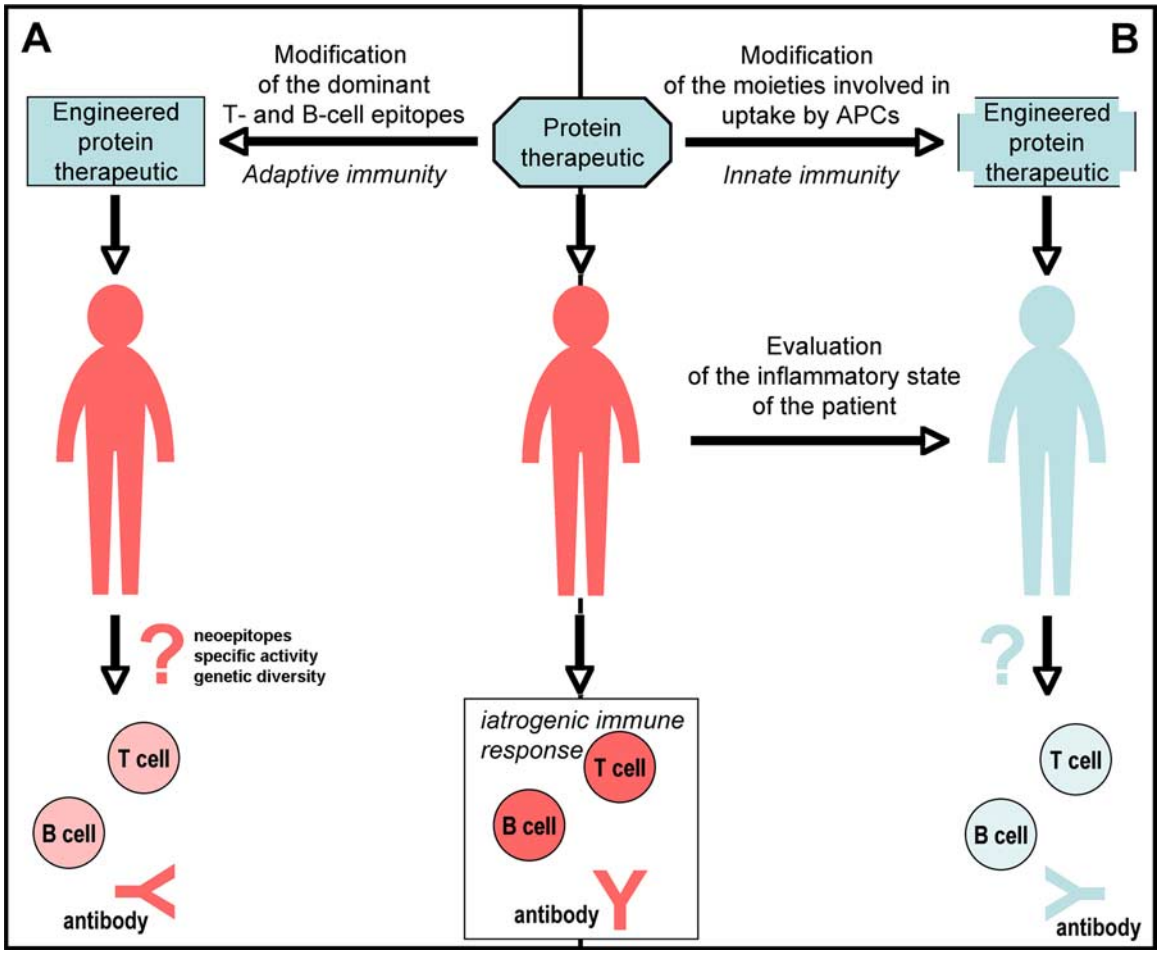

FIGURE 1. Prevention of immune responses to protein therapeutics involves auditing the intrinsic immunogenicity of the protein as well as the inflammatory status of the patients. *A*, Classical approaches for reducing immunogenicity of protein therapeutics consist in identifying and altering the immunodominant epitopes that are implicated in the recognition of protein therapeutics by the adaptive immune system, i.e., BCRs and TCRs. Such approaches are however hampered by the risk of decreasing the specific activity of protein therapeutics and the possible creation of novel immunogenic moieties, along with difficulties associated with patients' genetic diversity. *B*, We suggest a shift in the paradigm. Ideal treatment with protein therapeutics should encompass evaluation of the inflammatory state of the patient and its appropriate management (anti-inflammatory treatment, use of alternative nonimmunogenic protein therapeutics, or delay in the use of the protein therapeutics), in synergy with minute and targeted alterations of the specific features of protein therapeutics that are involved in their early recognition by cells of the innate immune system (i.e., APCs).

The use of rational design and engineering of protein therapeutics is being used to balance protein activity and immunogenicity (44). In a study by Tangri et al., rationally designed and engineered erythropoietin, with epitopic regions modulated for HLA affinity, were found to be both bioactive and less immunogenic (78).

Reducing the immunogenicity of protein therapeutics by altering the "management" of protein therapeutics by APCs

We propose several strategies for consideration to intervene at the level of management of protein therapeutics by APCs to reduce their iatrogenic immunogenicity (Fig. 1). Inflammatory mediators are known to induce maturation and activation of professional APCs (79). In addition, an exacerbated expression of endocytic receptors and internalization of Ags have been demonstrated on APCs from patients with diverse pathological backgrounds (80, 81).

The inflammatory status of the patients may be monitored by analyzing the levels of circulating cytokines and the polymorphisms of immune genes (e.g., TNF, IL-10, IFN-α). Introduction of protein therapeutics in such patients at a high risk of developing neutralizing Ab responses could be delayed or avoided and alternative drugs administered. Thus, activated factor VII could be used instead of factor VIII in patients with hemophilia A who are at elevated risk of developing anti-factor VIII inhibitory Abs. An alternative approach could be to neutralize the inflammatory environment of the patients before or at the time of administration of protein therapeutics. This may be achieved by using therapeutic mAbs to inflammatory cytokines, including TNF, or immunosuppressive agents (e.g., steroids). Importantly, although such approaches can have usual side effects, they would not only reduce the activation state and endocytic capacity of APCs and impart anergy to T cell effectors but also restore normal functions of Tregs (82). Further, tailoring the dose and frequency of administration of therapeutic mAbs to inflammatory cytokines should minimize adverse reactions in patients.

Identification of specific motifs on protein therapeutics that facilitate interactions with professional APCs may lead to the development of structurally modified molecules that are "invisible" to immune sentinels and devoid of iatrogenic immunogenicity. Thus, therapeutic factor VIII, both that of recombinant origin or that purified from normal human plasma, has been found to contain two mannose-terminating glycans that allow

specific endocytosis by DCs through mannose-sensitive receptors (83). Quenching mannose residues or removing glycosylation sites on factor VIII by site-directed mutagenesis within limits that are compatible with functional integrity should lead to the conception of less immunogenic hemostatic drugs. The possibility for revealing previously cryptic epitopes upon the removal of glycan chains, thus creating neoantigens, remains a possibility/concern however. Alternatively, as demonstrated in the factor VIII-von Willebrand factor model, the use of a protein therapeutics in the presence of its physiological chaperon may reduce its endocytosis and presentation by APCs (84) to immune effectors.

Disclosures

The authors have no financial conflict of interest.

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