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Review

Immunogenicity risk mitigation of therapeutic proteins with translational immunogenicity, analytical characterization, and regulatory insight

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ABSTRACT

The immunogenicity of therapeutic proteins remains a key challenge, leading to early and late-stage clinical failures, posing a significant hurdle in the development of safe and efficacious biopharmaceuticals. This review highlights the main categories of factors influencing the immune response-mediated impacts of biotherapeutics: 1) patient-related factors, 2) product-related factors, and 3) administration-related factors. It provides a comprehensive overview of these immune response-mediated impacts, ranging from the development of anti-drug antibody (ADA) responses, injection site reactions (ISR), and injection site pain (ISP). Using immune response-mediated impacts as a focal point, the review discusses tools and strategies that can be used to evaluate the potential critical quality attributes (pCQAs) of therapeutic proteins as they have an impact on immunogenicity. These tools include various immunogenicity assessment assays spanning *in silico, in vitro, ex vivo, in vivo* animal models, and clinical tools. It also highlights a comprehensive repertoire of analytical characterization methods. Emphasis is placed on the importance of combined stage-appropriate use of these tools to minimize the risk of immunogenicity. Additionally, regulatory guidance, forums, and consortium landscapes are outlined to inform immunogenicity risk assessment strategy for therapeutic proteins. Ultimately, this integrated comprehensive immunogenicity testing strategy aims to advance the field of immunogenicity risk assessment to develop safe and efficacious protein therapeutics.

Introduction

The immunogenicity of therapeutic proteins has been a constant challenge in developing safe and efficacious drug products. Despite the rise in approvals of therapeutic protein drug products, the attrition rate of biotherapeutics also remains high[1,2]. While the reasons for the high attrition rates may vary, product safety and efficacy consistently emerge as central themes.[3] Failures occurring as a result of efficacy or safety can often be attributed to the immunogenicity of the drug product.[1] Addressing this challenge requires an integrated approach that combines meticulous analytical characterization with thorough and relevant translational testing for effective immunogenicity risk evaluation. While immunogenicity is a concern across a broad range of therapeutic modalities like cell and gene therapies, this review specifically focuses on

List of abbreviations: ADA, Antidrug antibody, or anti-drug antibody; ISR, Injection site reaction(s); HSR, Hypersensitivity reactions; ISP, Injection site pain; pCQA, potential Critical Quality Attributes; CQA, Critical Quality Attributes; HLA, Human Leukocyte Antigen; MHC, Major Histocompatibility Complex; PTMs, Post-translational modifications; CMs, Chemical modifications; HCPs, Host cell proteins; CDR, Complementarity-determining regions; PD1, PD-L1, Programmed Cell Death Protein 1, Programmed Death Ligand 1; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; PEG, Polyethylene glycol; mAb, Monoclonal antibody; APCs, Antigen presenting cells; CRS, Cytokine release syndrome; PBMCs, Peripheral blood mononuclear cells; DC, DDC, Dendritic cells, Dermal dendritic cells; MAPPs, MHC-associated peptide proteomics; MoDC, Monocyte derived dendritic cells; FRET, Förster resonance energy transfer; TPI, Therapeutic Product Immunogenicity; CFSE, Carboxyfluorescein diacetate succinimidyl ester; HuALN, Human artificial lymph node; 3Rs, Replace, reduce, and refine; FDA, Food and drug administration; EMA, European Medicines Agency; ICH, International Council for Harmonization; VAS, visual analog scale; NRS, numeric rating scale; VRS, verbal rating scale; HADA, high-affinity ADAs; SEC, Size exclusion chromatography; QC, Quality control; PK, Pharmacokinetic; ABIRISK, Anti-Biopharmaceutical Immunization: Prediction and Analysis of Clinical Relevance to Minimize the Risk; EIP, European Immunogenicity Platform; AAPs, American Association of Pharmaceutical Scientists; PRCA, Pure red cell aplasia; GLP, Good Laboratory Practices.

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protein-based therapies. Emerging modalities could have distinct immunogenicity mechanisms that are outside the scope of this review.

Clinical immunogenicity is given significant importance, with reverse translation emerging as a primary tool for managing this concern.[3] Reverse translation involves using immunogenicity data collected during clinical studies to assess the impact of critical quality attributes (CQAs). While it is evident that reverse translation is crucial during clinical stages, it can be a powerful tool if implemented earlier during drug selection and development in conjunction with forward translation, termed mixed translation.[4]

Using early reverse translation through the perspective of mixed translational approaches necessitates a thorough understanding of various factors influencing immunogenicity and the nuances thereof. It also necessitates addressing the limitations and risks of different analytical and immunogenicity tests used in evaluations. It is crucial to carefully and rigorously document the CQAs as understood at the time of all drug batches used in preclinical and clinical studies. This practice can include analyzing the ADA incidence with changes in chemical modifications such as glycosylation, oxidation, or sequence variants across clinical lots,[3] which could inform a specification setting strategy. Reverse translation can be used to justify more relaxed specifications for certain CQAs, such as subvisible particles, in cases where only high thresholds are known to break immune tolerance, as evidenced for specific products.[32] Additionally, while admittedly *in vivo* studies have limited translatability to humans with respect to immunogenicity, immune monitoring in non-GLP and GLP toxicology studies can serve as early indicators of clinical risk, especially when performed in relevant species. Beyond later-stage pre-clinical applications, reverse translation can also be leveraged during early discovery, where epitope mapping and *in silico* immunogenicity predictions guide early sequence optimization to minimize immunogenic potential.[26,27] Taken together, although complex in nature, these applications can demonstrate the utility of this mixed translation approach as a cross-development decision-making tool, especially when timely monitoring and correlation analysis are conducted.

The regulatory heritage behind immunogenicity assessment adds an additional layer of complexity that should be factored into decision-making. While examples of the mixed translation approach have been successfully used in improving the treatment response,[5] integrating reverse translation within a mixed translational framework could further support immunogenicity mitigation. The approach could enable potentially high-throughput, high-fidelity immunogenicity predictions, and support the broader shift toward reducing reliance on *in vivo* models, in alignment with emerging regulatory trends.[4]

This review aims to provide a comprehensive spectrum of tools for assessing the immune consequences of various factors impacting the immunogenicity of therapeutic proteins. The intentional broad reach of immune response-mediated impacts to include injection site reactions (ISR) and injection site pain (ISP), along with clinical immunogenicity (ADA development), aims to capture the complex interplay of factors that could potentially affect immunogenicity risk. The discussion here is anchored on key categories of factors influencing the immunogenicity of therapeutic proteins. The review proposes an integrated approach towards immunogenicity assessment at various stages tailored to specific factors. A key emphasis is placed on understanding that no single assay can capture the entirety of the immune response or the breadth of analytical factors involved in the assessment. This limitation becomes particularly apparent when considering the nature of the readout and the stage of testing. Finally, the review summarizes collective recommendations from regulatory authorities, immunogenicity forums, and consortia to facilitate informed, stage-appropriate decision-making regarding the immunogenicity risk assessment of therapeutic proteins.

The immune impact of factors affecting immunogenicity

The immune system orchestrates sophisticated coordinated responses via various cells, signaling chemicals such as cytokines and chemokines, and plasma proteins, amongst other elements, to combat foreign molecules and abnormal molecules of self-origin.[6] This elicitation of immune responses to pathogens and vaccine therapies is immensely beneficial. Conversely, aberrant immune reactions to self-antigens in autoimmunity, as well as against peptide and protein therapeutics, are undesirable and are referred to as immunogenicity.[3,7–9] The desired and undesired immune responses present myriad physiological and pathological implications. Despite the differing nature of these implications, the fundamental contributors to immune responses remain consistent. Thus, to identify and mitigate the immunogenicity of therapeutic proteins, an understanding of the key contributors to immune responses, both in relation to human immunology and the drug-related factors, is essential.

While various factors relating to the immunogenicity of therapeutic proteins have been captured in detail elsewhere,[10–13] a brief insight into the landscape of the immune impacts from these factors paves the way to better inform the tools for comprehensively assessing immunogenicity. Table 1 summarizes examples of these patient, product, and administration-related factors, highlighting the complexity of immunogenicity (ADA) and associated responses such as injection site reactions (ISRs) and injection site pain (ISP).

Patient-related factors

The human immune system comprises consistent key players across individuals, yet exhibits high variability influenced by various heritable and non-heritable factors. While a healthy individual's immune system can remain relatively stable over long periods of time (up to six years in case of yearly sampling done by Brodin et. al.), excluding acute infection-dependent spikes, significant inter-individual variability exists in immune cell populations.[14] Table 1 highlights the variability in immune cell populations of seemingly healthy individuals. This highlights that the immune system employs complex adaptive strategies to balance the increased or decreased counts of immune cells in outlier individuals.[14] While not an immunological organ by itself, blood serves as a reliable medium for circulating immune cells and can offer a practical, but admittedly incomplete, snapshot of the immune activity.[14] This variability can lead to a lack of correlation between larger human populations in clinical trials. Understanding these baseline compensatory factors is crucial for interpreting immunogenicity data and improving the prediction capabilities of clinical trial outcomes. Additionally, the genetic influence of HLA haplotypes, which is another patient-related factor, plays an important role in determining the immune responses against the protein therapeutics of different populations. The variability in immune responses can be further affected by age and sex, as seen in the examples in Table 1.

Similarly, the disease status of a patient can significantly influence their immunogenicity risk. The frequency of immune response to the same drug may be a function of the disease it is targeting, as seen from the examples in Table 1. For instance, the variability of immunogenicity to infliximab and adalimumab when used to treat rheumatoid arthritis, Crohn's disease, and ankylosing spondylitis indicates a disease-specific break of immune tolerance.[15] While each of these diseases differs in clinical presentation, their underlying cause of inflammation forms a framework for comparison between the incidences of immunogenicity as a function of disease context. Moreover, patients with a longer duration of the disease and a higher activity of the disease are more likely to develop ADA against the treatment being administered, as observed in the infliximab and adalimumab cases.[16] While these data lack nuance regarding individual patient factors, their consideration is important for understanding immunogenicity risk. ADA development could be influenced by concomitant use of immunosuppressive agents (*e.g.*, methotrexate or azathioprine), prior treatments the patients have been under, and genetic predispositions such as HLA haplotypes, particularly HLA-DQA1*05 for infliximab and adalimumab.[17] Notably,

the immunogenicity data reported in Table 1 may include studies involving both monotherapy and combination therapy with the above-mentioned combination of factors. This could further complicate direct comparisons across disease contexts. While it is challenging to disaggregate these influences in the available literature, the data presented is the best estimate that can be used for comparative purposes across different disease settings. Overall, the above considerations underscore that the immune system can mount a variable immune response dependent on the state of distress, which can contribute to the immunogenicity of therapeutic proteins.

While post hoc monitoring of these factors is common for immunogenicity risk assessments, systematic studies can aid in identifying and subsequently using these factors for informed patient selection to minimize risk.[10] Such studies could help identify markers and stratify patients based on risk of immunogenicity to achieve the best treatment outcomes.

Administration-related factors

Except route of delivery, the associated immunogenicity risk of other administration-related factors have not been studied. This review aims to touch upon other administration factors such as the site of administration, volume, viscosity, and needle gauge used, and their non-ADA immunological impacts such as ISP, ISRs, and hypersensitivity.

While the subcutaneous route by itself is not necessarily more immunogenic than its intravenous counterpart,[7,18] subcutaneous injection sites such as the abdomen, thigh, and arm show significant differences in pharmacokinetic profile depending on the molecule being administered, though no concomitant immunogenicity data exists.[19,20] Although there are differences in the pharmacokinetic profile, these injection sites also display variable levels of ISP and volume-dependent ISRs[18] (Table 1). While no direct correlation between injection site pain and immunogenicity has been observed in therapeutic protein literature, vaccine studies have indicated some link between ISP or reactogenicity and immunogenicity.[21,22] Further clinical research is needed to explore this relationship, as studying ISP in pre-clinical models presents challenges. It is also important to note that the path of therapeutic protein transfer into systemic circulation varies with different injection sites, influenced by the number of lymph nodes encountered and the residence time due to adipose tissue differences.[23,24] These factors could potentially impact the overall immunogenicity, though no current data confirms this.

Similarly, selecting the appropriate needle gauge (higher gauge) and geometry is crucial to minimize injection site trauma and subsequent immune response.[23] Volume, viscosity, and site of administration together have an impact on ISRs and ISPs as seen in Table 1.

The above-mentioned administration-related factors can significantly impact the ISP associated with protein therapeutic injections. Additional research possibly correlating ISP to reactogenicity and immunogenicity in the therapeutic protein field is needed to understand the complex interactions between these variables to optimize administration strategies.

Product-related factors

Various product-related factors play a key role in driving immunogenicity (ADA development), ISR, and ISP (Table 1). Some of these factors have been classically well understood, such as the "humanness" of the antibody. As a result, the first-generation murine antibodies were quickly replaced by chimeric (human constant region and non-human variable region), humanized (non-human CDR), and fully human antibodies over the years.[25] This shift is reflected in the Antibody Society database, which tracks all approved and 'under regulatory review' antibodies.[25] The database shows that, in contrast to 19 monospecific chimeric antibodies, 75 monospecific humanized antibodies, and 65 monospecific human antibodies approved or under review, only 7 monospecific murine antibodies have undergone evaluation.[25] While the limited number of murine antibodies captured by the database is unsurprising due to an expected high xenogeneic immune response, the data nevertheless highlight the industry's strategic shift toward reducing immunogenic risk. Additionally, factors such as T-cell and B-cell epitopes present on the antibody must be systematically evaluated through *in silico* and *in vitro* methods to predict, identify, and, if needed, engineer them as part of a potential mitigation strategy.[26,27]

The target and function of the drug are other key factors that can affect the immunogenicity profile of a protein therapeutic. For example, drugs targeting CD20 on B-cells can lower immunogenicity, likely due to the killing of B-cells, and some checkpoint inhibitors are known to drive up the immunogenicity, possibly due to the release of the "brake", as evidenced in Table 1.[7,8,17] However, it is important to note that many checkpoint inhibitors have "standard" immunogenicity profiles with less than 10 % ADA rates (*e.g.*, Pembrolizumab: 1.80 %, Avelumab: 4.10 %, Cemiplimab: 1.30 %, and Durvalumab: 2.90 %)[8,17]. One contributing factor may be that checkpoint inhibitors are often administered as a late line of therapy. In such cases, patients more often than not have undergone prior immunosuppressive treatments such as chemotherapy or radiotherapy.[28] These interventions can contribute to the dampening of the immune response, potentially resulting in lower observed ADA rates.[28,29] However, as checkpoint inhibitors get increasingly used in earlier treatment lines, sans the immune suppression features of other treatment lines, the immunogenicity profiles may shift. While the above-mentioned factors are patient-related factors, they can heavily influence the impact of how the product influences immunogenicity. This indicates that the mechanisms leading to the breakdown of tolerance towards the protein sequence and modifications are complex and not solely attributed to the drug's mechanism of action. In summary, while the target and function of a biotherapeutic drug play a critical role in determining its immunogenicity profile, multiple other factors likely contribute to this phenomenon, as discussed in this review.

The less well-understood product-related factors are the protein's biophysical and biochemical instabilities resulting in immunogenicity, both independently and as a combination of the two. Systematic studies delineating the effect of immunogenicity of each of these factors have been challenging, as inducing one type of CQA might result in a concurrent increase in another type of CQA (Table 1). It is therefore important to explore the nuances of these instabilities, particularly focusing on their implications for immunogenicity. For example, despite their varying characteristics, aggregates are typically classified by size: soluble oligomeric aggregates (<100 nm), submicron particulates (100 nm–1000 nm), subvisible particulates (1 μm–100 μm), and visible particulates (>100 μm).[30,31] Evidence for each size bin driving immunogenicity is well-documented in the literature (Table 1). While size-based links are frequently studied, contrasting and ambiguous data suggest that other aggregate properties may also play a role in immunogenicity development. Aggregates can be reversible or non-reversible, self-associated or covalently bound, and may or may not have chemical modifications (CMs). Similarly, CMs and post-translational modifications (PTMs) may be accompanied by aggregation as seen in the examples from Table 1. It is therefore important to study these variables both as independent factors and in combination to understand their impact on the immunogenic potential of therapeutic proteins. A comprehensive analytical and immunogenicity characterization approach may be able to delineate the product-specific impact of these factors. Finally, the threshold of higher-order aggregates required to induce immunogenicity may be much greater than what is perceived as acceptable by the field currently, depending on the protein therapeutic in question(32). While this is not always well understood in the course of the development of the biotherapeutic, having this knowledge can significantly append the timelines for development and change the outlook on the impact of biophysical instabilities of protein therapeutics.[32]

A strategy to comprehensively look at the product-related factors

Table 1
Factors affecting immunogenicity of therapeutic protein.

Category	Key points	Key examples	Immune impact	Citations
Patient related factors				
Variability of immune responses	High inter-individual variability in the immune system	The frequency of B cells, expressed as a percentage of total lymphocytes, ranges from 4–69 %. Monocytes and NK cells, reported as a percentage of total PBMCs, range from 0–49 % and 0–59 %, respectively. CD4+ and CD8+ T cells, calculated as percentages of total CD3+ T cells, range from 22–93 % and 6–65 %, respectively. Total T cells (CD3+) comprise 13–90 % of PBMCs in seemingly healthy individuals.	This can lead to a lack of correlation between smaller clinical trial data when scaled to a larger human population.	[14]
HLA haplotypes	The presence of certain haplotypes can become a key immune response driver	DRB1*0701-DQA1*0201 haplotype is predominant in Caucasian populations and is associated with higher incidences of ADA against interferon β	Higher ADA	[35]
		HLA-DQB1*05, HLA-DRB1*01, and HLA-DRB1*07 were identified as protective/ tolerizing alleles and resulted in patients with fewer ADA incidences against adalimumab	Lower ADA	[11,36]
		The HLA-DRB1*03 allele was more prevalent in patients with anti-adalimumab and anti-infliximab antibodies	Higher ADA	[11,36]
		Patients with HLA-DQ-05 and HLA-DRβ-11 showed a correlation with ADA development against anti-TNF antibodies	Higher ADA	[11]
Age	Age contributes to the overall variability of the immune system in different populations	In elderly patients, pro-inflammatory TNF-α may be upregulated, leading to a state of low-grade inflammation, while many in this demographic also experience lymphopenia, resulting in downregulated immune responses	Heightened or depressed immune responses	[14]
Sex	Peak and intensity of immune response varies in men and women	Immune response peaks at day 1 in men and day 3 in women, based on transcriptional signatures that have been shown to correlate with subsequent IgG responses. Women also generally exhibit stronger immune responses due to the upregulation of humoral immunity induced by estrogen	Sex-dependent variability in the peak and intensity of immune responses	[14]
Disease context	The frequency of immunogenicity of the same biologics drug may be a function of the disease it is targeting	Infliximab has been recorded to have ADA incidences ranging from 10–51 % when treating rheumatoid arthritis, 6–61 % when treating Crohn's disease, and 15–29 % when treating ankylosing spondylitis. Similarly, for adalimumab, this rate is 0.7–87 %, 0.04–17 %, and 31 % for rheumatoid arthritis, Crohn's disease and ankylosing spondylitis, respectively.	Disease-specific variations in ADA	[15]
		A correlation between anti-infliximab antibodies and anti-adalimumab antibodies was found in patients with longer disease durations and baseline disease activity observed before treatment.	Disease duration can affect ADA incidences and titers	[8,15,16,37]
Administration-related factors				
Route of administration	SC *versus* IV route immunogenicity	Immunogenicity of therapeutic proteins administered subcutaneously is either comparable to or only moderately higher than that of intravenous administration.	When moderate immunogenicity was observed with the subcutaneous route, it did not adversely affect safety and efficacy.	[7,18]
Site of administration	Abdomen *versus* arm *versus* thigh	Pharmacokinetic differences were observed with no correlation to immunogenicity. Injections in the thigh are consistently more painful compared to the abdomen.	Site of administration can cause higher ISP	[18]
		The path of therapeutic protein transfer into systemic circulation varies with different injection sites, influencing the number of lymph nodes encountered and the residence time due to adipose tissue differences.	Lymph node encounter could potentially impact the incidence and titer of anti-drug antibodies (ADA), though no current data confirms this.	[23,24]

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Table 1 (*continued*)

Category	Key points	Key examples	Immune impact	Citations
		Patient related factors		
Volume of injection in the context of the site of injection	Site-specific volume can impact ISP and ISR	In one study, the abdomen was able to tolerate a 3 mL injection without any pain, while the injections in the thigh were painful, even at 1–1.5 mL volume of 0.9 % NaCl post single administration.	Site-specific volume tolerability with respect to ISP	[38]
		In another study, maximum volumes of up to 10 mL were injected into the abdomen and 5 mL in the thigh. The higher volume had a slightly higher perception of pain. The scores were deemed clinically insignificant, higher volume was also accompanied by wheal/ urticaria and erythema formation but resolved within 2 hours.	Larger volumes can be associated with transient ISRs and clinically insignificant ISPs	[39]
Volume and viscosity of the drug product administered	High volume in combination with high and low viscosity	Higher volumes were more painful when administered in the abdomen when the viscosity of the liquid was lower (1.1 cP) but were less painful when the viscosity was higher.	High ISP with high volume and low viscosity of the drug product administered	[39]
Needle gauge and tip geometry	Needle gauge and tip geometry related to ISP	Higher gauge needles with 5-bevel tips cause less pain.	A higher gauge needle can result in a lower ISP	[23,40,41]
		A reduction in needle gauges from 23 to 31 decreased the perception of pain.	A higher gauge needle can result in lower ISP	[23,40]
		Product-related factors		
Protein sequence	"Humanness" of a protein impacts immunogenicity	Murine antibodies administered to humans elicited a strong human anti-mouse antibody (HAMA) response, resulting in compromised safety, faster clearance, and low efficacy. The first-generation murine antibodies were quickly replaced by chimeric (human constant region and non-human variable region), humanized (non-human CDR), and fully human antibodies.	More “humanness” results in a lower likelihood of ADA and resulting complications in efficacy, safety, and immunogenicity.	[25,42,43]
	Presence of T-cell and B-cell epitopes	The presence of T-cell epitopes can result in higher immunogenicity through effector T-cell function or lower immunogenicity due to Treg function. Presence of B-cell epitopes can similarly impact immunogenicity.	Possibility of tolerogenic or immunogenic response	[8,26,44]
Immunogenicity to endogenous molecules	Sequence and non-sequence related factors driving immunogenicity against endogenous molecules	Antibody-mediated pure red cell aplasia (PRCA) following rHuEPO administration due to development of neutralizing anti-erythropoietin antibodies to endogenous proteins	Possibly fatal transfusion-dependent anemia in patients with chronic kidney disease	[33]
		GLP-1 analogues like Liraglutide, semaglutide, and exenatide resulting in immunogenicity against endogenous GLP-1	Impaired endogenous GLP-1 activity, mostly observed with greater divergence in sequence from endogenous GLP-1	[34]
Function of the drug and its target	Biotherapeutics that target the immune system cells and cytokines can affect the immunogenicity profile of the drug	Rituximab, a monoclonal antibody that targets CD20 on B-cells in non-Hodgkin lymphoma and autoimmune diseases, leads to antibody-dependent cellular cytotoxicity (ADCC) of B-cells, resulting in lower incidences of antidrug antibodies (ADA).	Lower ADA as a result of the drug target	[7]
		Checkpoint inhibitors targeting PD1, PD-L1, CTLA-4, and other molecules can exhibit high ADA incidences, particularly in monotherapies (*e.g.*, Atezolizumab: 30–48 %, Ipilimumab: up to 26 % and even more so in combination therapies (*e.g.*, Nivolumab-Ipilimumab: 24–38 % ADA). Depletion of Treg tolerance against the drug epitopes was hypothesized as a cause for their enhanced immunogenicity. Not true for all checkpoint inhibitors, though.	Higher ADA as a function of the drug	[8,17]

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Table 1 (*continued*)

Category	Key points	Key examples	Immune impact	Citations
		Patient related factors		
Biochemical instability: Post-translational and chemical modifications	PTMs and CMs impact the immunogenicity of biotherapeutics	PEGylation, the PTM itself can mask immunogenic epitopes and drive immunogenicity down	Lower immunogenicity	3,45
		Preexisting antibodies to PEG can be the drivers of compromised efficacy and adverse reactions	Preexisting ADAs to PEGs drive up immunogenicity.	11,46,47
		Glycosylation can either increase or decrease the immunogenicity of therapeutic proteins	Shielding of the epitope lowers ADA, while preexisting antibodies to non-human glycans can drive up ADA	3,48
		CMs like oxidation, deamidation, and isomerization have been implicated in the immunogenicity of biotherapeutics. Isomerization increased the immunogenicity of Elotuzumab. Oxidation of therapeutic factor VIII increases immunogenicity.	Increased immunogenicity as a result of CMs with or without underlying aggregation.	49–52
		Celiac disease: deamidated variants of wheat proteins had better T-cell recognition in comparison to the chemically unmodified protein	Increased immunogenicity from the disease example as a result of CM.	53
Biophysical instability	Aggregation impacts the immunogenicity of therapeutic proteins	Aggregates have been linked to longer residence times in the subcutaneous space when administered via the S.C. route, with a size-based higher accumulation in lymph nodes and the spleen during *in vivo* murine studies.	Longer residence time due to aggregation resulted in immunogenicity	54
		The increase in retention time in the case of precipitated antibodies delayed the onset and the magnitude of the immune response	Longer residence time due to aggregation resulted lowering immunogenicity response	55
		Native oligomeric aggregates exhibit high immunogenicity compared to subvisible non-native counterparts, regardless of administration route	Native aggregation is a driver of immunogenicity	56
		Submicron particles were more immunogenic than oligomeric or subvisible aggregates in a murine antibody study	Submicron particles were the driver of immunogenicity	57
		Preclinical and clinical evidence linking subvisible particles to immunogenicity	Subvisible particles were the driver of immunogenicity	58–60
		Roche's study demonstrated that chemical modifications (oxidation) combined with aggregate size could result in neo-epitope formation, leading to immunogenicity of monoclonal antibodies	CMs in combination with aggregation drive immunogenicity	50
		Recombinant mouse growth hormone aggregates adsorbed on glass and alum particles maintained more of their native structure than those produced by mechanical or freeze-thaw stress. This structural preservation led to a higher degree of immune tolerance disruption in the mouse model	Native aggregation drives immunogenicity	61
		The threshold of higher order aggregates required to induce immunogenicity may be much greater than what is perceived as acceptable by the field currently depending on the protein therapeutic in question	High threshold of subvisible particles may be required to induce immunogenicity	32
Impurities	Host cell proteins (HCPs) can result in immunogenicity	Sandoz biosimilar clinical trial for human growth hormone, produced in *Escherichia coli*, which contained residual *E. coli* proteins due to inadequate HCP detection assays	Presence of HCPs was correlated with higher immunogenicity	62
	Extrinsic and intrinsic particulates can result in immunogenicity	Protein aggregates adsorbed to particulates, especially metal particles, increased antidrug antibody incidences	Extrinsic and intrinsic particulates increase immunogenicity and the chances of capillary clogging	63
		Protein aggregates adsorbed to glass particles heightened immunogenicity due to an adjuvant-like response.		61
		Adsorption of therapeutic protein mAb1 on glass, silicone oil, or aluminum hydroxide acted as adjuvants in terms of the immunological response elicited in mice		64

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Table 1 (*continued*)

Category	Key points	Key examples	Immune impact	Citations
		Patient related factors		
Formulation factors	pH and buffering species can impact ISP and ISR	The presence of citrate buffer and sodium chloride, in a concentration-dependent manner, has been linked to increased ISP	pH and buffering species were implicated in ISP	[65–67]
		The pH of buffers significantly affects tissue damage and pain, with pH levels above 9 causing tissue damage and levels below 3 causing pain upon injection	Extreme pHs can cause tissue damage and ISP	[68]
		Maintaining a physiological pH or minimizing buffer concentration (ideally around 10 mM) is recommended to prevent ISP	pH and buffer concentration play a key role in ISP	[68]
	Osmolarity impact ISP	A limit of 600 mOsm/kg for subcutaneous and 1000 mOsm/kg for intravenous products is advised to minimize discomfort	Osmolarity can drive up ISP	[69]
	Excipients can impact hypersensitivity and ISPs	Excipients, such as polysorbates (PS), have also been associated with ISR and hypersensitivity reactions	Excipients can increase ISR and hypersensitivity	[3,67,70]
		Products with polysorbates like recombinant erythropoietin and darbepoetin increased hypersensitivity reactions		[71]
		Polysorbates in adalimumab and Ustekinumab caused urticaria and other hypersensitivity indicators		[72]
		PS20 has been linked to ISRs with alirocumab, and PS80 with evolocumab.		[70]
		Cross-sensitization to PEGs can cause polysorbate hypersensitivity		[46]
		PEGs themselves, used as excipients in some biologics, warrant close monitoring for anaphylactic reactions and anti-drug antibody formation.		[46]
		Mannitol, another commonly used excipient, has been noted to cause anaphylactic incidences, though not specifically in the context of protein therapeutic administration		[46]
Coadministration with immunomodulatory agents and other antibodies	Immunogenicity can be affected by the coadministration of therapeutic proteins with immunomodulatory agents and other antibodies	The use of methotrexate and prednisone has been shown to reduce the ADA incidences of various TNF inhibitors	Coadministration of antibodies with other therapeutics can drive the immunogenicity up or down.	[73]
		Rituximab usage as an anti-CD20 antibody suppresses B-cell expression and has also been used in modulating the immunogenicity of biotherapeutics		[8]
		Azathioprine resulted in immunogenicity reduction when administered with infliximab or adalimumab		[17,74]
		Independently administered Nivolumab had an ADA response of 12 %, which jumped to 24–38 % when administered with Ipilimumab. Similarly, Ipilimumab, independently administered, had an immunogenicity of 1.1 % that increased to 5.4 % upon coadministration with Nivolumab.		[75]

also includes product process-related impurities. These impurities include host cell proteins (HCPs), endotoxins, CpG oligodeoxynucleotides, intrinsic and extrinsic particulates, among other molecular patterns and foreign species that can induce an immune response (Table 1). Each of these can cause immune implications at varying levels and may or may not have compendial limits. The stringent control strategy to minimize these impurities can mitigate the immunogenicity risk profiles of the therapeutics.

A combination of formulation-related factors, sequence, and biophysical instability can cause immunogenicity to endogenous proteins, which is the most devastating consequence of immunogenicity (Table 1). The most notable example of this is that of neutralizing antibodies attacking endogenous erythropoietin following the administration of rHuEPO, resulting in potentially fatal antibody-mediated pure red cell aplasia (PRCA).[33] A relatively recent example of this can be found in the immunogenicity against endogenous Glucagon-Like Peptide-1 (GLP-1) with the administration of GLP-1 analogs.[34] However, the low antibody titers of transient and non-neutralizing antibodies do not result in loss of efficacy or compromised safety.[34] An increased risk might be presented with a greater sequence divergence or neoepitope formation, as in the case of the exendin-4 from the Gila monster.[34] These cases underscore the importance of careful control over product-related factors, especially with respect to significant immune responses against endogenous protein.

Analytical characterization tools

Various analytical assays have been utilized to identify and study the

potential critical quality attributes (pCQA) of protein therapeutics and their impact on immunogenicity. Table 3 lists some of these analytical characterization techniques along with the immunogenicity assays performed in conjunction. The use of multiple analytical characterization techniques along with patient-related factors in the clinic has been proposed as an approach for reverse translation.[3] Using a similar approach with multiple analytical characterization techniques and multiple immunogenicity assessment assays can be viewed as a proactive translation of protein therapeutics in a comprehensive manner. However, an industry-wide need for standardization of both immunogenicity assessment tools and tools for analytical characterization are required for meaningful advances in this strategy of proactive translation. These analytical characterization assays can be broadly classified into tools for biophysical analysis, biochemical analysis, and quality control assays as tools for pCQA identification.

Biophysical analysis tools

Tools for biophysical analysis are a vital part of identifying and characterizing pCQAs associated with the structure of the protein in biotherapeutics. One or more of these tools have been used in various immunogenicity investigations to identify the extent of aggregation of the protein, changes in the secondary structure that could result in protein instability, and the type of aggregate (Table 3). While each of these assays provides a unique perspective on the above-mentioned criteria, each of these assays has shortcomings or limitations that can be bridged by using a comprehensive approach of using other assays along with the readout. For example, turbidity measurement, a simple and non-destructive technique offering insight into insoluble global aggregation, has low sensitivity and is prone to interference from bubbles or non-proteinaceous particles. If used in conjunction with techniques like size exclusion chromatography (SEC), where the larger particles are filtered out as a result of sample preparation, would provide information about the soluble aggregates missed out by turbidity analysis. Furthermore, layering particle counting techniques like flow imaging microscopy, light obscuration, and/ or membrane microscopy would provide a readout on the particle size population and, in some cases, on the morphology of these particles. Similarly, more techniques could be layered on this strategy to build a more comprehensive analytical framework for characterizing the various types of aggregations.

Many analytical techniques used to characterize higher-order aggregates focus primarily on size and extent of aggregation but often fall short in distinguishing between inherent proteinaceous aggregates, extrinsic particulates introduced from the environment, and intrinsic particulates generated during the production process. For example, light obscuration, a tool with the most regulatory heritage is not adept at identifying the translucent or aspherical proteinaceous aggregates resulting in undercounting these inherent particulates.[76] Some instruments such as the Halo labs Aura have the ability to have a fluorescence component with the use of dyes like Thioflavin T (ThT) to identify proteinaceous aggregates.[77] However, ThT can have non-specific concentration-dependent aggregation or adsorption to non-proteinaceous surfaces which could potentially lead to inaccurate counts.[78] Raman microscopy offers some advantages in this area of high-fidelity aggregate identification. However, this technique suffers from low throughput and potential overlap of protein spectra with that of the polycarbonate membranes at 2930 cm^{-1}[79] Thus, a concerted effort in this field would be required to build high throughput, high fidelity tools for aggregate identification. These tools could then potentially be adopted by regulatory agencies for product characterization and by researchers in the early stages of product development to minimize immunogenicity risk arising from protein instability due to structural changes.

Another key factor, as mentioned above, is that evidence exists for each size bin of aggregation driving immunogenicity. For instance, Fathallah et al. found that native oligomeric aggregates exhibit high immunogenicity compared to subvisible non-native counterparts, regardless of administration route.[56] Kijanka et al. observed that submicron particles were more immunogenic than oligomeric or subvisible aggregates in a murine antibody study.[54] Additionally, Ahmadi et al., Barnard et al., and Ratanji et al. provide both preclinical and clinical evidence linking subvisible particles to immunogenicity.[58–60] It is key to note that each study used a different analytical characterization approach for aggregate quantitation. While the proteins used in each of these studies could have independent effects on immunogenicity post-aggregation, the analytical characterization strategies could result in a potential gap in knowledge on what truly may be driving immunogenicity. An industry-wide approach was attempted for *in vitro* immunogenicity assays and their use in an attempt to contextualize and standardize early-stage immunogenicity data.[80] A similar consensus on the use of these analytical tools, based on the limitations of each of these tools, may be useful to contextualize data and its correlation to immunogenicity.

Biochemical analysis tools

Biochemical analysis and characterization tools are used to assess the chemical alterations to a protein's structure that could impact the immunogenic potential of the protein. Charge profiling tools such as cation exchange chromatography (CEX) and charged isoelectric focusing (cIEF) can provide some level of insight into the possible PTMs or CMs causing charge alterations. Charge-altering modifications, such as deamidation, increase acidic species, while C-terminal lysine retention increases basic species. Modifications like isomerization of aspartic acid to isoaspartic acid, which are linked to immunogenicity, typically do not alter the isoelectric point due to no net charge change unless intermediates like succinimide are detected, which can increase basic species in these assays.[49,81] Therefore, while useful as research tools, these would likely benefit from the supplementation with peptide mapping and other mass spectrometry tools, where identification of site-specific modifications can be conducted. These tools and associated best practices are reviewed in detail elsewhere.[82–84] Similarly, chromatographic tools such as hydrophobic interaction chromatography (HIC) and protein A chromatography provide insights into the global oxidation of antibodies.[85–88] Oxidation of methionine residues in the Fc region of the antibodies reduces the hydrophobicity of the antibodies and results in earlier elution in a high salt gradient of HIC.[85,87] In Protein A chromatography, oxidized residues of monoclonal antibodies can reduce the protein A binding affinity, resulting in faster elution.[86,88] While each of these techniques provides insights into global oxidation, they are not able to differentiate between methionine and tryptophan oxidation and are not able to pinpoint the specific residues as sites for oxidation. Using a mass spectrometry-based approach, using peptide mapping enables users to overcome the shortcomings of these techniques.[85] It is also important to view the modifications in the context of other pCQAs such as aggregation. For example, Roche's study demonstrated that chemical modifications (oxidation) combined with aggregate size could result in neo-epitope formation, leading to immunogenicity of monoclonal antibodies.[50] Thereby highlighting a need for a comprehensive assessment to enable the development of low immunogenicity products.

Quality control tools

Quality control tools in this manuscript are the tools used to identify and characterize the impurities found in protein therapeutics and ensure a consistent quality of product. Endotoxin, host cell proteins, extrinsic and intrinsic particulates, among others, can all contribute to the immunogenicity profiles of products. Some CQAs, like endotoxin, have clearly defined compendial limits, associated endotoxin tests, and are consistently used in assays assessing the immunogenicity of therapeutic

proteins.[89,90] As mentioned earlier, HCPs and other potential innate immune response modifiers (IIRMIs), on the other hand, do not have well-defined limits but pose a significant safety and immunogenicity risk at likely a similar level as endotoxins.[91] Verthelyi et al. demonstrated that even trace levels of multiple IIRMIs, like CpG DNA, can synergistically activate the immune system and break tolerance to therapeutic proteins and, even more seriously, to their endogenous proteins.[92] This underscores the need for more comprehensive impurity profiling. While significant efforts are devoted to high-quality sample purification for the removal of HCPs, some HCPs, like phospholipase B-like 2 (PLBL2), can co-purify in antibody therapeutics produced in Chinese hamster ovary (CHO) cells.[93] The use of a proteomics-based analysis via mass spectrometry with concomitant *in vitro* immunogenicity tests can establish the risk profile for the pCQA.[93]

The analysis for both extrinsic and intrinsic particulates has a significant overlap with the biophysical analysis of proteinaceous higher-order aggregates. The compendia-defined tools for the analysis of these particulates are light obscuration (LO), membrane microscopy, flow imaging microscopy, and visual inspection for particles in visible ranges. The key limitation for all of these techniques in the categorical identification of extrinsic and intrinsic particulates is the inability of these methods to identify the nature and chemical makeup of the particulates that could distinguish them from the inherent proteinaceous particles. As mentioned, techniques such as LO can result in gross undercounting of particles with a low contrast, which not only include proteinaceous particles but also plastic particles.[76] Techniques such as flow imaging microscopy and membrane microscopy offer higher resolution but, again, fail to distinguish between contaminants *versus* the inherent proteinaceous particles. For a thorough characterization of such contaminants, techniques that enable the building of spectra libraries for various contaminants, like Raman microscopy, can be used.[94] However, Raman microscopy is limited when it comes to metal analysis due to weak Raman scattering, where should the source of the contamination be the aluminum from crimp caps of vials, additional techniques would be required in combination to do a comprehensive analysis. Overall, similar to the other analysis tools, QC tools require an all-encompassing analysis strategy considering the limitations of each tool to ensure a minimized immunogenicity risk of protein therapeutics.

Tools for preclinical and clinical immunogenicity assessment

Immunogenicity assays are critical tools employed to evaluate and potentially predict the immunogenicity of therapeutic proteins, both in terms of injection site reactions and humoral responses. Fig. 1 outlines the various methodologies employed to access the immunogenicity of therapeutic proteins. Each of these methodologies is used in a stage-appropriate manner throughout the development of therapeutic proteins and has varying degrees of predictive capacities or can be used as a measure of relative immunogenicity.[120]

In silico predictions

In silico prediction tools have been widely utilized in early phases of drug product development to down-select candidates with a low immunogenicity risk profile. Proteins, when digested into peptides in antigen-presenting cells (APCs), can be presented on Major Histocompatibility Complex (MHC) molecules. These presented peptides have the ability to potentially engage T-cells and induce an associated immune response. T-cell engagement and the generation of ADA in response are correlated, making *in silico* predictions a critical upstream tool in

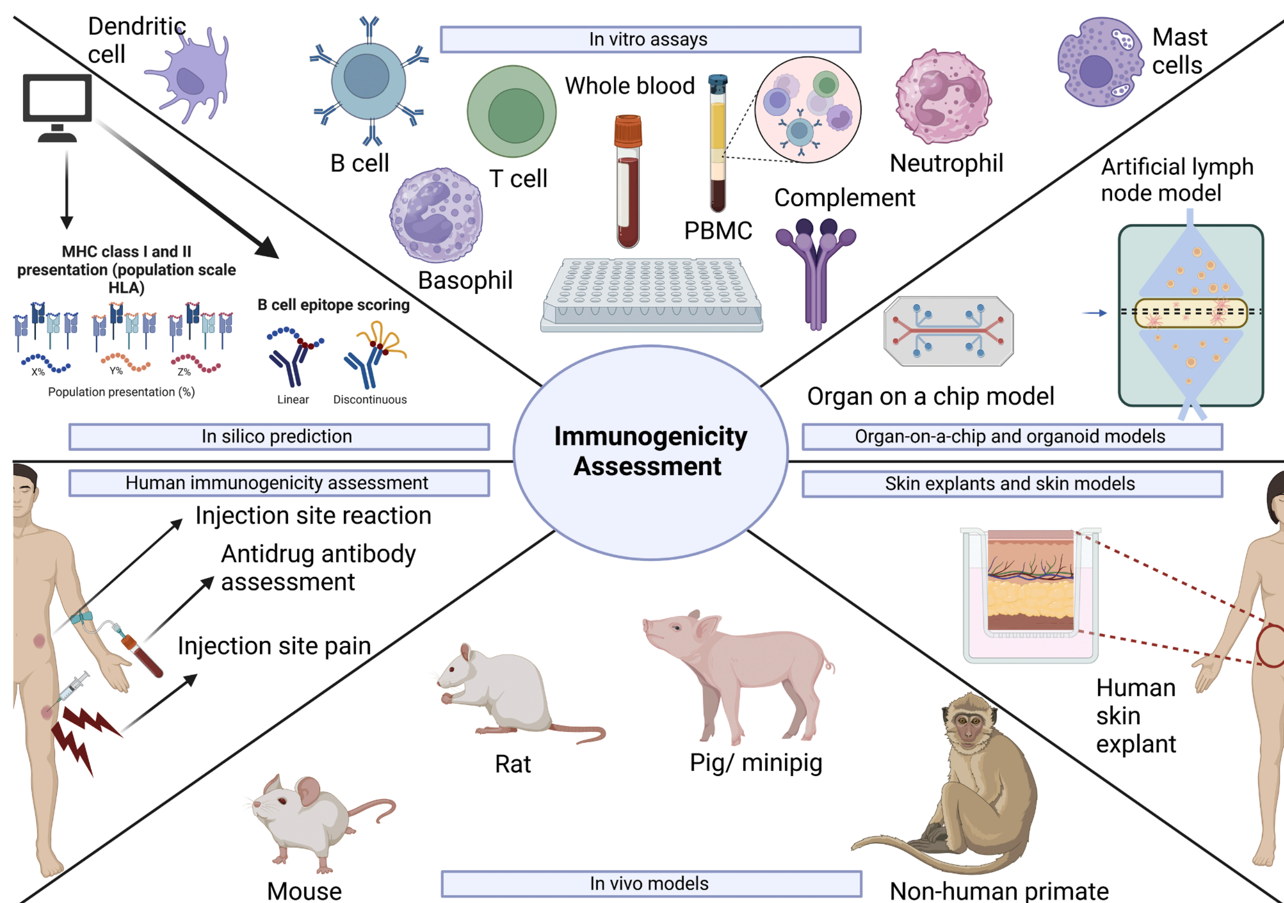

Fig. 1. Methodologies employed to assess immunogenicity.

immunogenicity risk assessment and minimization. Some notable tools that enable the prediction of T-cell epitopes are as follows: ISPRI, (Immunogenicity Screening and Protein Re-engineering Interface) is a combination of multiple tools used in sequence where EpiMatrix (for epitope mapping) is used first, followed by ClustiMer (epitope cluster mapping) and JanusMatrix (to account for Treg specific epitopes), while also providing a potential ADA prediction.[121] Some other tools include NetMHCII and NetMHCIIpan,[122,123] Tepitope.[124] SYFPEITHI,[123,125] Epibase,[8,126] MCHPRED,[8] MHC2PRED,[8] RANKPREP,[8] SVRMHC,[8] and iTope.[8] Many of these T-cell prediction tools use the Immune Epitope Database (IEDB) sponsored by NIAID. IEDB also has useful epitope identification and visualization tools that can be used to run rapid analyses of the immunogenic potential of the protein molecules.[127] While T-cell epitope identification alone can be a powerful asset in immunogenicity risk assessment, it also enables the subsequent deimmunization of therapeutic proteins by modifying immunogenic epitopes, as long as such changes do not alter function. T-cell epitope editing has been employed successfully to reduce immunogenicity potential by using tools such as OptiMatrix.[121]

While many tools have been developed for T-cell epitope prediction, B-cell epitope prediction continues to be challenging not only *in silico* but also *in vitro*. T-cell epitopes are linear and can range from 12 to 29 amino acids (sometimes up to 30 amino acids), typically, as a result, making their detection relatively straightforward. While B-cell epitopes could sometimes be linear, a large majority of the epitopes are conformational or discontinuous in nature.[123,128] The conformational nature of these epitopes makes them challenging to predict.[123,128] Despite this challenge, some tools have been developed to predict B-cell epitopes: 1) PREDITOP[123,129] 2) PEOPLE[123,130] 3) BepiPred[123,131] 4) BCPREDS[123,132] 5) CEP[123,133] 6) PEPITO[123,134] 7) EPITOPIA[123,135] 8) EPSVR.[123,135] A detailed review of these methods is provided elsewhere.[123]

While *in silico* epitope prediction tools can be useful in upstream drug development to optimize for immunogenicity, the reliability and accuracy of these tools continue to be an evolving target.[8,44,123,128] Although relatively advanced, T-cell epitope prediction tools are known for overestimating immunogenic epitopes.[123] The conformational nature of B-cell epitopes makes their prediction using *in silico* tools limited in comparison to T-cell epitope tools and mostly unreliable.[123,128] The utility of these tools currently does not extend very well beyond early development. Some efforts have been made to predict aggregation propensity using *in silico* tools.[126] These tools, however, may not be able to do so with high fidelity in late-stage development as aggregation and its impacts on immunogenicity in the presence of other parameters are not well understood. Parameters such as PTMs and CMs that a molecule might undergo independently or in combination with aggregation, in post-discovery stages are not well accounted for by these tools.[8] In short, *in silico* tools currently fail to meet expectations in late-stage research where immunogenicity can be influenced by process and formulation parameters.[8]

In vitro assays

In vitro assays serve as an indispensable tool for evaluating the immunogenicity of therapeutic proteins, both in the early and late stages of development. These methods enable the investigation of various CQAs by modeling specific immune pathways. Despite their limitations, these assays provide high throughput and relatively reproducible means of evaluating immunogenicity, especially in the context of human cells.

Whole blood assay

Whole blood assays have primarily been utilized to study cytokine release syndrome (CRS), however, their use in evaluating the immunogenicity potential of CQAs of protein therapeutics is gaining momentum.[80,136] Whole blood assays do encounter challenges with long-term experiments, especially the decrease in the viability of the cells after 48 hours.[137] While proinflammatory cytokines like IL-1β, IL-6, IFN-γ, and TNF-α, from the supernatant, can be used to measure the response of immune cells in blood post-stimulation, each therapeutic protein can have a unique signature with regards to CRS prediction.[80,136] For example, FcγRIIIa-dependent IFN-γ release is associated with the risk of CRS with regard to IgG1 therapies.[136] This signature may vary for other biotherapeutics. The whole blood assay has the major advantages of being a high-throughput method, where no additional processing of the blood samples is required, and the ability to capture the population heterogeneity with the HLA haplotypes. However, the short time of operation between the sample collection and conducting the assay due to the loss of viability of cells, and the high variability in the responses makes the assay a less frequent tool for the analysis of immunogenicity.[137,138]

PBMC stimulation assay

PBMC stimulation assays are commonly utilized to predict CRS and to assess the impact of CQAs, like aggregation, on the immunogenicity potential of therapeutic protein.[119,139,140] This assay, in its unmodified form, involves stimulating isolated PBMCs from the buffy coat of spun-down whole blood samples with the test articles for 20–48 h, followed by collection of the supernatant and a multiplexed cytokine release analysis.[80,140] Donor-to-donor variability of what constitutes whole PBMCs can make the interpretation of results difficult.[14]

Developments in PBMC-based assays have led to the measurement of responses of specific cells, like in the case of the DC assays or the T cell assays described below. PBMC assays have the advantage of being high-throughput in nature and can act as a launchpad for further testing using other means. The PBMC-based assays also hold advantages over whole blood assays due to their higher stability and relative ability to culture these cells for up to a week.

DC-based assays: activation, migration, internalization, presentation, and MHC-associated peptide proteomics (MAPPs)

Dendritic cells are the professional APCs of the immune system and mediate T-cell-dependent immunity. As a result, dendritic cell-based assays have been widely used to assess the immunogenicity potential of therapeutic proteins. As APCs, all the key steps to antigen presentation have been captured by currently available assays. These steps include DC migration in the direction of the immune insult, DC activation measured by cytokine release, and increased cell surface markers,[141] DC internalization of fluorescently labeled therapeutic proteins, and the subsequent presentation of the internalized immunogenic peptides. The DC internalization and presentation process and its implications on immunogenicity are reviewed in detail elsewhere.[142]

A monocyte-derived DC (MoDC) assay developed on behalf of the ABIRISK Consortium, which measured various DC maturation endpoints, was able to detect the effect of aggregation on DC activation.[141] The assay found that antibodies like infliximab that aggregated upon induction of stress resulted in high markers of DC activation, like cytokine (IL-1β, IL-6, IL-8, IL-12, and TNFα), chemokine (CCL3 and CCL4) upregulation, and release along with cell surface marker upregulation.[141] Whereas Natalizumab, a monoclonal antibody with low aggregation propensity, caused no DC activation, and adalimumab and rituximab aggregates induced a moderate response via DC activation.[141] In another study, a DC activation assay was utilized (activation markers CD40 and IL-12) along with chemokine receptor CXCR4 levels to delineate DC migration potential in response to protein therapeutics.[143] The data generated as a part of the DC activation assay was used in conjunction with a DC migration assay, where migration occurred in the gradient of the therapeutic protein and chemokine ligand as an indication of the immunogenic potential of the protein.[143] The assay format found a strong positive correlation (Pearson $r = 0.87$) between the *in vitro* total response index and the highest percentage of clinical immunogenicity, indicating a strong clinical immunogenicity predictive potential.[143] While not studied specifically for this purpose, migration

assay can also have applicability in studying immunogenicity induced by aggregates, as demonstrated by the propensity of DCs to migrate toward immune complexes.[144]

Unlike DC activation and migration, DC internalization assays require the therapeutic protein entity to be fluorescently labeled. While 'always-on' fluorescence labels can be utilized for internalization assays, they do not provide specific information on whether the protein is internalized or adsorbed on the surface.[145] Fluorescence as a result of an interaction indicative of internalization is a more specific measurement for this kind of assay. A Förster resonance energy transfer (FRET) based quenching pair approach and a PHrodo dye-based approach were evaluated simultaneously to study internalization. The measured signals emerged based on the arising fluorescence as a result of cleaving the FRET quencher pair or change in pH, respectively.[145] The assay developed provided a high throughput platform for evaluating the immunogenicity risk of monoclonal antibodies.[145]

MHC-associated peptide proteomics (MAPPs) is a DC-based assay that is used in combination with the *in silico* methods to verify the immunogenic sequences identified by the algorithms.[146,147] A version of the MAPPs assay was used to study the effect of aggregation on the HLA presentation.[148] The findings indicated that a marked increase in peptide presentation by HLA-DR molecules was observed for aggregated proteins when compared to unstressed proteins.[148] This indicates the versatility of the assay can be used to study CQAs beyond screening for immunogenic antibody sequences in early-stage discovery and development.[148] It is, however important to note that the assay alone cannot distinguish between immunogenic and tolerogenic epitopes and likely requires subsequent T-cell assays to assess the ability of peptides to trigger a T-cell response.[147] The assay also depends on the protein concentration, which in turn influences uptake and presentation, which can lead to a risk of over-predicting immunogenicity in comparison to what is observed in the clinic.[147]

Given the current optionality that DC-based assays provide, they can be used in a comprehensive toolkit manner for assessing immunogenicity, where DC is the APC for the protein therapeutic. Using one or a combination of the assays can prove to augment immunogenicity risk assessment at various stages of development.

DC-T cell assay

Dendritic cell-T cell co-stimulation assay is another *in vitro* assay that attempts to capture cell-mediated immunity/ immunogenicity of protein therapeutics and the CQAs that might govern their immune profiles.[80,137,149,150] In this assay, dendritic cells, the APCs, are differentiated from PBMC-isolated monocytes and are exposed/ pulsed with the protein therapeutic.[8,80,137,149,150] The cells are allowed to mature, which leads to the upregulation of HLA(8,80,137,149,150). This is followed by the removal of the protein therapeutic, followed by the co-culturing of protein therapeutic exposed mature DCs with autologous CD4+ T-cells or CD8+ T-cells depleted- PBMCs for 7–10 days.[8,80,137,149,150] The final readout can include a cytokine analysis for the upregulation of cytokines like IL-2 and interferon-gamma (INFγ) and/or assessment of proliferation by incorporating Carboxyfluorescein succinimidyl ester (CFSE) stain.[8,80,137,149,150]

As the assay is used widely for immunogenicity risk assessment by the industry, as per a survey conducted by the Therapeutic Product Immunogenicity (TPI) community,[80] some efforts are being made to validate the assay to increase the sensitivity and have a consensus on the assay specifics. In one such assay validation attempt, the DC–T cell restimulation assay demonstrated an 83 % accuracy, correctly identifying the immunogenicity risk of 20 out of 24 antibodies when compared to clinical immunogenicity rates.[151] Of the four misclassified antibodies, two were false positives (daratumumab and pembrolizumab) and two were false negatives (brentuximab and atezolizumab).[152] This resulted in an assay accuracy of 83 %.[152] However, in another study, modifying the assay by adding a restimulation step to increase sensitivity did not improve the detection capabilities of immunogenic antibodies.[151] Instead, false-positive responses were observed for an antibody with low clinical immunogenicity, an issue not observed with the single-stimulation format of the assay.[151] Therefore, while the DC-T cell co-stimulation assay can be a powerful assay for the assessment of immunogenicity, it should be coupled with the use of other immunogenicity assays to account for the false-positive and false-negative readouts. It should also be noted that the many steps of the assay make it a tedious tool for screening, and the assay must be utilized at an appropriate stage in the immunogenicity risk assessment of therapeutic proteins.

T cell assays

The immunogenicity of protein therapeutics can be highly dependent on T-cell responses, especially those from CD4+ T cells. Evaluation of various processes leading to T-cell dependent immunogenicity can be broadly classified into 1) cytokines released from T-cells upon stimulation from therapeutic proteins, 2) T-cell activation by cell surface markers, and 3) evaluation of T-cell proliferation in the presence of peptide-MCH complex.

Cytokine released by T-cells in response to therapeutic proteins can be characterized using ELISA or ELISpot measurements.[153] Where ELISA provides information on the cytokines released in the supernatant, whereas the information regarding the number of cells producing cytokines is provided by ELISpot.[153] The assay can be performed independently or in conjunction with other T-cell-based assays mentioned above.

T-cell activation and proliferation assays are both widely used to predict clinical immunogenicity risk. T-cell activation assay involves the upregulation of receptors like CD134 (OX40) and CD137 (4–1BB) in response to therapeutic proteins of varying degrees of clinical immunogenicity rates. In one such study, the assay was found to be highly predictive of clinical ADA rates of 13 out of 14 therapeutic proteins.[154] The assay was able to correctly classify antibodies into 3 categories of high (> 30 % ADA), intermediate/high (15–30 % ADA), and low (< 15 % ADA) rates of immunogenicity, barring one antibody, adalimumab.[154] Within the study, a T-cell proliferation assay was also conducted using anti-Ki67 staining of the Ki67 protein, a marker of cell proliferation.[154] However, the T-cell activation assay was deemed to be more sensitive and has a higher specificity to predict the risk of immunogenicity of therapeutic proteins tested.[154] Other methods of conducting T-cell proliferation include Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, which measures the dilution of the dye by half as a result of each cell division, and a lesser utilized method of pulsing [^{3}H] thymidine into the DNA of dividing cells.[140,151,153] Limitations in the sensitivity of the T-cell proliferation assay were noted in another study where CD8+ depleted cultures of PBMCs were used with the CFSE labeling method.[151] The assay was only able to correctly predict 4 out of 8 highly immunogenic antibodies.[151] A version of the assay termed as *in vitro* comparative immunogenicity assessment assay (IVCIA) was utilized by J. R. Cohen et al., 2024 to test the effects of aggregates on diseased state donors and healthy donors, where the magnitude of response was highest in allergy patients.[32]

The limited sensitivity and specificity warrant the careful selection and combination of T-cell-based assays, despite their utility, with other platforms/ assays for accurate predictions of therapeutic protein immunogenicity. Another consideration is the lack of factoring in the Treg bystander response that can considerably alter the immunogenicity as elicited by T-cell-based assays.[155] T-cell responses that can take into account this Treg bystander effect can likely lead to improved evaluation of T-cell immunogenicity of proteins in the future.

B-cell assays

B-cells play an important role in both T-cell-dependent and T-cell-independent immune activation and are the primary drivers of an antibody response.[120] However, *in vitro* B-cell assays are currently both overlooked and not well developed to predict ADA risk in response to

therapeutic protein stimuli.[120] The major issue arises when the *in vitro* formats of B-cell assays are not able to capture B-cell class switching from IgM to IgG antibodies, which are the main category of ADA.[120] Current strategies in measuring and mitigating B-cell responses are limited to *in silico* linear epitope prediction,[123,156] discontinuous epitope prediction using electron-microscopy density map and X-ray crystallography,[156] T-cell-dependent and T-cell-independent stimulation using PBMC,[120,157] PBMC-derived B-cell assays,[120,157] and B-cell line assays.[158] The most commonly used format of the assay involves using an ELISpot/FluoroSpot-based assay to assess the memory B-cell response to pre-existing and potentially cross-reactive antibodies against protein therapeutics.[120] While an assay format may be developed for non-specific antigen internalization and activation of B-cells, the amount of antigen required in this process is 1000–10,000 times more as compared to the specific antigen uptake upon binding with the B-cell receptors.[159] As a result, an assay format that assesses the response of naïve B-cells in terms of ADA formation may be challenging to incorporate into the *in vitro* assay repertoire.

Mast cell degranulation

Mast cells have been implicated in IgE and non-IgE mediated injection site reactions (ISR) and hypersensitivity reactions (HSR) of protein therapeutics.[67,160] Mast cells are most abundantly found in the skin in the dermal layer, and stimulation-induced degranulation can result in the release of inflammation-inducing substances like histamine, tryptase, TNFα, IL-6, etc.[160] Mast cells have various surface receptors, including the IgE-binding FcεRI receptor, which can trigger degranulation when bound by IgE or directly by specific proteins.[160] Additionally, non-IgE-mediated activation and degranulation can occur through receptors such as toll-like receptors (TLR), protease-activated receptors (PARs), and Mas-related G-protein coupled receptor member X2 (MRGPRX2).[160] Using the mast cell degranulation assay can inform on the extent of ISR or HSR that a protein therapeutic might cause. Mast cells can either be isolated from skin tissue, from CD34+ myeloid progenitor cells, or cell lines like HMC-1 and LAD-2 can be used for conducting *in vitro* degranulation assays.[161] Common strategies involve measuring products of degranulation like beta-hexosaminidase, tryptase, or histamine. MRGPRX2 *in vitro* β-arrestin assay has also been utilized for detecting β-arrestin recruitment post-stimulation by test articles.[162] While no explicit examples of the usage of *in vitro* mast cell degranulation for studying protein therapeutics in the literature, Anakinra (Kineret) and its association with ISR and mast cell degranulation is well-studied in patients.[67]

Complement activation assay

Complement activation assays have been used to study the immunotoxicity of protein therapeutics, especially type II and type III hypersensitivity reactions.[163] No direct links can be drawn between complement activation and the development of ADAs. However, when protein therapeutics combine with ADA to form immune complexes, they can then activate the complement pathways, creating a cascade of inflammatory responses.[163–165] This can ultimately result in tissue damage.[163] As per the June 2023 guidance by the FDA: Nonclinical Evaluation of the Immunotoxic Potential of Pharmaceuticals, complement activation assessment has been recommended.[166] Commercially available whole blood assays can be used for this purpose. One such assay utilizes activation products of C3 and C5, C3a-des-Arg, and C5a-des-Arg, respectively, using ELISA.[167] Other whole blood assays may look at split products of C3a, C5a, SC5b-9, and/or Bb.[168–170] 1. An increase in the presence of one or more of these markers is an indication of complement activation.[163] While the tool can be valuable in evaluating early innate immune system responses, bridging gaps left by DC-T-cell assays or PBMC assays, complement activation evaluation alone cannot capture the immune system complexity, thereby having limited predictive capabilities.[171]

Organoid models and organ-on-a-chip

Organoids such as lymph node-mimicking models have witnessed an advance in recent years.[137,172] These models can be used to study the immune responses of lymph nodes to stimulation by therapeutic proteins. The Human artificial lymph node (HuALN) model is one such model that utilizes primary human immune cells and stromal cells in the format of a bioreactor.[173] Cytokine release, cell activation, and potentially antibody secretion can be monitored using this model.[173] The HuALN model has been used to study the immunogenicity of bevacizumab and adalimumab aggregates generated by inducing various stresses.[96] A Th-1 and proinflammatory immune response was noted using the model with heat-induced bevacizumab aggregates, while no response was seen for adalimumab aggregates.[96] However, since no correlation was drawn to clinical incidences of immunogenicity, such tools hold value in terms of relative measurements only. As multiple cells are being stimulated in the context of an organoid system, the data generated could be relatively complex to interpret for such models. Appropriate benchmarking against other *in vitro* immunogenicity assays and clinical immunogenicity data could make these models a useful potential tool in immunogenicity risk assessment.

Organ-on-a-chip models of the lymph node, while currently not utilized in the study of immunogenicity risk of therapeutic proteins, can potentially be a simpler version of the organoid model for evaluating antigenicity.[174,175] The lymphoid follicles (LFs) model in particular shows promise.[174] This model contains B-cells that express Activation-Induced Cytidine Deaminase (vital for class switching) and also demonstrates activation-induced plasma cells.[174] In the evaluation of the commercial influenza vaccine, the model produced plasma cells, anti-hemagglutinin IgG, and cytokine secretion akin to the human response to the vaccine.[174] Such a model could therefore be evaluated for studying protein therapeutics and the potential instabilities of the therapeutics that induce immunogenicity.

Skin models and skin explants

Skin explants and skin models are envisioned to help capture the immunogenicity risk of biotherapeutics administered subcutaneously, especially concerning ISRs. While skin models are easier to procure and have longer viability than skin explants, they have a limitation with their design as it stands currently. The current availability of models can be classified as 1) reconstructed human epidermis (RHE) models, that contain only normal human epidermal keratinocytes (*e.g.* SkinEthic™ RHE (Episkin, Lyon, France), EpiDerm™ (MatTek Corporation, Ashland, MA), EpiCS® (Phenion, Düsseldorf, Germany)), 2) Pigmented reconstructed human epidermis (RHE) models that contain normal human epidermal keratinocytes and melanocytes (*e.g.* MelanoDerm™ (MatTek Corporation, Ashland, MA), MEL/001 (StratiCELL, Gembloux, Belgium), and EpiCS®-M (Phenion, RHPE, Düsseldorf, Germany)) and 3) human skin equivalent (HSE) models that contain normal human epidermal keratinocytes with fibroblast cells (*e.g.* EpiDermFT™ (MatTek Corporation, Ashland, MA), Phenion® FT (Henkel AG & Co. KgaA, Düsseldorf, Germany) and T-Skin™(Episkin, Lyon, France)).[176] In all the different models, there is an apparent lack of adipose cells of the hypodermis, the site of the subcutaneous injections, thus making it impossible to test injection site reactions upon actually making the subcutaneous injections.[176] The other key lacking feature of the models is the absence of key immune cells of the skin, like Langerhans cells, dermal dendritic cells, T-cells, macrophages, B-cells, etc., grossly limiting the interpretation of the injection site reactions to protein therapeutics.[176] The models also currently lack skin appendages like hair follicles and sweat glands, and do not have any evidence of vasculature either.[176] Even so, the EpidermFT™ skin model has been utilized to test the critical quality attributes of protein therapeutics like aggregation and process-related impurities, however, the exposure to the analytes was through the media and not upon subcutaneous injection, and the interpretation of

the data concerning cytokine release would be limited by the above--mentioned shortcomings.[103] Having said this, the full-thickness models or the human skin equivalent (HSE) models can be utilized for screening early candidates, similar to *in vitro* cell-based assays.[103] Enhancing these models by incorporating subcutaneous adipose tissue from liposuction material could improve their immune competence and more closely mimic subcutaneous injection environments, thus expanding their utility in immunogenicity assessments.[177]

Human skin explant-based tests, unlike the skin models, are derived from human donor biopsies or as waste products of surgical procedures like abdominoplasty, panniculectomy, or breast reduction surgeries.[178–183] These skin explants can be sourced directly from hospitals or are commercially available from Biopredic International (https://wepredic.com/en/biopredic), BioIVT (https://bioivt.com/human-skin-tissue), Genoskin (https://genoskin.com/tissue-samples/human-skin-models-nativeskin/) and from Xenoskin (https://www.xenometrix.ch/shop/XenoSkin-H-Squares-Ex-vivo-dermatomed-Skin-Explants-for-in-vitro-Dermal-Absorption-Permeation-and-Skin-Penetration-Tests-OECD-428-EU-Reg-2017–745). While skin explants have been used extensively by the cosmetic industry for tests like dermal absorption, permeation and skin penetration, their use in evaluating immunogenicity risk of therapeutic proteins is relatively recent. A recent study demonstrated a strong correlation (13 out of 16 antibodies) between results obtained using skin biopsies co-cultured with PBMCs over 10 donors obtained from the corresponding skin donors for 16 different antibodies, and compared the results with the associated clinical immunogenicity outcomes for the same.[183] While the study did not involve injecting the skin directly with the test articles and used samples at a very low concentration of 1 μg/mL, the proposed method provides a useful tool to bridge the gap between pre-clinical studies and clinical trials, especially with protein therapeutics administered subcutaneously.[183] Further studies done using injection into the skin can prove to be crucial in understanding injection site reactions in the context of the skin's immune system and further complement the existing infrastructure of immunogenicity testing.

While skin models have shown promise in their ability to potentially predict clinical ADA response and ISRs of subcutaneously delivered protein therapeutics, their use may be limited due to certain shortcomings. One such key shortcoming is the post-excision viability of the skin. While the epidermal integrity is maintained over 20 days, necrosis was observed at day 9 and in some cases as early as 7 days (as reported by vendors).[181] Therefore, all tests post the shipment of the skin sample and equilibration have a window of 3–7 days, limiting the readouts to short-term stimulation and primarily the innate immune response. It is therefore important to stage the testing appropriately to measure both cytokine response and the histopathology of the skin. The other factor that further enhances the considerations of using this model is the availability of skin samples and appropriate diversity of donors. Therefore, to make this model a widely used assay tool to assess immunogenicity, it would be important to overcome certain shortcomings and design around others.

In vivo models

While immunogenicity assessment using *in vivo* models is not always predictive of human responses, these models can provide critical insights into the relative immunogenicity of biotherapeutics and can be useful in the overall risk assessment. The human immunogenicity prediction abilities of these models can be highly product-specific. Animal models, unlike *in vitro* models, have the advantage of the dynamic interplay of various immune cells. Advances in the development of transgenic species can, in the future, improve the relevance of these models to evaluate immunogenicity. Additionally, the strongest rationale for the use of these models is conserved endogenous proteins, where biotherapeutics can be evaluated to study the severe consequences of unintended immunogenicity against endogenous molecules, such as in the case of erythropoietin.[33,184]

Mouse

Mouse models have laid the foundation of both basic immunology research and the evaluation of immunogenicity of therapeutic proteins in the context of a closed immune system. Mouse models offer a significant advantage like low variability as a result of genetic inbreeding.[185] They are convenient to source or breed and can be easily housed as a result of their small size.[185] Despite their many advantages, the inherent immune system differences between wild-type mice and humans limit the model to evaluating either the relative immunogenicity of therapeutic proteins or the immunogenicity of murine proteins as surrogates to human proteins.[185,186] The innate and adaptive immune systems differ significantly between mice and humans in several aspects, including leukocyte subset levels, IgG subclasses, and IgG class switching, which are critical to humoral immune responses.[187,188]

To overcome the shortcomings of the wild-type mouse models, various other mouse models were developed to evaluate the immunogenicity of protein therapeutics, while maintaining the utility of the wild-type mouse models. Jiskoot et al., 2016 describe wild-type mice, transgenic mice, XenoMouse, Xeno-heterozygous mice, and SCID mice with human blood grafts to name a few, detailing their use, advantages, and limitations.[186] While the non-wild-type strains can bring the advantages of having human antibody production (XenoMouse) and genetic tolerance to human proteins (transgenic mouse), they can be expensive and tedious to develop and still lack the humanness to translate immune responses to the clinical immunogenicity rates.[186] Another review by Kraus and colleagues describes that when it comes to evaluating CQAs like protein aggregation, the most commonly used mouse models are wild-type and transgenic mouse models.[189] In recent years, a proprietary model developed by grafting human PBMCs into a mouse to create the XenoImmune™ Mouse Model has shown human immunogenicity predictive potential for rhFVIII, rhGAA, and rh-Interferon-β, and AAV9 when compared to wild-type C57BL/6 mouse.[190] This indicates that further development on the mouse model has the potential to not only provide relative immunogenicity rates but also clinical predictions of human immunogenicity.

Rat

The rat model, although widely used in the toxicology space, is less frequently used for the immunogenicity assessment of therapeutic proteins compared to the mouse model.[191] Although both mouse and rat models fall under the class of rodent models, their immune systems have distinct differences that can impact immunogenicity assessments. Historically, mice have been preferred for immunogenicity assessment due to ease of operation governed by availability and size considerations to support the large sample size for immunogenicity assessment. However, when the immune systems are under consideration the rat model has more similarities to the human immune system compared to wild-type mice.[192] For example, both rat and human T-cells express MHC-II molecules, which are thought to regulate immune response by presenting antigens to other T-cells.[192,193] This antigen presentation seen in rats can induce anergy, downregulating the immune response and presenting a more balanced view of potential immunogenicity similar to humans but is absent in mice(192). Additionally, both humans and rats exhibit transient expression of Foxp3 in activated T cells, indicating a nuanced balance between immune activation and suppression not observed in mice, where Foxp3 is solely a marker for regulatory T cells (Tregs).[192] Furthermore, CD4+/CD8+ macrophages in rats and humans play a role in processing and presenting antigens on MHC-I and MHC-II molecules, a feature limited or absent in mice models.[192] Antibody production also varies, with rat IgG1 binding to complement similarly to human IgG1, whereas mouse IgG1 does not.[193] Some studies have recorded the use of Sprague Dawley rats for studying the immunogenicity impact of therapeutic proteins; however, specific CQAs were not assessed independently as often seen with mice models.[194–197] Although rat models are

not claimed to be the most accurate for representing human immunogenicity, based on the immune differences presented above, they may offer a better evaluation of relative immunogenicity between products than wild-type mouse models.[198]

Pig and minipig

The porcine model is considered to be the closest in skin architecture to humans(199,200). This has led to the usage of the model extensively in evaluating the pharmacokinetic profiles of protein therapeutics; however, human translatability is still questionable.[199,200] Amongst the

Table 2

Non-exhaustive list of bioassays used in ADA assessment, their salient features, advantages and disadvantages, and examples of use in the detection of ADA in pre-clinical and clinical samples.

Type of assay	Salient features	Advantages	Disadvantages	Recorded examples of use	
				ADA detection from preclinical samples	ADA detection from clinical samples
pH-shift-anti-idiotype antigen binding test (PIA)	Antigen-antibody complexes are disrupted by pH shift. Drug-binding Fab fragments are added to prevent rebinding to ADAs. Labeled Fab fragments are added to measure anti-idiotypic antibody binding to ADA.	Drug-tolerant assay. pH-shift-anti-idiotype antigen binding test (PIA)	Complex to standardize. Careful pH control is required to prevent the unfolding of the ADA and the drug.	–	226,227
Acid-dissociation lanthanide-fluorescence immunoassay (ALFIA)	Uses lanthanide chelates for detection after acid dissociation of antibody-antigen complexes.	Drug tolerant. High sensitivity and specificity.	Specialized equipment and reagents required.	–	228
Electrochemiluminescence Assays	Detection is done using electrochemiluminescent labels.	Drug tolerant. Wide dynamic range and high sensitivity.	Specialized equipment is required	229–232	230,231,233,234
Surface plasmon resonance (SPR)	Refractive index change near the sensor surface is measured.	Real-time detection with kinetic data.	Expensive equipment.	235	233,236
Biolayer interferometry (BLI)	Optical technique measuring interference pattern changes.	Label-free, real-time detection.	Expensive equipment and lower throughput.	231	233
Bridging ELISA	The drug is used as a capture and detection reagent.	High specificity and sensitivity. Commonly used.	Not drug tolerant, requires acid dissociation or a drug to wash out.	231,237	234,238,239
Direct ELISA	Direct detection of ADAs from the sample.	Easy to perform assay.	High background due to ubiquitous protein binding from samples; Change in the conformation of ADAs bound to the plate, leading to masking, lowering sensitivity of assay. Intolerant to the presence of drug.	240	241,242
Indirect ELISA	Uses a secondary antibody for detection.	Higher sensitivity than direct ELISA.	Change in the conformation of ADAs bound to the plate leading to masking compromising sensitivity of assay.	64	241
Sandwich ELISA	Capture antibody binds ADA, and then a secondary antibody detects it (likely species specific).	High sensitivity and specificity. Preferred assay for preclinical research.	Not utilized in detecting ADAs from human samples as secondary antibody, usually a human anti-Fc can detect other IgGs in the samples	237,243,244	–
Capillary Electrophoresis	Separation is based on their size and charge.	High resolution and sensitivity.	Relatively new, use in human samples is not established.	245,246	–
Radioimmunoassays (RIA)	ADA detection done using radiolabel.	High sensitivity and specificity.	Create radioactive waste. Complicated scaling and automation.	247	238,248
Immunological multi-parameter chip technology (IMPACT)	Microarray-based platform for simultaneous detection of multiple analytes.	Multiplexing capabilities	Not well established or frequently used in ADA detection	–	249
label-free immunoassay (LFIA-thin-film interferometry (TFI)	Thin film interference patterns are measured	Can monitor drug and ADA simultaneously. Label-free and real-time analysis.	Recent technique. Not well established in ADA detection.	–	250
Homogeneous mobility shift assay (HMSA)	Mobility of antibody (drug)-ADA complexes measured using fluorescent-tagged drug elution on SEC.	No separation steps. High sensitivity.	Expensive equipment required.	251	252,253
Cell-based reporter gene assay (RGA)	Cells with reporter gene bind drug unbound to ADA. Reduction in signal intensity indicative of ADA presence and quantity.	Used for measuring neutralizing ADA, reflects biological activity. High specificity.	Complex assay setup with long lead times associated with establishing cell lines. Cell viability can confound results.	–	254,255
LC-MS/MS	Pull down or immunocapture of acid-dissociated ADA sample, followed by tryptic digestion for ADA peptide mapping using LC-MS.	Highly quantitative. High drug tolerance especially when immunocapture is utilized with the LC-MS detection. Multiple classes of antibodies can be analyzed in single assay.	Complex data interpretation. Requires technical expertise. Expensive equipment is required	256,257	258,259

non-rodent models, the porcine model also has immune system similarities to the human immune system.[193,201] While some studies have shown promise with the prediction of a human immune response in the case of administering adalimumab and infliximab,[202] this model falls short on the front of predicting human immune response largely as it lacks MHC complex haplotypes that play a key role in driving humoral immunogenicity through antigen presentation.[172,203] However, recent advances with the genetically modified Göttingen pig model have shown promise in terms of evaluating immune responses for clinically well-tolerated molecules (daratumumab, bevacizumab) and clinically highly immunogenic molecules (atezolizumab, engineered interleukin cergutuzumab amunaleukin).[204]

Non-human primate

Non-human primates have been considered a valuable preclinical tool as they have a genetic homology to humans.[198,205] The use of non-human primates for nonclinical safety evaluations for biologics has been common but is gradually slowing down due to a lack of availability and acceleration of replace, reduce, and refine (3Rs) by the FDA and industry due to ethical constraints.[206] With respect to absolute immunogenicity, the antidrug antibody response of NHPs and humans was comparable only in 59 % of the cases with the type of response being significantly different.[207] However, for cross-species conserved proteins, the NHP model still may offer advantages in terms of its predictive value for relative immunogenicity.[198] One such example of the relative immunogenicity of methionyl-human growth hormone (hGH), pituitary-derived hGH, and recombinant natural sequence hGH had antibody incidences of 81 %, 69 %, and 5–23 %, respectively, which correlated with relative human immunogenicity.[208,209]

In cases where subcutaneous delivery of antibodies is the primary route of administration, NHPs can be a useful model with respect to the similarities between the model with human skin. The model contains similar immune cell populations of Langerhans cells (LCs), dermal dendritic cells (DDCs), macrophages, and T cells that are crucial to skin immunity.[210] The model also has shared immune cell markers with human skin, *e.g.*, CD1a, CD11c, and HLA-DR.[210] The antigen recognition, activation, internalization, and migration of the dendritic cells to lymph nodes are also conserved across the two species.[210] However, some differences in the timing and magnitude of the innate immune responses were observed with TLR agonists between macaque and human skin.[211]

While some efforts have been made to develop transgenic marmoset non-human primates due to their short gestation period and easy availability, no studies have been conducted to test their feasibility for models for evaluating the immunogenicity of therapeutic proteins.[212] Therefore, as per the status quo, the current directions indicate that while NHPs can be a useful model for both subcutaneous and relative immunogenicity, ethical and availability concerns result in the infrequent utilization of this species.

Human immunogenicity assessment

Anti-drug antibody assessment

The nature of the therapeutic protein determines the study design considerations of ADA monitoring in the clinic. Low-risk therapeutics are monitored for ADA in the pre-treatment phase, throughout the treatment period until the end of treatment, and in the treatment-free period until the end of the study.[213] High-risk therapeutics, however, require extended monitoring of ADA beyond the end of the study as per the FDA.[214] While the FDA does not specifically state the conditions under which the extended monitoring is stipulated, the European Immunogenicity Platform (EIP) suggests utilizing tailored testing strategies beyond the standard approaches.[213] ADA monitoring as per the standard approach involves the collection of samples in a tiered fashion. Tier 1 involves a screening assay aimed at minimizing false negatives, tier 2 is an approach to minimize false positives by utilizing a confirmatory assay, and tier 3 is a characterization-based approach to ascertain the titer of the ADA, the isotype, subclass, and other features.[213,215] Advanced testing strategies are proposed for high-risk therapeutics where pharmacokinetics/pharmacodynamics (PK/PD), safety biomarkers, and neutralizing ADA and ADA cross-reactivity with endogenous proteins must be evaluated.[213] The perspective is to consider a patient-centric approach in extending the monitoring of ADAs for ethical reasons and an appropriate risk-benefit analysis, which can be done using a criteria-based decision tree. The EIP has also emphasized being subjective with the end criteria for monitoring ADA as one where the ADA may not necessarily return to baseline, but where there is no compromised safety risk as a result of the often-observable fluctuations in the long-term monitoring of ADAs.[213]

Similar to the study design considerations, the assay format for the assessment of ADAs may also vary between studies, the type of therapeutic protein, and the tier of testing. Ligand binding assays are used in tier 1 and tier 2 settings, some of which are captured in Table 2, and a wide variety of assays have been used in the tier 3 setting.[215] Readouts from each of these assays are crucial in understanding the product profile with regard to PK, PD, efficacy, and safety; hence, the FDA guidance to appropriately develop and validate these assays.[214] However, standard cutoffs cannot be assigned for ADAs, nor can comparisons between two therapeutic protein products or even the same protein product in two studies be made. This arises from the fact that unique positive controls are used in these assays of non-human species in origin, where the response may vary from animal to animal and species to species.[215] Additionally, different assay formats, sensitivity, specificity, and any other differentiating critical assay parameters further limit the ability to compare ADA responses across different molecules and studies. An appropriately tailored approach to ADA assessment is crucial for determining the immunogenicity of the molecules in a clinical setting.

Injection site reaction and injection site pain

ISR and ISP can both be viewed as standalone parameters and at the same time interconnected consequences of one on the other. ISP can be an inflammation-triggered subset of ISR.[216] However, not all incidences of ISP are a result of ISR.[216] While the recorded data on the administration of protein therapeutics does not show any evidence of ISP correlating with immunogenicity,[216] the vaccine literature has some examples of reactogenicity, inclusive of ISP correlating with the magnitude of the immune response.[21,22,217] Therefore, continued data collection on ISP can inform us of potential links between ISRs and/or immunogenicity for protein therapeutic products. However, doing so might be challenging in the current format, as pain perception is highly subjective, and the data collected is in variable formats, which can become challenging to normalize to one another. The three methods primarily utilized to measure pain intensity are the visual analog scale (VAS), numeric rating scale (NRS), and verbal rating scale (VRS).[216,218] The VAS utilized a 10 cm line where patients could pick a point along the line indicating their pain level. Mild pain on the VAS is characterized as a score $\leq$ 3 cm.[216] The NRS is a score is a commonly used scoring system that ranks pain between 1 to 10. A score of 1 indicates that the patient has "no pain", and a score of 10 indicates the highest pain level.[216] VAS scores can sometimes be converted to NRS scores for normalization purposes. The third method, the verbal rating scale (VRS), uses verbal descriptors of the severity of pain, bucketing them in categories such as "none", "light", "moderate", or "severe".[218] The NRS method has shown the highest sensitivity and compliance as a scoring method for pain, and efforts should be made to standardize this as the measurement of injection site pain.[219] The NRS method is likely high in compliance and sensitivity, as broad descriptors can be supplemented with specific patient experiences. Thus, advocacy for standardized ISP measurement can potentially help with better correlation of ISPs with other indicators of immunogenicity and improve patient experience of protein therapeutic products.

Similar to ISP, ISRs are a locally occurring phenomenon. ISRs,

Table 3
Analytical techniques used to evaluate pCQAs and their recorded use in assessing the immunogenicity of therapeutic proteins.

Type of pCQA	Analytical technique(s)	Key limitation(s) and challenge(s)	Associated immunogenicity assay(s)	References
		Biophysical characterization tools		
Global insoluble aggregation and particulate analysis	Turbidity	Prone to interference from non-proteinaceous particles and bubbles in the sample. The technique is nonspecific and semi-quantitative. Sample preparation (mixing) can determine the outcome.	*In vivo* mouse ADA analysis, Human artificial lymph node-based immunogenicity analysis	95,96
Soluble aggregates	Size exclusion chromatography (SEC)	Sample filtration using a 0.22 μm filter to prevent column clogging results in potential sample loss. This limits the technique to the analysis of aggregates < 220 nm.	*In vivo* mouse ADA analysis, human IgG transgenic mice, human artificial lymph node-based immunogenicity analysis, clinical immunogenicity rates, *in vitro* T cell activation, CD8+-Depleted PBMC cytokine release assay, DC activation	58,95–98
Soluble aggregates/submicron particles	Analytical ultracentrifugation (AUC)	While sample filtration is not required, the technique is very low throughput in nature.	Clinical immunogenicity rates	59,99
Submicron particles	Nanoparticle tracking analysis (NTA)	Relative brightness-based analysis results in significantly drowning out the ability to visualize smaller particles in the presence of larger particles, depending on the abundance of the larger particles.	Clinical immunogenicity rates, *in vivo* mouse ADA analysis	59,98
Submicron particles	Dynamic light scattering (DLS)	Particle shape is always assumed to be spherical, making the analysis less accurate for other shapes of irregular aggregates. Significant sample dilution is required. Bulk measurement.	*in vitro* T cell activation, CD8+-depleted PBMC cytokine release assay, DC activation, *in vivo* immunogenicity analysis in Balb/C mice	58,100
Submicron particles	Microfluidic Resistive Pulse Sensing (MRPS) Spectradyne nCS1	Low throughput, and susceptible to sample preparation sensitivities. May struggle with accuracy and resolution issues. Also has less established validation protocols for widescale adoption.	Proposed use	101,102
Proteinaceous particulate analysis/ submicron particle analysis	Resonant Mass Measurements (Archimedes)	Low accuracy for low-concentration samples. Low throughput.	Human IgG transgenic mice, clinical immunogenicity rates	59,97
Insoluble subvisible particulates: proteinaceous and non-proteinaceous (impurities (overlap with QC tools)	Light obscuration	Proteinaceous particles can be translucent and can be missed by the LO resulting in severely undercounting particles in comparison to other subvisible particle analysis techniques. Cannot distinguish particle type. Sample dilution can result in artifactual readings as a result of stress. Low throughput	*In vivo* mouse ADA analysis, Human IgG transgenic mice, human artificial lymph node-based immunogenicity analysis, *in vitro* T cell activation, CD8+-depleted PBMC cytokine release assay, DC activation, IVICIA PBMC assay	32,58,95–97,103
Insoluble subvisible particulates: proteinaceous and non-proteinaceous (impurities) (overlap with QC tools)	Fluid imaging microscopy (FlowCam, Microflow imaging, Digital particle analyzer (DPA) 4100)	Higher sensitivity can result in high particulate counts in comparison to compendial methods like LO. Flow cell-based techniques can result in clogs based on the nature of the sample. Dilution-dependent sample preparation artifacts.	Clinical immunogenicity rates, *in vivo* mouse ADA analysis	32,59,98
Insoluble subvisible particulates: proteinaceous	Flow cytometry analysis	Dependence on fluorescent dyes for detecting aggregation that may have nonspecific binding to non-proteinaceous particles or lead to inaccurate proteinaceous particle size detection.	*in vivo* mouse ADA analysis	98,104,105
Insoluble subvisible particulates: proteinaceous and non-proteinaceous (impurities)	Membrane microscopy: Horizon and Aura	Membrane saturation for samples with a large number of particulates. Restricted area of imaging analyzed by software for the Horizon. Possible sampling bias due to low sampling volume. Limited scope for complete sample analysis.	Proposed use	106
Insoluble subvisible particulates: proteinaceous and non-proteinaceous (impurities)	Light microscopy	Relies on the high contrast of particles compared to the membrane. Sample preparation procedures can alter particle size distribution. Tedious manual technique with difficulty in calibration approaches.	Clinical immunogenicity rates	59,107
High-fidelity aggregation analysis	Raman spectroscopy	Low throughput: can be dependent on scanning the field of view one particle at a time. Membranes used in filtration can have Raman protein spectrum overlap convoluting data analysis.	Proposed use	108–110
Changes in secondary structure	Infrared (IR) Spectroscopy	Relatively low resolution in comparison to techniques like NMR. May have water interference in signal deconvolution.	*In vivo* mouse ADA analysis, human artificial lymph node-based immunogenicity analysis	95,96,100
Changes in secondary structure	Extrinsic Fluorescence Spectroscopy	Data interpretation can be marred by large number of particulate in sample. Reliance on fluorophores that can cause protein structure impacts. Not a direct measurement for	Human artificial lymph node-based immunogenicity analysis	96

(*continued on next page*)

Table 3 (*continued*)

Type of pCQA	Analytical technique(s)	Key limitation(s) and challenge(s)	Associated immunogenicity assay(s)	References
		secondary structures, and depends on the environment of the protein as opposed to CD.		
Protein aggregation and secondary structure analysis	Circular dichroism	Requires purified sample in appropriate buffer; buffer exchange may result in sample stress, causing artifactual readouts.	Proposed use	[111]
Protein unfolding analysis	Differential Scanning Fluorimetry	The possibility of non-specific binding with dyes that can be dye concentration dependent, affecting readout.	Proposed use	[112]
		Biochemical characterization tools		
Charge profiling	Cation exchange chromatography (with mass spectroscopy)	Possible overlap of isoelectric points that may lead to inadequate resolution of charge variants.	Proposed use	[113,114]
Charge profiling	Capillary isoelectric focusing (IEF)	Reproducibility issues are marred by small changes to running conditions. Sensitivity of the sample to ampholytes can lead to PI shifts.	Proposed use	[115]
Potential oxidation profiling	Hydrophobic interaction chromatography (HIC)	Reliance on high salt gradients may induce artifactual aggregation. Unable to resolve minor effects in oxidation.	Proposed use	[116]
Potential oxidation profiling	Protein A chromatography	Low resolution: Only higher levels of oxidation are known to be detectable by this method.	Proposed use	[117]
Post-translational and chemical modifications, aggregation analysis	Nuclear magnetic resonance	Complex data interpretation. Low throughput. Expensive instrumentation. A large amount of samples is required, which may be inappropriate for screening stages.	Proposed use	[118]
Post-translational and chemical modifications	Peptide mapping	Complex data analysis. Loss of modifications due to sample preparation. Disulfide bind scrambling.	Human IgG transgenic mice	[97]
		Quality control tools		
Impurities/ QC	Endotoxin	Sample dilution to prevent excipient-related endotoxin masking.	*In vivo* mouse ADA analysis, Human artificial lymph node-based immunogenicity analysis, IVICIA	[92,95,96,119]
Impurities/ QC	Host cell protein analysis via mass spectrometry	Requires significant sample enrichment and can face saturation from the drug substance due to low abundance of HCP.	PBMC IVCIA Assay, T cell proliferation assays, Multiplex Cytokine Analysis	[93]

commonly observed with subcutaneous delivery of protein therapeutics, are the local presentation of one or more of the symptoms like erythema, edema, pain, wheals, and pruritis.[70,220] These reactions can be immediate or delayed and transient or long-lasting. These ISRs are classified into type α, type β, and recall ISRs.[70,220] Type α immunostimulation reactions are immediate ISRs caused as a result of proinflammatory cytokines released in response to the injection and are generally not correlated with immunogenicity.[70,220] Type β reactions on the other hand have an immunogenicity correlation.[70,220] Rapid or immediate type β is generally linked to IgE release and delayed reactions are linked to the presence of ADA.[70] Although a definitive correlation between all ISRs and the development of ADA cannot be drawn, examples exist where a higher frequency of ISRs was found in patients with higher ADA.[70] For example, patients with high-affinity ADAs (HADAs) against alirocumab had a higher frequency of ISRs (15.9 %) compared to patients who were either transiently HADA-positive (11.6 %) or HADA-negative (5.9 %).[221] Similar findings were observed with rheumatoid arthritis (RA) patients treated with TNF inhibitors (infliximab, etanercept, adalimumab) where a higher frequency of ISRs was observed in patients who developed ADAs.[222] Therefore, monitoring ISRs and ADA incidences can help establish if there is an interdependency between the two responses and inform further steps on the line of treatment. It is noteworthy to mention that while ISRs can be severe in some cases, pre-treatment or concomitant treatment with corticosteroids, antihistamines, acetaminophen, and in some less severe cases, cold compresses can offer symptomatic relief.[271] Monitoring such cases for impact on ADA can provide further insights into the interdependence of ADA and ISRs.

Bioassays for assessment of humoral immunogenicity

Various formats for the assessment of ADAs from both preclinical and clinical samples can be used. A few detailed reviews capture the nuances of these techniques, and the prevalence of their use to detect ADA can be referenced elsewhere.[223–225] Table 2 provides a non-exhaustive list of the assays that are utilized in testing ADA from preclinical and clinical samples.

Guidance on immunogenicity assessment

Perspective of the regulatory bodies

Clinical immunogenicity assessment and reporting

Clinical assessment of immunogenicity for biotherapeutics is well established, both under the guidelines of the United States Food and Drug Administration (USFDA or FDA) and the European Medicines Agency (EMA). Both agencies underscore the importance of measuring immunogenicity and further reporting it under the ‘adverse effects’ section of their respective drug labeling requirements. The FDA guidance titled “Immunogenicity Testing of Therapeutic Protein Products — Developing and Validating Assays for Anti-Drug Antibody Detection-Guidance for Industry” details the specifics of ADA testing in the clinic. While this guidance is not legally binding, the methodology and the suggestions made as a part of this document are widely followed by the biopharmaceutical industry.[260] The guidance advocates for a tiered approach toward immunogenicity testing. The first step to this tiered approach is a screening assay to detect binding ADAs. The screening

assay is followed by a competitive confirmatory assay to eliminate false positives. The final tier involves a neutralization assay to detect neutralizing antidrug antibodies (NAbs) as a marker to assess the impact of ADAs on efficacy. The FDA also recommends tittering assays to assess the magnitude of ADA responses. To substantiate the clinical incidences of immunogenicity for a drug product, the FDA document details the steps that should be taken to validate the assay used by investigators. This validation involves, but is not limited to, detecting antibody isotypes, demonstrating domain specificity, sensitivity, and minimum required dilution (MRD), among others.

The assay workflow stated above for the FDA has a vital risk-based testing and characterization approach, where "Immunogenicity Assessment for Therapeutic Protein Products, 2014″ goes into sufficient detail on factors affecting the immunogenicity of therapeutic proteins and associated mitigation approaches(13). The EMA guidance follows the same strategy in terms of the tiered approach to testing for immunogenicity and applying a Risk Management Plan (RMP).[261]

Collectively, these guidelines offer a comprehensive framework for immunogenicity in clinical stages. The guidelines serve as a structured roadmap for both new biotherapeutics and novel formulations of existing approved biotherapeutics.

Preclinical immunogenicity risk assessment: use of in vivo studies

Immunogenicity risk assessment in the preclinical stages using *in vivo* assessments is not as well defined as the clinical immunogenicity risk assessments by the FDA and EMA. The preclinical risk assessment varies greatly based on the biotherapeutic and the specific risk being evaluated. As noted above, while immunogenicity assessments in animal models can offer valuable insights, their predictive value for clinical immunogenicity is variable and influenced by multiple factors.[172,186,198,203] Despite this, evaluating important CQAs, such as the impact of aggregation, in a relative manner can provide valuable insights into the alterations to the baseline characteristics of the protein therapeutics by the CQA and can provide critical insights into immunogenicity against endogenous molecules conserved across species.

Animal models, therefore, serve as essential tools in the broader context of relative immunogenicity evaluation and immunogenicity risk assessment. Context-based evaluation of ADA responses in these models is considered important by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) S6 guidance (ICHS6(R1)).[262] Although ICH S6(R1) was developed specifically to guide the conduct and interpretation of toxicology studies for biotechnology-derived pharmaceuticals, certain principles within the guidance can offer indirect relevance to broader immunogenicity risk assessment strategies. The guidance adopts the approach of collecting immunogenicity samples during pharmacokinetic and pharmacodynamic evaluations. The guidance indicates that immunogenicity assessments should be carried out should there be an impact on PK, where unexpected changes in exposure are observed, like the reduction in area under the curve (AUC), especially in the absence of pharmacodynamic markers. In other cases, such as where immune reactions like anaphylaxis or aberrant changes in efficacy are observed, testing of the immunogenicity samples is also advised. It is important to note that the impact of ADAs on pharmacokinetic exposure has been found to be more telling and prophetic of future impact on efficacy.[263]

In this sense, the principles from ICH S6(R1) can be leveraged in the broader ecosystem of immunogenicity assessment in conjunction with work done by consortia and forums. Together, these can inform the immunogenicity risk assessment strategy for biotherapeutic products.

Perspective of forums and consortia

Preclinical immunogenicity risk assessment: use of in vivo studies

Various consortia and forums offer guidance on refining preclinical immunogenicity assessment strategies with respect to *in vivo* studies. The focus of such industry-wide initiatives spans balancing scientific

Table 4
Various factors affecting immunogenicity and a stage-appropriate map of tools that can be used to access them.

Category of factor	Type of factor	Immune impact assessment								Analytical characterization tools		
		In silico	In vitro	Organoids and Organ-on-chip	Skin models and skin explants	*In vivo* models	Clinical ADA evaluation	Clinical ISR and ISP evaluation	Post Hoc analysis	Biophysical characterization tools	Biochemical characterization tool	QC tools
Patient related factors	Variability of immune responses								✓			
	HLA haplotypes						✓	✓	✓			
	Age						✓	✓	✓			
	Sex						✓	✓	✓			
	Disease status						✓	✓	✓			
Product related factors	Protein sequence	✓	✓								✓	✓
	Function of the drug and its target		✓	✓	✓	✓	✓	✓	✓		✓	✓
	Biochemical instability: Post-translational and chemical modifications		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Biophysical instability		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Impurities		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Formulation factors		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Coadministration with immunomodulatory agents and other antibodies						✓	✓	✓			✓
Administration related factors	Route of administration						✓	✓	✓			
	Site of administration						✓	✓	✓			
	Volume of injection in the context of site of injection					✓	✓	✓	✓			
	Volume and viscosity of drug product administered					✓	✓	✓	✓			
	Needle gauge and tip geometry					✓	✓	✓	✓			

rigor, the utility of models, and assay-specific guidance to inform effective risk assessment approaches. For instance, the Health and Environmental Sciences Institute (HESI), through its Immuno-Safety Technical Committee (ITC), has focused on evaluating humanized mouse models to de-risk immunogenicity. Other groups have focused efforts on assay development for preclinical ADA evaluations.[264] The European Bioanalysis Forum, drawing on extensive literature and ICH S6 guidance, recommends a "lean" approach to preclinical immunogenicity testing.[254] As opposed to the clinical immunogenicity testing guidelines, where three tiers of analysis exist for testing, the forum recommends only a screening assay-based approach.[254] The screening assay is applied with stringent cut points (0.1–1 % false positive rate), thereby eliminating the need for a confirmatory assay.[254] A decision tree-based approach is proposed for the immunogenicity risk assessment of an asset by the European Bioanalysis Forum.[254] Various aspects are built into the process of decision-making, spanning business considerations to the extent of impact on the toxicokinetic and pharmacodynamic results.[254]

Although the regulatory and consortium consensus on the *in vivo* preclinical evaluation of immunogenicity remains subjective and less standardized, *in vivo* testing continues to provide valuable insights for the development of biotherapeutics. While these immunogenicity results are relative and not predictive, recommendation by the above expert guidelines provides for a streamlined approach and a context-driven *in vivo* evaluation of immunogenicity risk.

Preclinical immunogenicity risk assessment: use of cell-based assays

While admittedly the regulatory guidance on the *in vivo* tools is sparse, some directionality on when their application is appropriate does provide a useful foundation for the *in vivo* preclinical immunogenicity assessments.[172] This sparse yet basic foundation is notably absent with cell-based assays for preclinical immunogenicity risk assessment.[172] While regulatory agencies encourage the use of cell-based assays for in-depth characterization of protein therapeutic products, the variability and choice of these assays can result in imprecise and non-standardized assessments.[172]

Consortia, working groups, and platforms play a key role in acting as a bridge between the regulatory agencies and the drug developer entities to provide guidance to fill the gap in the *in vitro* assay space. Organizations such as Anti-Biopharmaceutical Immunization: Prediction and Analysis of Clinical Relevance to Minimize the Risk (ABIRISK) and the Non-Clinical Immunogenicity Risk Assessment working group of the EIP are actively working to identify cell-based assays that could be predictive of clinical immunogenicity and useful for preclinical risk assessment of biotherapeutics.[120,265,266] These organizations focus on developing new predictive immunogenicity assays. They also focus on the harmonization and standardization of existing assays. The initiatives advise against the use of a single assay to determine immunogenicity, as individual cell-based assays could provide a partial and myopic view of the immunogenicity risks when used independently.[120]

It is also notable to point out that other consortia have focused on specific questions associated with immunogenicity. For example, the Subcutaneous Drug Delivery and Development Consortium, or the Subcutaneous Consortium, focuses on immunogenicity challenges and predictions associated with subcutaneous delivery.[267] The International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) also has working groups to tackle the immunogenicity concerns of novel drug modalities using *in vitro* immunogenicity assays.[268] Through its collaborative initiatives, the HESI ITC has contributed to the development and qualification of a reference antibody panel for cytokine release assays, along with validation of the assay for industry-wide use in immunogenicity risk assessment.[269] These efforts represent just a few examples of the broader body of work conducted by such organizations to bolster risk assessment through *in vitro* assay use. Work done by these consortia can be utilized to create a selection of standardized approaches, especially in cases where specific parameters like the impact of the delivery mode or new modality are being investigated.

Some groundwork laid down by the US FDA in the space of peptide drug products could be informative in ascertaining standardization parameters for protein therapeutics. An FDA poster, "*In vitro* Immunogenicity Assays for Evaluating Generic Peptide Drug Products," outlines some assay-related specifics that can serve as a template for this standardization and harmonization.[270] The poster provides various useful details such as the number of donors required for various assays and the parameters that need optimization, such as sensitivity, specificity, cell viability, the effect of excipients, and the positive standards used in these assays.[270]

While more work needs to be done in identifying a set of immunogenicity assays and then further standardizing them, the Therapeutic Product Immunogenicity (TPI) community of the American Association of Pharmaceutical Scientists (AAPS) has made some progress in this direction.[80] The TPI conducted a limited survey of key industry and academia members on the *in vitro* tools utilized to conduct immunogenicity risk assessments.[80] The team has put together data on the frequency of use of assays, the stage in which they are utilized, assay specifics, and the consensus *versus* variability in their use across the industry.[80] More such undertakings can lead to standardization and harmonization of *in vitro* assays. Such efforts will become crucial in regulatory agencies adopting *in vitro* immunogenicity tools as standard practices for reliable preclinical immunogenicity risk assessments.

Conclusions

The immunogenicity of therapeutic protein is a multifaceted problem. The understanding and subsequent mitigation of immunogenicity exists at the interface of characterizing the product performance from an analytical and translational perspective. The internal complexities of protein therapeutics and the immune system's complex interactions with them necessitate bridging gaps and plugging the shortcomings in existing translational and analytical methodologies. Additionally, pushing the envelope to develop new techniques will bring us closer to our goal of being able to accurately predict clinical immunogenicity. No single technique can be used analytically or translation-wise, and a comprehensive approach is the way forward in immunogenicity risk assessment. Table 4 provides an example of what a comprehensive strategy might look like to develop therapeutic proteins with a minimized immunogenicity risk profile. Continued efforts in advancing standardization and stage-appropriate use of the methodologies for pCQAs are likely to pave the road toward developing safer protein therapeutics and new and upcoming modalities. Ultimately, a comprehensive characterization approach, along with input from regulatory bodies, can ensure a potentially appended product approval timeline and successful clinical outcomes for patients.

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References

1. Zurdo J, Arnell A, Obrezanova O, Smith N, Gómez de la Cuesta R, Gallagher TRA, et al. Early implementation of QbD in biopharmaceutical development: a practical example. *Biomed Res Int*. 2015;2015:1–19.
2. Sun D, Gao W, Hu H, Zhou S. Why 90% of clinical drug development fails and how to improve it? *Acta Pharm Sin B*. 2022;12(7):3049–3062. Jul.
3. Wen Y, Jawa V. The impact of product and process related critical quality attributes on immunogenicity and adverse immunological effects of biotherapeutics. *J Pharm Sci*. 2021;110:1025–1041.
4. Vanmeerbeek I., Naulaerts S., Garg A.D. Reverse translation: the key to increasing the clinical success of immunotherapy? 2023; Available from: https://doi.org/10.1038/s41435.
5. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature*. 2018;554(7693):544–548. Feb 22.
6. Abbas AH, Lichtman AH, Pillai S. *Cellular and Molecular Immunology*. 10th ed. Elsevier Inc.; 2021:123–150.
7. Jarvi NL, Balu-Iyer SV. Immunogenicity challenges associated with subcutaneous delivery of therapeutic proteins. *BioDrugs*. 2021;35(2):125–146. Mar.
8. Jawa V, Terry F, Gokemeijer J, Mitra-Kaushik S, Roberts BJ, Tourdot S, et al. T-cell dependent immunogenicity of protein therapeutics pre-clinical assessment and mitigation–Updated consensus and review 2020. *Front Immunol*. 2020:11. Jun 30.
9. Dingman R, Balu-Iyer SV. *Immunogenicity of Protein Pharmaceuticals*. 108. Journal of Pharmaceutical Sciences. Elsevier B.V.; 2019:1637–1654.
10. Carter PJ, Quarmby V. Immunogenicity risk assessment and mitigation for engineered antibody and protein therapeutics. *Nat Rev Drug Discov*; 2024 [Internet] Oct 18; Available from: http://www.ncbi.nlm.nih.gov/pubmed/39424922.
11. Harris CT, Cohen S. Reducing immunogenicity by design: approaches to minimize immunogenicity of monoclonal antibodies. *BioDrugs*. 2024;38(2):205–226. Mar 23.
12. Tourdot S, Hickling TP. *Nonclinical immunogenicity risk assessment of therapeutic proteins*. 11. Bioanalysis. Future Medicine Ltd; 2019:1631–1643.
13. FDA. CDER. Guidance for Industry immunogenicity assessment for therapeutic protein products [Internet]. Available from: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htmand/orhttp://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm; 2014.
14. Brodin P, Davis MM. Human immune system variation. *Nat Rev Immunol*. 2017;17(1):21–29. Jan 5.
15. Prado MS, Bendtzen K, Andrade LEC. Biological anti-TNF drugs: immunogenicity underlying treatment failure and adverse events. *Expert Opin Drug Metab Toxicol*. 2017;13(9):985–995. Sep 2.
16. Spinelli FR, Valesini G. Immunogenicity of anti-tumour necrosis factor drugs in rheumatic diseases. *Clin Exp Rheumatol*. 2013;31(6):954–963.
17. Vaisman-Mentesh A, Gutierrez-Gonzalez M, DeKosky BJ, Wine Y. The molecular mechanisms that underlie the immune biology of anti-drug antibody formation following treatment with monoclonal antibodies. *Front Immunol Front Media S.A.* 2020;11.
18. Davis JD, Bravo Padros M, Conrado DJ, Ganguly S, Guan X, Hassan HE, et al. *Subcutaneous administration of monoclonal antibodies: pharmacology, delivery, immunogenicity, and learnings from applications to clinical development*. In: *Clinical Pharmacology and Therapeutics*. 115. John Wiley and Sons Inc; 2024:422–439.
19. Zou P, Wang F, Wang J, Lu Y, Tran D, Seo SK. Impact of injection sites on clinical pharmacokinetics of subcutaneously administered peptides and proteins. *J Control Rel*. 2021;336:310–321. Aug 10.
20. Richter WF, Bhansali SG, Morris ME. Mechanistic determinants of biotherapeutics absorption following SC administration. *AAPS J*. 2012;14:559–570.
21. Callegaro A, Burny W, HervCrossed DS©C, Hyung Kim J, Levin MJ, Zahaf T, et al. Association between immunogenicity and reactogenicity: a post hoc analysis of 2 phase 3 studies with the adjuvanted recombinant zoster vaccine. *J Infect Dis*. 2022;226(11):1943–1948. Dec 1.
22. Takano T, Morikawa M, Adachi Y, Kabasawa K, Sax N, Moriyama S, et al. Distinct immune cell dynamics correlate with the immunogenicity and reactogenicity of SARS-CoV-2 mRNA vaccine. *Cell Rep Med*. 2022;3(5). May 17.
23. Iris U, Martinez R, Festini T, Peris J. Subcutaneous injection of drugs: literature review of factors influencing pain sensation at the injection site. *Adv Ther*. 2019;36:2986–2996 [Internet]Available from: doi. 10.6084/.
24. Varkhede N, Forrest ML. Understanding the monoclonal antibody disposition after subcutaneous administration using a minimal physiologically based pharmacokinetic model. *J Pharm Pharmaceut Sci*. 2018;21(1S):130s–148s.
25. The Antibody Society. Therapeutic monoclonal antibodies approved or in review in the EU or US [Internet]. [cited www.antibodysociety.org/resources/approved-antibodies; 2024.
26. De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, et al. Activation of natural regulatory T cells by IgG Fc–derived peptide "tregitopes. *Blood*. 2008;112(8):3303–3311. Oct 15.
27. De Groot AS, Roberts BJ, Mattei A, Lelias S, Boyle C, Martin WD. Immunogenicity risk assessment of synthetic peptide drugs and their impurities. *Drug Discov Today*. 2023;28(10), 103714. Oct.
28. Hwang WL, Pike LRG, Royce TJ, Mahal BA, Loeffler JS. *Safety of combining radiotherapy with immune-checkpoint inhibition*. In: *Nature Reviews Clinical Oncology*. 15. Nature Publishing Group; 2018:477–494.
29. van Meir H, Nout RA, Welters MJP, Loof NM, de Kam ML, van Ham JJ, et al. Impact of (chemo)radiotherapy on immune cell composition and function in cervical cancer patients. *Oncoimmunology*. 2017;6(2). Feb 1.
30. Narhi LO, Schmit J, Bechtold-Peters K, Sharma D. Classification of protein aggregates. *J Pharm Sci*. 2012;101(2):493–498. Feb.
31. Zölls S, Tantipolphan R, Wiggenhorn M, Winter G, Jiskoot W, Friess W, et al. Particles in therapeutic protein formulations, part 1: overview of analytical methods. *J Pharm Sci*. 2012;101(3):914–935. Mar.
32. Cohen JR, Brych SR, Prabhu S, Bi V, Elbaradei A, Tokuda JM, et al. *A High Threshold of Biotherapeutic Aggregate Numbers is Needed to Induce an Immunogenic Response In Vitro*. Vivo, and in the Clinic. Pharm Res; 2024.
33. Schellekens H. *Immunologic mechanisms of EPO-associated pure red cell aplasia*. In: *Best Practice and Research: Clinical Haematology*. 18. Bailliere Tindall Ltd; 2005:473–480.
34. Staby A, Steensgaard DB, Haselmann KF, Marino JS, Bartholdy C, Videbæk N, et al. Influence of production process and scale on quality of polypeptide drugs: a case study on GLP-1 analogs. *Pharm Res*. 2020;37(7). Jul 1.
35. Barbosa MDFS, Vielmetter J, Chu S, Smith DD, Jacinto J. Clinical link between MHC class II haplotype and interferon-beta (IFN-β) immunogenicity. *Clin Immunol*. 2006;118(1):42–50. Jan.
36. Liu M, Degner J, Davis JW, Idler KB, Nader A, Mostafa NM, et al. Identification of HLA-DRB1 association to adalimumab immunogenicity. *PLoS One*. 2018;13(4), e0195325. Apr 3.
37. Atiqi S, Hooijberg F, Loeff FC, Rispens T, Wolbink GJ. Immunogenicity of TNF-inhibitors. *Front Immunol*. 2020;11. Feb 26.
38. Berteau C, Filipe-Santos O, Wang T, Roja HE, Granger C, Schwarzenbach F. Evaluation of the impact of viscosity, injection volume, and injection flow rate on subcutaneous injection tolerance. *Med Dev*. 2015;8:473–484. Nov 11.
39. Woodley WD, Morel DR, Sutter DE, Pettis RJ, Bolick NG. Clinical evaluation of large volume subcutaneous injection tissue effects, pain, and acceptability in healthy adults. *Clin Transl Sci*. 2022;15(1):92–104. Jan 10.
40. Arendt-Nielsen L, Egekvist H, Bjerring P. Pain following controlled cutaneous insertion of needles with different diameters. *Somatosens Mot Res*. 2006;23(1–2):37–43. Jan 10.
41. Hirsch L, Gibney M, Berube J, Manocchio J. Impact of a modified needle tip geometry on penetration force as well as acceptability, preference, and perceived pain in subjects with diabetes. *J Diabetes Sci Technol*. 2012;6(2):328–335. Mar.
42. LoBuglio AF, Wheeler RH, Trang J, Haynes A, Rogers K, Harvey EB, et al. Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. *Proc Natl. Acad Sci*. 1989;86(11):4220–4224. Jun.
43. Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Adv Drug Deliv Rev*. 2006;58(5–6):640–656. Aug.
44. Sun R, Qian MG, Zhang X. T and B cell epitope analysis for the immunogenicity evaluation and mitigation of antibody-based therapeutics. *MAbs*. 2024;16(1). Dec 31.
45. Mora JR, White JT. *DeWall SL. Immunogenicity risk assessment for PEGylated Therapeutics*. In: *AAPS Journal*. 22. Springer; 2020.
46. Caballero ML, Krantz MS, Quirce S, Phillips EJ, Stone CA. Hidden dangers: recognizing excipients as potential causes of drug and vaccine hypersensitivity reactions. *J Allergy Clin Immunol Pract*. 2021;9(8):2968–2982. Aug.
47. Zhang P, Sun F, Liu S, Jiang S. Anti-PEG antibodies in the clinic: current issues and beyond PEGylation. *J Control Rel*. 2016;244:184–193. Dec.
48. Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, Morse M, et al. *Cetuximab-Induced Anaphylaxis and IgE Specific for Galactose-α-1,3-Galactose*. 358. New England Journal of Medicine; 2008:1109–1117. Mar 13.
49. Zeunik R, Ryuzoji AF, Peariso A, Wang X, Lannan M, Spindler LJ, et al. Investigation of immune responses to oxidation, deamidation, and isomerization in therapeutic antibodies using preclinical immunogenicity risk assessment assays. *J Pharm Sci*. 2022;111(8):2217–2229. Aug 1.
50. Tourdot S, Abdolzade-Bavil A, Bessa J, Broët P, Fogdell-Hahn A, Giorgi M, et al. European immunogenicity platform open symposium on immunogenicity of biopharmaceuticals. *MAbs*. 2020;12(1). Jan 1.
51. Gervais D. Protein deamidation in biopharmaceutical manufacture: understanding, control and impact. *J Chem Technol Biotechnol*. 2016;91(3):569–575. Mar 8.
52. Peyron I, Dimitrov JD, Delignat S, Gangadharan B, Srivastava A, Kaveri SV, et al. Oxidation of factor VIII increases its immunogenicity in mice with severe hemophilia A. *Cell Immunol*. 2018;325:64–68. Mar.
53. Arentz-Hansen H, Körner R, Molberg Ø, Quarsten H, Vader W, Kooy YMC, et al. The intestinal T cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med*. 2000;191(4):603–612. Feb 21.

54. Kijanka G, Prokopowicz M, Schellekens H, Brinks V. Influence of aggregation and route of injection on the biodistribution of mouse serum albumin. *PLoS One*. 2014; 9(1), e85281. Jan 22.
55. Kinderman F, Yerby B, Jawa V, Joubert MK, Joh NH, Malella J, et al. Impact of precipitation of antibody therapeutics after subcutaneous injection on pharmacokinetics and immunogenicity. *J Pharm Sci*. 2019;108(6):1953–1963. Jun.
56. Fathallah AM, Chiang M, Mishra A, Kumar S, Xue L, Russell Middaugh C, et al. The effect of small oligomeric protein aggregates on the immunogenicity of intravenous and subcutaneous administered antibodies. *J Pharm Sci*. 2015;104(11):3691–3702. Nov.
57. Kijanka G, Bee JS, Korman SA, Wu Y, Roskos LK, Schenerman MA, et al. Submicron size particles of a murine monoclonal antibody are more immunogenic than soluble oligomers or micron size particles upon subcutaneous administration in mice. *J Pharm Sci*. 2018;107(11):2847–2859. Nov.
58. Ahmadi M, Bryson CJ, Cloake EA, Welch K, Filipe V, Romeijn S, et al. Small amounts of sub-visible aggregates enhance the immunogenic potential of monoclonal antibody therapeutics. *Pharm Res*. 2015;32(4):1383–1394. Apr 16.
59. Barnard JG, Babcock K, Carpenter JF. Characterization and quantitation of aggregates and particles in interferon-β products: potential links between product quality attributes and immunogenicity. *J Pharm Sci*. 2013;102(3):915–928. Mar.
60. Ratanji KD, Dearman RJ, Kimber I, Thorpe R, Wadhwa M, Derrick JP. Editor's highlight: subvisible aggregates of immunogenic proteins promote a Th1-type response. *Toxicol Sci*. 2016;153(2):258–270. Oct.
61. Fradkin AH, Carpenter JF, Randolph TW. Glass particles as an adjuvant: a model for adverse immunogenicity of therapeutic proteins. *J Pharm Sci*. 2011;100(11): 4953–4964. Nov.
62. Vanderlaan M, Zhu-Shimoni J, Lin S, Gunawan F, Waerner T, Van Cott KE. Experience with host cell protein impurities in biopharmaceuticals. *Biotechnol Prog*. 2018;34(4):828–837. Jul 10.
63. Van Beers MMC, Gilli F, Schellekens H, Randolph TW, Jiskoot W. Immunogenicity of recombinant human interferon beta interacting with particles of glass, metal, and polystyrene. *J Pharm Sci*. 2012;101(1):187–199.
64. Shomali M, Tanriverdi S, Freitag AJ, Engert J, Winter G, Siedler M, et al. Dose levels in particulate-containing formulations impact anti-drug antibody responses to murine monoclonal antibody in mice. *J Pharm Sci*. 2015;104(5):1610–1621. May.
65. Shi GH, Pisupati K, Parker JG, Corvari VJ, Payne CD, Xu W, et al. Subcutaneous injection site pain of formulation matrices. *Pharm Res*. 2021;38(5):779–793. May 3.
66. Pilunni D, Santuccio C, Sottosanti L, Felicetti P, Navarra P. Relationship between injection site reactions and different adalimumab formulations. Analysis of the adverse events reported in Italy in the period 2016-2019. *Eur Rev Med Pharmacol Sci*. 2021;25(8):3300–3305. Apr.
67. Kaiser C, Knight A, Nordström D, Pettersson T, Fransson J, Florin-Robertsson E, et al. Injection-site reactions upon Kineret (anakinra) administration: experiences and explanations. *Rheumatol Int*. 2012;32(2):295–299. Feb 1.
68. Usach I, Martinez R, Festini T, Peris JE. Subcutaneous injection of drugs: literature review of factors influencing pain sensation at the injection site. *Adv Ther*. 2019;36 (11):2986–2996. Nov 5.
69. Wang W. Tolerability of hypertonic injectables. *Int J Pharm*. 2015;490(1–2): 308–315. Jul.
70. Singh SK, Mahler HC, Hartman C, Stark CA. Are injection site reactions in monoclonal antibody therapies caused by polysorbate excipient degradants? *J Pharm Sci*. 2018;107(11):2735–2741. Nov.
71. Steele RH, Limaye S, Cleland B, Chow J, Suranyi MG. Hypersensitivity reactions to the polysorbate contained in recombinant erythropoietin and darbepoietin (Case Report). *Nephrology*. 2005;10(3):317–320. Jun 14.
72. Pérez-Pérez L, García-Gavín J, Piñeiro B, Zulaica A. Biologic-induced urticaria due to polysorbate 80: usefulness of prick test. *Br J Dermatol*. 2011;164(5):1119–1120. May.
73. Jani M, Barton A, Warren RB, Griffiths CEM, Chinoy H. The role of DMARDs in reducing the immunogenicity of TNF inhibitors in chronic inflammatory diseases. *Rheumatology*. 2014;53(2):213–222. Feb 1.
74. Colombel JF, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, et al. *Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease*. 362. New England Journal of Medicine; 2010:1383–1395. Apr 15.
75. Davda J, Declerck P, Hu-Lieskovan S, Hickling TP, Jacobs IA, Chou J, et al. *Immunogenicity of immunomodulatory, antibody-based, oncology therapeutics*. In: *Journal for ImmunoTherapy of Cancer*. 7. BioMed Central Ltd; 2019.
76. Matter A, Koulov A, Singh S, Mahler HC, Reinisch H, Langer C, et al. Variance between different light obscuration and flow imaging microscopy instruments and the impact of instrument calibration. *J Pharm Sci*. 2019;108(7):2397–2405. Jul.
77. Halo Labs. Rapidly Distinguish Protein from Non-Protein Particles in Biologic Formulations.
78. Khurana R, Coleman C, Ionescu-Zanetti C, Carter SA, Krishna V, Grover RK, et al. Mechanism of thioflavin T binding to amyloid fibrils. *J Struct Biol*. 2005;151(3): 229–238. Sep.
79. Wong B, Zhao X, Su Y, Ouyang H, Rhodes T, Xu W, et al. Characterizing silicone oil-induced protein aggregation with stimulated raman scattering imaging. *Mol Pharm*. 2023;20(8):4268–4276. Aug 7.
80. Gokemeijer J, Wen Y, Jawa V, Mitra-Kaushik S, Chung S, Goggins A, et al. Survey outcome on immunogenicity risk assessment tools for biotherapeutics: an insight into consensus on methods, application, and utility in drug development. *AAPS J*. 2023;25(4). Jul 1.
81. Yi M, Sun J, Sun H, Wang Y, Hou S, Jiang B, et al. Identification and characterization of an unexpected isomerization motif in CDRH2 that affects antibody activity. *MAbs*. 2023;15(1).
82. Leitner A. *A review of the role of chemical modification methods in contemporary mass spectrometry-based proteomics research*. In: *Analytica Chimica Acta*. 1000. Elsevier B. V.; 2018:2–19.
83. Doll S, Burlingame AL. *Mass spectrometry-based detection and assignment of protein posttranslational modifications*. In: *ACS Chemical Biology*. 10. American Chemical Society; 2015:63–71.
84. Donnelly DP, Rawlins CM, DeHart CJ, Fornelli L, Schachner LF, Lin Z, et al. Best practices and benchmarks for intact protein analysis for top-down mass spectrometry. *Nat Methods*. 2019;16(7):587–594. Jul 1.
85. Wei B, Han G, Tang J, Sandoval W, Zhang YT. Native hydrophobic interaction chromatography hyphenated to mass spectrometry for characterization of monoclonal antibody Minor variants. *Anal Chem*. 2019;91(24):15360–15364. Dec 17.
86. Gaza-Bulseco G, Faldu S, Hurkmans K, Chumsae C, Liu H. Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008;870(1):55–62. Jul 1.
87. Fekete S, Veuthey JL, Beck A, Guillarme D. Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products. *J Pharm Biomed Anal*. 2016;130:3–18. Oct 25.
88. Loew C, Knoblich C, Fichtl J, Alt N, Diepold K, Bulau P, et al. Analytical protein a chromatography as a quantitative tool for the screening of methionine oxidation in monoclonal antibodies. *J Pharm Sci*. 2012;101(11):4248–4257.
89. Candau-Chacon R. FDA perspective on recombinant endotoxin detection systems 2021 USP virtual open forum alternatives to compendial reagents used in the bacterial endotoxins test. 2021.
90. United States Pharmacopeia (USP). *〈85〉 Bacterial Endotoxins Test*. United States Pharmacopeial Convention; 2018.
91. Auclair J, Rathore AS. *Analytical Characterization of Host Cell Proteins (HCPs)*. LCGC North America; 2022:493–495. Oct 1.
92. Verthelyi D, Wang V. Trace levels of innate immune response modulating impurities (IIRMIs) synergize to break tolerance to therapeutic proteins. *PLoS One*. 2010;5(12).
93. Jawa V, Joubert MK, Zhang Q, Deshpande M, Hapuarachchi S, Hall MP, et al. Evaluating immunogenicity risk due to host cell protein impurities in antibody-based biotherapeutics. *AAPS J*. 2016;18(6):1439–1452. Nov 1.
94. Anger PM, von der Esch E, Baumann T, Elsner M, Niessner R, Ivleva NP. Raman microspectroscopy as a tool for microplastic particle analysis. *TrAC Trends Analyt Chem*. 2018;109:214–226. Dec.
95. Freitag AJ, Shomali M, Michalakis S, Biel M, Siedler M, Kaymakcalan Z, et al. Investigation of the immunogenicity of different types of aggregates of a murine monoclonal antibody in mice. *Pharm Res*. 2015;32(2):430–444.
96. Kraus T, Lubitz A, Schließer U, Giese C, Reuschel J, Brecht R, et al. Evaluation of a 3D Human artificial lymph node as test model for the assessment of immunogenicity of protein aggregates. *J Pharm Sci*. 2019;108(7):2358–2366. Jul 1.
97. Bessa J, Boeckle S, Beck H, Buckel T, Schlicht S, Ebeling M, et al. The immunogenicity of antibody aggregates in a novel transgenic mouse model. *Pharm Res*. 2015;32(7):2344–2359. Jul 30.
98. Uchino T, Miyazaki Y, Yamazaki T, Kagawa Y. Immunogenicity of protein aggregates of a monoclonal antibody generated by forced shaking stress with siliconized and nonsiliconized syringes in BALB/c mice. *J Pharmacy Pharmacol*. 2017;69(10):1341–1351. Sep 5.
99. Healey JF, Parker ET, Lollar P. Identification of aggregates in therapeutic formulations of recombinant full-length factor VIII products by sedimentation velocity analytical ultracentrifugation. *J Thrombosis Haemostasis*. 2018;16(2): 303–315. Feb.
100. Thorlaksen C, Schultz HS, Gammelgaard SK, Jiskoot W, Hatzakis NS, Nielsen FS, et al. *In vitro* and *in vivo* immunogenicity assessment of protein aggregate characteristics. *Int J Pharm*. 2023;631, 122490. Jan.
101. Grabarek AD, Weinbuch D, Jiskoot W, Hawe A. Critical evaluation of microfluidic resistive pulse sensing for quantification and sizing of nanometer- and micrometer-sized particles in biopharmaceutical products. *J Pharm Sci*. 2019;108(1):563–573. Jan.
102. Stelzl A, Grabarek A, Schneid S, Jiskoot W, Menzen T, Winter G. Comparison of submicron particle counting methods with a heat stressed monoclonal antibody: effect of electrolytes and implications on sample preparation. *J Pharm Sci*. 2022; 111(7):1992–1999. Jul.
103. Tokuda JM, Xie J, Jawa V, Hawkins JM, Ferbas J, Joh NH, et al. Use of *In vitro* Human skin models to assess potential immune activation In response to biotherapeutic attributes and process-related impurities. *J Pharm Sci*. 2022;111(4): 1012–1023. Apr 1.
104. Lubich C, Malisauskas M, Prenninger T, Wurz T, Matthiessen P, Turecek PL, et al. A flow-cytometry-based approach to facilitate quantification, size estimation and characterization of sub-visible particles in protein solutions. *Pharm Res*. 2015;32 (9):2863–2876. Sep 8.
105. Mach H, Bhambhani A, Meyer BK, Burek S, Davis H, Blue JT, et al. The use of flow cytometry for the detection of subvisible particles in therapeutic protein formulations. *J Pharm Sci*. 2011;100(5):1671–1678. May.
106. Schleinzer F, Strebl M, Blech M, Garidel P. Backgrounded membrane imaging—a valuable alternative for particle detection of biotherapeutics? *J Pharm Innov*. 2023; 18(4):1575–1593. Dec 1.
107. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, et al. *An industry perspective on the monitoring of subvisible particles as a quality attribute for*

protein therapeutics. In: *Journal of Pharmaceutical Sciences*. 99. John Wiley and Sons Inc.; 2010:3302–3321.
108. McAvan BS, Bowsher LA, Powell T, O'Hara JF, Spitali M, Goodacre R, et al. Raman spectroscopy to monitor post-translational modifications and degradation in monoclonal antibody therapeutics. *Anal Chem*. 2020;92(15):10381–10389. Aug 4.
109. Nitika N, Chhabra H, Rathore AS. Raman spectroscopy for *in situ*, real time monitoring of protein aggregation in lyophilized biotherapeutic products. *Int J Biol Macromol*. 2021;179:309–313. May.
110. Wong B, Zhao X, Su Y, Fu D. *Imaging protein aggregation in biopharmaceutical formulation with stimulated Raman scattering microscopy*. SPIE-Intl Soc Optical Eng; 2023:35.
111. Kuroda Y. *Biophysical studies of amorphous protein aggregation and in vivo immunogenicity*. In: *Biophysical Reviews*. 14. Springer Science and Business Media Deutschland GmbH; 2022:1495–1501.
112. Pham NB, Meng WS. Protein aggregation and immunogenicity of biotherapeutics. *Int J Pharm*. 2020;585, 119523. Jul.
113. Shi RL, Xiao G, Dillon TM, Ricci MS, Bondarenko PV. Characterization of therapeutic proteins by cation exchange chromatography-mass spectrometry and top-down analysis. *MAbs*. 2020;12(1). Jan 1.
114. Ma F, Raoufi F, Bailly MA, Fayadat-Dilman L, Tomazela D. Hyphenation of strong cation exchange chromatography to native mass spectrometry for high throughput online characterization of charge heterogeneity of therapeutic monoclonal antibodies. *MAbs*. 2020;12(1). Jan 1.
115. Kaur H, Beckman J, Zhang Y, Li ZJ, Szigeti M, Guttman A. Capillary electrophoresis and the biopharmaceutical industry: therapeutic protein analysis and characterization. *TrAC Trends Analyt Chem*. 2021;144, 116407. Nov.
116. Khalil S, Patel N, Bevillard-Kumar F, Chéry C, Burkitt W, O'Hara J, et al. Characterization and validation of a middle-down hydrophobic interaction chromatography method to monitor methionine oxidation in IgG1. *J Pharmaceut Biomed Anal Open*. 2023;1, 100008. Jun.
117. Fischer P, Merkel OM, Siedler M, Huelsmeyer M. Development of a high throughput oxidation profiling strategy for monoclonal antibody products. *Eur J Pharmaceut Biopharmaceut*. 2024:199. Jun 1.
118. Wang G, Zhang ZT, Jiang B, Zhang X, Li C, Liu M. *Recent advances in protein NMR spectroscopy and their implications in protein therapeutics research*. In: *Analytical and Bioanalytical Chemistry*. 406. Springer Verlag; 2014:2279–2288.
119. Joubert MK, Deshpande M, Yang J, Reynolds H, Bryson C, Fogg M, et al. Use of *in vitro* assays to assess immunogenicity risk of antibody-based biotherapeutics. *PLoS One*. 2016;11(8). Aug 1.
120. Ducret A, Ackaert C, Bessa J, Bunce C, Hickling T, Jawa V, et al. Assay format diversity in pre-clinical immunogenicity risk assessment: toward a possible harmonization of antigenicity assays. *MAbs*. 2022;14(1). Dec 31.
121. Mattei AE, Gutierrez AH, Seshadri S, Tivin J, Ardito M, Rosenberg AS, et al. *silico methods for immunogenicity risk assessment and human homology screening for therapeutic antibodies*. 16. mAbs. Taylor and Francis Ltd.; 2024.
122. Jensen KK, Andreatta M, Marcatili P, Buus S, Greenbaum JA, Yan Z, et al. Improved methods for predicting peptide binding affinity to MHC class II molecules. *Immunology*. 2018;154(3):394–406. Jul 1.
123. Sanchez-Trincado JL, Gomez-Perosanz M, Reche PA. *Fundamentals and methods for T- and B-cell epitope prediction*. In: *Journal of Immunology Research*. 2017. Hindawi Limited; 2017.
124. Zhang L, Chen Y, Wong HS, Zhou S, Mamitsuka H, TEPITOPEpan ZS. Extending TEPITOPE for peptide binding prediction covering over 700 HLA-DR molecules. *PLoS One*. 2012;7(2), e30483. Feb 23.
125. Rammensee HG, Bachmann J, Emmerich NPN, Bachor OA, Stevanović S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999;50 (3–4):213–219. Nov 22.
126. Obrezanova O, Arnell A, de la Cuesta RG, Berthelot ME, Gallagher TR, Zurdo J, et al. Aggregation risk prediction for antibodies and its application to biotherapeutic development. *MAbs*. 2015;7(2):352–363. Mar 4.
127. Martini S, Nielsen M, Peters B, Sette A. *The Immune Epitope Database and Analysis Resource Program 2003–2018: reflections and outlook*. In: *Immunogenetics*. 72. Springer; 2020:57–76.
128. Potocnakova L, Bhide M, Pulzova LB. *An introduction to B-cell epitope mapping and in silico epitope prediction*. In: *Journal of Immunology Research*. 2016. Hindawi Limited; 2016.
129. Pellequer† JL, Westhof E. PREDITOP: a program for antigenicity prediction. *J Mol Graph*. 1993;11(3):204–210. Sep.
130. Alix AJP. Predictive estimation of protein linear epitopes by using the program PEOPLE. *Vaccine*. 1999;18(3–4):311–314. Sep.
131. Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res*. 2017;45(W1):W24–W29. Jul 3.
132. EL-Manzalawy Y, Dobbs D, Honavar V. Predicting linear B-cell epitopes using string kernels. *J Mol Recognit*. 2008;21(4):243–255. Jul 22.
133. Kulkarni-Kale U, Bhosle S, Kolaskar AS. CEP: a conformational epitope prediction server. *Nucleic Acids Res*. 2005;33:W168–W171. Jul 1(Web Server).
134. Sweredoski MJ, Baldi P. PEPITO: improved discontinuous B-cell epitope prediction using multiple distance thresholds and half sphere exposure. *Bioinformatics*. 2008; 24(12):1459–1460. Jun 15.
135. Rubinstein ND, Mayrose I, Martz E, Pupko T. Epitopia: A web-server for predicting B-cell epitopes. *BMC Bioinform*. 2009;10:287. Sep 14.
136. Alakhras NS, Qiu J, Rocha GV, Witcher DR, Koester A, You J, et al. FcγRIIIa-dependent IFN-γ release in whole blood assay is predictive of therapeutic IgG1 antibodies safety. *MAbs*. 2018;10(6):913–921. Aug 18.
137. Duke BR, Mitra-Kaushik S. *Current In vitro assays for prediction of T cell mediated immunogenicity of biotherapeutics and manufacturing impurities*. In: *Journal of Pharmaceutical Innovation*. 15. Springer; 2020:202–218.
138. Liebers V, Kendzia B, Stubel H, Borowitzki G, Gering V, Monsé C, et al. *Advances in Experimental Medicine and Biology*. 1108. Current Trends in Immunity and Respiratory Infections; 2018:25–36. Vol. Volume.
139. Ito S, Miwa K, Hattori C, Aida T, Tsuchiya Y, Mori K. Highly sensitive *in vitro* cytokine release assay incorporating high-density preculture. *J Immunotoxicol*. 2021;18(1):136–143.
140. Joubert MK, Hokom M, Eakin C, Zhou L, Deshpande M, Baker MP, et al. Highly aggregated antibody therapeutics can enhance the *in vitro* innate and late-stage T-cell immune responses. *J Biol Chem*. 2012;287(30):25266–25279. Jul 20.
141. Morgan H, Tseng SY, Gallais Y, Leineweber M, Buchmann P, Riccardi S, et al. Evaluation of *in vitro* assays to assess the modulation of dendritic cells functions by therapeutic antibodies and aggregates. *Front Immunol*. 2019;10(MAR).
142. Lteif M, Pallardy M, Turbica I. *Antibodies internalization mechanisms by dendritic cells and their role in therapeutic antibody immunogenicity*. In: *European Journal of Immunology*. 54. John Wiley and Sons Inc; 2024.
143. Jarvi NL, Balu-Iyer SV. A mechanistic marker-based screening tool to predict clinical immunogenicity of biologics. *Communications Medicine*. 2023;3(1). Dec 8.
144. Clatworthy MR, Aronin CEP, Mathews RJ, Morgan NY, Smith KGC, Germain RN. Immune complexes stimulate CCR7-dependent dendritic cell migration to lymph nodes. *Nat Med*. 2014;20(12):1458–1463. Dec 1.
145. Wen Y, Cahya S, Zeng W, Lin J, Wang X, Liu L, et al. Development of a FRET-based assay for analysis of mAbs internalization and processing by dendritic cells in preclinical immunogenicity risk assessment. *AAPS J*. 2020;22(3). May 1.
146. Di Ianni A, Fraone T, Balestra P, Cowan K, Sirtori FR, Barbero L. Assessing MAPPs assay as a tool to predict the immunogenicity potential of protein therapeutics. *Life Sci Alliance*. 2024;7(1). Jan 1.
147. Karle AC. *Applying MAPPs Assays to Assess Drug Immunogenicity*. 11. Front Immunol; 2020. Apr 21.
148. Rombach-Riegraf V, Karle AC, Wolf B, Sordé L, Koepke S, Gottlieb S, et al. Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses *in vitro*. *PLoS One*. 2014;9 (1). Jan 21.
149. Gokemeijer J. *Immunogenicity risk assessment for biotherapeutics*. *Bioanalytical Aspects in Biological Therapeutics*. John Wiley and Sons Inc.; 2022:141–158.
150. Di Ianni A, Barbero L, Fraone T, Cowan K, Sirtori FR. *Preclinical risk assessment strategy to mitigate the T-cell dependent immunogenicity of protein biotherapeutics: state of the art, challenges and future perspectives*. In: *Journal of Pharmaceutical and Biomedical Analysis*. 234. Elsevier B.V.; 2023.
151. Walsh RE, Nix A, Ackaert C, Mazy A, Schockaert J, Pattyn S, et al. Preclinical immunogenicity risk assessment of biotherapeutics using CD4 T cell assays. *Front Immunol*. 2024:15.
152. Siegel M, Steiner G, Franssen LC, Carratu F, Herron J, Hartman K, et al. Validation of a dendritic cell and CD4+ T cell restimulation assay contributing to the immunogenicity risk evaluation of biotherapeutics. *Pharmaceutics*. 2022;14(12). Dec 1.
153. Jawa V, Cousens LP, Awwad M, Wakshull E, Kropshofer H, De Groot AS. *T-cell dependent immunogenicity of protein therapeutics: preclinical assessment and mitigation*. In: *Clinical Immunology*. 149. Academic Press Inc.; 2013:534–555.
154. Cohen S, Myneni S, Batt A, Guerrero J, Brumm J, Chung S. Immunogenicity risk assessment for biotherapeutics through *in vitro* detection of CD134 and CD137 on T helper cells. *MAbs*. 2021;13(1).
155. De Groot AS, Rosenberg A, Miah SMS, Skowron G, Roberts BJ, Lélias S, et al. Identification of a potent regulatory T cell epitope in factor V that modulates CD4+ and CD8+ memory T cell responses. *Clin Immunol*. 2021:224. Mar 1.
156. Lollier V, Denery-Papini S, Larré C, Tessier D. A generic approach to evaluate how B-cell epitopes are surface-exposed on protein structures. *Mol Immunol*. 2011;48(4): 577–585. Jan.
157. Marsman C, Verhoeven D, Koers J, Rispens T, ten Brinke A, van Ham SM, et al. Optimized protocols for In-vitro T-cell-dependent and T-cell-independent activation for B-cell differentiation studies using limited cells. *Front Immunol*. 2022; 13. Jun 29.
158. Van Belle K, Herman J, Boon L, Waer M, Sprangers B, Louat T. Comparative *In vitro* immune stimulation analysis of primary Human B cells and B cell lines. *J Immunol Res*. 2016;2016:1–9.
159. Cohen S, Chung S. *In vitro* immunogenicity prediction: bridging between innate and adaptive immunity. *Bioanalysis*. 2021;13:1071–1081.
160. Porebski G, Kwiecien K, Pawica M, Kwitniewski M. Mas-related G protein-coupled receptor-X2 (MRGPRX2) in drug hypersensitivity reactions. *Front Immunol*. 2018;9. Dec 20.
161. Hermans MAW, van Stigt AC, van de Meerendonk S, Schrijver B, van Daele PLA, van Hagen PM, et al. Human mast cell line HMC1 expresses functional mas-related G-protein coupled receptor 2. *Front Immunol*. 2021;12. Mar 15.
162. John LM, Dalsgaard CM, Jeppesen CB, Conde-Frieboes KW, Baumann K, Knudsen NPH, et al. *In vitro* prediction of *in vivo* pseudo-allergenic response via MRGPRX2. *J Immunotoxicol*. 2021;18(1):30–36.
163. Leach MW, Rottman JB, Hock MB, Finco D, Rojko JL, Beyer JC. Immunogenicity/ hypersensitivity of biologics. *Toxicol Pathol*. 2014;42(1):293–300. Jan.
164. Krishna M, Nadler SG. *Immunogenicity to biotherapeutics - the role of anti-drug immune complexes*. In: *Frontiers in Immunology*. 7. Frontiers Research Foundation; 2016.
165. Brennan FR, Kiessling A. *In vitro* assays supporting the safety assessment of immunomodulatory monoclonal antibodies. *Toxicol Vitro*. 2017;45:296–308. Dec.

166. FDA. CDER. Nonclinical evaluation of the immunotoxic potential of pharmaceuticals guidance for industry [Internet]. Available from: https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs; 2023.
167. Sanquin. Whole blood complement activation assay [Internet] [cited 2024 Jun 10]. Available from: https://www.sanquin.org/products-and-services/immunomonitoring-services/clinical-immunomonitoring/assays/whole-blood-complement-activation-assay; 2021.
168. Immuneed. Complement activation [Internet] [cited https://immuneed.com/our-services/complement-activation-and-inhibition-cdc-adcc/?utm_term=complement%20activation%20assay&utm_campaign=EU+%7C+Complement+Activation&utm_source=adwords&utm_medium=ppc&hsa_acc=6116467023&hsa_cam=9248714565&hsa_grp=93780347477&hsa_ad=699741276956&hsa_src=g&hsa_tgt=kwd-351718533584&hsa_kw=complement%20activation%20assay&hsa_mt=e&hsa_net=adwords&hsa_ver=3&gad_source=1&gclid=CjwKCAjw65-zBhBkEiwAjrqRMPCTCeBS_ekkYPwtDMSj41AzqHFlVbVgrD1sCdGAu4H4lvdgFXKU0hoCYrUQAvD_BwE; 2024.
169. BD Biosciences. BD™ cytometric bead array (CBA) Human anaphylatoxin kit [Internet] [cited https://www.bdbiosciences.com/en-us/products/reagents/immunoassay-reagents/cba/cba-kits/human-anaphylatoxin-kit.561418; 2024.
170. Wessig AK, Hoffmeister L, Klingberg A, Alberts A, Pich A, Brand K, et al. Natural antibodies and CRP drive anaphylatoxin production by urate crystals. *Sci Rep*. 2022;12(1). Dec 1.
171. Ricklin D, Reis ES, Lambris JD. *Complement in disease: a defence system turning offensive*. In: *Nature Reviews Nephrology*. 12. Nature Publishing Group; 2016: 383–401.
172. Groell F, Jordan O, Borchard G. *In vitro models for immunogenicity prediction of therapeutic proteins*. In: *European Journal of Pharmaceutics and Biopharmaceutics*. 130. Elsevier B.V.; 2018:128–142.
173. Lubitz A, Giese C. *Human artificial lymph node (HuALN). Encyclopedia of Immunotoxicology*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014:1–6.
174. Goyal G, Prabhala P, Mahajan G, Bausk B, Gilboa T, Xie L, et al. Ectopic lymphoid follicle formation and Human seasonal influenza vaccination responses recapitulated in an organ-on-a-chip. *Adv Sci*. 2022;9(14). May 14.
175. Mitra B, Jindal R, Lee S, Dong DX, Li L, Sharma N, et al. Microdevice integrating innate and adaptive immune responses associated with antigen presentation by dendritic cells. *RSC Adv*. 2013;3(36), 16002.
176. Hofmann E, Schwarz A, Fink J, Kamolz LP, Kotzbeck P. *Modelling the complexity of Human skin In vitro*. In: *Biomedicines*. 11. MDPI; 2023.
177. Vidal SEL, Tamamoto KA, Nguyen H, Abbott RD, Cairns DM, Kaplan DL. 3D biomaterial matrix to support long term, full thickness, immuno-competent human skin equivalents with nervous system components. *Biomaterials*. 2019;198: 194–203. Apr 1.
178. Ghose S, Ju Y, McDonough E, Ho J, Karunamurthy A, Chadwick C, et al. 3D reconstruction of skin and spatial mapping of immune cell density, vascular distance and effects of sun exposure and aging. *Commun Biol*. 2023;6(1):718. Jul 19.
179. Eskes C., Van Vliet E., Maibach H.I., Goldberg A.M. Alternatives for Dermal Toxicity Testing.
180. Zhou L, Ji W, Dicolandrea T, Finlay D, Supp D, Boyce S, et al. An improved human skin explant culture method for testing and assessing personal care products. *J Cosmet Dermatol*. 2023;22(5):1585–1594. May 1.
181. Neil JE, Brown MB, Williams AC. Human skin explant model for the investigation of topical therapeutics. *Sci Rep*. 2020;10(1). Dec 1.
182. Dickinson A, Wang XN, Ahmed S. *An In vitro Human skin test for assessing adverse immune reactions and sensitization potential. Alternatives for Dermal Toxicity Testing*. Cham: Springer International Publishing; 2017:437–448.
183. Ahmed SS, Ahmed MM, Ishaq A, Freer M, Stebbings R, Dickinson AM. An *In vitro* Human skin test for predicting skin sensitization and adverse immune reactions to biologics. *Toxics*. 2024;12(6):401. May 30.
184. Wen D, Boissel JPR, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals [Internet]. Available from: http://ashpublications.org/blood/article-pdf/82/5/1507/611147/1507.pdf; 1993.
185. Tao L, Reese TA. *Making mouse models that reflect Human immune responses*. In: *Trends in Immunology*. 38. Elsevier Ltd; 2017:181–193.
186. Jiskoot W, Kijanka G, Randolph TW, Carpenter JF, Koulov AV, Mahler HC, et al. *Mouse models for assessing protein immunogenicity: lessons and challenges*. In: *Journal of Pharmaceutical Sciences*. 105. Elsevier B.V.; 2016:1567–1575.
187. Mestas J, Hughes CCW. IMMUNOLOGY of mice and not men: differences between mouse and Human immunology [Internet]. *J Immunol*. 2004;172. Available from: http://journals.aai.org/jimmunol/article-pdf/172/5/2731/1181081/2731.pdf.
188. Pan-Hammarström Q, Zhao Y, Hammarström L. Class switch recombination: a comparison between mouse and human. *Adv. Immunol.* 2007;93:1–61.
189. Kraus T, Winter G, Engert J. Test models for the evaluation of immunogenicity of protein aggregates. *Int J Pharm*. 2019;559:192–200.
190. Ramadan A., Fathallah A.M. AAPS National Biotechnology Conference. 2023 [cited 2024 Jul 20]. (M1130-01-04) XenoImmune™ mouse model, a novel and proprietary animal model that recapitulates clinical immunogenicity rates of biologics. Available from: https://www.eventscribe.net/2023/NBC//fsPopup.asp?efp=Rk1PU0dYVkgxOTA1OA&PosterID=561329&rnd=0.533424&mode=posterinfo.
191. Prior H, Haworth R, Labram B, Roberts R, Wolfreys A, Sewell F. Justification for species selection for pharmaceutical toxicity studies. *Toxicol Res (Camb)*. 2021;9(6):758–770.
192. Wildner G. Are rats more human than mice? *Immunobiology*. 2019;224(1):172–176. Jan 1.
193. Haley PJ. Species differences in the structure and function of the immune system. *Toxicology*. 2003;188(1):49–71. Jun.
194. Herskovitz J, Ryman J, Thway T, Lee S, Zhou L, Chirmule N, et al. Immune suppression during preclinical drug development mitigates immunogenicity-mediated impact on therapeutic exposure. *AAPS J*. 2017;19(2):447–455. Mar 1.
195. Jeon EJ, Han JH, Seo Y, Koh EM, Han KH, Hwang K, et al. Implementation of systematic bioanalysis of antibody–drug conjugates for preclinical pharmacokinetic study of Ado-Trastuzumab Emtansine (T-DM1) in rats. *Pharmaceutics*. 2023;15(3). Mar 1.
196. Kim YJ, Koh EM, Song CH, Byun MS, Choi YR, Jeon EJ, et al. Preclinical immunogenicity testing using anti-drug antibody analysis of GX-G3, Fc-fused recombinant human granulocyte colony-stimulating factor, in rat and monkey models. *Sci Rep*. 2021;11(1). Dec 1.
197. Thway TM, Magana I, Bautista A, Jawa V, Gu W, Ma M. Impact of anti-drug antibodies in preclinical pharmacokinetic assessment. *AAPS J*. 2013;15(3): 856–863. Jul.
198. Brinks V, Weinbuch D, Baker M, Dean Y, Stas P, Kostense S, et al. Preclinical models used for immunogenicity prediction of therapeutic proteins. *Pharm Res*. 2013;30:1719–1728.
199. Richter WF, Bhansali SG, Morris ME. Mechanistic determinants of biotherapeutics absorption following SC administration. *AAPS J*. 2012;14:559–570.
200. Richter WF, Grimm HP, Gouy MH, Søgaard S, Kreuzer C, Wessels U, et al. Subcutaneous site-of-absorption study with the monoclonal antibody tocilizumab in minipigs: administration behind ear translates best to humans. *AAPS J*. 2020;22(3). May 1.
201. Rubic-Schneider T, Christen B, Brees D, Kammüller M. *Minipigs in translational immunosafety sciences*. In: *Toxicologic Pathology*. 44. SAGE Publications Inc.; 2016: 315–324.
202. van Mierlo GJD, Cnubben NHP, Wouters D, Wolbink GJ, Hart MHL, Rispens T, et al. The minipig as an alternative non-rodent model for immunogenicity testing using the TNFα blockers adalimumab and infliximab. *J Immunotoxicol*. 2014;11(1): 62–71. Jan 5.
203. Hamuro L, Kijanka G, Kinderman F, Kropshofer H, xiu BuD, Zepeda M, et al. Perspectives on subcutaneous route of administration as an immunogenicity risk factor for therapeutic proteins. *J Pharm Sci*. 2017;106:2946–2954.
204. Flisikowska T, Egli J, Flisikowski K, Stumbaum M, Küng E, Ebeling M, et al. A humanized minipig model for the toxicological testing of therapeutic recombinant antibodies. *Nat Biomed Eng*. 2022;6(11):1248–1256. Nov 1.
205. Messaoudi I, Estep R, Robinson B, Wong SW. Nonhuman primate models of Human immunology. *Antioxid Redox Signal*. 2011;14(2):261–273. Jan 15.
206. Ackley D, Birkebak J, Blumel J, Bourcier T, de Zafra C, Goodwin A, et al. FDA and industry collaboration: identifying opportunities to further reduce reliance on nonhuman primates for nonclinical safety evaluations. *Regul Toxicol Pharmacol*. 2023;138, 105327. Feb.
207. Van Meer PJK, Kooijman M, Brinks V, Gispen-De Wied CC, Silva-Lima B, Moors EHM, et al. Immunogenicity of mAbs in non-human primates during nonclinical safety assessment. *MAbs*. 2013;5(5):810–816. Sep.
208. Lee HJ, Riley G, Johnson O, Cleland JL, Kim N, Charnis M, et al. *In vivo* characterization of sustained-release formulations of human growth hormone. *J Pharmacol Exp Ther*. 1997;281(3):1431–1439. Jun.
209. Zwickl C. Comparison of the immunogenicity of recombinant and pituitary human growth hormone in rhesus monkeys*1. *Fundam Appl Toxicol*. 1991;16(2):275–287. Feb.
210. Adam L, Rosenbaum P, Cosma A, Le Grand R, Martinon F. Identification of skin immune cells in non-human primates. *J Immunol Methods*. 2015;426:42–49. Nov 1.
211. Puig M, Tosh KW, Schramm LM, Grajkowska LT, Kirschman KD, Tami C, et al. TLR9 and TLR7 agonists mediate distinct type I IFN responses in humans and nonhuman primates *in vitro* and *in vivo*. *J Leukoc Biol*. 2011;91(1):147–158. Nov 4.
212. Sasaki E, Suemizu H, Shimada A, Hanazawa K, Oiwa R, Kamioka M, et al. Generation of transgenic non-human primates with germline transmission. *Nature*. 2009;459(7246):523–527. May 28.
213. Lotz GP, Benstein K, Bloem K, Buddiger H, Calonder C, Elm S, et al. When to extend monitoring of anti-drug antibodies for high-risk biotherapeutics in clinical trials: an opinion from the European Immunogenicity Platform. *AAPS J*. 2022;24.
214. Fda, Cder. Immunogenicity testing of therapeutic protein products —developing and validating assays for anti-drug antibody detection guidance for industry [Internet]. Available from: https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htmand/orhttps://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm; 2019.
215. Shakhnovich V, Meibohm B, Rosenberg A, Kierzek AM, Hasenkamp R, Funk RS, et al. Immunogenicity in clinical practice and drug development: when is it significant? *Clin Transl Sci*. 2020;13:219–223.
216. St Clair-Jones A, Prignano F, Goncalves J, Paul M, Sewerin P. Understanding and minimising injection-site pain following subcutaneous administration of biologics: a narrative review. *Rheumatol Therapy*. 2020;7:741–757.
217. Zhuang CL, Lin ZJ, Bi ZF, Qiu LX, Hu FF, Liu XH, et al. Inflammation-related adverse reactions following vaccination potentially indicate a stronger immune response. *Emerg Microbes Infect*. 2021;10(1):365–375.
218. Taghizadeh B, Jaafari MR, Zarghami N. New insight into the importance of formulation variables on parenteral growth hormone preparations: potential effect on the injection-site pain. *Front Endocrinol Front Media S.A.* 2022;13.
219. Hjermstad MJ, Fayers PM, Haugen DF, Caraceni A, Hanks GW, Loge JH, et al. Studies comparing numerical rating scales, verbal rating scales, and visual

analogue scales for assessment of pain intensity in adults: a systematic literature review. *J Pain Symptom Manag*. 2011;41:1073–1093.
220. Thomaidou E, Ramot Y. Injection site reactions with the use of biological agents. *Dermatol Ther*. 2019;32(2), e12817. Mar 29.
221. Roth EM, Goldberg AC, Catapano AL, Torri A, Yancopoulos GD, Stahl N, et al. *Antidrug Antibodies in Patients Treated with Alirocumab*. 376. New England Journal of Medicine; 2017:1589–1590. Apr 20.
222. Benucci M, Gobbi FL, Meacci F, Manfredi M, Infantino M, Severino M, et al. Antidrug antibodies against TNF-blocking agents: correlations between disease activity, hypersensitivity reactions, and different classes of immunoglobulins. *Biologics*. 2015;9:7–12. Feb 17.
223. Suh K, Kyei I, Hage DS. Approaches for the detection and analysis of antidrug antibodies to biopharmaceuticals: a review. *J Sep Sci*. 2022;45:2077–2092.
224. Gorovits B, Baltrukonis DJ, Bhattacharya I, Birchler MA, Finco D, Sikkema D, et al. Immunoassay methods used in clinical studies for the detection of anti-drug antibodies to adalimumab and infliximab. *Clin Exp Immunol*. 2018;192(3): 348–365. Jun 1.
225. Partridge MA, Purushothama S, Elango C, Lu Y. Emerging technologies and generic assays for the detection of anti-drug antibodies. *J Immunol Res*. 2016;2016.
226. Bloem K, van Leeuwen A, Verbeek G, Nurmohamed MT, Wolbink GJ, van der Kleij D, et al. Systematic comparison of drug-tolerant assays for anti-drug antibodies in a cohort of adalimumab-treated rheumatoid arthritis patients. *J Immunol Methods*. 2015;418:29–38.
227. van Schouwenburg PA, Krieckaert CL, Rispens T, Aarden L, Wolbink GJ, Wouters D. Long-term measurement of anti-adalimumab using pH-shift-anti-idiotype antigen binding test shows predictive value and transient antibody formation. *Ann Rheum Dis*. 2013;72(10):1680–1686. Oct.
228. van Strien J, Dijk L, Atiqi S, Schouten R, Bloem K, Wolbink GJ, et al. Drug-tolerant detection of anti-drug antibodies in an antigen-binding assay using europium chelate fluorescence. *J Immunol Methods*. 2023;514, 113436. Mar.
229. Mano Y. An electrochemiluminescence assay for quantification of Denileukin Diftitox and its anti-drug antibodies in rat serum. *J Pharmacol Toxicol Methods*. 2023;119, 107239. Jan.
230. Aoyama M, Mano Y. Quantification of anti-drug antibodies against E6011, an anti-fractalkine monoclonal antibody, in monkey and human serum, by an electrochemiluminescence assay. *J Pharmacol Toxicol Methods*. 2023;124, 107470. Nov.
231. Li J, Schantz A, Schwegler M, Shankar G. Detection of low-affinity anti-drug antibodies and improved drug tolerance in immunogenicity testing by Octet® biolayer interferometry. *J Pharm Biomed Anal*. 2011;54(2):286–294. Jan.
232. Van Roy M, Ververken C, Beirnaert E, Hoefman S, Kolkman J, Vierboom M, et al. The preclinical pharmacology of the high affinity anti-IL-6R Nanobody® ALX-0061 supports its clinical development in rheumatoid arthritis. *Arthritis Res Ther*. 2015; 17(1). May 20.
233. Shibata H, Nishimura K, Miyama C, Tada M, Suzuki T, Saito Y, et al. Comparison of different immunoassay methods to detect human anti-drug antibody using the WHO erythropoietin antibody reference panel for analytes. *J Immunol Methods*. 2018;452:73–77. Jan.
234. Wyant T, Yang L, Rosario M. Comparison of the ELISA and ECL Assay for Vedolizumab anti-drug antibodies: assessing the impact on pharmacokinetics and safety outcomes of the phase 3 GEMINI trials. *AAPS Journal*. 2021;23(1). Jan 1.
235. Barbosa M, Gokemeijer J, Martin AD, Bush A. Altering drug tolerance of surface plasmon resonance assays for the detection of anti-drug antibodies. *Anal Biochem*. 2013;441(2):174–179. Oct.
236. Beeg M, Nobili A, Orsini B, Rogai F, Gilardi D, Fiorino G, et al. A surface Plasmon resonance-based assay to measure serum concentrations of therapeutic antibodies and anti-drug antibodies. *Sci Rep*. 2019;9(1). Dec 1.
237. Stubenrauch K, Mackeben K, Vogel R, Heinrich J. Generic anti-drug antibody assay with drug tolerance in serum samples from mice exposed to human antibodies. *Anal Biochem*. 2012;430(2):193–199. Nov.
238. Jani M, Isaacs JD, Morgan AW, Wilson AG, Plant D, Hyrich KL, et al. Detection of anti-drug antibodies using a bridging ELISA compared with radioimmunoassay in adalimumab-treated rheumatoid arthritis patients with random drug levels. *Rheumatology*. 2016;55(11):2050–2055. Nov.
239. Li M, Li H, Gao K, Wang M, An W, Zhu Y, et al. A simple and cost-effective assay for measuring anti-drug antibody in human patients treated with Adalimumab. *J Immunol Methods*. 2018;452:6–11. Jan.
240. van Mierlo GJD, Cnubben NHP, Kuper CF, Wolthoorn J, van Meeteren-Kreikamp AP, Nagtegaal MM, et al. The Göttingen minipig as an alternative non-rodent species for immunogenicity testing: a demonstrator study using the IL-1 receptor antagonist anakinra. *J Immunotoxicol*. 2013;10(1):96–105. Mar 7.
241. Vande Casteele N, Buurman DJ, Sturkenboom MGG, Kleibeuker JH, Vermeire S, Rispens T, et al. Detection of infliximab levels and anti-infliximab antibodies: a comparison of three different assays. *Aliment Pharmacol Ther*. 2012;36(8):765–771. Oct 28.
242. Bourdage JS, Cook CA, Farrington DL, Chain JS, Konrad RJ. An Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. *J Immunol Methods*. 2007;327 (1–2):10–17. Oct.
243. Boado RJ, Hui EKW, Lu JZ, Pardridge WM. IgG-Enzyme fusion protein: pharmacokinetics and anti-drug antibody response in Rhesus monkeys. *Bioconjug Chem*. 2013;24(1):97–104. Jan 16.
244. Boado RJ, Ka-Wai Hui E, Zhiqiang Lu J, Pardridge WM. Insulin receptor antibody-iduronate 2-sulfatase fusion protein: pharmacokinetics, anti-drug antibody, and safety pharmacology in Rhesus monkeys. *Biotechnol Bioeng*. 2014;111(11): 2317–2325. Nov 5.
245. Wiswell D, Neupane D, Chen M, Bowman EP, Linn D, Sawant A, et al. A capillary electrophoresis based approach for the identification of anti-drug antibodies against camelid VHH biologics (Nanobodies®). *J Pharmacol Toxicol Methods*. 2020; 103, 106872. May.
246. Zhang S, Chen M, Zhou S, Raoufi F, Wiswell D, Hsieh S, et al. Development of a novel capillary electrophoresis-based approach for detection of anti-drug antibody responses. *J Immunol Methods*. 2021;494, 113047. Jul.
247. Poulsen S, Jørgensen L, Galle PS. Heat pre-treatment can abolish anti-drug antibody interference in ligand binding pharmacokinetic assays. *AAPS Open*. 2022; 8(1). Dec.
248. Kneepkens EL, Wei JCC, Nurmohamed MT, Yeo KJ, Chen CY, van der Horst-Bruinsma IE, et al. Immunogenicity, adalimumab levels and clinical response in ankylosing spondylitis patients during 24 weeks of follow-up. *Ann Rheum Dis*. 2015;74(2):396–401. Feb.
249. Krintel SB, Grunert VP, Hetland ML, Johansen JS, Rothfuss M, Palermo G, et al. The frequency of anti-infliximab antibodies in patients with rheumatoid arthritis treated in routine care and the associations with adverse drug reactions and treatment failure. *Rheumatology*. 2013;52(7):1245–1253. Jul 1.
250. Luo YR, Chakraborty I, Lazar-Molnar E, Wu AHB, Lynch KL. Development of label-free immunoassays as novel solutions for the measurement of monoclonal antibody drugs and antidrug antibodies. *Clin Chem*. 2020;66(10):1319–1328. Oct 1.
251. Rojas JR, Taylor RP, Cunningham MR, Rutkoski TJ, Vennarini J, Jang H, et al. Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in Cynomolgus monkeys. *J Pharmacol Exp Therap*. 2005;313(2): 578–585. May.
252. Wang SL, Hauenstein S, Ohrmund L, Shringarpure R, Salbato J, Reddy R, et al. Monitoring of adalimumab and antibodies-to-adalimumab levels in patient serum by the homogeneous mobility shift assay. *J Pharm Biomed Anal*. 2013;78–79:39–44. May.
253. Wang SL, Ohrmund L, Hauenstein S, Salbato J, Reddy R, Monk P, et al. Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum. *J Immunol Methods*. 2012;382(1–2):177–188. Aug.
254. Pavlov IY, Carper J, Lázár-Molnár E, Delgado JC. Clinical laboratory application of a reporter-gene assay for measurement of functional activity and neutralizing antibody response to infliximab. *Clin Chimica Acta*. 2016;453:147–153. Jan.
255. Lallemand C, Kavrochorianou N, Steenholdt C, Bendtzen K, Ainsworth MA, Meritet JF, et al. Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNFα antagonists. *J Immunol Methods*. 2011;373 (1–2):229–239. Oct.
256. Roos D, Chen L, Vesapogu R, Kane C, Duggan J, Norris S. Detection of cynomolgus monkey anti-protein XYZ antibody using immunocapture-LC/MS. *J Appl Bioanal*. 2016;2(4):117–128. Oct 13.
257. Heinig K, Wirz T, Schick E, Guenzi A. Bioanalysis of therapeutic peptides: differentiating between total and anti-drug antibody bound drug using liquid chromatography–tandem mass spectrometry quantitation. *J Chromatogr A*. 2013; 1316:69–77. Nov.
258. El Amrani M, Göbel C, Egas AC, Nierkens S, Hack CE, Huitema ADR, et al. Quantification of neutralizing anti-drug antibodies and their neutralizing capacity using competitive displacement and tandem mass spectrometry: infliximab as proof of principle. *J Transl Autoimmun*. 2019;1, 100004. Apr.
259. Smeijsters EH, van der Elst KCM, Visch A, Göbel C, Loeff FC, Rispens T, et al. Optimization of a quantitative anti-drug antibodies against Infliximab assay with the liquid chromatography-tandem mass spectrometry: a method validation study and future perspectives. *Pharmaceutics*. 2023;15(5):1477. May 12.
260. FDA. CDER. Immunogenicity testing of therapeutic protein products —developing and validating assays for anti-drug antibody detection guidance for industry [Internet]. Available from: https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htmand/orhttps://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm; 2019.
261. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP) 3 4 5 Guideline on Immunogenicity assessment of 6 biotechnology-derived therapeutic proteins [Internet]. Available from: www.ema.europa.eu/contact; 2015.
262. Committee for medicinal products for human use (CHMP). ICH guideline S6 (R1)-preclinical safety evaluation of biotechnology-derived pharmaceuticals part I (Parent guideline) preclinical safety evaluation of biotechnology-derived pharmaceuticals [Internet]. Available from: www.ema.europa.eu; 2011.
263. Wadhwa M. Harmonisation of immunogenicity testing: the EU perspective. 2018.
264. HESI. Immuno-Safety Technical Committee (ITC) [Internet] [cited 2025 Mar 27]. Available from: https://hesiglobal.org/immuno-safety/; 2025.
265. Rup B, Pallardy M, Sikkema D, Albert T, Allez M, Broet P, et al. Standardizing terms, definitions and concepts for describing and interpreting unwanted immunogenicity of biopharmaceuticals: recommendations of the Innovative Medicines Initiative ABIRISK consortium. *Clin Exp Immunol*. 2015;181(3):385–400. Sep 1.
266. Innovative Medicine Initiative, ABIRISK [Internet]. [cited 2024 May 27]. Available from: Innovative Medicine Initiative, ABIRISK.
267. Sánchez-Félix M, Burke M, Chen HH, Patterson C, Mittal S. *Predicting bioavailability of monoclonal antibodies after subcutaneous administration: open innovation challenge*. In: *Advanced Drug Delivery Reviews*. 167. Elsevier B.V.; 2020:66–77.
268. Yuan YT, Braun M, Lembke W, McBlane F, Kamerud J, DeWall S, et al. *Immunogenicity assessment of AAV-based gene therapies: An IQ consortium industry white paper*. 26. Molecular Therapy Methods and Clinical Development. Cell Press; 2022:471–494.

269. Vessillier S, Fort M, O'Donnell L, Hinton H, Nadwodny K, Piccotti J, et al. Development of the first reference antibody panel for qualification and validation of cytokine release assay platforms – report of an international collaborative study. *Cytokine X*. 2020;2(4). Dec 1.
270. Liu H, Pang E. *In vitro* immunogenicity assays for evaluating generic peptide drug products [Internet] [cited 2024 May 27]. Available from: https://www.fda.gov/science-research/fda-science-forum/in-vitro-immunogenicity-assays-evaluating-generic-peptide-drug-products; 2021.
271. Matsui T, Umetsu R, Kato Y, Hane Y, Sasaoka S, Motooka Y, et al. Age-related trends in injection site reaction incidence induced by the tumor necrosis factor-α (TNF-α) inhibitors etanercept and adalimumab: the Food and Drug Administration adverse event reporting system, 2004–2015. *Int J Med Sci*. 2017;14(2):102–109. Jan 15.